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"On-Off" RGD Signaling Using Azobenzene Photoswitch-Modified Surfaces

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"ON-OFF" RGD SIGNALING USING AZOBENZENE PHOTOSWITCH-MODIFIED SURFACES

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Dedicated to whom was,
is and will be always close to me.

CHAPTER 1

New Challenges for Cell Instructive Materials: Design and Controlled Exposure of Biological Cues through Azobenzene-Modified Platforms

Abstract. When engineering new biomaterials for tissue engineering, cell-material interface is one of the most important module of a biomedical device to care about. A big part of biomaterials science research is focused on the development of new cell instructive materials (CIMs) that can stimulate cells and guide them to express specific biological features. Recent advances have introduced a variety of smart engineered matters, which are sensitive to external stimuli like pH, temperature, light or applied electric or magnetic fields, to provide an *in situ* dynamic control of the interactions between these artificial substrates and cell behavior. The novelty of these "dynamic CIMs", indeed, is the possibility to also achieve a temporal control of their bioactivity, with respect to the spatial organization of their ligands provided by the well-engineered "static CIMs". In this chapter an overview on both static and dynamic approaches will be thoroughly discussed, presenting some interesting examples. With respect to the "dynamic CIMs", our work focused on azobenzene-modified platforms to induce a photocontrolled cell adhesion. An outline of the thesis is given at the end of this chapter.

1.1 Biomaterials in Tissue Engineering

Tissue engineering is a multidisciplinary research area, which involves several contributions from biological, medical and engineeristic point of view, that are all merged in the field of regenerative medicine. The main goal of tissue engineering is the reconstruction of an injured tissue through the regenerative process involving a new biomaterial as scaffold to assist in rebuilding a well functioning tissue.[1] The use of biomaterials, indeed, helps to restore the structure and the functions of a damaged tissue and provides a transient two- or three-dimensional support to interact at biomolecular level with cells, thus guiding the complex processes involved in tissue formation and regeneration.[2] Actually, the intercommunications between cells and these artificial matrixes play a central role in the modern strategies adopted to develop new biomaterials. It is well established, indeed, that cell interact specifically with their surrounding extracellular matrix (ECM), thus regulating the biological processes. The regeneration of lost or damaged tissues requires that cells adhere, migrate, proliferate, and differentiate promoting both the reparation and the synthesis of a new tissue.[3] The most promising results are achieved when the normal physiological environment of cells is closely reproduced, as shown in Figure 1. Hence current strategies are focusing on the basic principles of cell-ECM interaction to develop implantable matrices, which are able to mimic natural tissues.[4] Such biomimetic materials allow cellular interactions with specific cell responses, according to the selected tissue engineering application.[5] It is really important to understand and reproduce the physiological environment in which the regeneration of a damaged tissue occurs, especially for what concerns

the angiogenesis processes. One of the major requirements of a biomaterial, indeed, is the ability to promote and sustain vascularization events, which are of vital importance for the regeneration processes.[6]

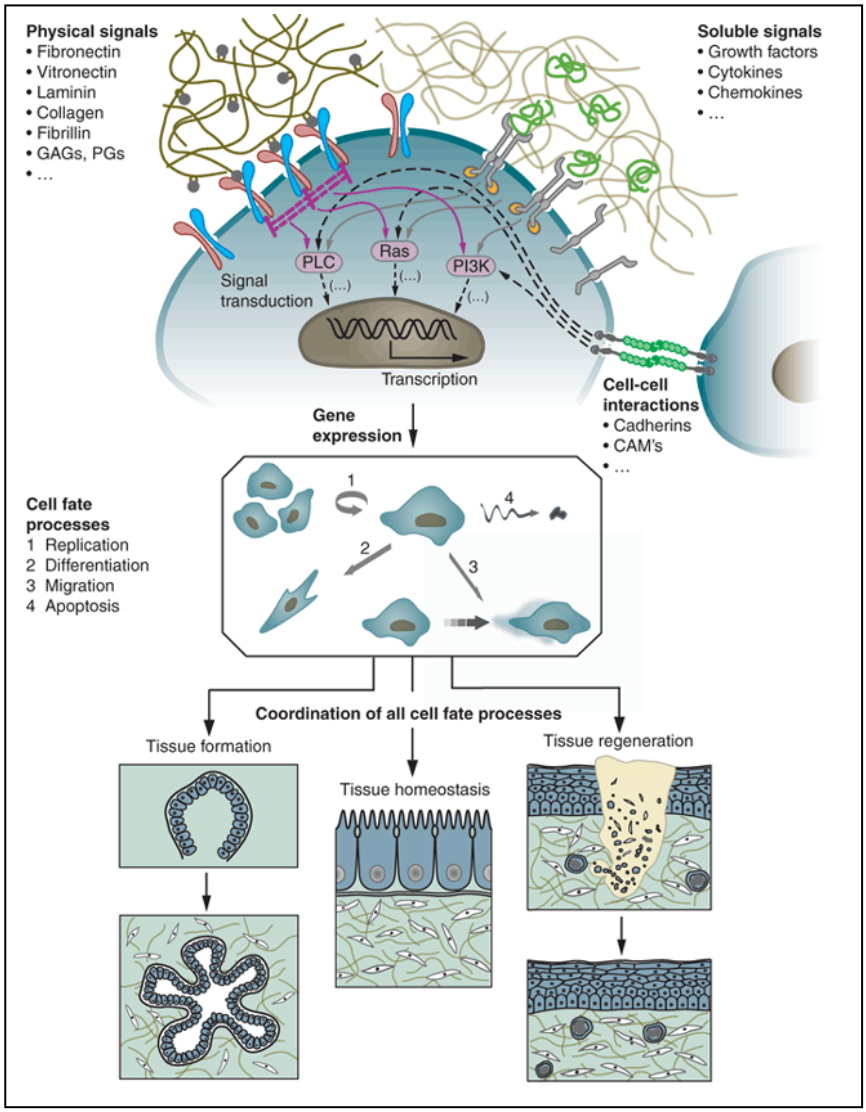

Figure 1. The behavior of an individual cell and the dynamic state of multicellular tissues are regulated by intricate reciprocal molecular interactions between cells and their sorroundings.[2]

In this respect, human umbilical vein endothelial cells (HUVECs) can provide an important *in vitro* models to study the endothelial cell behavior during angiogenesis processes.[7] So far, both biologically derivatives and synthetic materials have been extensively explored in regenerative medicine and tissue engineering.[8] Natural materials are for example collagen, gelatine derivatives, alginate and fibrin-based materials; while as synthetic matters the most exploited are biocompatible polymers like polyglycolic acid (PGA), poly-L-lactide (PLLA), as well as copolymers of lactic and glycolic acids (PLGA), polyurethane and polytetrafluoroethylene (PTFE). Hybrid materials, instead, which combine naturally derivatives and synthetic polymers, have been developed to exploit advantages of both material types as well as to overcome their limitations.

1.2 Cell Instructive Materials

When engineering new biomaterials, cell-material interface is one of the most important module of a biomedical device to care about. Artificial matrixes have to interact with cells and instruct them in order to promote tissue regeneration events. Instructing cells, indeed, is a crucial aspect for the field of regenerative medicine and new biomaterials.[9] As matter of fact, a big part of this science research is focused on the development of new cell instructive materials (also known as CIMs), as smart engineered matters that can stimulate cells and guide them to express specific biological features.[2, 10] Cell adhesion and intracellular events are strictly related to the intercommunication process that cells can establish with the ECM.[11] *In vivo*, the interface between cell surface receptors and the ECM represents a complex but really efficient microsystem. Actually, cell

adhesion is mediated by integrins, a big family of cellular receptors composed of two noncovalently linked heterodimers, named α and β subunits. Integrins are the crosstalking players involved during the biological recognition and the signaling events with the outer environment.[12] The process of integrin-mediated cell adhesion comprises a cascade of four different, partly overlapping, events: cell attachment, cell spreading, organization of actin cytoskeleton and formation of focal adhesion. During these steps integrins are involved in physical anchoring as well as in signal transduction processes through the cell membrane.[13] Cell biologists have become awared of the fact that understanding cell behavior in their intricate physiological environment requires a systematic study of cell responses that can be facilitate by exploiting specific microenvironments as models. These ECM-model systems could significantly reduce the complexity of the real ECM, but at the same time can mimic within a certain high degree the *in vivo* situation. In general, when cells interact with the surface of a material, a combination of biochemical and topographic signals occurs.[10] Thus, surfaces equipped with molecular signals mimicking certain aspects of the natural ECM can offer new opportunities to understand the pathway by which cells sense, integrate and respond to environment changes (Figure 2).

Hence, the use of bioactive components can instruct cells to adhere, migrate, proliferate or even differentiate. Specific binding of these cues with cell surface receptors induces complex intracellular signaling cascades that converge to regulate gene expression, establish cell phenotype and direct tissue formation and regeneration.

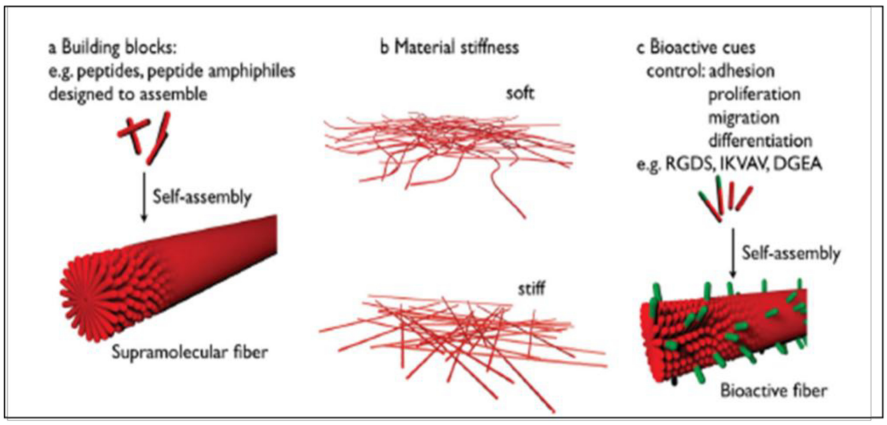

Figure 2. Important parameters that can influence and instruct cells: (a) supramolecular building blocks similar to the structural components of the ECM; (b) the overall stiffness of a material and (c) tethering of bioactive cues.[9]

1.3 Cell Signaling through Bioactive Peptide Sequences

One of the most exploited approach to improve the bioactivity of a new biomaterial is the modification through biological cues, like short peptide sequences or adhesive proteins.[14-15] In this way, it is possible to get a better interaction with cells, mimicking as much as possible the real physiological condition. Peptides can activate integrin modulation mechanisms to elicit precise biological responses and, in this respect, the arginine-glycine-aspartic acid (RGD) tripeptide[16-17] is one of the most well-known sequence used for recognition purposes, which was discovered by Pierschbacher and Rouslahti in fibronectin almost three decades ago.[18] RGD is often used to improve cell adhesion on artificial materials because of its extensive distribution, its ability to recognize several receptors and its biological influence on cell anchoring, proliferation and survival. For this reason, the modification of biomaterials with RGD sequence to regulate cell adhesion boosted the interest of bioengineering researchers on designing and optimizing

biocompatible features in synthetic matrixes.[4,14] An exhaustive literature confirmed that the RGD motif is highly efficient in promoting the attachment of several cell types to a vast class of materials.[19-22] Besides RGD, also other peptide sequences have been investigated. As given examples, PHSRN[23] found in fibronectin domain, has been identified as a synergy ligand that, when attached to substrates in which the RGD peptide is also present, enhances cells spreading; IKVAV sequence,[24] derived from laminin, is known to promote cell adhesion and induce neurite outgrowth of neural progenitor cells (NPC); while DGEA sequence, which is derived from collagen type-1, can induce differentiation of bone marrow stromal cells (BMSCs) into bone-producing osteoblasts.[25] These few reported examples clearly demonstrate that the bioactivity of synthetic biomaterials can be enhanced by grafting biological peptide sequences, which were directly derived from ECM proteins. In general, dealing with short peptide sequences instead of proteins has the advantage to ensure a feasible and reasonable cost of synthesis and use, together with the preserved chemical stability during the sterilization and other processing steps required for biological assays. Furthermore, peptides can be coupled with a good controlled density and orientation over the material, if compared to the less favorable control achieved when using full-length native proteins, such as fibronectin, laminin or vitronectin.[26] The degree of functionalization is an important parameter to take into account in order to improve cell interactions. Earlier studies performed by Massia and Hubbell demonstrated that the surface density of immobilized RGD peptides can elicit different cellular response for fibroblasts, either to simply adhere or to form integrin clusters called

focal adhesions.[27] A recent work proved that hosteoblasts adhesion was effectively promoted when RGD and PHSRN together covered at least 30% of the available surface of alginate scaffolds.[28] All these studies stressed the importance of a controlled modification with biological cues to improve cell adhesion, thus significantly affecting cell functions, such as proliferation but also migration as well as differentiation.

1.4 Stimuli Responsive Substrates

The majority of the modified materials present biomolecules grafted in a well-organized and engineered manner. However, their position and availability remained still static in time. Nowadays, the new frontiers are wide open to versatile systems where external stimuli can tune the accessibility of cell ligands at wish. A research area under investigation, indeed, concerns the improvement of novel substrates that can change their properties in a dynamic way to better mimic the real biological environment.[9, 29] Introducing this novel aspect provides the opportunity to design new materials where the strategy to "switch" the composition of cell-binding ligands between an active and inactive state lead to a dynamic control over the properties of such substrates. Current developments have created a variety of materials responsive to light,[30] pH,[31] magnetic or electric field,[32-34] enzymes[35] and also to host-guest chemistry approach.[36] The novelty of these "dynamic CIMs", indeed, is the control of their bioactivity in time through applied external stimuli, in this way the aspect of the spatial control provided from the "static CIMs" is implemented with the temporal parameter. Another important feature of these new generation of biomaterials is the reversibility of their feedback to the external stimuli.[37-38] Moreover,

the dynamic control over cell adhesion with non-invasive methods, such as photochemical or electrical stimuli are potentially interesting since the properties of the susbstrate can be easily switched between the inactive/active states, without affecting cell viability.[39] Stimuli responsive systems that allow a reversible controlled bioactivity are suitable for the next generation of biomaterials in regenerative medicine.

1.4.1 Photoresponsive Platforms

So far, many smart platforms have been investigated to direct cell fate with light as external trigger. The development of such light-responsive systems basically stand on the modification of the biomaterial with specific biomolecules that have a light-sensitive moiety.[40] In some recent works, the availability of biological signals is shown to be modulated to promote either cell adhesion or detachment through UV-photocleavable moieties that provoke a change in the surface properties.[41] When the *o*-nitrobenzyl group is introduced at the amide bond (linked to the nitrogen atom) between the glycine and the arginine residues of the RGD sequence, a "caged"-RGD is achieved. RGD exposure can be tuned at wish upon UV stimulus and hence cell adhesion is promoted only after the removal of the photocleavable group.[42] Furthermore, if *o*-nitrobenzyl groups are engrafted all over the glass surface it is possible to induce localized cell adhesion or even cell migration using some particular masks during the UV exposure step (Figure 3a).[43] In a different example, the photocontrolled modification can allow the adsorption of adhesive or non-adhesive proteins over the surface after the light treatment.[44] It is even possible to get the opposite effect, in terms of cell detachment. Silicon wafer

modified with these UV-photocleavable groups, which were introduced before the biological sequences, are able to release cells after short UV exposure time, without affecting cell viability (Figure 3b).[45]

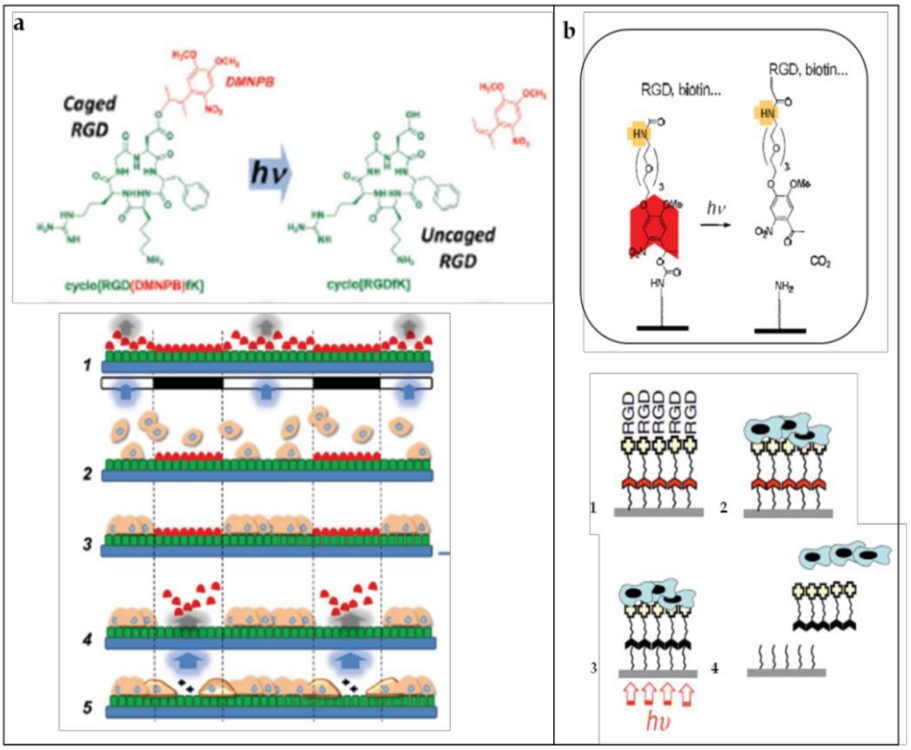

Figure 3. (a) Photo-activatable cell migration assay with "caged" RGD molecule. The RGD adhesive pattern and the relative cell migration response were achieved with consecutive irradiation steps, to generate new light-exposed areas.[43] (b) Working principle of phototriggered cell release from the surface through a photocleavable linker (inset with the chemical structure of the photocleavable linker before and after light exposure).[45]

The presented strategies were all based on the "caging" approach, in which the involved biomolecules are linked to a light-sensitive group (or protected with a "caging" group) that act as a "phototrigger", but they lack of reversibility.[40] An alternative approach can be found in the

host-guest chemistry, which is based on non-covalent bonding and thus allow to restore the original interaction present before the light treatment.[46] A promising strategy to confer reversibility concerns the use of "photoswitches", which are a class of chemical compounds that undergo reversible photochemistry reactions that alter their structures and so many rounds of active/inactive states of the biological cues can be produced.[47] Azobenzenes, are a well-known class of "photoswitches" thanks to their characteristic isomerization mechanism.[48] In this respect, azobenzene-modified surfaces can provide an interesting research line as photoresponsive platforms, by introducing the concept of reversibility through the azobenzene moiety.[48-49]

1.5 Azobenzene as Photoresponsive Moiety

The light-induced transformation between two interconvertible states is one of the key aspect for a molecule to behave as a molecular switch. Among several photoswitches, azobenzenes are a well-known class of aromatic molecules (an azo linkage N=N connects the two phenyl rings) capable to change their configuration upon light irradiation, which reveal also a strong resistance to optical fatigue.[48] Hence, they are excellent candidates as molecular "photoswitches", since they can exist in two (*trans* and *cis*) isomeric forms.[50] The first evidence of the formation for the *cis* isomer dates back to 1937, when Hartley observed a different absorbance for the azobenzene in solution after its exposure to light.[51] The isomerization process involved can induce a significant geometric change in the position of the two phenyl rings (Figure 4). In general, the *trans* isomer is the most stable and has a flat geometry because of the two aromatic rings laying on the same plane;

its strongest absorption band is around 365 nm, due to the π→π* transition. The *cis* isomer, instead, presents an angular geometry and has a relatively weak absorption around 445 nm related to the n→π* transition.[52] Because of the higher stability attributed to the *trans* isomer, azobenzene derivatives are predominantly present as *trans* form and when exposed to UV radiation, the *trans*-to-*cis* isomerization occurs. This process is completely reversible, and the back-isomerization can take place either spontaneously (eventually accelerated by heating) or by exposure to visible light.

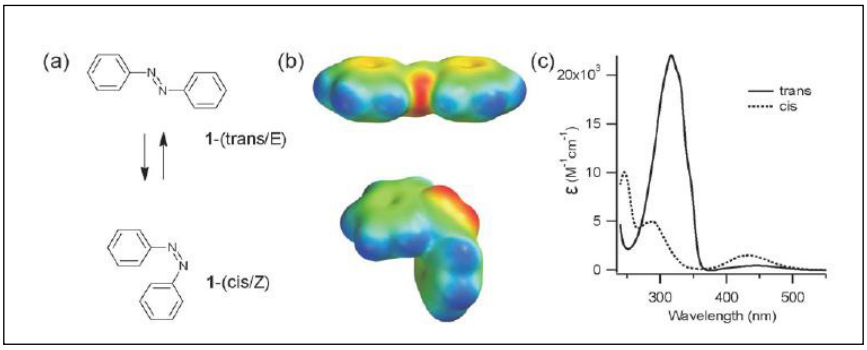

Figure 4. (a) Structures of *trans* and *cis* isomers of azobenzene. (b) Spacefilling models are coloured by electrostatic potential (red-negative to blue-positive). (c) Electronic absorption spectra of the *trans* and *cis* isomers of azobenzene dissolved in ethanol.[47]

Several chemical and physical parameters can influence the isomerization mechanism. Solvents and temperature can slightly affect the isomerization mechanism, while the nature and the position of the susbstituents on the phenyl rings can strongly modify the related absorption bands, especially the half-life of the related *cis* isomer.[53] The absorption spectrum, indeed, can be tailored among several substitution patterns, from the ultraviolet to the visible-red region. The

presence of an electron-donor group (e.g. amino goup, -NH_2) in *ortho*- or *para*-position of the phenyl ring can provoke a shift of the $\pi \rightarrow \pi^*$ transition band of the *trans* isomer to higher wavelenghts (red-shifted band), thus laying closer to the $n \rightarrow \pi^*$ band of the *cis* isomer. Azobenzenes, which have a red-shifted absorbance for the *trans* isomer and a shorter half-life of the related *cis* isomer, are identified as aminoazobenzenes. Moreover, if the susbstituents present in the two *para*-positions are an electron-donor and an electron-acceptor groups (e.g. an amino and a nitro group, -NO_2) a "push-pull" azobenzene is formed, which has the two characteristic bands completely red-shifted and quite overlapped.[50, 53] In this last case a single wavelength is able to stimulate both mechanisms, thus leading to a continuous interconversion between the two isomers upon light irradiation.[54] This feature becomes really intersting when dealing with amorphous azopolymer films that can undergo a large and reversible topographic modification when exposed to light with a specific polarization state. The photoinduced isomerization mechanism of azobenzene units generates a motion at the molecular level, which is capable to induce a large-scale modification, in terms of modulation of the thickness of the polymer films. In this way, a regular pattern is formed, which is better known as surface relief gratings (SRGs reported in Figure 5).

1.6 Azobenzene-Modified Surfaces

Once azobenzenes are confined on the solid support, it is important to ensure the required space for the isomerization to take place. One way to create the free volume suitable for the isomerization mechanism on the designed platform, is to introduce a short linker bound to the azobenzene moiety, which is acting like a spacer.[52] A large number of

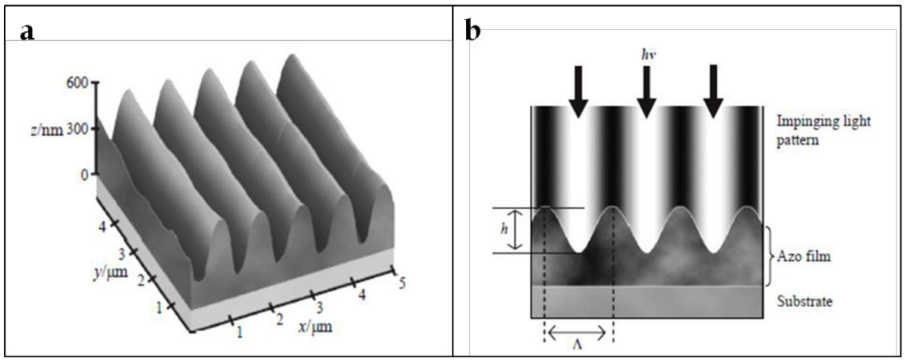

Figure 5. (a) AFM image of a typical surface relief grating (SRG) optically inscribed into an azopolymer film. (b) Schematic representation of a grating and the relative phase correlation with the incident light pattern.[54]

method can then be exploited to monitor the switching behavior of immobilized azobenzene moieties. This is possible because the reversible mechanism of isomerization lead to a variation in physical and chemical properties of the surrounding environment. For example, azobenzene molecules tethered to a surface can provoke a local alteration in wettability once they modify their conformation, mainly due to the change in the dipole moment of the molecules going from *trans* to *cis* isomer.[55] The photocontrolled wettability of a solid surface has been investigated monitoring the contact angle behavior in the presence of polar solvents, mainly water, that spread more readly on *cis*-modified surfaces, thanks to the higher dipole moment of this isomer. Moreover, Ichimura and co-workers demonstrate the possibility of a light-induced motion for an olive oil droplet over an azobenzene-modified silica surface.[56] For what concerns biological applications, there is a large number of efficient light-switchable systems involving the modification of susbstrates with photoisomerizable components to control biological activities through

light. Most of these approaches exploited the direct attachment of the photoresponsive moieties to the investigated biomolecules.[57] In this respect, when azobenzene moiety is introduced in a molecule with a specific biological activity, it promotes the desiderable spatial and temporal control of a variety of biological processes through illumination, such as enzyme activities,[35, 58-59] and especially peptides,[47, 60-61] proteins,[62-63] nucleic acids[64-65] or ion channels[66-67] regulations. Recent works have investigated synthetic matrix coated with azoderivatives that led to photocontrolled bacterial[68] or cell[49, 69-70] adhesion, with some examples reported in Figure 6.

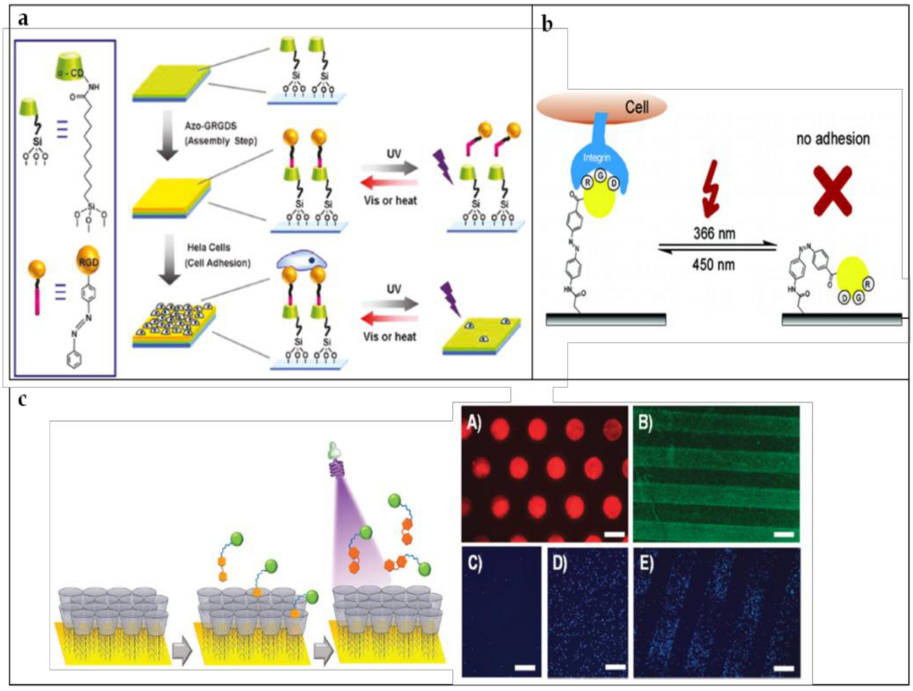

Figure 6. (a) Host-guest strategy for cell adhesion on SAM (inset with the chemical structure of α-cyclodextrin and azobenzene GRGDS).[46] (b) Photoregulated cell adhesion on surfaces modified with RGD ligand bound to the azobenzene unit.[69] (c) Assembly of bioactive ligand with photoswitchable properties on a β-cyclodextrin monolayer (inset with fluorescence microscopy images. First raw images of patterned β-cyclodextrin surfaces. Second raw images of adhered *E. Coli* strains).[68]

Moreover, some azobenzenes (see section 1.5) offer the opportunity to tune the isomerization in the visible region instead of the UV range, and this is largely preferred when exploiting azobenzene for *in vivo* applications, because UV wavelenghts can penetrate cells and cause apoptosis.[71]

1.7 Aim and Outline of the Thesis

This research work describes the design of a two-dimensional platform modified with an azobenzene unit, thus based on an "On-Off" photoswitching mechanism useful for the dynamic exhibition of the well-known GRGDS sequence. The final aim of the work concerns the realization and the optimization of such surface, which can be exploit for phototriggered cell adhesion studies. In **Chapter 2** the optimized synthesis of the azobenzene moiety, covalently bound to a short oligo(ethylene glycol) spacer on one side and to the peptide sequence on the other one, is described in detail. Synthesized compounds are characterized with the help of mass spectrometry and nuclear magnetic resonance (NMR) analyses. The photoresponsive behavior is proved with UV-Vis spectroscopy measurements, and some preliminary photocontrolled experiments in which the isomerization is induced using different light sources are shown. **Chapter 3** describes the grafting of the target photoresponsive molecule (presented in Chapter 2) to silanized glass surfaces through the chemoselective "click" approach. Chemical and topological characterizations of such surfaces are provided by Raman spectroscopy, atomic force microscopy (AFM) and confocal laser scanning microscopy (CLSM). Photoresponsive properties are shown to be reliable by monitoring the change in wettability behavior through water contact angle (WCA) technique.

Finally, in **Chapter 4**, the bioactivity of such characterized substrates is evaluated by some preliminary biological assays with human umbilical vein endothelial cells (HUVECs). Final conclusions and future perspectives, with some parameters that still need to be optimized are sintetically presented and discussed in **Chapter 5**.

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CHAPTER 2

Synthesis of GRGDS Azobenzene-Based Photoswitch

Abstract. The chance to control the biological activity of protein and peptide ligands, exploiting light as trigger, can be achieved coupling them with a light-sensitive moiety. A peptide sequence containing the RGD motif was linked to an azobenzene unit to obtain a new azoderivative in which the bioactivity of the peptide could be controlled through light stimuli. Here the synthesis of the azoderivative is presented together with an extensive investigation of the involved photoswitching behavior by spectroscopic measurements.

This work has partly been submitted for publication: E. Vaselli; C. Fedele; S. Cavalli; P. A. Netti; "On-Off" RGD Signaling Using Azobenzene Photoswitch-Modified Surfaces.

2.1 Introduction

When dealing with new biomaterials for *in vitro* or *in vivo* applications, one of the first prerequisite is the design of versatile systems with the chance of a dynamic control over their bioactivity. The capability to regulate the spatial and the temporal presentation of peptide or protein ligands endowed over the surface of such biomaterials, provides the possibility to instruct cell and guide them through their complex processes.[1-2] To achieve a programmable responsiveness of these biological cues, several strategies have been exploited such as altering pH[3] or temperature,[4] as well as exposing to electric[5] or magnetic fields;[6] in some cases irradiation with light can also elicit a response.[7] Light regulation, in particular, is one of the most attractive strategy, since it can provide the desired dynamic temporal control without a direct contact with the involved system, as occurs instead in the other cases.[8] The general approach is to couple a specific biomolecule with a light-sensitive moiety, hence the photoinduced modification can be translate into a significant change of the activity for the involved biomolecule. In particular, it is well desired to regulate these light-triggered events between two possible conditions, in one case the maximum of the bioactivity is promoted, while in the other one a "silent" bioactivity is induced, like a sort of "On-Off" mechanism that can be regulated at wish through light. Another required feature concerns also the possibility of controlled photochemistry events with the related good efficiency, thus providing the required light dose and avoiding extended exposure times. The light dose necessary to trigger the photocontrolled event is an important parameter, especially in the case of biological assays

involving cells. In this case, in fact, the photoregulation should be possible using "biocompatible" wavelengths to prevent undesired toxic effects that can lead to cell apoptosis.[9] When dealing with light-sensitive molecules, there are different available strategies to explore and an interesting approach involves the use of "photoswitches", which can undergo reversible photochemistry reactions. One of the most exploited is azobenzene, which belong to a class of aromatic molecules where an azo linkage N=N connect two phenyl rings capable to change their configuration as a consequence of a photoinduced isomerization mechanism.[10] Indeed, azobenzenes are really excellent candidates as molecular "photoswitches" since they can exist in the two *trans* and *cis* isomeric forms, which can interconvert both photochemically or thermally.[14] Several methods can be used to prepare azobenzenes, which are related to the starting material employed for the synthesis.[11] The majority of azobenzenes are produced by azo coupling reactions, in which a diazonium salt can be coupled with a nucleophilic aromatic compound (e.g. aniline or phenol) through the electrophilic aromatic substitution mechanism.[12] Despite its large application, this method is a little bit critical because of the potential explosivity of the diazonium salt when the temperature of the reaction is not under proper control. Other methods, which do not require the use of diazonium salt, are the Mills' reaction (which employs electrophilic aromatic nitro derivatives and anilines) or the Wallach's method (which involves the conversion of azoxybenzene units in *p*-hydroxy susbsituted azoderivatives using acidic conditions). Moreover, also the reductive coupling of an aromatic nitro derivative

an provide an azobenzene molecule as product, both in acid or basic conditions.

For what concerns the use of the azobenzene coupled with biological molecules, many strategies have been widely explored to control peptides,[13-14] protein,[15-16] nucleic acids[17-18] or ion channels[19-20] activities. In some cases, the modification of such biomolecules with azobenzene moieties has been exploited to mediate and control cell adhesion response for *in vitro* experiments.[21-23] Also in our case, the short GRGDS peptide was coupled to the azobenzene unit to provide a tunable exposure of such sequence once grafted on the solid substrate, and thus regulate cell adhesion response with light. The strategies adopted to obtain the designed azoderivative were presented and discussed step by step, from the synhtesis of the azobenzene unit by reductive coupling method of a nitro aromatic derivative, to the conjugation with the peptide sequence performed on solid support. Then, the photoswitching behavior of each azoderivative was characterized and monitored by means of spectroscopic measurements.

2.2 Experimental Section

General Materials and Method. All chemicals and solvents were furnished from Sigma-Aldrich and were used without further treatments. 9-(*N*-Boc-amido)-4,7-dioxanonanoic acid was purchased from Cyanagen. Amino acids and Rink Amide AM resin were provided by Iris Biotech GmbH. Column chromatography was performed with granular silica gel (60-100 mesh). Milli-Q water (resistivity 18.2 MΩ cm) was obtained with a Millipore system.

Mass Spectrometry. Spectra were acquired with an Agilent Technologies 6530 Accurate Mass instrument. An ESI source, operating at 4 kV needle voltage and 320 ºC, with a complete HPLC system, equipped with MS pump, an auto-sampler was connected to a Q-TOF detector, which can be also coupled with a photo diode array detector (DAD). An extend-C18 column (2.1×50 mm) was used for the analyses. Buffer solutions employed were: known MS reference buffer in acetonitrile; water and acetonitrile with 0.1% in volume of formic acid as eluent solutions and isopropanol with 30% of acetonitrile for column washing. Each sample was prepared at the concentration of 0.5 mg/ml, injecting 5-20 µl each run. For MALDI analyses a MALDI Voyager-DE STR workstation of Applied Biosystems was used. For each measurement an equal volume of the sample (1 µl) was mixed with the matrix over the plate. DHB (2,5-dihydroxybenzoic acid) 20 mg/ml solubilized in a mixture of 10% in volume of acetonitrile in water was used as matrix. Analyses were performed in reflectron mode using a positive ion detection in the range of 200-550 m/z.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H and ^{13}C spectra were acquired with an Agilent Premium Compact+ instrument (600 MHz). Chemical shifts were reported in ppm and relative calibration was done on the deuterate solvent used in the experiments with the related software function. The abbreviations used were: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet and br = broad. 2D homonuclear and heteronuclear experiments (TOCSY, ^{1}H-^{13}C HSQC) were performed when required for peak assignment. Spectra were collected at room temperature solubilizing 2-3 mg of each compound in deuterated solvents.

UV-Visible Spectroscopy. Measurements were performed using a Cary 100 UV-Vis spectrometer, recording from 800 to 200 nm in double beam mode, using two quartz cuvettes (10 mm optical path) and *N*,*N*-Dimethylformamide (DMF) as solvent. Previously, a zero-baseline correction was performed recording 100% and 0% of transmittance signal. A step of 1 nm and an average time of 0.1 s were used for all measurements.

Illumination experiments. For UV radiation a Spectroline® E-Series UV lamp was used illuminating at 365 nm (after 20 minutes of warm-up). Light source was placed 5 cm far from the samples. The fluence rate[24] (1.2 mW/cm^2) was measured with a power meter (Thorlabs). For visible light radiation a normal desk lamp equipped with an halo bulb was used and placed 10 cm far from the samples. Fluence rate (130 mW/cm^2) was measured providing the sensor of the power meter instrument with a proper filter (435-443 nm). During irradiation experiments, temperature was monitored with a thermocouple (K-Type thermometer).

Synthesis of azobenzene 4,4'-dicarboxylic acid (**1**). *p*-Nitrobenzoic acid (0.9 mmol, 150 mg) was dissolved in a Milli-Q water solution of sodium hydroxide (14 equiv.) at 60 °C, under stirring. A hot solution of glucose D(+) in Milli-Q water (6 equiv., 1 g) was added when reagents were completely solubilized. After few minutes, the yellow precipitate became dark orange. The reaction was performed for 5 hours at 60 °C, then it was left under stirring conditions overnight at room temperature. The precipitate was washed by centrifugation twice with water and acetic acid, and twice only with water. At the end azobenzene 4,4'-dicarboxylic acid (**1**) was dried in

vacuum oven at 30 °C for 3 hours to give a pink powder (yield 68%). MS for $C_{14}H_{11}N_2O_4$: calculated $[M+H]^+$= 271.07 m/z; found (MALDI) $[M+H]^+$= 271.13 m/z. Other peak found: $[M+Na]^+$= 293.1 m/z. ^1H NMR (DMSO, 2 drops of TFA, δ): 8.09 (d, C*H* Ar, 4H); 8.16 (d, C*H* Ar, 4H). ^{13}C NMR (DMSO, 2 drops of TFA, δ): 122.84, 130.72, 133.44 (*C*H Ar); 154.22, 166.65 (*C*O).

Synthesis of azobenzene 4,4'-Bis (2,5-dioxo-pyrrolidin-1-yl ester) (**1a**). Compound **1** (0.6 mmol, 160 mg) was dissolved in DMF at 60 °C for 1 hour at least to allow complete solubilization. Then *N*-Ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl; 2.4 equiv., 273 mg), *N*-Hydroxysuccinimide (NHS; 2.4 equiv., 163 mg) and a catalytic amount of 4-(dimethylamino) pyridine (DMAP) were added to the solution. The reaction was left under stirring conditions overnight at room temperature. The mixture was washed by centrifuge three times with water and then dried in vacuum oven at 30 °C for two hours. Product **1a** was purified by silica gel column chromatography and eluted with 2% of methanol (MeOH) in dichloromethane (DCM) to give an orange powder (yield 40%). MS for $C_{22}H_{17}N_4O_8$: calculated $[M+H]^+$= 465.09 m/z; found (MALDI) $[M+H]^+$= 465.1 m/z. Other peak found: $[M+Na]^+$= 487.07 m/z. ^1H NMR ($CDCl_3$, δ): 2.91 (s, CH_2, 8 H); 8.04 (d, C*H* Ar, 4 H); 8.30 (d, C*H* Ar, 4 H). ^{13}C NMR ($CDCl_3$, δ): 25.88 (*C*H_2); 123.61, 127.73, 131.99 (*C*H Ar); 155.92, 161.43, 169.22 (*C*O).

Synthesis of compound **2**. 9-(*N*-Boc-amido)-4,7-dioxanonanoic acid (0.361 mmol, 100 mg), 1-Hydroxybenzotriazole hydrate (HOBt·H_2O; 1.2 equiv., 58.5 mg), EDC·HCl (1.2 equiv., 83 mg) and triethylamine (TEA; 2 equiv., 100 µl) were dissolved in 10 ml of DCM

in a round-bottom flask under magnetic stirring for 3 minutes of pre-activation. After that time, propargylamine (1.2 equiv., 25 µl) was added and the mixture was left under magnetic stirring overnight. The reaction was followed by TLC on silica with 10% of MeOH in DCM and stained with a solution of ninhydrin. Product **2** was purified by silica gel column chromatography and eluted with 3% of MeOH in DCM as a white oil (90% yield). MS for $C_{15}H_{27}N_2O_5$: calculated $[M+H]^+$= 315.18 m/z; found (ESI) $[M+H]^+$= 315.02 m/z. Other main peak found: $[M+Na]^+$= 337.17 m/z. ^{1}H NMR ($CDCl_3$, δ): 1.42 (s, C*H*$_3$, 9 H); 2.20 (s, ≡C*H*, 1 H); 2.48 (t, COC*H*$_2$CH$_2$, 2 H); 3.30 (dd, OCH$_2$C*H*$_2$NH, 2 H); 3.53 (t, OC*H*$_2$CH$_2$NH 2 H); 3.59 (s, OC*H*$_2$C*H*$_2$O, 4 H); 3.71 (t, COCH$_2$C*H*$_2$, 2 H); 4.03 (m, ≡CC*H*$_2$NH, 2 H); 5.00 (br s, CH$_2$CH$_2$N*H*CO, 1 H); 6.75 (br s, ≡CCH$_2$N*H*CO, 1 H). ^{13}C NMR ($CDCl_3$, δ): 28.61 (C*C*H$_3$); 29.16 (*C*CH3, ≡C*C*H$_2$NH); 36.88 (CO*C*H$_2$CH$_2$); 40.52 (OCH$_2$*C*H$_2$NH); 67.16 (COCH$_2$*C*H$_2$); 70.14, 70.46 (O*C*H$_2$*C*H$_2$O*C*H$_2$); 71.51 (≡*C*H); 80.02 (*C*≡CH); 156.18, 171.48 (*C*O).

Synthesis of compound **3**. Compound **2** (64 µmol, 20 mg) was treated for 2 hours with a solution of 50% trifluoroacetic acid (TFA) in DCM in order to remove BOC protecting group and treated by co-evaporation at rotavapor with toluene. Meanwhile, azobenzene **1a** (1 equiv., 30 mg) was dissolved in 2 ml of dry DMF at 60 °C for 1 hour under stirring conditions. After that, a solution of deprotected compound in DCM was added dropwise, still at 60 °C. Once the reaction started, the mixture was left under magnetic stirring at room temperature overnight. The reaction was followed by TLC at 5% of MeOH in DCM. Final product **3** was purified performing silica gel

column chromatography and eluted at 2% of MeOH in DCM (40% yield). MS for $C_{28}H_{30}N_5O_8$: calculated $[M+H]^+$= 564.21 m/z; found (ESI) $[M+H]^+$= 564.21 m/z. ^{1}H NMR ($CDCl_3$, δ): 2.18 (s, ≡C*H*, 1H); 2.44 (t, CO*C*H_2CH_2O, 2H); 2.91 (s, COC*H*$_2$C*H*$_2$CO, 4H); 3.64-3.74 (m, C*H*$_2$OC*H*$_2$C*H*$_2$OC*H*$_2$C*H*$_2$, 10 H); 4.05 (m, ≡CC*H*$_2$, 2H); 6.50 (s, N*H*, 1H); 6.84 (s, N*H*, 1H); 7.96-8.02 (m, C*H* Ar, 4H); 8.28 (d, C*H* Ar, 4H). ^{13}C NMR ($CDCl_3$, δ): 25.87 (CO*C*H$_2$*C*H$_2$CO); 29.52 (≡C*C*H$_2$); 40.16 (O*C*H$_2$*C*H$_2$O); 67.07 COCH$_2$*C*H$_2$O); 69.99, 70.36 (O*C*H$_2$*C*H$_2$NH); 71.65 (≡*C*H); 79.89 (*C*≡CH); 123.40, 128.38, 131.97 (*C*H Ar); 154.16, 156.12, 161.51, 166.89, 169.27 (*C*O).

Solid-phase peptide synthesis of GRGDS sequence **4**. GRGDS peptide was manually synthesized using standard Fmoc solid-phase peptide synthesis (SPPS). Fmoc-protected amino acids were loaded onto Rink Amide AM resin (100-200 mesh, 0.48 mmol/g, 120 μmol) as follows: Fmoc-Ser-(tBu)-OH; Fmoc-Asp-(OtBu)-OH; Fmoc-Gly-OH; Fmoc-Arg-(Pbf)-OH; Fmoc-Gly-OH. The steps performed for each coupling were: 1) Fmoc deprotection with a solution of 20% of piperidine in DMF; 2) coupling reaction of each amino acid (4 equiv. with respect to the resin) in the presence of HOBt·H_2O (4 equiv.), *N*,*N'*-diisopropylcarbodiimide (DIC; 4 equiv.) and *N*,*N'*-diisopropylethylamine (DIPEA; 8 equiv.) in 50% of DCM in DMF; 3) after each coupling, a capping step for unreacted amine groups was performed with a solution of acetic anhydride (0.5 M) and DIPEA (0.125 M) in DMF. Each reaction step was controlled and confirmed using a standard Kaiser test. Peptide sequence was checked by cleavage of a testing sample (10% in weight) using a solution of 2.5% H_2O and 2.5% triisopropylsilane in TFA, precipitated in diethyl ether,

recollected by centrifugation and finally freeze-dried. MS for $C_{17}H_{32}N_9O_8$: calculated $[M+H]^+$= 490.24 m/z; found (ESI) $[M+H]^+$= 490.24 m/z.

Synthesis of compound **5**. Azoderivative **3** (32 µmol, 18 mg) was coupled to the anchored peptide **4** (1 equiv. with respect to compound **3** and according to the nominal loading of the resin). First, anchored peptide **4** was put in a syringe with a filter and washed several times using DCM and DMF, in order to swell the resin and then Fmoc deprotection was done using a solution of 20% of piperidine in DMF. Then the coupling with **3** was performed, in the presence of a mixture of NHS (1 equiv. with respect to compound **3**, 4 mg), DIC (1 equiv., 5 µl) and DIPEA (2 equiv., 11 µl) in DMF. As last step, the cleavage from the resin was performed using a solution of 10% of H_2O in TFA for 2 hours. Product **5** was precipitated in cold diethyl ether, recollected by centrifugation and finally freeze-dried. Crude product was used directly for the next reaction, without any further purification (71% yield). MS for $C_{41}H_{55}N_{13}O_{13}$: calculated $[M+H]^+$= 938.41 m/z; found (ESI) $[M+H]^+$= 938.44 m/z. ^{1}H NMR (DMSO, δ): 1.5-1.7 (m, $C_\beta H_2$, $C_\gamma H_2$ Arg, 4H); 2.34 (t, $COCH_2$, 2H); 2.73 (m, $C_\beta H_2$ Asp, 2H); 2.9 (s, OCH_2CH_2O, 4H); 3.0-3.1 (m, $C_\delta H_2$ Arg, ≡C*H*, CH_2CH_2NH, 5H); 3.4-3.9 (m, $C_\alpha H_2$ Gly, $C_\alpha H_2$ Gly, $C_\beta H_2$ Ser, CH_2NH, CH_2OCH_2, CH_2CH_2NH, 13 H); 4.16 (m, $C_\alpha H$ Ser, 1H); 4.31 (m, $C_\alpha H$ Arg, 1H); 4.59 (m, $C_\alpha H$ Asp, 1H); 6.5 (t, C*H* Ar, 2H); 7.15 (s, $CONH_2$, 2H); 7.4 (m, CH_2NH Arg, 1H); 7.5-7.6 (m, C*H* Ar, 2H); 7.8 (t, N*H* Ser, 1H); 7.9-8.1 (m, N*H* Arg, C*H* Ar, 5H); 8.15-8.30 (m, N*H* Asp, N*H* Gly, N*H* Gly, 3H). ^{13}C NMR (DMSO, δ): 28.29, 29.02 (C_β, C_γ Arg); 35.7 ($COCH_2$); 36.01 (C_β Asp); 40.36 (C_δ Arg);

40.03 (O*C*H_2*C*H_2O); 40.36 (C_α Gly); 41.89 (≡C*C*H_2); 42.64 (C_α Gly1); 49.54 (C_α Asp); 55.27 (C_α Ser); 57.00 (C_α Arg); 66.55 (C_β Ser); 68.7 (*C*H_2CH_2NH); 69.46 (CH_2*C*H_2NH); 72.89 (≡*C*H); 81.12 (*C*≡CH); 112.51, 122.53, 128.67 (*C*H Ar); 156.54, 165.77, 166.68, 168.83, 168.96, 169.79, 170.31, 171.77, 171.91 (*C*O).

2.3 Results and discussion

The modification of peptide or protein ligands with light-sensitive moieties represents a powerful method to achieve the temporal control over their biological functions. These modified biomolecules, indeed, can undergo significant modifications regarding their bioactivity or their conformation upon light irradiation.[9] In our specific case, an azobenzene molecule was covalently tethered to the GRGDS sequence, in order to exploit the photocontrolled isomerization mechanism of the azobenzene moiety to tune the peptide exposure. The photocontrolled exposure of the GRGDS sequence was really interesting for our purposes, because gave the opportunity to regulate its availability for cell adhesion experiments, once it was immobilized on solid substrate. From this point of view, our target molecule **5** (Chart 1) was designed taking into account three important features: presenting the azobenzene photoswitchable unit, containing a short spacer and bearing the GRGDS signal.

First of all, azobenzene 4,4'-dicarboxylic acid (**1**) was chosen, among other azobenzenes, in order to achieve a *cis* isomer stable for several hours. This feature was related to the chemical substituent pattern of the two phenyl rings, which provided slow rate for the back-isomerization mechanism.[25] Second, a short oligo(ethylene glycol) linker was introduced as spacer, to allow an adequate degree of

freedom for the isomerization to take place, even after confinement of the azobenzene to the solid support.[7, 26] In addition, an alkyne was attached at the end of the spacer, which was required for the "click" reaction that will be used to graft the azoderivative on the solid substrate. The GRGDS peptide was attached directly to the azobenzene, thus its availability could be regulated upon azobenzene isomerization.

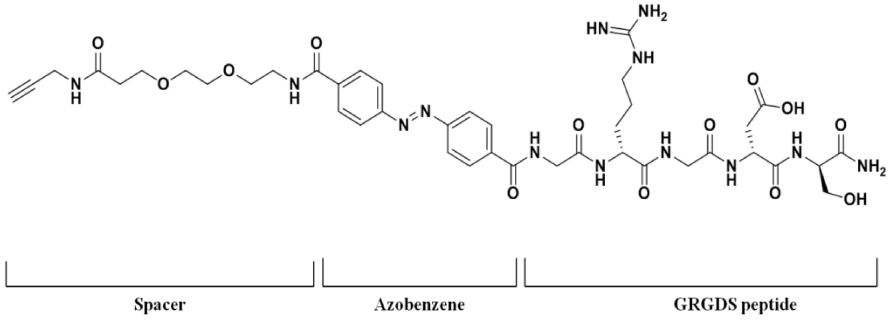
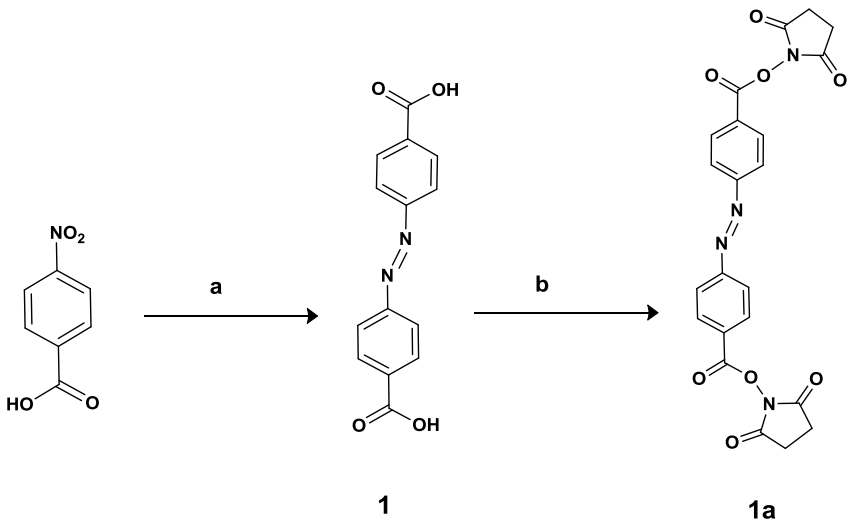


Chart 1. Chemical structure of compound **5**.

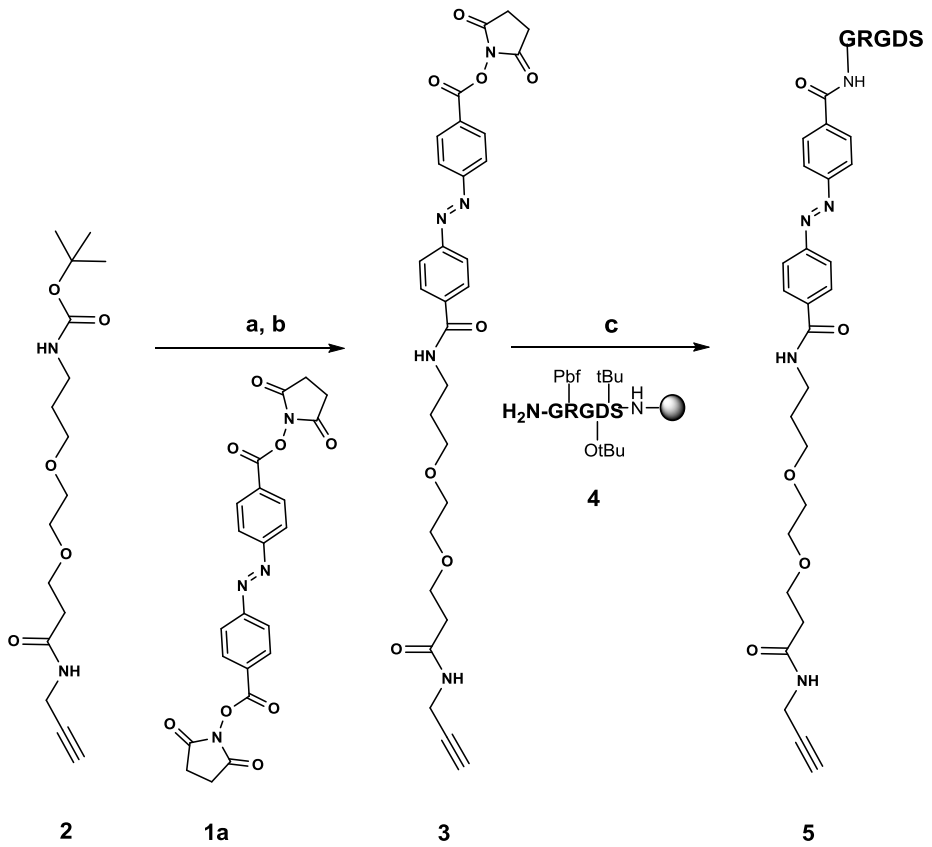
Azobenzene unit was synthesized according to an already reported procedure[22] to obtain 4,4'-dicarboxylic acid (compound **1** in Scheme 1). The strategy involved in the synthesis of the azobenzene unit was a reductive coupling of an aromatic nitro derivative, which occurred in basic aqueous solution in the presence of a large excess of glucose as reducing agent *in situ*.[27] The reaction provided a symmetric azobenzene with high yield, in addition the obtained byproducts were water soluble, so they were easily removed with water washing steps. Then the NHS group was introduced on the carboxylic acid functions of azobenzene **1**, to obtain the ester-activated azobenzene **1a** to use for subsequent coupling reactions.



Scheme 1. Reagents and conditions. (a) Milli-Q water and NaOH (14 equiv.), glucose solution (6 equiv.), 5 hours at 60 ºC and then overnight at RT. (b) NHS (2.4 equiv.), EDC·HCl (2.4 equiv.), DMAP (catalytic amount), in DMF overnight at RT.

Afterward, BOC protecting group of PEG linker **2** (Scheme 2) was cleaved with a solution of 50% TFA in DCM. Then, the amine salt was treated with TEA to restore its nucleophilic feature, and finally it was conjugated with the azobenzene **1a** to get the azoderivative **3**. GRGDS sequence was synthesized by standard solid-phase peptide synthesis (SPPS) via Fmoc strategy. The conjugation between the azoderivative **3** and the fully protected peptide **4**, still anchored to the resin, was performed as a final coupling to prevent side reactions and simplify the work-up of the target molecule **5**.

The final azoderivative **5** was checked with mass spectrometry (Figure 1) and nuclear magnetic resonance analyses (^{1}H NMR in Figure 2 and ^{13}C NMR in Figure 3), that confirmed the expected chemical structure for the designed compound.



Scheme 2. Reagents and conditions. (a) 50% TFA in DCM, 2h at RT; (b) **1a** (1 equiv. with respect to compound **2**) in DMF, 24 h at RT. (c) **4** (1 equiv. with respect to compound **3**), NHS (1 equiv.), DIC (1 equiv.), DIPEA (2 equiv.) in DMF, overnight at RT.

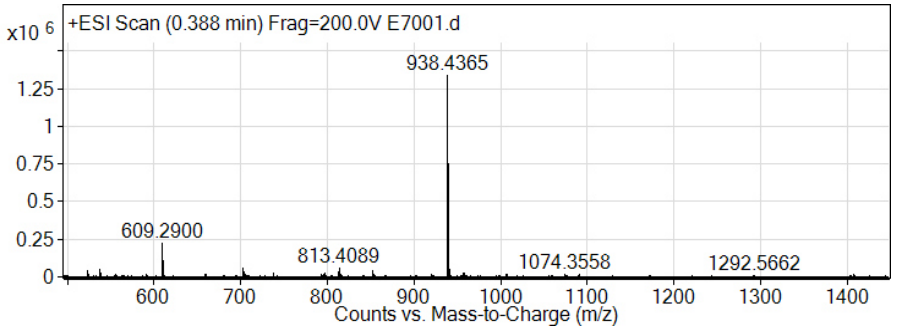


Figure 1. Mass spectrum for compound **5**.

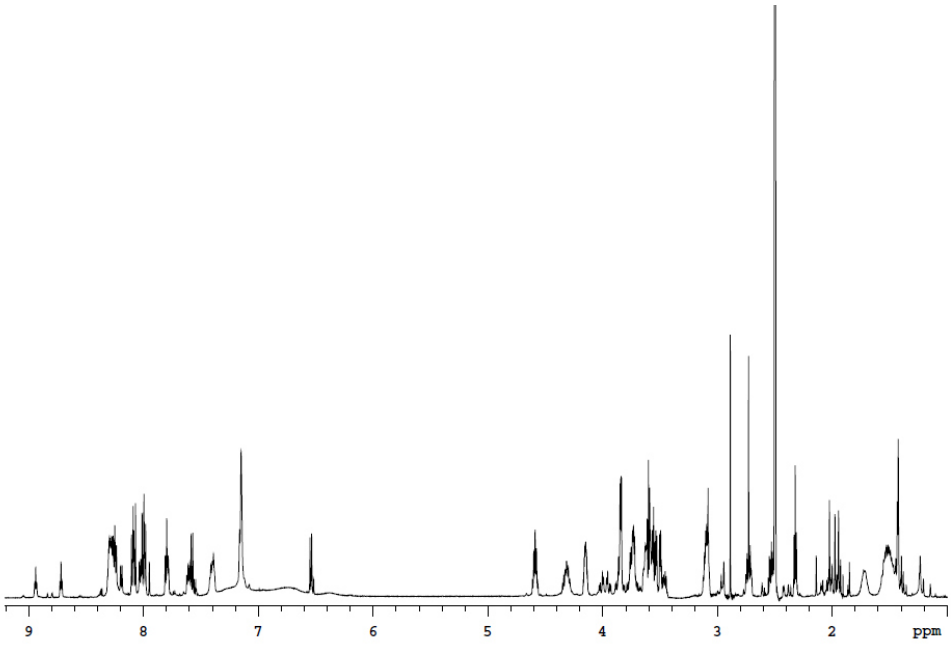


Figure 2. ^{1}H NMR (DMSO, 600 MHz) spectrum for compound **5**.

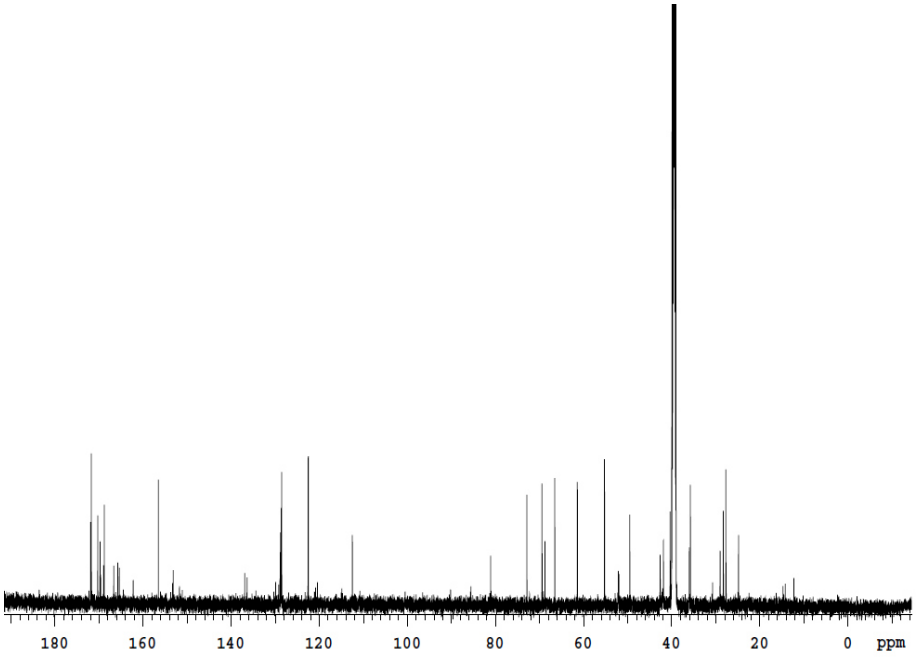


Figure 3. ^{13}C NMR (DMSO, 600 MHz) spectrum for compound **5**.

As already discussed above, the introduction of a short spacer in the chemical structure of the developed azoderivative, seemed to be required in order to ensure the proper degree of freedom for azobenzene isomerization, once grafted on the solid substrates.[28] This concept became really important when the final purpose of these photoresponsive surfaces concerned biological applications. As a matter of fact, an efficient exhibition of the biological cue can be achieved only when the RGD sequence was far enough from the surface of the artificial platform or scaffold, in order to reach better the integrin binding sites.[2, 26] This concept was introduced for the first time in 1992 by Beer *et al*.,[29] and later was considered also in other studies. Lee *et al*. investigated the response in adhesion for G_nRGDSP sequences with different glycine units (G_n) introduced as spacer between the surface of an alginate scaffold and the peptide sequences.[30] Cell adhesion and spreading was enhanced when increasing the number of the introduced glycine units, and below four glycine residues any significant increase in spreading on the surface was observed, which can be compared to the response obtained without glycine units. Auernheimer *et al*. investigated cell adhesion response for a set of immobilized cyclic and linear RGD peptides containing also an azobenzene unit. An ε-amino hexanoic acid was introduced as spacer, once tethered between the azobenzene unit and the peptide and once introduced before the azobenzene unit. An higher adhesion rate was observed for those surfaces where the azobenzene and the peptide were separated at least with one spacer unit, and with the azobenzene present as *trans* isomer.[21] Taking into account all these experimental evidences reported in literature, we decided to introduce

the short oligo(ethylene glycol) spacer in the chemical structure of our target molecule. This choice was performed, on one side, to ensure the occurrence of azobenzene isomerization mechanism over the surface, and on the other one to provide a better exposure of the GRGDS sequence for cell recognition as well.

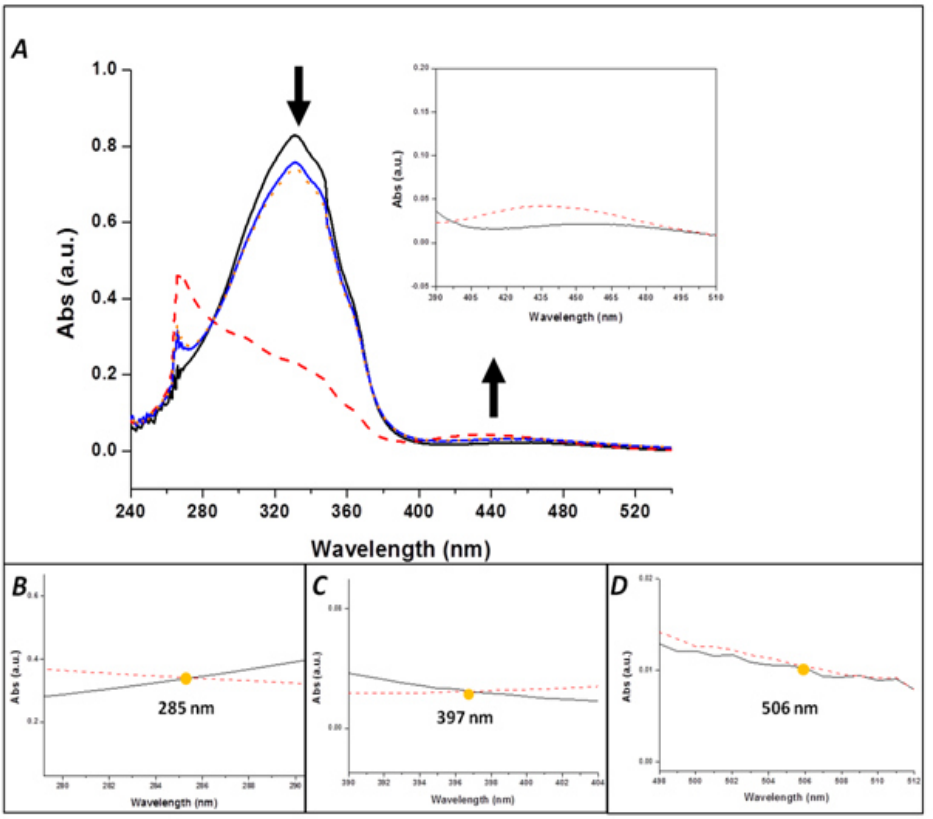


Figure 4. (A) UV-Visible absorption spectra of **1** in DMF (black line) before and (red dashed line) after 20 minutes of UV irradiation; thermal back-isomerization (blue line) after two days in the dark and photocontrolled back-isomerization (orange dotted line) after 20 minutes of white light illumination. (Inset) Magnification of the peak at 430 nm related to the *cis* isomer. (B-D) Isosbestic points referred to spectra in Figure 4A.

The isomerization mechanism of azobenzene molecules in solution can be monitored by UV-Vis spectroscopy, thanks to the fact that each

isomer presents a characteristic absorption spectrum.[25, 28]

As reported in Figure 4, the spectrum of azobenzene **1** exhibited an intense $\pi \rightarrow \pi^*$ band in the UV region at 331 nm, related to the more stable *trans* conformation (black line), that decreased upon irradiation with the UV lamp. Meantime, the red-shifted absorption broad band at 430 nm, related to the $n \rightarrow \pi^*$ transition of the *cis* isomer raised (red dashed line, also reported in the inset magnification). After 20 minutes of UV radiation exposure, a photostationary state was reached in which the two isomer were both present but with a predominant composition for the *cis* isomer. In addition, the presence of the isosbestic points (285, 397 and 506 nm) ensured that the different peaks observed in each spectrum belong to the two absorbing species (*trans* and *cis*) in equilibrium with each other.[24]

The thermal back-isomerization was achieved just after two days (blue line), keeping the sample at room temperature in the dark. The photocontrolled back-isomerization was induced using a common desk lamp provided with an halo bulb (orange dotted line).

As shown in Figure 5, a partial recovery of *trans* isomer was evident just after one minute after irradiation with the desk lamp (blue line). After 20 minutes of white light irradiation (orange line in Figure 5) a photostationary state was reached. In this experiment, the desk lamp was placed far enough to avoid some local heating of the solution, and the temperature was monitored with a thermocouple, reaching 37 ºC after 20 minutes of continuous irradiation.

To verify the absence of any heating enhancement when using the desk lamp to induce the photocontrolled back-isomerization, immediately after the UV illumination step, we left sample **1** for 20 minutes in the

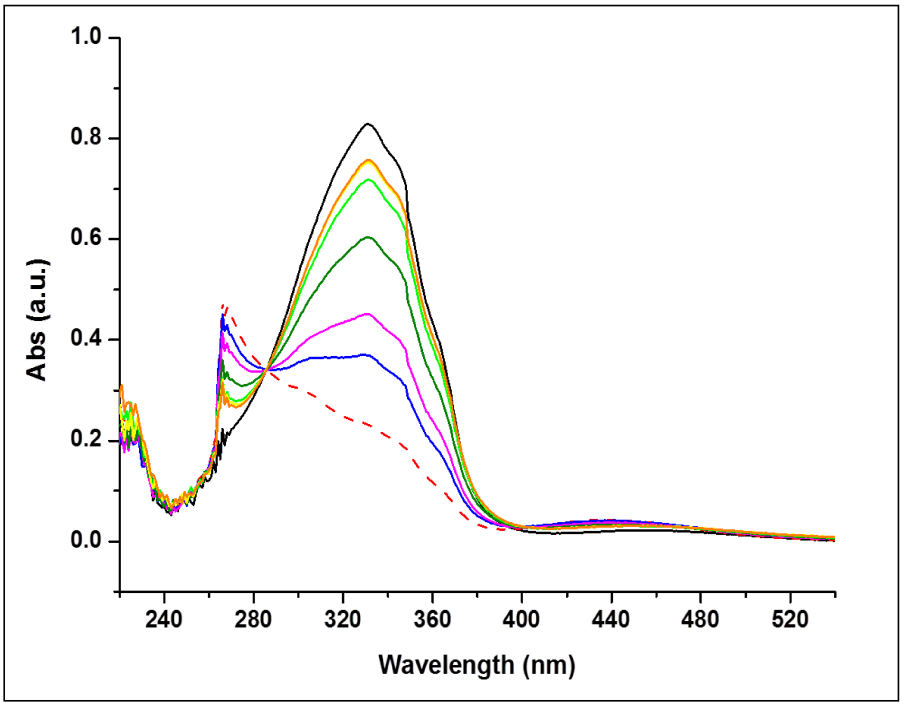


Figure 5. UV-Vis spectra of azobenzene **1** in DMF. (Black line) before and (red dashed line) after 20 minutes of UV radiation. Photoinduced back-isomerization of consecutive irradiation times with the desk lamp are reported with different colors: (blue line) after 1 minute, (pink line) 2 minutes, (olive line) 5 minutes, (green line) 10 minutes, (yellow line) 15 minutes and (orange line) 20 minutes.

dark in a preheated oven at 37 ºC. UV-VIS absorption spectra collected after the UV illumination (red dashed line in Figure 6) and after the oven incubation (blue line in Figure 6) did not exhibit a significant variation in intensity for the *trans* isomer band, proving that the main effect on the photocontrolled back-isomerization with the desk lamp was only related to the light stimulus.

The NHS-activated azobenzene **1a** exhibited an UV-Vis absorption spectrum similar to azobenzene **1** (data not shown).

For what concerns molecule **3**, where the azobenzene moiety is bound

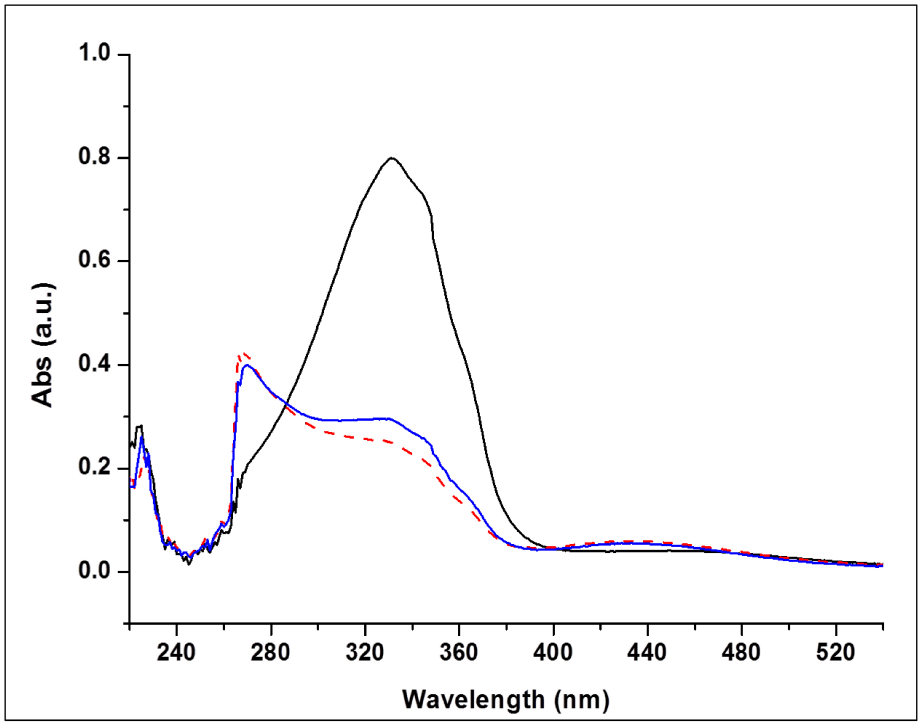


Figure 6. UV-Vis spectra of azobenzene **1** in DMF. (Black line) before and (red dashed line) after 20 minutes of UV irradiation. (Blue line) after 20 minutes in a preheated oven at 37 °C in the dark.

to the short oligo(ethyilen glycol), a slightly red-shifted peak at 334 nm for the *trans* isomer was observed (black line in Figure 7). This azoderivative was able to undergo the *trans*-to-*cis* isomerization with the same characteristic previously observed for the azobenzene **1**, hence the spacer linked to the azobenzene unit did not affect the isomerization mechanism.

Finally, azoderivative **5** was investigated, in which the peptide sequence was linked to the azobenzene moiety. Azoderivative **5** exhibited a strong $\pi \rightarrow \pi^*$ band in the UV region (between 320 and 380 nm) related to the more stable *trans* conformation, which decreased

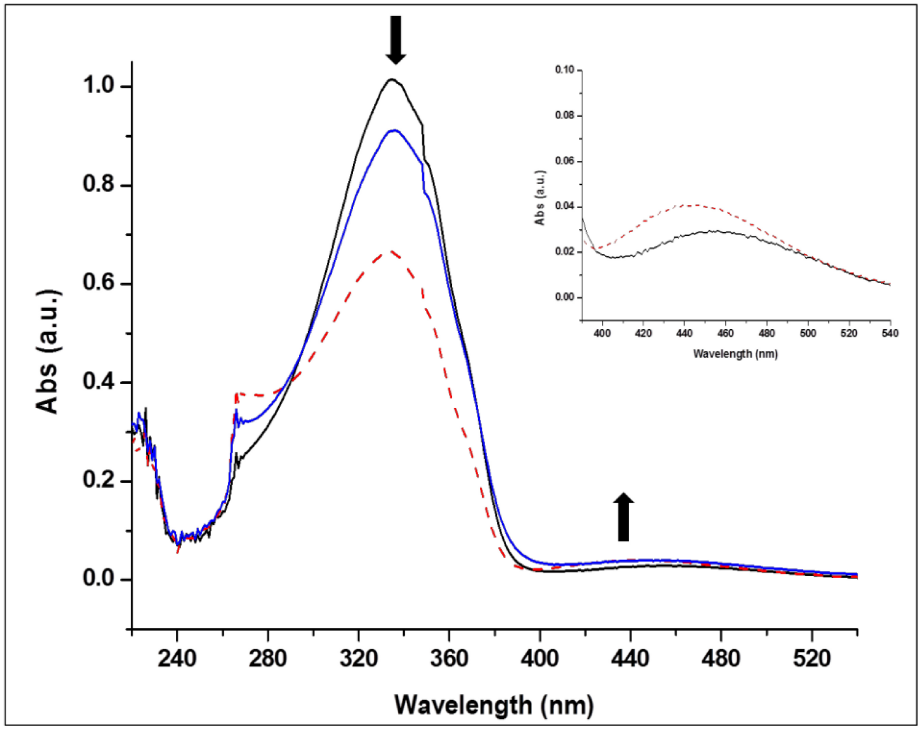


Figure 7. UV-Vis spectra for compound **3** in DMF. (Black line) before and (red dashed line) after 20 minutes of UV irradiation. (Blue line) thermal back-isomerization after two days in the dark. Inset magnification of the peak related to the *cis* isomer.

upon UV irradiation, as reported in Figure 8. Meantime, the red-shifted broad band at 420 nm, related to the n→π* transition of the *cis* isomer, raised and after 20 minutes of UV irradiation the photostationary state was reached. Also in this case the presence of isosbestic points (263, 387 and 490 nm) ensured that all different peaks observed in each spectrum belong to both absorbing species in equilibrium with each other. The thermal back-isomerization was achieved after two days, keeping the sample at room temperature in the dark, while the photoinduced back-isomerization was reached using again the desk lamp. As already observed for the azobenzene **1**, this simple tool was

able to restore quite completely the *trans* isomer just after 20 minutes of exposure to white light radiation.

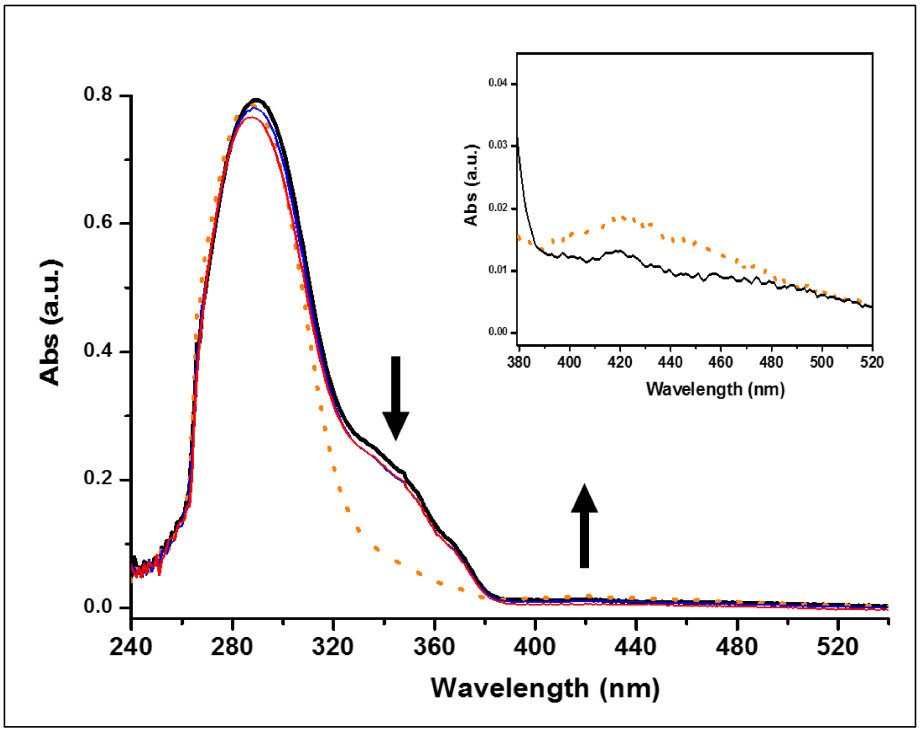


Figure 8. UV-Vis spectra for compound **5** in DMF. (Black line) before and (orange dotted line) after 20 minutes of UV irradiation. (Blue line) thermal back-isomerization after two days in the dark and (red line) photocontrolled back-isomerization after 20 minutes of halo lamp illumination. Inset magnification of the peak related to the *cis* isomer.

In general, UV-Vis measurements were a powerful method to probe the photoswitching behavior of these modified biomolecules, many other works investigated the intrinsic features of azoderivatives before grafting them on solid surfaces.[21, 23, 31] In our case, the performed measurements established that the modified azobenzene moiety, tethered on one side to the short spacer and on the other one to the GRGDS sequence, maintained its characteristic photoswitching

behavior and underwent both the *trans*-to-*cis* and the reverse *cis*-to-*trans* isomerization processes. Furthermore, the reversibility of the photoinduced isomerization was proved for each azoderivative, by repeating the cycle of UV and visible light illumination for two times and monitoring the related switching by spectroscopic measurements. In this section, only the data related to the azoderivative **5** were discussed and reported in Figure 9. The maximum absorption band at 341 nm related to the *trans* isomer, underwent a cyclic variation according to applied light stimuli, decreasing in intensity after the UV irradiation and raising again after the visible light illumination with the desk lamp (Figure 9A). The same cyclic behavior was also observed for the band at 420 nm, related to the *cis* isomer, which exhibited an opposite variation in intensity, thus increasing upon UV irradiation and decreasing after visible light stimulation (Figure 9B), in line with the involved isomerization mechanism. Illumination steps were performed taking into account the time required to reach both involved photostationary states. This last experiment proved the reversibility of the photoregulated isomerization mechanism and at the same time the resistance to optical fatigue typical of azobenzene molecules was also confirmed.[32] Moreover, experiments performed with these synthesized azoderivatives provided useful information about the light-driven isomerization mechanism in solution, which were used as model to investigate the photoswitching behavior of such azoderivatives after their immobilization on the glass surface. The related results were presented and discussed in the next chapter (Chapter 3).

It is also important to discuss about the light sources employed. The desk lamp used for the *cis*-to-*trans* back-isomerization was a simple

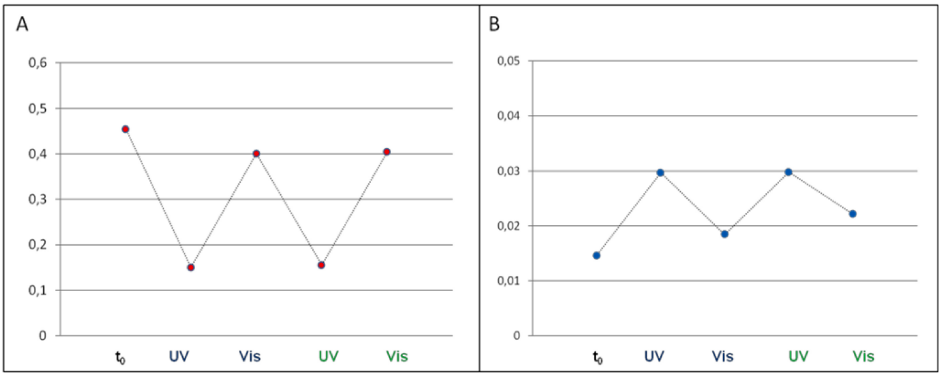


Figure 9. Intensity of absorption for azoderivative **5** during two cycle of UV-VIS illumination: (A) *trans* isomer band around 341 nm and (B) *cis* isomer band at 420 nm. Each cycle is illustrated as "UV-Vis" with different colors.

equipment able to regulate the isomerization mechanism in a very feasible and little expensive manner with respect to other available light sources. The only negative feature of the desk lamp was related to the broad band of wavelength emission, typical for an halo bulb source. Despite the absence of a narrow range of light emission, it is even possible to achieve the back-isomerization event with reasonable short exposure time. Furthermore, another way to provide shorter light exposure can be achieved using a LED, which has a narrow band of emission in the range of interest. When exploiting the azobenzene isomerization mechanism for biological application, it would be likely to tune such conformational changes without using UV light. It is well-known, indeed, that UV wavelengths are critical respect to other red-shifted, because they can induce apoptotic events affecting cell viability.[33-34] Most of the developed azobenzene-modified biomolecules, and also our synthesized azoderivatives, required the use of UV light to regulate the photoisomerization mechanism. Among the different available classes, there are other azobenzenes which can

undergo isomerization events with more "biocompatible" wavelenghts. The main difference in their photoswitching behaviors is based on the chemical structure involved in the substituent pattern of the two phenyl rings, that influences the absorption spectra of the two isomers.[25] In general, an electron-donor group can provoke the shift of $\pi \rightarrow \pi^*$ transition of the *trans* isomer band from the UV range to higher wavelengths near the visible region, where there is also the $n \rightarrow \pi^*$ band of the *cis* isomer. When an electron-acceptor and an electron-donor groups are present in the two *para*-positions of the two phenyl rings, a "push-pull" azobenzene is formed with the two characteristic bands completely red-shifted and quite overlapped in the visible region, hence a single wavelength is able to stimulate both isomer.[9] The only drawback for this class of azobenzene concerns the short lifetime of the related *cis* isomer, which is in the range of seconds or less. Woolley and co-workers explored the possibility to tune the photoisomerization mechanism of the azobenzene unit introducing different functional groups in the *ortho*-position of the two phenyl rings.[35] In this way, a red-shifted absorption of the azobenzene unit was achieved, together with an increased lifetime of the involved *cis* isomer. Under this point of view, the opportunity to use such modified class of azobenzenes represents a further improvement for the design of our azoderivatives. The chance to control the photoisomerization mechanism with more "biocompatible" wavelengths is really interesting, especially for biological application. A new generation of azoderivatives can be designed, synthesized and exploited to control cell response upon different light stimuli, also with the perspective of a dynamic photoswitching mechanism *in situ*.

2.4 Conclusions

The modification of peptide or protein ligands with light-sensitive moieties represents a powerful method to also achieve the temporal control on their bioactivity by using light. In our work, the azobenzene unit was coupled to the GRGDS sequence to achieve the possibility of a photocontrolled exposure of such peptide, after tethering on the solid substrate (that will be discussed in the next chapter). The azoderivative was designed taking into account some important features. The linkage between the azobenzene unit and the peptide sequence was required to encourage cell recognition and thus to promote cell adhesion on the azoderivative-modified surfaces. In addition, the azobenzene unit was provided with a short oligo(ethylene glycol) as spacer, which was important to ensure the isomerization mechanism of azobenzene once grafted on the solid substrate. UV-Visible spectroscopic measurements demonstrated that the modified azobenzene moiety, tethered on one side with the short spacer and on the other one with the GRGDS sequence, maintained its characteristic photoswitching behavior. The azoderivative underwent both the *trans*-to-*cis* isomerization upon UV stimulation, and the reverse *cis*-to-*trans* isomerization processes. In particular, the back-isomerization mechanism was achieved not only as a thermal process (according to the higher stability of the *trans* isomer), but it was also induced upon visible light irradiation using a normal desk lamp, with a very short illumination time. Furthermore, the reversibility of the photoinduced isomerization was proved repeating the cycle (UV-Vis) for two times. The related switching was monitored following the cyclic variation of intensity in absorption of the characteristic maxima for the two involved isomers. An

improvement that concerns light sources could be the use of a LED, which provides a more specific and narrow band of emission if compared to the broad band of the halo bulb of the desk lamp, and thus promoting the isomerization event with shorter exposure time. The range of wavelength employed to regulate the isomerization events become really important when going towards biological applications. It is well desired to tune the involved conformational changes without using UV light, because these high-energy wavelengths are more critical respect to those more red-shifted, because can induce apoptotic events and thus affecting cell viability. A further improvement regarding the use of "biocompatible" wavelengths, could be achieved exploiting a different azobenzene, which do not require UV light to induce the photoisomerization events. Azobenzenes with a particular substituent pattern on the two phenyl rings can provide a red-shifted and "biocompatible" photoswitching behavior, in terms of required light dose. In this way, exploring all the possible chemical modifications of azobenzene unit for light-controlled *in vivo* applications, can help to prevent undesired side effects that can compromise cell viability.

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CHAPTER 3

"On-Off" GRGDS Signaling Using Azobenzene Photoswitch-Modified Surfaces

Abstract. Photoresponsive surfaces have been developed modifying glass slides with switchable GRGDS peptide, in order to investigate light-controlled cell adhesion. GRGDS sequence has been attached to an azobenzene moiety and then "clicked" to silanized glass substrates. After a chemical and topographical characterization of such substrate with Raman spectroscopy and atomic force microscopy, the photoresponsive behavior of such platform was investigated by contact angle technique, recording local changes in wettability due to the isomerization of the azobenzene domain upon light stimuli. The "On-Off" switching behavior was observed for at least five cycles when illuminated consecutively with UV and visible light sources.

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3.1 Introduction

Material surface decoration with peptide or protein ligands represents a powerful method to extend the functionality of synthetic materials presenting active signals to the cell surface able to elicit specific cell functions.[1-2] This approach has been proved as practical and effective in improving cell recognition on synthetic materials.[3] Furthermore, the degree of bioactivation can be controlled and modulated by the orientation, the density as well as the spatial distribution of the bioactive signal.[4-6] However, native extracellular matrix (ECM) guides cellular processes providing a tight regulation in temporal and spatial presentation of cell adhesive motifs, whereas most of the bioactivation strategies lack of the possibility to modulate the temporal presentation of the signal.[7] Therefore, novel classes of biomaterials that better mimic the native ECM call for programmable platforms able to regulate presentation of matricellular cues in time and space to instruct the complex cellular processes.[8-9] This advancement requires the implementation of versatile systems where external stimuli can tune the accessibility of cell ligands at wish. The sensitive response can be triggered by altering pH[10] or temperature,[11] as well as exposing to electric or magnetic fields;[12-13] in some cases irradiation with light can also elicit a response.[14] Another important feature of these new generation of biomaterials is the reversibility of their feedback to the external stimuli.[15-16] So far, many smart platforms have been already exploited to direct cell fate with light. In recent works, the availability of biological signals was modulated trough photocleavable moieties, promoting either cell adhesion[17-18] or detachment.[19] In this respect, azobenzene-modified surfaces[20-21] can provide a further improvement

introducing the concept of reversibility through the isomerization mechanism. Indeed, azobenzenes are excellent candidates as molecular photoswitches, since they can exist in two isomeric forms, *trans* and *cis*, which can interconvert both photochemically and thermally.[22-23] In addition, among several photoswitches, azobenzene is one of the most resistant to optical fatigue.[24] The isomerization process involved is reversible and can induce a significant geometric change in the position of the two phenyl rings. In general, the *trans* isomer is the most stable, and has a flat geometry with the two aromatic rings laying on the same plane, with a strong absorption band around 365 nm due to the $\pi \rightarrow \pi^*$ transition. The *cis* isomer, instead, presents an angular geometry and has a relatively weak absorption around 445 nm, which is related to the $n \rightarrow \pi^*$ transition.[25] Because of the higher stability attributed to the *trans* isomer, azobenzene derivatives are predominantly present as *trans* form and, when exposed to UV radiation, the *trans*-to-*cis* isomerization occurs. This process is completely reversible, and the back-isomerization can take place either spontaneously (eventually accelerated by heating) or by visible light exposure. This reversible mechanism can lead to a variation in physical and chemical properties of the surrounding environment. For example, azobenzene molecules that are linked to a surface provoke a local alteration in wettability once they modify their conformation, mainly due to a change in the dipole moment of the molecules going from *trans* to *cis* isomer. Actually, such local variation in wettability can be monitored by water contact angle technique, providing useful information about the isomerization process.[26] Furthermore, if the azobenzene brings substituents on the two phenyl rings, during the

isomerization the position of these groups changes as well as their distance.[27-28] Inspired by recent works, which investigated synthetic matrixes coated with azoderivatives that led to photocontrolled cell[20-21, 29] or bacterial adhesion,[30] we designed a two-dimensional platform, based on an "On-Off" photoswitching mechanism, useful for the dynamic exhibition of a well-known peptide sequence (GRGDS), and exploitable for phototriggered cell adhesion studies. The azobenzene moiety was firstly linked to the GRGDS peptide, then grafted to glass surfaces with the chemoselective "click" chemistry approach.[31-32] Here, the synthesis of our dynamic GRGDS-modified platform is discussed, followed by an extensive characterization of the photoresponsive properties using several techniques.

3.2 Experimental Section

General Materials and Method. All chemicals and solvents were purchased from Sigma-Aldrich and were used without further treatments. Glass slides were provided by Thermo Fisher Scientific-MENZEL. Milli-Q water (18.2 MΩ·cm) was obtained with a Millipore system.

Raman Spectroscopy. Spectra were acquired at room temperature with a DXR Raman spectrometer (Thermo Fischer Scientific) with 20 mW laser power. Measurements were performed using a diode laser at 532 nm with different intensity for each investigated sample (1÷5 mW). For 2D measurements on solid substrates a 50×/0.75 NA objective was utilized to focus the laser beam on the surface. The investigated area was chosen as a map of

950×600 µm size, with a step of 75 µm between each point (13×8 points map).

Confocal Laser Scanning Microscopy. Images were collected with a Leica confocal microscope SP5 (Leica Microsystems), provided with an HCX IRAPO L 25.0×0.95 WATER objective and an Argon laser (514 nm as selected wavelength) set at 10% of power. For each sample 5 frames have been collected, exploring different region all over the sample. Images have been collected with a speed of 400 Hz, 8 lines average, and the relative size was 206.7×206.7 µm.

Atomic Force Microscopy. AFM measurements were performed with a JPK-Nanowizard instrument in contact mode in air, under ambient conditions. MLCT tips were used (spring constant 0.01 N/m).

Water Contact Angle. Water contact angle values were acquired by the sessile drop technique using a KSV-CAM 200 instrument. Measurements were performed at room temperature deposing Milli-Q water drops of 4 µl of volume. Contact angle measurements were executed after each synthesis step on glass surfaces in order to verify the occurrence of the chemical modifications. Obtained values were determined as the average of several frames for different drops. While, in the case of the experiment to evaluate the wettability change upon azobenzene photoinduced isomerization, a specific protocol was developed. In this case, samples were investigated by monitoring the local change in wettability in the same area of the sample, before and after illumination, with the help of some landmarks drawn on the opposite side of the glass. The analysis

was performed by collecting five frames for each drop, and an average value was considered for every single drop.

Illumination experiments. For UV radiation a Spectroline® E-Series UV lamp was used illuminating at 365 nm (after 20 minutes of warm-up). Light source was placed 5 cm far from the samples. The fluence rate[33] (1.2 mW/cm^2) was measured with a power meter (Thorlabs). For visible light radiation a normal desk lamp equipped with an halo bulb was used and placed 10 cm far from the samples. Fluence rate (130 mW/cm^2) was measured providing the sensor of the power meter instrument with a proper filter (435-443 nm).

Protocol of silanization with 3-(Bromopropyl) trimethoxysilane (BPTMS) for the preparation of glass surface **6**. Glass coverslips were washed in acetone under sonication and dried in oven for about 30 minutes at 30 °C. Then, oxygen plasma treatment (Electronic Diener Plasma surface technology) was performed for 3 minutes, and immediately after the activation step, glasses were put in a solution of 4% in volume of BPTMS in toluene on an oscillating plate overnight. After reaction, glass slides were washed with toluene, ethanol, acetone and finally dried under vacuum.

Bromine-azide nucleophilic substitution for the preparation of glass surface **6A**. Bromo silanized glasses **6** were put in a glass petri dish containing a saturated solution of sodium azide (50 mM in DMF) and left overnight on an oscillating plate. The day after all glass coverslips were washed with DMF, ethanol, acetone and finally dried under vacuum.

Synthesis of compound **5A**. Compound **5A** was synthesized according to the protocol reported for molecule **5** (Chapter 2). A slight

difference in the peptide sequence was the introduction of the Lysine (Fmoc-Lys-(Mtt)-OH) residue at the end of the peptide bound to the resin, to obtain the KGRGDS sequence. A selective cleavage of the Mtt protecting group was performed with 3% of volume of TFA in DCM, to allow the coupling with 5(6)-Carboxytetramethylrhodamine (4 equiv. with respect to the resin) in the presence of DIC (4 equiv.), HOBt (4 equiv.) and DIPEA (8 equiv.). All the following steps were conducted in accordance to the procedure adopted also for molecule **5**.

"Click" chemistry protocol for sample **7C**. In a petri dish with glass sample **6A**, a solution containing compound **5A** (1.1 μmol, 1 mg), sodium ascorbate (10 equiv. with respect to compound **5A**) and $CuSO_4 \cdot 5H_2O$ (0.5 equiv.) dissolved in 3 ml of DMF was added. The reaction was left on an oscillating plate overnight at room temperature and the day after samples were washed with DMF, ethanol, acetone and finally dried under vacuum.

"Click" chemistry protocol for sample **7A**. In a petri dish with glass sample **6A**, a solution containing compound **5** (1.1 μmol, 1 mg), sodium ascorbate (10 equiv. with respect to compound **5**) and $CuSO_4 \cdot 5H_2O$ (0.5 equiv.) dissolved in 3 ml of DMF was added. The reaction was left on an oscillating plate overnight at room temperature and the day after samples were washed and finally dried under vacuum.

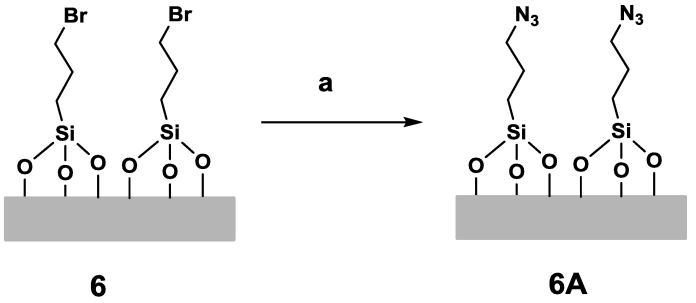
"Click" chemistry protocol for sample **7B**. In a petri dish with glass sample **6A**, a solution containing compound **3** (1.1 μmol, 1 mg), sodium ascorbate (10 equiv. with respect to compound **5**) and $CuSO_4 \cdot 5H_2O$ (0.5 equiv.) dissolved in 3 ml of DMF was added. The reaction was left on an oscillating plate overnight at room temperature

and the day after samples were washed and finally dried under vacuum.

3.3 Results and discussion

The work presented here aimed to develop glass modified surfaces where the exposure of GRGDS peptide sequence could be triggered by light, in order to tune cell adhesion in a photocontrolled manner. To build up such photoresponsive platforms, azobenzene was introduced in the chemical structure of our system and covalently immobilized on the substrate. In general, it is important that the biomolecules maintain their bioactivity in terms of conformation and chemical stability, once immobilized on a solid surface, in order to ensure the proper RGD signaling. For this reason, the target molecule was grafted with a chemoselective method, exploiting the Huisgen 1,3-dipolar cycloaddition[34-35] between azides and terminal alkynes, also known as "click" reaction, which has been largely established as a useful chemical handle for bioconjugation in both non copper-mediated[36] and copper (I) catalyzed[31-32] manners. The unreactive character of azides and alkynes towards other functional groups present in the synthesized molecules, together with the thermal and hydrolytical stability of their bonding, represent optima features in our specific case, as well as in the general field of bioconjugations.[37-40] The density of packaging is another important parameter to consider while grafting azobenzenes on a solid substrate. In fact, a sufficient free volume for the isomerization to occur has to be taken into account dealing with azopolymer coatings or unsubstituted azobenzene monolayers.[25-26] Before use, glass slides were washed, activated by oxygen plasma treatment and then immediately modified with a short bromo silane. After silanization, the

bromine head-group was displaced by sodium azide (**6A** in Scheme 1) in DMF.



Scheme 1. Reagents and conditions. (a) Sodium azide solution in DMF (50 mM) overnight at RT.

Water contact angle values were evaluated before the plasma treatment and after each modification step, in order to verify the occurrence of the reaction. In general, collected values were in good agreement with those already reported in literature,[41] in particular for bromo silanized glass was found an average angle θ_{av} of 80°, while after displacement with azide the average contact angle decrease to 76° (Figure 1).

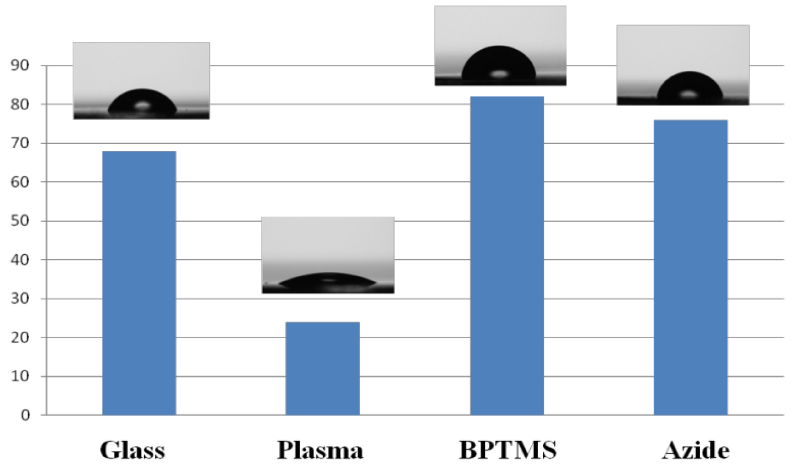
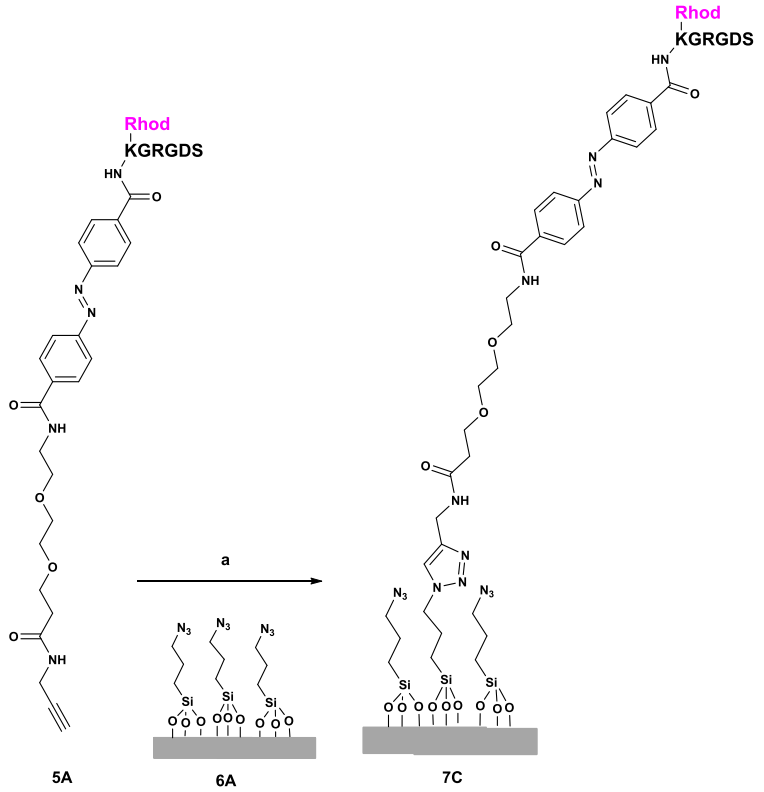


Figure 1. Water contact angle values for glass slides after each modification step.

Finally, azoderivatives were attached to silanized glasses via "click" chemistry approach. In particular, we used a catalytic amount of copper (II) salt ($CuSO_4 \cdot 5H_2O$) with a large excess of a water soluble reducing agent (sodium ascorbate) to produce *in situ* copper (I). A glass coverslip (**6A**) treated without the Cu (I) catalyst (condition in which reaction did not occur) was used as negative control to verify the occurrence of the "click" reaction. Preliminary investigations were performed with substrate **7C**, where the rhodamine tagged azoderivative **5A** was grafted on the glass surface (Scheme 2).



Scheme 2. Reagents and conditions. (a) $CuSO_4 \cdot 5H_2O$ (0.5 equiv. with respect to compound **5A**), sodium ascorbate (10 equiv.) in DMF, overnight at RT.

Images collected with confocal laser scanning microscopy experiments evidenced a non homogeneous functionalization for this substrate (Figure 2A). The lower intensity of fluorescence signal for the negative control (**6A** substrates treated without Cu catalyst; Figure 2B) ensured the occurrence of the covalent grafting for the azoderivative on the surface by "click" chemistry, hence any potential physical absorption of the dye could be excluded.

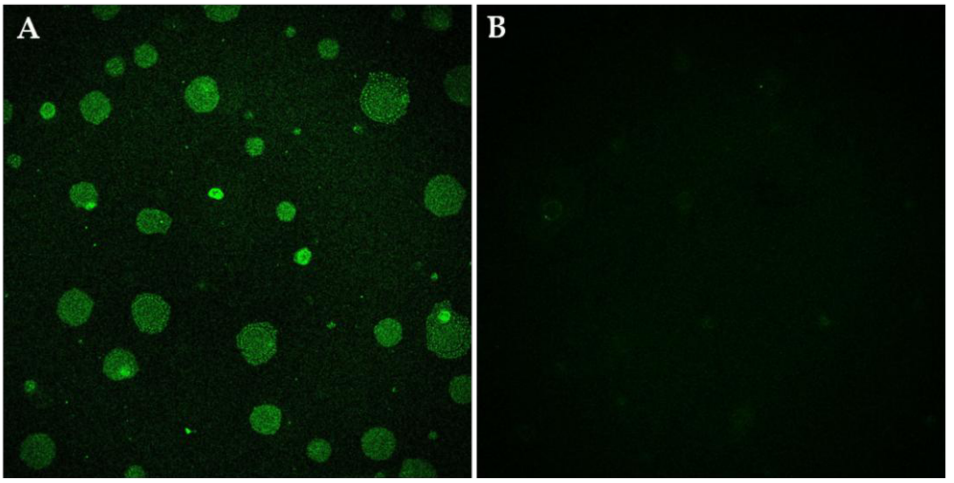
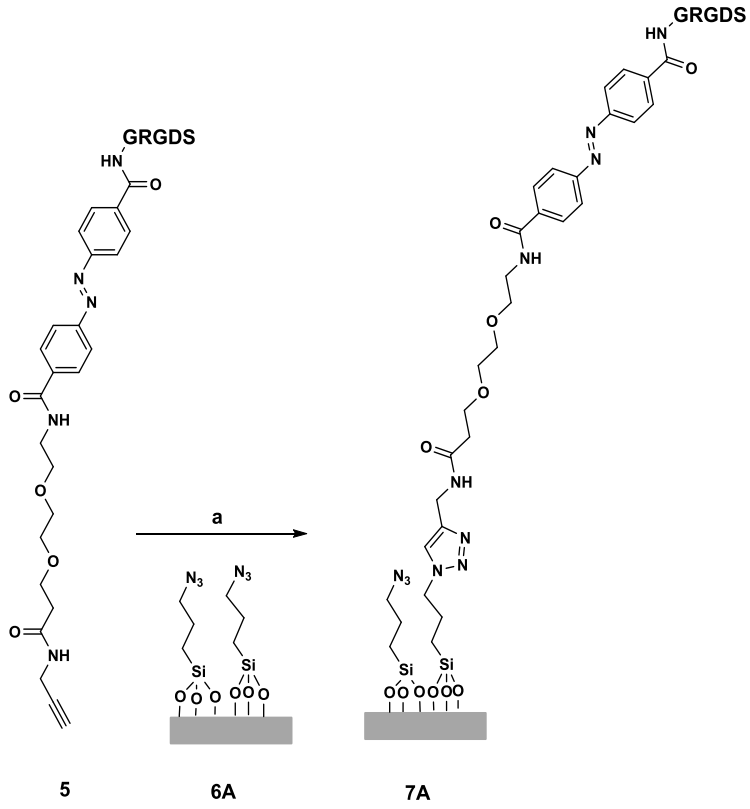


Figure 2. Confocal laser scanning microscopy images of (A) sample **7C** with Rhodamine dye and (B) negative control **6A**. Size of images (206×206 µm).

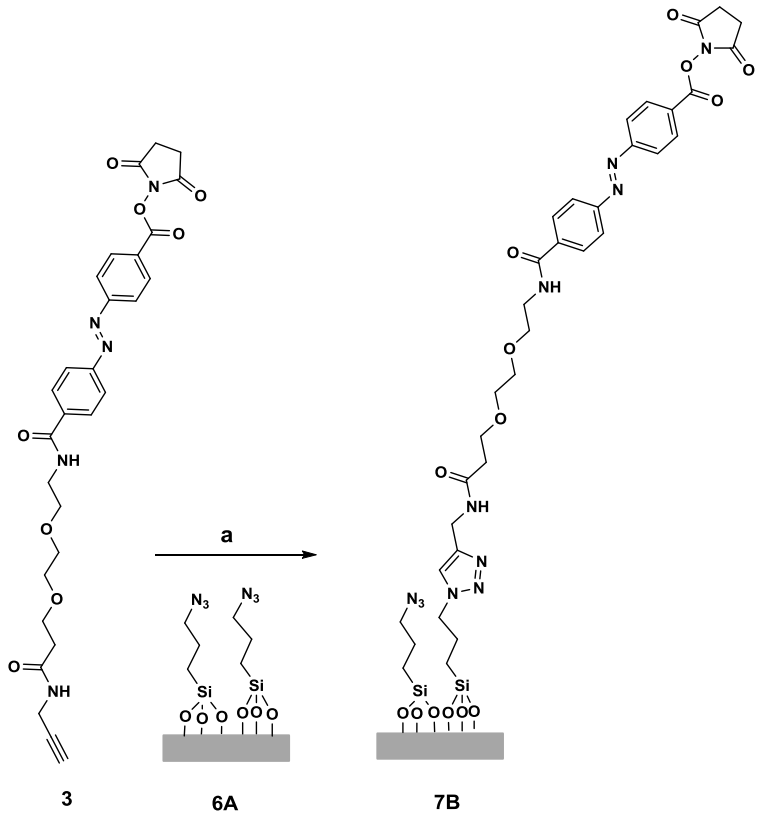
Sample **7C** represents an important first step for the presented work, because confirmed the efficiency of the "click" chemistry approach used to graft our azoderivative on the glass silanized surface. Following investigations were performed on substrates where the rhodamine dye was not present. Even if the rhodamine could help to better visualize the organization of our azoderivative on glass surfaces, it could interfere with the final biological application, preventing cell recognition mechanism for the peptide sequence. In particular, we will refer to sample **7A** where the GRGDS peptide is involved in the

chemical structure of the grafted azoderivative (Scheme 3) and to sample **7B**, where the peptide sequence is not present (Scheme 4). These two samples were prepared following the same "click" chemistry protocol already exploited for sample **7C**.



Scheme 3. Reagents and conditions. (a) $CuSO_4 \cdot 5H_2O$ (0.5 equiv. with respect to compound **5**), sodium ascorbate (10 equiv.) in DMF, overnight at RT.

Raman measurements were performed to characterize the chemical distribution of the azoderivative grafted on our substrate. As first step, the Raman spectrum for the starting material was collected, in order to determine the typical Raman scattering bands for our azoderivatives **5** which were reported in Figure 3.



Scheme 4. Reagents and conditions. (a) $CuSO_4 \cdot 5H_2O$ (0.5 equiv. with respect to compound **3**), sodium ascorbate (10 equiv.) in DMF, overnight at RT.

The band at 1460 cm^{-1} was assigned to the characteristic Raman shift for the N=N stretching vibration mode of the azobenzene moiety. Other characteristic bands were the peak at 1140 cm^{-1} related to the symmetric stretching of C-O-C bonds of the short oligo(ethylene glycol) spacer linked to the azobenzene, the band at 1400 cm^{-1} of the C-N amide vibration (amide III bond) and finally the peak at 1600 cm^{-1} related to the stretching vibration for C=C aromatic bonds of the phenyl rings. Raman measurements were then performed on the glass surfaces after grafting the azoderivatives.

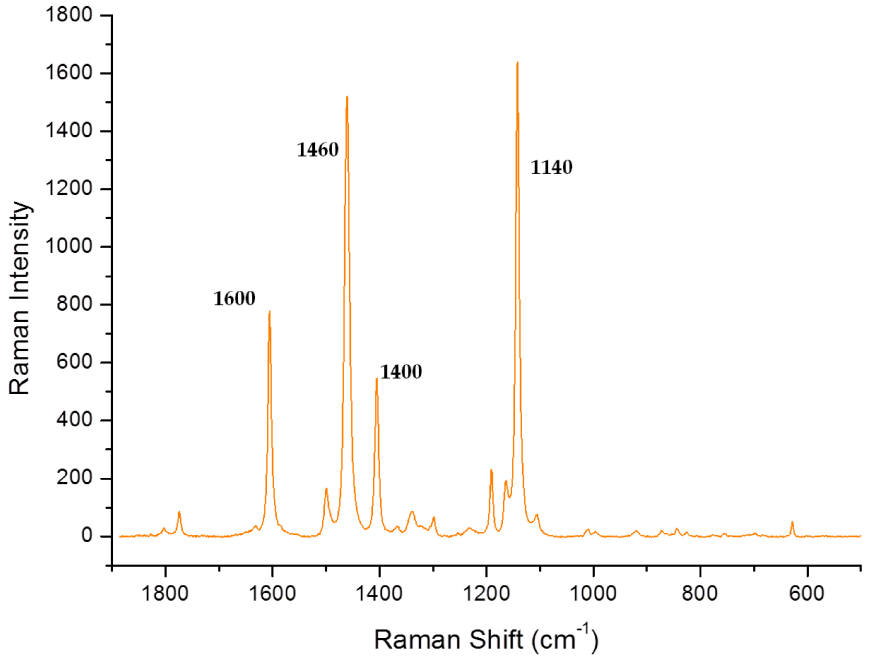


Figure 3. Raman spectrum for azoderivative **5**.

After this preliminary characterization, Raman 2D mapping were performed on azoderivative-modified samples, measuring all over the surface. As reported in Figure 4, sample **7A** exhibited the peak related to the N=N vibration band at 1466 cm^{-1} with diverse intensities in different points of the sample, thus confirming a non homogeneous functionalization for the investigated platform.

Moreover, this information was also confirmed from the topographic point of view as evidenced from the AFM images reported in Figure 5. For sample **7A** a different topography between more functionalized areas (Figure 5C) and less functionalized ones (Figure 5D) was observed while exploring several region of the surface. Specifically, less functionalized areas were similar to the topography of the negative

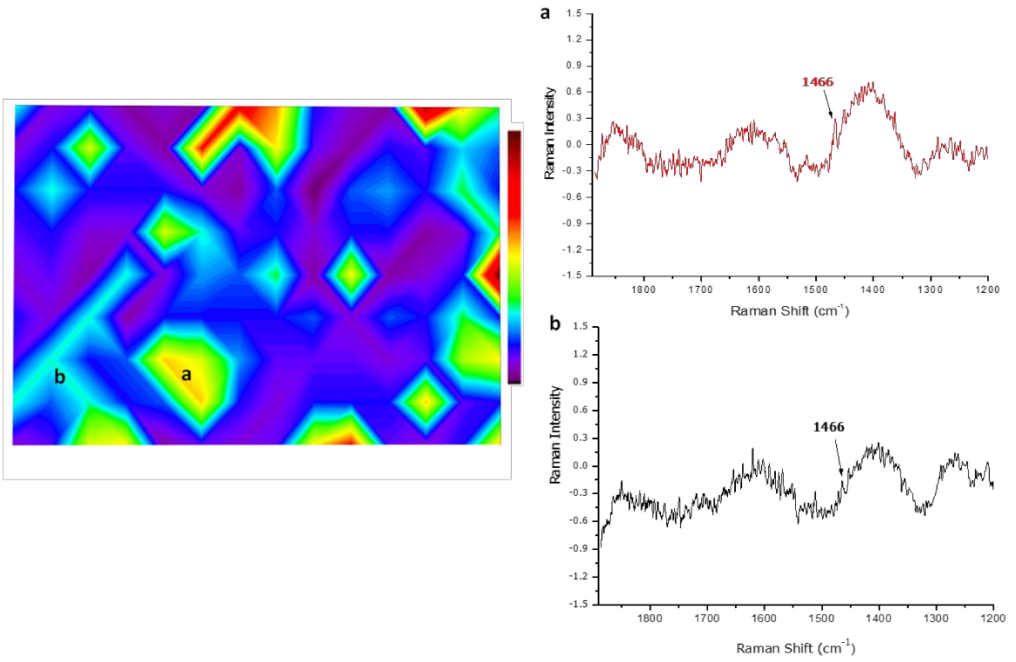


Figure 4. Raman 2D measurements on sample **7A**. Raman intensity values of the peak at 1466 cm^{-1} related to the N=N bond of the azobenzene unit were reported with different color ranging from points of (a) higher intensity to (b) lower intensity of the band. Size of image (950×600 µm).

control **6A** (Figure 5A). After the chemical and the topographical characterization of such synthesized platform, the photoswitching behavior of grafted azoderivatives was then investigated by water contact angle technique. Previous experiments performed for the azoderivatives in solution with UV-Visible spectroscopy technique (Chapter 2) gave important information on how substituents linked to the pehnyl rings of the azobenzene affected the isomerization.[27-28] In addition, these measurements provided an useful starting point to set up water contact angle measurements by using the same parameters, such as employed light sources and exposure. In particular, contact angle technique has been exploited as a powerful tool to investigate

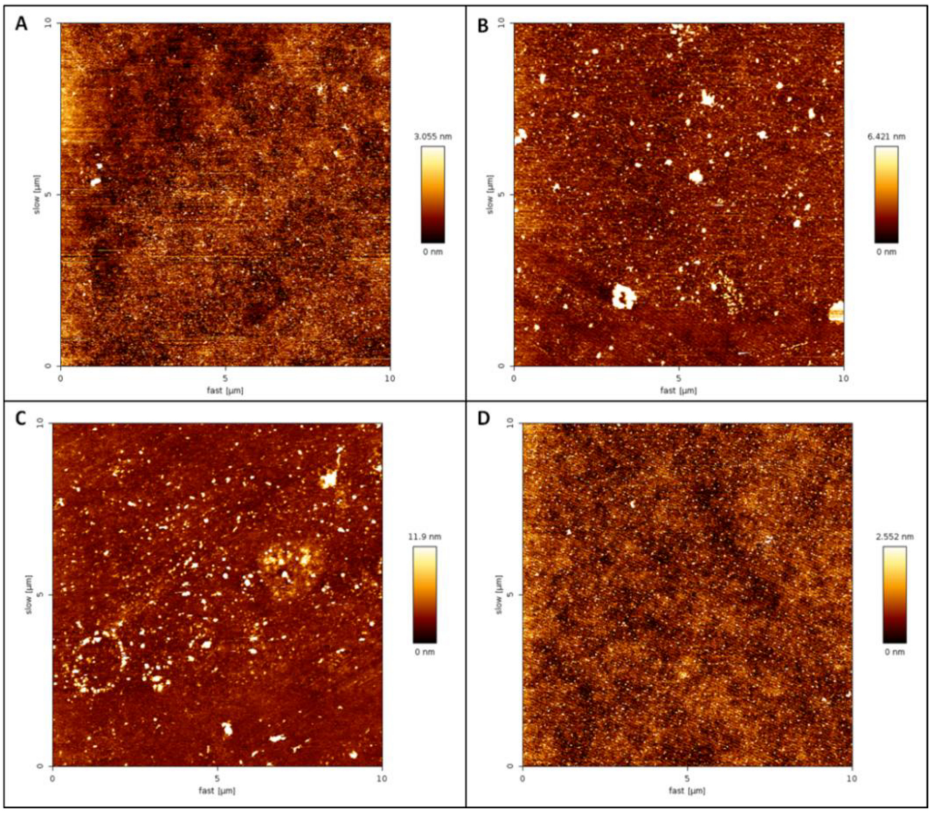


Figure 5. AFM images of (A) negative control **6A** (B) sample **7B**. For sample **7A** the images reported are related to (C) more functionalized and (D) less functionalized areas of the substrate. Size of images (10×10 µm).

local changes in wettability.[25] According to literature, the concept of wettability in azobenzene-modified surfaces is related to the change of the dipole moment of the N=N double bond that occurs upon isomerization, with a concomitant switch in the geometry of the molecule itself.[27] In a general case, when an unsubstituted azobenzene is present as *trans* isomer, the surface appears more hydrophobic. Then, upon UV irradiation, the *cis* isomer is promoted and an enhanced hydrophilic feature is exhibited, according to an increase in the dipole moment of this isomer.[25] The two different samples **7A** and

7B were both investigated in order to understand the role of the short peptide sequence linked to the azobenzene unit.

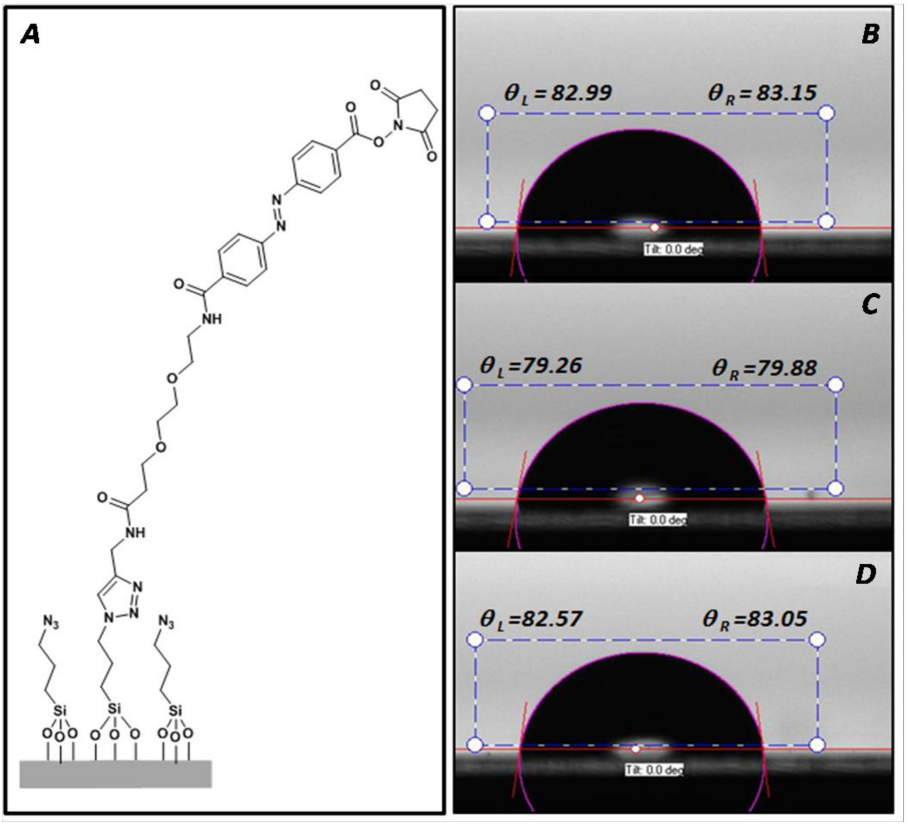


Figure 6. (A) Chemical structure of sample **7B**. WCA images of a drop (B) before UV illumination, (C) after UV illumination and (D) after three days in the dark for thermal back-isomerization. For each drop, left and right contact angles were reported as raw data. WCA data were obtained analyzing the same region of the sample.

First of all, contact angle values were collected before any light treatment. Then, samples were irradiated for 20 minutes at 365 nm with a UV lamp. After this illumination step, the local contact angle was measured as explained in details in the experimental section. Thermal back-isomerization was verified for each sample after leaving

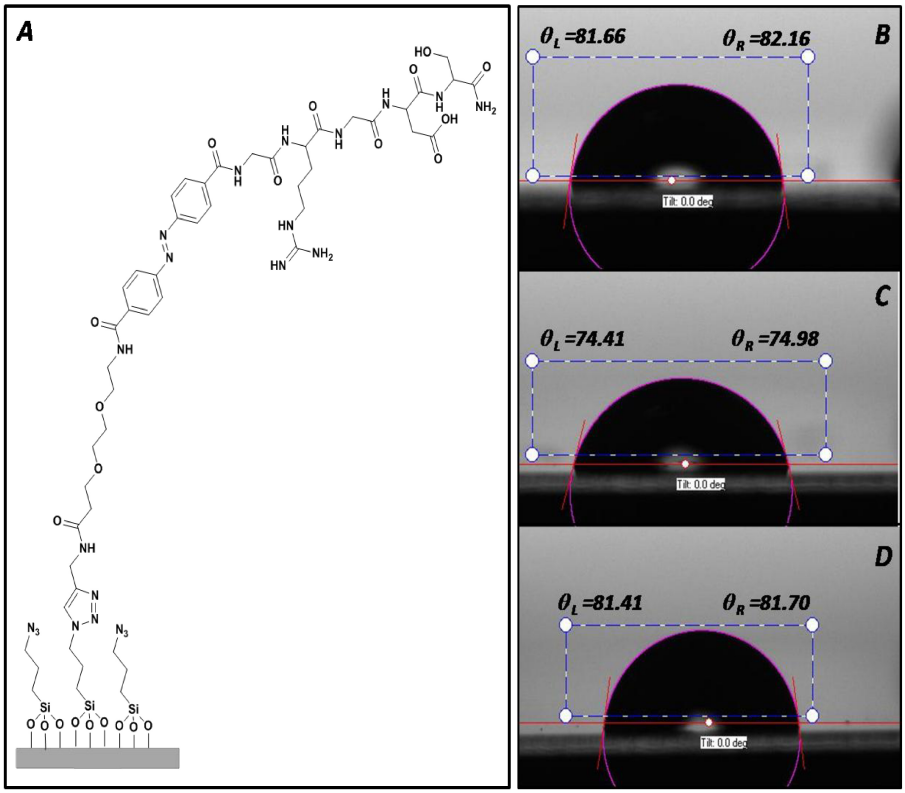


Figure 7. (A) Chemical structure of sample **7A**. WCA images of a drop (B) before UV illumination, (C) after UV illumination and (D) after three days in the dark for thermal back-isomerization. For each drop, left and right contact angles were reported as raw data. WCA data were obtained analyzing the same region of the sample.

it for three days in the dark at room temperature, while the photoinduced back-isomerization was confirmed after just 30 minutes of irradiation with the desk lamp. Experimental data collected for the two samples proved that both of them underwent photodriven isomerization. Moreover, they experienced a decrease in water contact angle value after UV illumination, even if with some differences.

In the case of sample **7B**, in which the azobenzene unit bore the NHS group (Figure 6A), it was detected a lower variation in contact angle

value after UV illumination that reached 4°, as reported in Figure 6B-C. On the other hand, sample **7A** (Figure 7A) revealed a decrease in contact angle value up to 8° (Figure 7B-C), because of the presence of charged polar amino acid residues (arginine, serine and aspartic acid). In fact, it is known that not only the change in dipole moment, but also the water affinity of the group present at the outermost part of the monolayer affects the change in WCA.[42] It is even possible to have an opposite variation of wettability (from a more hydrophilic monolayer in the *trans* state to a less hydrophilic in the *cis* one), as an example by placing an hydrophobic moiety in the *meta* position of the phenyl rings.[43] In our specific case, however, the observed variations were not uniform all over the substrate. Each drop exhibited its own change in WCA due to the different degree of local functionalization of the surface. In fact, some drops did not reveal significant changes in WCA, as reported in Figure 8A-B. Comparing these data with those related to the negative control **6A**, that did not present any azobenzene unit, no contact angle alteration occurred (maximum 1° in few cases), proving once more that wettability variations were strictly related to conformational changes of the azobenzene. Variations in WCA values that occurred after UV stimulation were completely recovered after three days in the dark. In early experiments of photodriven back-isomerization, instead, we observed a partial recovery of the initial value; here visible illumination time was 2 hours and the intermediate washing step was included (Figure 8C). Thus, we supposed that the washing step between the UV and visible light exposure affected the final value and to overcome this problem, we decided to skip the

intermediate measurement between the two irradiation steps, relying on the fact that UV-induced variations had always been observed.

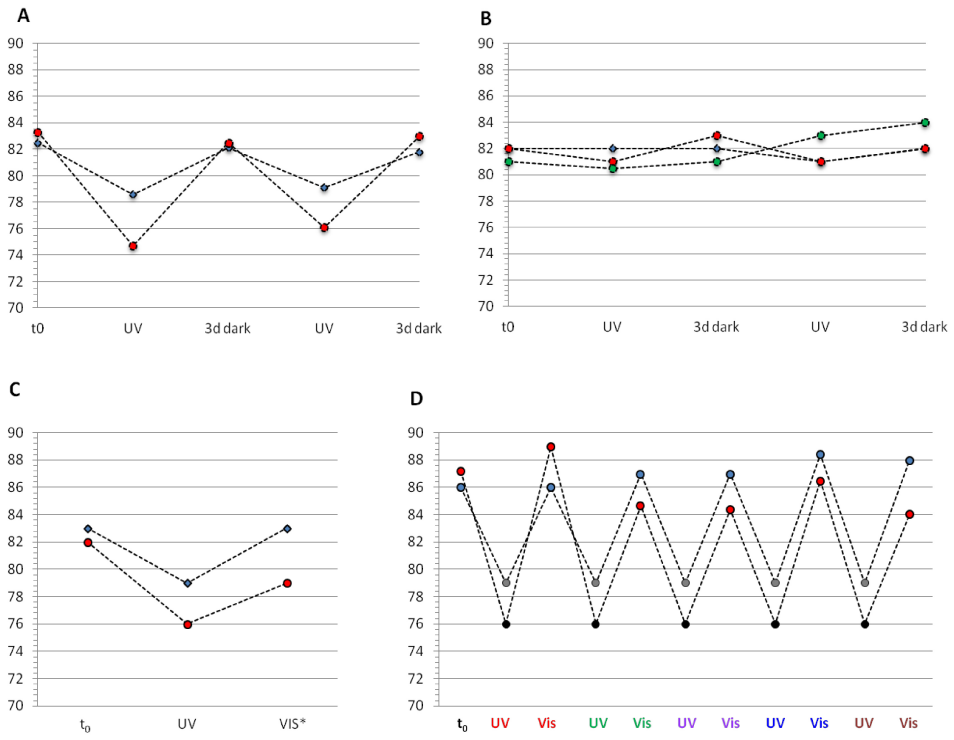


Figure 8. (A) WCA values for sample (red dots) **7A** and (blue rhombic shape) **7B**; (B) WCA variation for a different area of samples **7A** and **7B**, together with negative control sample **6A** (green dots). (C) WCA values for sample (red dots) **7A** and (blue rhombic shape) **7B** after visible illumination (VIS* refers to 2 hours of illumination with visible light and comprehends an intermediate washing step). (D) Five cycles of consecutive UV-Visible irradiations for the samples (red dots) **7A** and (blue dots) **7B**. The WCA values after UV stimulation (black and gray dots) were reported in the graph as reference ("UV" data in 4C). Each cycle is illustrated as "UV-Vis" with different colors.

Therefore, WCA experiments were performed just collecting values before any light treatment and after the final visible illumination step, yielding reproducible data. In this case, the photocontrolled back-isomerization was also achieved with reasonable short time of

illumination, using a common desk lamp as trigger. The reversibility of photocontrolled isomerization was confirmed irradiating the samples with UV and visible light at least for five consecutive cycles (Figure 8D), where the UV value reported as reference has been collected in previous measurements ("UV" in Figure 8C). As already mentioned, WCA data reported were obtained analyzing the same area of the solid substrate. The switching behavior revealed the possibility to tune at will the availability of the peptide sequence with an "On-Off" mechanism typical of switch regulators.

3.4 Conclusions

In this work a photoresponsive surface has been developed by "clicking" azobenzene-based photoswitches bearing GRGDS sequence to silanized glass. The unreactive character of azides and alkynes towards other functional group present in the synthesized azoderivative was exploited in the chemoselective "click"approach, in order to preserve not only the bioactivity but also the chemical and the conformational stability of the introduced biological sequence, once immobilized on glass slides. Preliminary investigations by confocal microscopy with a rodhamine-modified azoderivative grafted on the glass surface (sample **7C**) were performed to validate the "click" chemistry protocol. Moreover, a non homogeneus functionalization of the glass slides was exhibited, confirmed later on by chemical and topographical characterizations of our target platforms (**7A** and **7B**), using Raman spectroscopy and AFM analysis. The photoswitching behavior of our platforms was proved using contact angle technique, monitoring local changes in wettability upon light stimuli. Experimental data confirmed that each platform underwent

photodriven isomerization events, even with some differences mainly related to the chemical structure of the grafted azoderivative.When no peptide was present (sample **7B**), the decrease in WCA reached 4° after the exposure to UV light. This response has been mainly attributed to a change in dipole moment of the N=N double bond. When GRGDS sequence was present (sample **7A**) water contact angle variation reached 8°, thus showing a further contribution to the change in wettability from the peptide itself. Back-isomerization was achieved either by thermal relaxation in time, or by illumination with white light using a common desk lamp. The switching was observed for at least five cycles when illuminated consecutively with UV and visible light, thus revealing the possibility of tuning at will the availability of the peptide sequence with an "On-Off" mechanism typical of switch regulators.

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CHAPTER 4

Controlling Cell Adhesion through Photoswitchable GRGDS-Modified Surfaces

Abstract. In order to investigate light-controlled cell adhesion, photoresponsive surfaces have been developed modifying glass slides with the GRGDS peptide bound to a photoresponsive azobenzene moiety. The presence of azobenzene, according to its isomerization mechanism induced upon light irradiation, allows a different exposure of the peptide that in turn can promote or prevent cell adhesion. Human umbilical vein endothelial cells (HUVECs) were selected to test the possibility of a controlled adhesion behavior upon external light stimuli.

This work has partly been submitted for publication: E. Vaselli; C. Fedele; S. Cavalli; P. A. Netti; "On-Off" RGD Signaling Using Azobenzene Photoswitch-Modified Surfaces.

4.1 Introduction

When engineering new biomaterials, cell-material interface is one of the most important module of a biomedical device to care about.[1] It is there that the crosstalk between the biological entities and the artificial counterpart takes place. Actually, a big part of materials science research is focused on the development of new cell instructive materials (also known as CIMs) as smart engineered matters that can stimulate cells and guide them to express their specific biological features.[2-3] Cell adhesion and intracellular events are strictly related to the intercommunication process that cells can establish with the extracellular matrix (ECM).[4] Most of them are regulated by integrins, adhesion receptors of the cell membrane, which are the real crosstalking players involved during the biological recognition and signaling with the outer environment.[5] Several molecular interactions can mediate cell-material interface, and one of the most exploited approach to improve the biological activity of a biomaterial is the modification trough peptide sequences that can activate integrin modulation mechanisms, thus mimicking as much as possible the real physiological condition.[1] Peptides, in fact, target specific cell-membrane receptors to control precise biological responses, such as the adhesion process. One of the most well-known adhesion-responsible tripeptide is the RGD motif.[6-7] In the past decades, the modification of biomaterials with RGD sequence to regulate cell adhesion boosted the interest of bioengineering researchers on designing and optimizing biocompatible features of synthetic matrixes.[8-11] So far, the majority of the modified surfaces presented biomolecules grafted in a well-organized and engineered manner.

However, their position and availability remain still static in time. Nowadays, the new frontiers are wide open to versatile systems where external stimuli can tune the accessibility of cell ligands at wish. A research area under investigation, indeed, concerns the development of novel substrates that can change their properties in a dynamic way to better mimic the real biological environment.[12-13] The sensitive response can be triggered by altering pH[14] or temperature,[15] as well as exposing to electric or magnetic fields;[16-17] in some cases also irradiation with light can elicit a response.[18] Another important feature of these new generation of biomaterials is the reversibility of their feedback to the external stimuli.[19-20] So far, many smart platforms have been already exploited to direct cell fate with light. In recent works, the availability of biological signals was modulated trough photocleavable moieties, promoting either cell adhesion[21-22] or detachment.[23] In this respect, azobenzene-modified surfaces[24-25] can provide a further improvement introducing the concept of reversibility through the isomerization mechanism. Inspired by recent works, which investigated synthetic matrixes coated with azoderivatives that led to photocontrolled cell[24-26] or bacterial adhesion,[27] we designed a platform based on an "On-Off" photoswitching mechanism useful for the dynamic exhibition of the well-known GRGDS peptide sequence, and exploitable for phototriggered cell adhesion studies with human umbilical vein endothelial cells (HUVECs).

4.2 Experimental section

General Materials and Method. All chemicals and solvents were purchased from Sigma-Aldrich and were used without further treatments. HO-PEG_6-COOH was supplied from Creative PEGWorks.

BulletKit (M200 cell medium and LSGS kit Cascade Biologics), phosphate buffered saline (PBS 1×, pH=7.4), HEPES buffer (1M), Cell Dissociation Buffer enzyme-free (PBS), were all provided by GIBCO® (Life Technologies). Sterile polystyrene dishes, flasks and multiwell culture plates were furnished from Corning Incorporated (Corning, NY, USA).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from LONZA. Cells were cultured in gelatin-coated flasks in an incubator at 37 °C and humidified atmosphere with 5% of CO_2. Cell medium M200 supplemented with LSGS Kit (fetal bovine serum 2% v/v, hydrocortisone 1 µg/ml, human epidermal growth factor 10 ng/ml, basic fibroblast growth factor 3 ng/ml, heparin 10 µg/ml) was used. Cells were detached with trypsin/EDTA (0.25% w/v trypsin/0.02 mM EDTA) and counted with the Neubauer cell counting chamber before seeding on solid substrates.

Cell adhesion experiments. For each cell adhesion experiment 3×10^4 cells were seeded (8 to 12 passages) on each sample and left overnight in incubator at 37 °C and 5% of CO_2. In all experiments selected positive and negative controls were introduced together with the azoderivative-modified glass surfaces bearing the GRGDS sequence. Each sample was present as triplicate in the same experiment. All substrates were sterilized for one hour under the UV lamp of the biological safety cabinet (30 mW/cm^2) one week before the test. Once sterilized, azoderivative samples were stored seven days in the dark before the experiment, in order to achieve the *trans* configuration of azobenzene moieties (**7Atrans** and **7Btrans**, according to the thermal back-isomerization mechanism). The *cis*

configuration (**7Acis** and **7Bcis**), instead, was induced lightening again the samples for one hour under the UV lamp of the biological safety cabinet, immediately before seeding cells. The photoinduced back-isomerization (**7Atrans'**) was achieved leaving the sample under the visible light radiation of a normal desk lamp (Fluence rate 130 mW/cm^2) for one hour, immediately after UV irradiation just before seeding cells.

Cell Imaging. After overnight incubation, all substrates were moved to a new multiwell plate, washed twice with PBS, refreshed with new medium supplemented with HEPES buffer (24 mM) and imaged with an inverted phase contrast microscope at 10× magnification. Images were collected all over the substrate and shown without any modification.

Gelatin coating. Gelatin-coated substrates were used as nonspecific cell adhesion substrates (nonspecific positive control). Before the experiment glass slides were washed, sterilized, then coated with a sterile solution of 1% gelatin in water and left in incubator at 37 °C and 5% of CO_2 for at least one hour. Excess of gelatin was removed immediately before seeding cells.

Bromo silanized glasses (**6**). Bromo silanized glasses were used as negative controls for cell adhesion experiments. Plasma-activated glass slides were covered with a solution of 4% in volume of 3-(Bromopropyl)trimethoxysilane (BPTMS) in toluene and left shaking on an oscillating plate overnight. After reaction, glass slides were washed with toluene, ethanol, acetone and finally dried in vacuum conditions.

Bromine-azide nucleophilic substitution for silanized glasses (**6A**). Bromo silanized glasses (**6**) were put in a glass petri dish containing a saturated solution of sodium azide (50 mM in DMF) and left overnight on an oscillating plate. The day after, all glass slides were washed with DMF, ethanol,acetone and finally dried in vacuum conditions.

APTES silanized glasses (**6c**). A solution of 0.5% in volume of 3-(Aminopropyl)triethoxysilane (APTES) in ethanol, with the addition of 3% in volume of an acid aqueous mixture (10% in volume of acetic acid in water), was poured in a petri dish with glass slides previously activated by plasma treatment. After 5 minutes, slides were accurately rinsed with ethanol, dried in air and cured for two hours in vacuum oven at room temperature.

PEG-modified glasses (**9**). APTES silanized substrates (**6c**) were put in a petri dish and covered with a solution of HO-PEG_6-COOH (8 µmol, 3 mg), DIC (1.5 equiv., 2µl), HOBt (1.5 equiv., 2 mg) and TEA (2 equiv., 2.5µl) in DMF and left shaking on an oscillating plate overnight at room temperature. Then substrates were washed with DMF, ethanol, acetone and dried in vacuum conditions. PEG-modified glasses were used as negative control for cell adhesion experiments.

GRGDS-modified glasses (**8**). GRGDS-modified glasses were used as specific cell adhesion surfaces (specific positive control). APTES silanized glass slides (**6c**) were put in a petri dish with a solution of succinic anhydride (62 µmol, 6 mg) and TEA (1.2 equiv. with respect to amine functions, 12 µl) in DMF, overnight. The day after, glass slides were washed three times with DMF, ethanol and

acetone as last washing step and finally dried in vacuum conditions. Before grafting GRGDS sequence, the carboxylic functions of the glass surface (**6d**) were activated for 30 minutes in water medium in the presence of EDC·HCl (1 equiv. with respect to carboxylic acid, 3 mg), NHS (1 equiv., 2 mg) and DMAP (catalytic amount). After the activation step, GRGDS peptide **4** (synthesis protocol reported in Chapter 2; 1 equiv. with respect to carboxylic functions, 1.6 mg) and TEA (2 equiv., 1 µl) were added and the reaction was left overnight. Washing steps with water, ethanol and acetone were performed before final drying in vacuum oven at room temperature.

Azoderivatives samples. Investigated azoderivative samples refer to **7A** and **7B** (all synthetic protocols are described in Chapter 3). **7Amix** and **7Bmix** refer to glass modified surfaces **7A** and **7B** with the addition of a surrounding monolayer of PEG. Briefly, sample **7A** and **7B** were immersed in a solution of triphenylphosphine (7 µmol, 1.8 mg) in tetrahydrofuran (THF) in the presence of 30% in volume of water overnight at room temperature, in order to reduce azides to free amines. The day after, glasses were washed with THF, water, ethanol and acetone and dried in vacuum oven at room temperature. In the next step, amines were coupled with HO-PEG_6-COOH (7 µmol, 3 mg) in the presence of DIC (1 equiv., 1 µl), HOBt (1 equiv., 1 mg) and TEA (2 equiv., 2 µl) in DMF overnight at room temperature. Finally glass slides were washed with DMF, ethanol, acetone and dried in vacuum conditions.

Sample **8mix**. GRGDS-modified glass (**8**) was modified coupling the available free amines with HO-PEG_6-COOH following the same protocol reported for azoderivative samples.

4.3 Results and discussions

One of the most exploited approach to improve the bioactivity of a biomaterial is the modification through peptide sequences that can activate integrin modulation mechanisms.[1] The RGD sequence, found in the adhesive proteins of the extracellular matrix, can mediate and promote cell adhesion.[7] Several works attested that this universally recognized biological sequence was efficient in promoting also endothelial cell adhesion.[28-31]

Human umbilical vein endothelial cell (HUVECs) line is an important *in vitro* model, which is mainly exploited to study the behavior of endothelial cells during angiogenesis processes.[32] In this work, biological assays with HUVECs were performed to test the response in adhesion through photoswitchable RGD-modified surfaces. The coupling between peptide sequences and light-sensitive units provides the chance to control the bioactivity of these biomolecules using light as external trigger. A photoresponsive ligand was prepared tethering the GRGDS sequence to the azobenzene unit (synthetic details were reported in Chapter 2). The synthesized azoderivative was then grafted on azide-modifed surface (**6A**) to obtain the final sample (**7A** in Chart 1), exploiting the chemoselective "click" approach[33-34] (the reaction protocol was explained in Chapter 3). Substrates modified with the azoderivative but lacking of the peptide sequence were also tested as negative control (**7B** in Chart 1).

As reported in other studies, a good interaction for the cells with a substrate could be achieved only providing the proper degree of functionalization with the adhesive RGD sequence.[35] The surface density of immobilized ligands, indeed, was important for cell

response, either to simply adhere or form the integrin clusters called focal adhesion.[36] In this respect, the platform investigated in this study was modified with the azoderivative providing more than 30% of coverage of the surface.

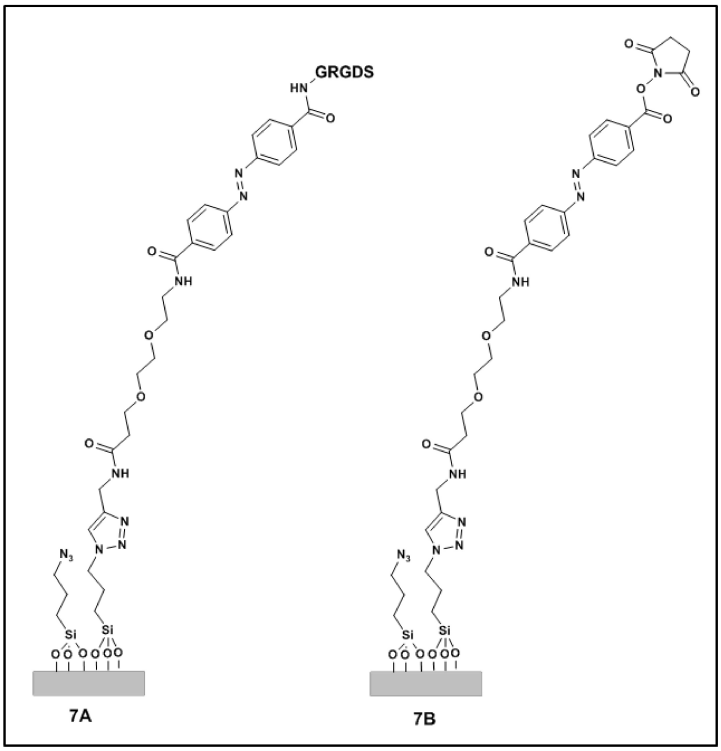

Chart 1. Azoderivative samples investigated for cell adhesion experiments with HUVECs.

The main goal of the designed platform was the dynamic exposure of the peptide, like a sort of "On-Off" mechanism that can regulate cell adhesion at wish, in a photocontrolled and reversible manner. In this respect, the presence of the azobenzene moiety ensured the possibility of light modulation for the GRGDS sequence. In a general case, azobenzenes, indeed, undergo a reversible *trans*-to-*cis* isomerization upon light irradiation, with a concomitant change in geometry that can

help to either present or mask the peptide and consequently modulate cell adhesion as schematically illustrated in Figure 1. Actually, when azobenzene is not stimulated with the UV light, it is present in its *trans* configuration (**7Atrans** in Figure 1). In this situation the peptide sequence is well exposed over the surface and available for cell recognition. When irradiation with a UV source occurs, azobenzene switches from *trans*-to-*cis* conformation (**7Acis** in Figure 1). In this last case, the peptide is no longer exposed as in the previous situation, and this can preclude cell recognition. To validate this assumption several experiments were carried out. In each experiment **7A** and **7B** samples were treated in order to have at least one substrate for each configuration (that is **7Atrans** and **7Acis** as well as **7Btrans** and **7Bcis** obtained as discussed in the experimental section).

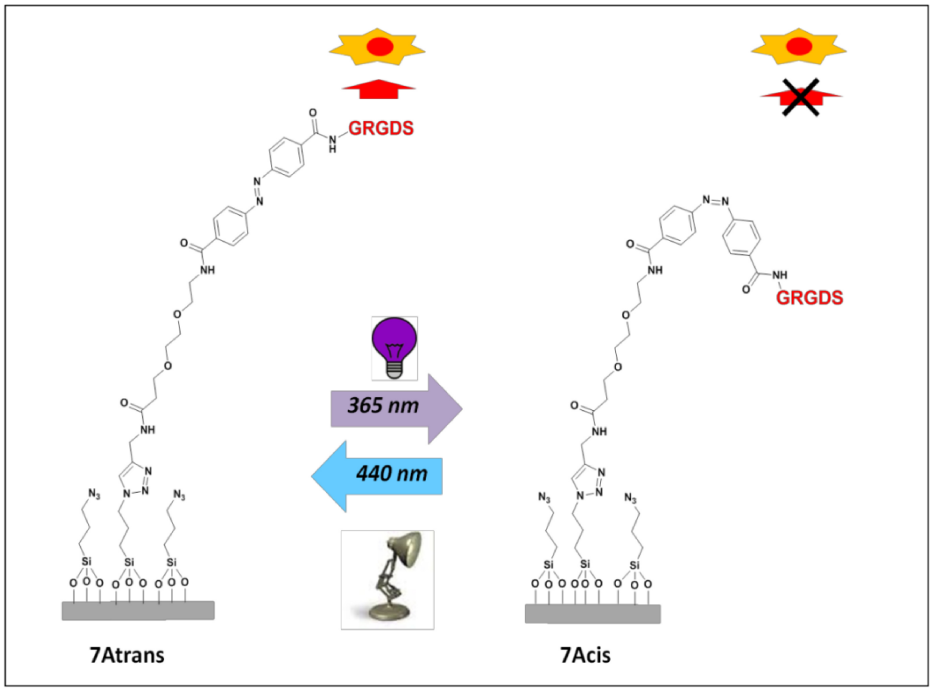

Figure 1. Azobenzene isomerization upon light stimulation.

Performed experiments revealed that sample **7Atrans** exhibited a stronger adhesion with respect to the sample **7Acis**, as also reported in Figure 2a-b. A similar result was observed after repeating the test inverting the two samples, **7Atrans** became **7Acis** and the way around. This inversion was planned in order to confirm the real involvement of RGD exhibition in driving cell adhesion response. Indeed, HUVEC adhesion was promoted for sample **7Atrans**, in which the peptide was well exposed on the surface according to the *trans* configuration of the azoderivative. For **7Acis**, which was stimulated with UV light before cell seeding, the azobenzene underwent the *trans*-to-*cis* isomerization, thus, GRGDS was no longer available for cell recognition, hence HUVEC adhesion decreased.

It is known that generally UV-irradiated azobenzene moieties are not present as pure *cis* isomers, actually a photostationary state is reached, which comprised the *cis* isomer (usually up to 90%) and the *trans* form, according to the thermal relaxation of the former to the latter.[37-38] The presence of this photostationary state could help to explain why few cells were still able to adhere on the surface, even after UV exposure. GRGDS-modified glasses (**8**) were introduced in each test as specific positive control of adhesion. Even if the photosensitive moiety was not present, sample (**8**) was treated as described above for **7Atrans** and **7Acis**. In this case, as expected, a great adhesion was present on both surfaces and no differences were evidenced. As a matter of fact, GRGDS sequence was always available on the surface and did not change its exposure upon applied light irradiation.

For what concerns the negative control **7B**, a very low adhesion was observed in both *trans* (**7Btrans** in Figure 2e) and *cis* (**7Bcis** in Figure

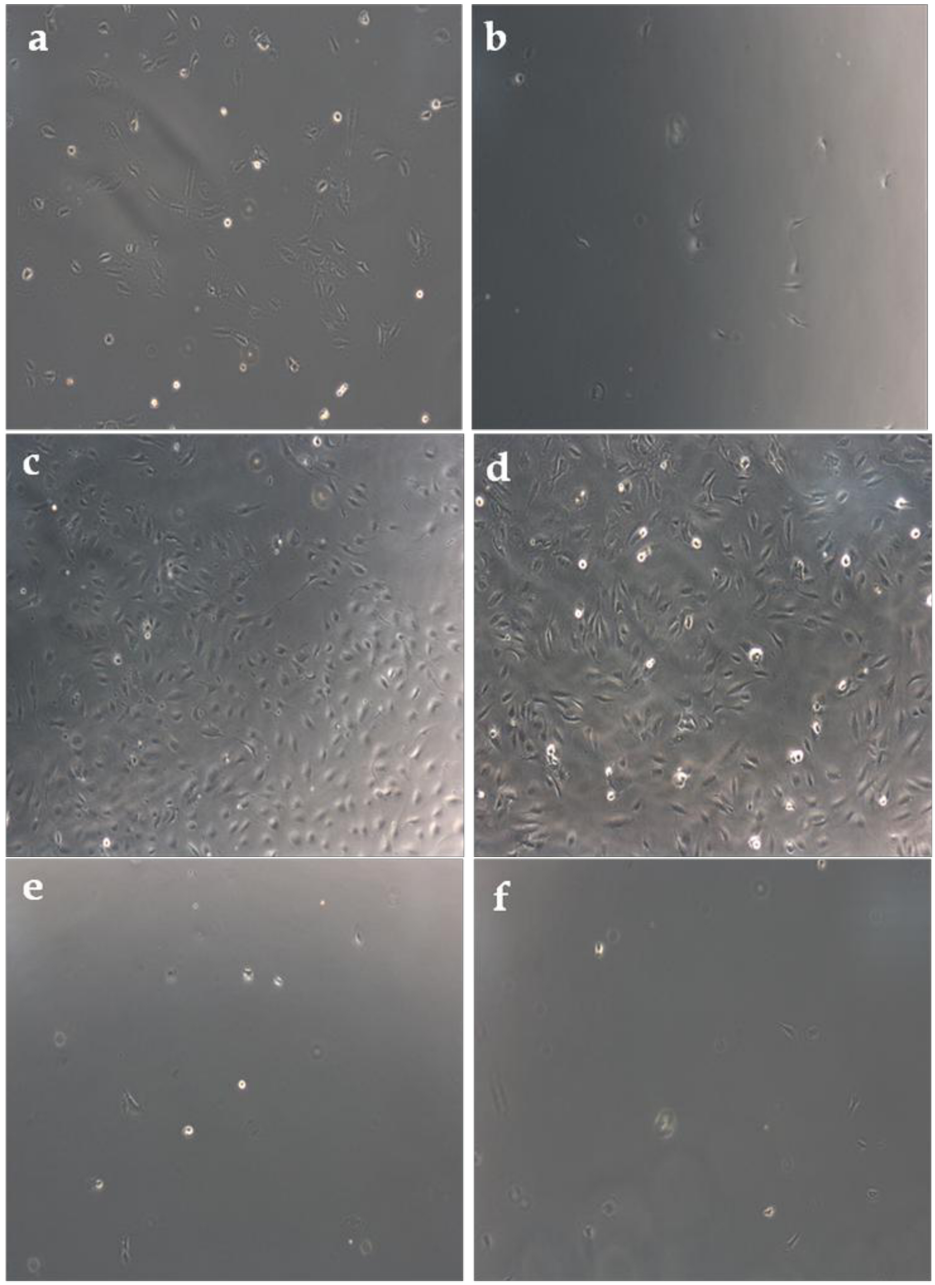

Figure 2. Phase contrast images of HUVECs (10× magnification) of **s**ample (a) **7Atrans** and (b) **7Acis**. Positive controls (c) **8trans** and (d) **8cis**. Negative controls (e) **7Btrans** and (f) **7Bcis**.

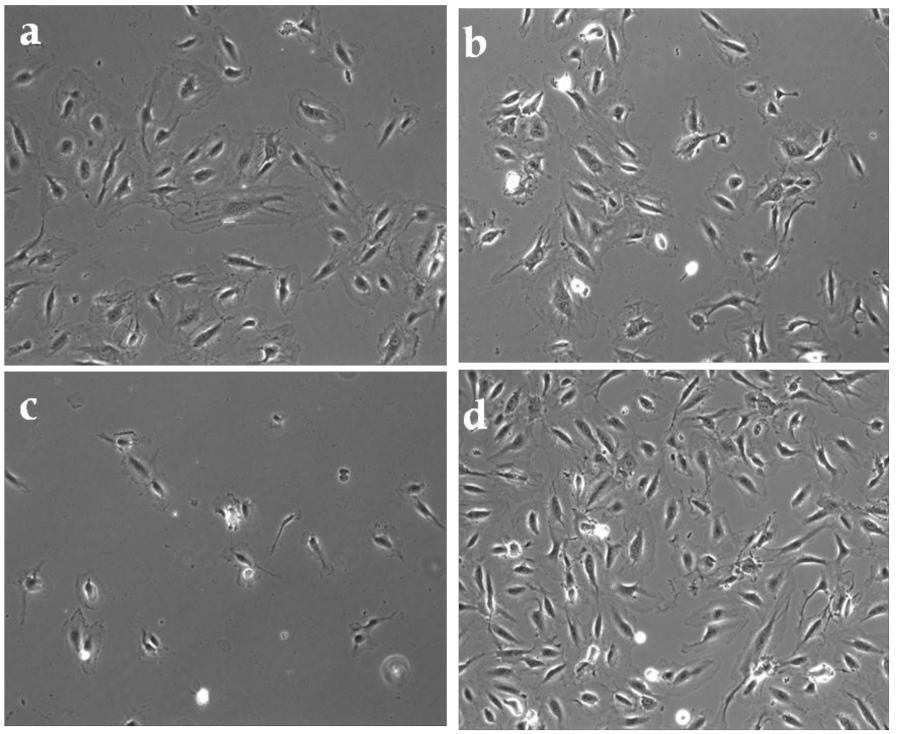

Figure 3. Phase contrast images of HUVECs (10× magnification) of sample (a) **7Atrans** (thermal back-isomerization), (b) **7Atrans'** (photoinduced back-isomerization with visible light immediately after UV illumination), (c) **7Acis** (UV stimulated) and (d) positive control (**8**).

2f) configurations, according to the lack of GRGDS ligand. The capability to regulate GRGDS exposure was also tested using visible light illumination to induce the photoregulated back-isomerization. HUVECs were seeded on sample **7Atrans** (where the thermal back-isomerization was achieved after leaving the sample one week in the dark) and on sample **7Atrans'**, in which the photocontrolled back-isomerization was induced applying UV and then visible light, one straight immediately after the other. The response in adhesion for **7Atrans'** (Figure 3b) was comparable to that observed for **7Atrans** (Figure 3a). Proving that photoinduced back-isomerization obtained using visible light irradiation was as consistent as the thermal back-

isomerization itself. In fact, both samples exhibited higher adhesion with respect to **7Acis** (Figure 3c) in which the UV light was applied immediately before seeding cells.

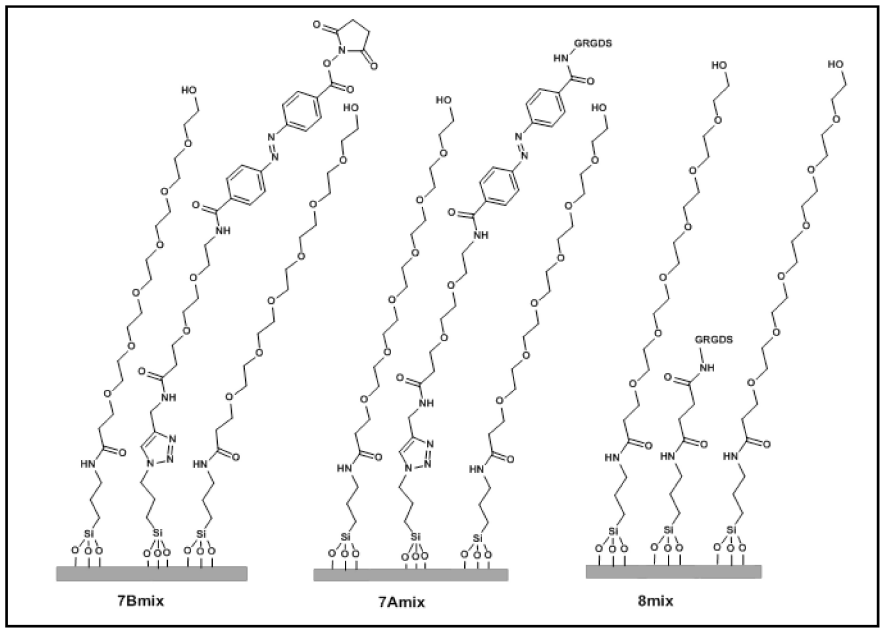

Chart 2. Mixed monolayer with azoderivatives **7Bmix**, **7Amix** and the positive control **8mix**.

Even if promising, these results were not always completely reproducible, probably due to the fact that GRGDS peptide was not completely masked for integrin recognition when the azobenzene was in its *cis* configuration, thus compromising the "Off" signaling. In addition, it is well known that cells after seeded on a substrate can deposit a new layer of adhesive proteins, according to the process called "remodeling".[39] To overcome this problem we included a surround of non adhesive PEG monolayer, that could provide an inert background and could more efficiently hide the peptide once the *cis*

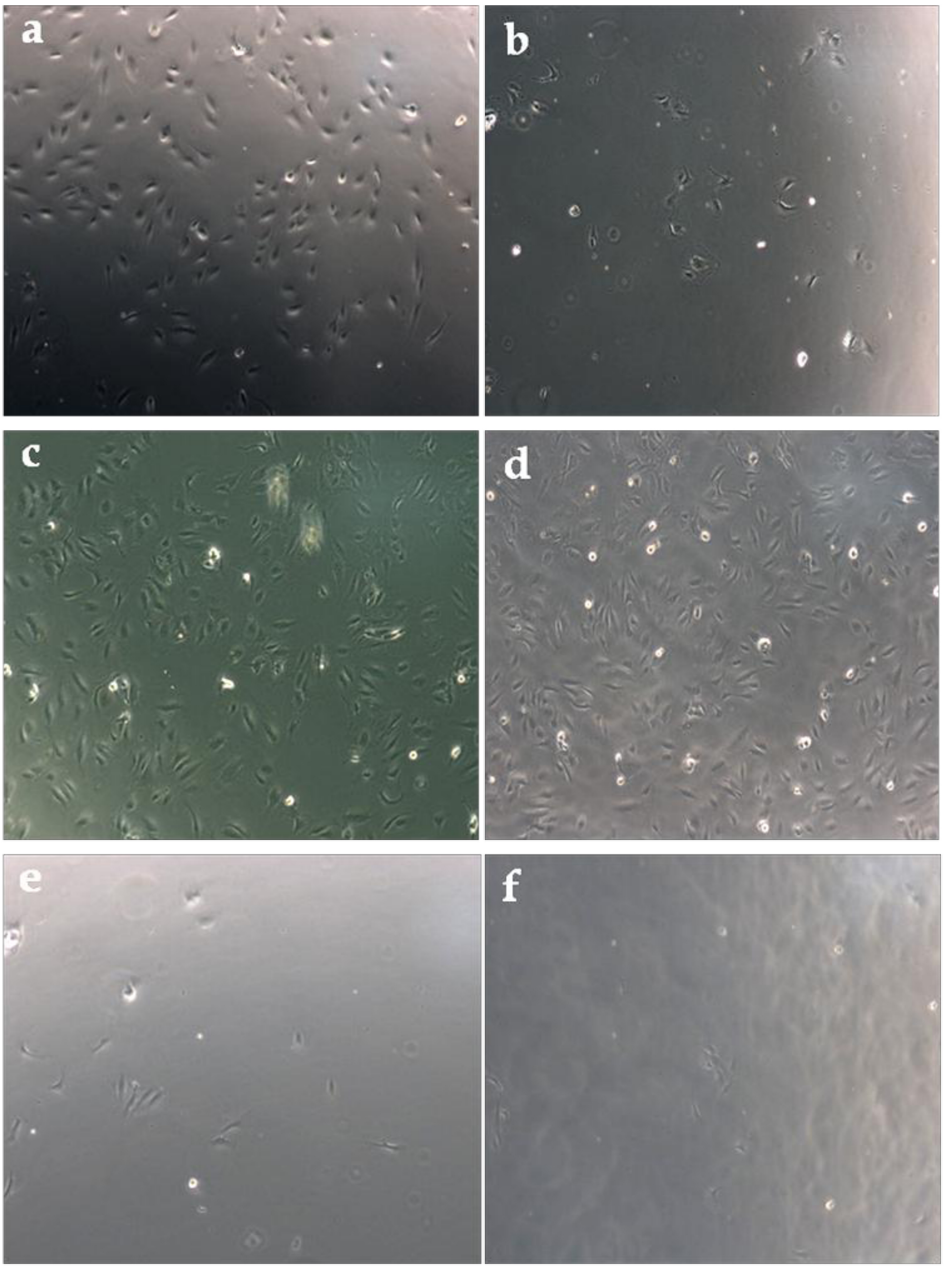

Figure 4. Phase contrast images of HUVECs (10× magnification) for samples (a) **7AmixT** and (b) **7AmixC**. Positive controls (c) **8mix1** (d) **8mix2** (UV stimulated) and negative controls (e) **7BmixT** (f) **7BmixC**.

azoderivative was formed.[25, 39] Previously prepared **7A** and **7B** substrates, were later modified in order to convert the azides functions available on solid substrates into free amines, in the presence of triphenylphosphine and water according to the Staudinger reaction. After this step, amines were conjugated with HO-PEG_6-COOH through the carboxylic acid-amine condensation. After these steps, substrates **7Amix** and **7Bmix** (Chart 2) were used for adhesion test with HUVECs. Collected images for **7AmixT** sample (where the *trans* isomer was achieved after one week in the dark, Figure 4a) showed an enhanced adhesion with respect to **7AmixC** (in which the *cis* isomer was induced with UV irradiation, Figure 4b), where again few adhering cells were observed. GRGDS-modified glasses used as positive control (**8mix** in Chart2) revealed a negligible variation in cell response even with or without applied UV stimulation (**8mix2** and **8mix1** in Figure 4c-d, respectively), while a scarce adhesion was observed for the negative controls **7BmixT** and **7BmixC** (Figure 4e-f).

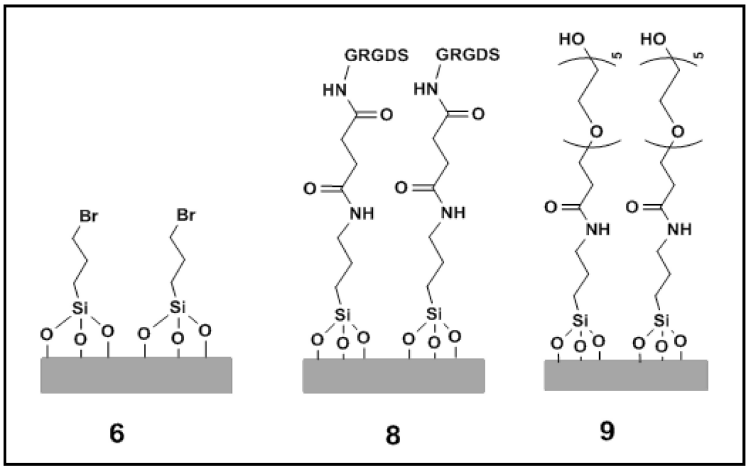

Chart 3. GRGDS-modified glasses (**8**) as positive specific controls; bromo silanized (**6**) and PEG-modified (**9**) glasses as negative controls for cell adhesion experiments.

Positive and negative controls (Chart 3) were always present in each experiment, in order to have a reliable accomplishment of the performed biological test. Gelatin-coated glasses were used as nonspecific positive controls (Figure 5a), while GRGDS-modified glasses (**8**) were used as specific positive controls (Figure 5b). Negative controls were PEG-modified glasses **9** (Figure 5c) and silanized glasses (bromo silanized glasses **6** in Figure 5d).

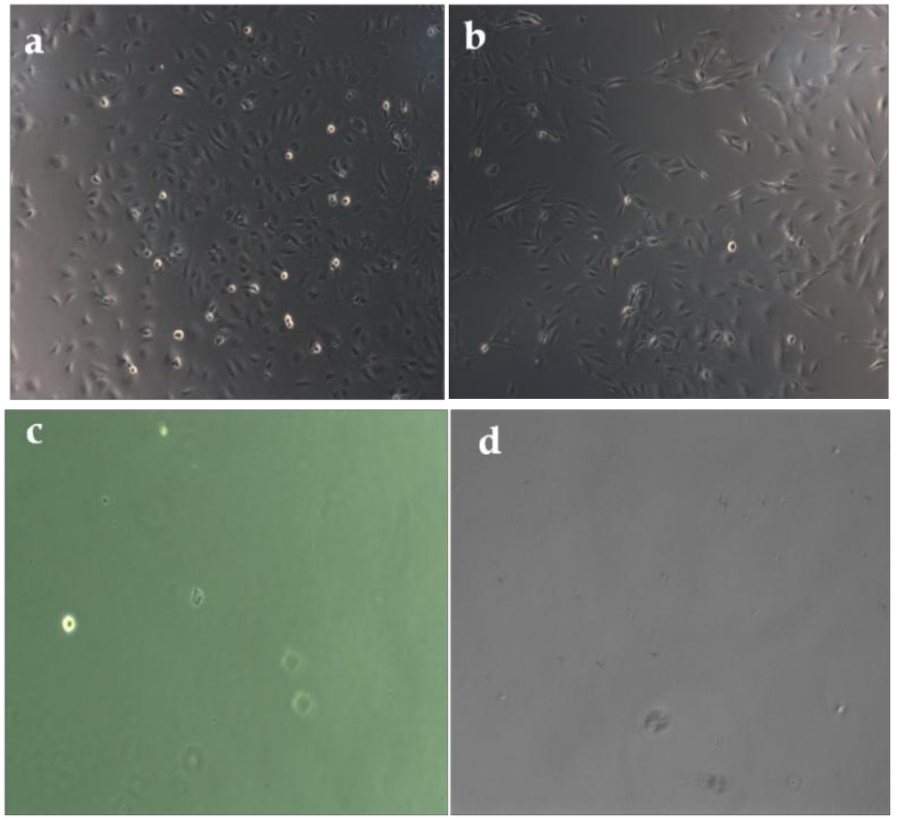

Figure 5. Phase contrast images of HUVECs (10× magnification) for positive controls: (a) gelatin-coated glass and (b) GRGDS-modified glass (**8**). Negative controls: (c) PEG-modified glass (**9**) and (d) bromo silanized glass (**6**).

The use of light to regulate cell adhesion was investigated by several groups. For example, Del Campo's group exploited the use of UV-

photocleavable molecules, such as the *o*-nitrobenzyl group. "Caged" RGD sequences obtained by introducing the *o*-nitrobenzyl group on the carboxylic acid side chain of the aspartic acid residue, were used to trigger photocontrolled HUVEC[22] or fibroblast[40] adhesion to modified silicon slides. The adhesion was promoted only when the *o*-nitrobenzyl group was removed upon UV irradiation. When the same photocleavable group was introduced between the surface and the RGD sequence, it was possible to induce as well HUVEC detachment after short UV exposure time, without affecting cell viability.[23] Cell-repellent surfaces modified with the "caged" peptide sequence could be converted into cell-adhesive substrates upon UV irradiation. The light-sensitive "caging" group linked to the biomolecule worked as a "phototrigger" that was able to switch from an inactive state to an active one. Since the "caging" group was removed with the breaking of a chemical bond, it could not be regenerated through another light stimulus. Therefore, in these systems the reversibility for the "On-Off" mechanism was missed, because it was no more possible to restore the pre-existing condition. Other approaches, in terms of reversibility, seemed to be more promising to control the exposure of such biological cues and thus to regulate cell fate with light. The reversibility provided by the isomerization mechanism of the azobenzene units represented an interesting application, which was exploited also in other similar works.[24-25] Besides azobenzenes, also the host-guest approach, which was based on non-covalent bonding interactions, seemed to be a good method in this research line. Some recent studies combined both strategies. Zhangh and co-workers described a platform consisting of (α-cyclodextrin)-terminated

alkanesilane for the host-guest interaction with an azobenzene-GRGDS moiety, which was able to control HeLa cell adhesion in a reversible manner.[41] Jonkheijm and colleagues also published an interesting result with a similar system that exploited the interaction between (β-cyclodextrin) monolayers and photoresponsive azobenzene-glycoconjugates to immobilize bacterial cells.[27] Inspired by all these works, in which several strategies to achieve a light-responsive platform have been explored, we decided to focus our attention on azobenzene-modified surfaces. The development of a platform in which the azobenzene unit allowed to present in a reversible manner GRGDS ligands with an "On-Off" mechanism was achieved with promising preliminary results. Performed experiments demonstrated the possibility to regulate the isomerization mechanism of the azoderivatives grafted on the glass surfaces through light stimuli, thus the availability of GRGDS ligands and their recognition from integrin binding sites. HUVEC adhesion was promoted with high efficiency only when the azoderivative was present in its *trans* configuration, while when the *cis* isomer was induced upon UV stimulation, adhesion was attenuated. It is important to explain that in this case adhesion was reduced, but not completely excluded. This feature was probably related to the photostationary state achieved after a certain UV exposure time, as already explained above. However, better results were achieved in the presence of a non adhesive PEG layer, which prevented nonspecific adhesion events. Furthermore, from a qualitative point of view, our substrates exhibited a good bioactivity in terms of specific response to GRGDS ligands. In general, HUVEC spreading was similar for surfaces modified with the synthesized azoderivatives

and for substrates directly modified with GRGDS sequence, without the azobenzene unit. Cooper and co-workers demonstrated that for endothelial cells the specific recognition in the presence of GRGDS was really efficient, if compared to the response obtained in the presence of GRGES sequence.[29] The slight difference between the two sequences (an extra methylene group in the side chain of the glutamic acid residue) was enough to induce a different behavior in terms of adhesion and spreading, thus proving the specific response with GRGDS. Our interest in dealing with HUVECs was based on their importance as *in vitro* model to study angiogenesis process. The designed platform could be used as a starting point for future investigations concerning the development of more specific applications. According to these potential perspectives, the use of other ligands could be exploited, which are able to induce other specific functions beside the already explored adhesion response. For what concerns angiogenesis several growth factors can mediate the expression of HUVEC phenotype, enhancing or inhibiting the involved mechanisms.[42]

Current investigations with these photoresponsive molecules required most of the time the treatment with high-energy UV light, which could provoke undesired side effects for *in vivo* applications. Some new research lines proposed to take advantage of near-infrared (NIR) light, which allowed to penetrate tissues with enough intensity and minimal damage.[43] A similar opportunity could be achieved working with a different class of azobenzenes, that allowed to tune the isomerization in the visible region, instead of the UV range.[44] In this way, cells viability could be preserved and a wide range of possible

investigations could lead toward the chance to control cell response with a dynamic light-modulation *in situ* using "biocompatible" wavelengths.

4.4 Conclusions

A photoresponsive cell-instructive platform has been developed using azobenzene-based photoswitches bearing GRGDS sequence, that were covalently "clicked" to silanized glasses. HUVEC cells, known as important *in vitro* model to study angiogenesis processes, were selected to test the photoregulated exposure of the peptide over the designed platform. When the grafted azoderivative was present in its *trans* configuration, GRGDS sequence was available for cell recognition, thus promoting HUVEC adhesion. If the azobenzene was UV stimulated to undergo *trans*-to-*cis* isomerization, GRGDS sequence was not well exposed for cell recognition, thus decreasing HUVEC adhesion response. To have a better reproducibility of such results, it was necessary to include a non adhesive layer of PEG that could improve the masking of GRGDS sequence once the azobenzene was in its *cis* configuration. These experiments proved that azoderivatives grafted on the solid surfaces were able to tune the exposure of GRGDS ligands upon light stimuli, and that the azobenzene unit was the light-mediated trigger for the "On-Off" exposure mechanism. Furthermore, azoderivative-modified substrates exhibited a good bioactivity in terms of HUVEC adhesion and spreading. The response in adhesion for such substrates was similar to that observed for surfaces, which were directly modified with the GRGDS sequence, without the azobenzene unit. However, current investigations with these photoresponsive molecules required high-

energy UV wavelengths which could affect cell viability. These preliminary results could be optimized with some further improvements, concerning the use of azobenzene units with a red-shifted absorption, in order to achieve the photoisomerization events in a range of more "biocompatible" wavelengths. In this way, it could be possible to open up a wide range of investigations with new azoderivatives that could guide to the *in situ* dynamic light-modulation of cell behavior, excluding UV wavelengths.

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CHAPTER 5

5.1 Summary, Conclusions and Future Prospects

When engineering new biomaterials, one of the first requirement is the design of versatile systems where the accessibility of engrafted biomolecules can be regulated at wish. The chance to regulate the presentation of such biological cues stand on some new strategies adopted to achieve controlled responsiveness in a dynamic way, exploiting several external triggers. The modification of peptide or protein ligands with light-sensitive moieties represents a powerful method to achieve the temporal control of their bioactivity using light. In our work GRGDS sequence was coupled with the azobenzene unit to provide the possibility of a photocontrolled exposure of such peptide, after tethering it on a solid substrate. The final aim of this research, indeed, concerns the development of a smart platform in which the capability to tune cell adhesion response can be achieved exploiting light as trigger.

The developed azoderivative was designed taking into account several important features. The presence of GRGDS sequence was required to promote specific cell recognition and adhesion on our platform and, in addition, the azoderivative was provided with a short oligo(ethylene glycol) as spacer between the azobenzene unit and the surface to enable the free space required for the isomerization azobenzene to occur, once it was confined to the solid substrate. In Chapter 2, the synthesis of such azoderivative and the relative characterization by means of UV-Visible spectroscopy confirmed that the typical photoswitching behavior of the azobenzene unit was preserved, even

after the introduction of the peptide. In fact, this azoderivative underwent both *trans*-to-*cis* and reverse *cis*-to-*trans* isomerization processes. The former was stimulated under the UV light, while the latter could be achieved either as a thermal process (according with the higher stability of the *trans* isomer) or upon visible light irradiation using a normal desk lamp. Furthermore, the reversibility of the photoinduced isomerization was proved repeating UV-Vis cycles for two times and the related switching was confirmed by the cyclic variation in absorption intensity for the two involved isomers. After an exhaustive characterization of the switching behavior in solution the synthesized azoderivative was grafted on a silanized glass surface, exploiting the "click" chemistry strategy (Chapter 3). A comprehensive chemical and topographical characterization of such substrate with Raman spectroscopy and atomic force microscopy, was followed by the investigation of the photoresponsive behavior on solid substrate through the water contact angle technique, recording local changes in wettability related to the isomerization of the azobenzene domain upon light stimuli. The back-isomerization was achieved either by thermal relaxation in time, or by illumination with the white light using the desk lamp. The "On-Off" switching behavior was observed for at least five cycles when illuminated consecutively with UV and visible light sources, thus revealing the possibility of tuning at will the availability of the peptide sequence with an "On-Off" mechanism typical of switch regulators. Finally, the developed photoresponsive platform was tested for biological applications, through adhesion assays with human umbilical vein endothelial cells (HUVECs), in order to evaluate the possibility of a controlled adhesion upon external light stimuli

(Chapter 4). Performed experiments revealed that when the grafted azoderivative was present in its *trans* configuration, GRGDS sequence was effectively available for cell recognition, thus promoting cell adhesion. While, once the *trans*-to-*cis* isomerization was induced upon UV stimulation, GRGDS sequence was not well exposed for cell recognition, so decreasing cell adhesion response. However, to have a more evident and reproducible difference in cell adhesion for our platform, it was necessary to include a non adhesive layer of PEG that could help to mask GRGDS sequence once the azobenzene was in its *cis* configuration. These experiments proved that the azoderivatives grafted on glass surfaces were able to photoregulate the "On-Off" mechanism involved with the exposure of GRGDS ligand upon light stimuli, hence activating the specific recognition with integrin receptors. Furthermore, the azoderivative-modified substrates exhibited a good bioactivity for HUVECs adhesion and spreading.

An interesting research line connected with this project concerns the introduction of a different biological cue. The RGD motif is a sort of universal code that can interact with all type of cells, our experimental results also validate the well-established knowledge that this sequence is able to promote cell adhesion response. Anyway, besides RGD other peptide sequences could be exploited to induce or enhance specific cell functions different from adhesion. Each cell line, indeed, has its own peculiarity and its characteristic phenotype, which can be regulated through specific cell recognition motifs. In this respect, angiogenesis processes for endothelial cell (like HUVECs) are mediated through a wide series of growth factors, such as the vascular endothelial growth factor (VEGF), which is able to induce the basic mechanisms for the

creation of new blood vessels in a tissue. QK peptide, one of the new developed biological sequence, is able to mimic VEGF activity enhancing angiogenesis response and inducing endothelial cell migration and proliferation.[1-2] Interesting would be to link the QK sequence to the azobenzene unit in order to control HUVEC response in developing new blood vessels, exploiting the photoswitching mechanism proposed in this work.

Although our results are quite promising, the discussed "dynamic CIMs" still need optimizations to improve the photocontrolled bioactivity for *in vivo* cellular environment. It would be well desired to induce the conformational changes without using UV light, because this range of high-energy wavelengths is more harmful and can induce cell apoptosis, if compared to those red-shifted. An important improvement in this respect, could be achieved using a diverse azobenzene, with a specific substituent pattern on the two phenyl rings that provoke a red-shifted absorption spectrum, thus not requiring UV light to undergo photoisomerization events. As a given example, Woolley and co-workers[3] investigated the photoswitching behavior of an amidoazobenzene derivative, which was modified in the *ortho*-positions of the two phenyl rings with methoxy groups (Chart 1). These azoderivatives underwent a significant red-shifted absorption, especially when all the *ortho*-positions were functionalized. The *trans*-to-*cis* isomerization, indeed, could be stimulated using green light (530-560 nm), while the back-isomerization could be induced using blue light (460 nm) and the related *cis* isomer had a lifetime of two days. Exploring the wide possibilities of chemical modifications of the azobenzene unit, open up a wide range of investigations with new

azoderivatives that can lead to the light-modulation of cell behavior *in situ*, excluding UV wavelengths and preventing undesired side effects that can compromise cell viability. Such platforms could afford biocompatible "dynamic CIMs" for providing light-triggered "On-Off" signaling control for *in vivo* tissue engineering applications.

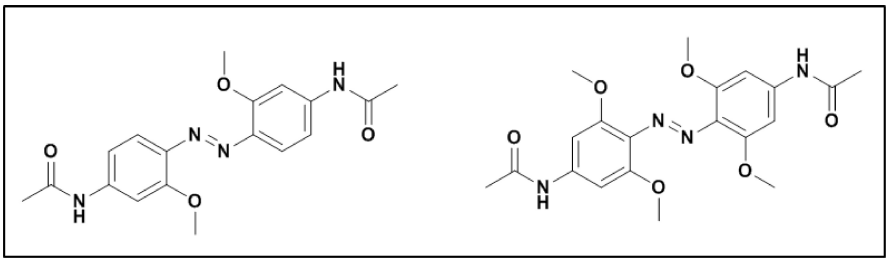

Chart 1. Structures of azobenzene with methoxy groups in the *ortho*-positions of the two phenyl rings.[3]

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