# Università degli Studi di Napoli Federico II

# SCUOLA POLITECNICA E DELLE SCIENZE DI BASE



Multifunctional hybrid materials from biowaste valorization

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# Contents

### Introduction

1.	Aim of PhD research work	1
2.	Thesis structure	5
3.	Publications and other achievements	8

### Chapter 1: General overview and state of the art

Humic Acids	9
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### Chapter 2: Hybrid multifunctional materials

1.	Hybrid nanostructured materials
2.	Sustainable hybrid biopolymeric materials for food packaging
	applications47
3.	Design of hybrid multifunctional 3D hydrogels55
4.	Design of silicon-modified epoxy resin containing functional bio-
	waste flame retardant additives for epoxy-based systems60

# Chapter 3: Hybrid humic acids-based nanoparticles: from the design to the functional properties

# Chapter 4: Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks

Chapter 5: Conclusions	
Chapter 6: Appendix	

# Introduction

# Introduction

# Contents

1. Aim of PhD research work	1
2. Thesis structure	5
3. Publications	8
3.1 Published	8
3.2 Submitted	9
4. Oral and poster presentation to national and international conferences	10
5. Academic activities	12
6. Other relevant achievements	13
7. References	14

# 1. Aim of PhD research work

The waste to wealth concept is a challenging research field that promotes a future sustainable lifestyle valorising waste into a wide range of valuable compounds and products. This approach is particularly promising for bio-wastes (BWs) because of their large abundance as well as remarkable chemical and biological richness. As a result, industry and academic researchers are working to increase the economic and environmental value of BWs by developing strategies for recycling and converting them into added-value compounds and materials (Glasing et al., 2016; Xu et al., 2019).

Among BWs, Humic acids (HA) are the alkali-soluble fraction of natural organic matter that survives the biological and chemical degradation of both vegetal and animal biomasses (Luo et al., 2019; Spaccini et al., 2019). Furthermore, they are the most abundant bioproduct of biorefinery processes. According to a circularchemistry approach, they are viewed as a promising and cost-effective source for high-value products and novel materials because of their intriguing properties. Indeed, phenolic, and carboxylic groups in HA are primarily responsible for improved plant growth and nutrition (Chen, 1996; Chen and Stevenson, 1986; Piccolo et al., 1992; Tahir et al., 2011), flame retardancy features due to char formation in combustion processes (Vahabi et al., 2021), and even antiviral and inflammatory activity (Junek et al., 2009; Klöcking and Helbig, 2005; Schols et al., 1991). Furthermore, their amphiphilic nature determines self-assembly in aqueous environment (Klavins and Purmalis, 2010; Nuzzo et al., 2013; Piccolo, 2001) building up supramolecular structures which can act as metal chelating agents (Li et al., 2020; Yates and Von Wandruszka, 1999) and can interact with organic contaminants (Afzal et al., 2019; Liu et al., 2020; Savy et al., 2020). Moreover, quinone moieties confer redox (antioxidant and/or pro-oxidant) behavior to these bioavailable mixtures thanks to their ability to generate, stabilize, or scavenge Reactive Oxygen Species (•OH, •O<sub>2</sub>-, •OOH), also known as ROS.

Despite the substantial potential offered by bioavailable HA materials, their full technological exploitation is strongly limited by their segregation leakage and/or degradation phenomena in aqueous environment. Accordingly, despite their large abundance, they are mainly considered as waste and only a small amount is employed for low value processes including soil amending. Furthermore, their presence in water environment raises big issues to decontamination processes, since they contribute to fouling phenomena and improve pollutant persistence preventing efficient removal.

In this context, the PhD project aims at defining different synthetic approaches to turn this environmental issue into a technological tool, through the design of multifunctional materials based on HA biomolecules.

The project has been developed with the support of the company Verde Vita s.r.l. based in SASSARI, where the composting plant is located. This firm provides for quality compost certified by "Consorzio Italiano Compostatori" as HA source.

The PhD thesis has been focused on two main parts:

- Design of hybrid nanostructured HA-based materials.
- Design of HA-based polymeric materials.

The molecular combination of these heterogeneous moieties with an inorganic matrix (e.g., SiO<sub>2</sub>, TiO<sub>2</sub>, ZnO) is an effective method for limiting HA aqueous degradation phenomena, thus improving physicochemical stability, mechanical properties, and even enhancing intrinsic HA features in the final hybrid nanostructured HA-based materials (Pota et al., 2020; Venezia et al., 2022a, 2022b; Vitiello et al., 2021). This approach has been combined with electrospinning technology to produce bioactive and sustainable nanocomposite films for active

packaging applications made of electrospun biodegradable and bioderived polymers and hybrid nanoparticles.

Furthermore, HA have been explored as functional additives for polymeric materials. In particular, proper synthesis strategies have been designed to use these biomolecules as functional biowaste flame retardant for epoxy-based systems (Venezia et al., 2021b) or as an additive for gelatine hydrogels (Venezia et al., 2021a) to obtain hybrid HA-3D network with tunable rheological features and significant biological activity, including antimicrobial and antioxidant efficacy, for a broader range of biotechnological applications.

From a scientific point of view, the thesis project has contributed to clarify the chemistry of HA with a special focus on their interaction with either organic or inorganic components as well as on the physico-chemical features of HA-based hybrid materials.

From a technological point of view, the research activity has provided viable routes for HA valorization, by developing different materials with intriguing features and concrete application perspective in food-packaging, biomedical as well as automotive fields. Furthermore, developed synthesis strategies could be easily extended to BWs valorisation other than HA, thus significantly contributing to the challenging mission of giving waste a new value.

The following graphical abstract summarizes the main objectives achieved in this thesis project.

## Introduction



# **Graphical abstract**

# 2. Thesis structure

The following PhD work is divided into seven chapters.

**Introduction** contains the aim of the PhD thesis and the thesis structure. This chapter also provides the collection of articles already published or under review, the academic activities and other relevant achievements achieved during this PhD project.

**Chapter 1** provides a general overview on Humic Acids (HA), in respect to their chemical and physical features, their role in circular bioeconomy, their limitations and, therefore, the challenge underlying their valorization.

**Chapter 2** deals with hybrid multifunctional materials, discussing on (**a**) the design and development of HA/SiO<sub>2</sub>, HA/TiO<sub>2</sub> and HA/ZnO nanostructured materials through in situ methods based on wet chemistry approach (sol-gel, hydrothermal and solvothermal routes), (**b**) the use of biopolymers and biopolymer nanocomposites (i.e. electrospun hybrid nanofibers) as multifunctional active food packaging materials, (**c**) the development of naturally-derived hydrogels based on HA and (**d**) the incorporation of HA as biowaste materials providing flame retardant properties in epoxy resin systems.

**Chapters 3** and **4** are structured as a collection of original versions of scientific papers deriving from the present PhD thesis.

The first article of Chapter 3 (published in *Polymers*) deals with the synthesis, the physico-chemical and functional characterization of hybrid organic-inorganic HA/SiO<sub>2</sub> nanostructures synthesized via an in-situ sol-gel route, exploiting both physical entrapment and chemical coupling.

The second one (published in *Chemosphere*) highlighted that hybrid HA/SiO<sub>2</sub> nanostructures acted as dynamic systems which exhibit structural supramolecular reorganization during aging in aqueous environment with marked effects on physico-chemical and functional properties, including improved antioxidant activity.

The third one (published in *Environmental Research*) demonstrated that hybrid HA/TiO<sub>2</sub> nanomaterials, prepared via a versatile *in situ* hydrothermal strategy, displayed promising antibacterial activity against various pathogens and behaved as selective sequestering agents of amoxicillin and tetracycline antibiotics from wastewater.

The fourth one (published in *Journal of Environmental Chemical Engineering*) highlighted that hybrid HA/ZnO nanoparticles, synthetized through a bottom-up strategy, exerting an improved prooxidant behavior by generating Reactive Oxygen Species, even without light irradiation and favouring an enhanced photocatalytic and antimicrobial activity against different bacterial pathogens.

The fifth one (published in *Journal of Environmental Chemical Engineering*) deals with eco-designed HA-doped/ZnO nanoparticles synthesized via solvothermal route and tested as photocatalysts for the degradation of linear low-density polyethylene (LLDPE) and polylactic acid (PLA) thin films, under UVA/light irradiation.

The first article of Chapter 4 (submitted to ACS Sustainable Chemistry & Engineering) demonstrated that electrospun composites made by including hybrid nanostructures  $TiO_2_HS$  (synthetized through hydrothermal route using humic substances) into PHBV matrix can be converted into hybrid fiber-PHBV based films with antimicrobial properties against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli), as well as good mechanical, moisture/light barrier, optical and thermal properties, suggesting electrospinning methodology as a promising sustainable approach to convert biowaste into multifunctional materials for active packaging applications.

The second article of Chapter 4 (published in *Biomacromolecules*) exploring the chance to convert biowaste into a valuable resource, testing the potential role of HA as physical agents to improve gelatin's mechanical and thermal properties and, therefore, extending HA exploitation for a large set of technological applications.

The third article of Chapter 4 (published in *ACS Applied Polymer Materials*) deals with the incorporation of HA as flame retardants in silicon-modified epoxy resins, thanks to the presence of oxygen-containing functional groups, carbon-enriched chemical composition, and supramolecular architectures of these biomolecules.

**Chapter 5** contains the general conclusions and outlook of this PhD Thesis. Moreover, future perspectives were also included in the same chapter.

**Chapter 6** describes the synthesis procedures and the experimental methods used in this PhD research.

Introduction

# **3.** Publications

### **3.1 Published**

- <u>Venezia, V.</u>, Pota, G., Silvestri, B., Vitiello, G., Di Donato, P., Landi, G., ... & Luciani, G. (2022). A study on structural evolution of hybrid humic Acids-SiO2 nanostructures in pure water: Effects on physico-chemical and functional properties. *Chemosphere*, 287, 131985.
- Vitiello, G., <u>Venezia, V.</u>, Verrillo, M., Nuzzo, A., Houston, J., Cimino, S., ... & Luciani, G. (2021). Hybrid humic acid/titanium dioxide nanomaterials as highly effective antimicrobial agents against gram (-) pathogens and antibiotic contaminants in wastewater. *Environmental research*, 193, 110562.
- Pota, G., <u>Venezia, V.</u>, Vitiello, G., Di Donato, P., Mollo, V., Costantini, A., ... & Luciani, G. (2020). Tuning Functional Behavior of Humic Acids through Interactions with Stöber Silica Nanoparticles. *Polymers*, *12*(4), 982.
- <u>Venezia, V.</u>, Avallone, P. R., Vitiello, G., Silvestri, B., Grizzuti, N., Pasquino, R., & Luciani, G. (2021). Adding Humic Acids to Gelatin Hydrogels: A Way to Tune Gelation. *Biomacromolecules*, 23(1), 443-453.
- <u>Venezia, V.</u>, Matta, S., Lehner, S., Vitiello, G., Costantini, A., Gaan, S., ... & Bifulco, A. (2021). Detailed Thermal, Fire, and Mechanical Study of Silicon-Modified Epoxy Resin Containing Humic Acid and Other Additives. *ACS Applied Polymer Materials*, 3(11), 5969-5981.
- <u>Venezia, V.</u>, Sannino, F., Costantini, A., Silvestri, B., Cimino, S., & Califano, V. (2020). Mesoporous silica nanoparticles for β-glucosidase immobilization by templating with a green material: Tannic acid. *Microporous and Mesoporous Materials*, 302, 110203.
- <u>Venezia, V.</u>, Pota, G., Silvestri, B., Costantini, A., Vitiello, G., & Luciani, G. (2022). Tailoring Structure: Current Design Strategies and Emerging Trends to Hierarchical Catalysts. *Catalysts*, *12*(10), 1152.

- <u>Venezia, V.</u>, Verrillo, M., Gallucci, N., Di Girolamo, R., Luciani, G., D'Errico, G., ... & Vitiello, G. (2022). Exploiting bioderived humic acids: a molecular combination with ZnO nanoparticles leads to nanostructured hybrid interfaces with enhanced pro-oxidant and antibacterial activity. *Journal of Environmental Chemical Engineering*, 108973.
- Amato, P., Muscetta, M., <u>Venezia, V.</u>, Cocca, M., Gentile, G., Castaldo, R., ... & Vitiello, G. (2022). Eco-sustainable design of Humic Acids-doped ZnO nanoparticles for UVA/light photocatalytic degradation of LLDPE and PLA plastics. *Journal of Environmental Chemical Engineering*, 109003.

# 3.2 Submitted

<u>Venezia, V.</u>, Prieto, C., Evtoski, Z., Silvestri, B., Vitiello, G., Lucian, iG., Lagaron, J. M. Electrospun hybrid TiO<sub>2</sub>/Humic Substance PHBV films for active food packaging applications, under submission to ACS Sustainable Chemistry & Engineering in December 2022.

# 4. Oral and poster presentation to national and international conferences

- Oral Communication entitled "Immobilizzazione di β-glucosidasi su nanoparticelle di silice mesoporosa: effetto della morfologia dei pori sulle prestazioni catalitiche" at the "VIII workshop AICIng 2019", 27-29 June 2019 – Lipari, Italy.
- 2. Oral Communication entitled "The challenge of humic acid valorization: biohybrid multifunctional materials for biomedical and environmental applications" at the joint 5th International Conference on Bioinspired and Biobased Chemistry and Materials (N.I.C.E. Conference 2020) and 2nd International Conference on Optics, Photonics, & Materials ONLINE, 12-14 October 2020.
- Oral Communication entitled "From waste to valuable sources: Humic acids valorization into multifunctional nanostructured materials" at the 5<sup>th</sup> Green and Sustainable Chemistry ONLINE", 10-11 November 2020.
- 4. Green-week Festival della Green Economy, 9-11 July 2021, Parma, Italy.
- 5. Contest "CIG 2021, Catalisi in Gioco", 27-30 July 2021, Reggio Calabria, Italy.
- Poster Communication entitled "Biowaste as valuable resource: humic acids valorization into hybrid multifunctional nanomaterials" at "AICIng 2021", 5-8 September 2021, Reggio Calabria, Italy.
- Poster Communication entitled "Tannic acid as green templating agent for mesoporous silica nanoparticles to β-glucosidase immobilization" at AICIng 2021, 5-8 September 2021, Reggio Calabria, Italy.
- Oral Communication entitled "Biowaste as valuable resource: humic acids valorization into multifunctional materials", at SCI 2021 ONLINE, 14-23 September 2021.

- Oral Communication entitled "Resilience degree of Humic Acids: An indicator for valorisation as polymer additives" at "API Congress ONLINE", 11-12 November 2021.
- Oral Communication entitled "From waste to valuable sources: humic acids valorization into biohybrid multifunctional materials" at the 6<sup>th</sup> Green and Sustainable Chemistry ONLINE", 16-18 November 2021.
- Oral Communication entitled "Electrospun TiO<sub>2</sub>/Humic Acid poly(3hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) composite nanofibers with enhanced antibacterial activity for food packaging applications" at the 5th European Symposium on Electrohydrodynamic Atomization and Electrospinning, 27-29 April 2022, Naples, Italy.
- Poster Communication entitled "Antibacterial electrospun hybrid TiO<sub>2</sub>/humic acid PHBV fibers for active packaging", at the "1° Congresso Nazionale della Divisione di Chimica per le Tecnologie", 4-7 September 2022, Naples, Italy.
- Flash Communication entitled "Antibacterial electrospun hybrid TiO<sub>2</sub>/humic acid PHBV fibers for active packaging", at the "1° Congresso Nazionale della Divisione di Chimica per le Tecnologie", 4-7 September 2022, Naples, Italy.
- 14. Oral Commucation entitled "Combination at molecular scale of humic acids and ZnO nanoparticles via wet-chemistry route: hybrid nanomaterials with enhanced pro-oxidant and antibacterial activity", at the "Seventh International Conference on Multifunctional, Hybrid and Nanomaterials", 19-22 October 2022, Genova, Italy.
- 15. Oral Communication entitled "Electrospun hybrid TiO<sub>2</sub>/Humic Acid poly(3hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanofibers with improved antibacterial activity for active packaging" at the "Seventh International Conference on Multifunctional, Hybrid and Nanomaterials", 19-22 October 2022, Genova, Italy.

- Oral Communication entitled "From Waste to Wealth: Humic Acids as additives to tune polymer rheological and functional features" at the "BIOPOL 2022", 14-16 November 2022, Alicante, Spain
- Oral Communication entitled "Electrospun hybrid TiO<sub>2</sub>/Humic substance PHBV Films for active packaging" at the "BIOPOL 2022", 14-16 November 2022, Alicante, Spain.
- Oral Communication entitled "ELECTROSPUN HYBRID TiO<sub>2</sub>/HUMIC SUBSTANCE PHBV FILMS: Antimicrobial materials for food packaging applications" at the 4<sup>th</sup> International Congress Advances in the Packaging Industry "Sustainability: products and processes", 24-25 November 2022, Naples, Italy.

# 5. Academic activities

1. Co-supervisor in chemical engineering thesis entitled: "Nanostructured materials from biowastes valorization for water micropollutants removal"

2. Co-supervisor in chemical engineering thesis entitled: "Immobilization of  $\beta$ -Glucosidase over structured cordierite monoliths washcoated with SiO<sub>2</sub> nanoparticles "

3. Co-supervisor in chemical engineering thesis entitled: "Photodegradation of polymeric matrices of LLDPE through hybrid nano-catalysts of ZnO and a biomass-derived organic component"

4. Co-supervisor in chemical engineering thesis entitled: "Biowaste to wealth: nanostructured materials for the recovery of noble metals from electronic waste"

5. Co-supervisor in chemical engineering thesis entitled: "Synthesis of lignin nanoparticles: top-down, bottom-up and hybrid approaches"

6. Co-supervisor in chemical engineering thesis entitled: "Lignin - cellulose aerogel: design and characterization for water remediation"

# 6. Other relevant achievements

- Visiting period at the Novel Materials and Nanotechnology for Food Related Applications Spanish Council for Scientific Research (CSIC), Instituto de Agroquimica y Tecnologia de Alimentos (IATA) (Paterna Valencia), 02/11/2021-31/01/2022, 02/05/2022-31/07/2022
- 2. Expert on the subject Chemistry approved in May 2020
- 3. Winner of The Best Scientific Poster Award at the XII Convegno Nazionale AICIng – Reggio Calabria 2021
- 4. Grant award to attend the XXVII Congresso Nazionale della Società Chimica Italiana
- 5.3rd place at the Contest Catalisi in gioco CIG 2021
- 6. Presenting activity at Futuro Remoto 2022.

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# **Chapter 1**

*General overview and state of the art* 

# Contents

Humic acids 1	9
1. The concept of Circular Bioeconomy 1	9
2. Biowastes recycling and conversion 2	21
3. The challenge of humic acids valorization	21
4. Chemical structure of humic acids	23
5. Solubility and pH dependence 2	24
6. Amphiphilic behavior 2	25
7. Binding cationic metals	25
8. Humic acids paramagnetic properties 2	26
9. Limitations of humic acids	27
10. References	28

## Humic acids

### 1. The concept of Circular Bioeconomy

Circular economy and bioeconomy aim to transform the current linear economic system into a more sustainable one.

According to the previously stated definitions, achieving a circular economy implies primarily slowing, narrowing, and closing material resource loops, relying on renewable energy and non-toxic materials, as well as long-term design, maintenance, repair, reuse, remanufacturing, refurbishing, and recycling (Tan and Lamers, 2021) (as illustrated in **Figure 1A**).

In this context, the concept of bioeconomy implies the use of renewable biological resources, such as biomass, to produce renewable biofuels, bioproducts, and biopower for economic, environmental, and social benefits (**Figure 1B**).

The circular bioeconomy, as shown in **Figure 1C**, can be seen as the intersection of the two emerging concepts of the circular economy and the bioeconomy.

However, a sustainable bioeconomy will also necessitate more than the replacement of fossil resources with renewable biological resources, since it will also require a sustainable supply chain, which includes sustainable biomass feedstock production and conversion processes, as well as sustainable products. As a result, the success of bioeconomy will be highly dependent on the development of breakthrough biorefining technologies for the transformation of bio-based and renewable resources into high-value bio-based products, materials, and fuels.

Some argue that the bioeconomy is already "circular by nature," because it is based on the renewable and circular nature of the planet's ecosystems (Leipold and Petit-Boix, 2018). For example, biobased products derived from renewable biological carbon, the main component of biological compounds such as biomass, are recycled and reused throughout the biosphere (i.e., carbon cycle). Therefore, the bioeconomy

is only associated with the biological cycle, in which materials are made from biological nutrients (i.e., bio-based products) that can be safely reintroduced into the biosphere. As a result, a bio-based circular carbon economy is a framework for increasing circularity within the economic system.



Figure 1. (A) Components of a circular economy definition; (B) Scheme of a sustainable bioeconomy renewable biological resources to renewable products (i.e., biopower, biofuels, bioproducts); (C) Scheme of a circular bioeconomy resulting from the intersection between circular economy and bioeconomy concepts (Tan and Lamers, 2021).

### 2. Biowastes recycling and conversion

The waste to wealth concept is a challenging research field that promotes a future sustainable lifestyle by valorizing wastes and enabling the development of closed-loop waste-free processes, which are at the heart of the circular economy. In particular, the management and valorization of biowastes (BWs) is one of the most pressing issues for circular bioeconomy development (Clark et al., 2016; Keijer et al., 2019), because of the massive amount of bio-residues produced by biogenic and non-biogenic transformations (Clark et al., 2016; Keijer et al., 2019). Indeed, BWs are a largely available source of chemical, physical, and biological richness that can be transformed into a wide variety of valuable products and functional novel materials.

As a result, industry and academic researchers are working to increase the economic and environmental value of BWs and develop strategies for converting them into added-value compounds and materials (Glasing et al., 2016; Xu et al., 2019), either through recycling or conversion (Xu et al., 2019).

### 3. The challenge of humic acids valorization

Among BWs, humic acids (HA) are an alkali-soluble fraction of natural organic matter that is commonly found in water, soil, and sediments (Keijer et al., 2019). They are crucial to preserving plant growth and controlling the fate of environmental pollutants as well as the biogeochemistry of organic carbon in global ecosystems (MacCarthy, 2001; Piccolo et al., 2019; Stevenson, 1994).

HA can be obtained from biomass through natural chemical and biological transformations and are also an abundant byproduct of biorefinery processes (Luo et al., 2019; Savy et al., 2020; Spaccini et al., 2019). More specifically, control oxidative degradation or stabilization treatments of biomass derived from organic

fraction of Urban or agro-industrial waste usually occurs in composting plants, providing a product known as compost (**Figure 2**), from which HA can be extracted.



Figure 2. Extraction of humic acids from compost

HA are rich in functional groups, including carboxyl, phenol, hydroxyl, and quinone moieties, which confer them many useful functional properties, including metal ions chelation, organic pollutants absorption, and regenerable red-ox behavior in terms of antioxidant, antimicrobial and anti-inflammatory activity (Aeschbacher et al., 2012; Scott et al., 1998; Van Rensburg et al., 2000).

Considering these features, HA are expected to play a leading role on the stage of circular-chemistry as an accessible and inexpensive source for the design and development of multifunctional materials for potential bio-sustainable applications (de Melo et al., 2016).

### 4. Chemical structure of humic acids

Humic substances (HS) are made up by a collection of heterogeneous compounds and are conventionally classified according to their molecular solubility in aqueous solution at different pH, into fulvic acids (FA) soluble in all pH conditions, humic acids (HA) soluble only under alkaline conditions, and humin (HU) insoluble at any pH.

HA are considered as heterogeneous mixtures of relatively small molecules (<1000 Da) with different polarity and rich in functional groups including quinone, phenol, carboxyl and hydroxyl moieties. Supramolecular theory (Piccolo, 2001) indicates that HS are supramolecular associations of self-organizing, relatively small heterogeneous molecules held together by weak dispersive forces, such as van der Waals,  $\pi$ -  $\pi$ , CH-  $\pi$  interactions.

In these superstructures, hydrophobic groups are in the interiors whereas hydrophilic parts make up the exterior surfaces. Due to the weakness of stabilizing interactions, HA supramolecular conformations are reversible and very sensitive to chemical changes of the surroundings. Indeed, different independent studies (Bezuglova, 2019; Myneni et al., 1999; Piccolo, 2001; Sutton and Sposito, 2005; Wershaw, 1986) have provided evidence that even materials extracted by alkali are not macromolecular.

The relative amount of functional groups in these biomolecules depend on the origin, age, climate, and environmental conditions of extraction/production of HA (Sposito and Weber, 1986; Stevenson, 1994), even if the phenolic and carboxylic moieties are the most prevalent in HA structures. Their presence provides HA with many capabilities, such as the improvement of plant growth and nutrition (Chen, 1996; Chen and Stevenson, 1986; Piccolo et al., 1992; Tahir et al., 2011; Yates and Von Wandruszka, 1999), complexation with heavy metals (Yates and Von Wandruszka, 1999), antioxidant, antiviral and inflammatory activity (Junek et al., 2009; Klöcking

and Helbig, 2005; Verrillo et al., 2022; Yates and Von Wandruszka, 1999). Besides, quinones moieties are responsible for the redox behavior of these materials because they can generate, stabilize, or quench Reactive Oxygen Species (•OH, •O<sub>2</sub>-, •OOH), the so-called ROS.

### 5. Solubility and pH dependence

HA are generally considered soluble in neutral or alkaline conditions. This behaviour varies with the chemical composition of these substances and thus with their origin. In alkaline media, phenolic and carboxylic groups are deprotonated, and the repulsion between these negatively charged groups causes the molecules to assume a stretched configuration. When the pH is reduced, the functional groups are protonated and the effects of repulsion are reduced, causing the molecule to adopt a coiled and compact structure. At this point, the hydrophobic portions of the structure are inside the domain, and the hydrophilic portions are exposed to the aqueous medium. This behavior is responsible for the surfactant properties of HA and their micelle-like organization. Indeed, these molecules can form aggregates on an intramolecular level, followed by intermolecular aggregation and ultimately precipitation (Von Wandruszka et al., 1997), as shown in **Figure 3**.



**Figure 3.** Behavior of HA molecules in alkaline conditions and the aggregation process upon pH reduction. High pH: charge repulsion (A). Decreasing pH: ntramolecular aggregation (B) and intermolecular aggregation (C). Acidic pH: precipitation (D) (de Melo et al., 2016).

# 6. Amphiphilic behavior

HA compounds form supramolecular aggregates stabilized by noncovalent interactions, containing hydrophobic and hydrophilic domains, and showing intrinsic amphiphilic behavior (Li et al., 2020; Nuzzo et al., 2013).

Indeed, in neutral to acidic conditions, HA forms micelle-like structures known as pseudo-micelles due to their amphiphilic behavior (Von Wandruszka, 2000; Von Wandruszka et al., 1997). This property has been investigated for applications in pollution remediation (Johnson and John, 1999; Lassen and Carlsen, 1999, 1997; Van Stempvoort and Lesage, 2002) as well as increasing the water solubility of hydrophobic drugs (Martini et al., 2010; Mirza et al., 2011).

# 7. Binding cationic metals

The ability of HA to bind cationic metals and form complexes makes them useful in a plethora of applications, including micronutrient transport from soil to plants (Chen and Stevenson, 1986; de Melo et al., 2016), heavy metal removal from soil and water (Yates and Von Wandruszka, 1999), inhibition of free radical formation by metal catalysis (Rice-Evans et al., 1997), and reduction and stabilization of metal nanoparticles (Litvin and Minaev, 2013). Multivalent cations can interact with phenolic and carboxylic groups on neighbouring chains, enhancing the detergent effect and pseudo-micellar domains. The interaction between HA molecules and metal cations is initially completely electrostatic, with the cations moving to their thermodynamically preferred locations within the structure. As shown in **Figure 4** 

(Von Wandruszka et al., 1997), this process produces spherical HA-metal complexes.



**Figure 4.** Interaction of  $Mg^{2+}$  ions with an HA molecule and its folding around the hydrophobic region. Figure taken with permission from Engebretson & von Wandruszka (Engebretson and von Wandruszka, 1994) Copyright (1994) American Chemical Society.

Further research on HA-metal binding has revealed that this type of interaction depends on the kind of metal and on its concentration as well as the origin, molecular weight, and concentration of HA (Christl and Kretzschmar, 2001; Yates and Von Wandruszka, 1999). Christl et al. (Christl and Kretzschmar, 2001) demonstrated that the binding capacity of HA to metals depends to their molecular size. <sup>13</sup>C NMR studies confirmed that HA fractions with the lowest molecular weight have the largest abundance of phenolic and carboxylic groups and, consequently, can bind metals most efficiently. Besides, higher metal concentrations could improve the interaction effectiveness, as demonstrated by Christl and Kretzschmar (Christl and Kretzschmar, 2001).

# 8. Humic acids paramagnetic properties

HA present stable organic radicals in their structure that confer them intrinsic paramagnetic properties of polyphenols-like materials. These radicals, which are

centered on the carbon or oxygen atom in the aliphatic and/or aromatic functional groups of HA, play a key role in defining their redox (antioxidant/pro-oxidant) equilibria (Rex, 1960).

# 9. Limitations of humic acids

Despite the great potential offered by bioavailable HA-like materials, several challenges still need to be overcome, due to their poor selectivity and great instability (browning) under aerated conditions (Allard et al., 1994). Their rapid conformational dynamics and, hence, segregation leakage and/or degradation phenomena in aqueous environment make HA poorly useful for applications in aqueous media (de Melo et al., 2016). Moreover, although many studies on interactions between HA and pollutants have been proposed, the difficulty in distinguishing between soluble and insoluble HA often hinders the assessment of their adsorption features (Afzal et al., 2019; Du et al., 2020). Indeed, HA have shown high solubility in aqueous media, thereby preventing the possibility of efficiently removing pollutants from aqueous environments. HA are controversial moieties since their interactions with nanomaterials and organic pollutants can increase the solubility and bio-persistence of these species in aqueous solutions (de Melo et al., 2016). Consequently, the nonnegligible chemical lability and heterogeneous self-assembly behaviour of HA strongly prevents the full exploitation of these intriguing biomolecules, which are often regarded as an environmental issue rather than a valuable resource due to their abundance and their function as carriers of pollutants.

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## Chapter 2

Hybrid multifunctional materials

#### Contents

1. Hybrid nanostructured materials
1.1 Nanomaterials and nanoparticles
1.2 Hybrid Materials and Nanomaterials
1.2.1 Class I Hybrid Materials
1.2.2 Class II Hybrid Materials
1.2.3 Inorganic Components
1.2.4 Synthesis of inorganic nanomaterials
1.2.5 Design of hybrid nanostructured humic acids-based materials (CERAMIC
TEMPLATED APPROACH)
2. Sustainable hybrid biopolymeric materials for food packaging applications 47
2.1 Biopolymers Based Food Packaging Materials
2.2 Active food packaging
2.3 Antimicrobial packaging 50
2.4 Electrospinning for Food Packaging
2.5 Electrospun hybrid nanostructured humic acids-biopolymer films for active
packaging
3. Design of hybrid multifunctional 3D hydrogels
3.1 Polyphenols-like gels
3.2 Gelatin-based hydrogels
3.3 Humic acids-based 3D hydrogels
4. Design of silicon-modified epoxy resin containing functional bio-waste flame
retardant additives for epoxy-based systems

4.1 Humic acids as biowaste flame retardant additive	61
5. References	63

#### 1. Hybrid nanostructured materials

#### **1.1 Nanomaterials and nanoparticles**

The term "nanomaterials" refers to materials with at least one dimension unit between 1-100 nm (the usual definition of nanoscale). Nanoparticles, with a size between 2 and 200 nm, have all three dimensions on the nanoscale and belong to this class.

Nanoparticles and nanomaterials are classified according to their morphology, size and physico-chemical features. Based on their shape and size, they can be classified into: nanospheres, nanotubes, nanorods, nanowires, nanoribbons, and nanocrystals. Chemically, nanoparticles and nanomaterials can be classified based on their different nature in inorganic (metals, oxides), organic and metallorganic. The nanomaterials physico-chemical properties (i.e. optical, electrical and magnetic) of are particularly interesting as they differ from those of the bulk, i.e. a sample of the same material but a macroscopic extension. These characteristics are often intermediate between those of the bulk material and those of the atomic structure and depend on the nanostructure dimensions. Conversely, in bulk materials the physicochemical properties are fixed and independent of the size. This difference in the physico-chemical properties of nanomaterials is attributed to their high surface/volume ratio (Suryanarayana, 2005). The optical properties, such as the refractive index and absorbance, of a bulk material are constant, independent of mass and volume. Conversely, in the case of nanoparticles, the optical properties are directly related to size and shape. For example, light absorption properties of noble metal nanoparticles strictly depend on the nanostructure size and show an intense absorption band in the UV-visible region, which is not in the spectrum of the bulk material. A similar behavior is exhibited by some semiconductor nanoparticles. It has been amply demonstrated that the wavelength of the absorption spectrum peak depends on the size, shape, and dielectric properties of these nanoparticles and those of the local environment, including substrates, solvent and adsorbates<sup>7</sup>. For their unique properties, nanomaterials find a great e application in different technological fields, including photocatalysis.

#### **1.2 Hybrid Materials and Nanomaterials**

Hybrid organic-inorganic (nano)materials represent one of the scientific frontiers currently most explored in many disciplinary fields, from chemistry to physics, from engineering to materials science, biology, and biomedicine. The main idea behind the development of this type of materials is to associate, on molecular scale, organic and inorganic components to obtain a synergistic combination of typical properties of the two classes of constituents (**Figure 1**) (Sanchez et al., 1999).



Figure 1: Schematic representation of hybrid (nano)materials.

The incorporation of inorganic molecules or aggregates (in the form of clusters) within an organic matrix or, viceversa, the organic molecule dispersion or embedding into an inorganic matrix can give the material the typical properties of organic materials (elasticity, flexibility, processability, multiple functionalities) but also the typical characteristics of inorganic ones (thermal stability, optical, magnetic, electrical properties).

Moreover, the dispersion of the two components on atomic or molecular scale can determine the occurrence of peculiar properties not manifested by the two phases alone. Both the chemical nature and the relative quantity of the constituents allows to modulate the chemical, physical, mechanical, morphological, and functional properties of the final hybrid material. An important role in defining the properties is also played by the morphology of the components, therefore by the structure of the material itself, as well as by the interactions between the two phases (Schubert and Hüsing, 2019). The wide variety of hybrid materials and nanomaterials does not allow for a rigid classification. It is possible to identify two classes of organo-inorganic hybrid materials, also valid at nanoscale, based on the type of interaction between the constituents:

- Class I Hybrid Materials
- Class II Hybrid Materials

These classes are schematized in Figure 2:



Figure 2: Hybrid materials of I (a, b) and II class (c,d).

#### 1.2.1 Class I Hybrid Materials

The organic and inorganic components are held together by noncovalent interactions such as van der Waals forces or hydrogen bonds. This class includes materials consisting of inorganic particles (e.g., metal clusters) embedded in a polymer matrix or, vice versa, organic molecules with special properties (colouring, fluorescent) embedded in inorganic matrices such as silica (**Figure 2 a**). This class of systems also includes the interpenetrated lattice (blend), in which the inorganic and organic lattice are intimately integrated without interacting with each other (**Figure 2 b**).

However, these materials, homogeneous at macroscopic level exhibit phase separation at microscopic level and therefore simply retain the properties of the components.

#### 1.2.2 Class II Hybrid Materials

This class is characterized by the presence of chemical bonds (ionic or covalent) between the species constituting the system. Class II hybrid materials include mixed inorganic-organic lattices (**Figure 2 d**) or organic molecules, particles stably bound to an inorganic matrix or, vice versa, inorganic clusters suitably anchored to an organic phase (**Figure 2 c**) by means of appropriate functional groups (Gross, 2011). This approach ensures an intimate and uniform distribution of species in the material matrix and no phase separation at microscopic level. Accordingly, the features of the final material can be easily controlled and optimized them for the required application. Hence, it is necessary to implement synthetic routes to obtain a highly homogeneous distribution of the component species and to tune the final properties of the material.

At nanoscale, the opportunity to design and realize a large variety of hybrid nanomaterials with tunable chemical, physico-chemical and functional properties is opening new perspective in the development of systems with enhanced properties, also in the photocatalytic field.

#### 1.2.3 Inorganic Components

Hybrid nanomaterials offer a large versatility of compositions, thanks to the possibility to choose both organic and inorganic components in a wide class of compounds. In the context of inorganic nanostructures, zinc oxide nanoparticles (ZnO NPs), titanium dioxide nanoparticles (TiO<sub>2</sub> NPs) and silicon dioxide nanoparticles (SiO<sub>2</sub> NPs) are attractive interfaces due to their biocompatibility, tunable size, shape, and porosity as well as functional versatility and are valid candidates to design multifunctional nanomaterials through a synergistic

combination with organic moieties (**Figure 3**). Indeed, they can act as structure directing agents and/or catalysts in biopolymers building up (ceramic templated approach), ultimately boosting their properties (Giulio Pota et al., 2020; Silvestri et al., 2012; Venezia et al., 2022a, 2022b; Vitiello et al., 2021, 2017, 2015).



Figure 3. Organic/Inorganic Nanoparticles (Zhao et al., 2018) 1.2.4 Synthesis of inorganic nanomaterials SiO<sub>2</sub> nanoparticles synthesis.

The Sol-Gel technique is one of the most widely used synthesis techniques for silicate systems, particularly silica nanoparticles. This method consists of hydrolysis and condensation reactions of metal alkoxides (i.e. tetrapropylorthosilicate or tetraetylorthosilicate) in hydroalcoholic solutions (**Scheme 1**) (Brinker and Scherer, 2013; Hench and West, 1990).

## Hydrolysis reactions: $=M-OR+H_2O \iff =M-OH+ROH$ Condensation reactions: $=M-OR+HO-M= \iff =M-O-M=+R-OH$ $=M-OH+HO-M= \iff =M-O-M=+H_2O$

#### M= metal component R= alkyl group of the metal precursor

Scheme 1. Hydrolysis and condensations reactions

The possibility to vary the relative amounts of metal alkoxide and water in the hydrolysis reaction, the type of solvent, the ionic strength of the reaction medium, the type and quantity of catalyst, modulating the shape, size, porosity, and surface chemistry make this process very convenient. Furthermore, the mild synthesis conditions, in terms of temperature and pH, allow for the incorporation of organic constituents into the synthesis phase without causing denaturation or decomposition, making this methodology ideal to produce hybrid materials. Using the Stöber method, it is possible to obtain monodisperse systems of spherical silica gel particles ranging in size from a few nanometers to a few microns as part of the Sol-Gel methodology. This method is based on the hydrolysis and polycondensation reactions of silicon alkoxides in ammonia-containing hydroalcoholic solutions. The latter is used as a morphological catalyst, in fact, in its absence the particles produced have irregular shapes.

Currently, silicon dioxide (SiO<sub>2</sub>) is one of the most used matrices for the fabrication of nano-structured hybrid systems. High purity, porosity, transparency, homogeneity, and low cytotoxicity are among the characteristics that motivate this choice.

TiO<sub>2</sub> and ZnO nanoparticles synthesis.

40

The wet-chemistry approach is a useful strategy that allows for the fine design of titanium dioxide (TiO<sub>2</sub>) or zinc oxide (ZnO) nanosystems with tuned physicochemical and functional properties (Rao et al., 2012; Yu, 2001). To this purpose, the solvothermal route, based on hydrolysis and condensation reactions under mild conditions of metallic salts or organometallic (i.e. Titanium (IV) isopropoxide or Zinc acetate) (Scheme 1), is a versatile and simple synthetic route for the preparation of this kind of nanomaterials. Solvothermal synthesis is a technique for producing crystalline ceramic materials under controlled temperature and pressure. In general, the synthesis is carried out at "autogenous" pressure, which corresponds to the saturated vapor pressure of the solution at the assigned temperature and composition. The ability to obtain already crystallized powders precipitated from the solution by adjusting thermodynamic (reaction temperature, pressure, nature and concentration of reagents) and non-thermodynamic variables (speed of agitation), results in a better control of the size and morphology of the crystallites, also decreasing significantly aggregation phenomena, which are commonly present in other synthesis processes. The intense research activity of the last decades has led to a better knowledge of the chemistry of solvothermal processes that has significantly reduced the reaction time, as well as temperature and pressure for crystallization of materials (T< 200 °C, P<1.5 MPa). This innovation has made solvothermal synthesis more economical and very effective for the realization of crystalline nanomaterials, but also has allowed the use of organic components in the synthesis, aimed at obtaining organic/inorganic hybrids employed in numerous technological fields. The hydrothermal synthesis is a variant of the solvothermal synthesis that involves the same steps as the hydrothermal synthesis but uses aqueous solutions.

Currently,  $TiO_2$  and ZnO nanostructures are two of the most intriguing inorganic materials because they are environmentally friendly, inexpensive, nontoxic, and photostable, as well as highly efficient photo-oxidative catalysts.

## 1.2.5 Design of hybrid nanostructured humic acids-based materials (CERAMIC TEMPLATED APPROACH)

Nanotechnology has the potential to provide strategic solutions to promote the conversion of biowastes (BWs) into useful hybrid functional materials with modulable properties (Glasing et al., 2016; Heuer-Jungemann et al., 2019; Luchini and Vitiello, 2019; Xu et al., 2019).

The ability to combine at the molecular scale bio-derived organic moieties with inorganic nanoparticles represents a valuable strategy for improving the intrinsic properties of both the inorganic and organic components and selectively driving their function (Deligiannakis et al., 2012; Silvestri et al., 2019; Vitiello et al., 2019). At the same time, this approach should allow us to overcome some intrinsic limits of such organic fractions, such as poor stability, fast conformational dynamics, or rapid reactivity in aqueous media, which severely limit its full exploitation.

To this regard, my research group recently proposed a novel synthetic strategy to produce hybrid nanostructures inspired to biologically active natural products, whereby an inorganic nanostructured phase can act as morphological agent for the building up of the organic component. Following this approach, a titanium dioxide (TiO<sub>2</sub>) sol has been exploited as a catalyst and templating agent for 5,6-dihydroxyindole-2-carboxylic acid (DHICA) polymerization to eumelanin (Pezzella et al., 2013; Vitiello et al., 2016, 2015). The inorganic phase tunes eumelanin supramolecular structure, leading to TiO<sub>2</sub>-eumelanin hybrids with unique antimicrobial features, sensibly better than intrinsic melanin biocide activity (Vitiello et al., 2018, 2017, 2015).

In the same way, inorganic nanostructured phase is envisaged to act as templating agent for humic acids (HA) supramolecular organization and consequently tune their functional features, even boosting their intrinsic properties (Deligiannakis et al., 2012; G. Pota et al., 2020; Venezia et al., 2022a, 2022b; Vitiello et al., 2021).

Therefore, combining HA with a foreign inorganic support appears as a promising strategy to improve their physicochemical stability in aqueous solution (Oliveira et al., 2016; Wang et al., 2020). Indeed, thanks to the amphiphilic character of their supramolecular structure (Piccolo, 2001), HA can interact with an inorganic surface charged phase through electrostatic and covalent forces, conferring a good colloidal stability in aqueous medium (Giulio Pota et al., 2020; M. Zhang et al., 2020). Scientific research on HA modified inorganic nanomaterials has exploded in recent years, providing proof of concept for these nanomaterials' enormous potential in a wide range of applications, including plant bio-stimulants (Adamiano et al., 2021; de Melo et al., 2016) and high efficacy sorbents for pollutants and even noble metal ions in aqueous solutions (Amer et al., 2021; Santosa et al., 2021). Indeed, the combination of organic and inorganic components into a hybrid nanostructure has resulted in far greater improvements in properties than the simple encapsulation/binding of organic compounds to an inorganic support.

Therefore, in this work synthetic strategies for producing organo-inorganic nanomaterials (**Figure 4**) in which HA from different sources (G. Pota et al., 2020; Venezia et al., 2022a; Vitiello et al., 2021) are combined with an inorganic matrix, including  $TiO_2$ ,  $SiO_2$  or ZnO are proposed. The obtained nanostructures showed good physico-chemical stability in aqueous solution, even boosted functional properties such as antioxidant and/or antibacterial behavior, as well as sequestering capability towards organic pollutants.



Figure 4. Hybrid nanostructures HA-based materials

Design of HA/SiO<sub>2</sub> Nanoparticles. Among nanostructured inorganic oxides, SiO<sub>2</sub> nanoparticles are functionally versatile biocompatible platform for the development of hybrid materials with modulable properties (Deligiannakis et al., 2012; Venezia et al., 2020). In particular, the Stöber method is an effective approach to design SiO<sub>2</sub> based hybrids, because it leads to tunable sized nanoparticles by using mild reaction conditions, thus preventing any degradation of the organic component (Deligiannakis et al., 2012; Venezia et al., 2020).

In this thesis (see **Chapter 3, Paragraphs 1 and 2**) an in-situ strategy was implemented, according to which silica nanoparticles were synthetized through solgel reaction occurring in the presence of HA. This approach was successfully used to combine HA with  $SiO_2$  component producing hybrid HA based nanostructures, with uniform size distribution and a good chemical and colloidal stability in aqueous solution as well as relevant antioxidant behavior (Giulio Pota et al., 2020; Venezia et al., 2022a). Two different coupling strategies, based on physical trapping and covalent conjugation respectively, were explored and their effect on the physical-chemical properties of obtained nanostructures was assessed to define the most promising combination approach (Giulio Pota et al., 2020). Then, different HA compositions were combined with Silica nanostructures in order to evaluate the effect of HA chemical nature on morphology, structure and functional properties of hybrid nanoparticles. In addition, since HA are extremely dynamic systems in

aqueous environment, SiO<sub>2</sub>\_HA nanostructures were employed to controlled aging in water and their physical chemical properties were then investigated.

Design of HA/TiO<sub>2</sub> Nanoparticles.

Titanium dioxide (TiO<sub>2</sub>) is an inexpensive, nontoxic and photostable material and can be proposed as biomimetic templating agent that improve HA intrinsic activity of HA, including ROS production ability, the sequester capability towards metal cations and organic pollutants and their size selective absorption features (de Melo et al., 2016; Tang et al., 2014; Vitiello et al., 2021). Furthermore, thanks to their amphiphilicity behavior (Piccolo, 2001), HA can interact with an inorganic surface charged phase through electrostatic forces and can confer satisfactory colloidal stability in aqueous media (G. Pota et al., 2020; Vitiello et al., 2021; M. Zhang et al., 2020).

In this thesis project (see **Chapter 3, Paragraph 3**), hybrid humic acid/titanium dioxide nanomaterials were designed and prepared through an *in-situ* method that was based on the hydrothermal wet chemistry approach. Then, the functionality of these novel nanohybrids was investigated, and two main applications for water remediation were considered: i. Antibacterial activity against gram-negative and gram-positive pathogens of various strains and ii. sequestering action towards three antibiotics, namely, amoxicillin, tetracycline and metronidazole, in an aqueous solution.

Design of HA/ZnO Nanoparticles. Among the semiconductor metal oxides, zinc oxide (ZnO) nanoparticles are particularly interesting thanks to a wide versatility because of non-toxicity, biocompatibility, low-cost and, above all, good optical, photocatalytic (Mallika et al., 2014; Mary Jacob et al., 2014; Van Hoecke et al., 2011), and antimicrobial (Madhumitha et al., 2016; Maryanti et al., 2014) properties, which favor the involvement in several technological fields. Synthetic approaches based on the intimate mixing of inorganic and organic precursors should lead to

nanostructured hybrid materials and interfaces with novel, peculiar and enhanced assets, which are directly determined by the molecular combination.

In this thesis project (see **Chapter 3**, **Paragraph 4**), novel multifunctional hybrid Humic Acid/Zinc Oxide (HA/ZnO) nanoparticles were synthesized through a bottom-up approach under solvothermal condition to finely combine at molecular level the precursor of semiconductor component with humic acids. The functional properties of hybrid HA/ZnO nanoparticles were tested in terms of redox behavior (i.e., anti-oxidant and pro-oxidant), photocatalytic degradation of Rhodamine B (as model dye molecule) and biocide activity against different bacterial pathogens. Besides, hybrid Humic Acids-doped/Zinc Oxide (HAs/ZnO) nanoparticles have been designed and synthetized and tested in the photodegradation of linear lowdensity polyethylene (LLDPE) and polylactic acid (PLA) films under UVA/light irradiation at different reaction times and experimental conditions (see **Chapter 3**, **Paragraph 5**).

# 2. Sustainable hybrid biopolymeric materials for food packaging applications

#### 2.1 Biopolymers Based Food Packaging Materials

Plastic consumption far exceeds recycling rates, therefore huge quantities of plastic waste are dumped into landfills and oceans, causing serious environmental concerns (Jogi and Bhat, 2020; Rech et al., 2020). Thus, in recent years, academic and industrial researchers have focused their efforts on the development of sustainable food packaging materials to reduce the use of conventional plastics while maintaining food quality.

To this purpose, the use of biopolymers in packaging has increased significantly in recent years due to their sustainable feedstock, biodegradability, and similar processing features to existing thermoplastics and to the non-biodegradable petroleum-based plastics (Braunegg et al., 1998). Biopolymers are a category of materials with a wide range of properties and applications. They include polymers with a "bio-based" origin, polymers that are "biodegradable," and polymers that combine both properties. Bio-based polymers are any polymer derived from bioavailable renewable resources, including both naturally occurring polymers and synthetic polymers derived from biological monomers (Ashter, 2016; Tharanathan, 2003).

Nature available polymers also include biomacromolecules, which are high molecular weight molecules produced by living organisms and plants. When exposed to the enzymatic action of microorganisms, carbon dioxide (aerobic process), methane (anaerobic process), water (aerobic and anaerobic processes), inorganic compounds, and biomass, the physical and chemical properties of biodegradable polymers deteriorate and completely degrade (Coelho et al., 2021; Ejaz et al., 2018; Wu et al., 2019).

Among biopolymers, the best known are the polylactic acid (PLA) and polyhydroxyalkanoates (PHAs), although the use of the former is limited due to its poor mechanical and thermal properties, as well as low ductility and bad oxygen barrier properties (Boufarguine et al., 2013).

Polyhydroxyalkanoates (PHAs), bio-based and biodegradable aliphatic polyesters synthesized by a wide variety of Gram-negative (G-) and Gram-positive (G+) microorganisms, on the other hand, have a high potential to replace polyolefins in packaging applications (Mutlu et al., 2018; Torres-Giner et al., 2018).

PHAs are classified according to the number of carbons in their repeating units, such as short-chain-length PHAs (scl-PHAs) with 3 to 5 carbon atoms (e.g., poly-3hydroxybutyrate (PHB) and poly-3-hydroxybutyrateco-3-hydroxyvalerate (PHBV)); medium-chain-length PHAs (mcl-PHAs) with 6 to 14 carbon atoms (e.g., poly-3hydroxyhexanoate (PHHx) and poly-3-hydroxyoctanoate (PHO)); and long-chainlength PHAs (lcl-PHAs) with more than 14 carbon atoms (Poltronieri and Kumar, 2017). The resulting polymers have suitable properties, including biodegradability, thermoplasticity, resistance to UV degradation, and water resistance and mechanical strength comparable to polypropylene and polystyrene (Reddy et al., 2003). However, because of the high costs of fermentation and downstream processes, their production is expensive. The use of industrial byproducts and waste, as well as mixed microbial cultures, represents a viable option for lowering PHA production costs (Choi and Lee, 1997).

Among PHAs, poly(3-hydroxybutyrate) (PHB) is the most widely studied and the first identified bacterial member of this family. Dye to its high crystallinity (>50%), this isotactic homopolyester has a high melting temperature ( $T_m$ ) and good stiffness. Nevertheless, the use of this biopolymer is limited because of its low impact strength and thermal stability. To address these issues, its copolymers, including those made with 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) to produce poly(3-

hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and poly(3HB-co-4hydroxybutyrate) (P(3HB-co-4HB) (Díez-Pascual and Diez-Vicente, 2014; Torres-Giner et al., 2016), can overcome these limitations, broadening its processing window. Indeed, PHBV is a suitable candidate for use in the packaging of films, or paper coatings (Khosravi-Darani and Bucci, 2015; McChalicher and Srienc, 2007; Yeo et al., 2018). To that end, various studied considered the use of this biopolymer as a sustainable, renewable, and biodegradable packaging material with high water vapor barrier properties (Kulkarni et al., 2011).

Furthermore, the incorporation of antimicrobial and/or antioxidant substances into a PHA-based packaging material can result in a high interest in improving both food protection and shelf life during storage (Requena et al., 2017; Torres-Giner et al., 2018).

#### 2.2 Active food packaging

Traditional food packaging was originally designed to contain food and protect it from external conditions (such as moisture, oxygen, dust, light, microorganisms, and mechanical forces) (C. Zhang et al., 2020). Active food packaging has grown in recent years as a successful method of extending food shelf life and improving consumer safety (Rech et al., 2020). Indeed, the use of an active compound may contribute to the development of active packaging that extends product shelf life through a mechanism that delays or inhibits the microbial, enzymatic, and oxidative reactions that are the primary causes of food deterioration (Coelho et al., 2021; Ejaz et al., 2018; Wu et al., 2019).

Active packaging plays a key role in quality and food preservation during the distribution process by protecting food products from environmental conditions and mechanical forces provoking contamination and deterioration and providing a significant contribution to sustainable goals, by reducing food wastes. Materials to reach the scope is intended to contain active compounds capable of releasing and

absorbing substances into or from the environment or packaged food (Ozdemir and Floros, 2004). Indeed, the most researched active food packaging types are those that contain antimicrobials and antioxidants, since they can preserve food against biocide contamination and oxidative degradation, which are the main causes for its deterioration. To functionalize packaging materials, various active compounds have been tested against a wide range of foodborne associated organisms (Appendini and Hotchkiss, 2002). Despite their efficacy most of them rises serious concerns related to their toxicity and often reduced recycling rates, which consequently increase the amount of plastic waste. Therefore, replacing synthetic chemicals with nature available compounds is highly recommended to avoid safety and environmental issues (Istiqomah et al., 2022).

#### 2.3 Antimicrobial packaging

Foodborne microorganisms are the most common cause of food spoilage. The primary functions of antimicrobial packaging are to reduce, inhibit, or delay the growth of foodborne bacteria. Antimicrobial substances are classified as organic or inorganic (Sharma et al., 2020), with both showing high activity against Gramnegative and Gram-positive bacteria. Incorporating these bioactive substances into polymer matrixes improves their stability and antimicrobial activity. Polymeric antimicrobial materials are classified according to their mode of action and include contact-active antimicrobial polymers with cationic charges that directly inhibit bacterial growth through interactions with negatively charged bacterial membranes. Physical interaction or chemical bonding is used to incorporate an active molecule or ion into the polymer matrix (Chen et al., 2017).

The mechanism of action of an antimicrobial agent depends on its nature and the interaction with bacterial cell, which can result in membrane damage or cell wall synthesis inhibition, cell membrane function inhibition, protein synthesis inhibition,

nucleic acids (DNA and RNA) inhibition, and suppression of other metabolic processes (Ren et al., 2017).

Inorganic nanoparticles with sizes ranging from 1 to 100 nm have found widespread use in biomedicine, biotechnology, chemical sensing, agriculture, and active food packaging.

Metal oxide nanoparticles, including silver (Ag), copper (Cu), gold (Au), titanium oxide (TiO<sub>2</sub>), zinc oxide (ZnO), silicon dioxide (SiO<sub>2</sub>), magnesium oxide (MgO), and calcium oxide (CaO) are the most common antibacterial inorganic/metallic nanoparticles (**Figure 5**).



Figure 5. Film containing antimicrobial nanoparticles (Bahrami et al., 2020)

The Food and Drug Administration (FDA) has classified some of them as Generally Recognized as Safe (GRAS) (Bradley et al., 2011; Emamifar et al., 2011; Espitia et al., 2012).

Mechanochemical processing, sol-gel or solvothermal methods, spray pyrolysis, green chemistry routes, photochemical methods, chemical reduction, electron irradiation, gamma irradiation, and laser ablation can all be used to synthesize these nanoparticles (Mahmud et al., 2006; Mavaei et al., 2020; Turner et al., 2010).

They can have a large surface area, good colloidal and mechanical stability, and high temperature and pressure resistance. Furthermore, some of them are biocompatible, low in cost and toxicity, and even contain essential minerals for human health (Jones et al., 2008; Moezzi et al., 2012; Vaid et al., 2020). The antimicrobial effects of Ag, Cu, Au, and ZnO are accomplished through various mechanisms of action, such as ion release and interaction with the bacterial cell wall and membrane, or through other mechanisms, such as reactive oxygen species (ROS) production, which leads to bacterial death. Besides, nanoparticles could confer an improvement in tensile strength, gas, and UV barrier material properties.

Among active and natural substances, polyphenol compounds (i.e. flavonoids, tannins, lignins) present promising antimicrobial and antioxidant performance thanks to their ability to generate or scavenge ROS species. However, they have a poor long-term stability, as they are affected by pH variation, presence of metal ions, light, temperature, oxygen, and enzymatic activities (Bąkowska et al., 2003). Moreover, due to low water solubility, they often present a poor bioavailability (Dube et al., 2010) and they are unstable in alkaline conditions. The combination between these moieties and either organic or inorganic components (i.e. inorganic nanostructures or polymer matrix) can allow to overcome these limitations, even boosting the intrinsic functional properties of the organic phase.

#### 2.4 Electrospinning for Food Packaging

Electrospinning is a simple and versatile technique for producing ultrathin fibers from a wide range of polymers solutions, allowing for improved material and structural design thanks to its ability to generate nano and micro-scale structures with variable fiber diameters and porosity (Bognitzki et al., 2001). The electrospinning equipment comprises a capillary tube with a needle that contains polymer solution, a power voltage supply that provides electrical forces that exceed the surface tension of viscoelastic polymer solutions, and a collector (e.g., copper plate), as shown in **Figure 6.** 



Figure 6. Scheme of a laboratory scale electrospinning equipment.

An electric field is applied to the needle tip where it is possible to observe the drop of the polymer. This latter is subjected to the repulsive force between its surface charges and the Coulomb force exerted by the external electric field. When the electric field overcomes the surface tension of the drop, the latter assumes a conical shape called Taylor's cone and there is the expulsion of the liquid jet which is conveyed to the collector (Wen et al., 2017).

The main parameters that can be controlled during the electrospinning process are the polymer molecular weight, the solvent type, and the polymer solution properties (viscosity, conductivity, dielectric constant, and surface tension), as well as process parameters including electric potential, flow rate, distance between the syringe needle and the collector plate, and environmental conditions (e.g., temperature, humidity, and air flow). Furthermore, even the polymer solution and its concentration affect the fiber morphology, the diameter distribution and the overall structures of obtainet mats (Chronakis, 2005). Produced nanofibers exhibit structural and functional properties, such as submicron to nanoscale diameters, high surface to volume ratio, suitable porosity, and uniform fiber diameters.

Furthermore, since electrospinning process occurs at room temperature it can leads to an efficient encapsulation of active compounds, avoiding antimicrobial and antioxidant activity loss. All these advantages make electrospinning a potential and promising technique for active food packaging design (Bognitzki et al., 2001; Cerqueira et al., 2016; Pérez-Masiá et al., 2014; Torres-Giner et al., 2016).

Once obtained electrospun mats, it is possible to apply a thermal the annealing posttreatment below the biopolymer's melting temperature, finally obtaining continous and highly transparent films of more interests in medical, or food packaging fields.

## 2.5 Electrospun hybrid nanostructured humic acids-biopolymer films for active packaging

Humic substances HS derived from biowaste oxidative processes exhibit intrinsic antioxidant and antimicrobial features which can be significantly boosted by molecular combination with an inorganic nanostructured phase, following the ceramic templated strategy (Giulio Pota et al., 2020; Venezia et al., 2022a, 2022b; Vitiello et al., 2021).

In this thesis, this approach has been integrated with electrospinning technology to design bioactive nanocomposite films for food packaging applications made of electrospun biodegradable and bioderived PHBV in the presence of hybrid nanostructured HS based materials based on the molecular combination between the bioderived HS and  $TiO_2$  nanostructures (**Figure 7**, see **Chapter 4**, **Paragraph 1**).



Figure 7. Electrospun fibers based on nanostructured HA-materials

#### 3. Design of hybrid multifunctional 3D hydrogels

Hydrogels are materials that stand out for their swelling properties, morphology and resistance to compression and can be classified in chemical or physical, depending on the nature of the interactions that form the three-dimensional network. Chemical hydrogels are made up of covalent bonds and this gives to the final product more resistant mechanical properties. Instead, physical hydrogels are made up of weaker interaction (i.e. hydrogen bonds).

#### 3.1 Polyphenols-like gels

Novel bioinspired approaches in biocompatible and biodegradable hydrogel fabrication involved in the use natural polyphenols-like molecules, such as tannic acid (TA), lignin (LIG), gallic acid (GA), tea polyphenols (TP), quercetin (QN), curcumin (CUR), caffeic acid (CA), etc (Xu et al., 2022). Natural polyphenols have been considered suitable candidates for the preparation of biomaterials with a wide range of fascinating functions (e.g., antioxidant, antimicrobial, antiviral features). They can be easily conjugated with many different kinds of building blocks to develop multifunctional and different dimensional composite materials, which broadens the multiple applications to obtain three-dimensional (3D) polyphenolic particles/hydrogels, owing to rich dynamic covalent interactions and strong noncovalent interactions between catechol/pyrogallol groups and other structures (Xu et al., 2022). Indeed, polyphenols had multibinding sites, and were capable of complexing or cross-linking with other hydrophilic macromolecules to form the hydrogels, thus beneficially integrating the advantages of polyphenols and hydrogels, as well as showing great vitality in the field of environmental applications.

Among these nature polymers, making lignin into hydrogels has great potential for upgrading lignin into functional materials (Meng et al., 2019; Passauer et al., 2015;

Xue et al., 2015). Lignin helps the cell wall and internal fibers to transport water and nutrients, protects them from microbial attack and provides mechanical support to the plant. This biopolymer is well-known for being inexpensive and sustainable, for its antioxidant, non-toxicity, antifungal and antibacterial properties. Its chemical structure contains many functional hydrophilic and active groups, such as hydroxyls, carbonyls and methoxyls, which provides a great potential to be employed in the synthesis of biodegradable hydrogels and materials that are recognized for their applicability in soil and water treatment, or in agriculture. Besides, the abundant polar sites on lignin's backbone can be employed for physical crosslinking of hydrophilic polymers by H-bonding. Rico-Garcia et al., 2020 studied the main methods for the preparation of lignin-based 3D hydrogels based on the chemical and/or physical interaction with polymers.

Ionotropic gelation was observed between alkaline lignin and chitosan leading to physical hydrogels by simple mixing of both polymers. This gelation was a consequence of the electrostatic interactions between phenoxide anions of lignin and the protonated cationic amine groups of chitosan. Moreover, the hydroxyl groups of lignin enable chemical reaction via hydroxymethylation and epoxidation by reaction with formaldehyde and epichlorohydrin, leading to crosslinked structures. Indeed, when lignin reacts with formaldehyde its hydromethylation takes place and introduced -OH groups, after reaction with phenol moieties led to lignin based polymerized networks. Hydroxymethyl groups are introduced in the lignin's reactive positions through the electrophilic substitution of formaldehyde. Therefore, ligninbased hydrogels could be considered as emerging materials with potential applications as drug delivery systems for agriculture or medicine, in water remediation applications and in heavy metal ions chelating activity. In fact, the phenolic rings present in the lignin derivatives could enhance the retention of dyes and other organic pollutants in water, thanks to  $\pi$ -  $\pi$  reaction with aromatic pollutants.

#### 3.2 Gelatin-based hydrogels

One of the most used polymers in the preparation of hydrogels is gelatin, a natural peptide macromolecule derived from partial hydrolysis of collagen (Poppe, 1992) in which the crystalline structure is due to the presence of both ordered triple and  $\alpha$ -helices domains. It is suitable for a wide range of applications in the food, biomedical, and pharmaceutical fields thanks to its low cost, biocompatibility, biodegradability, widespread availability, and low antigenicity (Rubini et al., 2020). Gelatin properties can be improved by the addition of co-solutes through chemical of physical interactions, thus increasing mechanical and rheological strength or conferring unusual properties such as water resistance or thermal stability (Campiglio et al., 2019).

Several studies deal with the interaction behavior of films of gelatin and other polymers, such as carboxymethylcellulose, CMC. The results evidenced the decrease of framework in triple helix of gelatin in the presence of polysaccharide, due to electrostatic interaction between gelatin and CMC. However, the addition of crosslinking agent, such as glutaraldehyde (GTA) to gelatin/CMC has been established to produce covalent three-dimensional networks. The use of GTA allows to modulate the physico-chemical properties of gelatin since it can act as bridge between CMC and gelatin, since it can bind to CMC forming acetals and to the gelatin through Schiff base formation, to obtain a 3D gel with a wide range of possible biomedical and environmental applications (Bigi et al., 2020, Asma et al.,2014).

Despite the high efficacy of commonly used aldehyde cross-linkers (formaldehyde and glutaraldehyde), their toxicity poses health and safety concerns and severely limits their use, particularly in the biomedical and food industries (Rubini et al., 2020): As a result, there is growing interest in developing more sustainable and safe cross-linking options based on natural moieties. Polyphenols, for example, are known to interact with proteins via physical and chemical conjugation, providing increased thermal stability as well as antioxidant and antimicrobial properties (Quan et al., 2019).

#### 3.3 Humic acids-based 3D hydrogels

Turning HA incoherent powders into self-standing and mechanically stable 3D is a promising strategy to scale up the range of HA possible applications to industrial plants. 3D porous architectures (hydrogel, aerogel) can be obtained by gelification with natural organic molecules (i.e. gelatin from porcine skin or chitosan) and/or by chemical cross-linking with natural phenolic compounds or through EDC chemistry. Among bioavailable compounds, HA, like polyphenols, are abundant in nature, but they are also more stable to degradation (Buurman et al., 2002), since they underwent to partial oxidation processes and less expensive than more common polysaccharides, such as pectin and agar, which are also widely used as additives for gelatin solutions. Furthermore, these supramolecular superstructures are greatly influenced not only by the chemistry of their surroundings, such as pH, co-solutes, and biological molecules, and they behave as dynamic systems, but they also undergo self-restructuring in water (Venezia et al., 2022a). As a result, a focused investigation is required to provide a concrete opportunity for HA technological application using a waste to wealth approach.

Following this goal, our research has focused on understanding the gel behavior, physicochemical properties, and functional properties of gelatin modified with HA (**Figure 8**, see **Chapter 4**, **Paragraph 2**).



Figure 8. Humic acids-based 3D hydrogels

### 4. Design of silicon-modified epoxy resin containing functional bio-waste flame retardant additives for epoxybased systems

Epoxy resins are among the polymer systems that are used to obtain a wide range of high-performance products, such as linings and components in the aircraft industry, where, in addition to the usual requirements, stringent fire safety standards and regulations must be considered (Markwart et al., 2019).

One of the most promising approaches for moving toward a circular economy concept is the reuse of biowaste materials as functional flame retardant additives for polymer-based systems. (Glasing et al., 2016; Xu et al., 2019).

Halogen-free additives, such as ammonium polyphosphate (APP), as well as biobased and biomass-based raw materials, such as chitosan, lignin, cellulose and cyclodextrins, have been researched in this area as more environmentally friendly alternatives to halogen-based flame retardants (**Figure 9**) (Markwart et al., 2019; Vahabi et al., 2021).



Figure 9. Bio-based flame retardant systems (Vahabi et al., 2021)

However, to get satisfying fire performances, a noticeable amount of these chemicals must be added to the epoxy matrix.

#### 4.1 Humic acids as biowaste flame retardant additive

Among biowastes, humic acids (HA) have a skeleton of aliphatic or aromatic units and exhibit amphiphilic behavior. These moieties are supramolecular architectures stabilized in by weak hydrophobic, hydrogen, and metal-bridged electrostatic bonds, able change their functionalities depending on the chemical environment (Matykiewicz et al., 2017). Because of the presence of oxygen-containing functional groups, a carbon-enriched chemical composition, and supramolecular architectures, HA can forming good physical interactions with the polymer matrix and promote charring phenomenon during the epoxy degradation process when exposed to a flame or an irradiative heat flux.

As a result, HA can provide flame retardant action to epoxy resins (Lee et al., 2019), even if this kind of application of HA in polymer systems has received little attention until now. Although Liu et al. (Liu et al., 2016) chelated HA with four different metal ions and incorporated it into a modified DGEBA resin to improve its flame retardance, no self-extinguishing materials based on HA have been developed thus far. Furthermore, a comprehensive characterization of the mechanical and fire behavior of HA-containing epoxy systems remains lacking, which is critical for any technological exploitation.

To that end, the reaction between DGEBA and (3-aminopropyl)-triethoxysilane can produce hybrid silicon-containing epoxy moieties capable of improving the interphase between matrix and filler due to their ability to interact with polar and apolar additive additives, allowing for fine dispersion (Liu et al., 2016). Given their amphiphilic nature, HA are expected to achieve very good distribution into siliconmodified epoxy resins and to exhibit improved charring behavior and fire performance. In this thesis (see **Chapter 4, Paragraph 3**), a thorough examination of the role of HA as a flame retardant for epoxy resin was conducted.

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### **Chapter 3**

#### Contents

1. Tuning functional behaviour of Humic Acids through interactions with Stöber
silica nanoparticles
1.1 Abstract
1.2 Introduction
1.3 Results and discussion
1.4 Conclusions
1.5 References
2. A study on structural evolution of hybrid Humic Acids-SiO <sub>2</sub> nanostructures in
pure water: effects on physico-chemical and functional properties 107
2.1 Abstract
2.2 Introduction
2.3 Results and discussion
2.3.1 Antioxidant activity: FOX and DPPH assays 114
2.3.2 Determination of NPs Yield and HA binding efficacy 116
2.3.3 NMR analysis116
2.3.4 SEM Analysis
2.3.5 EPR analysis
2.3.6 TGA Analysis
2.3.7 DSC Analysis 124
2.3.8 FT-IR analysis
2.3.9 Zeta potential measurements

2.3.10 Structural evolution of HA_SiO <sub>2</sub> NPs in water: main features and
effects on chemical and functional properties
2.4 Conclusions
2.5 Supplementary information
2.5.1 Antioxidant activity: Fox and DPPH assays
2.5.2 Determination of NPs Yield and HA binding efficacy 138
2.5.3 NMR Analysis 139
2.5.4 TGA Analysis
2.5.5 FT-IR Analysis
2.6 References
3. Hybrid humic acid/titanium dioxide nanomaterials as highly effective
antimicrobial agents against gram(-) pathogens and antibiotic contaminants in
wastewater
3.1 Abstract
3.2 Introduction 152
3.3 Results and discussion 157
3.3.1 Physicochemical features of the TiO <sub>2</sub> /HA-NDL nanohybrids157
3.3.2 Antibacterial activity of the TiO <sub>2</sub> /HA-NDL nanohybrids 169
3.3.3 Antibiotic sequestering activity
3.4 Conclusions
3.4 Conclusions1763.5 Supporting information177

4.	Exploiting bioderived humic acids: a molecular combination v	with	ZnO
na	noparticles leads to nanostructured hybrid interfaces with enhanced p	ro-ox	idant
an	d antibacterial activity.		. 187
	4.1 Abstract		. 188
	4.2 Introduction		. 190
	4.3 Results and Discussion		. 193
	4.3.1 Morphology and size distribution in aqueous medium		. 193
	4.3.2 Chemical and structural properties	•••••	. 195
	4.3.3 Paramagnetic properties		. 199
	4.3.4 Redox activity		. 201
	4.3.5 Antimicrobial activity		. 206
	4.4 Conclusions		. 209
	4.5 Supporting Information		. 210
	4.6 Acknowledgements		. 210
	4.7 References		. 211
5.	Eco-sustainable design of Humic Acids-doped ZnO nanoparticles for	UVA	/light
ph	otocatalytic degradation of LLDPE and PLA plastics		. 219
	5.1 Abstract		. 220
	5.2 Introduction		. 222
	5.3 Results and Discussion		. 225
	5.3.1 Morphological, dimensional, and surface properties		. 225
	5.3.2 Chemical and structural properties		. 227

5.3.3 Paramagnetic properties and ROS-generating activity	229
5.3.4 Photodegradation of LLDPE films	232
5.3.5 Photodegradation of PLA films	239
5.4 Conclusions	243
5.5 Acknowledgements	244
5.6 References	244

# **1.** Tuning functional behaviour of Humic Acids through interactions with Stöber silica nanoparticles

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#### **1.1 Abstract**

Humic acids (HA) exhibit multifunctional fascinating features, yet degradation phenomena as well as poor stability in aqueous environments strongly limit their use. Inorganic nanoparticles are emerging as powerful interface for the development of robust HA biohybrid materials with enhanced chemical stability and tunable properties. Hybrid organic-inorganic SiO<sub>2</sub>/HA nanostructures were synthesized via in-situ sol-gel route exploiting both physical entrapment and chemical coupling. The latter was achieved through amide bond formation between carboxyl groups of HA and amino group of 3-aminopropyltriethoxysilane (APTS). as confirmed by FTIR and NMR spectroscopy. Monodisperse hybrid nanoparticles about 90 nm in diameter were obtained in both cases, yet Electron Paramagnetic Resonance spectroscopy highlighted different supramolecular organization of HA. The altered HA conformation was reflected into a different antioxidant property of the conjugated nanoparticles that resulted however higher than for pure HA. Our findings proved the key role of both components in defining the morphology of the final system, as well as the efficacy of ceramic component in templating the HA supramolecular organization and consequently tuning their functional features, thus defining a green strategy for bio-waste valorization.



#### **1.2Introduction**

Biowaste management and valorization is one of the most challenging issues for sustainable development, due to the enormous amount of bioresidues resulting by biogenic and non-biogenic transformations [1,2]. The great abundance and chemical richness bio-waste make their valorization one of the most promising approaches to move toward circular economy. In this field, Nanotechnologies hold a key role in defining processes and innovative solutions to allow conversion of residues into added-value nanostructured materials, with relevant properties for a huge range of applications, from medicine, to sensing, packaging, environmental protection, electronics [1,2]. Among bio-wastes, Humic Acids (HA) are the alkalisoluble fraction of natural organic matter consisting in a multitude of heterogeneous organic molecules surviving the biological and chemical degradation of both vegetal and animal biomasses [3,4]. The associations of the amphiphilic HA components are stabilized by weak hydrophobic, hydrogen and metal-bridged electrostatic bonds in supramolecular architectures [5]. HA are considered as a promising and inexpensive source for high value products and novel materials through green chemistry approach [1,6]. The chemical heterogeneity and the metastable conformation of humic matter, as well as their different reactive functional groups are responsible for a wide range of useful properties, including the adsorbing capacity towards metals and organic pollutants [7-13]. Moreover, they exhibit a strong attitude toward Reactive Oxygen Species (ROS) quenching, due to their intrinsic paramagnetic properties, resulting as effective antioxidant agents. The red-ox behavior of humic substances is strongly related to the phenol/quinone moieties in their complex supramolecular structures [14,15]. However, their rapid conformational dynamics and, hence, fast reactivity in water environments make humic substances poorly useful for several applications in aqueous media [7,16].

A suitable solution may rely on the immobilization/encapsulation of HA onto an organic either inorganic support, providing for mechanical stability and steady reactivity. The embedding matrices act as a physical barrier to oxygen and other moieties thus reducing HA oxidation processes and prolonging their shelf-life [17]. Inorganic nanoparticles are emerging as powerful interface for the development of robust biohybrid materials with enhanced chemical stability and tunable properties [18]. In fact, the combination of HA with magnetic nanoparticles allows their easy reuse and recycle and provides effective environmental solutions to heavy metals remediation from water [19].

Recent studies proved that the inorganic phase was able to tune biopolymeric supramolecular structures, boosting their intrinsic properties and tuning their overall biological activity [20-23]. In this route, silica has been identified as an ideal support, due to its strong hydrophilicity, acknowledged biocompatibility, as well as tunable size, shape, porosity, and surface chemistry [21,24]. Immobilization of HA onto silica appears as a valid approach to exploit the large reactive potential of these moieties for several technological solutions ranging from separation chromatography [25], water remediation [9,26-28], and antioxidant products [14,15].

Two different strategies have been usually explored to combine HA with a silica support. Physical entrapment of humic molecules into silica matrices provides a simple but effective route to obtain HA-SiO<sub>2</sub> composites with high sorption capacity towards heavy metals cations [26] as well as organic hydrophobic contaminants [27]. Alternatively, chemical immobilization has been exploited to bind humic acids onto silica surface [28-31].

Generally, the first step for the immobilization is the surface functionalization of the silica support with amino groups by silanization with 3aminopropyltriethoxysilane. Binding can be carried out by electrostatic bonds

(silylation) or by chemical coupling, thereby unavoidably involving the functional groups responsible for the HA multiple reactivity [31-33]. Therefore, both physical and chemical methods can influence the conformation of the HA supramolecular structure, thus defining the availability of reactive centers and ultimately determining the HA reactivity.

In this scenario, this study aims at elucidating the influence of physical entrapment and chemical coupling strategies onto the physical-chemical properties of HA-SiO2 nanomaterials.

To this purpose two-types of silica-HA hybrid nanoparticles were designed and prepared through in-situ one-pot sol-gel route based on the modified Stöber method. Notably, the former (SiO<sub>2</sub>/HA\_p) was obtained through hydrolysis and polycondensation of tetra propyl orthosilicate in the presence of HA in the reacting mixture. The latter (SiO<sub>2</sub>/HA\_c) was prepared exploiting 3 aminopropyltriethoxysilane as coupling agent for HA, to allow chemical immobilization.

Both synthesized samples were submitted to an in-depth physical-chemical characterization, integrating different techniques, such as Scanning Electron Microscopy (SEM), to assess morphology and average size of obtained nanoparticles, BET porosimetry to evaluate specific surface area and pore size distribution. Furthermore, Thermogravimetric Analysis (TGA) provided the amount of organic content in nanohybrids, while chemical immobilization was investigated through Fourier Transform Infrared Spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR).

Electron Paramagnetic Resonance (EPR) spectroscopy gave information about the presence of HA intrinsic radical species and their distribution within the nanostructures. The antioxidant potential of hybrid nanostructures was finally tested through the ferrous oxidation-xylenol orange FOX assay. Our results proved

the key role of both components in defining the morphology of the final hybrid system, as well as the efficacy of ceramic component in templating the supramolecular organization of HA, consequently driving functional properties and setting a green strategy for biowaste valorization.

#### 1.3 Results and discussion

Hybrid HA/Silica nanoparticles were obtained by a sol-gel methodology which relies on the nanoparticles formation in the presence of HA (in-situ route). Two synthetic approaches were followed: the former based on a physical entrapment of HA superstructures and the latter based on a chemical coupling between the organic and inorganic phase.

Morphological investigation of all synthesized nanoparticles was performed through Scanning Electron Microscopy (SEM) reported in **Figure 1**.



*Figure 1.* SEM micrographs of SiO<sub>2</sub>\_p (A) and SiO<sub>2</sub>\_c (B) nanoparticles and SiO<sub>2</sub>/HA\_p (C) and SiO<sub>2</sub>/HA\_c (D) hybrid nanoparticles.

SEM images of bare  $SiO_2_p$  sample (**Figure 1A**) revealed nanoparticles with spherical shape and a narrow size distribution of about 400 nm in diameter. A bimodal size distribution was clearly observed in bare  $SiO_2_c$  sample (**Figure 1B**) made of two nanoparticles populations: the former composed by few large nanoparticles (400 nm in diameter) and the latter made of a significant number of small nanoparticles (100 nm in diameter).  $SiO_2_p$  sample was prepared following a modified Stöber method [34]. As expected, the use of only one alkoxide combined

to the choice of appropriate amounts of all reagents, allow for deep control over the nucleation and growth processes, leading to spherical and highly monodispersed nanoparticles. Widely recognised mechanism for Stöber silica NPs formation is reported in **Scheme 1** (route A) [34-37].



Scheme 1. Formation mechanism of  $SiO_2_p(A)$  and  $SiO_2_c(B)NPs$ 

First, hydrated monomers obtained from hydrolysis of precursors undergo condensation reactions, forming silicate polymer chains and then small nuclei. These grow through silica monomers and polymers bonding, forming primary particles, usually 5-7 nm in mean diameter [38,39]. Then, primary particles aggregate producing larger SiO<sub>2</sub> particles that grow up to a stationary critical size, able to generate a double electrical layer that hinders further aggregation of primary particles onto their surface. Starting from one monomeric precursor, such as TPOS, brings a uniform size distribution of nuclei and subsequently of primary particles,

finally providing narrow size distribution of nanoparticles. This mechanism leads to monodisperse particles [35,36,40,41].

Two different pathways are expected to concur to formation of  $SiO_2_c$  nanoparticles. On one hand, pre-hydrolysis of APTS is supposed to form a certain number on nuclei, that should act as binding sites for hydrolysed TPOS moieties and lead to fast formation of big nanoparticles. On the other hand, residual hydrolysed TPOS, not involved in the previous process, must give rise to a different population of nuclei and subsequently of primary particles, which are thermodynamically more favoured to aggregate into new secondary particles, rather than join the surface of big nanoparticles. This process should give rise to a second wide population of smaller nanoparticles than  $SiO_2_p$  samples, as expected for a lower concentration of TPOS. Overall process results in bimodal particles size distribution as revealed from SEM micrographs (**Figure 1B**) [35].

SEM micrographs of both SiO<sub>2</sub>/HA hybrid nanoparticles (**Figure 1C and 1D**) revealed a narrow size distribution of pseudospherical particles of about 90 nm is mean diameter, lower than bare SiO<sub>2</sub> (SiO<sub>2</sub>\_p, SiO<sub>2</sub>\_c). These results were confirmed by TEM micrographs reported in **Figure 2**, also revealing an intimate mixing of both components, as no differences in contrast were observed. Furthermore, at high magnification the hybrid nanoparticles clearly showed a cluster architecture consisting of smaller primary particles in accordance with proposed formation mechanism of particles growth through aggregation [36].

These clusters could be also formed through the previously reported intermolecular interactions among HA molecules [42].



Figure 2. TEM micrographs at different magnifications of SiO<sub>2</sub>/HA\_p (A,C) and SiO<sub>2</sub>/HA\_c (B,D).

Nanoparticles size of both hybrid samples was also confirmed by DLS investigations. Figure 3 reported size distribution of both  $SiO_2/HA_p$  and  $SiO_2/HA_c$  nanoparticles.



Figure 3. DLS analysis reported as number distribution of SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c NPs

The hydrodynamic diameter of SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c NPs were of about 97 nm (PDI = 0.155) and 105 nm (PDI = 0.183) respectively and appeared slightly larger than the dry particle diameters observed through TEM analysis. Furthermore, the  $\zeta$ -potential values were -36 ±8 mV and -40±7 mV, respectively, showing no significant surface charge differences between the two systems. Thus, the electrostatic repulsion kept HA functionalized nanoparticles stable dispersed in water.

Both synthetic routes were based on the formation of silica particles in a HA containing solution. Both morphological and DLS investigations revealed that the HA phase actively participated in the nanoparticles formation controlling their growth. Notably, HA superstructures limited diffusion of both silica oligomers and nanoparticles in solution. Furthermore, adsorption of HA moieties onto nanoparticles results in a high negatively charged surface which can hamper their aggregation, due to electrostatic repulsions [43]. Overall, these phenomena resulted into a smaller size of hybrid nanoparticles than bare SiO<sub>2</sub>\_p and SiO<sub>2</sub>\_c samples.

FT-IR investigation of both SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c hybrid NPs, reported in **Figure 4**, revealed the main characteristic bands of silica phases. The band at  $\sim$ 3500 cm<sup>-1</sup> was assigned to OH stretching vibration of surface silanol groups and absorbed water. The bands at 1100 cm<sup>-1</sup> were attributed to Si–O–Si stretching vibration modes in SiO<sub>4</sub> units, while the band at 950 cm<sup>-1</sup> was related to non-bridging Si–O stretching vibration. The bands at 800 and 470 cm<sup>-1</sup> were attributed to Si–O–Si stretching vibration between two adjacent tetrahedral and Si–O–Si bending, respectively. The assignments were reported in **Table 1** [44].



Figure 4. FT-IR spectra of SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c hybrid NPs

Peak Position (cm <sup>-1</sup> )	Vibrational Mode
~1100	Si-O-Si stretching
~950	Si-O-Si bond between two adjacent tetrahedra
~800	Si-O-Si stretching
~470	Si-O-Si bending

Table 1. Meaningful FT-IR bands of SiO<sub>2</sub>/HA hybrids NPs

To confirm the presence of HA component in the hybrid system, Thermogravimetric Analysis (TGA) measurements were carried out and the thermal behavior of the SiO<sub>2</sub>/HA samples was compared with those of pure HA and bare SiO<sub>2</sub> nanoparticles. The resulting TGA curves are reported in **Figure 5**. TG curve of pure HA showed a first weight loss below 150 °C (10wt%) related to the release physically adsorbed water and above 250 °C attributed of oxidative decomposition of both labile and recalcitrant components [28].

Furthermore, a total mass loss of approximately 80wt% was evaluated.



Figure 5. Thermogravimetric curves of pure HA, silica NPs and SiO<sub>2</sub>/HA hybrid NPs.

The TG curve of both SiO<sub>2</sub>\_c and SiO<sub>2</sub>\_p samples showed a low temperature weight loss (about 7%) in the range 25-120 °C, related to removal of physically adsorbed water. The second weight loss, in the range 250-600 °C, was attributed to the degradation of alkyl chains, more pronounced in the SiO<sub>2</sub>\_c sample with non-hydrolysable aminopropyl group, and dehydroxylation of Si-OH residual groups, forming Si-O-Si groups.

The TG curves of both SiO<sub>2</sub>/HA\_p an SiO<sub>2</sub>/HA\_c showed an additional visible weight loss in the range 200-580 °C, related to the decomposition of HA. A different amount of HA was estimated in the hybrid NPs: 15wt% in the case of SiO<sub>2</sub>/HA\_p NPs and 30wt% of HA for SiO<sub>2</sub>/HA\_c NPs. This result suggests that a chemical coupling between the two phases allowed to obtain a greater amount of HA in the final system.

Furthermore, no differences in the thermal degradation behavior of HA were appreciated in both hybrid samples, since changes in neither starting degradation temperature nor inflection point were observed.

The porosity of the hybrid nanoparticles was assessed by means of N<sub>2</sub> adsorption/desorption measurements (data not shown). BET analysis revealed a very similar surface area for both bare SiO<sub>2</sub> NPs of about 20.10 m<sup>2</sup>/g. A slightly increase of surface area was observed in both SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c NPs:  $33.54 \text{ m}^2/\text{g}$  and  $50.48 \text{ m}^2/\text{g}$ , respectively. This was attributed to the presence of humic components which usually shows highly narrow microporosity [45,46].

To investigate chemical coupling between the two components, the HA\_APTS hybrid precursor, produced during the first step of the synthesis of SiO<sub>2</sub>/HA\_c sample, was analysed. FT-IR spectra of APTS, bare HA and HA\_APTS hybrid monomer were carried out and reported in **Figure 6**. The typical HA FT-IR spectrum (black curve) revealed a broad adsorption band in the region 3700-2500 cm<sup>-1</sup> attributed to the -OH stretching vibration, the bands in the range from 2800 to 3200 cm<sup>-1</sup> was attributed to C-H symmetric and asymmetric stretching vibrations of alkyl structures. Additional bands are also visible in the region 1580-1660 cm<sup>-1</sup>, related to C=C bond in aromatics and olefins as well as carboxyl C=O bond, ketone and quinone groups, whereas the band at 1514 cm<sup>-1</sup> was attributed to the ring vibrating modes of ortho-substituted aromatic compounds. The bands around 1280-1070 cm<sup>-1</sup> corresponded to stretching vibration of phenolic C-O and aliphatic OH. Finally, the bands in the region 1460-1390 cm<sup>-1</sup> corresponded to OH of phenols, COO- and -CH<sub>3</sub> bending vibration mode [40,47,48].



Figure 6. FT-IR spectra of APTS, bare HA and HA-APTS hybrid precursor

The FT-IR spectrum of HA-APTS hybrid precursor showed a marked decrease of the bands in the region between 1400 and 1650 cm<sup>-1</sup>, including COO<sup>-</sup> asymmetric stretching vibration modes [49-51]. Furthermore, the spectrum showed a shoulder at around 1560 cm<sup>-1</sup>, which can be assigned to out-of-phase combination mode of the N-H in plane bend and the C-N stretching vibration of Amide II band [52,53]. Finally, by comparing the FTIR spectra of bare HA and HA-APTS precursor, changes in the adsorption band of OH vibrations in the range of 3500 cm<sup>-1</sup> were clearly visible. The differences in this region were attributed to N-H stretching in amide groups [51,54] **Scheme 2**). Furthemore, the changes in the range of 3500 cm<sup>-1</sup> and weak bands in the 1400-1600 cm<sup>-1</sup> region, were also visible in the FTIR spectrum of SiO<sub>2</sub>/HA\_c sample (**Figure 4**), confirming the presence of HA into hybrid nanoparticles [55].



Scheme 2. Amide bond between HA and APTS by EDC activation

In order to further support FT\_IR results, NMR investigation was carried out. Figure 7 reported the <sup>13</sup>C CPMAS NMR spectra of pure HA (**Figure 7A**) and HA-APTS hybrid precursor (**Figure 7B**).



*Figure 7.* <sup>13</sup>*C*-*Cross Polarization Magic Angle Spinning NMR spectrum of HA (A),* <sup>13</sup>*C spectrum extrapolated from the HSQC-HRMAS spectrum of HA-APTS hybrid precursor(B)* 

The total area of the bare HA spectrum was considered as 100% and divided into six regions (i.e., 190–160, 160–145, 145–110, 110–60, 60–45, 45–0 ppm). The percentage of each resonant carbon nucleus was reported in **Table 2**.

Range (ppm)	Attribution	НА
0-45	Alkyl C	19.52
45-60	Methoxyl C	12.78
60-110	<i>O</i> -Alkyl C	55.00
110-145	Aryl C	12.08
145-160	<i>O</i> -aryl C	4.47
160-190	Carboxyl	8.23

**Table 2.** Relative C distribution (%) in chemical shift regions (ppm) in <sup>13</sup>C-CPMAS -NMR spectrum of HA isolated from mature manure compost made

In **Figure 7B** a reduction of the peak at 173 ppm, related to carboxyl groups region, was clearly visible, suggesting an involvement of these groups in the reaction with APTS. Comparison of the results obtained by <sup>13</sup>C CP/MAS spectrum and twodimensional  $^{1}\text{H}-^{13}\text{C}$  HSQC, reported in **Figure 8**, revealed the presence of a signal at 170/9 ppm, supporting the formation of an amide bond between the carboxyl groups of HA and amine groups of APTS, in accordance with FTIR results (**Figure 6**).



Figure 8. Two-dimensional <sup>1</sup>H-<sup>13</sup>C HSQC NMR spectrum ( $\delta C/\delta H$  0-220/0-12 ppm) of the HA-APTS hybrid precursor

EPR analysis of hybrid SiO<sub>2</sub>/HA nanoparticles was performed according to the procedure recently used in the characterization of melanin-like nanomaterials [20-22], to provide important information about the nature of radical species as well as the supramolecular properties of the humic acid moiety forming the hybrid SiO<sub>2</sub>-based material. EPR spectra of hybrid samples are reported in **Figure 9**, whereas the corresponding spectral parameters are summarized in **Table 3**. The pure HA was also analyzed as reference.



Figure 9. EPR spectra (panel A) and power saturation curves obtained by plotting the normalized amplitude  $(A/A_0)$  values versus the square root of microwave power intensities (P) of free radicals (panel B) in HA powder and SiO<sub>2</sub>/HA hybrid nanoparticles.

Sample	g-factor	ΔΒ	Spin $\times$ g <sup>-1</sup> $\times$ 10 <sup>17</sup>
	(± 0.004)	(±0.2 G)	(err. ≤ 10%)
НА	2.0035	$6.5 \pm 0.2$	2.10
SiO <sub>2</sub> /HA_p	2.0034	$6.2 \pm 0.2$	0.32
SiO <sub>2</sub> /HA_c	2.0037	$7.7 \pm 0.2$	5.80

Table 3. Spectral parameters obtained by EPR spectra of HA and SiO<sub>2</sub>/HA hybrid nanoparticles

EPR spectra, **Figure 9**, showed a similar line-shape formed by a single and roughly symmetric peak at a g-factor of ~2.0035, typical of carbon-centered radicals of polyaromatic molecules, as reported in literature [20,56]. By comparing the EPR spectra, no significant difference was observed in the line-shape of SiO<sub>2</sub>/HA\_p sample in respect to that of pure HA. Indeed, this evidence was validated by the quantitative determination of the signal amplitude,  $\Delta B$ , which is related to the line-width of EPR peak, directly measurable by the experimental spectra (see **Figure 9A**).

The line-width of EPR signals reflects the relaxation time of spinning electrons and is primarily affected by the unresolved hyperfine interaction between unpaired electrons with the neighboring-atoms [56,57]. Consequently, it can furnish information about the presence of different carbon-based radical species as well as

on the supramolecular organization of polyaromatic structures hosting unpaired electrons. For this reason, the  $\Delta B$  parameter is usually considered indicative of the mean distance between the radical centers. As reported in **Table 3** the determined  $\Delta B$  value of SiO<sub>2</sub>/HA\_p sample resulted quite similar to that of pure HA, suggesting that the chemical and structural organization of the organic moiety was not particularly perturbed after combination with the inorganic matrix.

On the other hand, a great  $\Delta B$  increase (~1.2 G) was observed for the SiO<sub>2</sub>/HA\_c sample indicating that the chemical coupling, due to the APTS use during the synthesis, strongly influenced the local organization of the humic macromolecules in which the radical centers appeared closer within the hybrid nanoparticles. This behavior was also confirmed by the power saturation curves, reported in **Figure 9B** and obtained by plotting the normalized peak amplitude (A/A<sub>0</sub>) of spectra recorded increasing the incident microwave power (P) as function of the square root of P itself. In all cases, a monotonic trend was observed, indicating that the free radical spins did not exhibit the same relaxation times, and saturated independently.

This behavior is a mark of a chemical inhomogeneous distribution of radical centers characterizing the HA component, and probably associable the presence of different aromatic moieties on which the unpaired electrons were stabilized. More specifically, no changes were observed in the case of PS curve of SiO<sub>2</sub>/HA\_p in respect to pure HA.

On the other hand, a decrease in the inhomogeneity character was detected in the PS curve of  $SiO_2/HA_c$  sample, supporting the idea of changes in the HA supramolecular organization in which the organic component tended to be more confined within the hybrid nanoparticles, because of a different growth mechanism induced by the chemical conjugation of HA macromolecules with APTES and its consequently combination with  $SiO_2$  matrix. During silica formation on APTS

nuclei, HA hydrophobic chains tend interact and segregate in the interior of the born particles [31].

Finally, the spin-density values demonstrated a higher concentration of radical species in the SiO<sub>2</sub>/HA\_c sample in respect to SiO<sub>2</sub>/HA\_p one, confirming the presence of a higher organic content in agreement with TG analysis.

To assess the effect of immobilization onto silica phase, as well as of different combination strategies on functional behavior of HA, the antioxidant potential of the nanoparticles was evaluated by means of the FOX assay i.e the ferrous ions oxidation in the presence of the ferric ions indicator xylenol orange. The FOX assay is currently used to detect the levels of hydroxyperoxides in different kind of matrixes. The assay allows to measure the ability of a compound to act as an antioxidant by causing a decrease in the levels of peroxides like  $H_2O_2$ .

Antioxidant properties of both hybrid systems (SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c NPs) were evaluated in the presence of H<sub>2</sub>O<sub>2</sub> and in comparison, with those of the free acid (HA) and bare SiO<sub>2</sub> (reported results were representative of both SiO<sub>2</sub>\_c and SiO<sub>2</sub>\_p). The antioxidant potential was measured by adding hydrogen peroxide at three concentrations of i.e. 25  $\mu$ M, 12.5  $\mu$ M and 10  $\mu$ M: the results obtained are shown in **Figure 10A**, **10B and 10C**, respectively. The standard calibration curve of absorbance plotted versus H<sub>2</sub>O<sub>2</sub> respective concentrations was also reported in **Figure 10D**.

For all the  $H_2O_2$  concentrations used and for all the time interval, the highest antioxidant potential was shown by the SiO<sub>2</sub>/HA\_p sample; moreover, both the NP samples SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c showed to be more active than free HA tested in the same weight amount of the nanoparticles. Notably, the amount of HA in the hybrid systems was lower than that employed for tests as free acid thus confirming that the inclusion of the HA in the NP systems, enhanced its antioxidant properties thus requiring lower amounts of material to exploit its action.



**Figure 10**. Antioxidant properties SiO<sub>2</sub>, SiO<sub>2</sub>/HA\_p and SiO<sub>2</sub>/HA\_c NPs and pure HA at different hydrogen peroxide concentrations: 10  $\mu$ M (A), 12.5  $\mu$ M (B) and 25  $\mu$ M (C); standard calibration curve for hydrogen peroxide determination (D).

Furthermore, physical entrapment (SiO<sub>2</sub>/HA\_p sample) ensured higher radical scavenging activity than chemical coupling. As widely assessed, the red-ox behavior of these mixtures was strongly related to the phenol/quinone moieties of their complex molecular structures. Indeed, these functional groups can act as electron donors: after losing one electron per stage, they span from hydroquinone to quinone state. In SiO<sub>2</sub>/HA\_c samples these species are confined into the interior of silica due to chemical coupling with APTS. These features probably decreased availability of these reactive species, ultimately reducing the antioxidant activity.

#### **1.4 Conclusions**

This study reports the synthesis of hybrid SiO<sub>2</sub>/HA nanostructures via in-situ solgel route, following two combination approaches between organic and inorganic components. Physical entrapment was achieved through in-situ formation of silica nanoparticles in a HA containing solution, whereas 3-aminopropyltriethoxysilane was exploited as chemical coupling agent between HA and the inorganic matrix, through amide bond formation.

Our results proved that combination with inorganic component at the molecular scale is successful in addressing poor stability in aqueous solutions due to aggregation and degradation phenomena, while concurrently boosting the intrinsic antioxidant properties of HA. In fact, the HA components actively participated in nanoparticles formation, thus controlling their growth and leading to smaller size than bare  $SiO_2$  in the same synthesis conditions.

Furthermore, as shown by FT-IR, NMR and EPR results, the combination strategy of humic biomaterials with inorganic component has a great influence on the HA supramolecular organization, radical properties as well as nature and availability of functional groups. These features ultimately impact on antioxidant activity of final hybrid systems.

Notably, chemical coupling involves reactivity of HA moieties and strongly influences the local organization of humic superstructures. This decreased availability of reactive centers towards free radical species, resulted into a lower antioxidant activity than for hybrid systems obtained by physical entrapment of HA molecules.

These findings disclose the great potential of an inorganic templated approach as an effective strategy to turn bio-wastes into functional valuable materials.
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# 2. A study on structural evolution of hybrid Humic Acids-SiO<sub>2</sub> nanostructures in pure water: effects on physicochemical and functional properties.

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### 2.1 Abstract

Humic acids (HA) are considered a promising and inexpensive source for novel multifunctional materials for a huge range of applications. However, aggregation and degradation phenomena in aqueous environment prevent from their full exploitation. A valid strategy to address these issues relies on combining HA moieties at the molecular scale with an inorganic nanostructured component, leading to more stable hybrid nanomaterials with tunable functionalities. Indeed, chemical composition of HA can determine their interactions with the inorganic constituent in the hybrid nanoparticles and consequently affect their overall physico-chemical properties, including their stability and functional properties in aqueous environment. As a fundamental contribution to HA materials-based technology, this study aims at unveiling this aspect. To this purpose, SiO<sub>2</sub> nanoparticles have been chosen as a model platform and three different HAs extracted from composted biomasses, manure (HA Man), artichoke residues (HA\_Art) and coffee grounds (HA\_Cof), were employed to synthetize hybrid HA-SiO<sub>2</sub> nanoparticles through in-situ sol-gel synthesis. Prepared samples were submitted to aging in water to assess their stability.

Furthermore, antioxidant properties and physico-chemical properties of both as prepared and aged samples in aqueous environment were assessed through Scanning Electron Microscopy (SEM), Simultaneous Thermogravimetric (TGA) and Differential Scanning Calorimetric (DSC) Analysis, Fourier Transform Infrared (FT-IR), Nuclear Magnetic Resonance (NMR), Electron Paramagnetic Resonance (EPR) spectroscopies.

The experimental results highlighted that hybrid HA-SiO<sub>2</sub> nanostructures acted as dynamic systems which exhibit structural supramolecular reorganization during aging in aqueous environment with marked effects on physico-chemical and

functional properties, including improved antioxidant activity. Obtained results enlighten a unique aspect of interactions between HA and inorganic nanoparticles that could be useful to predict their behavior in aqueous environment.

Furthermore, the proposed approach traces a technological route for the exploitation of organic biowaste in the design of hybrid nanomaterials, providing a significant contribution to the development of waste to wealth strategies based on humic substances.



### **2.2 Introduction**

Waste management and valorization is imperative to enable development of closed-loop waste-free processes at the core of circular economy (Clark et al., 2016; Keijer et al., 2019). Particularly, bio-wastes (BWs) represent a largely available source of chemical richness, thus rising increasing attention by industry and academic researchers aiming at setting up strategies for their conversion into added-value compounds and materials (Glasing et al., 2016; Xu et al., 2019). To this purpose, nanotechnologies can offer intriguing solutions to promote upgrading of BWs into valuable nanomaterials (Glasing et al., 2016; Pota et al., 2020; Xu et al., 2019).

Humic acids (HA) are the alkali-soluble fraction of natural organic matter, usually found in water, soil and sediments. They are also a product of the composting process of BWs since they are produced by biological and chemical conversion of biomasses by natural transformations (Luo et al., 2019; Spaccini et al., 2019) and are also a massive co-products in biorefinery processes (Savy et al., 2020).

HA are heterogeneous mixtures of relatively small molecules (<1000 Da) with different polarity and rich in functional groups including quinone, phenol, carboxyl and hydroxyl moieties, which confer them many useful properties encompassing metal ions chelation, organic pollutants absorption, antioxidant and antiinflammatory activity as well as regenerable red-ox behavior (Afzal et al., 2019; Canellas et al., 2015; de Melo et al., 2016; Lowell et al., 2004; Milne et al., 2017; Nuzzo et al., 2020b; Piccolo, 2002; Pukalchik et al., 2019; Sangregorio et al., 2018; Tang et al., 2014). These features make HA intriguing feedstocks for the design of eco-sustainable multifunctional materials for a huge number of applications including medicine, sensing, packaging as well as environmental remediation (Glasing et al., 2016; Xu et al., 2019).

Yet, poor selectivity and short life-time, due to segregation leakage and/or degradation phenomena in aqueous environment, strongly limit their full exploitation (Allard et al., 1994). To address these issues, valuable inspiration comes from nature. In fact, HA mixtures proved to interact with inorganic nanoparticles increasing their mobility in aqueous environment and determining their fate in water and soil (Angelico et al., 2014; Stepka et al., 2018). Furthermore, interactions with inorganic compounds in soil provided stabilization towards degradation (Angelico et al., 2014; Grillo et al., 2015) ].

Therefore, combining HA with a foreign inorganic support appears as an promising strategy to improve their stability (Oliveira et al., 2016; Wang et al., 2020). Thanks to the amphiphilic character of their supramolecular structure (Piccolo, 2001), HA can favorably interact with an inorganic surface charged phase through electrostatic and covalent forces, conferring a good colloidal stability in aqueous medium (Pota et al., 2020; Zhang et al., 2020). Scientific studies on HA modified inorganic nanomaterials have been flourishing in the last years, providing proof of concept for the great potentialities of these nanomaterials in a huge number of applications including plant bio-stimulants (Adamiano et al., 2021; de Melo et al., 2016) as well as high efficacy sorbents for pollutants and even noble metal ions in aqueous solutions (Amer et al., 2021; Santosa et al., 2021). Indeed, far larger improvement of properties than those observed by the mere encapsulation/binding of organic compound to an inorganic support has been achieved by the combination at the molecular scale between the organic and inorganic components into a hybrid nanostructure.

In this system, the inorganic phase can successfully act as a templating agent and tune oligomer supramolecular structure; not only this boosts its intrinsic properties, but it can give rise to new features driving the organic phase function (Deligiannakis et al., 2012; Vitiello et al., 2021).

Among nanostructured inorganic oxides, SiO<sub>2</sub> have been emerging as functionally versatile biocompatible platform for the design of hybrid materials with tailored properties (Deligiannakis et al., 2012; Venezia et al., 2020).

Particularly, the Stöber method has emerged as an effective approach to design  $SiO_2$  based hybrids, since it allows to obtain tunable sized nanoparticles, under mild reaction conditions, thus preventing any degradation of the organic component (Deligiannakis et al., 2012; Venezia et al., 2020).

In this contest, in-situ strategy, according to which silica nanoparticles were synthetized through sol-gel reaction occurring in the presence of HA, was successfully used to combine HA with  $SiO_2$  component producing hybrid HA based nanostructures, with uniform size distribution and a good chemical and colloidal stability in aqueous solution as well as relevant antioxidant behavior (Pota et al., 2020).

Furthermore, it was recently assessed that chemical coupling between inorganic nanostructures and HA markedly influence their supramolecular organization ultimately determining their reactivity and functional properties (Pota et al., 2020). Indeed, chemical composition of HA must play a key role in determining their interactions with inorganic nanostructures and it consequently affects their supramolecular structure in the hybrid nanoparticles, thus influencing overall physico-chemical properties.

As a key contribution to the development of a mature technology based on HA, the present study aims at elucidating this aspect. To this purpose three different types of silica–HA hybrid nanoparticles were synthetized using HA extracted from three different composted biomasses (cow manure (HA\_Man), artichoke residues (HA\_Art), coffee grounds (HA\_Cof). Silica–HA hybrid nanoparticles were obtained, through an in-situ one-pot sol-gel route based on the modified Stöber method.

The synthesized systems were evaluated for their antioxidant behavior, by the ferrous oxidation-xylenol orange (FOX) and DPPH assays.

An in-depth physico–chemical characterization was carried out combining different techniques, including Scanning Electron Microscopy (SEM), Simultaneous Thermogravimetric (TGA) and Differential Scanning Calorimetric (DSC) Analysis, Fourier Transform Infrared Spectroscopy (FT-IR), solid-state Cross-Polarization Magic Angle Spinning Carbon-13 Nuclear Magnetic Resonance (<sup>13</sup>C-CPMAS-NMR) and Electron Paramagnetic Resonance (EPR) spectroscopies. This exhaustive investigation as well as antioxidant activity assessment were carried out on samples soon after synthesis, after 3 weeks' and even 3 months' aging in water. Furthermore, differences in physico-chemical and functional properties of the hybrid nanostructures, due to their evolution dynamics were related to HA chemical structures. From a technological point of view this study provides a versatile approach to engineer hybrid nanoparticles starting from otherwise insoluble and poor colloidally stable HA. Notably, obtained nanoparticles exhibit intriguing properties for their application as additives in food packaging.

This study is expected to fill a relevant gap in the chemistry of HA based hybrid materials, thus providing strategic guidelines to set up efficient waste to wealth strategies and exploit HA technological potential. Furthermore, it can provide a useful contribution to clarify the dynamics of interactions between HAs and inorganic nanoparticles which often control their fate in aqueous environment and interactions with biological systems.

### 2.3 Results and discussion

#### 2.3.1 Antioxidant activity: FOX and DPPH assays

The results of FOX and DPPH assays (**Figures S1, S2 and S3 and Table 1**) were obtained after normalization in respect to the HA weight % content in the SiO<sub>2</sub>\_HA samples, as driven by TGA measurements: 15.0 %, 8.8% and 6.7 % for SiO<sub>2</sub>\_HA\_Man, SiO<sub>2</sub>\_HA\_Art and SiO<sub>2</sub>\_HA\_Cof, respectively. These amounts kept constant in the aged samples, as driven by thermogravimetric measurements on aged samples.

FOX assay proved that  $SiO_2$  HA Man sample shows the highest antioxidant power among as prepared NPs: indeed 1h incubation resulted in about 70% consumption of the added hydrogen peroxide that was almost depleted after 4h incubation (Figure S1, Table 1). The SiO<sub>2</sub>\_HA\_Art and SiO<sub>2</sub>\_HA\_Cof NPs induced a decrease of about 50% H<sub>2</sub>O<sub>2</sub> already after 30' incubation, but no significant change was found with prolonged incubation for SiO<sub>2</sub> HA Art NPs, whereas SiO<sub>2</sub>\_HA\_Cof sample showed a further consumption (Figure S1, Table 1). The aging of samples that were left in water for three weeks, had different effects on the antioxidant activity of hybrid systems (Figure S2, Table 1).  $SiO_2$  HA Man did not show any significant change in the antioxidant activity. In fact, the rate of hydrogen peroxide scavenging and the final consumption yields were comparable to those of the fresh sample. In the case of the other NPs, aging induced an improvement of the antioxidant properties, in contrast to what usually expected due to degradation phenomena involving HA. Notably, SiO<sub>2</sub> HA Cof 3weeks sample showed the fastest kinetics of H<sub>2</sub>O<sub>2</sub> scavenging already after 30' incubation a 70% consumption of the added peroxide was observed. Overall, antioxidant activity of SiO2\_HA\_Art\_3weeks and SiO2\_HA\_Cof \_3 weeks grew comparable to SiO<sub>2</sub>\_HA\_Man systems.

Moreover, hydrogen peroxide scavenging activity (FOX) of Trolox in the same concentration as NP<sub>S</sub>, was assessed for comparison. Obtained results (**Figure S3**) show poor efficacy of Trolox, at investigated concentration, as hydrogen peroxide scavenger, revealing the better performance of  $SiO_2$ \_HA NPs.

DPPH results (**Figures S4, Table 1**) confirm the same trend as those obtained by FOX assay (**Figure S1, S2, Table 1**). In fact, antioxidant activity of SiO<sub>2</sub>\_Man\_NPs doesn't show any relevant changes over time (**Figure S4**). Instead, each of the other hybrid NPs, has a lower antioxidant activity than SiO<sub>2</sub>\_Man\_NPs at the beginning, but it grows rapidly over time, showing a much higher DPPH radical scavenging rate (up to a maximum of 70% at 3 months, **Figures S4**) than SiO<sub>2</sub>\_Man\_NPs after three months' aging. DPPH results of SiO<sub>2</sub>\_HA\_Man and SiO<sub>2</sub>\_HA\_Art is comparable to those of Trolox (**Figure S3**), whereas SiO<sub>2</sub>\_HA\_Man and SiO<sub>2</sub>\_HA\_Man and SiO<sub>2</sub>\_HA\_Art showed an even higher antioxidant performance. than the reference.

Furthermore, statistical comparison of antioxidant activity of different samples at each time evidenced high level of significance for both FOX (p<0.005 and DPPH assay (p<0.001).

FOX assay								
		Control°	SiO <sub>2</sub> _HA_Man		SiO <sub>2</sub> _HA_Art		SiO2_HA_Cof	
time			fresh	3weeks	fresh	3weeks	fresh	3weeks
	240'	92.8 ±	6.5 ±	$5.6 \pm 0.3$	47.6 ±	10.3 ±	33.1 ±	10.8 ±
		4.6	2.6		0.5	1.3	2.5	1.1
			****	****	**	****	****	****
DPPH assay								
	90'		20±1.1	21.3±1.2	11±1.4	34±1.3	20±0.9	59.7±0.8
					**	***	ns	****
%HA			15.0 ±		$8.8 \pm$		6.7 ±	

content			1.0		0.4		1.0	
Legend: °Control experiments were carried out on H <sub>2</sub> O <sub>2</sub> solution for FOX Assay: the reported results are related to the								
experiments carried out in the presence of $H_2O_2 25\mu M$ (further results are shown in the supplementary material, Figures S1,								
S2, S3). Control experiments were carried out on DPPH methanol solution for DPPH assay (further results are shown in the								
supplementary material, Figures S4). All the experiments were run in triplicate. Results are reported as the mean of obtained								

supprenentially internal, rights 34). An the experiments were funn in inpredict. Results are reported as the mean of obtained values  $\pm$  standard deviation of the mean. Values obtained from FOX assay compared to the control were significantly different at p < 0.01 (\*\*), p < 0.001 (\*\*\*) and p<0.0001) (\*\*\*\*). For DPPH assay, differences compared to SiO<sub>2</sub>\_HA\_Man sample at each time were considered not significant (ns) or significant at p < 0.01 (\*\*), p < 0.001 (\*\*\*) and p<0.0001) (\*\*\*\*).

#### Table 1. NPs antioxidant properties and %HA content measured as hydrogen peroxide scavenging activity.

These results indicate that HA combination with SiO<sub>2</sub> into hybrid nanostructures allows for their use as natural antioxidants. The antioxidant activity of investigated hybrid systems keeps constant or even increases with aging, in the case of SiO<sub>2</sub>\_HA\_Cof and SiO<sub>2</sub>\_HA\_Art. To assess the reasons of this unusual behaviour, a detailed physico-chemical characterization was carried out on bulk HA samples as well as on as prepared and aged hybrids.

#### 2.3.2 Determination of NPs Yield and HA binding efficacy

NPs effective concentration is 4.7 mg/ml for  $SiO_2_HA_Art$  and  $SiO_2_HA_Cof$ , and 1 mg/ml for  $SiO_2_HA_Man$  respectively (**Table S1**). NPs nominal concentration evaluated from employed precursors amounts (TPOS and HA) is 13.8 mg/ml for each sample. These values were employed to evaluate NPs yield (**Table S1**). Moreover, binding efficiency (BE) was calculated.

Therefore, process evidenced a good efficiency for SiO<sub>2</sub>\_HA\_Art and SiO<sub>2</sub>\_HA\_Art in terms of NPs yield and HA binding.

#### 2.3.3 NMR analysis

NMR analysis <sup>13</sup>C CPMAS NMR spectra of HA extracted from manure (HA\_Man), artichoke residues (HA\_Art) and coffee ground (HA\_Cof) are reported in **Figure S5**.

The NMR spectrum of HA\_Man was characterized by a predominance of O-alkyl carbons (60-110 ppm) due to polysaccharides whose amounts accounted for

46.56% of total spectra. Moreover, the signals in the alkyl-C region (0–45 ppm), confirmed the presence of methylene (CH<sub>2</sub>) chains in lipid compounds, such as cutin and wax, as well as of CH<sub>2</sub> groups in  $\beta$  position to carbonyls in aliphatic esters. The resonances in the aryl-C region (110–145 ppm) were assigned to unsubstituted phenyl carbons in either lignin monomers, lignans, flavonoids or unsaturated lipids structures, whereas those in the phenolic region (145–160 ppm) indicated the presence of O- and C- substituted aromatic structures, in lignin and lignan backbone. Finally, the intense signal in the carbonyl region (160–190 ppm) at about 174 ppm derived from carboxyl and amide groups in aliphatic acids and peptides, respectively. Relative carbon distribution in main carbon groups was obtained by signals integration over chemical shift regions. NMR signal assignment is reported in Table S2 (Nuzzo et al., 2020b, 2020a).

Spectral data also provided the hydrophobicity (HB), hydrophilicity (HI) as well as the hydrophobicity index (HB/ HI) of samples (**Table S2**).

HA\_Art showed the largest content of aromatic carbon (aryl + phenol) which accounted for 25% of the total signal area. Furthermore, carboxyl-C resulted more abundant in both HA\_Cof and HA\_Art samples than in HA\_Man, accounting for 9.26% and 11.89% of the overall spectral area, respectively. Finally, HA\_Cof and HA\_Art showed a distinct hydrophobic character as revealed by their larger hydrophobicity index (HB/HI) than HA\_Man sample (**Table S2**).

Bare HA samples were suspended in distilled water as well as in microbiological filtered distilled water, then recovered after three weeks and submitted to <sup>13</sup>C CPMAS NMR spectroscopy, to check whether the dispersion in aqueous media conveyed chemical or microbial-induced degradation phenomena. The <sup>13</sup>C CPMAS NMR spectra (**Figure S6 A, B, C**) did not show any significant difference with those recorded on raw samples, proving that no degradation or chemical changes occurred during wet aging.

### 2.3.4 SEM Analysis

SEM micrographs of SiO<sub>2</sub>\_NPs (**Figure 1 A**) nanoparticles showed a spherical morphology with a mean diameter of about 250 nm. Instead, SEM micrographs of SiO<sub>2</sub>\_HA NPs (**Figures 1 B, C, and D**) revealed a pseudo spherical morphology with a narrow size distribution of about 80 nm for SiO<sub>2</sub>\_Man and 30nm for SiO<sub>2</sub>\_Art and SiO<sub>2</sub>\_Cof in diameter, smaller than bare SiO<sub>2</sub> nanostructures (**Figure 1C, D**).

The synthetic route of all hybrid samples was based on the formation of silica nanoparticles in a HA-containing solution. Therefore, HA phase is expected to actively participate in the nanoparticle formation through two different phenomena: HAs limit diffusion of both silica oligomers and nanoparticles in solution.

HAs adsorption onto primary silica particles' surface hinders their further aggregation due to a highly negatively charged surface.

These phenomena lead to a smaller size of hybrid nanoparticles than bare SiO<sub>2</sub> samples (**Figures 1 B, C, D**) (Pota et al., 2020; Zhang et al., 2020).



Figure 1. SEM micrographs of SiO<sub>2</sub>\_NPs (A), SiO<sub>2</sub>\_HA\_Man (B), SiO<sub>2</sub>\_HA\_Cof (C), SiO<sub>2</sub>\_HA\_Art (D).

### 2.3.5 EPR analysis

EPR spectra of all obtained hybrid samples are shown in Figure 2 A-C, whereas the corresponding spectral parameters are summarized in **Table 2**. HA\_Man, HA\_Art and HA\_Cof powders were also analyzed as references. The EPR spectra shown in **Figure 2** showed a line shape formed by a single peak at a g-factor of ~2.0035, which is typical of carbon-centered radicals of polyaromatic molecules, as reported in the literature (Pota et al., 2021; Vitiello et al., 2021). However, some differences in the EPR signals were observed in dependence of HA chemical nature and of their combination with SiO<sub>2</sub> nanoparticles before and after aging treatment. Indeed, by comparing the EPR spectra of pure HA with those of SiO<sub>2</sub>\_HA NPs, significant differences were observed in the line shapes, as detectable in **Figure 2 A-C**. Particularly, these changes can be more clearly appreciated by the quantitative

determination of the signal amplitude,  $\Delta B$ , which is related to the linewidth of the EPR peak, directly quantifiable by the experimental spectra (see Figure 2 A-C). Being the peak line-width related to the relaxation time of free-electrons and affected by the unresolved hyperfine interaction between unpaired electrons with the neighboring atoms (Buszman et al., 2006; Panzella et al., 2018), it can furnish information about the presence of different carbon-based radical species, as well as about the supramolecular organization of polyaromatic structures hosting unpaired electrons (Alfieri et al., 2020; Moccia et al., 2020; Zykova et al., 2018). Consequently,  $\Delta B$  is typically linked to the mean distance between the radical centers, providing indirect information about the organization of the organic moiety. As summarized in **Table 2**, the  $\Delta B$  values of SiO<sub>2</sub>\_HA samples increased with respect to those of pure HA, indicating that the chemical and structural organization of the organic moiety was particularly perturbed after combination with the inorganic matrix. It could be argued that the chemical coupling, due to the APTS use during the synthesis, strongly influenced the local organization of the humic supramolecular structure, in which the radical centers appeared closer within the hybrid nanoparticles than in bulk HA. More specifically, a greater  $\Delta B$  increase was observed for both SiO<sub>2</sub> HA Art (~1.1 G) and SiO<sub>2</sub> HA Cof (~1.7 G) samples with respect to SiO<sub>2</sub>\_HA\_Man (~0.6 G) one. This behavior was also confirmed by the power saturation curves, reported in **Figure 2 D-F** and obtained by plotting the normalized peak amplitude  $(A/A_0)$  of spectra recorded while increasing the incident microwave power (P) as a function of the square root of P itself. Differently to the trends observed for pure HA samples, a monotonic trend was observed for all hybrid SiO<sub>2</sub>\_HA samples, indicating that the free radical spins did not exhibit the same relaxation times and saturated independently. It is a mark of a chemical inhomogeneous distribution of radical centers characterizing the HA component, and probably associable with the presence of different aromatic

moleties on which the unpaired electrons were stabilized and confirming possible changes due to the chemical conjugation with the inorganic component. Interestingly, different behaviors were observed after 3 weeks in dependence of the humic acid nature (see **Table 2**). Indeed, if  $\Delta B$  value of SiO<sub>2</sub> HA man 3weeks did not show any significant changes with respect to as prepared  $SiO_2$  HA man, the aging treatment caused a significant decrease in the  $\Delta B$  values of EPR spectra of both SiO<sub>2</sub> HA Art 3weeks (~0.5 G) and SiO<sub>2</sub> HA Cof 3weeks (~0.3 G) samples and an increase of inhomogeneous character of their PS curves (Figure 2D-F). These indicated that the radical's centers got more distanced, suggesting a possible re-organization of the organic component, due to the long exposure in an aqueous environment. At the same time, a deeper analysis of the experimental spectra of hybrid nanomaterials after three weeks indicates the appearance of a second component at lower magnetic field, which is much more detectable in the case of SiO<sub>2</sub>\_HA\_Art\_3weeks (Figure 2B, line c) and can be related to the presence of different radical centers (such as oxygen species), accordingly with the chemical heterogeneity shown by PS curves. These results suggest that aging causes a structural evolution of aromatic and polyphenolic moieties, which account for paramagnetic features evolution measured by EPR spectroscopy. This is in line with the HA supramolecular structure whose conformation is stabilized by weak dispersive forces and can be modified by the rearrangement of hydrophobic and hydrophilic domains when in prolonged contact with water (Piccolo et al., 2019). Finally, no significant variation in the spin-density values was observed, confirming that the amount of organic radical species was not altered after the combination of HAs with SiO<sub>2</sub>-based nanoparticles.



Figure 2. EPR spectra (panels A-C) and power saturation curves (panels E-F) of bulk HA (black lines a, black full squares), SiO<sub>2</sub>\_HA (red lines b, red full squares), and SiO<sub>2</sub>\_HA\_3weeks (blue lines c, blue full triangles).

Samples	ΔΒ	g-factor	Spin×g <sup>-1</sup> ×10 <sup>16</sup>	Spin×g <sup>-1</sup> ×10 <sup>16</sup>
	(± <b>0.2</b> )	(± 0.0003)		normalized
				for HA content
HA_Man	6.4	2.0034	5.8±0.6	
SiO <sub>2</sub> _HA_Man	7.0	2.0033		3.6± 0.4
SiO <sub>2</sub> _HA_Man_3weeks	6.9	2.0034		3.5±0.3
HA_Art	6.0	2.0034	4.3±0.4	
SiO <sub>2</sub> _HA_Art	7.1	2.0036		7.9±0.7
SiO <sub>2</sub> _HA_Art_3weeks	6.6	2.0037		7.8±0.7
HA_Cof	4.9	2.0034	$7.7 \pm 0.7$	
SiO <sub>2</sub> _HA_Cof	6.6	2.0035		8.0± 0.8
SiO <sub>2</sub> _HA_cof_3weeks	6.3	2.0036		10± 0.9

Table 2. Spectral parameters obtained by EPR spectra of HA and SiO2\_HA NPs

### 2.3.6 TGA Analysis

Thermogravimetric Analysis (TGA) was carried out on both SiO<sub>2</sub>\_NPs (**Figure S8 A**) and hybrid nanoparticles. To assess any release of humic material from the hybrid nanoparticles during water storage, TGA measurements were carried out on SiO<sub>2</sub>\_HA and SiO<sub>2</sub>\_HA\_3weeks samples. **Figure S8 B, C and D** report TGA curves of bulk HAs and SiO<sub>2</sub>\_HA NPs, both after synthesis and after three weeks' aging.

The values of high temperature weight loss were reported in **Table S3** and depended on the type of HA employed during the synthesis. The higher decomposition temperature of all hybrid samples than each bulk HA suggested a

better thermal stability of humic substances because of combination with  $SiO_2$  matrix.

Furthermore, from the comparison between TGA curves of as prepared and aged samples, which showed comparable weight losses (**Figure S8, B, C and D**) it can be inferred that no relevant HA release from hybrid NPs occurred during aging. TGA plots (**Figure S8**) were obtained from replicates from different independent experiments, carried out on samples from different synthesis batches, evidencing the high level of reproducibility of proposed synthesis.

#### 2.3.7 DSC Analysis

Differences in composition among investigated HA were confirmed by DSC analysis, which was carried out both on as prepared and aged samples to assess their evolution. Figure 3 shows DSC curves of bare HA Man, HA Cof and HA\_Art, respectively. Oxidative decomposition occurs in the temperature range 200-600 °C, as already driven by TGA curves, through several thermo-oxidation steps, corresponding to different exothermic peaks and appreciated through slope changes in TGA curves. The first peak, occurring at about 320 °C is usually related to decomposition of aliphatic groups, carbohydrate components and to some oxygen bonded carbons (catechol, phenolic) and amino-acids groups (Kucerik et al., 2004). A second exothermic peak can be observed in DSC curves of all HA compositions, at about 460 °C. It is usually assigned to oxidative decomposition of aromatic moieties and -COOH groups in humic samples. Notably, the peak at 460 °C has the highest intensity in HA\_Art sample, suggesting it is the richest in aromatic/polyphenolic compounds and probably has a more heterogeneous nature. This result agrees with <sup>13</sup>C NMR spectra (Figure S5, Table S2). A third exo peak occurs at higher temperatures, at about 530 and 570 °C, in DSC thermograms of both HA\_Art and HA\_Cof sample, respectively. This peak in the high temperature

region is usually related to oxidation and polycondensation of most stable aromatic moieties and cyclic groups containing C and N in humic substances.

DSC curves of hybrid nanostructures show different trends from bulk HA (**Figure 3 B, C, D**). Particularly, in  $SiO_2$ \_HA\_Man samples the first exo peak shifts to lower temperatures, whereas the second peak can be barely appreciated as a bump almost in the same temperature range. DSC thermograms of  $SiO_2$ \_HA\_Man\_3weeks appears quite similar.

In SiO<sub>2</sub>\_HA\_Cof the first peak does not shift with respect to that of bare HA\_Cof. Indeed, the bump representative aromatic oxidative decomposition looks more evident after three weeks' aging.

A shift to higher temperature of the second exo peak can be observed in DSC curves of  $SiO_2_HA_Art$  samples. However, as a major difference from the other investigated samples, the second oxidative peak can be clearly appreciated in DSC profiles, especially in  $SiO_2_HA_Art_3$ weeks, suggesting a higher content in polyphenolic moieties, in accordance with <sup>13</sup>C NMR results (**Table S2**).

Analysis of DSC thermograms of as prepared hybrid nanoparticles in comparison with those of bulk HA, suggests that only some fractions of HA suprastructure are entrapped into the silica backbone during *in situ* synthesis. Particularly, it can be assumed that most of the highly hydrophobic fraction of aromatic components were lost in suspension as suggested by the marked decrease of DSC peak corresponding to their decomposition, in the DSC curve of as-prepared SiO<sub>2</sub>\_HA samples. This should be expected due to the hydrophilic nature of Stöber SiO<sub>2</sub> nanoparticles. Analysis of DSC curves shows a change in decomposition trend of aged SiO<sub>2</sub>\_HA\_Cof and SiO<sub>2</sub>\_HA\_Art samples with respect to as prepared NPs suggesting that both samples undergo a conformational change with aging. Giving the constant amount of HA component within the samples, as shown by TGA curves (**Figure S8**), this evolution must essentially involve a structural

modification of the samples and particularly of HA component, with change their supramolecular organization during aging, resulting in a different decomposition trend. Obtained results are in accordance with EPR spectroscopy evidence (**Figure 2**).

Particularly, the changes of the peak at about 500 °C, related to thermal modifications of aromatic and polyaromatic moieties, that are clearly evident in the DSC curve of SiO<sub>2</sub>\_HA\_Art\_3weeks and SiO<sub>2</sub>\_HA\_Cof\_3weeks, suggest that supramolecular restructuring must involve the aromatic and polyphenolic species in HAs, which were trapped in the samples and should also account for their antioxidant activity (Aeschbacher et al., 2012).



Figure 3. DSC curves of bulk HA (A), fresh and aged hybrids for HA\_Man (B), HA\_Cof (C) and HA\_Art (D).

#### 2.3.8 FT-IR analysis

FT-IR spectra of bare HA\_Man, HA\_Art, HA\_Cof (black curve, **Figure 4 A, B, C**) reveal the typical absorption bands of HA mixtures as assigned in **Table S4**.

FTIR spectra of all SiO<sub>2</sub>\_HA NPs, reveal the peaks in the range between 1750 cm<sup>-1</sup> and 1400 cm<sup>-1</sup>, as well as the bands in the interval 3000-2800cm<sup>-1</sup>, corresponding to C-H stretching in methyl groups, proving the presence of HA in hybrid nanoparticles (blue curve, **Figure 4 A, B, C**).

FT-IR spectrum of SiO<sub>2</sub>\_NPs nanoparticles revealed the main characteristic bands of silica. Focusing on SiO<sub>2</sub>\_HA\_Art nanoparticles (**Figure 4 B, C**), the band at about 1100 cm<sup>-1</sup> (Si-O-Si stretching) occurs at lower wavenumbers than in bare SiO<sub>2</sub> nanoparticles, suggesting a less cross-linked SiO<sub>2</sub> network in these hybrid nanostructures.

Formation of Stöber SiO<sub>2</sub> nanoparticles occurs through the nucleation-aggregation mechanism. Notably, hydrolysis and condensation reactions lead to a certain number of nuclei, which grow producing primary nanoparticles, which further aggregate forming larger spherical SiO<sub>2</sub> nanoparticles (Pota et al., 2020). Primary particles are initially held together by weak reversible interactions (van der Waals forces, hydrogen bonds), that are gradually replaced by irreversible covalent necks, forming the final microstructure (Branda et al., 2015).

Humic molecules can strongly interact with SiO<sub>2</sub> primary nanoparticles, adsorbing on their surface (Pota et al., 2020; Zhang et al., 2020). Notably, the nature of HA is expected to determine the number and intensity of interactions. This behavior hinders NPs aggregation as well as the formation of covalent bridges among nanoparticles. Particularly, using APTS as a precursor, may allow HA chemical coupling with inorganic support through possible by amide bond formation between carboxyl groups of HA and amine moieties of SiO<sub>2</sub> nanoparticles (Pota et al., 2020). This process results, in SiO<sub>2</sub>\_HA\_Art samples, into a less crosslinked

silica, as driven by the shift of the band at about 1100 cm<sup>-1</sup>, towards lower wavenumbers in the FT-IR spectrum. HA\_3weeks spectra (red curve, **Figure 4 A**, **B**, **C**) are very similar to HA spectra except for the band in the region 3700-2500 cm<sup>-1</sup>, that appear sharper due to water sorption. Obtained results, in accordance with NMR, EPR and TGA/DSC evidence confirm that bulk HA did not experience any significant physico-chemical transformation during water soaking for three weeks, except for water sorption process.



*Figure 4.* FTIR spectra of HA\_Man, HA\_Man\_3 weeks, SiO<sub>2</sub>\_HA\_Man, SiO<sub>2</sub>\_HA\_Man\_3weeks, SiO<sub>2</sub>\_NPs (A), HA\_Art, HA\_Art\_3 weeks, SiO<sub>2</sub>\_HA\_Art, SiO<sub>2</sub>\_HA\_Art\_3weeks and SiO<sub>2</sub>\_NPs (B),HA\_Cof, HA\_Cof\_3 weeks, SiO<sub>2</sub>\_HA\_Cof, SiO<sub>2</sub>\_HA\_Cof and SiO<sub>2</sub>\_NPs (C).

Typical bands of HA in SiO<sub>2</sub>\_HA\_Art and SiO<sub>2</sub>\_HA\_Cof spectra in the region 1760-1500 cm<sup>-1</sup> (red box), corresponding to quinone groups, grow more intense after aging (**Figure 4 B, C**). This result is in accordance with DSC and EPR evidence, further supporting that HA\_Art and HA\_Cof components within the SiO<sub>2</sub>\_NPs undergo conformational changes during aging, which involve modification of HA superstructures. In SiO<sub>2</sub>\_HA\_Art sample, Si-O-Si stretching band slightly shifts to higher wavenumbers after three weeks soaking in water, suggesting that also silica component undergoes a structural reorganization in water (**Figure 4 B, C**).

### 2.3.9 Zeta potential measurements

Zeta potential values are reported in **Table 3**. The presence of HA in the hybrid nanostructures strongly influences their surface charge, which was lower in as prepared SiO<sub>2</sub>\_HA NPs than in bare SiO<sub>2</sub> (-34 mV), while it becomes more negative after aging. Surface charge of HA is a very dynamic parameter (Angelico et al., 2014). This is strongly influenced by the degree of humic supramolecular association, whose conformational expansion due to aging in water may reduce tight intermolecular hydrogen bonds and promote dissociation of acidic functions, thus establishing more negative surface charges (Li et al., 2015; Tang and Cheng, 2018).

Zeta potential values of as prepared SiO<sub>2</sub>\_HA NPs suggest a less negative surface than bare SiO<sub>2</sub>, probably due to partial charge neutralization through interactions between NH<sub>4</sub><sup>+</sup> ions from APTS precursor and HA carboxylic groups and tighter supramolecular conformations. Charge evolution towards more negative values during aging could be ascribed to HA structural unfolding, promoting ionization of phenolic groups (Angelico et al., 2014). This phenomenon is particularly evident in SiO<sub>2</sub>\_HA\_Art and SiO<sub>2</sub>\_HA\_Cof further confirming that these sample undergo marked conformational reorganization, exposing phenolic groups to water interfaces.

Sample	Zeta Potential as prepared	Zeta Potential 3weeks		
	( <b>mV</b> )	(mV)		
SiO <sub>2</sub> _HA_Man	-1.7±1.1	-18.1±0.7		
SiO <sub>2</sub> _HA_Art	7.9±0.6	-25.5±1.9		
SiO <sub>2</sub> _HA_Cof	10.0±0.2	-25.5±1.1		

Table 3. Zeta potential values for as-prepared and aged SiO<sub>2</sub>-HA nanoparticles

# 2.3.10 Structural evolution of HA\_SiO<sub>2</sub> NPs in water: main features and effects on chemical and functional properties

HA are regarded as supramolecular associations of self-organizing, relatively small and heterogeneous molecules, held together by weak dispersive forces, such as van der Waals,  $\pi$ -  $\pi$ , CH-  $\pi$ , interactions.

Indeed, different independent studies (Myneni et al., 1999; Piccolo, 2001; Sutton and Sposito, 2005; Wershaw, 1993, 1986) have provided evidence that even materials extracted by alkali are not macromolecular.

In aqueous environment (Bezuglova, 2019; Piccolo, 2001) these supramolecular clusters are organized in a way that hydrophobic moieties are shielded in the interior. Moreover, due to the presence of H-bonds, these superstructures keep a folded configuration and most of phenol groups are protonated (Angelico et al., 2014). Due to the weakness of stabilizing interactions, HA supramolecular conformations are reversible and very sensitive to chemical changes of the surroundings. Therefore, this configuration is strongly dependent on solution features, including ions concentration, nature of chemical species, pH (Angelico et al., 2014). At neutral or alkaline pH, unfolding of HA superstructures leads to a

more expanded structure. These modifications could be influenced by the topological and chemical constraints set by  $SiO_2$  framework.

Our results suggest that, during aging, HA undergo structural reorganization within silica matrix, since water molecules can diffuse into the pores of hybrid nanostructures through swelling phenomena (Qu et al., 2019; Sawadkar et al., 2020). In SiO<sub>2</sub>\_HA\_Art sample structural reorganization is also promoted by SiO<sub>2</sub> restructuring in water hydrolytic environment. In fact, Stöber mesoporous silica can also undergo spontaneous restructuring during water incubation, that does not change size and morphology, but exclusively its microstructure resulting into a different porosity and a higher condensation degree after aging (Teng et al., 2013). Moreover, HA is expected to play an active role in nanoparticles formation, not only do they control aggregation of primary nanoparticles and final size of hybrid nanostructures (Pota et al., 2020), but they also influence SiO<sub>2</sub> reticulation. Particularly, tighter interactions between HA and SiO<sub>2</sub> result into a less crosslinked silica, which is also more exposed to water hydrolytic etching during aging and subsequent spontaneous restructuring during water incubation. These phenomena must occur in SiO2\_HA\_Art\_3weeks as driven from shifting of the -Si-O-Si stretching band to higher wavenumbers in the FT-IR spectrum with respect to SiO<sub>2</sub>\_HA\_Art sample (Figure 4 B).

Structural evolution in SiO<sub>2</sub>\_HA\_Art and SiO<sub>2</sub>\_HA\_Cof, produces an expanded supramolecular organization, with larger distance between radical centres, accounting for the narrow signal amplitude ( $\Delta$ B) in EPR spectrum. Furthermore, it increases surface exposure of HA reactive functional groups, including quinones and phenols. Not only do these moieties determine surface charge, but they also are responsible of HA antioxidant activity, since they are considered the highest reducible species within HAs mixtures (Aeschbacher et al., 2012).

In aqueous environment, HA undergo to reversible red-ox reactions, with hydroquinone (HQ) moieties interconverting into quinone (Q) species and semiquinone (Q) radicals through electron transfer reactions coupled to proton exchange, according to disproportionation equilibria in water (Aeschbacher et al., 2011; Vitiello et al., 2018). This reversible red-ox behavior accounts for their ability to scavenge reactive oxygen species (ROS), exerting antioxidant action (Aeschbacher et al., 2012; Scott et al., 1998). Structural reorganization of hybrid samples is strongly dependent on water absorption kinetics of HA, which are influenced by their nature as well as by their supramolecular arrangement (Kučerík et al., 2012). Water uptake as well as swelling kinetics of HA\_Art and HA\_Cof must be slower than that of HA\_Man due their greater hydrophobicity, as indicated by NMR data elaboration (**Table S1**) and to their different pore morphology, as evidenced by N<sub>2</sub> physisorption (**Table S2**).

Furthermore, swelling kinetics should be further slowed in hybrid nanostructures due to topological constraints of inorganic component but also to the possible covalent network formed by amidic bonds. This effect is more evident in hybrid nanostructures containing HA\_Art and HA\_Cof, due to their tighter interaction with SiO<sub>2</sub>. Overall, high hydrophobic features combined with tighter interactions with inorganic component, lead to a slower dynamic evolution in SiO<sub>2</sub>\_HA\_Art and SiO<sub>2</sub>\_HA\_Cof than in SiO<sub>2</sub>\_HA\_Man hybrid NPs, accounting for improved antioxidant activity in SiO<sub>2</sub>\_HA\_Art\_3 weeks and SiO<sub>2</sub>\_HA\_Cof\_3 weeks samples.

In the present study, HA have been combined with a biocompatible and bioactive inorganic nanostructured phase. Obtained hybrid nanostructures highlighted a relevant antioxidant activity.

At the same time,  $SiO_2$  nanoparticles have been widely explored as safe filler to improve mechanical properties (Wu et al., 2019). Furthermore, their combination

132

with bioavailable compounds, such as polyphenols, are promising to design active food packaging materials. On these bases, combining HA with SiO<sub>2</sub> component into hybrid nanostructures, is envisaged as a promising technological route for food packaging applications.

### **2.4 Conclusions**

In this study, hybrid SiO<sub>2</sub>\_HA NPs were synthetized using HA extracted from three different composted biomasses. Altogether, tests of antioxidant activity and the indepth investigation on the synthesized nanostructures unveiled the relationship between HA molecular composition and conformational stability and the structural and functional properties of obtained hybrid systems.

Experimental findings proved that ceramic templated approach can be exploited as an effective and broad synthesis strategy to manage intrinsic heterogeneity of HA mixtures, addressing poor stability issues caused by degradation and aggregation processes. Notably, it offers the chance to engineer hybrid nanoparticles starting from otherwise insoluble and poor colloidally stable HA. In fact, obtained SiO<sub>2</sub>\_HA hybrids used in this study showed uniform size distribution, good colloidal stability as well as constant or even increased antioxidant behaviour after prolonged aging.

Zeta potential, FT-IR and EPR spectroscopic evidence suggest that  $SiO_2_HA$  NPs are dynamic systems, which can undergo structural reorganization during aging in aqueous environment due to hydration process. This process improves surface exposure of HA component in the hybrid nanostructures and in consequent enhanced availability of reactive centers, thus accounting for improved antioxidant activity in aged samples.

Kinetics of structural evolution in water during aging appeared strongly dependent on the specific HA molecular and conformational nature, as well as to topological and chemical constraints imposed by inorganic component. In fact, they were slower for larger hydrophobic compositions and tighter conformations, also possibly due intermolecular crosslinking driven by multilayered adsorption on inorganic surfaces or even by possible covalent bridging with inorganic

134

components. Such conformational rearrangement involves a change in the reciprocal distance between reactive groups, as shown in EPR results.

These findings shed light on the molecular features that drive the interactions between inorganic and organic components and the conformational dynamics of the hybrid systems, providing a useful contribution to clarify the interacting dynamics between HA and inorganic nanoparticles which control their fate in ecosystem.

Hence, this study contributes to the development of strategic guidelines to exploit the useful HA molecular properties to turn recycled biomass into a wealth technology.

## 2.5 Supplementary information



### 2.5.1 Antioxidant activity: Fox and DPPH assays

Figure S1. Residual  $H_2O_2$  versus time for SiO<sub>2</sub>\_HAs measured at different  $H_2O_2$  concentrations:  $25\mu M$  (A), 12.5  $\mu M$  (B), 10  $\mu M$  (C).  $H_2O_2$  calibration curve is reported in D. All the experiments were run in triplicate (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; \*\*\*\*p < 0.0001).






Figure S2. Residual  $H_2O_2$  versus time for SiO<sub>2</sub>\_HAs\_3weeks hybrids measured at different  $H_2O_2$  concentrations:  $25\mu M$  (A),  $12.5 \mu M$  (B),  $10 \mu M$  (C). All the experiments were run in triplicate (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; \*\*\*\*p < 0.0001).



Figure S3. Antioxidant activity also of Trolox



Figure S4. Antioxidant activity of SiO<sub>2</sub>\_HA\_NPs evaluating using DPPH assay. All the experiments were run in triplicate (ns: not significant; \*\*\*\*p<0.0001)

#### 2.5.2 Determination of NPs Yield and HA binding efficacy

НА	NPs Concentration (mg/ml)	NPs Nominal Concentration (mg/ml)	NPs Yield %	BE %
HA_Art	4.7	13.8	34	50
HA_Cof	4.7	13.8	34	50
HA_Man	1	13.8	7	18

Table S1. NPs concentration, NPs Yield and binding efficacy (BE)

### 2.5.3 NMR Analysis



Figure S5. <sup>13</sup>C CPMAS NMR Spectra of HA\_Man (A), HA\_Art (B) and HA\_Cof (C) samples

Sample	Carboxyl-	Phenol-	Aryl-C	O-alkyl-	C-0/C-	Alkyl-C	HB/HI*
	С	С		С	Ν		
	190-160	160-145	145-110	110-60	60-45	45-0	
	ppm	ppm	ppm	ppm	ppm	ppm	
HA_Man	5.30	3.38	13.40	46.56	13.79	17.57	0.52
HA_Art	9.26	5.70	19.37	37.50	12.43	15.75	0.69
HA_Cof	11.89	3.22	13.36	22.73	14.26	34.55	1.05

HB/HI\*= hydrophobicity index= $\sum[(0-45) + (110-160)]/\sum[(45-60) + (60-110) + (160-190)]$ 

**Table S2.** Relative Distribution (%) of Signal Area over Chemical Shift Regions (ppm) in <sup>13</sup>C–CPMAS-NMR Spectra of HAs extracted from Different Composted biomasses

Chapter 3- Hybrid humic acids-based nanoparticles: from the design to the functional properties



*Figure S6.* <sup>13</sup>*C*-*CPMAS NMR spectra of HA\_Man (A), HA\_Art (B) and HA\_Cof (C) after dispersion in non-filtered (blue) and filtered distilled water (red)* 



Figure S7. Size distribution of hybrid SiO2\_HA NPs (SiO2: 211.46  $\pm$  29.21 nm; SiO2\_Man: 84.55  $\pm$  13. 53 nm, SiO2\_Cof: 34.31  $\pm$  3.50 nm, SiO2\_Art: 32.84  $\pm$  2.77 nm)

## 2.5.4 TGA Analysis

The TGA curves of bare HAs (**Figure S8 A,B,C** black curves) show a first weight loss below 200 °C (10wt%) related to physical water desorption and a second weight loss starting from 200 °C, attributed to oxidative decomposition of both labile and recalcitrant components. For HA\_Man and HA\_Art (**Figure S8 A and C**, black curves) an 94% total loss in weight was estimated, while HA\_Cof (**Figure S8 B**, black curve) was completely degraded at about 600 °C. The TGA curves of SiO<sub>2</sub>\_HAs show a low temperature weight loss (about 7%) in the range of 25–200 °C, related to the removal of physically adsorbed water. A second weight loss was observed in the range of 250-600 °C for all SiO<sub>2</sub>\_ HAs and was attributed to the decomposition of organic components, including HA fractions, entrapped organic solvents and aminopropyl moieties in the nanoparticles.



**Figure S8.** Thermogravimetric profiles of bare HA, fresh and aged hybrids for manure (B), artichoke (C) and coffee (D). The weight loss curve of nude SiO2\_NPs is reported in A. TGA plots were obtained from replicates of different independent experiments, which were carried out on samples obtained from different synthesis processes

		junchonal properties
Sample	Organic content %	HA content %
SiO <sub>2</sub> _NPs	$10.0 \pm 0.1$	/
SiO <sub>2</sub> _HA_Man / SiO <sub>2</sub> _HA_Man_3	$25.0 \pm 1.0$	$15.0 \pm 1.0$
weeks		
SiO <sub>2</sub> _HA_Art / SiO <sub>2</sub> _HA_Art_3weeks	$18.8 \pm 0.4$	8.8 ± 0.4
SiO <sub>2</sub> _HA_Cof / SiO <sub>2</sub> _HA_Cof_3weeks	$16.7 \pm 1.0$	$6.7 \pm 1.0$

Chapter 3- Hybrid humic acids-based nanoparticles: from the design to the functional properties

Table S3. Organic material content in each sample

## 2.5.5 FT-IR Analysis

Bands (cm <sup>-1</sup> )	Assignments
3700-2500	Phenolic -OH hydroxyl groups
3000-2800	Asymmetric -CH <sub>2</sub> stretching
3000-2800	Symmetric -CH <sub>2</sub> stretching
1760-1580	C=C bond in aromatics and olefins,
	carboxyl C=O bond, ketone and quinone groups
1508	Ring vibration moes of ortho-substituted aromatic compounds
1460-1400	OH of the phenols, COO– and -CH <sub>3</sub> bending vibration mode
1280-1020	Stretching vibration of C-O and C-O-R structures
600	Out-of-plane bending vibration of aromatic -CH groups

Table S4. Assignment of FTIR bands of HA

# **2.6 References**

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# **3.** Hybrid humic acid/titanium dioxide nanomaterials as highly effective antimicrobial agents against gram(-) pathogens and antibiotic contaminants in wastewater

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# **3.1 Abstract**

Humic acids (HAs) provide an important bio-source for redox-active materials. Their functional chemical groups are responsible for several properties, such as metal ion chelating activity, adsorption ability towards small molecules and antibacterial activity, through reactive oxygen species (ROS) generation. However, the poor selectivity and instability of HAs in solution hinder their application. A promising strategy for overcoming these disadvantages is conjugation with an inorganic phase, which leads to more stable hybrid nanomaterials with tuneable functionalities. In this study, we demonstrate that hybrid humic acid/titanium dioxide nanostructured materials that are prepared via a versatile in situ hydrothermal strategy display promising antibacterial activity against various pathogens and behave as selective sequestering agents of amoxicillin and tetracycline antibiotics from wastewater. A physicochemical investigation in which a combination of techniques was utilized, which included TEM, BET, <sup>13</sup>C-CPMAS-NMR, EPR and DLS shed light on the structure-property-function relationships of the nanohybrids. The proposed approach traces a technological path for the exploitation of organic biowaste in the design at the molecular scale of multifunctional nanomaterials, which is useful for addressing environmental and health problems that are related to water contamination by antibiotics and pathogens.



# **3.2 Introduction**

The waste-to-wealth concept aims at encouraging a future sustainable lifestyle in which waste and biowaste valorisation is considered not only for its intrinsic environmental benefits but also to prompt the development of new technologies and jobs. To help rethink planetary well-being in terms of a circular economy, the main objective is to promote strategies for enhancing the economic and environmental value of biowaste through its recycling or conversion (Xu et al. 2019). Biowaste can be drastically transformed, either chemically, physically or biologically, into a plethora of end-use products and functional novel materials via several approaches.

In this context, humic acids (HAs) are an alkali-soluble fraction of natural organic matter, which are usually found in water, soil and sediments. They play central roles in sustaining plant growth and controlling both the fate of environmental pollutants and the biogeochemistry of organic carbon in global ecosystems (Maccarthy, 2001; Piccolo et al., 2019; Stevenson et al., 1994). They can be obtained via biomass conversion through natural biological transformations and are unavoidable co-products in biorefinery processes (Luo et al., 2019; Spaccini et al.,2019). Additionally, HAs are considered a promising and inexpensive source of novel materials for technological applications (de Melo et al., 2016; Dutta et al., 2019; Peña-Méndez et al., 2005; Perminova et al., 2019). HAs are attracting substantial interest as heterogeneous organic molecules due to their carbogenic diversity and tuneable redox properties, thereby providing an important source of inspiration for novel redox-active materials (de Melo et al., 2016; Nuzzo et al., 2013). Indeed, HAs present various functional groups in their chemical structures, such as quinone, phenol, carboxyl and hydroxyl moieties, which are directly responsible for regenerable redox properties (de Melo et al., 2016; Klüpfel et al., 2014). These properties enable HAs to generate or scavenge reactive oxygen

species (ROSs) (Aeschbacher et al. 2012; de Melo et al., 2016; Scott et al. 1998), thereby leading to various properties, such as antibacterial, antioxidant and antiinflammatory activities (van Rensburg et al. 2000; de Melo et al., 2016). Furthermore, they can easily act as metal chelating agents (Li et al., 2020) due to their amphiphilicity, which determines their self-assembly (Klavins et al., 2020; Nuzzo et al., 2013; Piccolo, 2001), and they can interact with contaminants (Afzal et al., 2019; Du et al., 2020; Liu et al., 2020). Considering these properties, HAs are expected to play a leading role in green chemistry as an accessible and inexpensive source for the design and development of multifunctional materials for potential bio-sustainable applications. HAs from North Dakota leonhardite (NDL) have attracted interest due to their low cost and marked amphiphilicity (Cozzolino et al., 2002; Nuzzo et al., 2016). The latter is due to the large amounts of both hydrophobic and hydrophilic functionalities within the humic components, such as fatty and phenolic residues (Drosos et al., 2017; Nebbioso et al., 2011).

Despite the substantial potential that is offered by bioavailable HA-like materials, several challenges remain to be overcome, which are related mainly to their poor selectivity and high instability (browning) under aerated conditions (Allard et al., 1994). Although intense research has focused on the production of radicals and trapping reactions of low-molecular-weight natural phenol-like derivatives, much less attention has been given to their possible exploitation in materials science. Moreover, although many studies on interactions between HAs and pollutants have been proposed, the difficulty in distinguishing between soluble and insoluble HAs often hinders the assessment of their adsorption features (Afzal et al., 2019; Du et al., 2020). HAs have shown high solubility in aqueous media, thereby preventing the possibility of efficiently removing pollutants from aqueous environments. HAs are controversial moieties since their interactions with nanomaterials and organic pollutants can increase the solubility and bio-persistence of these species in

aqueous solutions (de Melo et al., 2016). Consequently, the non-negligible chemical lability and heterogeneous self-assembly behaviour of HAs strongly prevent the full exploitation of these intriguing biomacromolecules, which are often regarded as an environmental issue rather than a valuable resource due to their abundance and their function as carriers of pollutants. In this context, a promising strategy for overcoming these disadvantages was proposed based on the conjugation of HA-organic moieties onto a foreign matrix that consisted of either organic or inorganic materials (de Oliveira et al., 2016). Inorganic nanoparticles have been highlighted as attractive bio-interfaces due to their biocompatibility and their tuneable size, shape and porosity with functional versatility and, therefore, are valid candidates for the realization of multifunctional nanomaterials with modulable properties (de Oliveira et al., 2016; Heuer-Jungemann et al., 2019; Luchini et al., 2019). Recently, following a bioinspired approach, we proposed a novel synthetic strategy for producing hybrid nanostructures, whereby a titanium dioxide (TiO<sub>2</sub>) sol acts as a catalyst and templating agent for 5,6-dihydroxyindole-2-carboxylic acid (DHICA) polymerization to eumelanin (Pezzella et al., 2013; Vitiello et al., 2015; Vitiello et al., 2016). The inorganic phase has a tuned eumelanin supramolecular structure, which leads to TiO<sub>2</sub>-eumelanin hybrids with unique antimicrobial properties that are sensibly better than the intrinsic melanin biocide activity (Vitiello et al., 2015; Vitiello et al., 2017; Vitiello et al., 2018). The combination of biomacromolecules and inorganic components into a hybrid nanostructure at the molecular scale has proven to be an extremely effective strategy for enhancing the intrinsic properties of the organic phase and selectively driving its function (Silvestri et al. 2019; Vitiello et al. 2019). On this basis,  $TiO_2$ can be proposed as a biomimetic templating agent for controlling and modulating the stability and reactivity of HAs, including the ROS production ability, the sequestering capability towards metal cations and organic pollutants and the size-

selective absorption features (de Melo et al., 2016; Tang et al., 2014). Additionally, due to the amphiphilicity of their supramolecular structure (Piccolo, 2001), HAs can favourably interact with an inorganic surface charged phase through electrostatic forces and can confer satisfactory colloidal stability in aqueous media (Pota et al., 2020; Zhang et al., 2020).

In this paper, hybrid humic acid/titanium dioxide nanostructures were designed and prepared through an *in situ* method that was based on the hydrothermal wet chemistry approach. Then, the functionality of these novel nanohybrids was investigated, and two main applications for water remediation were considered: i. antibacterial activity against gram-negative and gram-positive pathogens of various strains and ii. sequestering action towards three antibiotics, namely, amoxicillin, tetracycline and metronidazole, in an aqueous solution. To shed light on the relationship between the structural and functional features of the nanohybrids, a wide physicochemical investigation was conducted via a combined approach of various techniques, such as transmission electron microscopy (TEM), X-ray diffraction (XRD), thermogravimetry (TG), BET porosimetry, dynamic light scattering (DLS), Fourier-transform infrared (FT-IR) spectroscopy, solid-state cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance (13C-CPMAS-NMR) spectroscopy and electron paramagnetic resonance (EPR) spectroscopy. These analyses enable the investigation of the effects at the molecular scale of the combination of HA and TiO<sub>2</sub> components in defining the structural, surface and paramagnetic properties of the final TiO<sub>2</sub>/HA-NDL nanohybrids and in modulating both ROS generation for killing bacteria and the removal of antibiotics from wastewater.

Overall, this study defines a potential strategy for converting environmental challenges that are posed by the interaction of HAs with organic pollutants and inorganic nanoparticles into a technological opportunity through the design of

hybrid multifunctional nanomaterials. This approach offers the opportunity to match the objectives of giving biowaste a new life and managing water quality through pharmaceutical removal and pathogen disinfection, thereby addressing current environmental and health concerns.

# 3.3 Results and discussion

#### 3.3.1 Physicochemical features of the TiO<sub>2</sub>/HA-NDL nanohybrids

The formation of hybrid nanomaterials was verified by evaluating the presence of both inorganic and organic components. XRD patterns of the TiO<sub>2</sub>/HA-NDL nanohybrids primarily provided information about the properties of titanium dioxide. For all nanohybrids, the diffraction patterns showed the typical profile of a standard anatase lattice (JCPDS 84-1286) in terms of both peak positions (diffraction angles  $2\theta$ ) and relative intensities, as demonstrated by comparison with the pure anatase  $TiO_2$  XRD pattern (Fig. 1A) (JCPDS, International Centre for Diffraction Data, 1998). In contrast, HA-NDL was amorphous, and its partial contribution was detected by the presence of a broad halo around the most intense peak in the XRD profile of each TiO<sub>2</sub>/HA-NDL nanohybrid. These results demonstrated the formation of crystallized TiO<sub>2</sub> with a mean crystalline size of approximately 6 nm in all samples, as determined by the Scherrer equation; thus, during the hydrothermal treatment, the growth of TiO<sub>2</sub> was not hindered by the addition of HAs. Subsequently, a TG analysis was conducted to assess the organic component amount within the samples. The TG curves of the nanohybrids showed a weight loss in the range of 250-400 °C, which corresponded to the main decomposition of HA according to the TG curve of bare HA (as reported in Fig. **S1A**). This supported the presence of HAs, and their final content in the samples was measured in the range of 3.5 to 6.0 wt.% and depended on the HA amount that was used during the synthesis. These small changes among the samples could be due both to the presence of a saturated amount of HA that was conjugated with TiO<sub>2</sub> (TiO<sub>2</sub>/HA-NDL2 sample) and to the simultaneous presence of triethylamine, which was used as a capping agent, on the  $TiO_2$  surface during hydrothermal

synthesis, as demonstrated by the weight loss in the TG curve of the bare  ${\rm TiO_2}$  nanostructures.



*Fig. 1.* XRD spectra (Panel A) and <sup>13</sup>C-cross polarization magic angle spinning NMR spectra (Panel B) of pure HA-NDL (a), bare TiO<sub>2</sub> (b), and TiO<sub>2</sub>/HA-NDL\_0.1 (c), TiO<sub>2</sub>/HA-NDL\_0.2 (d), TiO<sub>2</sub>/HA-NDL\_1 (e), and TiO<sub>2</sub>/HA-NDL\_2 (f) nanohybrids.

The conjugation of HA-NDL with TiO<sub>2</sub> within the hybrid materials is also supported by FTIR and solid-state NMR analyses. The FTIR spectra of TiO<sub>2</sub>/HA-NDL showed a typical band in the 3700-3000 cm<sup>-1</sup> region, which is typical of -OH stretching vibrations. The bands in the range of 2800 to 3000 cm<sup>-1</sup> were attributed to C-H symmetric and asymmetric stretching vibrations of alkyl groups. The bands in the region 1580-1760 cm<sup>-1</sup> could be related to C=C bonds in aromatics and olefins, to carboxyl C=O bonds, and to ketone and quinone moieties (Fig. S1B, Table S1). A band at approximately 1400 cm<sup>-1</sup> was attributed to phenol -OH, COOand CH<sub>3</sub> bending vibration modes (Fig. S1 and Table S1) according to previous results (Pota et al., 2020). Another band can be distinguished in the range of 1280-1020 cm<sup>-1</sup>, which corresponds to the stretching vibration modes of phenolic C-O and aliphatic OH. Indeed, stretching of phosphate groups (PO<sub>4</sub>) could contribute to this band; this is supported by the presence of a sharp band at 620 cm<sup>-1</sup>, which was assigned to PO<sub>4</sub> bending. Typical bands of HAs were distinguished in the FTIR spectra of hybrid TiO<sub>2</sub>/HA-NDL nanostructures (Fig. S1), which were similar to the bands in the FTIR spectrum of bare HA-NDL, thereby supporting the presence of HAs in the samples. These bands appear at higher frequency in comparison to the FTIR spectrum of bare HA, which may be due to the increase in the force constant that was caused by complexation with Ti(IV) ions (Guan et al., 2007). Moreover, the FTIR spectra of both bare  $TiO_2$  and the  $TiO_2/HA-NDL$ nanostructures showed a clear band at 1621 cm<sup>-1</sup>, which can be assigned to the N-H bending vibration of the primary amine, thereby suggesting the presence of residual triethylamine (Fig. S1B).

<sup>13</sup>C-CPMAS spectra that were acquired for TiO<sub>2</sub>/HA-NDL samples were compared with spectra for the bare HA-NDL and TiO<sub>2</sub>. As shown in **Fig. 1B**, the HA-NDL NMR spectrum was principally characterized by three resonance regions (spectrum a) that were centred at i. ~170 ppm, which is typical of the carboxyl groups; ii. ~130 ppm, which was attributed to aromatic moieties, and iii. ~30 ppm, which was attributed to alkyl carbons. In the TiO<sub>2</sub>/HA-NDL spectra (**Fig. 1B, c-f**), the presence of humic acid was mainly proven by the appearance of the carboxyl group signal and, to a lesser extent, by aromatic carbon resonance. The additional signal in the 10-30 ppm range was ascribed to triethylamine that remained partially adsorbed on the surface of the hybrid nanostructures, which was also observed in the bare TiO<sub>2</sub> spectrum b of **Fig. 1B**. Therefore, NMR spectroscopy indicated the conjugation of titanium dioxide with humic acid.

A morphological analysis of the TiO<sub>2</sub>/HA-NDL samples was conducted via TEM. As shown in **Fig. 1A-B**, the TEM images indicated the formation of small nanoparticles of ~20 nm in size with a rod-like structure. These TiO<sub>2</sub>/HA-NDL nanoparticles appeared slightly smaller than the bare TiO<sub>2</sub> nanoparticles (~30-35 nm), which were prepared via the same synthesis route in the absence of HAs, as shown in Fig. 3C-D, and according to our previous studies (Vitiello et al., 2015; Vitiello et al., 2017). However, the presence of HA-NDL during TiO<sub>2</sub> growth seems to modulate the hierarchical organization of the TiO<sub>2</sub>/HA-NDL nanoparticles. This led to disordered hybrid clusters with an average size of ~200 nm (**Fig. 2A**) in comparison to the larger irregular aggregates that were obtained for the bare TiO<sub>2</sub> sample (**Fig. 2C**). This difference can be ascribed to the close interconnection at the molecular scale between the two components, in which TiO<sub>2</sub> nanoclusters/primary particles were likely held together by the HA-NDL molecules.



Fig. 2. TEM images of hybrid TiO<sub>2</sub>/HA-NDL (A, B) in comparison with bare TiO<sub>2</sub> (C,D) nanostructures.

Subsequently, the surface properties of the  $TiO_2/HA$ -NDL hybrids were investigated by obtaining N<sub>2</sub> adsorption isotherms, as shown in **Fig. 3A**. They were classified as Type IVb, which is typical of mesoporous materials that show capillary condensation that is accompanied by hysteresis on the desorption branch (Venezia et al., 2020). The values of the specific surface area (BET method) and total pore volume for each sample are reported in **Table 1**. The specific surface area of the reference TiO<sub>2</sub> sample was 169 m<sup>2</sup> g<sup>-1</sup> according to previous evidence

(Vitiello et al., 2015; Vitiello et al., 2017), while all samples that were prepared via HA-NDL addition showed larger values, which reached a maximum of approximately 240 m<sup>2</sup> g<sup>-1</sup> for samples with intermediate HA-NDL contents (TiO<sub>2</sub>/HA-NDL 0.2 and TiO<sub>2</sub>/HA-NDL 1). The slight decrease in the surface area of the  $TiO_2/HA$ -NDL 2 sample with respect to the others could be explained by the addition of a larger amount of organic matter in the reaction solution, which created a larger fraction of HA moieties on the surfaces of the primary colloidal TiO<sub>2</sub> nanoparticles. This caused an increase in the number of negative surface charges, thereby limiting the particle assembly and influencing the growth of hybrid materials, which were therefore characterized by a smaller surface area (Pota et al., 2020). In addition, as presented in Table 1, the changes in the surface areas of the hybrid materials with respect to that of bare TiO<sub>2</sub> nanoparticles were accompanied by a slight decrease in the total pore volume of the samples. The HA-NDL organic component on its own was characterized by a small specific surface area and a small pore volume. The pore size distribution analysis in **Fig. 3B** clearly shows that HA-NDL addition caused a significant decrease (~20 Å) in the average diameter of the mesopores.

A narrow, monomodal pore size distribution that extended from 20 to 60 Å with a sharp maximum centred at ~45 Å was exhibited by all TiO<sub>2</sub>/HA-NDL samples, in contrast to the broader distribution of the reference TiO<sub>2</sub> sample, which extended from 20 Å up to 100 Å and had a smaller peak at ~70 Å. This result seems to support the key role of HAs in modulating the growth and the consequent structural organization of TiO<sub>2</sub>-based hybrid nanostructures by modifying their final surface area and porosity.



Fig. 3. N<sub>2</sub> adsorption isotherms at 77 K (panel A) that correspond to TiO<sub>2</sub> (a), TiO<sub>2</sub>/HA-NDL\_0.1 (b), TiO<sub>2</sub>/HA-NDL\_0.2 (c), TiO<sub>2</sub>/HA-NDL\_1 (d) and TiO<sub>2</sub>/HA-NDL\_2 (e) and BJH pore distribution curves (panel B) for bare TiO<sub>2</sub> and TiO<sub>2</sub>/HA-NDL nanohybrids at various compositions.

Sample	Surface area (m²/g) (± 7%)	Pore volume (cm <sup>3</sup> /g) (± 0.02)	Average pore diameter (Å) (± 10%)
HA-NDL	15	0.02	-
TiO <sub>2</sub>	169	0.30	66
TiO <sub>2</sub> /HA- NDL_0.1	210	0.25	48
TiO <sub>2</sub> /HA- NDL_0.2	240	0.27	43
TiO <sub>2</sub> /HA-NDL_1	244	0.29	48
TiO <sub>2</sub> /HA-NDL_2	227	0.26	43

Table 1 Surface area and pore properties of HA-NDL, TiO2 and hybrid TiO2/HA-NDL nanostructures.

Exploiting the intrinsic paramagnetic properties of HA components, EPR measurements were also conducted on the  $TiO_2/HA$ -NDL samples by following an experimental approach that was already tested for the characterization of polyphenols and/or melanin-like materials (Cesareo et al., 2012; Panzella et al., 2018; Vitiello et al., 2015; Vitiello et al., 2017). The analysis of the EPR signal

provides significant information regarding the characteristics of the paramagnetic centres and the supramolecular properties of the organic moieties that constitute the TiO<sub>2</sub>/HA-NDL nanohybrids.

A similar line shape was observed for all EPR spectra in **Fig. 4A**, namely, a single, roughly symmetric signal at a g value of  $2.0031 \pm 0.0003$ , which is typical of carbon-centred radicals of HAs and similar polyaromatic molecules (Jezierski et al., 2000; Panzella et al., 2018), with a spin-density concentration that depends on the amount of HA-NDL that was used during the synthesis (Table 2). Upon closer inspection of the spectra, no differences in the spectral line shape among the TiO<sub>2</sub>/HA-NDL samples were observed, and the line shape was highly similar to that of pure HA-NDL. This finding was supported by the quantitative determination of the signal amplitude,  $\Delta B$ , which is related to the linewidth of the EPR peak, as detected from the experimental spectra (as shown in Fig. 4A). Typically, the linewidth of an EPR peak depends on the relaxation time of spinning electrons and is affected mainly by the unresolved hyperfine interactions between unpaired electrons and neighbouring atoms (Buszman et al., 2006). A broad line shape is due to the superposition of multiple signals that are associated with the presence of various carbon-based paramagnetic species. The narrowing of the line shape is related to the presence of stronger electron-electron interactions and can be associated with a more locally ordered organization of the polyaromatic structures that host the unpaired electrons. Hence, this parameter is usually considered indicative of the mean distance between the radical centers.



**Fig. 4.** EPR spectra (panel A) and power saturation curves that were obtained by plotting the normalized amplitude  $(A/A_0)$  values versus the square roots of microwave power intensities (P) of free radicals (panel B) in HA-NDL powder (a and  $\bullet$ ) and TiO<sub>2</sub>/HA-NDL\_0.1 (b,  $\blacktriangle$ ), TiO<sub>2</sub>/HA-NDL\_0.2 (c,  $\blacksquare$ ), TiO<sub>2</sub>/HA-NDL\_1 (d,  $\blacktriangleright$ ) and TiO<sub>2</sub>/HA-NDL\_2 (e,  $\blacklozenge$ ) nanostructures. The flat EPR spectrum of bare TiO<sub>2</sub> is reported for reference (f).

Sample	g-factor	ΔB/G	Lorentzian %	$spin \times g^{\text{-}1} \times 10^{16}$
HA-NDL	2.0031	5.6	35%	5.80
TiO <sub>2</sub> /HA-NDL_0.1	2.0032	5.6	15%	0.19
TiO <sub>2</sub> /HA-NDL_0.2	2.0031	5.5	20%	0.50
TiO <sub>2</sub> /HA-NDL_1	2.0031	5.6	23%	0.88
TiO <sub>2</sub> /HA-NDL_2	2.0032	5.5	20%	1.70

**Table 2** EPR spectral parameters of the obtained TiO<sub>2</sub>/HA-NDL hybrid nanostructures. The estimated experimental uncertainties are  $\pm 0.0003$  for the g-factor,  $\pm 0.2$  G for  $\Delta B$  and  $\pm 10\%$  for the spin-density.

As reported in **Table 2**, the determined  $\Delta B$  values are 5.6 ± 0.2 G for all samples; thus, the structural organization of the organic moiety was not perturbed in the nanohybrids. Then, EPR spectra of each sample were recorded by increasing the incident microwave power. The power saturation curves were obtained by plotting the normalized peak amplitude (A/A<sub>0</sub>) as a function of the square root of the microwave power (P), as shown in **Fig. 4B**. A non-monotonic trend with a decrease

in amplitude for microwave powers,  $P^{1/2}$ , of higher than 4 mW was observed for bare HA-NDL; thus, free radicals presented long relaxation times and were homogeneously located in the sample. In contrast, a monotonic increase was observed for TiO<sub>2</sub>/HA-NDL systems; hence, all free radical spins did not exhibit the same relaxation behaviour and saturated independently. This result demonstrates that the radical centres present a chemically inhomogeneous distribution, which is likely associated with the presence of aromatic moieties that were conjugated with Ti(IV) ions on which the unpaired electrons were stabilized. This suggests possible chemical changes due to the combination with the inorganic component during nanohybrid synthesis. The occurrence of this phenomenon was also supported by the Lorentzian (and Gaussian) contribution in the line shape of the recorded spectra, which was estimated by determining the best fitting curves of each EPR signal and using them to analytically discriminate the relative contributions. An increase in the Gaussian character of the EPR signals for all TiO<sub>2</sub>/HA-NDL nanohybrids was observed, which supports the presence of more radical centres than in HA-NDL with relaxation behaviours that differ from that in HA-NDL (Table 2).

In consideration of these paramagnetic properties of the TiO<sub>2</sub>/HA-NDL hybrid nanostructures, their ability to generate ROSs in aqueous environments was investigated via the EPR spin-trapping method. This is a useful method for detecting the formation of radical species with extremely high chemical reactivity, which typically have lifespans that are too short for direct detection (Davies et al., 2016; Villamena et al., 2004). These reactive free radicals are put in contact with a suitable amount of a DMPO spin-trap to extend the lifetimes of their spin-adducts, which can be monitored via EPR spectroscopy at room temperature. Following this approach, an initial analysis was conducted to preventively verify that DMPO in an aqueous environment did not produce any interfering signal (**Fig. 5a**). Then, an

EPR signal that was constituted by a quartet with a 1:2:2:1 intensity ratio (Fig. 5b) was detected for the supernatants of all nanohybrid suspensions, which corresponded to the DMPO-OH adduct that formed from the trapping of •OH radicals by the DMPO molecule. This finding was supported by the quantitative analysis results of the spectrum, according to which the hyperfine coupling constants for the nitroxide nitrogen and for the  $\beta$ -proton were  $a_N = a_{H}^{\beta} = 14.8 \pm 0.2$ G. These values are consistent with those reported in the literature for the DMPO-OH adducts (Dvoranová et al., 2014; Pirozzi et al., 2020), thereby supporting the formation of •OH radical species. No significant differences were detected in the EPR spectra that corresponded to the TiO<sub>2</sub>/HA-NDL nanohybrids, and no signal of DMPO- adducts was appreciated in the spectra of neat  $TiO_2$  or HA-NDL. This result suggests that the synergistic combination of HA-NDL and TiO<sub>2</sub> within hybrid nanostructures plays a key role in the ROS-generating ability of the final nanohybrids. HAs undergo reversible redox reactions, and under aerated conditions, semiquinone moieties are further oxidized, which causes electron transfer to  $O_2$  and, ultimately, the formation of •OH radicals (Pirozzi et al., 2020; Vitiello G. et al., 2018). This process could be more significant in  $TiO_2/HA-NDL$ nanostructures, which have larger exposed surface areas than bare HA-NDL. In addition, it can be assumed that catechol moieties within the HA backbone can act as ligands for Ti<sup>4+</sup> ions, thereby forming a ligand-to-metal charge transfer complex (LMCTC), which could cause ROS production by the  $TiO_2$  component in hybrid nanostructures, even upon visible light irradiation (Vitiello G. et al., 2016). This evidence underlines the key role of the molecular combination of the organic and inorganic components in improving the capability of the hybrid nanomaterials to generate radical species, in contrast to the behavior that is exhibited by the commonly designed humic acid-coated TiO<sub>2</sub> nanoparticles (Lin D. et al. 2012; He X. et al. 2016), which showed a drastic decrease in the ability of  $TiO_2$  to produce •OH or  $\bullet O_2^-$  radicals, thereby limiting their use in ROS-mediated applications (antimicrobial agents).

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Fig. 5. EPR spectra of a DMPO aqueous solution (a) and in the presence of TiO<sub>2</sub>/HA-NDL hybrid nanostructures (b) without light irradiation.

DLS experiments were conducted to evaluate the colloidal stability and the selfassembly behaviour of the TiO<sub>2</sub>/HA-NDL nanohybrids in aqueous environments. DLS measurements provide information about the sizes of the hybrid nanostructures. Since all suspensions were dilute, the Stokes–Einstein equation was used to evaluate the hydrodynamic radius,  $R_H$ , of the aggregates from their translation diffusion coefficient, *D*:

$$r_{H} = \frac{kT}{6\pi\eta_{0}D} \tag{1}$$

where k is the Boltzmann constant, T is the absolute temperature and  $\eta_0=0.89 \ cP$  is the solvent viscosity. For non-spherical particles,  $r_H$  represents the radius of a spherical aggregate with the same diffusion coefficient (Benoit, 1997; Pecora, 2003; Roscigno et al., 2001; Luchini et al., 2016). As shown in **Fig. 6**, a monomodal distribution curves for all samples were obtained via DLS analysis, which suggest the presence of a single population of hybrid nanostructures in an

aqueous environment with a mean  $r_H$  of between 80-85 ± 5 nm and a polydispersity index of approximately 0.5. In contrast, a larger average hydrodynamic radius (150 ± 5 nm) was observed for the sample that was prepared in the presence of the lowest HA-NDL amount, namely, TiO<sub>2</sub>/HA-NDL\_0.1, which suggests that the use of a small amount of the organic component during the synthesis favored the formation of larger nanostructures. Moreover, this sample was not highly stable in the aqueous medium, and it exhibited fast aggregation and precipitation. These results demonstrate the key role of HAs in modulating the agglomeration process of TiO<sub>2</sub> nanostructures, which is directly related to the presence of aromatic moieties in the HA matrix, thereby leading to a stronger stabilization effect (Zhao et al., 2019).



**Fig. 6.** Hydrodynamic radius distribution functions that were obtained at 25 °C and 90° via DLS measurements for hybrid TiO<sub>2</sub>/HA-NDL nanostructures that were prepared with various HA-NDL/titanium isopropoxide molar ratios: TiO<sub>2</sub>/HA-NDL\_0.1 (a), TiO<sub>2</sub>/HA-NDL\_0.2 (b), TiO<sub>2</sub>/HA-NDL\_1 (c) and TiO<sub>2</sub>/HA-NDL\_2 (d)

#### 3.3.2 Antibacterial activity of the TiO<sub>2</sub>/HA-NDL nanohybrids

The antibacterial activity of the TiO<sub>2</sub>/HA-NDL nanostructures was evaluated using four strains of gram-negative bacteria (*E. coli DH5a, E. coli* ATCC35218, *K. pneumonia* and *P. aeruginosa*) and two strains of gram-positive bacteria (*E.* 

*faecalis* and *S. aureus*). These strains were chosen due to their high diffusion in water and healthcare environments and their ability to develop strong resistance to common antibiotics. The experimental results are reported in **Fig. 7**. First, the bare  $TiO_2$  nanostructures are not active against all the bacterial strains (as reported in **Fig. S2**).

The TiO<sub>2</sub>/HA-NDL nanostructures were tested at various concentrations that ranged from 10 to 1000  $\mu$ g·mL<sup>-1</sup> to evaluate the possible influence of the concentration of the nanosystems on the survival of bacterial strains without irradiation. As shown in **Fig. 7**, an increasing trend of the antimicrobial activity with the nanoparticle concentration was observed against both *Escherichia coli DH5a* and *Escherichia coli* ATCC 35218. The observed biocide activity as a function of the HA-NDL content differed among the nanohybrids: the TiO<sub>2</sub>/HA-NDL\_0.1 sample showed satisfactory activity up to 50  $\mu$ g·mL<sup>-1</sup>, while the samples (0.2, 1 and 2) that were prepared with higher HA-NDL contents exhibited moderate activity already at the lowest concentration of 10  $\mu$ g·mL<sup>-1</sup> up to the maximum of 1000  $\mu$ g·mL<sup>-1</sup>.





**Fig. 7.** Antibacterial activities of the TiO<sub>2</sub>/HA-NDL\_0.1 (red columns,  $\blacksquare$ ), TiO<sub>2</sub>/HA-NDL\_0.2 (blue columns,  $\bullet$ ), TiO<sub>2</sub>/HA-NDL\_1 (purple columns,  $\blacktriangle$ ) and TiO<sub>2</sub>/HA-NDL\_2 (green columns,  $\bullet$ ) hybrid nanostructures at increasing concentrations against gram-negative bacterial strains Escherichia coli DH5a, Escherichia coli ATCC 35218, Klebisella pneumoniae ATCC 700503, and Pseudomonas aeruginosa ATCC 27355 and against gram-positive Staphylococcus aureus ATCC 5538P. The error bars indicate the standard error (n=3); the standard deviation was less than 5%.

Moreover, our results demonstrate that the best antibacterial performance was realized by the TiO<sub>2</sub>/HA-NDL\_2 sample, which was prepared with the highest content of HAs at all tested concentrations, except for the 200 and 1000  $\mu$ g·mL<sup>-1</sup> samples, for which no significant differences were identified between TiO<sub>2</sub>/HA-NDL\_1 and TiO<sub>2</sub>/HA-NDL\_2. As also shown in **Fig. 7**, pronounced activity was observed for the TiO<sub>2</sub>/HA-NDL samples against the *Pseudomonas aeruginosa* 

ATCC 27355 and Klebisella pneumoniae ATCC700503 strains of gram-negative bacteria. For these strains, the hybrid nanostructures showed a marked biocidal activity from 50  $\mu$ g·mL<sup>-1</sup>, and in these cases, the biocide activity of the TiO<sub>2</sub>/HA-NDL samples increased as a function of the HA-NDL content. Finally, antibacterial activity tests were conducted against the *Staphylococcus aureus* ATCC 5538P and *Enterococcus faecalis* ATCC 29212 strains of gram-positive bacteria. A low activity against *Staphylococcus aureus* ATCC 5538P was observed since the biocide effect of the nanohybrids became observable at a concentration of 100  $\mu$ g·mL<sup>-1</sup> (**Fig. 7**). No activity was detected against *Enterococcus faecalis* (data not shown). According to these results, the TiO<sub>2</sub>/HA-NDL nanohybrids exhibited lower biocide efficacy against gram-positive bacteria.

The antibacterial results are in accordance with the EPR results, according to which the spin-density values increase with the HA content. This finding supports the hypothesis that the large amount of free electrons that were stabilized in the organic structures that were combined with TiO<sub>2</sub> at the molecular scale were responsible for the generation of •OH in the bacterial cell environment, which is also supported by the results of EPR spin-trapping experiments, thereby producing biocidal activity through ROS-mediated cell damage (Nain et al., 2020; Vitiello et al., 2018). Indeed, HAs have been proven to interact with aquatic bacteria (Tikhonov et al., 2010) to promote ROS action against pathogens.

The antibacterial results that were obtained *in vitro* seem to suggest that the biocidal efficacy of the TiO<sub>2</sub>/HA-NDL nanohybrids depended strongly on the HA content (Huang et al., 2020; Rainer et al., 1978; van Rensburg et al., 2000), which directly contributed to the increase in the relative amount of generated •OH species, and on the concentration of nanohybrids that were in contact with the various bacterial cultures. However, the differences in behaviour between the gramnegative and gram-positive strains towards nanostructured materials are most likely
due to the differences in composition and structural organization of the external bacterial membranes. This experimental evidence supports the hypothesis of a synergistic role of the inorganic and organic components in determining the antibacterial activity of humic acid-based materials, even under visible light irradiation. Indeed, it is demonstrated that humic substances can promote bacterial growth (Tikhonov et al., 2010), and other studies have demonstrated high efficacies of humic materials, such as oxifulvic and humic acids, against microbial pathogens (van Rensburg et al., 2000), for example, by hindering the growth of Staphylococcus aureus and Candida albicans or decreasing the number of colonies in Escherichia coli and Salmonella enteritidis (Yarkova et al., 2011). Consequently, the antibacterial activity of HAs is related to the extraction method and to the molecular composition of the humic acid, which depends on the natural source from which it was obtained (Nebbioso and Piccolo, 2013). Therefore, the combination of these highly reactive substances with an inorganic phase provides a chemical strategy for modulating their chemical functionalities to obtain alternative bioinspired materials with enhanced biocide activity.

#### 3.3.3 Antibiotic sequestering activity

The capability of TiO<sub>2</sub>/HA-NDL hybrid nanostructures to sequester the three antibiotics, namely, amoxicillin, tetracycline, and metronidazole, was investigated by conducting HPLC analysis on the supernatants. These supernatants were separated via centrifugation from suspensions that contained a mixture of each antibiotic with TiO<sub>2</sub>/HA-NDL nanohybrids, as previously described for the adsorption of biomolecules by porous nanomaterials (Califano et al., 2018; Stan et al., 2017). The behaviour of the nanohybrids was also compared with that of bare TiO<sub>2</sub> nanoparticles. It was not possible to monitor the performance of neat HA-NDL in sequestering the three antibiotics because HA-NDL showed a high

solubility in the aqueous medium and a very low tendency to precipitate via centrifugation, thereby preventing antibiotic separation from the supernatant. The HPLC analysis showed that metronidazole was not sequestered by the TiO<sub>2</sub>/HA-NDL nanohybrids since the antibiotic concentration remained unaffected, which suggests a possible selectivity of these hybrid nanomaterials in antibiotic removal. A similar behaviour was also observed for bare TiO<sub>2</sub>. The HPLC results demonstrate satisfactory sequestering by all nanohybrids of both amoxicillin and tetracycline molecules, and these nanohybrids outperform TiO<sub>2</sub> in sequestering these antibiotics. Small residual amounts were detected in aqueous environments, as reported in **Table 3**. Increased selectivity towards tetracycline was observed for all TiO<sub>2</sub>/HA-NDL nanohybrids, while the best performance in sequestering both antibiotics was detected for the TiO<sub>2</sub>/HA-NDL\_1 system. Antibiotic removal occurs essentially through an adsorption mechanism that is based on electrostatic interactions and on complex formation with discrete sites in humic acid (Sibley et al., 2008).

Sample	Amoxicillin wt% residue	Tetracycline wt% residue	
	(±5%)	(±5%)	
TiO <sub>2</sub>	28.2	9.6	
TiO <sub>2</sub> /HA-NDL_0.2	25.4	10.2	
TiO <sub>2</sub> /HA-NDL_1	8.50	< 5.0	
TiO <sub>2</sub> /HA-NDL_2	12.7	13.5	

**Table 3** Residual percentages of amoxicillin and tetracycline antibiotics that were determined after their contact with TiO<sub>2</sub>/HA-NDL hybrid nanostructures in aqueous environments.

Thus, the high activities of the  $TiO_2/HA$ -NDL samples towards the removal of antibiotics could be due to the larger surface areas of the nanohybrids compared to that of bare  $TiO_2$  and, concurrently, to the presence of pores in the hydrated hybrid nanostructures with sizes that are compatible with the molecular dimensions of the

investigated antibiotics. Water uptake of HA moieties could play a key role in influencing the organization of their supramolecular structures, which undergo swelling (Kučerík et al., 2011). This phenomenon is expected to produce larger pore sizes that can host organic molecules. Both surface features, which are due to the presence of HAs and their combination with the inorganic phase at the molecular scale, induced increased sequestration behaviour in the final hybrid nanosystems. Ultimately, the obtained results agree with recent reports on the application of humic acid-based nanomaterials in the removal of pesticides and antibiotics (Mahmoud et al., 2020; Song et al., 2019; Xu et al., 2019). This opens new opportunities for the development of hybrid biomass-derived nanomaterials for pharmaceutical removal (Phoon et al., 2020) and, thus, contributes to solving one of the most prominent environmental problems that is faced in public health.

### **3.4 Conclusions**

This study establishes a synthetic approach that transforms environmental issues that are linked to the interactions of HAs with organic pollutants and inorganic nanoparticles into a technological tool. Hybrid multifunctional humic acid/titanium dioxide nanomaterials have been designed and prepared via solvothermal synthesis, which show substantial promise for environmental applications. The combination at the molecular scale of humic acids from North Dakota leonardite, HA-NDL and the inorganic  $TiO_2$  phase confers high sequestering efficiency towards amoxicillin and tetracycline antibiotics and enhances significant ROS-mediated biocide properties against many pathogenic gram-negative bacteria. Our findings demonstrate the feasibility of the proposed strategy for the design of hybrid nanomaterials with improved and tuneable properties. Moreover, the multiple applications of these nanomaterials offer a versatile approach for usefully employing molecules from either geochemical deposits or recycled biomasses. This strategy provides a technological route to biowaste valorisation and concurrently addresses environmental and health problems that are linked to antibiotic and pathogen water contamination in the furrow of a circular economy to enhance the quality of life.



#### 3.5 Supporting information

Fig. S1 TG (A) and FTIR (B) spectra of pure HA-NDL, bare TiO2 and TiO2/HA-NDL hybrid nanostructures.



Fig. S2. Antibacterial activity of bare TiO<sub>2</sub> nanoparticles as function of the increasing concentration on the following Gram-negative bacterial strains: Escherichia coli DH5a, Escherichia coli ATCC 35218, Klebisella pneumoniae ATCC 700503, Pseudomonas aeruginosa ATCC 27355. Error bars indicate standard error (n=3); standard deviation was less than 5%

Bands (cm <sup>-1</sup> )	Assignments		
3377	Phenolic -OH hydroxyl groups		
2921	Asymmetric -CH <sub>2</sub> stretching		
2849	Symmetric -CH <sub>2</sub> stretching		
1583	C=C bond in aromatics and olefins,		
	carboxyl C=O bond, ketone and quinone groups		
1375	OH of the phenols, COO– and -CH $_3$ bending vibration mode		
1113	C-O and C-O-R structures		
616	Out-of-plane bending vibration of aromatic -CH groups		

Table S1 Assignment of FTIR bands of HA-NDL

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### 4. Exploiting bioderived humic acids: a molecular combination with ZnO nanoparticles leads to nanostructured hybrid interfaces with enhanced prooxidant and antibacterial activity.

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### 4.1 Abstract

The waste-to-wealth strategy is encouraging the design of a plethora of new valueadded materials, by exploiting the chemical and biological richness of biowastes. Humic Acids (HA) are mostly intriguing because of their amphiphilic supramolecular associations which are responsible for several assets, such as adsorption ability towards small molecules, metal ion chelation, redox behavior, and antibacterial activity. The molecular combination of HA with semiconductor nanoparticles represents a valuable strategy to obtain nanostructured hybrid materials and interfaces with advanced features. Concurrently, it permits to overcome intrinsic limits of such organic fraction, including poor stability, fast conformational dynamics, or rapid reactivity in aqueous media. Herein, hybrid HA/ZnO nanoparticles are synthetized through a bottom-up strategy, exerting an improved pro-oxidant behavior by generating Reactive Oxygen Species, even without light irradiation, favoring an enhanced photocatalytic and antimicrobial activity against different bacterial pathogens. Several techniques, including TEM, DLS, XRD, FTIR, TGA/DSC, and EPR, allows to define the structure-propertyfunction relationships, thus highlighting the crucial role of a fine conjugation amongst the metal oxide precursor and bioderived fraction to drive the pro-oxidant activity. This study provides strategic guidelines to easily produce low-cost organoinorganic nanomaterials with redox and biocide properties, aimed at coping environmental and health issues.



### 4.2 Introduction

The waste-to-wealth concept encourages a future sustainable lifestyle in which waste and biowaste valorization is imperative for the development of closed-loop waste free processes at the core of circular economy [1]. Biowastes (BWs) represent a largely available source of chemical richness and can be chemically, physically, or biologically transformed into added-value compounds and materials [2]. Among BWs, Humic Acids (HA) are heterogeneous mixtures of relatively small molecules (<1000 Da) which are produced by biological and chemical conversion of biomasses through natural transformations [3,4]. They are proposing as intriguing and inexpensive sources of eco-sustainable multifunctional materials for a huge number of applications including medicine, packaging, and environmental applications [2,5,6]. Indeed, HA are rich in functional groups, such as quinone, phenol, carboxyl and hydroxyl moieties, which confer them many useful functional properties, including organic pollutants absorption, metal ions chelation and regenerable red-ox behavior in terms of antioxidant, antimicrobial and anti-inflammatory activity [5,7-13], which can be exploited for different technological applications within a circular economy framework [14].

In this context, nanotechnology can offer strategic solutions to promote the upgrading of BWs into useful functional materials [2,15]. More specifically, the opportunity to combine bio-derived organic moieties at a molecular level with semiconductor nanoparticles represents a valuable strategy for enhancing the intrinsic properties of both the inorganic and organic components and selectively driving their function [16–19]. At the same time, this approach should permit to overcome some intrinsic limits of such organic fraction, such as poor stability, fast conformational dynamics, or rapid reactivity in aqueous media which strongly brings down its full valorization as it is. In this way, we have recently proposed synthetic strategies for producing organo-inorganic nanomaterials, in which

eumelanin pigments [16,17] as well as polyphenol-like moieties from different sources [16,17,20] are combined with an inorganic matrix (i.e.,  $TiO_2$  or  $SiO_2$ ) leading to hybrid nano-bio-materials with good physicochemical stability in aqueous solution, even boosting functional properties such as antioxidant and/or antibacterial behavior [16,17] as well as sequestering capability towards organic pollutants [17,21].

Among the semiconductor metal oxides, zinc oxide (ZnO) nanoparticles are particularly interesting thanks to a wide versatility because of non-toxicity, biocompatibility, low-cost and, above all, good optical, photocatalytic [22–24], and antimicrobial [25,26] properties, which favor the involvement in several technological fields. Many synthetic methods are proposed to prepare these nanoparticles; however, sonochemical [27], solvothermal [28], hydrothermal [29,30], sol-gel [31], and mechanochemical [32] ones can lead to some disadvantages ascribable to low stability and self-aggregation phenomena. Even though nanoparticle surface coating with specific amphiphilic moieties contributes to avoid these issues, it does not consequently induce a real upgrading of the physicochemical and functional features. On the other hand, synthetic approaches based on the intimate mixing of inorganic and organic precursors should lead to nanostructured hybrid materials and interfaces with novel, peculiar and enhanced assets, which are directly determined by the molecular combination.

In this work, we design and synthetize novel multifunctional hybrid Humic Acid/Zinc Oxide (HA/ZnO) nanoparticles through a bottom-up approach under solvothermal condition to finely combine at molecular level the precursor of semiconductor component with the bio-derived HA from North Dakota Leonhardite (HA\_LEON), selected by the virtue of low cost and high accessibility as well as a marked amphiphilicity [11,17,33] related to the large amounts of both hydrophobic and hydrophilic chemical functionalities [34,35]. This approach

promotes a supramolecular design of the hybrid interfaces, based on the association of two or more chemical species and on the formation intermolecular bonds between them [36]. The functional properties of hybrid HA/ZnO nanoparticles are tested in terms of redox behavior (i.e., anti-oxidant and pro-oxidant), photocatalytic degradation of Rhodamine B (as model dye molecule) and biocide activity against different bacterial pathogens. An extensive combination of complementary techniques, including Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS). X-Rav Diffraction (XRD). Thermogravimetric Analysis/Differential Scanning Calorimetry (TGA/DSC), Fourier Transform InfraRed (FTIR) and Electron Paramagnetic Resonance (EPR) lead to define the structure-property-function relationships for HA/ZnO nanoparticles due the intimate conjugation between both organic and inorganic components.

Overall, this study provides strategic guidelines to design and prepare organoinorganic nanomaterials with improved redox and antimicrobial properties, favoring the valorization of organic molecules from biowastes, contributing at coping both environmental and health issues.

### 4.3 Results and Discussion

#### 4.3.1 Morphology and size distribution in aqueous medium

The morphological features of bare ZnO and hybrid HA/ZnO nanoparticles were determined by TEM analysis, as reported in **Figure 1.** TEM micrographs indicate that both types of ZnO-based nanoparticles were characterized by an irregular morphology and an average size ranging between 150 and 200 nm. No significant difference in the shape of hybrid HA/ZnO nanostructures look as they are less dense than bare ZnO ones. However, hybrid HA/ZnO seems to have less density than bare ZnO nanoparticles, probably because HA wrapped the surface of preformed primary inorganic particles before their further aggregation, acting as a structuring agent for inorganic phase.



Figure 1 - TEM images of bare ZnO (Panels A) and hybrid HA/ZnO (Panels B) nanoparticles and histograms of particle sizes (Panels C and D).

Moreover, the statistical analysis of nanoparticles size carried out on TEM images (**Figure 1**) indicated that a bimodal size distribution was obtained for bare ZnO sample suggesting the presence of two populations of nanoparticles with a mean diameter lower than 200 nm and larger than 240 nm, respectively. On the other hand, hybrid nanomaterial showed a single population ascribable to the presence of nanoparticles with an average diameter of about 170-180 nm. These results suggested that the presence of HA\_LEON in the reaction mixture drove the growth of ZnO-based nanoparticles, acting as templating agent which tuned the final size and favored a dimensional uniformity. However, HA\_LEON was not sufficiently able to control the shape of the hybrid nanoparticles, which resulted comparable to neat ZnO.

DLS analysis was performed to investigate the colloidal behavior of both bare ZnO and hybrid HA/ZnO interfaces in aqueous environment. As first, by comparing the autocorrelation functions of both samples as shown in **Figure 2A**, it could be observed that the curve of hybrid nanomaterial started to decay at lower values than those corresponding to bare ZnO, suggesting the formation of smaller nanoparticles than neat ZnO because of HA presence in the reaction batch. At the same time, the autocorrelation function of HA/ZnO sample tends to reach a plateau condition over time differently to the one of naked ZnO which showed a scarce colloidal stability and a high tendency to self-aggregate and, consequently, to form clusters.



Figure 2 - Correlation functions (A) and distribution curves (B) of bare ZnO and HA/ZnO hybrid nanoparticles in aqueous medium.

By a quantitative point of view, the hydrodynamic radius distribution of ZnO (**Figure 2B**, curve a) indicated a polydisperse system with the presence of two populations: the first was centered at 98  $\pm$  14 nm, while the second one was centered at about 400 nm, suggesting the presence of nanoparticle clusters of larger size. Conversely, HA/ZnO sample (**Figure 2B**, curve b) showed a monodisperse system with a narrow hydrodynamic radius distribution centered at 87  $\pm$  3 nm, which was clearly ascribable to the presence of a single population of uniform nanoparticles. The DLS analysis confirmed the results of microscopy analyses, indicating that the molecular combination of HA\_LEON to ZnO played a key role in tuning the growth and final size of the hybrid nanoparticles, conferring an improved colloidal stability because of reduced aggregation and precipitation phenomena.

#### 4.3.2 Chemical and structural properties

The formation of the hybrid nanomaterial as well as its chemical and structural features were verified by evaluating the presence and the main characteristics of both inorganic and organic components. First, XRD profiles were recorded to obtain information about the crystalline phases of zinc oxide. The pure HA\_LEON had a completely amorphous character [17], while both ZnO and HA/ZnO nanoparticles presented a crystalline structure (**Figure 3A**) corresponding to wurtzite, which is typical of the hexagonal crystal structure of zinc oxide [37,38].



Figure 3 - XRD profiles (A) and FTIR spectra (B) of bare ZnO and HA\_LEON, and hybrid HA/ZnO nanoparticles.

In particular, the mean crystallite size evaluated by Scherrer's equation was almost 69 nm, whereas it increased to about 84 nm in the case of HA/ZnO. A deep analysis of HA/ZnO pattern showed a splitting of the diffraction peaks for each crystalline plane (Figure 3A, red curve), which suggested the presence of two different nanoparticle populations exhibiting the same crystalline structure. This feature could be the result of the adsorbed organic molecules on the surface of the crystal [39]. This structural evolution could be ascribable to the close interaction of the aromatic moieties of HA with the zinc oxide primary particles occurring during the first phase of the synthetic route. Specifically, the crystallite size of ZnO in the HA/ZnO nanocomposite was higher than that of bare ZnO, probably because HA diffused to the surface of preformed ZnO crystallites and, consequently, adsorbed on the primary inorganic particles before their further assembly. The adsorption process of HA moieties on primary particles resulted in a highly negatively charged surface which probably hampered their aggregation due to electrostatic repulsions [20,40]. The conjugation of HA\_LEON to ZnO was also corroborated by FTIR spectrum of HA/ZnO sample is reported in the **Figure 3B** (red curve). Firstly, the band in the region 3700-3000 cm<sup>-1</sup> appears larger due to the presence of more -OH

hydroxyl moieties, as well as the bands at around 1580 cm<sup>-1</sup> and at 1400 cm<sup>-1</sup> become more intense due to C=C of aromatic and olefins and -OH of the phenols in organic component. Furthermore, ZnO stretching band at about 540 cm<sup>-1</sup> slightly shifts at lower wavenumber, suggesting a structural reorganization of inorganic component due to the addition of humic acid [16]. These results suggested that the aromatic moieties within HA supramolecular structure acted as ligands for  $Zn^{2+}$  ions, leading to the formation of a covalent complexation between HA and  $Zn^{2+}$  ions. Consequently, HA can be considered as a structuring agent of the inorganic phase, tuning the size and structure of the final hybrid system.

TGA measurements were carried out to assess the amount of organic component within the samples (**Figure 4A**), while the DSC analysis was realized to evaluate the thermal features of nanoparticles. TGA curve of HA/ZnO sample showed two greater weight loss in the ranges 750-900 °C and 150-700 °C, corresponding to the main decomposition of HA\_LEON in agreement with TGA curve [17]. According to the procedure described at the section 2.3.7, the specific amount of HA within hybrid HA/ZnO nanoparticles was estimated to be about 20 w/w%.



Figure 4 - TGA profiles (A) and DSC curves (B) of HA/ZnO hybrid nanoparticles, bare ZnO and pure HA\_LEON.

More in detail, **Figure 4B** shows the DSC curves of the samples HA\_LEON (orange), ZnO (blue) and HA/ZnO (red). Oxidative decomposition of HA\_LEON

(Figure 4B, orange curve) occurs in the temperature range 230–700 °C, as already indicated by TGA curve [17], through several thermo-oxidation steps, corresponding to different exothermic peaks and appreciated through slope changes in TGA curve. The first two peaks, at about 300 °C and 350 °C, are due respectively to the decomposition of aliphatic, catechol and phenol groups, in which carbon is bound to oxygen. In the range between 440 °C and 480 °C, there are two other peaks due to the oxidative decomposition of aromatic and carboxylic groups. A further exothermic peak related to oxidation and polycondensation of the hydrophobic aromatic component of the humic substance is observed at about 650 °C [41].

The DSC curve relative to the hybrid nanostructure, compared to the DSC curve of bare ZnO, highlights two broad peaks around 350 °C and 440 °C, in the temperature decomposition of HA, thus demonstrating the presence of humic acid in the nanostructures. In particular, the two peaks, corresponding to the oxidative decomposition of the aliphatic groups and of the aromatic and carboxylic groups, are found at higher temperatures than those evidenced in the spectrum of pure HA\_LEON. This suggests a better thermal stability of HA in the nanohybrids, thanks to the combination, at molecular scale, with inorganic matrix. In addition, the absence of the exothermic peak at 670 °C in the DSC of hybrid sample confirmed that only some fractions of HA were entrapped into the inorganic matrix during *in situ* synthesis [16]. Particularly, it can be assumed that most of the highly hydrophobic fraction of aromatic components were lost in suspension, as suggested by the marked decrease of the corresponding DSC band (T=430 °C), also because of the hydrophilic nature of ZnO nanoparticles.

#### 4.3.3 Paramagnetic properties

Exploiting the intrinsic paramagnetic properties of HAs and polyphenols-like materials [16,17,20], EPR measurements were recorded and furnished significant information about the nature (directly) and the structural organization (indirectly) of the paramagnetic centers localized on the organic moiety of HA, also within the hybrid nanomaterial. In Figure 5A, the EPR spectra of bare ZnO and hybrid HA/ZnO nanoparticles were recorded in a wide range of the magnetic field. In both cases, a large and not-intense signal centered at g-factor value of 1.9565±0.0003 was detected, which corresponded to bulk  $Zn^{2+}$  specie and typical of crystalline zinc oxide nano-powders [42,43]. In addition, the spectrum of hybrid nanoparticles showed a second intense and narrow signal at a greater g-value, ascribable to the presence of paramagnetic species belonging to HA component. In contrast, bare ZnO nanoparticles showed a very slight signal at  $g \sim 2.002$  corresponding to single ionized oxygen vacancies on the surface [44] indicating a very small presence of these defects. The nature of the second signal at the greater g-factor in the spectrum of HA/ZnO sample was analyzed by comparing it with that observed in spectrum of pure HA\_LEON. As shown in **Figure 5B**, it corresponded to the single peak centered at  $g = 2.0032 \pm 0.0003$  and typical of carbon-centered radicals found in HA and similar polyaromatic molecules [17,45]. However, some differences in the line shapes for the two sample were detected. In particular, the peak corresponding to HA/ZnO sample appeared more asymmetric and narrower than that of pure HA LEON, indicating that some chemical and structural changes were induced in the organic component because of the conjugation with the inorganic one. Specifically, this finding was supported by the quantitative determination of the signal amplitude,  $\Delta B$ , which is related to the line-width of EPR peak, directly detectable by the experimental spectra (as shown in **Figure 5A**).



*Figure 5* - *EPR* spectra of bare ZnO and hybrid HA/ZnO nanoparticles (A). Spectra (B) and power saturation curves (C) of hybrid HA/ZnO nanomaterial and pure HA-LEON.

Generally, line-width of EPR peaks depends by the relaxation time of spinning electrons and is mainly affected by the unresolved hyperfine interaction between unpaired electrons with the neighboring-atoms [46]. A broad line-shape is due to the superposition of multiple signals associated to the presence of different carbon-based paramagnetic species, while the narrowing of line-shape is related to the presence of stronger electron-electron interactions and can be associated to a more locally ordered organization of the polyaromatic structures hosting unpaired electrons. For this reason, this parameter is usually considered indicative of the mean distance between the radical centers. As summarized in **Table 1**, the  $\Delta$ B value determined for HA/ZnO sample was equal to  $4.8 \pm 0.2$  G while that of pure HA\_LEON was  $5.6 \pm 0.2$  G, confirming that the chemical and structural organization of HA was partially perturbed by the combination at molecular level with inorganic component.

Sample	g-factor (± 0.0003)	Δ <b>B</b> / <b>G</b> (± 0.2)	Gaussian %	$spin \times g^{-1}$ (<10%)
HA_LEON	2.0031	5.6	65	$5.80 imes10^{16}$
HA/ZnO	2.0032	4.8	77	$1.40 imes10^{16}$
	1.9567	10.5	-	-
ZnO	2.0024	~8.0	-	-
	1.9565	10.3	-	-

Table 1 - Spectral parameters obtained by the quantitative analysis of EPR spectra.

To clarify this behavior, EPR spectra were also recorded increasing the incident microwave power and the corresponding power saturation curves were built by plotting the normalized peak amplitude  $(A/A_0)$  as function of the square root of the normalized microwave power  $(P/P_0)$ , as represented in Figure 5C. Two different trends were obtained. A decrease in the amplitude for higher microwave powers was observed for bare HA\_LEON, indicating that free radicals presented long relaxation times and were homogeneously located in the sample. In contrast, HA/ZnO systems showed a monotonic increase indicating that the free radical spins did not exhibit the same relaxation behavior and saturated independently. This result indicates that radical centers present a chemical inhomogeneous distribution, probably associated to the presence of different aromatic moieties on which the unpaired electrons were stabilized. This suggests possible chemical changes due to the combination with the inorganic component during the nanohybrids synthesis. Such behavior was also confirmed by the estimation of the Gaussian contribution in the line shape of the recorded spectra, that was achieved by determining the best fitting curves of each EPR signal to analytically discriminate the relative contributions. An increase in Gaussian character of the EPR signal of HA/ZnO sample was also observed, confirming the presence of greater number of radical centers with different relaxation behavior than in HA LEON.

#### 4.3.4 Redox activity

Anti-oxidants are involved in several bioactivity mechanisms, such as inhibition of free radical generation, enhancement of scavenging capacity against free radicals, and activation of reducing power. In the case of humic matter, it has been conceived the presence of many different anti-oxidant sites which can be exposed in relation to humic supramolecular assemblies [47,48].

The results of anti-oxidant tests on hybrid HA/ZnO nanoparticles are reported in Table S1. DPPH assay highlighted that bare ZnO and hybrid HA/ZnO systems had the same anti-oxidant activity after having been in contact with the radical DPPH. Instead, HA LEON has already at low concentration (0.5 mg/mL) an antioxidant activity equal to about 57%. Similarly, the results of ABTS assay showed that the UV-Vis absorbance inhibition due to HA/ZnO and bare ZnO nanoparticles was 34.5 and 33.7%, respectively, whereas that of HA LEON was 72.4%. Additionally, antioxidant activity measured as TEAC (mmol of Trolox equivalent kg<sup>-1</sup> of sample) values confirmed that hybrid nanoparticles showed an antioxidant activity equal to ZnO, with a value of 10.7 and 10.4 respectively, while HA LEON showed the best antioxidant performance with a value of 40.1. In all cases, the antioxidant capacities were compared by applying a one-way analysis of variance (ANOVA) and the means (n = 3) were validated applying Tukey's range test at the 0.05 significance level using XLSTAT (Addinsoft, New York, USA). Although the complex supramolecular structures of HA were characterized by functional groups, like quinones and phenolic, that act as natural antioxidants [49], in the hybrid HA/ZnO nanoparticles, they were mainly located in the inner core of the hybrid nanomaterial, as evidenced by surface and morphological characterization and not fully-exposed to the water interface, thus limiting the antioxidant activity [16]. The behavior of hybrid HA/ZnO nanoparticles in the presence of DMPO was

investigated before and after light irradiation at 365 nm and compared to that of bare ZnO ones under the same conditions. As described in the preparation section, the supernatants of both HA/ZnO and ZnO suspensions were recuperated by centrifugation and 20  $\mu$ L of each one was analysed. Different results were obtained in dependence of the nanoparticles composition and light irradiation, as shown in **Figure 6**, while no signal of DMPO adducts was appreciated in the spectra of neat HA\_LEON.



**Figure 6** - EPR spectra of the aqueous supernatants containing DMPO-adducts after the contact with bare ZnO (a) and hybrid HA/ZnO (b) nanoparticles before (panel A) and after (panel B) irradiation at  $\lambda = 365$  nm.

In the absence of irradiation, no significant signal appeared in the spectrum of pure ZnO nanoparticles (Figure 6A, a) indicating that no ROS species were generated, while a complex multi-signal spectrum was obtained for HA/ZnO sample (Figure 6A, b). It was ascribable to the co-existence of different radical species: first, a signal composed by a peak quartet with a 1:2:2:1 intensity ratio and corresponding to the typical signal of DMPO-OH adduct formed because of the trapping of •OH by DMPO molecule. This finding was also supported by the quantitative analysis of the spectrum, indicating the hyperfine coupling constants for the nitroxide nitrogen and for the  $\beta$ -proton,  $a_N = a^{\beta}_H = 14.8 \pm 0.2$  G, in agreement with the literature [50,51]. A second signal consisted of a 1:1:1 triplet was also detected. This identified the DMPO-H adduct [52] signifying the formation of •H radicals starting from the water ionization. The obtained results were ascribable to the occurrence of competitive reactions between the spin-trap and hydroxyl or hydrogen radicals, indirectly indicating that DMPO molecules in solution were not fully involved in sequestering •OH species which formed only in a relatively small amount. These results proved that the synergistic combination at the molecular level of organic and inorganic components produced hybrid HA/ZnO nanoparticles with improved the generation of ROS in the dark. Notably, on the basis involving interactions of

polyphenol-based moieties with metal oxide semiconductors, it can be inferred that during the synthesis, the aromatic moieties proper of HAs acted as ligands for Zn<sup>2+</sup> ions as ligands for Zn<sup>2+</sup> ions, as also indicated by FTIR spectra, indicating a covalent complexation of HA with  $Zn^{2+}$  ions. This led to the formation of a ligandto-metal charge transfer complex (LMCTC) which was the main responsible of ROS production. Moreover, as previously described, HA undergo reversible red-ox reactions and, under aerated conditions, semiquinones moieties would further oxidize causing an electron transfer to  $O_2$  ultimately forming the •OH radicals [17,51,53]. Instead, after the irradiation at  $\lambda = 365$  nm, EPR spectra of both hybrid HA/ZnO and bare ZnO nano-systems showed the presence of the quartet signal typical of DMPO-OH adduct, indicating the generation of •OH radical species. These became thought a well-known mechanism by which the electron-hole pair was generated from the semiconductor ZnO by excitation of electrons from the appropriate band levels through absorption of UV or visible light, and these electrons-holes produced ROS on interaction with water [44]. However, the signal shown in the spectrum of hybrid HA/ZnO nanomaterial was clearly characterized by a greater intensity with respect to that of bare ZnO, suggesting a superior tendency of hybrid nanoparticles in generating •OH radicals. This result was also confirmed by the appearance of some additional not well resolved peaks in the spectrum of supernatant after the contact with bare ZnO, which possibly corresponded to DMPO-H adducts. Definitely, the spin-trapping measurements confirmed the improved ability of hybrid HA/ZnO nanoparticles in generating ROS before and after light irradiation, thus confirming their enhanced pro-oxidant activity.

In addition, the photocatalytic activity of bare ZnO and hybrid HA/ZnO nanoparticles was also investigated by monitoring the degradation of rhodamine B (RhB), which belongs to stable fluorescent xanthene, with the aim to evaluate the

pro-oxidant ability of the prepared nanomaterials [54]. After the ZnO or HA/ZnOmixed RhB solution was irradiated with ultraviolet (UV) light for a maximum time of 60 min, the solution became paler in color, and its absorbance decreased, as reported in **Figure 7**. According to the variations in absorption peaks and area under the curve, the RhB photodegradation with HA/ZnO nanoparticles was faster than that with bare ZnO ones. On the contrary, pure RhB irradiated under UV light didn't degrade. These results supported the enhanced photoactivity of hybrid nanoparticles due to the presence of HAs which probably improved the electron transfer, as confirmed by the greater tendency to generate ROS, thereby intensifying the performances in Advanced Oxidative Processes (AOPs).



A



*Figure 7 -* Absorption curves of RhB degradation by ZnO and HA/ZnO nanoparticles under UV irradiation for different times: 0, 15, 30 and 60 min (A). Trends in the absorbance peaks of the RhB curves at each UV exposure time point (B). Areas under the absorption curves at each time point (C).

#### 4.3.5 Antimicrobial activity

Antibacterial efficacy of HA/ZnO nanoparticles were measured against two strains of gram-negative bacteria (E. coli and P. aeruginosa) and two strains of grampositive bacteria (E. faecalis and S. aureus). These different bacterial cells were chosen due to their ability to develop strong resistance to common antibiotics [55,56]. The experimental results are reported in Figure 8. The HA/ZnO nanomaterial were tested at two different concentrations (1 and 2 mg mL<sup>-1</sup>) to evaluate the possible influence of the concentration of the nanoparticle on the survival of microorganisms. First, bare ZnO nanoparticles showed a good antibacterial action against both Gram-positive and Gram-negative bacteria and against spores, in accordance with previous studies [57]. However, the antimicrobial activity of ZnO nanoparticles was lower than that of pure HA\_LEON and hybrid HA/ZnO nanomaterial, as shown in Figure 8. This result was in agreement with the literature indicating that the antibacterial activity of pure ZnO nanoparticles in the dark is mainly attributed to the dissolution of  $Zn^{2+}$  ion or the internalization of NPs leading to cell death [44]. Consequently, the antibacterial activity of nano ZnO is strongly depended by the: i. size, ii. presence of surface

defects, and iii. the light irradiation, which favor the production of ROS as dominant mechanism for bacterial cell death due to the induced oxidative stress [44]. On the other hand, in the case of HA/ZnO nanoparticles, the best antimicrobial performance was observed against all Gram-positive bacterial strains (S. aureus and E. faecalis) and for E. coli by an inhibition zone of 8.2 and 7.3 mm, respectively. Conversely, a low antimicrobial activity was found for P. aeruginosa with an inhibition alone of 7.3 mm. These results agreed with previous ones reported for hybrid HA/TiO<sub>2</sub> nanoparticles [17] suggesting that, if the intrinsic antibacterial activity of HA depends on their molecular composition, extraction method and the natural resource [48,58-61], the biocide performances of hybrid HA/ZnO nanoparticles depended on the concentration of nanoparticles used in the treatment of different bacterial cultures [62,63]. Moreover, the antibacterial activity was strikingly related to the generation of •OH radicals which was favored by the conjugation of ZnO between ZnO and humic acids, as demonstrated by EPR spintrapping experiments, thus proposing as the main responsible for the enhanced antibacterial activity even also in the dark [44].



*Figure 8 -* Antibacterial activity of pure HA\_LEON, bare ZnO and HA/ZnO hybrid nanomaterials at two different concentrations (1 and 2 mg/mL) against Gram-negative and Gram-positive bacterial strains. The error bars indicate the standard error (n = 3); the standard deviation was less than 5%.

To this regard, several works suggested that phenolic groups inside humic substances (HSs) can directly exert significant antimicrobial performances against Gram-positive than Gram-negative bacteria [64]. Although the action mechanism is not yet fully understood, it was hypothesized that the presence of a higher amount of carboxylic and phenolics groups in HSs could increase the number of hydrogen bonds with cell bacterial membranes [59,64,65]; this higher affinity should induce irreversible modifications in its lipid structure [53], thus reducing wall integrity, increasing permeability to ROS and enhancing their action, with a consequential reduction of pathogenic strains growth and a bacterial cells death. Indeed, oxidative cellular damage or oxidative stress can occur when the regular balance in the living organisms between the generation and the detoxification of ROS (pro-oxidant vs anti-oxidant ability) was altered and, consequently, the viability of thcells would decrease. In particular, •OH radicals can attach to cell walls, thus causing a cell destruction through the rapid release of potassium ions or the oxidization and degradation of intracellular CoA enzyme [66,67]. On the other hand, the different behavior against Gram-negative and Gram-positive bacterial strains should be due to the different mechanisms of action, such as oxidative-stress induced by ROS, release of  $Zn^{2+}$  ions interacting with the genetic material, membrane dysfunction, nanoparticle penetration, interruption, and blockage of transmembrane electron transport [68,69], which could exert a dissimilar level of resistance to the action of ROS and Zn<sup>2+</sup> ions as well as to the internalization of nanoparticles causing the cells death [68–70]. The strength of each effect is hardly related to the different composition and structural organization of bacterial outer membranes: they could exert a dissimilar level of resistance to the action of ROS and Zn<sup>2+</sup> ions as well as to the internalization of nanoparticles which can cause irreversible damages leading to the disintegration of the membrane and, finally, to the cells death [68–70].
#### **4.4 Conclusions**

In this work, novel functional hybrid HA/ZnO nanoparticles were designed and synthetized through a bottom-up approach under solvothermal condition, with which the molecular precursor of the semiconductor component was mixed with humic acids from Leonhardite in North Dakota to achieve a chemical conjugation that simultaneously drives the growth and formation of hybrid nanomaterials. The effects of this molecular combination were evaluated by defining the morphological, structural, surface, and paramagnetic properties of the final hybrid HA/ZnO nanoparticles, which displayed an improved colloidal stability associated with a very low tendency to precipitate quickly. The distinctive chemical and physicochemical features of the obtained hybrid interfaces lead strongly to prooxidant behavior, by exerting an improved ability with respect to bare ZnO nanoparticles in generating Reactive Oxygen Species (ROS) in aqueous environment, before and after the irradiation, proposing as light-sensitive nanomaterials for (bio)technological applications involving Advances Oxidation Processes (AOPs). At the same time, the promising antibacterial activity against different pathogenic Gram-negative and Gram-positive bacteria proposed them as skilled biocide agents to be englobed within filter systems for air disinfection, to realize antibacterial surface coatings and/or for applications in food packaging, both in the absence and presence of light. The proposed approach, aimed to respond to the growing demand for environment purification as well as to fight the bacterial resistance problem, is intertwined with the development of circular and sustainable chemistry, thus favoring the valorization of (bio)active molecules from biowaste.

Sample	% DPPH	% ABTS	TEAC (mmol equivalent TROLOX
	inhibition	inhibition	Kg <sup>-1</sup> sample)
HA_LEON	70.00±0.03	72.40±0.02	40.1±0.1
ZnO	12.00±0.05	33.70±0.05	10.40±0.04
HA/ZnO	17.00±0.02	34.50±0.02	10.70±0.03

### **4.5 Supporting Information**

Table S1. Anti-oxidant results obtained from DPPH and ABTS assays

#### 4.6 Acknowledgements

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## 5. Eco-sustainable design of Humic Acids-doped ZnO nanoparticles for UVA/light photocatalytic degradation of LLDPE and PLA plastics

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## 5.1 Abstract

The mismanagement of plastics is associated with high levels of waste and release into the environment, where they can persist for long times due to the low degradation rate. Photocatalytic methods, based on the action of Reactive Oxygen Species (ROS) generated by semiconductor materials, are promising eco-friendly and low-cost process for the plastics degradation. Here, eco-designed Humic Acids-doped/ZnO nanoparticles were synthesized via solvothermal route and tested as photocatalysts for the degradation of linear low-density polyethylene (LLDPE) and polylactic acid (PLA) thin films, under UVA/light irradiation. LLDPE is one of the most common commercial polymers widely used in packaging field wherein PLA is also broadly used to replace conventional plastics. The combination of diagnostic techniques (TEM/SEM, XRD, TGA/DSC, ATR/FTIR, EPR and DRUV) allowed to define the structure-property-function relationships of the hybrid nanomaterials and to monitor the chemical, structural and morphological changes occurring on the polymeric films as a consequence of the photodegradation. Experimental results demonstrated the validity of this eco-sustainable approach to realize hybrid photocatalysts with enhanced ROS-generating ability suitable for an improved photo-oxidation of plastics in aqueous environment.



## **5.2 Introduction**

Plastics are semi-synthetic or synthetic organic polymers widely applied in different sectors owing to their unique performance such as durability, corrosion-, chemical- and light-resistance, hydrophobicity/hydrophilicity, lightweight, high thermal and electrical insulation, mechanical properties, and relatively low cost. Despite the benefits mentioned above, plastics are considered a new type of persistent contaminants, as they can persist into the environment for long time due to their degradation resistance (Ouyang et al., 2021). Plastic products entering the environment, exposed to natural forces, such as solar light, biological organisms, redox processes, etc., are continuously fragmented until they degrade into smaller sizes becoming micro- and nanoparticles (Enfrin et al., 2020; Uheida et al., 2021) Among the most widely used polymers (Andreeßen and Steinbüchel, 2019; Rahman and Bhoi, 2021), polyethylene (PE) stands out to be one of the most diffused commercial polymers in packaging field, for its good mechanical properties (i.e., high strength, elongation), biochemical resistance (barrier properties against water-borne organism responsible for food spoilage), and easy processing (Ganji, 2016; Shehzad et al., 2014).

In the recent decades, a growing attention has addressed to compostable and biodegradable polymers, such as polylactic acid (PLA), polybutylene succinate (PBS), polyhydroxyalkanoate (PHA), In particular, PLA is a biodegradable and compostable polymer derived from renewable sources (mainly starch and sugar) that appears to be an ideal material for consumer goods and packaging (Auras et al., 2004; Castro-Aguirre et al., 2016; Luo et al., 2018; Murariu and Dubois, 2016) replacing PE in many applications.

However, the evaluation of degradation performance of biodegradable polyesters in marine environment indicated that PLA is almost unaffected by aging in simulated marine environment, exhibiting no loss of material and limited degradation

phenomena on the film surface (de Falco et al., 2021; Deroiné et al., 2014). In this scenario, there is an increasing attention towards the development of innovative strategies to treat plastic wastes and to design innovative degradation and recycling plastic processes (Federici et al., 2022). The existing approaches to manage the plastic menace are thermal, catalytic, mechanical, chemical, incineration, landfills, ozonation and photo-oxidative degradation (Canopoli et al., 2020; Murata and Makino, 1975). However, these technologies employ large amount of energy and are often very expensive. Recently, biodegradation and photocatalysis have been proposed as eco-friendly green technologies to remove plastics from the environment (Ariza-Tarazona et al., 2020; Silva et al., 2018). In particular, the solar based photocatalytic methods are capable to generate Reactive Oxygen Species (ROS) such as hydroxyl (•OH) and superoxide ( $\bullet O_2^-$ ) radicals through an interaction of a photo-semiconductor with light (Nakata and Fujishima, 2012). These species initiate the polymer degradation process, leading to molecular chain scission and finally, complete mineralization into water and carbon dioxide (Tofa et al., 2019; Zhao et al., 2007).

The major benefit of this kind of process is related to the possibility to use sunlight as a clean energy source. For this reason, one of the standpoints is to design and realize suitable catalytic materials with improved ROS generating properties, even under light irradiation, and enhanced photocatalytic performances for the chemical degradation of polymeric matrices (Goh et al., 2022). For this purpose, the synthesis of organo-inorganic materials, in which a semiconductor inorganic oxide (i.e., TiO<sub>2</sub>, ZrO<sub>2</sub> or ZnO) can be combined with an organic molecule to form ligand-to-metal charge transfer complexes (LMCTCs), represents a promising strategy leading to hybrid nanomaterials able to stabilize and/or produce ROS. Among the organic molecules to be combined with, Humic Acids (HAs) are attracting a great interest. They are alkali-soluble fraction of natural organic matter,

which are usually found in water, soil and sediments ("Humus Chemistry: Genesis, Composition, Reactions, Second Edition (Stevenson, F. J.)," 1995; MacCarthy, 2007; Piccolo et al., 2019). HAs are rich in functional groups (quinones, phenols, carboxyl and hydroxylic species) which are directly responsible for regenerable redox properties (i.e., generation and scavenging ROS). They are characterized by the presence of radical centres as delocalized electrons which can be exploited for the preparation of nanostructured photocatalysts within a circular economy framework (Venezia et al., 2021).

In the present paper, hybrid Humic Acids-doped/Zinc Oxide (HAs/ZnO) nanoparticles have been synthetized through a bottom-up approach under solvothermal condition to combine at molecular level the precursor of semiconductor (ZnO) with two different bioderived HAs, thus promoting the creation of intermolecular bonds between them Photocatalytic properties of hybrid HAs/ZnO nanoparticles have been tested in the photodegradation of LLDPE and PLA films under UVA/light irradiation at different reaction times and experimental conditions. An extensive combination of complementary techniques, including Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Differential X-Rav Diffraction (XRD). Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA), Evolved Gas Analysis (EGA), Fourier Transform Infrared (FTIR)/Attenuated Total Reflection (ATR), Electron Paramagnetic Resonance (EPR) and UV-Vis Diffuse Reflectance (DRUV) spectroscopies, led to define the structure-property-function relationships for HAs/ZnO nanoparticles as well as to monitor the changes induced into polymeric films after their use as photoactive catalysts during the degradation treatments.

## 5.3 Results and Discussion

Before the use, a physicochemical analysis of bare and hybrid HAs/ZnO nanoparticles was carried out to analyse morphological, structural and chemical properties. The main results are summarized in **Table 1**.

Sample	Organic amount	Size	Surface area	ΔΒ
	(w/w %)	(diameter, nm)	$(\mathbf{m}^2/\mathbf{g})$	(G)
ZnO	-	$152 \pm 39$	16±1	-
HASS/ZnO	1.6	$148\pm46$	$10 \pm 1$	$4.7\pm0.2$
HA/ZnO	1.0	$140 \pm 42$	$14 \pm 1$	$5.6 \pm 0.3$
HASS	100	-	-	$4.2 \pm 0.2$
НА	100	-	-	$6.9 \pm 0.3$

**Table 1.** Main physicochemical properties of hybrid HASS/ZnO and HA/ZnO nanoparticles compared withthose of bare ZnO, HASS and HA samples

#### 5.3.1 Morphological, dimensional, and surface properties

TEM micrographs of the bare and hybrid ZnO nanoparticles are shown in **Figure 1**. ZnO-based nanoparticles showed a quasi-spherical shape (**Figure 1a-c**) and an averaged diameter of about 150 nm (**Figure 11-n**).



Figure 1. TEM micrographs and size histograms of bare ZnO (a-c, l), hybrid HASS/ZnO (d-f, m) and HA/ZnO (g-i, n) nanoparticles

TEM analysis indicated that bare ZnO and HAs/ZnO nanoparticles were characterised by similar average dimension:  $152 \pm 39$  nm for ZnO nanoparticles,  $148 \pm 46$  nm for HASS/ZnO and  $140 \pm 42$  nm for HA/ZnO. ZnO and HA/ZnO nanoparticles form aggregates larger than that observed in HASS/ZnO nanoparticles (**Figure 1a,b,g,h**). The presence of HASS during the synthesis tuned the nanoparticles formation process, thus favouring a reduction of the aggregation phenomena (**Figure 1 d,e**), improving the dispersion, which are crucial in determining the final properties (Cocca and D'Orazio, 2008). Indeed, around the ZnO nanoparticles, it is also possible to observe some shadows, implying the existence of the HASS layers. Then, the surface area of all nanostructured materials was evaluated by BET analysis: bare ZnO, HASS/ZnO and HA/ZnO nanoparticles showed specific surface areas of about  $(16\pm1)$  m<sup>2</sup>/g,  $(10 \pm 1)$  m<sup>2</sup>/g and  $(14 \pm 1)$  m<sup>2</sup>/g respectively. These results confirmed that HASS induced more changes than HA in the formation and aggregation of ZnO nanoparticles, thus slightly tuning the surface properties.

#### 5.3.2 Chemical and structural properties

XRD analysis allowed to define and quantify the crystalline phases of the inorganic components. The spectra of ZnO, HASS/ZnO and HA/ZnO samples are reported in **Figure 2a**. The Humic Acids showed an amorphous character (Venezia et al., 2022a), except for some weak diffraction peaks related to the presence of some inorganic materials, typical of clay soil, while ZnO-based nanoparticles were characterised by a crystalline structure corresponding to wurtzite, which is typical of the hexagonal crystal structure of zinc oxide. (Espitia et al., 2012; Hasnidawani et al., 2016).



Figure 2. XRD profiles (a) and TGA curves (b) of bare ZnO, hybrid HASS/ZnO and HA/ZnO nanoparticles

The quantitative determination of crystallite mean sizes by Scherrer Equation indicated value of  $(69 \pm 2)$  nm for bare ZnO,  $(80 \pm 2)$  nm for HASS/ZnO and  $(61 \pm 2)$  nm for HA/ZnO. The experimental results indicated that the addition of the organic HAs precursors in the synthetic mixture did not hinder the formation of wurtzite ZnO-based nanoparticles during the solvothermal treatment but induced some slight differences in the crystallites during their growth, thus determining the

dimensions of the final nanoparticles and indirectly confirming the molecular doping of ZnO structure with HA moieties.

TGA was carried out on HASS/ZnO and HA/ZnO samples under N<sub>2</sub> conditions, allowing a quantitative determination of the weight loss to estimate the content of organic component included in the nanomaterials (Figure 2b). TGA curves showed two marked weight loss (in the ranges of 1000-500 °C, and 500-300 °C for HA/ZnO or 400-100 °C for HASS/ZnO), corresponding to the main decomposition of HAs. According to the procedure described at the section 2.5.1, the specific amount of humic acids fraction within HASS/ZnO and HA/ZnO nanoparticles was estimated to be about 1.6 and 1.0 w/w%, respectively, confirming a molecular doping of the ZnO crystalline structure. However, the difference in weight loss percentages between the two hybrid samples could be attributed to a diverse chemical behaviour of HASS and HA fractions. In particular, being the first one a sodium salt, it was better dispersed than HA in the synthesis mixture, favouring an homogeneous conjugation with ZnO nanocolloids. Indeed, the fine combination between organic and inorganic components occurred in situ because the aromatic moieties of HAs acted as ligands for  $Zn^{2+}$  ions, leading to the formation of a covalent complexation (Vitiello et al., 2021). The greater dispersion of HASS in the synthesis mixture with respect to HA should be favour the conjugation of HASS to  $Zn^{2+}$  driving the nanoparticles growth and determining some differences in the morphology, polydispersity and size, as observed by TEM.

#### 5.3.3 Paramagnetic properties and ROS-generating activity

Exploiting the intrinsic paramagnetic properties of HAs, a specific characterization was also performed by EPR spectroscopy which provided information about the nature of the paramagnetic centres and the supramolecular organization of the organic moiety within the hybrid nanomaterials. EPR spectra of hybrid HASS/ZnO

and HA/ZnO nanoparticles showed a second single and roughly symmetric signal centred at a g-factor value of about  $2.0033 \pm 0.0003$ , which was ascribable to the presence of paramagnetic species belonging to HAs moiety (Figure 3a). The nature of this signal was confirmed by the comparison with those appearing in the spectra of both pure HASS and HA powders. As shown in Figure 3a, each signal can be ascribable to the single peak typical of carbon-centred radicals found in humic substances and/or similar polyphenol-like molecules (Panzella et al., 2018; Pota et al., 2021, 2020; Venezia et al., 2022b). However, some differences in the line shapes were detected. First of all, the peak corresponding to HASS/ZnO sample appeared more symmetric and broader than that of pure HASS, while the peak corresponding to HA/ZnO resulted more asymmetric and narrower than that of pure HASS. In both cases, this evidence indicated that some chemical and structural changes were induced in the organic component because of the conjugation with the inorganic one, thus determining peculiar properties to the hybrid nanoparticles. These findings were also confirmed by the quantitative determination of the signal amplitude,  $\Delta B$ , which is related to the line-width of EPR peak, and directly detectable by the experimental spectra (as shown in Figure **3a**). Being the signal line-width dependent on the relaxation time of spinning electrons and mainly affected by the interaction between unpaired electrons with the neighbouring-atoms, this  $\Delta B$  parameter is usually considered a qualitative indication of the distance between the radical centres and the chemical heterogeneity of the radical species. Lower  $\Delta B$  values, as observed for HASS/ZnO with respect to HA/ZnO, indicated that the radical centres are at greater distances, and vice versa for higher ones. This evidence indirectly confirmed that the distribution and structural organization of the two HAs within the hybrid ZnObased nanoparticles were different, thus tuning their growth. The estimated values of  $\Delta B$  are reported in **Table 1**.



Figure 3. EPR spectra of: (a) hybrid HASS/ZnO and HA/ZnO nanoparticles compared with pure HASS and HA powders; (b) DMPO spin-adducts formed in aqueous medium in the presence of ZnO, HASS/ZnO and HA/ZnO nanoparticles after UVA/light irradiation

Finally, EPR peak of HASS/ZnO sample showed a very high intensity with respect to that of HA/ZnO one, indicating a greater amount of radical centres in the HASS fraction with respect to HA and, at the same time, confirming higher content of organic fraction in the hybrid HASS/ZnO in agreement with TG results.

Subsequently, the EPR spin-trapping method was adopted to investigate the tendency of HAs-doped/ZnO nanoparticles in generating Reactive Oxygen Species (ROS) in aqueous environment under UVA/light irradiation, as key marker of their use in photocatalytic processes (**Figure 3b**). This approach allowed to reveal the formation of extremely unstable radical species with too short lifespan, such as ROS, through a direct reaction with a specific amount of DMPO spin-trap, which lead to longer-living spin-adducts and more easily detectable by EPR spectroscopy at room temperature. A first analysis was performed to preventively check that DMPO in aqueous environment did not produce any interfering signal. Then, after UVA/light irradiation, the appearance of an EPR signal constituted by a quartet with a 1:2:2:1 intensity ratio was detected for the supernatants of bare and hybrid

ZnO-based suspensions (**Figure 3b**). This corresponded to the typical signal of DMPO-OH adduct formed from the trapping of •OH radicals by DMPO molecule, as confirmed by the values of the hyperfine coupling constants for the nitroxide nitrogen and for the  $\beta$ -proton,  $a_N = a^{\beta}_H = 14.8\pm0.2$  G. These values agreed with those reported in the literature for DMPO-OH adduct, thus confirming the generation of •OH species after UVA/light irradiation. Interestingly, the DMPO-OH spectra corresponding to both hybrid HAs/ZnO nanoparticles showed a greater relative intensity than that of bare ZnO, thus indicating an enhanced ROS-generating ability in aqueous medium after UVA/light irradiation. This effect should be ascribable to the doping of ZnO with the organic moieties already stabilizing carbon-localized free-electrons which, thanks to the fine combination at molecular scale with the semiconductor material, could improve the photocatalytic activity.

#### 5.3.4 Photodegradation of LLDPE films

SEM micrographs allow to observe the effects of the photodegradation process on the surface morphology of LLDPE films after photocatalytic tests in the absence (not shown) and presence of bare and hybrid nanoparticles (**Figure 4a-d**). If not significant morphological changes were appreciated after 75 h of wet-photolysis (data not shown), a different behaviour was observed after 225 h, also as function of the nature of nanoparticles used as photocatalysts. It is possible to observe that the surface of LLDPE after 225 h of photocatalysis in presence of ZnO nanoparticles appeared uniform and smooth with no relevant degradation morphological effects (**Figure 4b**) with a surface roughness close to that exhibited by neat LLDPE (**Figure 4a**). Otherwise, after 225 h of UVA/light exposure in the presence of HASS/ZnO nanoparticles, degradation phenomena were visible: some spots and cracks appeared on LLDPE film surface (**Figure 4c**); at the same time, after 225 h of UVA/light exposure in presence of HA/ZnO nanoparticles, small holes appeared on the surface of LLDPE (**Figure 4d**).



**Figure 4.** SEM micrographs of LLDPE before (a) and after irradiation for 225 h in the presence of ZnO (b); HASS/ZnO (c); HA/ZnO (d) This evidence suggested a much more efficient photodegradation induced by hybrid HA/ZnO catalysts than that exerted by bare ZnO, because of the enhanced ROS-generating ability of HAs-doped nanoparticles as indicated by EPR spin-trapping results.

To monitor the chemical changes on the films surfaces induced by the photodegradation process in the presence of different nanoparticles, ATR-FTIR spectra of LLDPE films were recorded before and after 75 and 225 h under UVA/light irradiation, as shown in **Figures 5a,b**. Unexposed pure LLDPE films showed the characteristics IR bands corresponding to the stretching vibration of carbon-hydrogen bond (CH) at 2920-2850 cm<sup>-1</sup>, the scissoring and bending vibration of methylene (CH<sub>2</sub>) and methyl (CH<sub>3</sub>) groups in the range 1480-1430 cm<sup>-1</sup>, rocking vibration of methylene (CH<sub>2</sub>) groups at 710 cm<sup>-1</sup>. ATR-FTIR spectra of LLDPE under photocatalytic process without ZnO-based catalysts, recorded as references, did not show any significant change (data not shown).

Although these peaks remained in the films after the photocatalytic process, new significant IR bands appeared after UVA/light irradiation for both 75 and 225 hours. In particular, *i*) a broad band in the range 3550-3200 cm<sup>-1</sup>, attributed to the stretching of hydroxyl OH groups, and ascribable to the presence of hydrogen-bonded products, such as hydroperoxides or alcohols, formed during the photo-

oxidation; *ii*) a semi-broad band in the range 1700-1500 cm<sup>-1</sup>, corresponding to the stretching vibration of carbonyl group (C=O); *iii*) a band close to 1260-1240 cm<sup>-1</sup>, corresponding to C-O stretching in ether-type linkages or primary and secondary ROOH or esters and in the range 1100-1040 cm<sup>-1</sup>, corresponding to C-O stretching in tertiary alcohol or alkyl-substituted ether; iv) a small band in the range 880-910 cm<sup>-1</sup>, corresponding to vinyl groups (-CH=CH<sub>2</sub>). The appearance of these bands as well as the changes in their relative intensities were considered a clear hallmark of the LLDPE photo-oxidation (Baskaran and Sathiavelu, 2020; Hsu et al., 2012; Xie and Qu, 2001). Interestingly, after 75 h irradiation, LLDPE films treated with both hybrid HASS/ZnO and HA/ZnO nanoparticles showed very intense absorption bands as appeared in the carbonyl and all other ranges (Figure 5a), which resulted higher than those observed in the case of film treated with bare ZnO. After 225 h irradiation, no significant changes were detected in the bands of spectra corresponding to hybrid nanoparticles, while an increase of the bands intensity was observed in the spectra corresponding to bare ZnO (Figure 5b), which resulted quasi-overlapped with the other ones.



Figure 5. ATR-FTIR spectra of LLDPE films treated with bare ZnO, HASS/ZnO and HA/ZnO nanoparticles after 75 h (a) and 225 h (b) of the exposure to UVA/light irradiation

Then, the improved photoactivity of hybrid HAs/ZnO nanoparticles with respect to bare ZnO was also confirmed by XRD analysis on LLDPE films after the UVA/light irradiation, as shown in Figure 6. LLDPE was characterized by two main crystalline peaks, centred in the initial sample at 21.5 and 23.8 of 20, corresponding to (110) and (200) reflections of the orthorhombic form of polyethylene, respectively, and by a third crystalline peak centred at 36.3 of  $2\Theta$ , corresponding to (002) reflection, in agreement with literature (Guadagno et al., 2001). After 75 h irradiation (Figure 6a), a slight decrease in the peaks intensity was observed for all exposed samples and, at the same time, the values of the diffraction angles increased in the presence of photocatalysts (see insets of Figure **6a**). In particular, this effect was greater for polymeric films treated with hybrid HASS/ZnO ( $\Delta 2\Theta = 0.24^{\circ}$ ) and HA/ZnO ( $\Delta 2\Theta = 0.19^{\circ}$ ) nanoparticles than for that treated with ZnO ( $\Delta 2\Theta = 0.10^{\circ}$ ), suggesting a perfectioning of the crystalline cell probably due to the expulsion of chain parts containing the side group (Guadagno et al., 2001). After 225 h irradiation (Figure 6b), a similar behaviour was observed in the case of polymeric films treated with hybrid HASS/ZnO and HA/ZnO nanoparticles, while an opposite effect was detected in the presence of bare ZnO with a slight increase of the peaks intensity and a small shift at lower diffraction angle (see insets of **Figure 6b**), which indicated an enhanced photodegradation of the amorphous phase of the film.



*Figure 6.* XRD spectra of LLDPE exposed to UVA/light irradiation for 75 h (a) and 225 h (b) in the presence of bare ZnO, HASS/ZnO and HA/ZnO nanoparticles

The changes induced by the photodegradation process in the presence of ZnObased nanoparticles were confirmed by a quantitative analysis of XRD spectra. In particular, the crystallinity index,  $X_c$ , was calculated by comparing the areas of the crystalline peaks (A<sub>c</sub>) to the total areas (A<sub>c</sub> + A<sub>a</sub>) of each diffractograms as summa of the areas of crystalline and amorphous phases (Hsu et al., 2012), using the following equation:

$$Xc \% = \frac{Ac}{Aa+Ac} = \frac{A(100) + A(200) + A(020)}{Aa+A(100) + A(200) + A(020)}$$

As reported in **Table 2**, a slight decrease (~2%) in the crystallinity degree of LLDPE films was detected after the photocatalytic treatment in the presence of all catalysts. This trend did not agreed with some previous evidences for PE films which proposed an  $X_c$  increase related to a local reorganization of the mobile small chain fragments formed from chain scission reaction induced by the photo-oxidation (Hsu et al., 2012). On the other hand, the small observed effect could be ascribable to the presence of defects in the crystalline structure or of chemical impurities within the polymeric films, thus confirming that the photoactive nanoparticles induced the ROS attack against the surfaces not significantly altering the bulk structure after the considered irradiation times.

LLDPE samples	Crystallinity Index, X <sub>c</sub> (err <u>&lt;</u> 2%)		
	75h	225h	
bare	42.1 %		
treated with ZnO	41.5 %	42.4 %	
treated with HASS/ZnO	40.8 %	40.1 %	
treated with HA/ZnO	39.8 %	40.3 %	

**Table 2.** Values of crystalline index, Xc, of LLDPE before and after UVA/light irradiation, in the presence of bare ZnO or hybrid HASS/ZnO and HA/ZnO nanoparticles

#### 5.3.5 Photodegradation of PLA films

The photocatalytic degradation of PLA films in the presence of bare and hybrid ZnO-based nanoparticles was evaluated using two different experimental conditions, such as dry-photolysis or wet-photolysis, with the aim to investigate the role of water presence in tuning the photo-oxidation of PLA, being it particularly sensitive to humidity. First, the morphological changes were monitored by SEM analysis (Figure 7). As already observed for LLDPE films, the most significant differences were observed at longer times (225 h). In particular, under dryphotolysis condition, some superficial scraps appeared on PLA surface exposed in the presence of bare ZnO (Figure 7b), while only small surface damages appeared in the presence of HASS/ZnO (Figure 7c) differently from a larger and more diffuse corrosion of PLA surface observed in the presence of HA/ZnO (Figure 7d) with appearance of cracks and holes on the surface, strongly affecting the material morphology. A similar behaviour was detected for PLA films exposed to photocatalysts under wet-photolysis condition; moreover, in this case, the photodegradation seems to proceed faster with the appearance of very clear alterations of the PLA surfaces in the presence of all type of photocatalysts (Figure 7a'-d').



239



**Figure 7.** SEM micrographs of PLA films before (a) and after 225 h of irradiation: dry-photolysis (panel up) and wet-photolysis (panel down), in the presence of bare ZnO (b,b'), hybrid HASS/ZnO (c,c') and HA/ZnO (d,d') nanoparticles

ATR-FTIR spectra of PLA films under photocatalytic process without ZnO-based catalysts, recorded as references, did not show any change (data not shown).

**Figures 8** shows ATR-FTIR spectra of PLA polymer before and after the photocatalytic treatment for 75 and 225 hours in the presence of ZnO, HASS/ZnO and HA/ZnO nanoparticles under UVA/light irradiation.





*Figure 8.* ATR-FTIR spectra of PLA polymer treated with ZnO, HASS/ZnO and HA/ZnO nanoparticles, after 75h (a) and 225 h (b) of the exposure to UVA/light lamp.

The main bands typical of PLA polymers were observed (Mucha et al., 2014) at 2997 and 2946 cm<sup>-1</sup>, corresponding to C-H asymmetric and symmetric stretching vibrations, 1456 cm<sup>-1</sup> related to -CH<sub>3</sub> asymmetric bending vibration, 1382 cm<sup>-1</sup> related to C-H symmetric bending vibration and 868 cm<sup>-1</sup> corresponding C-C stretching vibration. Moreover, the intensive carbonyl bands C=O at 1752 cm<sup>-1</sup>, C-O-C at 1190 (asymmetric) and 1093 cm<sup>-1</sup> (symmetric) stretching vibration were also observed. Under dry-photolysis condition, any significant change was appreciated in ATR-FTIR spectra of PLA films in the presence of all photocatalysts at different times (data not shown). Under wet-photolysis condition, slight alterations of spectra were observed in all cases in early stages of photo-exposure (75 h), while marked changes appeared after longer time (225 h) of irradiation. As observable in **Figure 8**, the photocatalytic process induced the formation of a broad absorption band in the hydroxyl region in the range 3500-

3300 cm<sup>-1</sup>corresponding to products such as hydroperoxides or alcohols, a significant decrease of C=O band at 1752 cm<sup>-1</sup> and a strong intensity heightening of the bands at 1456 cm<sup>-1</sup> and 1382 cm<sup>-1</sup> related to  $-CH_3$  asymmetric and C-H symmetric bending vibration, respectively.

XRD provides an ideal method to monitor changes in the crystallization of PLA polymers during degradation. As shown in **Figure 9**, un-weathered PLA was characterized by a quasi-completely amorphous structure as indicated characterized by the intense broad peak with maximum approximately at  $2\theta$ ~17° and the second small peal approximately at  $2\theta$ ~32°. XRD spectra of the polymer films were not significantly affected by the presence of bare and hybrid nanoparticles during the photocatalytic process (**Figure 9 a,b**).



Figure 9. XRD spectra of PLA polymer treated with ZnO, HASS/ZnO and HA/ZnO nanoparticles, after 75h (a) and 225 h (b) of the exposure to UVA lamp

#### **5.4 Conclusions**

This work proposed an eco-inspired design of ROS-generating hybrid ZnO-based nanoparticles as efficient photocatalysts for the degradation of polymeric films under UVA/light irradiation. By following a waste-to-wealth approach combined to a solvothermal synthesis, ZnO nanoparticles were doped at molecular scale with small percentages of a bio-derived Humic Acids (HAs). A commercial HA at low molecular weight and its sodium salt (HASS) were chosen to investigate how the chemical features influence the conjugation with the zinc oxide precursor salt, leading to hybrid HASS/ZnO and HA/ZnO nanoparticles of about 150 nm. Hybrid nanoparticles showed an enhanced ability in producing •OH radicals in aqueous environment after UVA/light irradiation greater than that of the bare ones This was probably due to the intrinsic free-radicals of HAs, which strongly contributed to speed up the surface degradation of polyolefin LLDPE and biodegradable PLA films. For LLDPE film, HASS/ZnO nanoparticles showed an improved photocatalytic activity compared to bare ZnO, while the PLA ones were mainly altered by HA/ZnO, according to an effect quite depending by the irradiation times. Physicochemical analyses showed significant changes in the surface properties in terms of exposed functional groups (e.g., hydroxyl, carbonyl, and carbonhydrogen) as well as in the structural and morphological features of the polymeric films. This was a proof that the presence of bioderived organic components at low contents promoted the photo-oxidation thus acting as erosion initiators toward the surface of the polymeric films.

Overall, the present study shows a green and sustainable strategy which sees photoactive hybrid semiconductor nanoparticles with organic molecules from biowaste, promising candidates to face the serious issue of the treatment and removal of (micro)plastics from aqueous environment.

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## **Chapter 4**

Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks

### Contents

1. Electrospun hybrid TiO_2/Humic Substance PHBV films for active food packaging
applications
1.1 Abstract
1.2 Introduction
1.3 Results and discussion
1.3.1 Characterization of the hybrid nanoparticles
1.3.2 Morphology of the electrospun mats
1.3.3 Physico-chemical and functional characterization of fibers 261
1.3.4 Morphological, physico-chemical, and functional characterization of films
1.4 Conclusions
1.5 Supporting Information
1.6 Acknowledgements
1.7 References
2. Adding humic acids to gelatin hydrogels: a way to tune gelation
2.1 Abstract
2.2 Introduction
2.3 Results and discussion
2.3.1 Effect of HA on viscoelasticity and temperature ramps
2.3.2 Effect of HA on gelation kinetics
2.3.3 Effect of HA on compression tests

2.3.4 XRD Analysis	304
2.3.5 FT-IR spectroscopy	305
2.3.6 SEM analysis	309
2.3.7 Low temperature DSC	310
2.3.8 Swelling analysis	313
2.4 Conclusions	315
2.5 Supporting information	316
2.6 Acknowledgements	317
2.7 References	318
3. Detailed thermal, fire and mechanical study of silicon-modified epoxy re-	esin
containing humic acid and other additives	324
3.1 Abstract	325
3.2 Introduction	327
3.3 Results and discussion	331
3.3.1 Chemical study of the hybrid epoxy composites	331
3.3.2 Thermal analysis	332
3.3.3 Fire behavior of the hybrid epoxy composites	334
3.3.4 Mechanical behavior	342
3.4 Conclusions	345
3.5 Supporting Information	347
3.5.1 Preparation of silica-epoxy composites	347
3.5.2 Chemical characterization of the silica-epoxy composites	348
3.5.3 Fire behavior of the hybrid epoxy composites	349

3.6 References	1
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# **1. Electrospun hybrid TiO<sub>2</sub>/Humic Substance PHBV films** for active food packaging applications.

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#### **1.1 Abstract**

Sustainable packaging materials can play a key role in minimizing the environmental footprint of packaged food by preserving its quality and avoiding environmental persistence of plastic waste. In this context, waste-to-wealth approach can cope with these major challenges by providing for bioavailable active compounds from waste residues. To this regard, humic substances (HS) derived from biowaste oxidative processes exhibit intrinsic antioxidant and antimicrobial features, which can be significantly boosted by molecular combination with an inorganic nanostructured phase.

Herein, this approach has been integrated with the electrospinning technology to design composite films made of electrospun biodegradable and bioderived polymers in the presence of hybrid nanostructured HS based materials.

To this purpose, electrospun composites made by including hybrid TiO<sub>2</sub>\_HS nanostructures into PHBV matrix were first produced and then converted into homogeneous and continuous films. These were characterised to assess the morphology, thermal, crystallinity, optical, mechanical and barrier properties of the resultant PHBV-based films as well as antimicrobial properties against Staphylococcus aureus and Escherichia coli. The obtained results suggested that the combination of hybrid nanomaterials with electrospinning methodology is a promising sustainable approach to convert biowaste into multifunctional materials for active packaging applications.



#### **1.2 Introduction**

The development of sustainable food packaging materials is crucial to decrease the use of the conventional plastics and ensure food quality, thus minimizing the environmental footprint of packaged food <sup>1</sup>. Polyhydroxyalkanoates (PHAs), biobased and biodegradable aliphatic polyesters, hold high potential to replace polyolefins in packaging applications <sup>2,3</sup>. Among PHAs, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) shows greater flexibility, as well as better gas and water barrier properties than other polyesters, which broadens its industrial applicability <sup>4,5</sup>. At the same time, to confer active properties to PHAs, several substances can be incorporated to delay or inhibit the microbial, enzymatic, and oxidative reactions, which are the main causes of food deterioration <sup>6,7</sup>. To this regard, replacing synthetic chemicals with nature available compounds is highly recommended to avoid safety and environmental issues <sup>8,9</sup>. Therefore, the combination of a bio-based and biodegradable polymer with natural fillers, is the most coherent technological answer to sustainability issues <sup>10,11</sup>

Following waste to wealth approach, biowastes can be converted into added-value compounds and materials. Among these, humic substances (HS), the alkali soluble fraction of organic matter, usually found in water, soil and sediments <sup>12,13</sup>, provide an important bio-source for redox-active compounds and multifunctional materials, since they are rich in quinones, phenols, carboxyl, and hydroxyl moieties. Accordingly, HS can generate or scavenge reactive oxygen species (ROS), exhibiting antioxidant, antibacterial and anti-inflammatory features <sup>14–18</sup>. Main limitations to HS full exploitation, related to aggregation and segregation phenomena in aqueous environment <sup>19,20</sup>, have been recently addressed by ceramic templated approach. This involves molecular combination of these heterogeneous moieties with an inorganic nanostructured phase, including SiO<sub>2</sub> or TiO<sub>2</sub> nanoparticles <sup>20–22</sup>, which can act as a templating agent, tuning HS supramolecular structure and finally

boosting the intrinsic organic and inorganic properties. The synergistic combination of HS with  $TiO_2$  nanomaterials is crucial to get also a material with new features, including antimicrobial ones <sup>20,22,23</sup>.

The electrospinning technique provides for high-performance active and bioactive nanofiber mats with high surface-to-volume ratios <sup>24</sup>. The thermal post-treatment annealing below the biopolymer's melting temperature leads to continuous films for food packaging applications <sup>25</sup>.

This study proposed the design of composite films made of electrospun biodegradable and bioderived polymers in the presence of hybrid nanostructured HS based materials. For that, hybrid TiO<sub>2</sub>\_HS nanostructures were synthetized through ceramic templated approach, using HS extracted from the commercially available compost supplied by Verde Vita (s.r.l.). Then, synthesized nanoparticles were included into electrospun PHBV fibers, which were then converted into biopapers (PHBV/TiO<sub>2</sub>\_HS), and characterized in terms of physical, mechanical, moisture/light barrier, optical, thermal resistance, microstructure, as well as antibacterial and barrier properties to evaluate their potential application in food packaging materials.

#### **1.3 Results and discussion**

1.3.1 Characterization of the hybrid nanoparticles



Figure 1. TEM morphology of TiO2\_HS nanostructures.

TEM micrographs of TiO<sub>2</sub>\_HS nanostructures (**Figure 1**) evidenced the formation of hybrid clusters with an average size of about 200 nm and made by aggregated primary rod-like particles about 20 nm, in accordance with previous works <sup>22,26</sup>. Nanoparticles looked smaller than bare TiO<sub>2</sub> nanostructures (**Figure S1**), suggesting that HS can control TiO<sub>2</sub> nanoparticles growth and aggregation during solvothermal process <sup>22</sup>.

BET analysis suggested that hybrid TiO<sub>2</sub>\_HS nanoparticles showed a specific surface area (SSA) value of 170.0  $\pm$  1.0 m<sup>2</sup>/g with a mean pore volume of 0.40  $\pm$  0.05 m<sup>3</sup>/g, suggesting the formation of a mesoporous materials <sup>22</sup>. The close interconnection between the two phases is crucial to increase SSA value since HS has a SSA value of 2.0  $\pm$  0.5 m<sup>2</sup>/g with a negligible pore size.

FTIR spectrum of TiO<sub>2</sub>\_HS sample evidenced typical bands of humic substances (**Figure. S2 A, Table S1**), thus confirming their presence in the nanostructures  $^{22,27}$ .

The organic content within the samples was evaluated through TG analysis. The TGA curve of hybrid nanostructures shows a larger weight loss than bare TiO<sub>2</sub> (**Figure S2 B**), in the range 240-400°C, where HS main decomposition occurs (**Figure S2 B**), thus supporting the presence of HS in the nanostructures  $^{20,22}$ . Besides, the final HS content in the nanohybrids was about 20% wt.

For both bare and hybrid nanoparticles, the XRD patterns showed the typical profile of a standard anatase lattice in terms of both peak positions (diffraction angles 2 $\theta$ ) and relative intensities (**Figure S2 C**). In contrast, HS was amorphous, and its contribution was detected by the presence of a broad halo around the most intense peak in the XRD profile of TiO<sub>2</sub>\_HS nanohybrids <sup>22</sup>.

NMR analysis <sup>13</sup>C CPMAS NMR spectra of HS was acquired to determine the molecular structure and chemical composition of the sample (**Figure S3, Table S2**). HS was principally characterized by four resonance regions (**Figure S3**) that were centered at: i. ~170 ppm, which is typical of the carboxyl groups; ii. ~130 ppm, which was attributed to aromatic moieties, iii. ~100 ppm due to the presence of O-alkyl-C moieties and iv. ~30 ppm, which was attributed to alkyl carbons.

NMR signal assignment is reported in **Table S2**<sup>17,28</sup> and spectral data also provided the hydrophobicity index (HB/HI) of samples.

**Table S3** displays the MIC and MBC values of HS, bare and hybrid  $TiO_2$  HS nanoparticles against S. aureus and E. coli strains chosen as representative G+ and G- foodborne bacteria, respectively. The results suggested that the molecular combination between HS and  $TiO_2$  is crucial to obtain improved antimicrobial features, even boosting both organic and inorganic functionality. This evidence is consistent with our previous research <sup>22</sup>, in which the antimicrobial activity of nanohybrids is due to a synergistic combination of different effects.

#### 1.3.2 Morphology of the electrospun mats

Different amounts of hybrid  $TiO_2$ \_HS nanoparticles were added to PHBV solutions before electrospinning process to obtain composite mats with different nanostructure content (**Figure 2**).



**Figure 2.** A) SEM micrographs of the electrospun fibers of PHBV: Neat PHBV; PHBV/TiO<sub>2</sub>\_HS 1% wt; PHBV/TiO<sub>2</sub>\_HS 3% wt; PHBV/TiO<sub>2</sub>\_HS 6% wt; PHBV/TiO<sub>2</sub>\_HS 10% wt; Scale markers of 50 µm in all cases. B) Size distribution of PHBV and PHBV/TiO<sub>2</sub>\_HS 10% wt fibers.

The morphology of electrospun mats was assessed through SEM analysis. **Figure 2** reports SEM images and fiber size distribution of all mats. Neat PHBV fibers evidenced a mean diameter of about  $0.7 \pm 0.09 \,\mu$ m, as in previously studies (**Figure 2**) <sup>29,30</sup>. All fibers presented a uniform and smooth surface, showing no surface or structural defect, indicating that the NP addition doesn't prevent fiber formation. Furthermore, PHBV/TiO<sub>2</sub>\_HS 10% wt fibers show larger mean diameter of  $0.9 \pm 0.1 \,\mu$ m than neat PHBV mats (**Figure 2 B**). This might be due to the presence of nanoparticle aggregates on the fiber surface <sup>31</sup> even at low NP content (**Figure S4**) The nanostructure dispersion in the mats was evaluated through TEM analysis. TEM images (**Figure 3**) show that the nanoparticles were efficiently encapsulated in the mats even though some of them segregated forming aggregates underneath the

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks

surface, as shown also by SEM. The presence of titanium was also confirmed by EDAX analysis (**Figure 3B**) and the NP distribution was assessed by mapping from the SEM images.

The elemental mapping image of the **Figure 3B** confirmed the presence of nanostructures on fibers surface also in the sample with the lowest NP content (PHBV/TiO<sub>2</sub>\_HS 1 %wt).



*Figure 3.* A) TEM micrographs of the electrospun fibers of PHBV containing TiO<sub>2</sub>\_HS nanoparticles at a) 1%wt; b) 3%wt; c) 6%wt; d) 10% wt; B) Elemental mapping of coated system by EDAX from SEM image (Titanium in light blue).

#### 1.3.3 Physico-chemical and functional characterization of fibers

Water contact angle (CA) measurements were carried out on all electrospun mats to assess the material hydrophobicity or hydrophilicity. CA value is larger than 100° for all samples, typical of hydrophobic materials (**Table 1**)

Sample	Average Contact Angle (°) (±2)
PHBV	125
PHBV/TiO <sub>2</sub> _HS 1%wt	112

PHBV/TiO <sub>2</sub> _HS 3%wt	109
PHBV/TiO <sub>2</sub> _HS 6%wt	106
PHBV/TiO <sub>2</sub> _HS 10%wt	101

 Table 1. Water contact angle values of obtained fibers.

Furthermore, CA decreases at increasing filler content, reaching the lowest value of about 101° in the sample containing the highest amount of TiO<sub>2</sub>\_HS nanostructures. Both components in hybrid nanostructures exhibit a well assessed hydrophilic nature  $^{20,22,31}$ . Indeed, TiO<sub>2</sub> nanoparticles have been often exploited for hydrophilic modification of a widespread range of materials and surfaces, including polymers  $^{32}$ . At the same time, HS exhibit a relevant water affinity  $^{33}$ . Thus, the incorporation of TiO<sub>2</sub>\_HS nanoparticles causes a certain decrease of hydrophobicity and slightly affects CA values. However, all the investigated samples can be still classified as hydrophobic, being CA > 65°  $^{29}$ .

XRD analysis was carried out to evaluate the possible change in the crystalline structure of PHBV electrospun fibers due to the presence of TiO<sub>2</sub>\_HS nanoparticles (**Figure 4 A**).

The X-ray patterns of all samples showed the typical diffraction peaks of the PHB orthorhombic unit cell (**Figure 4**). No significant differences in the diffraction peak widths were observed between the neat and the nanocomposite fiber. However, a little shift of diffraction peaks towards higher  $2\Theta$  in the case of PHBV/TiO<sub>2</sub>\_HS fibers appeared, suggesting an increase in lattice parameters, which might be caused by the consequential reorganization of the crystalline part after nanoparticles addition (**Figure 4 B**) <sup>34,35</sup>. Indeed, crystallinity index values (X<sub>c</sub>) values, shown in **Table 2**, suggest an overall slight increase in the crystallinity of the nanocomposite fibers <sup>36</sup>, maybe because nanoparticles are bound to act as nucleating agents for polymer crystallization.

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks





Figure 4. A) XRD patterns of bare and hybrid electrospun PHBV fibers; B) XRD patterns at higher magnification of bare PHBV and hybrid electrospun PHBV fibers.

PHBV fibers	Crystallinity Index, X <sub>c</sub> (err <u>&lt;</u> 2%)
PHBV	48
PHBV/TiO2_HS 1% wt	47
PHBV/TiO2_HS 3% wt	51
PHBV/TiO2_HS 6% wt	48
PHBV/TiO2_HS 10% wt	56

Table 2. Crystallinity Index Xc of PHBV fibers.

The thermal behaviour of both bare and hybrid electrospun PHBV fibers was analysed by TG and DSC analyses. **Table 3** shows the values of the onset temperature ( $T_{5\%}$ ), degradation temperature ( $T_{deg}$ ), and residual mass at 700°C. All fibers first undergo a low-intense weight loss process (<1%) at 100°C due to absorbed moisture and/or volatiles leaving the samples, then the total thermal decomposition reaction occurred sharply from 270°C to 300°C (**Figure 5**).

Neat PHBV fibers were thermally stable up to 268.5°C and showed a  $T_{deg}$  value of 287.7°C with a residual mass of 1.4 wt% (**Figure 5 A, B**). These features are close to those reported for PHBV materials in previous works <sup>37</sup>, where the thermal decomposition reaction of the biopolymer chain occurred sharply in one single and sharp step from approximately 270 °C to 280 °C.

On the other hand, PHBV/TiO<sub>2</sub>\_HS 10% wt sample had the lowest temperature at 5% weight loss ( $T_{5\%}$ ) but the highest value of residual mass (10.1 %), confirming the highest amount of nanoparticles <sup>30,38</sup>. Indeed, all hybrid PHBV fibers showed a residual mass in the range of 2-10% wt, which increased with NP content.

 $T_{deg}$  values increased by incorporating nanoparticles into the polymer matrix (**Table 3**), resulting in enhanced thermal resistance of polymer materials, which could be attributed to the interaction between the polymer matrix and the nanoparticles, that acted as physical cross-linking sites for the polymer.<sup>39</sup> Thermal stabilization could be due also to the barrier effect of NPs, which can inhibit degradation by preventing the transport of decomposition products and heat diffusion from the bulk polymer matrix during thermal degradation, also because of the possible sorption of these products on the filler surface <sup>40–42</sup>.

Sample	T5% (°C)	T <sub>deg</sub> (°C)	Residual mass (%)	
	(err <u>&lt;</u> 2°C)	(err <u>&lt; 2</u> °C)	(err <u>&lt;</u> 1 %)	
PHBV	268.5	287.7	1.4	
PHBV/TiO2_HS 1% wt	264.3	283.9	2.2	
PHBV/TiO2_HS 3% wt	270.0	291.0	4.4	
PHBV/TiO <sub>2</sub> _HS 6% wt	270.0	291.0	4.9	

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks

PHBV/TiO2_HS 10%	248.6	294.9	10.1
wt			

**Table 3.** Thermal properties in terms of  $T_{5\%}$ , degradation temperature  $T_{deg}$  and residual mass at 700°C for all fibers.



**Figure 5.** A) Thermogravimetric analysis (TGA) curves and B) differential thermal analysis (DTA) curve of PHBV and PHBV/TiO<sub>2</sub>\_HS fibers at 1,3,6 and 10%wt of nanoparticles.

DSC was used to assess the thermal properties of electrospun bare and hybrid PHBV fibers. The thermal curves and parameters (melting/crystallization temperatures and melting enthalpy) determined from the first heating run, first cooling run, and second heating run, respectively, are shown in **Figure 6** and **Table 4**. Investigating the cooling curves, neat PHBV shows an intense crystallization peak at around 108 °C,

while nanocomposite fibers present an equally well defined but less broad crystallization peak at higher temperatures (**Figure 6 B**). The positions of the first melting temperature  $T_{m1}$ , second melting temperature  $T_{m2}$ , and crystallization temperature  $T_c$  peaks were almost independent of NP content but slightly shifted to higher temperatures than the typical  $T_m$  and  $T_c$  values for PHBV, <sup>36</sup> suggesting that NPs restricted the physical mobility of the polymer chains and acted as a nucleating agent for PHBV crystals <sup>36,43–45</sup>. This hypothesis is also supported by XRD results evidencing a higher crystal degree in nanocomposite mats. Other nanofillers, such as multi-walled carbon nanotubes (MWCNTs), SiO<sub>2</sub>, and ZnO, played an analogous nucleating role in PHBV crystallization <sup>45–47</sup>.

A slight decrease in melting enthalpy  $\Delta H_{m1}$  (**Table 4**) was observed, except for PHBV/TiO<sub>2</sub>\_HS 1%wt sample. According to Ahmad et al. <sup>48</sup>, the heat of fusion of PVA in nanocomposites decreased as TiO<sub>2</sub> content increased, implying that TiO<sub>2</sub> NPs have a significant effect on the crystal stability of PVA. The mobility of polymer chains was more restricted by NP presence that led to the formation of more non-perfect heterogeneous crystals easier to melt.

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks





Temperature/ °C

*Figure 6.* DSC of pure PHBV and PHBV/TiO<sub>2</sub>\_HS fibers at 1,3,6 and 10%wt of nanoparticles. A) First heating run from -20°C to 200°C; B) First cooling run from 200°C to -20°C; C) Second heating run from -20°C to 200°C.

Sample	T <sub>c</sub> (°C)	<b>T</b> <sub>m1</sub> (° <b>C</b> )	$\Delta H_{m1} (J/g)$	<b>T</b> <sub>m2</sub> (° <b>C</b> )
	(err <u>&lt;</u> 2°C)	(err <u>&lt; 2</u> °C)	(err <u>&lt;</u> 4J/g)	(err <u>&lt; 2</u> °C)
PHBV	107.6	172.3	99.4	161.6
PHBV/TiO2_HS 1%wt	111.9	173.2	104.0	165.5
PHBV/TiO <sub>2</sub> _HS 3%wt	113.5	175.3	87.1	169.5
PHBV/TiO <sub>2</sub> _HS 6%wt	111.0	173.2	86.8	166.0

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks

PHBV/TiO <sub>2</sub> _HS	112.0	173.8	78.9	164.6
10%wt				

**Table 4.** Thermal properties in terms of crystallization temperature,  $T_c$ , Melting temperature  $T_m$  and Melting  $(\Delta H_{m1})$ .

S. aureus and E. coli are two common microorganisms related to food-borne diseases. As a result, the incorporation of antimicrobial compounds within the biopolymer matrix can be necessary to avoid food safety issues. After confirming the superior antibacterial activity of hybrid nanoparticles over neat TiO<sub>2</sub> NPs, all electrospun fibers were tested for antimicrobial activity using JIS assay <sup>38</sup>.

**Figure 7** shows the antimicrobial activity of both bare and electrospun nanocomposite mats, containing different amounts of hybrid nanoparticles. The reduction R value is significant (~2) for the sample containing the highest nanoparticle concentration (PHBV/TiO<sub>2</sub>\_HS 10%wt) against both S.A. and E.C. strains, suggesting the dependence of the antimicrobial performance on hybrid nanoparticle content <sup>22</sup>. The greater amount of NPs on the fiber surface caused a more effective interaction with pathogens, leading to an improvement of antimicrobial performance. Since neat PHBV has no antimicrobial activity <sup>30</sup>, the biocide action of composite mats should be related only to the presence of hybrid NPs nanoparticles, through various possible concurring phenomena essentially based on ROS production and membrane interaction <sup>22,49</sup>.



Figure 7. Antimicrobial activity of the electrospun mats of bare PHBV and PHBV at different concentrations of TiO<sub>2</sub>\_HS nanoparticles against S. aureus (S.A.) and E. coli (E.C.).

Indeed, HS can undergo reversible redox reactions; under aerated conditions, semiquinone moieties oxides, causing electron transfer to  $O_2$  and the generation of  $\cdot$ OH radicals <sup>22,50</sup>. Furthermore, catechol moieties in HS, could act as ligands for Ti<sup>4+</sup> ions, forming a ligand-to-metal charge transfer complex (LMCTC) that could cause ROS production by inorganic phase, even under visible light. Several studies have also suggested that phenolic and carboxylic groups found in HS can directly exert significant antimicrobial activity against different bacterial strains <sup>51</sup>. These functional groups increase the number of hydrogen bonds with cell bacterial membranes <sup>51,52</sup>, causing irreversible changes in their lipid structure <sup>53</sup> and increasing permeability to ROS, resulting in a reduction in pathogenic strain growth and bacterial cell death.

Based on the results, PHBV/TiO<sub>2</sub>\_HS 10% wt sample demonstrated not only superior thermal stability, attributed to the heat barrier and nucleating effect of NPs, but also the highest antimicrobial performance. This sample was thermally post-treated to produce a continuous film and its optical, thermal, and functional properties were compared to those of bare PHBV film to assess its potential application as a bioactive material for food packaging.

#### 1.3.4 Morphological, physico-chemical, and functional characterization of films

Figure 8 shows the SEM images of the electrospun mats after annealing at  $150^{\circ}$ C, temperature below the biopolymer melting point, in their top views (A, C) and cross-section (B, D). The annealing process provides enough molecular mobility to undergo interfiber coalescence, to obtain continuous films for food packaging applications.

Both biopapers showed a homogeneous surface without cracks and/or pores, suggesting the optimal annealing treatment conditions. Some nanoparticles agglomerations were visible in the top view of  $TiO_2$ \_HS 10 % wt film sample at higher magnification (1  $\mu$ m).



**Figure 8.** SEM images of the films top view (left) of neat PHBV (a),  $TiO_2_HS 10 \% wt$  (c) and SEM images of the films cross-section (right) of neat PHBV (b),  $TiO_2_HS 10 \% wt$  (d). Scale markers of 50 µm and 1 µm for  $TiO_2_HS 10 \% wt$ .

Since the roughness and the SSA value of fiber samples decreased significantly after the thermal treatment, the average CA in both PHBV and PHBV/TiO<sub>2</sub>\_HS 10% wt samples decreased to about  $75^{\circ}$  after annealing.

**Figures 9 and 10** show TG and DSC curves of neat PHBV and PHBV nanocomposite films, while the values of thermal properties are reported in **Tables 5 and 6**. Both films showed a similar thermal decomposition process, even though the degradation stage in the PHBV/TiO<sub>2</sub>\_HS 10% wt sample began at lower temperatures (around 248°C versus 277°C for neat PHBV film). However, at 700°C,

the residual content in the hybrid sample was around 8%, whereas it was only about 1% in the neat PHBV. The char residue could be related to the presence of NPs  $^{37}$ .



Figure 9. Thermogravimetric analysis (TGA) curves (on the left) and differential thermal analysis (DTA) curve (on the right) of PHBV and PHBV/TiO2\_HS 10% wt films.

Sample	<b>T</b> 5%	T <sub>deg</sub>	Residual mass (%)
	(err <u>&lt; 2</u> °C)	(err <u>&lt; 2</u> °C)	(err <u>&lt;</u> 1 %)
PHBV Film	276.6	295.8	1.1
PHBV/TiO2_HS 10% wt Film	247.9	296.2	7.9

**Table 5.** Thermal properties in terms of  $T_{5\%}$ , degradation temperature  $T_{deg}$  and residual mass (%) at 700°C for PHBV and PHBV/TiO<sub>2</sub>\_HS 10% wt films.

The appearance of multiple melting peaks in DSC first heating run of bare PHBV (**Figure 10 A**) could be due to more than one crystallographic form or changes in morphology, such as lamellar thickening and more crystal perfection <sup>54</sup>.

With the presence of NPs, only one melting peak can be seen (Figure 10 A) and  $\Delta H_{m1}$  is lower than that of neat PHBV (Table 6).

Investigating the cooling curves, nanocomposite films presented an equally welldefined crystallization peak at higher temperatures (**Figure 10 B, Table 6**), confirming the NP nucleating effect for PHBV crystals <sup>36,44</sup>.



275



*Figure 10.* DSC of pure PHBV and PHBV/TiO<sub>2</sub>\_HS 10% wt Films: A) First heating run from -20°C to 200°C; B) First cooling run from 200°C to -20°C; C) Second heating run from -20°C to 200°C.

Sample	T <sub>c</sub> (°C)	T <sub>m1</sub> (°C)	$\Delta H_{m1} \left( J/g \right)$	$T_{m2}$ (°C)
	(err <u>&lt;</u> 2°C)	(err <u>&lt;</u> 2°C)	(err <u>≤</u> 4J/g)	(err <u>&lt;</u> 2°C)
PHBV Film	116.5	171.2	81.4	170.4
PHBV/TiO2_HS 10%wt Film	119.0	172.9	76.0	170.4

**Table 6.** Thermal properties in terms of crystallization temperature,  $T_c$ , Melting temperature  $T_m$  and Melting Enthalpy  $(\Delta H_{ml})$ 

Optical and barrier properties were evaluated to unveil the technological potential of produced nanocomposites as active food packaging. To this aim, the effect of nanoparticle addition on the color coordinates and values of color difference ( $\Delta E^*$ ), transparency (T), and opacity (O) of bare and hybrid electrospun PHBV films was

studied. NP presence reduced brightness while increasing opacity, as indicated by the values of L\* and O, respectively (**Table 7**). Nanocomposite films appeared much more "yellow," as suggested by the improvement in the coordinate b\*, and T value raised from  $5.44\pm0.05$  for neat PHBV film to  $17.70\pm0.72$  for nanocomposite film, indicating that NP addition significantly reduced the films' ability to transmit visible and UV light. In this regard, some authors have previously stated that this property may be extremely useful in some packaging materials for the protection of foodstuffs from light, particularly UV radiation, which can cause lipid oxidation in food products <sup>29</sup>.

Sample	L*	a*	b*	ΔΕ*	Transpar ency	Opacity
PHBV Film	90.03±0. 05	0.78±0.0 3	4.90±0.0 7	-	5.44±0.05	0.066±0. 02
PHBV/TiO <sub>2</sub> _	78.50±0.	2.50±0.0	28.30±0.	23.70±0.	17.70±0.7	0.13±0.1
HS 10% wt	05	2	07	09	2	0
Film						

*L*\*: luminosity (+*L* luminous; -*L* dark), a\*: red/green coordinates (+a red, -a green), b\*: yellow/blue coordinates (+b yellow, -b blue),  $\Delta E^*$ : color difference, T: transparency, and O: Opacity **Table 7.** Color parameters and transparency characteristics of the electrospun films of neat PHBV and PHBV

containing TiO<sub>2</sub>\_HS nanoparticles.

The Water Vapor Permeability (WVP), Limonene Permeability (LP), and Oxygen Permeability (OP) values are shown in **Table 8**.

Biopolyesters have lower WVP properties than conventional petrochemical polymers which however are harmful to food preservation <sup>55</sup>.

The incorporation of hybrid nanoparticles resulted in a slight decrease in WVP value, a significant decrease in LP value (about 100 times) while the OP value did not change with respect to neat PHBV. According to Nielsen <sup>56</sup>, the NP presence into polymer matrix can successfully act as barrier component forcing permeant molecules to travel through a longer path to permeate across. Melendez-Rodriguez et al. <sup>30</sup> demonstrated that large quantities of mesoporous silica nanoparticles could

improve barrier properties because of the formation of hydrogen bonds between the polymer hydroxyl groups and the oxygen atoms of silica and to the good dispersion of nanoparticles in the polymer matrix.

Considering that water vapor is a diffusivity-driven property in PHAs due to their low water sorption nature <sup>57</sup>, a slight decrease in WVP in PHBV/TiO<sub>2</sub>\_HS 10% wt could be attributed to the physical barrier provided by nanoparticles into the film, that promoted lower free volume available for diffusion <sup>30</sup>.

LP properties are important in packaging applications because limonene is used as a model permeant to assess aroma barrier. Limonene is a strong plasticizing component for PHAs, thus in this kind of permeation solubility is more important than diffusion.

In our case, the strong improvement in LP properties could be attributed to the hydrophilic nature of the nanofiller, which contributed to diminish the sorption and transport of the organic molecule through the material. Other authors reported an improvement to this property, but it had not revealed such a low value as in this case  $\frac{58-60}{2}$ .

Sample	WVP x 10 <sup>15</sup> (kg*m*m <sup>-2</sup> *Pa <sup>-1</sup> *s <sup>-1</sup> )	$\frac{LP \times 10^{14}}{(kg*m*m^{-2}*Pa^{-1}*s^{-1})}$	OP x 10 <sup>18</sup> (m <sup>3</sup> *m*m <sup>-2</sup> *Pa <sup>1</sup> *s <sup>-1</sup> )
PHBV Film	$9.99\pm0.46$	$1.37\pm0.21$	$0.38\pm0.05$
PHBV/TiO <sub>2</sub> _HS	$6.62\pm0.48$	$0.026\pm0.01$	$0.40\pm0.08$
10% wt Film			

 Table 8. Barrier properties of PHBV and PHBV/TiO2\_HS Films.

Tensile modulus (E), tensile strength at yield ( $\sigma_y$ ) and elongation at break ( $\epsilon_b$ ) of obtained films were evaluated (**Table 9**). The addition of nanoparticles to polymer matrix leads to a reinforcement because both E and  $\sigma_y$  increased to 3471 ± 181 MPa and 22.4 ± 2.9MPa, respectively, starting from 1250 ± 75 MPa and 18.1 ± 0.8 MPa, respectively, in neat PHBV.

The obtained results could indicate a good transfer of mechanical energy from the NPs and an interaction between the filler and the biopolymer, as previously reported <sup>30</sup>. Díez-Pascual et al. <sup>61</sup> reported an increase in the E and  $\sigma_y$  values with the addition of ZnO NPs in the range of 1-5 wt% in PHB. Load dispersion, polymer crystallinity, and NP interfacial adhesion to the biopolymer matrix could influence the achieved reinforcement. The advantage of adding NPs to improve food packaging materials in terms of mechanical properties is necessary for food packaging films to withstand the normal stress used during their application, subsequent shipping, and handling <sup>62</sup>.

Sample	E (MPa)	σy (MPa)	<b>Е</b> ь (%)	T (mJ/m <sup>3</sup> )
PHBV Film	$1250\pm75$	$18.1 \pm 0.8$	2.6 ± 0.2	0.3 ± 0.1
PHBV/TiO2_HS 10 %wt Film	3471 ± 181	22.4 ± 2.9	$1.1 \pm 0.4$	$0.36 \pm 0.2$

 Table 9. Mechanical properties of PHBV and PHBV/TiO2\_HS films.

The antimicrobial results suggested that NP presence reduced of about 100 times CFU/mL value (R~2) (**Figure 11**). Even if the strong decrease in SSA after annealing process, the greater NP amount on the surface of the whole composite material could make their contact with the bacterial strains more effective, allowing to preserve the final biocide performance. NP encapsulation into biopolymer matrix during solution preparation could control their migration, preventing a possible decrease in the functional activity after the thermal treatment <sup>63</sup>. Several studies demonstrated that the antimicrobial activity of polymeric films coated with bare TiO<sub>2</sub> particles is enhanced only by UV irradiation. Therefore, HS presence in the final material led to a significant antimicrobial activity even under visible light. <sup>63,64</sup>.

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks



Figure 11. Antimicrobial activity of obtained films of bare PHBV and PHBV/TiO<sub>2</sub>\_HS 10 %wt against S. aureus (S.A.) and E. coli (E.C.).
# **1.4 Conclusions**

The integration of electrospinning with ceramic templated approach is an effective and promising strategy to broaden the potential of nanotechnology and produce more sustainable materials with improved properties for food packaging.

In this study we designed composite films made of electrospun biodegradable and bioderived polymers and hybrid humic substances (HS) based nanoparticles, obtained by the molecular combination of HS extracted from the commercially available compost supplied by Verde Vita (HS) (s.r.l.) and titanium dioxide nanostructures, TiO<sub>2</sub>, through solvothermal wet chemistry approach. Different contents of synthesized nanoparticles were added to PHBV solutions, which were electrospun into nanocomposite homogeneous fibers and then converted into continuous hybrid biopapers (PHBV/TiO<sub>2</sub>\_HS) through the annealing process. Barrier, optical, thermal, mechanical, and antimicrobial properties confirmed the advantage of combining the electrospinning technology with hybrid TiO<sub>2</sub>\_HS nanomaterials to convert biowaste into bioactive films with significant potential applications in food packaging.



# **1.5 Supporting Information**

Figure S1. TEM morphology of bare TiO<sub>2</sub> nanoparticles.



**Figure S2.** A) FT-IR of HS (green curve) and TiO<sub>2</sub>\_HS (navy curve); B) TGA analysis of bare TiO<sub>2</sub> nanoparticles, TiO<sub>2</sub>\_HS nanohybrids and bare HS; C) XRD analysis of bare TiO<sub>2</sub> nanoparticles, TiO<sub>2</sub>\_HS nanohybrids and bare HS.

Bands (cm <sup>-1</sup> )	Assignments
3700-3000	Stretching vibration of O-H
2800-3000	Symmetric and asymmetric vibration of -CH <sub>2</sub>
1580-1760	C=C (aromatic and olefins), C=O (ketones and quinone)
1400	-OH (phenols), COO <sup>-</sup> and -CH <sub>3</sub> bending
1280-1020	Stretching vibration of phenolic C-O and alyphatic – OH groups
600	Stretching vibration of Ti-O-Ti

Table S1. Assignment of FTIR bands of TiO2\_HS nanoparticles.

Sample	Carboxyl-	Phenol-	Aryl-	O-alkyl-	C-O/C-	Alkyl-C	HB/HI*
	С	С	С	С	Ν	45-0	
	190-160	160-145	145-	110-60	60-45	ppm	
	ppm	ppm	110	ppm	ppm		
			ppm				
HS	10.40	3.64	12.50	38.9	6.50	28.05	0.79

 $HB/HI^* = hydrophobicity index = \sum [(0-45) + (110-160)] / \sum [(45-60) + (60-110) + (160-190)]$ 

**Table S2.** Relative Distribution (%) of Signal Area over Chemical Shift Regions (ppm) in 13C–CPMAS-NMR Spectra of HS extracted from compost Verde Vita.

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks



Figure S3. <sup>13</sup>C CPMAS NMR Spectra HS.

Active agent	Bacteria	MIC (µg/mL)	MBC (µg/mL)
TiO <sub>2</sub>	E. coli	$2.6\pm0.06$	$7.5 \pm 0.3$
TiO <sub>2</sub>	S. aureus	$6.3\pm0.01$	$4.5 \pm 0.2$
HS	E. coli	$0.04\pm0.02$	$1.9 \pm 0.06$
HS	S. aureus	$0.5 \pm 0.02$	$1.5 \pm 0.07$
TiO <sub>2</sub> _HS	E. coli	$0.03 \pm 0.01$	$1.7 \pm 0.02$
TiO <sub>2</sub> _HS	S. aureus	$0.4 \pm 0.03$	$1.4 \pm 0.04$

**Table S3**. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of bare TiO<sub>2</sub>, HS and hybrid TiO<sub>2</sub>\_HS nanoparticles against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli).



**Figure S4.** A) SEM micrographs of the electrospun hybrid fibers at higher magnification of PHBV: A) PHBV/TiO<sub>2</sub>\_HS 1% wt; B) PHBV/TiO<sub>2</sub>\_HS 3% wt; C) PHBV/TiO<sub>2</sub>\_HS 6% wt; D) PHBV/TiO<sub>2</sub>\_HS 10% wt.

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# 2. Adding humic acids to gelatin hydrogels: a way to tune gelation

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# 2.1 Abstract

Exploring the chance to convert bio-wastes into a valuable resource, this study tests the potential role of humic acids (HA), a class of multifunctional compounds obtained by oxidative decomposition of biomass, as physical agents to improve gelatin mechanical and thermal properties. To this purpose, Gelatin-HA aqueous samples were prepared at increasing HA content. HA/gelatin concentrations changed in the range 2.67(wt/wt)% - 26.67(wt/wt)%.

Multiple techniques were employed to assess the influence of HA content on the gel properties and to unveil the underlying mechanisms. HA increased gel strength up to a concentration of 13.33(wt/wt)% and led to a weaker gel at higher concentrations. FT-IR and DSC results proved that HA can establish non-covalent interactions through H- bonding with gelatin. Coagulation phenomena occur due to HA-gelatin interactions and, at higher concentrations than 13.33(wt/wt)%, HA established preferential bonds with water molecules, preventing them from coordinating with gelatin chains. These features were ac- companied by a change in the secondary structure of gelatin, which lost triple helix structure and exhibited an increase in the random coil conformation.

Besides, higher HA weight content caused swelling phenomena due to HA water absorption, contributing to a weaker gel.

Current findings may be useful to enable a better control of gelatin structures modified with composted bio-wastes, extending their exploitation for a large set of technological applications.





# **2.2 Introduction**

Gelatin, a natural peptide macromolecule obtained by partial hydrolysis of collagen, is one of the most employed polymers.<sup>1,2</sup> Its large availability, low cost, biocompatibility, biodegradability and poor antigenicity make it suitable for a wide number of applications in food as well as biomedical and pharmaceutical fields.<sup>3–5</sup> Gelatin is easily soluble in water at temperatures above 30°C, and a thermoreversible physical gel can be obtained by cooling down gelatin aqueous solutions,<sup>6–9</sup> as they undergo a sol-gel transition upon cooling, whose characteristics depend on various parameters (e.g., gelatin concentration, pH etc).<sup>10–13</sup> Gelation of gelatin aqueous solutions has been widely studied in literature.<sup>14,15</sup> Several works reported the study of the time to onset of gelation under isothermal conditions,<sup>16,17</sup> as well as the influence on the gelation temperature of a ramp rate ap- plied to the solution.<sup>10,13,18</sup>

Gelatin properties can be improved by adding co-solutes, which interact through either chemical or physical junctions in a way to increase, on the one side, rheological and mechanical strength, or to confer, on the other side, peculiar properties, such as water resistance or thermal stability.<sup>19–21</sup> Despite the great efficacy of the commonly used aldehyde cross-linkers (formaldehyde and glutaraldehyde), their toxicity poses health and safety issues and strongly limit their application, particularly in the biomedical field and food industry.<sup>3,4</sup> Thus, there is a growing interest to find more sustainable and safe cross-linking choices based on natural moieties.<sup>22</sup> Among these, polyphenols are known to interact with proteins, through physical and chemical conjugation, providing higher thermal stability as well as antioxidant features.<sup>23</sup> For such reasons, polyphenols have been explored as additives for gelatin, yet with contrasting results. In some systems, gelatin benefits from their addition, exhibiting improved mechanical properties as well as higher thermal stability.<sup>22,24</sup>

In other cases, however, polyphenols addition results in lower tensile strength of gelatin films.<sup>24</sup> Among bioavailable compounds, humic acids (HA), the alkalisoluble fraction obtained from oxidative degradation of biomass in either natural or biorefinery processes, are intriguing moieties, with various functionalities in their backbone, including quinone, phenol, carboxyl and hydroxyl groups, which confer them different properties, such as antioxidant, antibacterial and anti- inflammatory activity. Thus, HA are biowaste already available in nature in large quantities, as well as polyphenols, with the additional characteristic that HA are more stable in terms of degradation.<sup>25,26</sup> HA also cost less than more common polysaccharides, such as pectin and agar,<sup>27</sup> that are also widely used as additives for gelatin solutions, albeit obtained with a longer extraction. They hold, as such, a huge potential as a source for eco-sustainable materials. To this purpose, recent studies prove that HA blending with hydrogels leads to promising solutions, including biodegradable sorbents and delivery systems for a large number of applications.<sup>28,29</sup>

Despite similarity of chemical functional groups with polyphenols, HA have peculiar features.<sup>30–33</sup> In fact, according to currently accepted view they are made of relatively low molecular weight com- pounds which are self-organized into supramolecular structures, held together by weak dispersive forces, such as van der Waals,  $\Pi - \Pi$ , CH –  $\Pi$ , interactions.<sup>34–36</sup> Furthermore, due to the non-covalent nature of stabilizing interactions, these superstructures are greatly affected not only by the chemistry of surrounding environment, including pH, co-solutes, biological molecules and behave as dynamic systems, but they also undergo self-restructuring in water.<sup>33</sup> Since gel formation occurs in aqueous environment, these features may have great influence on the gel behavior, and cannot be disregarded. Therefore, the effect of HA on gel properties of gelatin cannot be forecast by analogy based on the available studies on polyphenols-gelatin systems, which however exhibit contrasting results.<sup>22–24</sup> A dedicated investigation is required to provide a concrete chance for

HA technological application, according to a waste to wealth approach. Prompted by this need, this study is focused on understanding the gel behavior and physicochemical properties of gelatin modified with HA. To this purpose, gelatin from porcin skin (Type A) was selected as the most exploited source for gelatin hydrogels,<sup>37</sup> whereas Aldrich Humic Acid has been selected as a model HA moiety. Rheological properties have been investigated over a wide range of compositions (i.e., by tuning HA content, keeping fixed the gelatin concentration) to study the effect of HA on the mechanical and thermal properties of the resulting hydrogel. Gelation kinetics and transition temperatures have been investigated as a function of the HA concentration. Hydrogels have been characterized in terms of both the resulting strength and the chemical inter- and intra- molecular interactions arising between gelatin and HA. The physico-chemical properties of Gelatin-HA systems were assessed through X-Ray Diffraction (XRD), Fourier Transform Infrared spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM) and swelling kinetics to investigate the non-covalent interactions between HA and gelatin and their role on conformational and rheological features.

# 2.3 Results and discussion

#### 2.3.1 Effect of HA on viscoelasticity and temperature ramps

Figure 1 shows the typical response of a dynamic temperature ramp test, both in cooling (blue curves) and in heating (red curves). The viscoelastic moduli are reported as a function of temperature for both solutions (Figure 1a: Gelatin; Figure 1b: Gelatin-HA 16). In both cases, at high temperature, the sample is a viscous liquid, as revealed by the very low viscoelastic moduli. Approaching a critical temperature, the viscoelastic moduli start to increase abruptly, pointing at an incoming gelation process. At low temperature, below the critical temperature just discussed, the elastic modulus exceeds the viscous modulus by two orders of magnitude. This behavior indicates that a gel-like structure is formed. Then, when the temperature ramp is reversed, a melting process takes place, characterized by a steep decrease of the viscoelastic moduli. Finally, at high temperature, the moduli return to their initial values, indicating that the gelation is thermoreversible. The difference between the cooling and the heating ramps is indicative of a hysteresis, which depends on ramp rate.<sup>10</sup> Various ramp rates were performed, and the transition temperatures extracted, following Avallone et al.<sup>10</sup> For more details, see Figure S1 in Supporting Information.



*Figure 1:* Viscoelastic moduli measured during cooling and heating ramps at 1°C/min, for (a) Gelatin and (b) Gelatin-HA 16

**Figures 2a and 2b** report the complex modulus during cooling and heating at 1°C/min, respectively, for different systems containing various HA amounts. Increasing HA content, the rheological curves shifts non-monotonically, suggesting the presence of a critical HA concentration,  $c*_{HA} \sim = 13.33$  (wt/wt)%, for which the complex modulus exhibits the highest values, both in cooling and in heating ramp.



*Figure 2:* Complex modulus as a function of temperature at  $1^{\circ}$ C/min and 10 rad/s for different concentrations of gelatin and humic acid aqueous solutions (a) cooling and (b) heating ramp at  $1^{\circ}$ C/min.

**Figure 3a** displays frequency sweep tests at 5°C for various samples at different HA concentrations. The linear rheological response is typical of an elastic network, with G' higher than G" and independent on frequency, whatever HA content is considered. **Figure 4b** reports the transition temperatures in cooling ( $T_{sol-gel}$ , blue squares) and in heating ( $T_{gel-sol}$ , red squares), measured at 1°C/min together with the elastic modulus at 5°C and 10rad/s as a function of HA content. The elasticity of the resulting gel changes in a non-monotonic way according to HA amount. A low content (up to 13.33 (wt/wt)%) increases the viscoelasticity of the resulting gel, whereas a higher HA amount builds a "softer" gel, characterized by a larger value of the viscous modulus and a lower value of the elastic modulus. In other words, when HA exceed a specific amount, their presence reduces the "distance" between G' and

G". Even the transition temperatures show the same non-monotonic trend as a function of the HA content.

Notably, HA addition to gelatin up to a concentration of 13.33 (wt/wt)% has both G' and G'' increased, resulting in |G\*| values higher than water/gelatin system. These features evidence that mechanical and thermal stability of the gel improve, due to the presence of HA. Notably, it must be argued that up to a limit concentration HA has a beneficial effect on the gelation process, promoting formation of a tighter network.



*Figure 3:* (a) Frequency sweep tests at  $5^{\circ}C$  for samples with different HA concentrations; (b) Transition temperatures and elastic modulus at  $5^{\circ}C$  and 10 rad/s as a function of HA content.

#### 2.3.2 Effect of HA on gelation kinetics

**Figures 4a and 4b** show the isothermal kinetics for Gelatin and Gelatin-HA 16 samples, respectively. In particular, the complex modulus as a function of time is reported at different temperatures. The gelation process can be followed with time by the transient increase of the complex modulus, being faster at lower temperatures. Moreover, by comparing the data plotted in **Figures 4a and 4b** at the same temperature (same curve color), it is evident that, at short times, the value of |G\*| is higher for the Gelatin-HA 16 sample. It means that HA affect the rheological properties, since they increase the viscosity of the final solution. Conversely, at long times, a weaker gel (characterized by a lower value of |G\*|) is obtained for the Gelatin-HA 16 sample.

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks



*Figure 4:* Complex modulus as a function of time for different temperatures for (a) Gelatin and (b) Gelatin-HA 16 samples.

**Figure 5** displays the temperature dependence of the gel time for Gelatin, Gelatin-HA 8 and Gelatin-HA 16 samples, highlighting that HA slightly affect  $t_{gel}$ , since the time necessary to obtain a gel is marginally influenced.



Figure 5: Temperature dependence of the gel time.

To investigate the reasons for this rheological behavior, a detailed physico-chemical characterization was carried out.

#### 2.3.3 Effect of HA on compression tests

The compression responses of gelatin gels with and without HA are shown in **Figure 6a**. Samples display nonlinear elasticity and strain hardening at large stretch ratio. The value of deformation at break increases on increasing HA concentration, showing a tighter network that must be broken under the effect of the uniaxial compression in the presence of HA. The stress at break, instead, shows a nonmonotonic behavior with HA content, although not significant. Such features can be easily detected by the inset in **Figure 6a**, which also reports the abrupt decrease of true stresses after breaking.



Figure 6: Uniaxial compression experiments; true stress plotted as a function of the stretch ratio for the samples reported in Table 1. The inset reports stress-strain curves

#### 2.3.4 XRD Analysis

**Figure 7** shows XRD profiles of Gelatin, HA, Gelatin-HA 16 and Gelatin-HA 8 samples. XRD patterns of bare gelatin and HA are reported in the same picture for comparison. HA XRD profile, displayed in **Figure 7** with black curve, exhibits a broad halo, revealing its amorphous structure except for some weak diffraction peaks related to the presence of some inorganic materials, typical of clay soil. Gelatin XRD pattern shows a sharp and a broad peak at  $2\theta$  of  $8^{\circ}$  and  $20^{\circ}$ , respectively, typical of a partially crystalline gelatin structure.<sup>38</sup> Notably, the former is assigned to the ordered triple-helical crystalline structure, whereas the latter confirms the presence

of  $\alpha$ -helix in the protein.<sup>39</sup> The peak at 20 of 8° is strongly reduced in XRD profile of Gelatin-HA 16 sample, indicating a decrease of the content of triple helices in the sample and, thus, a change in the secondary structure of gelatin due to its mixture with HA.<sup>38</sup> Instead, Gelatin-HA 8 XRD pattern shows that the peak at 20 of 8° is only slightly reduced, suggesting that in this case the conformation of the protein is better preserved after mixing with HA. These results agree with the rheological ones, which highlighted a weaker gel for the sample Gelatin-HA 16. In fact, at high amounts, HA influence the protein secondary structure, preventing gelatin chains from organizing into triple helix domains and causing formation of more disordered organization.



Figure 7: XRD patterns of Gelatin, HA, Gelatin-HA 8 and Gelatin-HA 16

#### 2.3.5 FT-IR spectroscopy

FT-IR analysis was carried out to identify functional groups of Gelatin, HA, Gelatin-HA and Gelatin-HA mix powders and to assess if any change occurred due to Gelatin-HA interactions (**Figure 8a**). **Tables 1 and 2** list the band assignment in the FT-IR spectra of Gelatin and HA, respectively.<sup>40,41</sup>

region	wavenumber (cm <sup>-1</sup> )	functional groups
amide A	3430	$\nu_{ m NH}, \nu_{ m OH}$
amide B	3060	$ u_{ m NH} $
amide B	2930	asymmetric $\nu_{\rm CH_2}$
amide I	1650	$\nu_{\rm C=O}, \nu_{\rm NH}$
amide II	1540	$\nu_{\rm NH^{\prime}} \; \nu_{\rm C-N^{\prime}} \; \nu_{\rm C-C}$
amide II	1450	$\nu_{CH_2}$
amide II	1410	$\nu_{\rm COO}$ -
amide II	1330	$\delta_{\rm CH_2}$ wagging
amide III	1235	$\delta_{\rm C-N}, \delta_{\rm NH}$
amide III	1080	$\nu_{\rm C-O}$

 Table 1. Infrared spectral characteristics of gelatin

wavenumber (cm <sup>-1</sup> )	functional groups
3690	$\nu_{ m NH_2}$
3390	phenolic –OH hydroxyl groups
2925	aliphatic C–H bands
1575	antisymmetric $\nu_{\rm COO}$ - of carboxyl salt
1380	symmetric $\nu_{ m COO}$ - of carboxyl salt
1100	$ u_{\rm CO} $ (phenolic), $ u_{\rm OH} $ (aliphatic)
1040	$\nu_{\rm C-N}$
1005	$\nu_{\rm CO}$
910	out-of-phase $\delta_{ m CH}$ (aromatic)



The FT-IR spectra of Gelatin and Gelatin-HA 8 are similar. This evidence is due to the high amount of Gelatin in the sample, which cover the HA FT-IR characteristic bands. On the other hand, in the FT-IR spectrum of Gelatin-HA 16 the band in the range 3500 - 3400 cm<sup>-1</sup>, assigned to N-H and O-H stretching vibration modes, shifts towards lower wavenumbers (3270 cm<sup>-1</sup>).<sup>42,43</sup>

This can be attributed to the H-bonds interactions between carbonyl groups of HA and H atoms in gelatin residues, further confirming the interaction between HA and gelatin (observed in the TGA analysis - **Figure S2**). At a closer look, in the FT-IR spectrum of Gelatin-HA 16, Amide I band changes its shape, moving towards lower wavenumbers, suggesting that the peaks, related to unordered structures, become

more prevalent, indicating protein restructuring due to the mixture with HA. Furthermore, the bands at 1653, 1540 and 1400cm<sup>-1</sup>, respectively, related to  $v_{C=O}$  and  $v_{NH}$  stretching vibrations in Amide I and to  $v_{NH}$ ,  $v_{C-N}$ ,  $v_{C-C}$  and  $v_{COO-}$  stretching modes in Amide II, grow more intense in the FT-IR spectrum in Gelatin-HA 16 sample.<sup>50,51</sup> This might be partly due to presence of HA, whose peculiar bands occur in the same ranges. These modifications are a further proof of a conformational change of the gelatin secondary structure.

More insight can be obtained by calculating the second derivative of FT-IR spectra in the range  $1600 - 1700 \text{cm}^{-1}$ , which is reported in **Figure 8b**. This enabled to resolve Amide I band into six main peaks at  $1693 \text{cm}^{-1}$ ,  $1663 \text{cm}^{-1}$ ,  $1658 \text{cm}^{-1}$ ,  $1652 \text{cm}^{-1}$ ,  $1630 \text{cm}^{-1}$  and  $1620 \text{cm}^{-1}$ , related to the presence of intermolecular associations: triple helix (1658,  $1663 \text{cm}^{-1}$ ), single  $\alpha$  – helix ( $1652 \text{cm}^{-1}$ ),  $\beta$  – sheets ( $1620 \text{cm}^{-1}$ ),  $\beta$  – turns ( $1693 \text{cm}^{-1}$ ) and random coil ( $1630 \text{cm}^{-1}$ ).<sup>44,45</sup>

In the secondary derivative of Gelatin-HA 16, the peak of unordered structure is prevalent, whereas those related to triple helix are no longer evident. These features suggest that the protein undergoes a conformational change due to its interaction with HA. This is also confirmed by the shift towards lower wavenumbers of the peaks at 1693cm<sup>-1</sup> and 1620cm<sup>-1</sup>, related to intermolecular associations in the gelatin matrix. Changes in the second derivative are more evident in the sample with the highest HA concentration (**Figure 8b**), confirming the key role of HA in modifying protein structure.

Notably, high concentration of HA leads to a random coil organization because the protein chains are not able to establish triple helix domains, typical of an ordered structure. This is also confirmed by XRD spectra that show a more unordered structure of gelatin due to the presence of HA in the matrix. On the contrary, the second

derivative of Gelatin-HA 8 suggests that the helical structures are much better recovered, pointing out that HA act through hydrogen bond interactions with gelatin, also improving the elastic properties of the resulting gel, as shown in Figure 3. HA are mixtures of relatively low molecular weight compounds, characterized by a great variety of functional groups, including quinone, phenol, carboxyl and hydroxyl moieties.<sup>35,46</sup> HA are organized in supramolecular soft and permeable clusters, exposing hydrophilic groups whereas hydrophobic species are shielded in the interior.<sup>47,48</sup> These chemical features make HA highly reactive with proteins.<sup>49</sup> Accordingly, they can interact with gelatin through non-covalent inter- action such as hydrophobic and electrostatic interactions, as well as H-bonds. Notably, H-bond interactions should be established between carbonyl groups of HA and hydroxyl groups of hydroxyproline and proline residues as well as amino moieties of glycine, which are the most abundant amino acids in gelatin chains.<sup>50</sup> This hypothesis is supported by previous studies, reporting interactions between gelatin and polyphenols, which bear similar functional groups as HA.<sup>42,51</sup> Indeed, electrostatic interactions between HA and gelatin should be negligible, since the pH value of HA-gelatin solutions is roughly 8 (close to gelatin isoelectric point) and both moieties are expected to be negatively charged. Thus, HA improve elastic properties of the final gels if their concentration does not exceed 13.33 (wt/wt)% dry gelatin. This kind of interactions accounts for gel formation in the investigated samples, as evidenced by the marked dissolution of the gel in urea solution, which is effective in breaking hydrogen bonds.44 More specifically, FT- IR spectra and XRD patterns (Figures 7 and 8a) clearly evidence that HA affect gelatin structure. Notably, the addition of higher HA concentrations hinders the protein structuring into the triple helix conformation, probably because water molecules are constrained by interactions with HA moieties, preventing H-bonds between gelatin and water.<sup>52,53</sup> In this way, they may interfere

with the hydrogen bonding among three  $\alpha$  – chains required to form the triple helix structure.



Figure 8: (a) FT-IR spectra (b)  $2^{nd}$  derivative spectra

## 2.3.6 SEM analysis

**Figure 9** shows SEM pictures of gelatin and Gelatin-HA surfaces. Bare gelatin exhibits a smooth surface (**Figure 9a, d**) whereas, the introduction of HA determines a significant change in gelatin structure, producing a rougher surface (**Figure 9b, c, e, f**) due to the presence of sub-micrometric particle aggregates. These results suggest that the interaction between gelatin and HA induces a partial coagulation of the protein, preventing the formation of the ordered structure.<sup>32,54</sup> This phenomenon occurs at a larger extent in the presence of higher HA content in the samples. In fact, Gelatin-HA 16 sample shows the most heterogeneous structure, with large aggregates and large voids. Observed aggregates might be produced by the precipitation phenomena caused by the interaction between HA and gelatin. A similar behavior was already observed by the addition of polyphenols, which caused gelatin coagulation. Precipitation could prevent gelatin chains from reverting to triple helix structure and causes a decrease in the gel strength.<sup>55,56</sup> These findings are in accordance with FT-IR and XRD results, which evidence that an excessive amount of HA in the gel causes the loss of triple helices organization in the gelatin network.



Figure 9: SEM Pictures of Gelatin (a,d), Gelatin-HA 8 (b,e), Gelatin-HA 16 (c,f)

## 2.3.7 Low temperature DSC

Several studies reports the key role played by water in the network formation of gelatin.<sup>16,17,57</sup> At the same time, HA show great affinity towards water because of their hydrophilic groups.<sup>33,58</sup> Therefore, HA and gelatin might compete for interactions with water molecules.To investigate if any selective affinity occurs between water and HA or gelatin, respectively, low temperature DSC analysis was carried out on gelatin films, containing different amounts of HA (20 wt% and 50 wt%). **Figure 10** shows the DSC curves, evidencing an endothermic peak at about 0°C for the bare gelatin (red curve), related to the melting point of crystallized water.<sup>57</sup> Instead, this effect is not present in the DSC curve of bare HA (black curve), suggesting that water molecules are constrained by intimate interactions with HA moieties, which prevent them from crystallization.<sup>58</sup> Water affinity to HA must be larger than to bare gelatin, whose DSC profile shows an evident ice melting peak,

suggesting the presence of relevant amount of crystallized water. To highlight a significant change in crystallization water by varying HA concentration, a sample in which HA concentration is equal to that of gelatin (green curve) has been prepared. Concerning with this aspect, it is possible to observe that the peak area decreases, and peak temperatures shift to lower values by increasing HA content in the samples, confirming that upon increasing HA amount, the content of crystallizable water decreases.



*Figure 10:* DSC curves of Sol 1 (red curve - pure gelatin), Sol 2 (blue curve), Sol 3 (green curve) and HA (black curve).

The great ability of HA to attract and interact with water molecules should enable formation of more protein-protein junctions, increasing the network tightness and gel viscosity. Similar results were obtained by introducing sugar molecules into gelatin solutions.<sup>22</sup> On these bases, it can be inferred that in Gelatin-HA samples, HA preferentially interact with water molecules and "sequester" them, preventing their coordination with gelatin chains. It is known that water plays a key role in stabilizing the triple helix structure and the arising gel network, since it can act as a bridging agent between gelatin chains through H-bond interactions.<sup>52,59</sup>

Elastic properties show a non-monotonic trend with the HA content in the samples. Both elastic and viscous moduli, G' and G", increase up to a HA critical concentration 13.33 (wt/wt)% dry gelatin, whereas G' decreases at higher HA content. This behavior is in accordance with other studies reporting the rheological and physico-chemical features of gelatin hydrogels including polyphenols.<sup>40,68,69</sup> However, the amount of HA that can be added to gelatin solution without impairing its elastic properties, is significantly larger than the usually used polyphenols content. This can represent an intriguing feature for technological applications in view of antioxidant or antimicrobial properties of HA. Indeed, the best performing composition of the system Gelatin-HA is equivalent to other studies, reporting the use of Galla Chinensis as an additive for gelatin.<sup>61</sup> Nevertheless, compared to natural polyphenols, HA have the great advantage to be more stable towards oxidation phenomena.

In order to explain the non-monotonic trend of gelatin rheological properties with HA content, we deduce that if HA do not exceed a critical HA concentration ( $c_{*HA} = 13.33 (wt/wt)$ %), the presence of tight physical interactions between HA and gelatin and the ability to attract water molecules improves gel strength. However, high HA content more likely produces coagulation of gelatin, and the further increase of viscosity prevents interchain interactions due to the inhibition of gelatin molecules from approaching each other required to achieve and ordered organization. At the same time, water absorption by HA can produce relevant swelling phenomena,<sup>62,63</sup> consequently protein chains less interact through inter-chain hydrogen bonds, thus producing a weaker gel, as confirmed by the decrease of the elastic modulus, previously shown in **Figure 3**.

## 2.3.8 Swelling analysis

Figure 11 shows swelling kinetics of neat gelatin and gelatin-HA samples. The swelling ratios of bare gelatin and Gelatin-HA 1.6 samples steadily increased reaching a constant value soon after 20 min, whereas Gelatin-HA 8 and Gelatin-HA 16 specimen achieved constant weight after 60 min, evidencing slower swelling kinetics with higher water absorption. In fact, constant swelling ratios of the control, Gelatin-HA 1.6, Gelatin-HA 8, Gelatin-HA 16, were 2500%, 1200%, 5000% and 5500%, respectively. Therefore, HA addition to gelatin resulted in a non-monotonic trend of swelling ratio, Gelatin-HA 1.6 showing the lowest value among investigated samples, revealing a decreased ability to absorb water. Furthermore, measured values for Gelatin-HA 8 and Gelatin- HA 16 specimen are much higher than the those reported in literature for polyphenol containing gelatin.<sup>40</sup> Obtained results evidence that Gelatin-HA 8 samples combine relevant water absorption with improved elastic properties. The swelling ratio is usually influenced by cross-linking density as well as hydrophilicity.<sup>32,64</sup> From the analysis of obtained result it can be inferred that at low HA concentration, physical junctions between HA and gelatin result in a lower ability to absorb water. On the other hand, HA evidence a marked hydrophilic behavior, proven by low temperature DSC analysis as well as relevant swelling phenomena in water, usually with slow kinetics.<sup>33</sup> These features must prevail at high content leading to a marked improved water uptake, but with slower swelling rates as shown in Figure 11.



Figure 11: Swelling kinetics of gelatin hydrogels with and without HA at different concentrations.

# **2.4 Conclusions**

The incorporation of HA into the gelatin network affects the viscoelastic properties as well as the thermal stability of the resulting gel. Rheological characterization and in-depth physico-chemical investigation of gelatin samples at various HA concentrations demonstrated that HA affect the final gel structure through additional H-bonds. Besides, gelation kinetics is only slightly influenced by HA concentration, proving that humic acids marginally affect the time needed to obtain a gel with respect to water/gelatin solution. Nevertheless, a non-monotonic behavior in the elastic, swelling and thermal properties of the final gels was found, by varying HA content in solution.

At low HA concentrations (up to 13.33 (wt/wt)%), HA improve both elastic and thermal properties of the obtained gels thanks to H-bonds intimate interactions with gelatin chains, as also suggested by FT-IR results.

Physical interactions between HA and gelatin also reduce water absorption ability of the final gel up to HA concentration of 2.67 (wt/wt)%.

Low temperature DSC analysis evidence that HA have higher water affinity than gelatin. Therefore, at higher HA concentrations, elastic properties, and thermal stability decrease, due to the HA- driven swelling phenomena. This implies that the inter-chains interactions with the gelatin matrix are, as such, inhibited. On the other hand, marked hydrophilic features of HA have swelling ratio increased.

Furthermore, XRD and second derivative of FT-IR spectra proved that a higher HA content in solution leads to a prevailing random coil gelatin organization since the protein chains are not able to establish wide triple helix domains.

Our results shed light on the key aspects that might contribute to a more conscious management of gelatin properties, in the presence of bio-available moieties, such as HA, contributing to expand their re-purpose in a wide range of applications.

# 2.5 Supporting information

**Figure S1** reports  $T_{sol-gel}$  and  $T_{gel-sol}$  as a function of the imposed ramp rate. As expected, the  $T_{sol-gel}$  has a larger dependence on the ramp rate with respect to  $T_{gel-sol}$ . Both  $T_{sol-gel}$  and  $T_{gel-sol}$  move to lower temperatures at higher HA concentration (2.67 (wt/wt)%). On the other hand, samples with low quantity of HA (13.33 (wt/wt)% and 26.67 (wt/wt)%) show higher transition temperatures compared to the systems Gelatin and Gelatin-HA 1.6.

TGA curves of investigated samples (**Figure S2**) clearly show lower degradation kinetics than bare gelatin.<sup>1,2</sup> This does not occur in a physical mixture of powdered HA and gelatin (magenta curve), which evidences a TGA curve comparable to that of bare gelatin. These results suggest that interaction must be established between HA and gelatin, due to their intimate mixing and accounting for improved thermal stability.<sup>3</sup>



*Figure S1:* Tsol-gel and Tgel-sol as functions of the imposed ramp rate for various ternary sys- tems (HA, gelatin and water - see legend for details). The horizontal dashed line marks the two transitions.
Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks



Figure S2: TGA curves of Gelatin, HA, Gelatin-HA 0.8, Gelatin-HA 1.6 and Gelatin-HA Mix Powders

# 2.6 Acknowledgements

This work is dedicated to the memory of Veronica Vanzanella.

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# 3. Detailed thermal, fire and mechanical study of siliconmodified epoxy resin containing humic acid and other additives

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# 3.1 Abstract

Following a waste-to-wealth approach, humic acid (HA) was exploited as a flameretardant additive. The effect of its addition alone and in combination with urea (UR) and ammonium polyphosphate (APP), on the thermal, fire and mechanical performances of a bisphenol A diglycidyl ether (DGEBA)-based epoxy resin modified with (3-aminopropyl)-triethoxysilane (AP) and cured with aliphatic isophoronediamine (IDA), has been investigated. Unlike in previous studies a UL 94-V-0 classification was achieved for epoxy resin containing HA at 6 wt.% and APP at only 1 wt.% phosphorous (P) loading. The presence of silicon-modified epoxy chains ameliorated the distribution of the bio-waste within the resin and the addition of HA alone avoided melt dripping. Besides, APP and UR promoted a remarkable reduction (up to 52%) of the peak heat release rate (pHRR) values and a significant delay (up to 21%) of the time to ignition in cone calorimetry tests, hence an increase (up to 1.8 min) of the time to flashover, without any detrimental effect on the overall mechanical behavior. Evolved gas, thermal and fire analysis were used to propose the combined mode of action of HA, UR, APP and silicon in the fire performance improvement of the hybrid epoxy system.



## **3.2 Introduction**

In recent years, human being has been responsible for serious environmental impacts in term of major environmental concerns related to the global warming, depletion of natural resources as well as increased waste production <sup>1, 2</sup>. However, global awareness and governmental actions are now focused on a transition from a linear economic model toward a more sustainable circular approach, which allows for resources savings and waste reduction as well. Indeed, thinking of waste as resource is mandatory to preserve human and environmental health, though, at the same time, it can provide a great opportunity to economic and technological growth <sup>3</sup>. Thus, worldwide governments have been provided economic support for this transition through effective policies of intervention <sup>4</sup>. At the same time, the design and exploitation of more sustainable products, with a low impact on health and environment, have been rising a great interest in the scientific community. Recently, it has been even recorded a huge production of bio-wastes because of the human activities spanning from households to industrial applications including food processing and bio-refinery<sup>5</sup>. In the manufacturing of polymer-based products, some additives must be included to fulfill physico-chemical and mechanical performance requirements as defined by standards and regulations <sup>6</sup>. However, such compounds are mostly produced from nonrenewable sources and they can rise serious ecological concerns mainly linked to the release of toxic species in the atmosphere <sup>7</sup>. This issue still poses huge limitations for recycling of polymers <sup>3</sup>. Among polymer systems, epoxy resins are used to develop a large array of high-performance products including linings and components in the aircraft sector, where, along with the typical requirements, severe fire safety standards and regulations must be fulfilled<sup>8</sup>. In this field, as greener alternative than halogen-based flame retardants, halogen-free additives (e.g. ammonium polyphosphate (APP)) as well as bio-based and biomassbased raw materials (e.g. chitosan, lignin, cyclodextrins) have been investigated <sup>8</sup>.

However, a noticeable amount of these compounds must be incorporated into the epoxy matrix to achieve satisfying fire performances. As an example, Zhang et al. prepared thermoplastic polyurethane composites through the addition of 6.25 wt.% of chitosan derivatives (CSD) and 18.75 wt.% of APP <sup>9</sup>. Further, extensive use of bio-based compounds can overcome the earth's regenerative capacity, rising further environmental issues. Thus, in a context of sustainable development, the reuse of bio-waste materials as functional flame retardant additives for polymer-based systems, may represent one of the most promising approaches for moving toward a circular economy concept <sup>5, 10</sup>. Among bio-wastes, humic acids (HAs) are the alkalisoluble fraction of natural organic matter obtained by the biological and chemical degradation of both vegetal and animal biomasses <sup>11</sup>. HAs consist of a skeleton of aliphatic or aromatic units, with a marked amphiphilic behavior. These moieties are stabilized by weak hydrophobic, hydrogen and metal-bridged electrostatic bonds in supramolecular architectures that can change their functionalities depending on the chemical environment <sup>12</sup>. Due to the presence of oxygen-containing functional groups, a carbon-enriched chemical composition and supramolecular architectures, HAs can promote the charring process during the epoxy degradation upon exposure to a flame or an irradiative heat flux and establish good physical interactions with the polymer matrix. Therefore, HAs can provide epoxy resins with a huge potential as flame retardants <sup>13</sup>. However, the use of HAs as effective flame retardants for polymer systems has been poorly investigated. Liu et al. chelated HAs with four different metal ions and incorporated them into a modified DGEBA resin to enhance its flame retardance. In particular, as a result of the incorporation of 10 wt.% of HA-Fe and HA-Mn into the epoxy matrix, the limiting oxygen index (LOI) increased from 21.2% for pristine resin to 26.6 and 25.3%, respectively; meanwhile, the pHRR was reduced by 36 and 35.5%, respectively <sup>14</sup>. Despite HAs were used from Liu et al. as a charring agent, as far as we know, no self-extinguishing materials using HA

have been developed so far. Furthermore, a complete characterization of mechanical and fire behavior of HA-containing epoxy systems is still missing, which is really needed to enable any technological exploitation. An excellent distribution of the flame retardants within the epoxy resin is crucial to achieve uniform overall performances. To this purpose, the reaction between DGEBA and (3-aminopropyl)-triethoxysilane can form hybrid silicon-containing epoxy moieties able to improve the interphase between matrix and filler, because of their capability to interact with polar and apolar components of additives, hence enabling a fine dispersion <sup>15</sup>. Considering their amphiphilic nature, HAs are expected to achieve a very good distribution into silicon-modified epoxy resins and promise to show enhanced charring behavior and fire performances.

In this study, a detailed study on the role of HA bio-waste as a flame retardant for epoxy resin has been undertaken. To this purpose, hybrid silicon-modified epoxy composites were synthesized via sol–gel procedure from DGEBA and AP and then cured with IDA as a cycloaliphatic hardener. HA, UR and APP were added, alone or in combination, to this resin system in order to assess their role in flame retardance. Finally, a composite material with superior fire performance (V-0 rated in UL 94 tests) and low P-loading (not exceeding 1.0 wt.%) was obtained. Then, the effect of P-N synergism on the flame retardance of the hybrid epoxy composites was investigated. Fourier-transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR) was exploited for assessing the chemical composition consistency of the epoxy samples and the completeness of the reactions occurring between HA and both epoxy and curing agent. The thermal and fire behaviors of the obtained composites were thoroughly investigated by means of thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), cone calorimetry, UL 94 vertical flame spread tests, DIP-MS (direct insertion probe-mass spectrometry).

Furthermore, the effect of various additives on the mechanical behavior of the epoxy resin was studied by three-point bending tests.

## 3.3 Results and discussion

#### 3.3.1 Chemical study of the hybrid epoxy composites

The completeness of the curing process was proved by the disappearance of the characteristic adsorption bands of uncured epoxy resin (ENC) at 970 cm<sup>-1</sup>, 912 cm<sup>-1</sup> and 870 cm<sup>-1</sup> in the ATR-FTIR spectra of all cured epoxy systems (**Figure S1**). Furthermore, an increase of adsorption bands at 1100 cm<sup>-1</sup>, 950 cm<sup>-1</sup> and 800 cm<sup>-1</sup> was observed in the ATR-FTIR spectra of the samples prepared using AP (i.e. for E12AP, E6AP\_6HAURAPP and E12AP\_12HAURAPP samples) <sup>16</sup>. These features might suggest the formation of a hybrid epoxy phase containing silicon (i.e. of an organic-inorganic structure) as already proved in previous papers <sup>1, 16</sup>. In addition, the increase of the FTIR signals at about 3400 cm<sup>-1</sup>, 2920 cm<sup>-1</sup> and 2870 cm<sup>-1</sup>, related to N-H asymmetric deformation vibration <sup>17</sup>, confirms the presence of APP in E6AP\_6HAURAPP and E12AP\_12HAURAPP samples.

The presence of HA in E6AP\_6HAURAPP, E12AP\_12HAURAPP, ENC+HA and HA+IDA samples can be supported by the appearance of the FTIR bands around 3300 cm<sup>-1</sup>, which are attributed to O-H stretching of oxygen-containing functional groups (i.e. carboxyl, phenol, and alcohol) <sup>1, 13, 18, 19</sup> and at 1580 cm<sup>-1</sup>, related to C=O bonds, ketone and quinone moieties (**Figures S1, S2 and S3**). Moreover, these bands grow more intense in the FTIR spectra of the samples containing UR proving its presence within the matrix. Furthermore, physical mixtures of HA with ENC or IDA, which underwent the same cure and post-cure treatments as the other hybrid epoxy composites, did not evidence any significant change in the related FTIR spectra, suggesting that no reaction occurred between HA and ENC or IDA, during the curing process (**Figures S2 and S3**).

#### 3.3.2 Thermal analysis

**Figures 1a and 1c** show the thermogravimetric curves of E, E12AP, E12AP\_12HAURAPP, E6AP\_6HAURAPP samples recorded in N<sub>2</sub> and air atmosphere, respectively. In N<sub>2</sub> atmosphere, a first weight loss occurs at about 280 °C; this finding is ascribed to the production of some volatile products, specifically acrolein, acetone and allyl alcohol, as already reported in the literature  $^{20, 21}$ . The main decomposition step of the crosslinked resin (beyond 340 °C) occurs through the release of high molecular weight products along with more complex phenolic compounds  $^{21}$ . Conversely, no weight changes were observed between 400 and 600 °C, due to the production of a very stable aromatic char, (**Figure 1 a, b**).

In air (**Figure 1 c, d**), two main degradation steps around 350 °C and 500 °C are evident, in agreement with the degradation pathways of an aliphatic epoxy resin  $^{21}$ ,  $^{22}$ .

Besides, the incorporation of AP leads to the appearance of a second degradation step at around 400 °C in N<sub>2</sub> atmosphere and to a shift of the oxidative degradation phenomena toward higher temperatures in air atmosphere, possibly due to the hybrid epoxy network that provides a higher thermal stability to the epoxy matrix (**Figure 1**)  $^{23, 24}$ .

The presence of any of the employed additives improves the overall thermal behavior of the samples, providing higher residues with respect to the neat resin E, which, conversely, decomposes completely.

HA positively affects the thermal behavior of the epoxy systems, because of the generation of a stable aliphatic char under inert atmosphere at high temperatures, resulting in an increase of the residue as compared to E <sup>14, 25</sup>. In fact, the addition of HA leads to a faster decomposition of the resin chains, due to the acidic features of HA, which favor the char formation during the pyrolysis process <sup>14, 17</sup>. Notably, the aromatic nature of HA allows the production of a more stable char during the

degradation of E in air atmosphere, preventing the full oxidation of the resin with the formation of low molecular weight molecules <sup>14, 22, 26</sup>. In addition, the combined presence of HA and each of other additives within the resin further improves the thermal behavior: in fact, the charring phenomenon is further supported by the presence of AP-modified DGEBA moieties containing siloxane groups that show a weak acidic character.

In air, the presence of AP in the epoxy matrix brings to the formation of a siliconrich char, which acts as a thermal shield and protective barrier, preventing oxygen diffusion, hence further enhancing the thermo-oxidative stability.

Besides, an abundant char formation is further expected by the degradation of the samples containing APP, whose decomposition produces acid phosphorus species and non-flammable volatiles (i.e. P-species and N<sub>2</sub>), promoting a strong dehydration of the polymer matrix. Then, phosphoric acids obtained by APP decomposition might react with amino groups of AP-modified DGEBA moieties, generating P-N-O substructures on the char surface, which improve its intumescence, as well as its efficacy as oxygen barrier during combustion  $^{26, 27}$ . Also, the decomposition of UR produces NH<sub>3</sub> that can be exothermically oxidized to N<sub>2</sub>, hence leading to a dilution of gas phase and a delay of the ignition time  $^{26, 28, 29}$ .

Therefore, E12AP\_12HAURAPP sample may combine the beneficial effects of HA, APP and AP on the overall thermal stability with those provided by UR, able to dilute the flammable volatiles in the gas phase and generate an intumescent char with excellent fire retardant properties <sup>27</sup>. This hypothesis is confirmed by experimental data showing that the sample E12AP\_12HAURAPP has the best thermal performance, combining one of the highest residues with self-extinguish properties.

Chapter 4 – Design of humic acids-based polymeric materials: from electrospun hybrid fibers to 3D networks



Figure 1. (a, c) TGA curves and (b, d) DTG curves of EPO and epoxy composites under  $N_2(a, b)$  and air (c, d).

3.3.3 Fire behavior of the hybrid epoxy composites

Vertical flame spread tests were carried out on all the epoxy composites to assess their flammability. E12AP\_12HAURAPP was self-extinguishing and V-0 rated (**Figure S4**). On the contrary, all the other investigated samples were not classifiable (**Table 1**), though the formation of an abundant and coherent char was observed in the composites containing both AP and HA. The highest production of char occurred in E12AP\_12HAAPP sample, where the acid phosphorus compounds released by APP during its decomposition contributed to dehydration of the epoxy resin, through a synergistic effect with hybrid chains and HA additives. In addition, the presence of

both AP and HA in the epoxy matrix prevented dripping (**Table 1**), due to increase of the melt viscosity of the burning system  $^{30}$ . Considering the afterflame times (t<sub>1</sub> and t<sub>2</sub> values, Table 2) of E\_URAPP, E12AP\_URAPP and E12AP\_12HAURAPP samples, the incorporation of HA into the epoxy resin was crucial to obtain V-0 rating; the sample containing AP, HA (6 wt.%), UR and APP burned partially. producing a very coherent char (Figure S4). This also suggests a combined condensed phase action exerted by HA and the hybrid epoxy moieties, which strongly affects the charring process from the early combustion stages. For E\_URAPP and E12AP\_URAPP samples, a decrease in flame inhibition after first application of flame was observed (Table 1). However, E12AP URAPP showed lower t<sub>1</sub> values, which may be due to the high char-forming character of AP-modified epoxy network, able to generate a silicon-rich char acting as a thermal shield and oxygen barrier for the underlying polymer matrix <sup>31</sup>. Conversely, the use of APP and UR blowing agents was fundamental to positively influencing  $t_1$  extinguishing time (sample E URAPP, **Table 1**), especially when the epoxy resin did not undergo solgel modification <sup>26, 28, 32</sup>. This finding was ascribed to decomposition of APP and UR, since the former acts in the gas phase forming phosphorus species, whereas the latter produces nitrogen-based volatiles, leading to the dilution of flammable volatiles and increasing t<sub>1</sub> extinguishing time with respect to the other unmodified formulations.

Sample	$t_1$ in Sec. $t_2$ in Sec.	UL 94 / Dripping		
E	-	Not classifiable		
		(NC)/Yes		
E_12HA	-	NC/No		
E_UR	-	NC/No		
E_APP	-	NC/No		
E_URAPP	8 7 6 7 8 -	NC/No		

E_12HAUR	-								NC/No		
E_12HAAPP	-								NC/No		
E_12HAURAPP	-							NC/No			
E12AP	-							NC/No			
E12AP_12HA	-							NC/No			
E12AP_UR	-						NC/No				
E12AP_APP	-						NC/No				
E12AP_URAPP	3 3 4 5 3 -					NC/No					
E12AP_12HAUR	-							NC/No			
E12AP_12HAAPP	-							NC/No			
E6AP_6HAURAPP	-							NC/No			
E12AP_12HAURAPP	0 0 0 0 0 0 0 0 0 0 0					V-0/No					

 $t_{1 \text{ and }} t_2 =$  Duration of flaming after 1<sup>st</sup> and 2<sup>nd</sup> flame application. The test was carried out 5 times for all the samples. *Table 1. Results of flame spread tests in vertical configuration* 

During CC tests, the decreases of pHRR (ranging from 39 to 52%) and THR (up to 23%) were more pronounced for the composites containing HA, UR and APP (**Figure 2, Table 2**), though the presence of AP alone (E12AP sample) significantly lowered the aforementioned parameters. However, an increase of the amount of AP, HA, UR and APP delayed the time to pHRR (**Table 2**). This effect can mainly be ascribed to decomposition of APP and UR, as clearly reported in the literature <sup>23, 33-35</sup>.

Cone calorimetry results (**Table 2**) confirm that the highest impact on the flame retardance of the composites is due to the acidic character of the hybrid epoxy chains, exerting a strong condensed phase activity through dehydration of the network and leading to a remarkable reduction of pHRR, mass loss rate (MLR) and FPI, but also to an increase of the residual mass and TTP. However, as previously mentioned for the vertical flame spread tests, the addition of HA in combination with AP, UR and APP was crucial to achieve self-extinction. Figure 2 shows a noticeable broadening

of HRR curve for E12AP 12HAURAPP, where phosphorous species stop the hydrocarbon oxidation reactions and slow down the branching and chain reactions occurring in the gas phase, thus reducing the heat production <sup>33</sup>. An additional of the residue observed in E12AP 12HAURAPP increase was and E6AP 6HAURAPP samples (**Table 2**), where the additives favor the formation of a coherent, swollen, and intumescent char (Figure 3). This can be ascribed to the release of nitrogen species and dehydration of the matrix by acidic hybrid epoxy moieties, HA and phosphorous species formed during combustion <sup>28, 36</sup>. The strong increase in  $CO/CO_2$ ratio observed for E12AP 12HAURAPP and E6AP 6HAURAPP (Table 2) may be due to APP that releases phosphorus species in gas phase, leading to an incomplete combustion and consequently to the formation of CO. These phosphorous species interrupt and slow down the branching and chain reactions of the hydrocarbon oxidation reactions in the gas phase, thus reducing the heat production and leading to flame inhibition <sup>33, 34</sup>.



Figure 2. Heat release rate (HRR) vs. time for E, E12AP, E6AP\_6HAURAPP, E12AP\_12HAURAPP samples

Sample	TT	pHRR	TT	THR	TSR	R	FP	TT	CO/C
	Ι	[kW/	Р	[MJ/	[m²/m	M	Ι	F	<b>O</b> <sub>2</sub>
	[s]	<b>m</b> <sup>2</sup> ]	[s]	<b>m</b> <sup>2</sup> ]	2]	[%	[-]	[mi	[-]
						]		n]	
E	28	1471	125	111	3755	3	52	-	0.29
E12AP	25	856	160	111	3493	7	34	0.5	0.26
E6AP_6HAURA PP	34	902	78	86	2913	9	26	1.6	0.44
E12AP_12HAUR APP	28	704	128	88	3145	11	25	1.8	0.41

TTI = Time To Ignition, pHRR = peak of Heat Release Rate, TTP = Time To Peak, THR = Total Heat Release, TSR = Total Smoke Release, RM = Residual Mass, FPI = Flame Propagation Index, TTF = Time To Flashover, FRI= Flame Retardancy Index. FRI is not reported for EPO, because this sample represents the neat polymer.

Table 2. Results from cone calorimetry tests for the investigated samples



**(a)** 



**(b)** 



**(d)** 

*Figure 3.* SEM images of residual char obtained after CC test of E (a), E12AP (b), E6AP\_6HAURAPP (c) and E12AP\_12HAURAPP (d) samples

To better elucidate the fire behavior of the epoxy samples, time to flashover (TTF) and flame propagation index (FPI) were calculated (**Table 2**) <sup>36, 37</sup>. The first parameter represents the time available to escape a fire in a confined space <sup>37-39</sup>, while the second one depends on the flammability (i.e. front flame movement). E12AP\_12HAURAPP and E6AP\_6HAURAPP samples significantly increased TTF and, therefore, were characterized by lower FPI, smoke production rate (SPR) and total smoke release (TSR) with respect to the pristine resin (**Table 2**). The decomposition of HA, UR, and APP generates greener compounds replacing several aromatic structures (e.g., benzene, naphthalene, anthracene, among a few to mention), which are normally produced during the last steps of the carbonization and gas-phase combustion processes of a DGEBA resin <sup>40-44</sup>. These phenyl-based

compounds are the main components of smoke during the degradation of an epoxy resin <sup>21, 41, 45</sup>.

Based SEM analyses of residual char (Figure 3), a combined effect of HA, UR, APP and AP acting in both condensed and gas phases is proposed (Figure 4). It is well known that epoxy resins are well-established charring-polymers and undergo carbonization processes during combustion <sup>46</sup>. As previously observed in thermogravimetric analysis, HA may boost the dehydration kinetics because of its acidic character, affecting decomposition temperatures and final residue under N<sub>2</sub> atmosphere. A similar condensed phase activity is due to the presence of the hybrid domains, which address the pyrolysis toward the formation a silicon-rich char on the material surface <sup>47</sup>. This latter works as a thermal shield toward the spread of gaseous species supplied from the decomposing polymer bulk and of oxygen <sup>45</sup>. The detection of silicon elements in addition to C, H and N, which are dominating in the char of a pristine epoxy (E sample, Figure 4a), proves the production of a silicon-rich char in the case of E12AP (Figure 4b). As regards SEM analysis (Figures 4a and 4b), it is evident that the char morphology of E12AP appears more compact and coherent with respect to that of E, in which fractures and holes are present. These findings justify the improved fire behavior for E12AP with respect to E observed in forcedcombustion tests (**Table 2**)<sup>16</sup>, as the hybrid epoxy network is responsible for a reduction of pHRR as high as 42%.

The scientific literature well reports that UR and APP degrade around 250 °C, releasing NH<sub>3</sub> that undergoes exothermic decomposition forming N<sub>2</sub><sup>48, 49</sup>. Besides, the degradation of APP produces acid phosphorous compounds during combustion, which, together with HA, are responsible for the generation of an intumescent and abundant char along the dehydration <sup>28, 32</sup>. In addition, NH<sub>3</sub> may also react with polyphosphoric acid to produce P-N-O substructures, hence providing insulating properties to the intumescent char <sup>26, 27</sup>. Further, the combined use of HA, UR and

APP (E6AP 6HAURAPP sample) is responsible for the formation of an intumescent char characterized by the presence of P-N-O-Si-O-P polymeric substructures on the surface (Figure 4c) <sup>27, 36</sup>. These latter are obtained through condensation between silanol groups (i.e. Si–O–Si) of hybrid epoxy network and P-N-O substructures. This char behaves as a thermal shield and oxygen barrier at the boundary layer <sup>32, 45, 50</sup>, leading to a high retention of P for E6AP 6HAURAPP and a strong delay in the ignition time and time to flashover (**Table 2**). However, the synergism of these effects was not enough to achieve self-extinction (Table 1). In this regard, E12AP\_12HAURAPP sample (Figure 4d), prepared with a higher amount of additives, showed a swollen multicellular intumescent char and higher retention of P as compared to E6AP\_6HAURAPP (Figures 3c and 3d). Therefore, it is reasonable to hypothesize that this swollen multicellular intumescent char contains a higher number of P-N-O-Si-O-P polymeric substructures on the surface <sup>26</sup>. SEM analysis supports this hypothesis, since the char morphology of E6AP 6HAURAPP appears intermediate between those produced by E12AP and by E12AP 12HAURAPP, because the multicellular structures are not uniformly formed yet (Figures 3 and 4). Hence, E12AP\_12HAURAPP may exert a strong condensed phase mechanism resulting in excellent performances in UL 94 (V-0 class) and CC tests (52% of pHRR reduction, **Table 2**), despite the use of an aliphatic amine (IDA) as hardener.

The interaction of hybrid moieties, phosphorus flame retardants and nitrogen additives to form a stable char and resulting in an improved fire performance is well known for other polymeric systems, though, based on the best knowledge of the authors, this is the first time that a bio-waste (i.e. HA) becomes a crucial component in the flame retardance of an epoxy-based system <sup>14, 27, 51, 52</sup>.



**Figure 4.** Proposed mechanism of E-AP/APP-UR-HA in oxygen  $(O_2)$  atmosphere. (a) E, (b) E12AP, (c) E6AP\_6HAURAPP, (d) E12AP\_12HAURAPP. Black arrows show the direction of rising bubbles (white balls) from the hot polymer matrix (borrow region). Yellow circles indicate the sol-gel hybrid epoxy moieties in the network. In particular, the yellow border line around the black carbonaceous char indicates the presence of a silicon-rich char (thermal shield and oxygen barrier) on the surface of burning sample.

#### 3.3.4 Mechanical behavior

Tensile properties of E, E12AP, E12AP\_12HAURAPP and E6AP\_6HAURAPP samples were measured according to the ASTM D638 standard (**Table 3**). It is well known that the formation of silicon-containing hybrid moieties can be considered an effective method for increasing the toughness of epoxies <sup>53</sup>. The presence of hybrid epoxy chains in the polymer matrix slows down the kinetic of energy dissipation mechanisms, as for example the crack growth occurring from filler debonding. Thus, the incorporation of a hybrid epoxy network into neat epoxies promotes an increase of fracture toughness <sup>53</sup>. Therefore, E12AP sample, which was synthetized by following the first step of a sol-gel method already exploited by the authors in several similar systems <sup>16, 26, 29, 41</sup>, is characterized by uniformly distributed hybrid moieties that increase its fracture strength " $\sigma_B$ " and elongation break " $\varepsilon_B$ " compared to E

sample (**Table 3**). The incorporation of HA, UR and APP into hybrid epoxy composites (E6AP\_6HAURAPP and E12AP\_12HAURAPP samples) does not seem to significantly affect the overall mechanical behavior of the cured epoxy resin (**Table 3**).

Sample	Et	σ <sub>B</sub>	ε <sub>B</sub>	
	[MPa]	[MPa]	[%]	
Ε	1900 ±157	38.3 ±17.7	3.7 ±2.3	
E12AP	1760 ±33	60.4 ±2.5	$10.0 \pm 3.2$	
E6AP_6HAURAPP	1810 ±156	36.3 ±15.1	3.3 ±2.1	
E12AP_12HAURAPP	$2030 \pm 68$	38.6 ±4.1	3.5 ±0.6	

*Table 3. Tensile tests result of pure epoxy resin* (E) *and epoxy composites for comparison.* 

The literature indicates that self-extinction can be achieved for DGEBA-based systems in a P-containing bio-resin, prepared with itaconic acid (IA) and 9,10dihydro-9-oxa-10-phosphaphenanthrene 10-oxide (DOPO), that was used as a biobased flame retardant in DGEBA system. <sup>54</sup>. This modification allowed the formation of a phosphorus-containing bio-epoxy resin system, where the synthetic resin completely changes its structure and can be cured by using methyl hexahydrophthalic anhydride. Even if this methodology was very effective in terms of fire behavior, it required a high concentration of phosphorous (~4 wt. %) to achieve V0 class in UL 94 vertical flame spread tests <sup>55</sup>. Conversely, the addition of HA in combination with AP, UR and APP (i.e. E12AP 12HAURAPP sample) allows achieving self-extinction with 1.1 wt. % of phosphorous only. Besides, it is well known from the literature that phenethyl-bridged DOPO derivatives in combination with organo-modified aluminum hydroxide (OATH) can provide DGEBA-based epoxy systems with flame retardant features <sup>56-58</sup>. In particular, 10 wt. % phenethyl-bridged DOPO derivative and 60 wt. % OATH were incorporated into DGEBA cured with 2-ethyl-4-methylimidazole. The resulting system achieved

UL 94 V0 rating thanks to the "sink effect" provided by OATH, which delayed the TTI of the composite <sup>59</sup>. Despite the achievement of self-extinction features, the high OATH loading showed a detrimental effect on the mechanical performances, which exhibited a significant loss of toughness <sup>58</sup>. On the contrary, our proposed strategy does not negatively affect the mechanical behaviour of the epoxy system, as clearly shown by the values of stiffness and elongation at break presented in **Table 3** (see E12AP\_12HAURAPP sample). In conclusion, E12AP\_12HAURAPP shows mechanical performances comparable to the neat epoxy matrix, as well as self-extinguishing capability, which make this formulation very promising for possible future industrial applications.

## **3.4 Conclusions**

In this work, humic acid (HA) as a bio-waste flame retardant (i.e. active in condensed phase), urea (UR) as nitrogen source and ammonium polyphosphate (APP) as phosphorous-based intumescent flame retardant were added to a silicon-modified epoxy resin to improve its fire performances. Thermal and microscopy analysis revealed that the incorporation of HA strongly promotes the thermal stability of the epoxy system by boosting the char-forming process, therefore HA was responsible for a strong condensed phase activity due to its chemical structure. Furthermore, the presence of humic acid in the epoxy system prevented melt dripping during vertical flame spread tests and the formulation containing HA (6 wt.%), UR (4 wt.%) and APP (6 wt.%) allowed to achieve self-extinguishing capability with an unprecedent very low phosphorous concentration (~1 wt.%). Forced combustion tests showed that the addition of HA, UR and APP to the hybrid epoxy system promoted a strong reduction of pHRR values up to 52%, together with an increase of the time to flashover and residue. The excellent flame-retardant results were ascribed to the formation of a N-P-O-Si containing intumescent char, which acts as thermal shield and oxygen barrier, and a gas phase activity of APP linked to a flame inhibition mechanism.

In conclusion, we demonstrated that a bio-waste flame retardant as humic acid can be well dispersed into a silicon-modified DGEBA resin, since silicon-containing hybrid epoxy moieties allow achieving a uniform distribution of the filler. In addition, a simple combination of inexpensive additives (HA, UR and APP) with a low amount of phosphorus accounts for excellent flame-retardant features for epoxy resins even cured with aliphatic hardener, without any negative effect on the overall mechanical performances. This study may contribute to the set-up of effective methodologies to exploit the flame-retardant features of HA toward the development

of new green effective composite materials. Finally, this strategy may inspire the design of more sustainable products, where the polymer matrix is also bio-based.

# **3.5 Supporting Information**

Sample composition (wt.%)	Code
Pristine epoxy resin (100%)	E
Epoxy resin (88%), HA (12%)	E_12HA
Epoxy resin (96%), UR (4%)	E_UR
Epoxy resin (96%), APP (4%)	E_APP
Epoxy resin (92%), UR (4%), APP (4%)	E_URAPP
Epoxy resin (80%), HA (12%), UR (8%)	E_12HAUR
Epoxy resin (80%), HA (12%), APP (8%)	E_12HAAPP
Epoxy resin (72%), HA (12%), UR (8%), APP (8%)	E_12HAURAPP
Epoxy resin (88%), AP (12%)	E12AP
Epoxy resin (88%), AP (6%), HA (6%)	E12AP_12HA
Epoxy resin (84%), AP (12%), UR (4%)	E12AP_UR
Epoxy resin (84%), AP (12%), APP (4%)	E12AP_APP
Epoxy resin (80%), AP (12%), UR (4%) APP (4%)	E12AP_URAPP
Epoxy resin (84%), AP (6%), HA (6%), UR (4%)	E12AP_12HAUR
Epoxy resin (84%), AP (6%), HA (6%), APP (4%)	E12AP_12HAAPP
Epoxy resin (90%), AP (3%), HA (3%), UR (2%), APP (2%)	E6AP_6HAURAPP
Epoxy resin (80%), AP (6%), HA (6%), UR (4%), APP (4%)	E12AP_12HAURAPP

3.5.1 Preparation of silica-epoxy composites

**Table S1.** Composition and codes of the investigated epoxy samples. HA = humic acid, UR = urea, APP = ammonium polyphosphate and AP = (3-aminopropyltriethoxy) silane

Code	Formulation			
Ε	Pristine epoxy resin			
E_12HA	Epoxy resin added with humic acid			

E_UR	Epoxy resin added with urea			
E_APP	Epoxy resin added with ammonium polyphosphate			
E_URAPP	Epoxy resin added with urea and ammonium polyphosphate			
E_12HAUR	Epoxy resin added with humic acid and urea			
E_12HAAPP	Epoxy resin added with humic acid and ammonium			
	polyphosphate			
E_12HAURAPP	Epoxy resin added with humic acid, urea and ammonium			
	polyphosphate			
E12AP	Epoxy resin modified with (3-aminopropyltriethoxy) silane (in-			
	situ silica-epoxy composite)			
E12AP_12HA	In-situ silica-epoxy composite added with humic acid			
E12AP_UR	In-situ silica-epoxy composite added with urea			
E12AP_APP	In-situ silica-epoxy composite added with ammonium			
	polyphosphate			
E12AP_URAPP	In-situ silica-epoxy composite added with urea and ammonium			
	polyphosphate			
E12AP_12HAUR	In-situ silica-epoxy composite added with humic acid and urea			
E12AP_12HAAPP	In-situ silica-epoxy composite added with humic acid and			
	ammonium polyphosphate			
E6AP_6HAURAPP	In-situ silica-epoxy composite added with humic acid, urea and			
	ammonium polyphosphate			

Table S2. List of acronyms and their meanings for all the prepared formulations

3.5.2 Chemical characterization of the silica-epoxy composites



Figure S1. ATR-FTIR spectra of ENC, E, E12AP, E6AP\_6HAURAPP and E12AP\_12HAURAPP



Figure S2. ATR-FTIR spectra of E, ENC, HA and "ENC + HA".



Figure S3. ATR-FTIR spectra of HA, IDA, "HA + IDA".

3.5.3 Fire behavior of the hybrid epoxy composites

TTF was calculated using equations to the formulas reported by Lyon et al. <sup>37, 39</sup> as follows:

Flame Propagation Index (FPI) = 
$$\frac{\text{pHRR}}{\text{TTI}}$$
 (S1)

**Time To Flashover** (**TTF**) =  $991 - 629 \cdot \log_{10}$  FPI (S2)

Flashover is a very important phenomenon that occurs in the case of compartment fires, where incomplete combustion products accumulate at the ceiling and ignite causing total involvement of the compartment materials and signaling the end to human survivability. Therefore, TTF represents the time available for escape and this is the single most important factor in determining the fire hazard. Equation (S2) allowing the calculation of TTF presupposes the knowledge of FPI <sup>37, 39</sup>. Equation (S2) provided the best fit ( $r^2$ = 0.94) to all of the EURIFIC full-scale fire test data <sup>38</sup> for 13 different lining materials obtained according to ISO9705 corner wall/room fire test using the 100/300 ignition option (100 kW fire for 10 minutes +300 kW fire for additional 10 minutes) in the corner of a 3.6-m-long x 2.4-m-wide by 2.4-m-high room <sup>37-39</sup>.



Figure S4. Residues after UL 94 VB tests of E (a) and E12AP\_12HAURAPP (b)

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# **Chapter 5**

Conclusions

# Contents

1. Conclusions and Future Perspectives
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# 1. Conclusions and Future Perspectives

According to a Circular Bioeconomy approach, humic acids (HA) can be viewed as a promising and cost-effective source for high-value products and novel materials, because of their chemical and biological richness. However, despite the substantial potential offered by these bioavailable materials, their full technological exploitation is strongly limited by their segregation leakage and/or degradation phenomena in aqueous environment which make these materials considered as waste nowadays, while their presence in water environment raises big issues to decontamination processes.

The present PhD work defined different synthetic approaches to turn HA environmental issues into technological opportunities through the design and the development of multifunctional hybrid materials based on HA biomolecules.

To this aim, two different strategies were followed:

1) Design of hybrid nanostructured HA-based materials;

2) Design of HA-based polymeric materials.

In the former, the molecular combination of these heterogeneous moieties with an inorganic matrix (e.g., SiO<sub>2</sub>, TiO<sub>2</sub>, ZnO) through a ceramic templated approach proved to be an effective method for limiting HA aqueous degradation phenomena, thus improving physicochemical stability, mechanical properties, and even enhancing intrinsic organic moieties features in the final hybrid nanostructured HA-based materials. This approach was also combined with the electrospinning technology to produce bioactive and sustainable nanocomposite films for active packaging applications made of electrospun biodegradable and bioderived polymers and hybrid nanoparticles.

In the latter, HA were explored as functional additives for polymer materials. Indeed, proper synthesis strategies were designed to use these biomolecules as functional biowaste flame retardant for epoxy-based systems or as an additive for gelatine

hydrogels to obtain hybrid HA-3D network with tunable rheological features and significant biological activity, including antimicrobial and antioxidant efficacy, for a broader range of biotechnological applications.

From a scientific point of view, this thesis has contributed to clarify the chemistry of HA with a special focus on their interaction with either organic or inorganic components as well as on the physico-chemical features of HA-based hybrid nanomaterials. From a technological point of view, it has provided viable routes for HA valorization by developing different materials of interest in diverse areas, especially environmental ones.

Future research will be conducted to increase of amount of regenerated moieties, and in particular of HA amount in prepared samples, in order to improve the circularity index of the produced multifunctional materials by optimizing both routes investigated in this thesis work.

Furthermore, the long-term toxicity of hybrid nanostructured HA-based materials will be assessed within the context of the proposed environmental applications. At the same time, more sustainable and biocircular alternatives to the ceramic templated approach will be proposed to produce nanomaterials based entirely on the organic matrix and in particular on biowaste. In this regard, HA nanoparticles will be produced using various chemical/physical/biological methods, resulting in different morphology, shape, size, yield, and physicochemical stability. The advent of HA nanoparticles productions will meet the green chemistry principles, since the production methods will be less environmental hazardous and waste prevention will be addressed.

Moreover, electrospinning technology is envisaged as a promising route to tune morphological and structural organization of biowaste moieties. Accordingly, this methodology will be exploited to build up nanostructured materials made entirely of biowastes and devices in which the amount of neat HA or HA-based nanomaterials will be maximized to boost the final functional properties. Notably, biopolymers used will also be derived from wastes (for example, food waste) will be used as organic template, with the goal of producing materials that will be completely biobased and biodegradable.

HA valorization will be further carried through supramolecular assembly in 2D and 3D systems (resins, matrix for composite materials, adhesive agent, aerogels and capturing devices). 3D porous architectures like aerogels, have piqued the interest of the research community due to their numerous applications in adsorption, oil/water separation, heat and sound insulation, and a variety of other engineering applications. Aerogels have enormous potential in a wide range of applications where high pore volume and surface area are important, because they are the lightest solid materials known. In addition, a huge composition range can be obtained using natural compounds. Turning HA incoherent powders into self-standing and mechanically stable 3D will be a promising strategy to scale up the range of HA possible applications to industrial plants.

As a result, considering the features and results of the thesis, HA are expected to play a leading role on the stage of circular-chemistry as an accessible and inexpensive source for the design and development of multifunctional materials for potential biobased and sustainable applications.

# **Chapter 6**

Appendix

# Contents

1.Synthesis and preparation of hybrid materials
1.1 Synthesis of silica-HA hybrid nanoparticles
1.2 Synthesis of hybrid TiO <sub>2</sub> /HA-NDL nanostructures
1.3 Synthesis of hybrid HA/ZnO nanoparticles
1.4 Preparation of photocatalytic nanostructured surfaces
1.5 Preparation of the electrospun mats
1.6 Preparation of neat gelatin, HA solution and gelatin-HA solutions
1.7 Preparation of hybrid epoxy composites
2. Physico-chemical characterization techniques
3. Functional characterization techniques
4. References

# 1.Synthesis and preparation of hybrid materials

This section describes the techniques of synthesis and the methods of preparation of the materials obtained in this PhD research and for which the results and discussions in Chapters 3 and 4 are described.

This section includes the description of synthesis nanoparticles that refers to the original version of the scientific papers described in Chapters 3 and 4.

## 1.1 Synthesis of silica-HA hybrid nanoparticles

Humic Acid functionalized silica nanoparticles were prepared *in situ* following two different sol-gel routes (Pota et al., 2020; Venezia, Pota, et al., 2022). The former based on physical entrapment of HA superstructures during the synthesis of the silica phase and the other one following a chemical addition between the two phases (Silvestri et al., 2017). Humic Acids were extracted from a mature compost made with manure (HA).

In the first synthesis route an aqueous solution of HA (2.7mg/mL) was prepared. After complete solubilization of HA, proper amounts of ammonia ( $120\mu L$ ) and 2propanol (13mL) were added, followed by the drop wise addition of TPOS (1.0mL). The obtained system was kept 2h at room temperature and the HA-functionalized silica particles were centrifuged and washed three times with distilled water. These nanoparticles will be indicated in the following as SiO<sub>2</sub>/HA\_p. Bare silica nanoparticles were produced following the same synthetic way, without the addition of HA, and they will be named as SiO<sub>2</sub>-p.

In the second synthesis procedure APTS-HA hybrid precursor was first prepared following the EDC chemistry. EDC (6.0 mM) and APTS (12.0mM) were added to the aqueous solution of HA. Reaction was allowed to proceed under stirring for 18 h. After this time, ammonia (120 $\mu$ L), 2-propanol (13mL) and TPOS (1.0mL) were added to the solution and the synthesis of hybrid nanoparticles was initiated. After

2h of reaction, the nanoparticles were centrifuged and washed three times with distilled water; they will be noted as  $SiO_2/HA_c$ . Bare silica nanoparticles were synthesized following the same procedure in absence of HA and this sample will be denoted as  $SiO_2_c$ .

Same synthesis procedure was adopted to obtain hybrid Silica–HA nanoparticles (SiO<sub>2</sub>\_HA NPs) described in Venezia et al., 2022 (Venezia, Pota, et al., 2022). In this case, humic acids from different sources were used and three sets of hybrid nanoparticles were prepared: SiO<sub>2</sub>\_HA\_Man, SiO<sub>2</sub>\_HA\_Cof and SiO<sub>2</sub>\_HA\_Art. In this case, three different humic acids (HA) were isolated, as previously reported (Spaccini et al., 2019), from a mature composts made with either cow manure (HA\_Man), or spent coffee grounds (HA\_Cof) or artichoke residues (HA\_Art). The composts were obtained in the composting facility of the Experimental Farm of University of Napoli Federico II at Castel-Volturno (CE).

#### 1.2 Synthesis of hybrid TiO<sub>2</sub>/HA-NDL nanostructures

TiO<sub>2</sub> and hybrid TiO<sub>2</sub> nanostructures were prepared in the absence and presence, respectively, of humic acid (HA) (Vitiello, Venezia, et al., 2021). The HAs were extracted from North Dakota leonardite (HA-NDL) and purified via common procedures, as described elsewhere (Piccolo et al., 2005). The average molecular weight (*Mwa*) of the purified HA was 1470  $\pm$  60 Da (Piccolo et al., 2005), and its elemental content and solid-state NMR spectrum were reported earlier (Piccolo, Spaccini, De Martino, et al., 2019). The synthesis was conducted via a hydrothermal process that was based on an experimental procedure that was previously developed by our group for producing antimicrobial hybrid melanin-based nanomaterials (Vitiello et al., 2015, 2017). Various molar ratios of the organic and inorganic components were used to evaluate the effects of the amount of the organic component on the structural and functional properties of the final hybrid nanostructures. First, a solution of the TiO<sub>2</sub> precursor was prepared via dropwise

addition of 6 mL of 1.69 M titanium(IV) isopropoxide (TTiP) in 2-propanol to 31.3 mL of an aqueous solution of acetic acid at pH=1.5. This mixture was stirred for two days, and a TiO<sub>2</sub> colloidal suspension was obtained. Then, suitable amounts of HA-NDL were added to the TiO<sub>2</sub>-sol suspension to produce four ratios: 0.1, 0.2, 1.0 and 2.0 mmol HA-NDL/mol TTiP. Subsequently, triethylamine (TEA) was added dropwise to the mixtures until pH=7.0. Then, the obtained suspensions were sealed within Teflon recipients, in which the liquid volume corresponded to 75% of the total volume, and they were placed into a circulating oven and maintained at 120 °C for 18 hours. The final samples were obtained by centrifugation at 12500 rpm for 15 min and washing 3 times with distilled water.

The obtained precipitates were resuspended in doubly distilled water to obtain four stable suspensions, which were named TiO<sub>2</sub>/HA-NDL\_0.1, TiO<sub>2</sub>/HA-NDL\_0.2, TiO<sub>2</sub>/HA-NDL\_1 and TiO<sub>2</sub>/HA-NDL\_2.

The same synthesis procedure was adopted to obtain hybrid TiO<sub>2</sub> nanomaterials in the presence of Humic Substance (HS) extracted from the compost supplied by the company Verde Vita (s.r.l.), according to the procedure described elsewhere (Spaccini et al., 2019). Briefly, 100 g of air-dried compost samples were suspended in 500 mL of 1 M NaOH solution in polypropylene containers and shaken overnight in a rotatory shaker. The supernatant containing the humic compounds was separated by centrifugation for 10 min at 11500 rpm and brought to neutral pH using 37 wt% HCl, dialyzed until Cl-free against distilled water, and freeze dried.

## 1.3 Synthesis of hybrid HA/ZnO nanoparticles

Bare ZnO and hybrid HA/ZnO nanoparticles were prepared in the absence and presence of Humic Acids (HA), respectively (Venezia, Verrillo, et al., 2022). HA from North Dakota Leonardite (HA\_LEON) was purified via common procedures, as described elsewhere (Piccolo et al., 2005). The average molecular weight (Mw) of the purified HA was 1470 ± 60 Da (Piccolo et al., 2005), and its elemental content

and solid-state NMR spectrum were reported earlier (Piccolo, Spaccini, Savy, et al., 2019).

Hybrid HA/ZnO nanoparticles were produced through a bottom-up synthetic approach under solvothermal conditions, taking inspiration from our previously defined protocol (Vitiello, Iervolino, et al., 2021; Vitiello, Venezia, et al., 2021). First, a solution of the inorganic precursor was prepared by adding a specific amount of Zinc Acetate (ZnAc, 2.64 g) to the organic mixture of ethanol (72 mL) and TEA (1.68 mL); then, it was alternately stirred and sonicated for about 15 minutes to allow a complete solubilization of the acetate salt. A definite amount of HA LEON powder (21 mg) was subsequently added to the solution to have a specific ratio between the organic and inorganic components equal to 1.0 mmol HA\_LEON/mol ZnAc. This molar ratio was defined according to the best composition used for hybrid TiO<sub>2</sub>based nanomaterials exerting the most effective functionality (Vitiello, Venezia, et al., 2021). Finally, a fixed volume of  $H_2O(8 \text{ mL})$  was added, thus resulting in a slight opacification of the suspension indicating that hydrolysis and condensation reactions started. A similar procedure was repeated without the addition of HA\_LEON to prepare bare ZnO nanoparticles to be used as reference. Therefore, the solvothermal treatment was carried out pouring both the final mixtures within Teflon mini reactors, in which the liquid volume corresponded to 75% of the total one and placing the vessels into a circulating oven at 120 °C for 2 hours. The final aqueous suspensions of bare ZnO and hybrid HA/ZnO nanoparticles were obtained by centrifugation at 12500 rpm for 15 min, after washing for 3 times with bi-distilled water.

The same procedure was adopted to obtain hybrid HAs-doped/ZnO nanoparticles (see Chapter 3, Paragraph 5). In this case commercial Humic Acids were purchased from Sigma-Aldrich (Milan, Italy).

#### 1.4 Preparation of photocatalytic nanostructured surfaces

Nanostructured surfaces with photocatalytic properties were prepared by dropcasting method: 0.4 mL of each suspension of bare ZnO or hybrid HASS/ZnO and HA/ZnO nanoparticles at a concentration 5 mg/mL were alternatively deposited on glass supports. Then, these supports were placed in an oven for 30 minutes at 90 °C allowing the solvent removal and leading to dry films.

#### **1.5 Preparation of the electrospun mats**

The PHBV solution for electrospinning was prepared by dissolving 10% (wt/vol) of the biopolymer PHBV in TFE. The solution was kept under vigorous stirring at 60°C overnight. Different solutions with contents of 1% wt, 3% wt, 6% wt, and 10% wt of TiO<sub>2</sub>\_HS with respect to PHBV were prepared.

Both bare and PHBV solutions containing NPs were electrospun in a Fluidnatek LE10, a benchtop electrospinning/electrospraying tool manufactured and commercialized by Bioinicia S.L. (Valencia, Spain). The equipment was operated in the lab mode using a motorized single needle injector, scanning horizontally towards a metallic fixed collector. The process conditions consisted of a flow-rate of 3 mL/h per single emitter, a medium voltage of 18 kV, a collector voltage of 1kV and a tip-to-collector distance of 20 cm. The obtained electrospun mats had a thickness of approximately 200  $\mu$ m. The obtained electrospun mats had a thickness of approximately 200  $\mu$ m. The resultant electrospun mats were subjected to annealing in a 4122-model press from Carver, Inc. (Wabash, IN, USA). The thermal post-treatment was performed at 150°C, for 20s, without pressure. The resultant film samples had an average thickness of approximately 100  $\mu$ m.

# **1.6 Preparation of neat gelatin, HA solution and gelatin-HA solutions**

Gelatin powder was dissolved in bi-distilled water at 5.7 wt% using a magnetic stirrer at 360rpm and 60°C for 30min to guarantee complete dissolution. Gelatin concentration was chosen to work in semi-dilute regime and avoid any precipitation during gel preparation. At the same time, this value is close to those reported in previous studies.(Avallone et al., 2021; Zhao & Sun, 2017).

Humic acid sodium salt (HA) was obtained from Sigma Aldrich (Milan, Italy). HA solution at 3wt% was prepared by dissolving HA powder, added gradually, in bidistilled water. In particular, the solution was continuously stirred, after each HA addition, for 15 minutes, and sonicated for 10 minutes. The obtained dispersion was kept for 2 days to separate the precipitate and the supernatant from the solution. The maximum water-soluble fraction of HA remained in solution is 2 wt%.

Gelatin-HA solutions were prepared according to the following procedure (Venezia et al., 2021). Gelatin solutions at different concentrations were prepared and an appropriate volume was mixed with HA solution at 2 wt% to achieve the final gelatin concentration equal to 5.7 wt% and different HA composition as reported in **Table 1**. The obtained solutions were stirred at 360rpm and 60°C overnight and the value of pH is roughly 8. Samples were stored in glass bottles at room temperature.

Sample	HA composition (mg/ml)	%HA/Gelatin (wt/wt)
Gelatin-HA 1.6	1.6	2.67
Gelatin-HA 8	8	13.33
Gelatin-HA 16	16	26.67

 Table 1: Composition of Gelatin-HA samples

## 1.7 Preparation of hybrid epoxy composites

Pristine epoxy resin was prepared following the procedure reported elsewhere (Bifulco et al., 2020). Briefly, bisphenol-A-based epoxy resin (E, 15 g) was mixed

with IDA (26 wt.% of E) at room temperature and then the resulting mixture was cured at 60 °C overnight, followed by post-curing at 80 °C for 4h. In the preparation of epoxy composites (**Table 2**), AP was chosen as coupling agent and exploited to improve dispersion of HA within the polymeric matrix. Samples containing AP were produced according to the following procedure:

- A specific amount of AP (6, 12 wt.% of "E + IDA" mass) was added to E (15 g);
- The mixture was stirred at 80 °C for 2h, followed by the addition of HA or HA/UR or HA/UR/APP with fixed weight ratio, as reported in Tables 1 and S1;
- The mixture was kept for 90 min at 80 °C, then it was cooled down to room temperature before the addition of 3.9 g of hardener (IDA) and finally mixed for 5 min;
- The resulting system was crosslinked at 60 °C overnight and then post-cured (80 °C for 4h).

Sample	E	IDA	HA	AP	UR	APP	Р	Si
	(g)	( <b>g</b> )	(g)	(g)	(g)	<b>(g</b> )	(%)	(%)
Ε	15	3.9	-	-	-	-	-	/
<b>E_12HA</b>	15	3.9	2.3	-	-	-	-	/
E_UR	15	3.9	-	-	0.8	-	-	/
E_APP	15	3.9	-	-	-	0.8	1.3	/
E_URAPP	15	3.9	-	-	0.8	0.8	1.3	/
E_12HAUR	15	3.9	2.3	-	1.5	-	-	/
E_12HAAPP	15	3.9	2.3	-	-	1.5	2.2	/
E_12HAURAPP	15	3.9	2.3	-	1.5	1.5	2.1	/
E12AP	15	3.9	-	2.3	-	-	-	1.4
E12AP_12HA	15	3.9	1.1	1.1	-	-	-	0.7
E12AP_UR	15	3.9	-	2.3	0.8	-	-	1.3

E12AP_APP	15	3.9	-	2.3	-	0.8	2.1	1.3
E12AP_URAPP	15	3.9	-	2.3	0.8	0.8	1.1	1.1
E12AP_12HAUR	15	3.9	1.1	1.1	0.8	-	-	0.6
E12AP_12HAAPP	15	3.9	1.1	1.1	-	0.8	1.1	0.6
E6AP_6HAURAPP	15	3.9	0.6	0.6	0.4	0.4	0.6	0.4
E12AP_12HAURAPP	15	3.9	1.1	1.1	0.8	0.8	1.1	0.6

 Table 2. Compositions of the epoxy samples

# 2. Physico-chemical characterization techniques

This section describes the physico-chemical characterization techniques of the materials obtained in this PhD research and for which the results and discussions in Chapters 3 and 4 are described.

This section includes the description of characterization techniques that refers to the original version of the scientific papers described in Chapters 3 and 4.

#### 2.1 Scanning electron microscope (SEM) analysis

The morphological investigation of nanoparticles and hydrogels was performed via SEM using the following tool: FEI Ispect S; source, 6-12.5 kV; filament, tungsten equipped with an Everhart-Thornley detector (Thermo Fisher Scientific, Waltham, USA). More specifically, a drop of NPs aqueous solution was deposited onto the surface of aluminium stub and sputter-coated with a Pt/Pd layer (5 nm) in a Cressington sputter coated 208HR.

The surface morphologies of LLDPE and PLA before and after photodegradation were analysed by means of SEM using a FEI Quanta 200 FEG SEM (Eindhoven, The Netherlands). Small specimens were cut from the middle of the sample and mounted on aluminium stubs by means of carbon adhesive disks and coated with a thin layer (about 10 nm thick) of an Au-Pd alloy by means of a sputter coating system (Emitech K575, Quorum Technologies LTD, UK). The analysed surfaces of the films were those exposed to UV light during photocatalytic tests.

Scanning electron microscopy (SEM), by means of a Hitachi S-4800 (Tokyo, Japan) was used to determine the morphology and the size (diameter) distributions of electrospun PHBV fibers. The samples for SEM analysis were previously sputtered with a gold-palladium mixture for 2 min under vacuum. The size distribution of the fibers was assessed by measuring 100 diameter fibers using ImageJ (National Institute of Health, Maryland, USA). For the SEM cross-section observations, the electrospun mats were cryo-fractured by immersion in liquid nitrogen.

EVO 15 scanning electron microscope (SEM) from Zeiss (Oberkochen, Germany), coupled to Ultim Max 40 energy dispersive X-ray (EDX) micro-analyzer by Oxford Instruments (High Wycombe, UK) with AZtecLive integrated software, was exploited to investigate the structure of the residual char after cone calorimetry (CC) combustion. Fragments of the compounds obtained by a brittle fracture were attached to conductive adhesive tapes and gold-metallized. The dispersion of HA in the epoxy matrix was assessed using a Wild M3Z binocular microscope using a resolution of 35–400 X (Wild, Heerbrugg, Switzerland), coupled to an Olympus digital camera (Olympus, Tokyo, Japan).

#### 2.2 Transmission Electron Microscopy (TEM) analysis

TEM images of nanoparticles were obtained using an FEI TECNAI  $G^2$  200 kV microscope (Thermo Fisher Scientific, Waltham, USA) that was equipped with a high-angle annular dark-field (HAADF) detector for high-resolution imaging.

To assess the presence of nanoparticles into electrospun mats (**Chapter 4**, **Paragraph 1**), microanalysis and elemental mapping conducted from SEM images with an EDAX detector and transmission electron microscopy (TEM), using a JEOL 1010 (Peabody, MA, USA) were carried out. An accelerating voltage of 10 kV and a working distance of 8–10 mm was used during SEM analysis. For TEM, an acceleration voltage of 100 kV was employed.

#### 2.3 Brunauer-Emmett-Teller (BET) analysis

The surface properties were determined via N<sub>2</sub> adsorption at -196 °C starting from  $P/P_0=5\times10^{-6}$  using a Quantachrome Autosorb 1-C instrument (Quantachrome, Anton Paar Italia, Rivoli, Italy). Before the analysis, the samples were degassed under high vacuum at 120 °C for 4 h. The BET method (P/P<sub>0</sub> range from 0.08 to 0.3) was adopted for the calculation of the specific surface area (S<sub>BET</sub>), while the total pore volume (V<sub>TOT</sub>) was computed using Gurvitsch's rule (at P/P<sub>0</sub>=0.995). Finally, the

pore size distribution was evaluated via application of the BJH model to the desorption branch of the isotherm.

#### 2.4 Fourier Transform Infrared Spectroscopy (FT-IR) analysis

Nicolet instrument (Nexus model) equipped with a DTGS KBr (deuterated triglycinesulfate with potassium bromide windows) detector was used to perform FT-IR investigation. IR absorption spectra of all samples were recorded in the 4000–400 cm<sup>-1</sup> range at 2 cm<sup>-1</sup> resolution on pressed disks of the powders previously diluted in KBr (1wt%). IR spectrum of each sample was corrected for the spectrum of blank KBr.

#### 2.5 Attenuated Total Reflectance (ATR) analysis

ATR nalysis on polymeric films and on hybrid epoxy sistems was carried out. The samples were placed on a ZnSe crystal. ATR absorption spectra were recorded in the 4000-400 cm<sup>-1</sup> range and with 8 cm<sup>-1</sup> spectral resolution. The spectrum of each sample was corrected for that of blank, which was carried out in air.

The chemical composition of the electrospun fibers was evaluated by attenuated total reflection Fourier transformed infrared spectroscopy (ATR-FTIR). Fourier transform infrared (FTIR) spectra were collected coupling the attenuated total reflection (ATR) accessory Golden Gate of Specac, Ltd. (Orpington, UK) to the Tensor 37 FTIR equipment (Bruker, Germany). Single spectra were collected in the wavelength range from 4000 to 600 cm<sup>-1</sup> by averaging 10 scans at a resolution of 4 cm<sup>-1</sup>.

#### 2.6 Zeta potential measurements

These analyses were carried out to evaluate surface charge of nanoparticles. All the measurements were evaluated by means of electrophoretic light scattering using a Zetasizer Nano ZSP (Malvern Instruments, England). All the measurements were performed using aqueous solution of hybrid NPs at a concentration of 0.1 mg/mL. Each measurement was performed at 25°C upon a 30 s equilibration time and the

average of three measurements at a stationary level was taken. The zeta potential was calculated by the Smoluchowski model.

#### 2.7 Dynamic light scattering (DLS) analysis

DLS experiments were performed to obtain the size of the nanoparticles in aqueous environment by using a homemade instrument composed of a Photocor compact goniometer (Moscow, Russia), SMD 6000 Laser Quantum 50 mW light source (Laser Quantum, Fremont, CA, USA) operating at 532.5 Å, a photomultiplier (PMT-120-OP/B) and a correlator (Flex02-01D) from Correlator.com (Shenzhen, China). The experiments were conducted at constant temperature 25.0 °C, by using a thermostatic bath and at the scattering angle  $\theta$  of 120°. The scattered intensity correlation function was performed with a regularization algorithm (Lomakin et al., 2005). The diffusion coefficient of each population of diffusing particles was calculated as the z-average of the diffusion coefficients of the corresponding distributions (Gallucci et al., 2021; Luchini et al., 2016; Zhang & Annunziata, 2008).

#### 2.8 Nuclear magnetic resonance spectroscopy (NMR) analysis

The solid-state <sup>13</sup>C-CPMAS-NMR spectrum of pure HA was acquired with a 300 MHz Bruker Avance wide-bore magnet (Bruker Bio Spin GmbH, Rheinstetten, Germany), equipped with a CPMAS probe, while the spectra of the HA-APTS hybrid precursor were obtained with a 400 MHz Bruker Avance magnet (Bruker Bio Spin GmbH, Rheinstetten, Germany), equipped with a <sup>1</sup>H-<sup>13</sup>C HRMAS probe. For CPMAS spectroscopy, the sample was loaded into 4-mm zirconia rotors, closed with Kel-F caps and spun at a rate of 13000  $\pm$  1 Hz. The spectrum was acquired by applying a cross-polarization technique and consisted of 1814 time domain points, a spectral width of 300 ppm (22,727.3 Hz), a recycle delay of 2 s, 5000 scans and 1 ms of contact time. The <sup>13</sup>C-CPMAS pulse sequence was conducted by using a <sup>1</sup>H RAMP pulse to account for the *non*-homogeneity of the Hartmann–Hahn condition. A TPPM15 scheme was applied to perform the <sup>13</sup>C-<sup>1</sup>H decoupling. The Free

Induction Decay (FID) was transformed by applying a 4k zero filling and an exponential filter function with a line broadening of 100 Hz. The NMR spectrum was acquired at a temperature of  $298 \pm 1$  K and processed by using MestReC NMR Processing Software (v.4.8.6.0, Cambridgesoft, Cambridge, Massachusetts, USA). Zero filling was applied during Fourier transform of free induction decays (FIDs). For the HRMAS probe, about  $15 \pm 2$  mg of the HA-APTS sample was packed into a HRMAS-NMR 4mm zirconia rotor, fitted with a perforated Teflon insert, soaked with approximately 15  $\mu$ L of D2O solution (99.8% D<sub>2</sub>O/H<sub>2</sub>O, Armar Chemicals) and sealed with a Kel-F cap (Rototech-Spintech GmbH). The 2D hetero-nuclear experiment, HSOC (Heteronuclear Single Quantum Coherence) was performed to identify the <sup>1</sup>H-<sup>13</sup>C correlations and assign the most intense NMR signals detected in samples. The HSQC experiment was acquired with a spectral width of 16 (6410.3 Hz) and 300 (30186.8 Hz) ppm for 1H and <sup>13</sup>C nuclei, respectively, a time domain of 2048 points (F2) and 256 experiments (F1), 16 dummy scans, 80 total transients and 0.5 µs of trim pulse length. The experiment was optimized by considering 145 and 6.5 Hz as the optimal  ${}^{1}H - {}^{13}C$  short- and long-range J-couplings, respectively. Spectra were processed by using both Bruker Topspin Software (v 2.1, BrukerBiospin, Rheinstetten, Germany) and MNOVA Software (v.9.0, Mestrelab Research, Santiago de Compostela, Spain). A two-fold zero-filling was applied during the Fourier Transformation of free induction decays whereas the apodization function was not necessary. Phase and baseline corrections were conducted to bidimensional spectra. <sup>13</sup>C and <sup>1</sup>H NMR frequency axes were calibrated by associating both carbon and proton peaks of  $\alpha$ -Glucose anomeric group to 94.73 and 5.22 ppm, respectively (Mazzei et al., 2016).

# 2.9 Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) analyses

Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC) were used to investigate the thermal behavior of samples by using a TA instrument simultaneous thermoanalyser SDT Q600 (TA Instrument, New Castle, DE, USA). 10 mg of each sample were placed in a platinum pan and tested under air atmosphere, in a temperature range between 25 and 1000°C with a heating rate of 10°C/min. The percentage in organic content in bare and each hybrid nanoparticles was evaluated according to the following equation:

Organic content (%) = 
$$\frac{W(200^{\circ}c) - W(1000^{\circ}c)}{W(200^{\circ}C)} * 100$$

where W(200°C) and W(1000°C) are the residual weight at 200°C and 1000°C, respectively, for the same sample.

The percentage in HA content in each sample was calculated by subtracting the organic content of bare inorganic nanoparticles to the one of the hybrids.

In some cases, to evaluate the weight loss of each sample and, therefore, HA content in hybrid nanoparticles, TGA analysis was carried out according to the Standard Test Methods for Proximate Analysis (Standard, 1998). Briefly, 10 mg of each sample were placed in a platinum pan and tested in inert atmosphere. First, to eliminate moisture contribution, each sample was raised at 110 °C with a heating rate of 20 °C/min. Then, the analysis was carried out in the temperature range between 110 °C and 970 °C with a heating rate of 50 °C/min and finally, the samples were kept at 970 °C for 15 minutes. The percentage in organic content in bare and hybrid materials was evaluated as following:

Organic content (%) =  $\frac{W(110 \,^{\circ}C) - W(970 \,^{\circ}C)}{W(110 \,^{\circ}C)} * 100$ 

where W(110 °C) and W(970 °C) are the weight at 110 °C and 970 °C of the same sample, respectively. Besides, the percentage in HA content in hybrid sample was calculated as following:

HA (%) = 
$$\frac{W_{bare NPs}(970 \,^{\circ}\text{C}) - W_{hybrid NPs}(970 \,^{\circ}\text{C})}{W_{bare NPs}(970 \,^{\circ}\text{C}) - W_{HA}(970 \,^{\circ}\text{C})} * 100$$

where  $W_{\text{bare NPs}}$  (970 °C),  $W_{\text{hybrid NPs}}$  (970 °C) and  $W_{\text{HA}}$  (970 °C) are the weight at 970 °C of bare nanoparticles, hybrid nanoparticles and humic acid HA, respectively, each calculated from equation.

The thermal behavior of hybrid epoxy sisyems was investigated through thermogravimetric analysis (TGA) using a Q500 system from TA Instrument (New Castle, DE, USA); the samples were heated from 50 to 800 °C at 10 °C/min, in nitrogen or air (gas flow: 60 mL/min). The tests were performed placing about 10 mg of sample in open alumina pans. T<sub>5%</sub> and T<sub>10%</sub> (temperatures of 5% and 10% of weight lost, respectively), T<sub>max</sub> (temperature, at which maximum weight loss rate is observed in dTG - derivative - curves) and residues were measured.

DSC measurements on hydrogels were carried out at lower temperatures, using a TA Instrument simultaneous thermo-analyser (DSC 2500 172.23.164.25) under nitrogen atmosphere (procedure: equilibrate at -120°C, isothermal 2.0min, ramp 10°C/min to 200°C). Samples were hydrated with a defined amount of water (15 wt%), to verify the interaction with water through the evaluation of its temperature melting/crystallization. Furthermore, TGA/DSC measurements were recorded using a TA Instrument simultaneous thermo-analyzer (SDT Q600, New Castle, Delaware, USA). About 10mg of each powder sample was put into a platinum pan and then heated up to 600°C, at a heating rate 10°C/min, under air atmosphere.

The thermal properties of bare PHBV and and its nanocomposite mats with TiO<sub>2</sub>\_HS nanoparticles were evaluated also by DSC using a PerkinElmer DSC 8000 thermal analysis system under nitrogen atmosphere. The sample treatment consisted of a first heating step from -20 °C to 200 °C, a subsequent cooling down to -20 °C and a second heating step up to 200 °C. The heating and cooling rates for the runs were 10 °C/min and the typical sample weight was ~3 mg. The first melting endotherm, and the controlled crystallization at 10 °C/min from the melt, was analyzed. To ensure

reliability of the data obtained, heat flow and temperature were calibrated using indium as a standard.

#### 2.10 X-ray diffraction (XRD) analysis

XRD experiments were carried out with a Malvern PANalytical diffractometer (Malvern, UK) with a nickel filter and Cu K $\alpha$  radiation, to define the crystalline phases of the inorganic component in the hybrid nanomaterial and to assess the crystalline structure of the biopolymer. The average crystal size ( $\tau$ ) was evaluated through the Scherrer formula:

$$\tau = \frac{K\lambda}{\beta \cos\theta}$$

where  $\tau$  is the mean size of the crystallite domains, K is a dimensionless shape factor,  $\lambda$  is the X-ray wavelength,  $\beta$  is the broadening at half the maximum intensity (FWHM),  $\theta$  is the Bragg angle.

XRD analysis on polymeric films was performed as previously described in the paragraph 2.5.1, to check the change in crystalline structure of each polymer after the photocatalytic treatment. In particular, the crystallinity index,  $X_c$ , was calculated by comparing the areas of the crystalline peaks ( $A_c$ ) to the total areas ( $A_c + A_a$ ) of each diffractograms as summa of the areas of crystalline and amorphous phases.

X-ray diffraction (XRD) analysis was performed all the obtained electrospun fibers using a Bruker AXS D4 Endeavour diffractometer from Bruker Corporation (Billerica, MA, USA). The samples were scanned, at room temperature, in reflection mode using incident CuK $\alpha$  radiation (k = 1.5406 Å), while the generator was set up at 40 kV and the filament current was set at 40 mA. The data were collected over the range of scattering angles (2 $\theta$ ) comprised in the 5–40° range. Peak fitting was carried out using Igor Pro software package and Gaussian function was used to fit the experimental diffraction profiles obtained.

#### 2.11 Electron paramagnetic resonance spectroscopy (EPR) analysis

EPR analysis was carried out following the approach previously defined to investigate the nature, organization and amount of radical species proper of HA component within the hybrid nanomaterial (Pezzella et al., 2013). Each sample was placed into a flame-sealed glass capillary which, in turn, was coaxially inserted in a standard 4 mm quartz sample tube. The spectra were recorded at 25 °C by using a X-band (9 GHz) Bruker Elexys E-500 spectrometer (Bruker, Rheinstetten, Germany), equipped with a super-high sensitivity probe head, and fixing the instrumental settings (sweep width, 100 G; resolution, 1024 points; modulation frequency, 100 kHz; and modulation amplitude, 1.0 G). Power saturation curves were first recorded by varying the microwave power from 0.004 mW to 128 mW. Then, EPR spectra of each sample were recorded at a fixed microwave power of 0.06mW selected to avoid the microwave saturation of the resonance absorption curve. 32 scans were accumulated to improve the signal to-noise ratio. Finally, a quantitative analysis was realized calculating the g-factor value and spin-density by using a mixed MnO/MgO powder as internal standard, which was placed in the quartz tube co-axially with each sample. The line-shapes analysis was performed by determining the signal linewidth,  $\Delta B$ , and by estimating the Gaussian character through the best fitting of the experimental curves.

#### 2.12 Water contact angle measurements

The water contact measurements ( $\theta$ ) were carried out to estimate the wettability of the electrospun mats with an optical tensiometer in a Video-Based Contact Angle Meter, Theta Lite TL 101 model (Biolin Scientific, Espoo, Finland) using the OneAttension v 3.1 software. Brielfy, a 5 µL droplet of distilled water was deposited on the material surface at room temperature and the image of the drop was recorded. Three different measurements were taken and averaged at different parts of the samples. The sliding angle was evaluated on a water droplet deposited over the surface whilst being tilted and analyzed with the online software Ergonomics Ruler of the Universitat Politècnica de València (UPV), Valencia, Spain (Pardo-Figuerez et al., 2018).

#### 2.13 Dynamic Rheological Measurements

Rheological measurements were performed in a rotational stress-controlled rheometer (Discovery Hybrid Rheometer 2, TA Instruments, USA) equipped with a Peltier cell for temperature control and 40mm diameter sandblasted parallel plates (thermal expansion coefficient  $0.957\mu$ m/°C). Tests are carried out by using a gap of 1mm, and a solvent trap to avoid evaporation at high temperature.

## 3. Functional characterization techniques

This section describes the functional characterization techniques of the materials obtained in this PhD research and for which the results and discussions in Chapters 3 and 4 are described.

This section includes the description of characterization techniques that refers to the original version of the scientific papers described in Chapters 3 and 4.

#### 3.1 Antioxidant activity

Ferrous oxidation-xylenol orange (FOX) assay. SiO<sub>2</sub>, SiO<sub>2</sub>-humic acids nanoparticles and free HA suspensions in distilled water (0.04 mg/ml) were incubated in the presence of H<sub>2</sub>O<sub>2</sub> 25.0 M, 12.5  $\mu$ M or 10.0  $\mu$ M at room temperature, under stirring. Aliquots (100  $\mu$ l) were withdrawn at times 0', 30', 1h and 4 hours. Each aliquot was then mixed with 900  $\mu$ L of reagent mixture containing 0.10 mM xylenol orange, 0.25 mM ammonium iron(II) sulfate hexahydrate in 250 mM H<sub>2</sub>SO<sub>4</sub> (10 mL) and 3.88 mM 1,1,3,3-tetramethoxypropane, butylated hydroxyanisole (BHA) in methanol (90 mL) (Nourooz-Zadeh et al., 1995). After 30 min under stirring at room temperature, absorbance at 593 nm was measured. Control experiments were performed in the absence of nanoparticles and by using non-bound HA in the same concentration. The antioxidant activity of all the samples was expressed as residual percentage of H<sub>2</sub>O<sub>2</sub> measured in the same conditions in the absence of nanoparticles.

(2,2-diphenyl-1-picryl-hydrazyl-hydrate) (DPPH) assay. DPPH free radical scavenging method was carried out according to the following procedure (Venezia, Pota, et al., 2022). Briefly, 1/5 mg/mL of each bare and hybrid nanoparticles was mixed with 2 mL of 100  $\mu$ M DPPH methanol solution. In addition, considering the amount of HA in hybrid sample determined by TGA analysis, a certain amount of HA was mixed with the same DPPH methanol solution. The mixture was incubated

in the dark for 90 min and the absorbance at 517 nm was measured by using a UV-2600i UV-VIS spectrophotometer, 230V (Shimadzu, Milan, Italy).

The percentage of DPPH free radical scavenging activity was calculated as follows:

DPPH inhibition (%) = 
$$\frac{A_{DPPH} - A_{sample}}{A_{DPPH}} * 100$$

where  $A_{DPPH}$  and  $A_{sample}$  are the absorbance of the methanolic solution of DPPH and the absorbance of the sample, respectively.

(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. ABTS test has been performed according to the procedure reported elsewhere (Re et al., 1999; Verrillo, Cozzolino, et al., 2021). This method is focused on the oxidation of 2,20azinobis-(3-ethyl benzothiazoline-6-sulphonic acid) diammonium salt (ABTS) by potassium persulphate to form a radical cation (ABTS<sup>•+</sup>). In detail, a concentration of 7 mM ABTS stock aqueous solution was prepared, and the ABTS radical cation (ABTS<sup>•+</sup>) was produced by adding 2.45 mM potassium persulfate and allowing the reaction solution to stand in the dark and at room temperature for 16 h before use. Then, a working ABTS<sup>++</sup> solution was prepared by diluting 10 mL of the stock solution in 800 mL of a water/ethanol (50:50, v/v) solution, whose final spectrophotometer absorbance was between 0.75 and 0.80 at 734 nm. For the preparation of samples, 2 mg of each sample were dissolved in 2 mL of water, and 100 µL of this solution was added to 1.9 mL of the ABTS<sup>++</sup> working solution. The mixture was shaken for 2 min in the dark to promote the reaction between humic molecules and free ABTS<sup>++</sup> radicals. The absorbances were measured at 734 nm by a Perkin Elmer Lambda 25 UV/Vis Spectrometer. Results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) based on a linear calibration curve of Trolox in the range between 0.1 and 80 mg×L<sup>-1</sup> (R<sup>2</sup> = 0.996).

#### **3.2 Antibiotic removal tests**

Amoxicillin, tetracycline and metronidazole were selected for removal tests by TiO<sub>2</sub>/HA-NDL nanohybrids due to their presence in wastewater as representatives

of the most commonly used antibiotics. Three independent aqueous antibiotic solutions were prepared at a concentration of 0.50 mg $\cdot$ mL<sup>-1</sup>. Then, 200  $\mu$ L of each antibiotic solution was added to 800 µL of each TiO<sub>2</sub>/HA-NDL suspension to obtain antibiotic and nanoparticle concentrations of 0.1 mg $\cdot$ mL<sup>-1</sup> and 1 mg $\cdot$ mL<sup>-1</sup>, respectively, in the final mixture. After mixing, each obtained suspension was gently stirred on a Roller Mixer SRT2 (Stuart Scientific) for 24 h at 33 rpm and subsequently centrifuged for 20 min at 12000 rpm to induce the precipitation of TiO<sub>2</sub>/HA-NDL. All supernatants were recuperated and analysed using a 1100 Agilent (Shimadzu Corp., Japan) high-performance liquid chromatography (HPLC) system that was equipped with a Gemini C18 110A column. The mobile phase was composed of 90% aqueous buffer (MeOH 5% v/v, H<sub>3</sub>PO<sub>4</sub> 1% v/v) and 10% acetonitrile that was flowing at  $1.0 \text{ mL} \cdot \text{min}^{-1}$ . Signals were acquired at 220 and 230 nm for amoxicillin, at 270 and 280 nm for tetracycline and at 320 nm for metronidazole. The retention times were 3.39 and 6.50 min for amoxicillin and tetracycline, respectively. Finally, the chromatograms were integrated using the LC Solution software (Shimadzu Corp., Japan).

#### **3.3 EPR-spin trapping**

The pro-oxidant activity of ZnO and HA/ZnO nanoparticles was investigated by performing EPR spin-trapping experiments to monitor the ability to generate ROS in aqueous environment before and after UV light irradiation. The following procedure was adopted: a specific amount of a stock aqueous solution of 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) spin-trap was added in 1 mL of the aqueous suspension of nanoparticles (both bare ZnO and hybrid HA/ZnO, alternatively) at a concentration of 2 mg×mL<sup>-1</sup>, with the aim to have a final spin-trap concentration of 20 mM. After 10 min from the dispersion preparation, the mixture was centrifuged and the supernatant was recuperated and placed in a capillary to be analyzed by EPR, as described in the section 2.3.8. This procedure was similarly repeated to investigate

the effect of light irradiation: the mixtures of ZnO-based nanoparticles and DMPO were exposed for 5 minutes to UV lamp ( $\lambda = 365$  nm) irradiation before to be centrifuged to recover the supernatants. The spectra were recorded with an attenuation of 15 dB and 64 scans were accumulated. A specific volume of supernatant, equal to 20 µL, was placed in a capillary to be analyzed by EPR as described in the section 2.3.8. This procedure was similarly repeated to investigate the effect of light irradiation: the mixtures of ZnO-based nanoparticles and DMPO were exposed for 5 minutes to UV lamp ( $\lambda = 365$  nm) irradiation before to be centrifuged to recover the supernatants. The spectra were recorded with an attenuation of 15 dB and 64 scans were accumulated.

#### **3.4 Antibacterial activity**

**Viable-count method.** An antibacterial activity of the TiO<sub>2</sub>/HA-NDL nanohybrids was conducted on various gram-negative and gram-positive bacterial strains, which included Escherichia coli DH5a, Escherichia coli ATCC 35218, Klebisella pneumoniae ATCC 700503, Pseudomonas aeruginosa ATCC 27355, Enterococcus faecalis ATCC 29212 and Staphylococcus aureus ATCC 5538P, which were kindly provided by the Department of Biology of the University of Naples Federico II. Antibacterial activity assays were conducted via the plate viable-count method (Vitiello et al., 2017, 2018). A single colony of each bacterial strain was suspended in 5 mL of Luria-Bertani (LB) broth medium (Becton, Dickinson) and incubated overnight at 37 °C. When the cell cultures reached an OD600 nm of 1 unit, they were diluted with a ratio of 1:100 in 20 mM sodium phosphate buffer (NaP) with pH 7.0. Then, 40 µL of the diluted bacterial cell suspension was alternatively added to the nanoparticle suspensions at various concentrations (10, 25, 50, 100, 200 and 1000  $\mu$ g·mL<sup>-1</sup>) to a final volume of 500  $\mu$ L using 20 mM NaP buffer with pH 7.0. Cells that were incubated with the ampicillin antibiotic  $(0.05 \text{ mg mL}^{-1})$  were used as a positive control, whereas cells that were incubated without any compound or with bovine serum albumin (BSA) at the same concentration of the nanoparticles were used as negative controls. The samples were incubated at 37 °C for 4 hours, and their dilutions (1:100 and 1:1000) were placed on an LB/agar medium and incubated overnight at 37 °C. This procedure was also conducted to analyse the antibacterial activity of bare HA-NDL, which was used as a reference. The following day, the number of surviving cells was estimated by counting the colonies on each plate, and they were compared with the controls. All samples were tested in triplicate, and their means were compared by applying one-way analysis of variance (ANOVA) and Tukey's HSD test. The statistical analysis was conducted by using XLSTAT software, and significant difference was set at 95% confidence.

Diffusion disc assay. Antibacterial activity of hybrid HA/ZnO nanoparticles, as well as of bare ZnO ones as reference, was estimated by diffusion disc assay as reported in (Verrillo, Salzano, et al., 2021). Briefly, the bacterial cells used in this study were stored at -80 °C in Luria Bertani (LB) broth with 20% glycerol and activated using agar transfer and incubation at 37 °C for 18 h. Antimicrobial screening was performed by testing two Gram-positive (Staphylococcus aureus ATCC 6538P and Enterococcus faecalis ATCC 29212) and two Gram-negative bacterial strains (Pseudomonas aeruginosa ATCC27355 and Escherichia coli ATCC 35218) applied a disk diffusion determination according to the National Committee for Clinical Laboratory Standards (NCCLS) standard method. This assay is based on the diffusion of nanoparticles in agar substrate. In fact, when the microorganism is susceptible to an antibacterial treatment, the proliferation of the bacterial cells will be limited and, consequently, an inhibition area becomes visible. If no inhibition zone arises, the bacterium is not sensitive to the tested substance. All materials were tested at two different concentrations of 1 and 2 mg mL<sup>-1</sup>. Culture of each bacterial strain was transferred to nutrient agar and incubated at 37 °C for 24 h. The inoculum was standardized by transferring colonies from the nutrient agar to sterile saline solution up to  $10^8$  CFU mL<sup>-1</sup> (0.5 McFarland). Then, 200 µL of the bacterial cells was placed onto the surface of Mueller–Hinton agar and different disks of 6.0 mm diameter were treated with 30 µL of each nanomaterial. Subsequently, all Petri dish contained tested nanomaterials were incubated at 37 °C for 24 h. Ampicillin, a common broad-spectrum antibiotic was used as positive control. The total diameters were measured analyzing the size (mm) of the inhibition zones. Each experiment was performed in triplicate. The significant difference between mean values was determined by the one-way analysis of variance (ANOVA), and the means (n = 3) were validated applying Tukey's test at the 0.05 significance level by using the XLSTAT software (Addinsoft, 2014).

**Broth microdilution method.** The antimicrobial assay on nanoparticles was performed by the broth microdilution method in a Mueller–Hinton broth medium by using sterile 96-well polypropylene microtiter plates. The microbial inoculum size used was  $1 \times 10^{6}$  cells mL<sup>-1</sup> (NCCLS, 1993). Two-fold serial dilutions of different samples were carried out to obtain concentrations ranging from 10 to  $1000 \mu \text{gmL}^{-1}$ . The bacterial cells were inoculated from an overnight culture at a final concentration of about  $5 \times 10^{5}$  CFUmL<sup>-1</sup> per well and incubated with different samples overnight at 37 °C. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) values were estimated against Staphylococcus aureus ATCC 6538P and Escherichia coli ATCC25922 by measuring the spectrophotometric absorbance at 570 nm (Verrillo et al., 2022).

**Japanese Industrial Standard JIS Z2801 assay.** The antimicrobial performance of the electrospun mats was determined based on the guidelines of the Japanese Industrial Standard JIS Z2801 (ISO 22196:2007) for film samples (Figueroa-Lopez et al., 2020). S. aureus CECT240 (ATCC 6538p) and E. coli CECT434 (ATCC 25922) strains were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). The bacterial strains were stored in phosphate buffered saline
(PBS) with 10 wt% tryptic soy broth (TSB) and 10 wt% glycerol at -80 °C. Before each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 °C for 24 h. A 100 µL aliquot from the culture was again transferred to TSB and grown at 37 °C to the mid-exponential phase of growth. The optical density showing an absorbance value of 0.20 and measured at 600 nm in a UV–Vis spectrophotometer VIS3000 from Dinko, Instruments (Barcelona, Spain) determined that the initial bacterial concentration was approximately a 10<sup>6</sup> colony-forming unit (CFU)/mL. The dimension of the samples was 1.5 cm × 1.5 cm. Onto the PHBV biopapers containing NPs (test samples) and bare PHBV electrospun mat (negative control sample), a microorganism suspension of S. aureus and E. coli was applied. Thereafter, the inoculated samples were placed in open bottles and incubated for 24 h at 24 °C and at a relative humidity (RH) of at least 95%. Bacteria were recovered with PBS, 10-fold serially diluted, and incubated for 24 h at 37 °C to quantify the number of viable bacteria by a conventional plate count. The antimicrobial activity reduction (*R*) was evaluated using the following equation:

$$R = \left[ Log\left(\frac{B}{A}\right) - Log\left(\frac{C}{A}\right) \right] = Log\left(\frac{B}{C}\right)$$

where *A* is the average of the number of viable bacteria on the control sample immediately after inoculation, *B* is the average of the number of viable bacteria on the control sample after 24 h, and *C* is the average of the number of viable bacteria on the test sample after 24 h. Next assessment was followed to evaluate the antibacterial activity of the biopapers (Torres-Giner et al., 2017): nonsignificant reduction if R < 0.5, a slight reduction when  $R \ge 0.5$  and < 1, a reduction that was significant when  $R \ge 1$  and < 3, and a reduction was strong if  $R \ge 3$ . Experiments were performed in triplicate.

#### 3.5 Rhodamine B (RhB) degradation assay

The photocatalytic activity of bare ZnO and hybrid HA/ZnO nanoparticles was investigated by carrying out a Rhodamine B (RhB) degradation assay, following a previously described protocol (Muscetta et al., 2021; Wang et al., 2020). Specifically, 1 mL of 8  $\mu$ g mL<sup>-1</sup> RhB solution and 100  $\mu$ g of each nanomaterial were added into ep tubes. A high-pressure Hg vapour lamp (Helios Italquartz) with a nominal power of 125 W was used to perform the photodegradation tests. The lamp emits in the UV range at 305 nm, 313 nm and 366 nm and in the visible range at 404.7 nm and 435.8 nm. The lamp was placed in a quartz sleeve, inside which cooling water at 25 °C circulated and was kept constant by using a thermostatic bath (Falc GTR 90) when the entire emission spectrum of the lamp was used. After tests, each suspension was centrifuged for 5 min at 1000 × g. The absorbance of the supernatants was determined by using an UV-2600i UV-VIS spectrophotometer, 230V (Shimadzu, Milan, Italy).

## 3.6 Swelling analysis

The swelling kinetics of both Gelatin and Gelatin-HA hydrogels were deter- mined following a well-known method reported in previous studies.<sup>40,45</sup> Briefly, the investigated samples were dried at 50°C, weighted and then re-hydrated in distilled water at room temperature. The samples were drained with filter paper to remove water in excess and weighted at 10, 20, 60 and 180 min. The swelling ratio was defined in the following equation:

Swelling ratio [%] = 
$$\frac{W_0 - W_d}{W_d} * 100$$

in which  $W_0$  and  $W_d$  are the hydrated and the dried weight of the hydrogel, respectively.

# 3.7 Optical properties of electrospun films

The light transmission of the biopapers was determined using an UV–Vis spectrophotometer VIS3000 from Dinko, Instruments (Barcelona, Spain). Therefore,

the absorption of light at wavelengths between 200 nm and 800 nm was evaluated in specimens sized 50 mm  $\times$  30 mm.

Transparency (T) and opacity (O) were determined using the following equations (Figueroa-Lopez et al., 2018):

$$T = \frac{A_{600}}{L}$$
$$O = A_{500}L$$

where *L* is the biopaper thickness (mm), while  $A_{500}$  and  $A_{600}$  are the absorbances at 500 and 600 nm, respectively.

Color of the biopapers was determined with a Chroma Meter CR-400 from Konica Minolta (Tokyo, Japan). The color difference ( $\Delta E^*$ ) was calculated using the following equation:

$$\Delta E * = [(\Delta L *)^{2} + (\Delta a *)^{2} + (\Delta b *)^{2}]^{0.5}$$

where  $\Delta L^*$ ,  $\Delta a^*$ , and  $\Delta b^*$  corresponded to the differences between the brightness and color parameters of the PHBV biopapers containing nanoparticles and the values of the neat PHBV biopaper. The color change was evaluated according to the following criteria (Agüero et al., 2019): the difference was unnoticeable if  $\Delta E^* < 1$ . Only an experienced observer can notice the difference for  $\Delta E^* \ge 1$  and < 2. An unexperienced observer notices the difference when  $\Delta E^* \ge 2$  and < 3.5. A clear noticeable difference is noted if n  $\Delta E^* \ge 3.5$  and < 5, and the observer notices different colors when  $\Delta E^* \ge 5$ . Tests were performed in triplicate.

## 3.8 Barrier properties

The water vapor permeability (WVP) of the film samples was determined using the gravimetric method ASTM E96-95 in triplicate. For this, 5 mL of distilled water was placed inside a Payne permeability cup (diameter of 3.5 cm) from Elcometer Sprl (Hermallesous-Argenteau, Belgium). The film was not in direct contact with water but exposed to 100% RH on one side and secured with silicon rings. They were placed within a sealed desiccator, with dried silica gel, at 0% RH in a cabinet at 25 °C. The control samples were cups with aluminum films to estimate solvent loss through the sealing. The cups were weighted periodically using an analytical balance  $(\pm 0.0001 \text{ g})$ . WVP was calculated from the regression analysis of weight loss data vs. time, and the weight loss was calculated as the total loss minus the loss through the sealing. The permeability was obtained by multiplying the permeance by the film thickness. Similar as described for WVP, **limonene permeability** (LP) was measured placing inside the Payne permeability cups 5 mL of D-limonene. The cups with the films were placed at room temperature and at 40% RH. The limonene vapor permeation rates were evaluated from the steady-state permeation slopes and the weight loss was calculated as the total cell loss minus the loss through the sealing. LP were calculated also considering the average film thickness in each case. All samples were tested in triplicate.

The **oxygen permeability coefficient** was derived from the oxygen transmission rate (OTR) measurements that were recorded at 60% RH and 25 °C, in duplicate, using an Oxygen Permeation Analyzer M8001 from Systech Illinois (Thame, UK). The humidity equilibrated samples were purged with nitrogen, before exposure to an oxygen flow of 10 mL/min. The exposure area during the test was 5 cm<sup>2</sup> for each sample. To obtain the oxygen permeability (OP), film thickness and gas partial pressure were considered.

# 3.9 Mechanical tests on electrospun films

Tensile tests were carried out on PHBV films according to ASTM standard method D638 using an Instron 4400 universal testing machine, using a 1 kN load cell, from Instron (Norwood, MA, USA). The measurements were carried out, at room temperature and humidity, with 5 x 25mm stamped dumb-bell shaped specimens

using a crosshead speed of 10 mm/min.. Three specimens were measured for each film sample and the average values with standard deviation (SD) were reported.

## 3.10 UL 94 vertical flame spread test and combustion behavior

The flammability of all composites was assessed by UL 94 vertical flame spread tests (IEC 60695-11-10; sample dimensions:  $13 \times 125 \times 3 \text{ mm}^3$ ).

The combustion behavior was studied by means of CC (Noselab Ats, Nova Milanese, Italy) according to the ISO 5660 standard, by using squared samples (100 x 100 x 3 mm<sup>3</sup>), with a heat flux of 35 kW/m<sup>2</sup>, in horizontal configuration. Time to ignition (TTI, s), total heat release (THR, MJ/m<sup>2</sup>), peak of the heat release rate (pHRR,  $kW/m^2$ ) were measured. Total smoke release (TSR, m<sup>2</sup>/m<sup>2</sup>) was also evaluated. For each sample, the experiments were repeated at least three times in order to ensure reproducible and significant data.

#### 3.11 Mechanical tests on hybrid epoxy systems

Tensile Tests were performed using a Zwick Z100 dynamometer (ZwickRoell S.r.l., Genova, Italy), following the ASTM D638 standard on rectangular specimens (50 x  $10 \times 4 \text{ mm}^3$ ) using a 100kN load cell at 1 mm/min rate until 0.2% deformation was reached. Then, the rate was increased up to 10 mm/min until the specimen broke. Five specimens were tested for each formulation and the average values of the tensile modulus (E<sub>t</sub>), the elongation at break ( $\epsilon_B$ ), and the tensile strength ( $\sigma_B$ ) were calculated.

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