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Peptides in motion

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Chapter 1

Photo-responsive peptides

The incorporation of optical switches in bio-macromolecules allows the non-invasive regulation of structure and function. In this chapter, the structure and the light responsiveness of photoswitchable molecules that can be inserted in peptides and protein are described, together with different strategies to incorporate the photoswitchable moiety and control function. Various examples of photoswitchable peptides and proteins are highlighted. Particular attention is given to specific peptide secondary structures, including photosensitive β -hairpins and zinc-fingers.

1.1 Introduction

The control of biological processes has always been a challenge for chemists and biochemists. The ability to interfere with a specific biological function helps scientists to achieve better understanding of the process itself. Especially if the process is associated with pathology, it might be possible to gain new insights in the development of diseases and eventually to discover new drugs and arrive at novel treatments.¹ Furthermore, this approach might lead to the development of new applications for the studied biological system. For example, the control of the aggregation properties of amyloid peptides may lead to the discovery of new biomaterials in which one can control the stiffness with light.

The control of biosystems can be achieved using different external stimuli,¹ e.g. temperature,² pH³ and redox potential.⁴ Lately, light has been preferred over other stimuli due to numerous advantages.⁵ Light is not invasive and bioorthogonal: it does not interfere with almost any biological function. The use of light usually doesn't lead to contamination, except when there is photodegradation. Finally, it can be delivered with high spatial and temporal precision to achieve control of function.

One of the key strategies for externally controlling biosystems is the use of molecular photoswitches, which can be interconverted between two forms by light irradiation.

In this chapter we describe strategies/methodologies how photoswitches can be inserted in peptides and which processes can be controlled in this way.

1.2 Methodological approaches to inserting photoswitches into peptides

In the past twenty years, many examples of photoswitchable peptides have been reported. The insertion of photoswitches into peptides has been proven to be a powerful tool to control the secondary structure, which is strictly associated with the function of the peptide. The photoswitches that have been used for this purpose are azobenzenes,⁶ stilbenes⁷ and diarylethenes (Figure 1).⁸

Azobenzenes are the most widely used switches in peptide photocontrol, because of the relatively easy synthesis and extensive data on the photochemical properties.⁹ In fact, the relationship between the nature of the substituents at the aromatic rings and the photochemical behavior has been well studied.¹⁰ Importantly, by using *push-pull* azobenzene¹¹ or by introducing four substituents into the ortho positions with respect to the diazo group,¹² it is possible to obtain azobenzenes that are switchable

with visible light,¹³ taking advantage of reduced toxicity and higher penetration depth as compared to UV light.

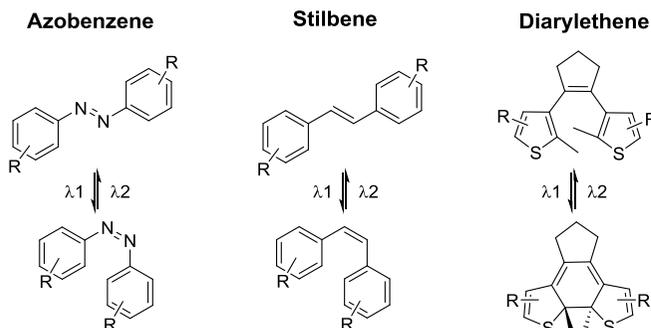


Figure 1: Photoswitches used in light-responsive peptides.

The methodologies to insert photoswitches into peptides are based on the following four approaches.

- 1- Insertion of a photoswitch into the peptide backbone (Figure 2). The photoswitch is part of the peptide backbone, therefore the photoisomerisation influences the secondary structure. Photoswitches with both an amine and a carboxylic acid functionality have been developed to be applied in solid phase peptide synthesis.¹⁴ This approach has been used, for example, to substitute the amino acids that are forming the turn in the β -sheet (Figure 2a).¹⁵ In this case the photoswitch has the ability to bring apart the two β -strands. The photoswitch can be inserted, as well, in the β -strand¹⁶ (Figure 2b) or in cyclic peptides (Figure 2c).¹⁷
- 2- Modification of an amino acid side chain with a photoswitch (Figure 3). This approach consists of introducing the photoswitch into the side chain of an amino acid.¹⁸ This methodology was used so far only for azobenzene switches.¹⁸ There are different ways to obtain a peptide containing an azobenzene in a side chain of an amino acid. It is possible to use non-natural amino acids that already contain an azobenzene, e.g. phenylazo-phenylalanine^{18a-e} or its derivatives (Figure 3, molecules **1-4**), some of which can further react with other functionalities in the peptide (see 4th approach). These amino acids have been genetically encoded, using the nonsense suppression method of Schultz,¹⁹ therefore it is possible to use them also in biosynthesized proteins. The alternative is to synthesize the entire peptide and connect the azobenzene using reactive groups in natural amino acids, e.g. glutamic acid,²⁰ or introducing an azidohomoalanine²¹ (Figure 3, molecule **5**) in

a sequence, which involves a copper-catalyzed cycloaddition (CuCCA) reaction or Staudinger-Bertozzi ligation with the desired photoswitch.

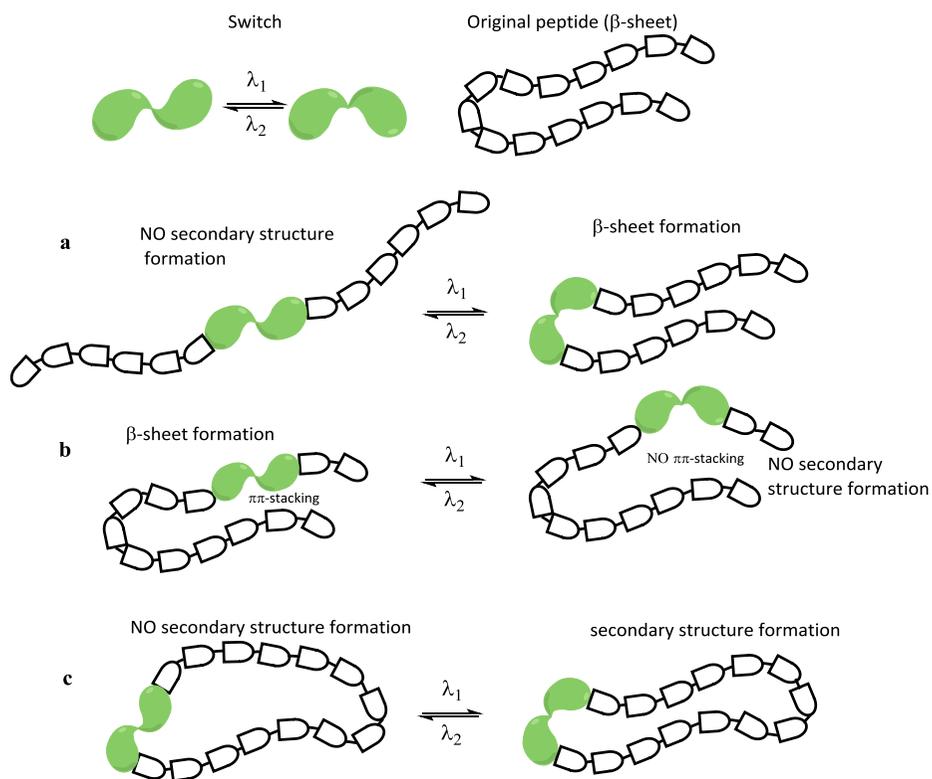


Figure 2: Insertion of a photoswitch into the backbone of a peptide, exemplified by the photocontrol of beta-sheet secondary structure. a) The photoswitch substitutes the amino acids that are forming the turn in the β -sheet. b) The photoswitch substitutes the β -strand. c) The photoswitch is inserted in cyclic peptides.

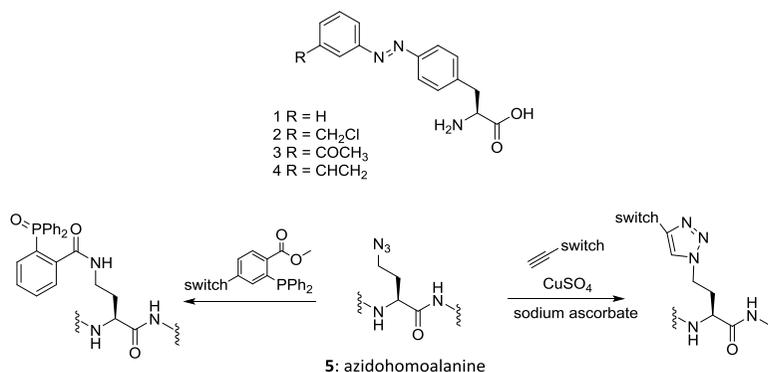


Figure 3: Insertion of a photoswitch into the side chain of an amino acid.

3- Cross-linking two cysteines with a photoswitch (Figure 4). Woolley and coworkers^{9,22} used azobenzene as photoswitchable unit to connect two side chains of different amino acids in a peptide (Figure 4). This methodology permits external control of the secondary structure. Practically, the irradiation changes the distance between the two cross-linked cysteines and, therefore, it affects the secondary structure. This approach has been successfully used for α -helical peptides. The choice of the position of the two cross-linked cysteines in the sequence of the peptide is important: depending of the length of the switch in the different isomers, it is possible to decide the positions of the two cysteines in the peptide sequence that favors the unfolded or folded structure preferentially in presence of one or the other isomers of the photoswitch. For example, when the azobenzene 3,3'-bis(sulfonato)-4,4'-bis(chloroacetamide)azobenzene (BSBCA)²³ (Figure 4) is used to cross-link two Cys with a distance $i,i+7$ in a peptide, the *trans*-form of the peptide will have an unfolded structure and the *cis*-isomer will have an α -helical structure. If BSBCA is used to cross-link two Cys with a distance $i,i+11$ in a peptide, the *trans*-form of the peptide will have an α -helical structure and the *cis*-isomer will not. Recently, an analysis of the effects of the photoswitchable cross-linker position on the degree of photocontrol was conducted. The more efficient control is achieved when BSBCA is cross-linked to a region with high propensity to adopt an α -helix.²⁴ Interestingly, in this case, the BSBCA promotes the formation of α -helix conformation in segments that are located in N-to-C direction.²⁴

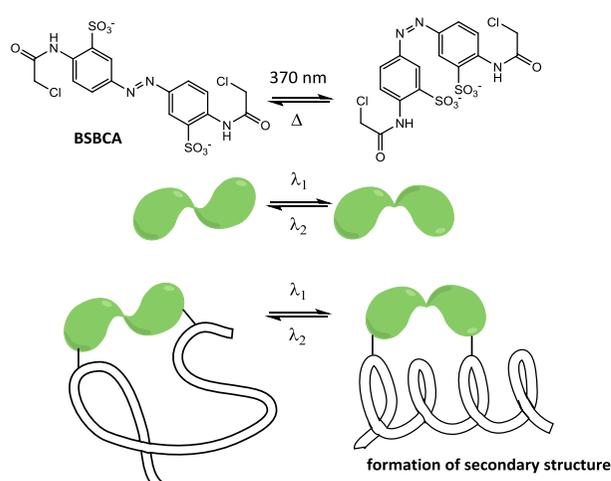


Figure 4: Cross-linking two cysteines, present in the sequence of the peptide, with a photoswitch.

- 4- Photoswitchable click amino acids (PSCaa) (Figure 5).²⁵ This methodology was introduced by Hoppmann²⁵ et al. and it is, practically, a combination of approaches 2 and 3: the idea is to insert in the sequence of the peptide an amino acid that has an azobenzene unit in the side chain. This azobenzene is modified to have a click functionality, like an alkene that can react with a thiol group of a Cys.²⁵ This yields a bridged photoswitchable linker. The photoswitch reacts in the *cis*-form, regardless if the Cys is in *i,i+4* or *i,i+7* distance. The click reaction occurs preferentially with Cys in *i,i+4* distance if a solvent that promotes α -helix formation, is used.²⁵

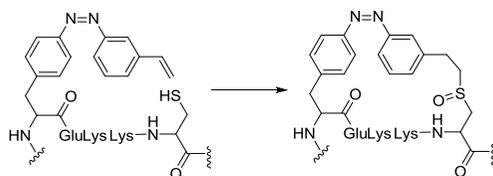


Figure 5: Incorporation of a photoswitchable amino acid into a peptide sequence and cross-linking of the photoswitch to another side chain of one amino acid in the sequence of the peptide, as proposed by Hoppmann et al.²⁵

1.3 New properties of photoswitchable peptides and their applications

1.3.1 Optimization of the design of photoswitchable peptides

Recently, considerable effort has been made to resolve problems connected to design and application of photoswitchable peptides.

1.3.1.1 Towards the use of visible light for the photoswitching

The first issue was related to the use of UV light to promote photoisomerization between the different isomers of the photoswitch. It has been shown that UV light is carcinogenic,²⁶ cause mutation²⁷ and can induce cell apoptosis.²⁸ Therefore, the groups of Temps^{12a} and Woolley^{12b} explored the use of recently-introduced photosensitive units or modified azobenzenes to achieve photocontrol using visible light. A benzylidene-pyrroline chromophore **6**²⁹ (Figure 6a) has been used as a cross-linker (*i, i+11*) (Figure 6a) to control the α -helix content of the model peptide SS11 by light (Figure 6a). The *E* isomer permits the formation of the α -helix, whereas the *Z* isomer is too short to permit the formation of the secondary structure. The wavelengths used to achieve the photostationary states are 400 nm and 446 nm, leading to 7/93 *E/Z* and 78/22 *E/Z* isomer ratio's, respectively.²⁹ One of the problems, related to this system

and others, is that there is overlap of the two absorption bands related to the two isomers, therefore complete photoswitching is not possible.

A different switch, compound **8** (Figure 6b), shows a better separation of the absorption bands of the two isomers. A new bridged azobenzene **7**³⁰ (Figure 6b), derived from compound **8**,³¹ was synthesized and used for cross-linking peptide FK-11, which is a peptide adopting an α -helical structure (Figure 6b).³⁰

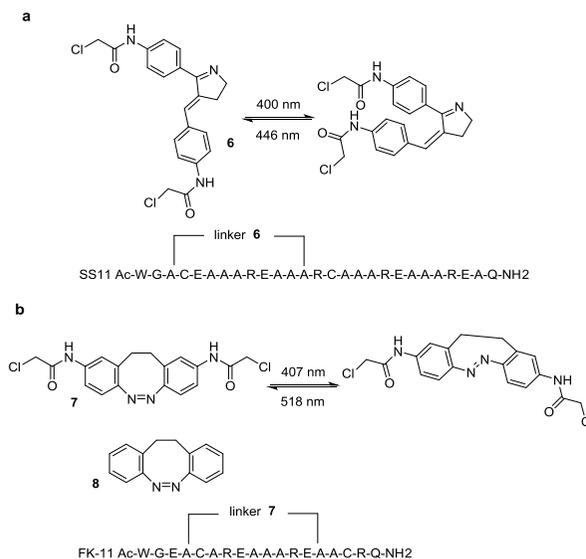


Figure 6: Cross-linker photoswitches sensitive to visible light. a) The benzylidene-pyrroline chromophore **6** used to cross-link peptide SS11.²⁹ b) The bridged azobenzene **7**, derived from compound **8**, used to cross-link peptide FK-11.³⁰

The *trans*-isomer of the C₂-bridged azobenzene **7** is less stable than the *cis*-isomer, and the $n\text{-}\pi^*$ absorption bands of the two isomers are separated by 85 nm. As a consequence, this photoswitch permits complete photoswitching using violet and green light to achieve control of the α -helix secondary structure.³⁰

Other azobenzenes, introduced by the group of Aprahamian,³² Hecht³³ and Woolley,³⁴ exhibit visible light switching properties. These molecules are, respectively, BF₂-coordinated azobenzenes, tetra-*ortho*-chloro-substituted and tetra-*ortho*-methoxy-substituted azobenzenes. Woolley and coworkers cross-linked the peptide FK11 and analogues with tetra-*ortho*-substituted azobenzenes and they achieved control over the peptide secondary structure using red light.³⁴ The tetra-*ortho*-chloro-substituted switch **9** was cross-linked to the synthetic peptide [D](Pro)₉-FK-11 modified with fluorescein (Figure 7).³⁴ Fluorescein has an emission spectrum that overlaps to a different extent with the $n\text{-}\pi^*$ bands of *cis* and *trans* isomers, therefore the two isomers can quench differently the fluorescence of fluorescein by FRET

(fluorescence resonance energy transfer). The photoswitchable peptide was microinjected into zebrafish embryos and by observing changes in fluorescence intensity it was possible to follow the photoswitching process induced by red light irradiation inside the living animal body.³⁴

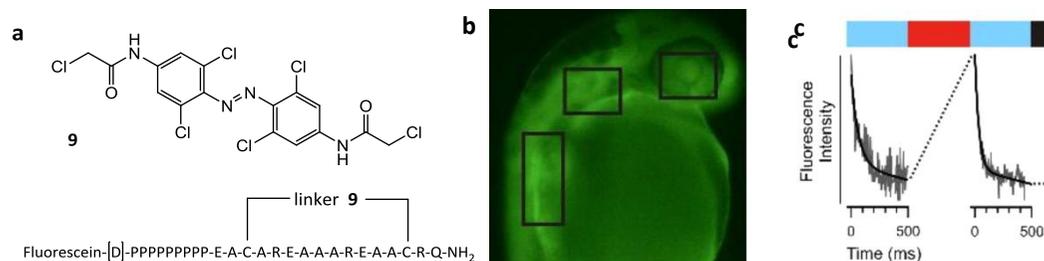


Figure 7: Photoswitchable peptide microinjected into zebrafish embryos. a) The tetra-*ortho*-chloro-substituted switch **9** cross-linked to the synthetic peptide [D](Pro)₉-FK-11 modified with fluorescein.³⁴ b) Fluorescence image of a zebrafish embryo (early pharyngula stage/~30 hpf) containing the peptide cross-linked with **9**. c) Measurements of fluorescence intensity at the zones indicated by black boxes in (b) as a function of time during blue light irradiation. A change in fluorescence emission was seen only if the peptide was first switched with red light (indicated by red bars). The figure was adapted with permission from 34. Copyright (2013) American Chemical Society.

1.3.1.2 Instability of azobenzenes under reducing conditions

Another important aspect of azobenzene photoswitches is their potential instability under reducing conditions: in particular, in cellular environment, azobenzenes may undergo reduction by intracellular glutathione or, if they are used in peptide ligation method, thiols may provoke the reduction.^{5a,35} It has been demonstrated that tetra-*ortho*-fluoro-substituted azobenzenes are less prone to give the reduced product in presence of glutathione, with respect to the tetra-*ortho*-methoxy-substituted one.³⁴ The mechanism of the reduction, presented initially by Kosower³⁶ is a two-step process, involving a nucleophilic attack of the thiol(ate) on the diazo bond to form an adduct hydrazo compound. The tetra-*ortho*-methoxy-substituted azobenzene should be much more stable due to the electron-donating properties of methoxy groups. However, four methoxy groups enhance the Bronsted basicity of the diazo moiety so that it gets protonated under physiological conditions. This makes it more electrophilic and prone to undergo the reaction with glutathione more easily for tetra-*ortho*-methoxy-substituted azobenzenes than for the tetra-*ortho*-fluoro-substituted one.

When azobenzenes are used as building blocks for solid phase peptide synthesis and chemical ligation methods are used, 4,4'-AMPB ((aminomethylphenylazo)benzoic acid) derivatives are less stable than the 3,4'-AMPB compounds under reducing condition of native chemical ligation (Figure 8).³⁷

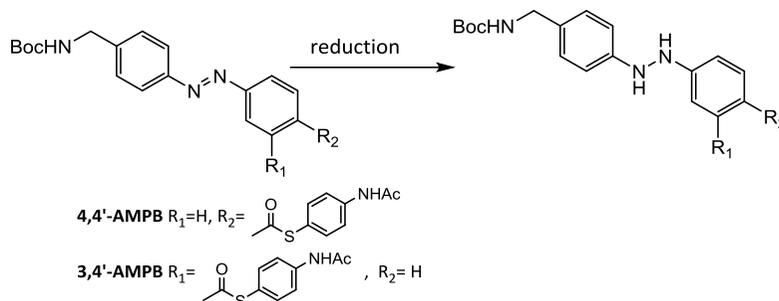


Figure 8: Reduction of the photoswitches 4,4'-AMPB and 3,4'-AMPB that occurs during the chemical ligation in peptide synthesis.³⁷

1.3.1.3 Improvements in the design of the photoswitchable peptides

Peptides are involved in many biological processes e.g. in protein-protein interaction, or in peptide-protein interaction, or as antibiotics. One of the issues of the developing of photoswitchable peptide ligands is the design of the photoactive system. Nowadays, the structure-guided approach is the most common: the switch is inserted or substitutes the part of the peptide that is involved in the function. Lately, the groups of Ito,³⁸ Derda³⁹ and Heinis⁴⁰ have proposed a different, directed evolution strategy to optimize the structure of peptide-switch conjugate for efficient photocontrol of biological process. Towards this goal, the photoswitchable molecules are introduced into the encoded peptides *via* phage display facilitating the selection of photoresponsive peptides. These approaches might improve the choice and selection of photoswitchable peptide ligand. The phage display⁴¹ is a technique that permits to identify peptides that can interact with a target (DNA, protein, peptide). A phage is a virus that contains the gene of the peptides that are displayed on the virus surface. These displaying phages can then be screened against other proteins, peptides or DNA sequences, in order to detect interaction. Derda³⁹ described a method that relies on cyclization of phage-encoded peptides with the sequence ACX₇CG and functionalization with azobenzenes. It was found that photoswitchable peptides bind streptavidin in the *trans*-form and 4.5-fold less effective in the *cis*-form. Heinis⁴⁰ also used the phage display to select BSBBA-cross-linked peptides CX₇C that bind to streptavidin in *cis*-form and are inactive in the *trans*-form (Figure 9). This selection was achieved by adding, in the process of phage display, a stage of depletion of *trans*-binders, followed by irradiation of the remaining phages and incubation with streptavidin beads (Figure 9). This approach yields a library of synthetic photoswitchable binders, which are inactive in the more thermodynamically stable state and can be activated by irradiation.

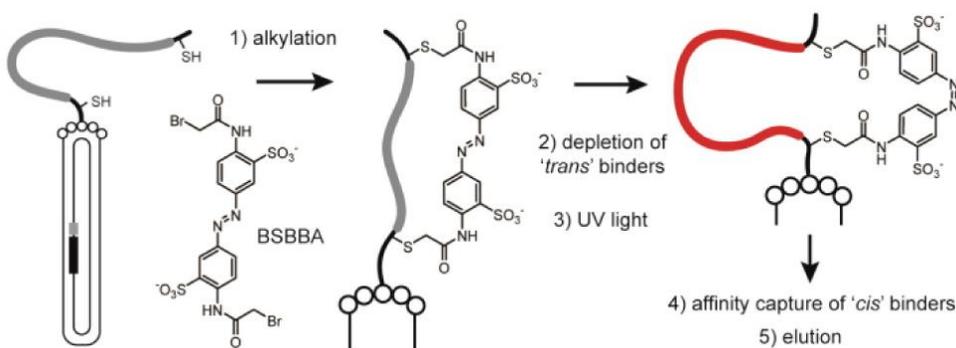


Figure 9: Phage selection of light-activated peptide ligands.⁴⁰ The phage-encoded peptides are modified with the photoswitch. The photoswitchable peptides that bind streptavidin in the *trans*-form are depleted and the remaining peptides are irradiated to give the *cis*-form. The photoswitchable peptides that bind streptavidin in *cis*-form and not in the *trans*-form are, therefore, identified. The figure was adapted with permission from ref 40. Copyright (2014) American Chemical Society.

1.3.2 Applications of photoswitchable peptides

The modification of peptides with photoswitchable molecules can be used to externally-control different biological functions. Here we report recent examples of photoswitchable peptides used for various applications, including cell signaling, control of secondary structure, self-assembly and cell-surfaces adhesion.

1.3.2.1 Photoswitchable peptides for DNA binding and protein-protein interaction

The cross-linking approach (Figure 4) was extended to the use of diarylethene switches by the group of Inouye.⁴² α -Helical peptides that bind to DNA have been modified with succinimidyl-diarylethene using ornithine residues as a cross-linking site. The positions of the ornithine determine whether the system works in a on/off or off/on manner.

Following the same approach to interfere with the α -helix structure of peptides, Nevola et al.⁴³ synthesized an azobenzene(BSBBA)-cross-linked peptide (Figure 10a,b) which binds AP2. This is one of the protein complexes that are involved in clathrin-mediated endocytosis (CME) in living cells. This process is related to many different functions, including the uptake of nutrients, cell signaling and surface expression of proteins.⁴⁴ Two photosensitive peptides have been identified to have opposite effects: TL-1 is an active inhibitor in the *trans*-form and TL-2 in the *cis*-form. This effect has also been shown in living cells, by following the uptake of the transferrin receptor (Figure 10d). The transferrin receptor is one of the cell-surface transmembrane proteins that undergo CME. When it is uptaken to the cell, cells internalize fluorescent transferrin

(Figure 10d, top); if the cells are pre-incubated with TL-1, the CME is inhibited and the fluorescence transferrin remains on the membrane (Figure 10d, bottom). This mechanism is affected in the opposite way using TL-2.

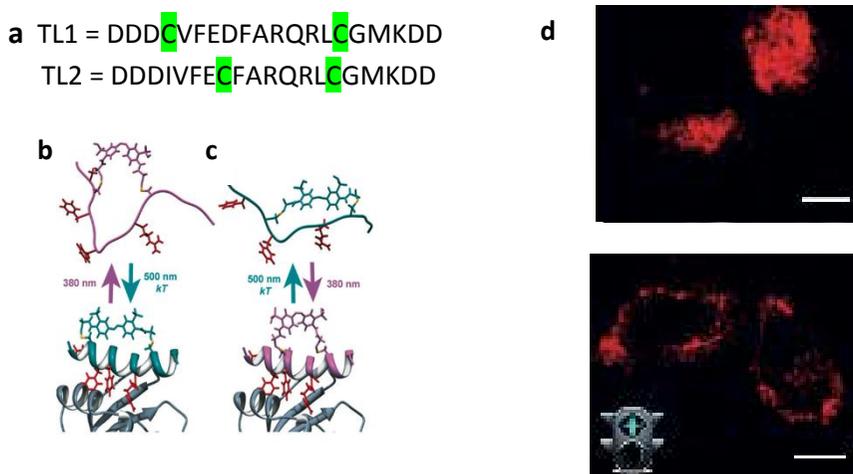


Figure 10: a) Sequences of peptides TL1 and TL2. b) Representation of the AP2 in grey and photoswitchable peptide inhibitors displaying key interacting residues in red, when the azobenzene crosslinker is attached to at positions i and $i+11$. c) Representation of the AP2 in gray and photoswitchable peptide inhibitors displaying key interacting residues in red, when the azobenzene crosslinker is cross-linked at positions i and $i+7$. d) Confocal microscopy (scale bars, 10 μ m), transferrin is diffused in untreated HeLa cells (top); transferrin is accumulated at the membrane of cells incubated with TL-1 in the dark (bottom). Adapted with permission from ref. 43. Copyright 2013 Wiley-VCH Verlag GmbH & Co. KGaA.

1.3.2.2 Photoswitchable peptides for nanotechnology

An example of an azobenzene-modified peptide that can be used in nanotechnology, was presented by Tamaoki (Figure 11).⁴⁵ This peptide was employed to regulate the motility of a kinesin-microtubule.⁴⁵ Kinesin^{46,47} is a motor protein⁴⁸ that moves along microtubules and can transport nanocargo's.^{49,50} The process is possible because kinesin transforms chemical bond energy (ATP) into mechanical work. Recently, surfaces coated with kinesin motors are used to visualize the movement of microtubules.^{51,52,53} These systems can be used to synthesize chips with controlled composition and morphology at the nano-level.⁵⁴ After many attempts^{51,55,56,57} at achieving efficient external control over this system on the surface, Kumar *et al.* introduced a method based on a photoswitchable peptide.⁴⁵ Peptide **1** (Figure 11),⁵⁸ derived from the C-terminus of kinesin, is a known inhibitor of kinesin. This peptide, together with other similar ones (Figure 11), has been synthesized with an azobenzene

in the N-terminus, and was added to the kinesin-functionalized chip.⁴⁵ It was found that the motility of microtubules can be effected by the presence of azo-peptides. The *trans* isomer acts as inhibitor, therefore the kinesin motor doesn't work and the microtubule doesn't move. On the other hand, the *cis* isomer is not active as an inhibitor and the microtubules can move on the surface (Figure 11). Among the three synthesized peptides, the best photocontrol can be achieved with peptide **2**, which is the one with the inverse sequence of the known inhibitor.

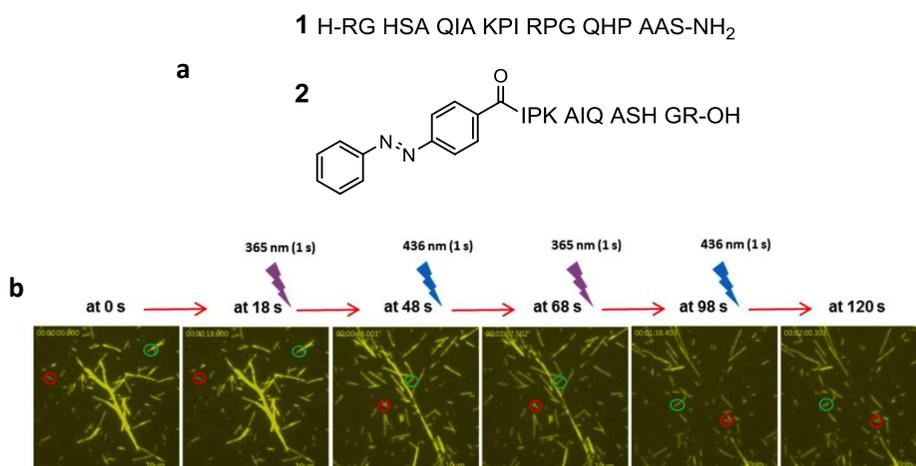


Figure 11: a) Sequences of peptides **1** and **2**. b) Fluorescence images of the gliding motility of microtubules on a kinesin-glass surface in presence of ATP and peptide **2**. Images were taken following the isomerization cycles of the azobenzene moiety. The figure was adapted with permission from ref 45. Copyright (2014) American Chemical Society.

With this peptide at the concentration of 2 mM in *trans* state, the velocity of microtubule is zero and in the *cis* form the velocity is up to 0.4 $\mu\text{m/s}$ (micro), similar at the velocity observed under the inhibitor-free conditions. This is the first example of complete on-off switching of the motility of microtubules.

1.3.2.3 Photoswitchable peptides as antibiotics

Some peptides are active as antibacterial agents; therefore, the introduction of a photoswitch could enable the photocontrol of their activity. The diarylethene switch has been inserted in the backbone of the peptidic antibiotic Gramicidin S to control its structure and activity with light (Figure 12).⁵⁹ Gramicidin can permeabilize the membranes of Gram-positive bacteria and kill them. A modified diarylethene switch that can be used for solid phase peptide synthesis was used to replace three different pairs of amino acids in the sequence. Molecular dynamic (MD) simulation revealed that the analogues with the “open” form of the switch resemble much more the conformation of the original gramicidin and that the “closed” form affects the structure required for biological activity. The determination of minimal inhibitory

concentration (MIC) confirmed the hypothesis and, within a certain range of concentration, the effect on the bacterial growth could be visualized (Figure 12).

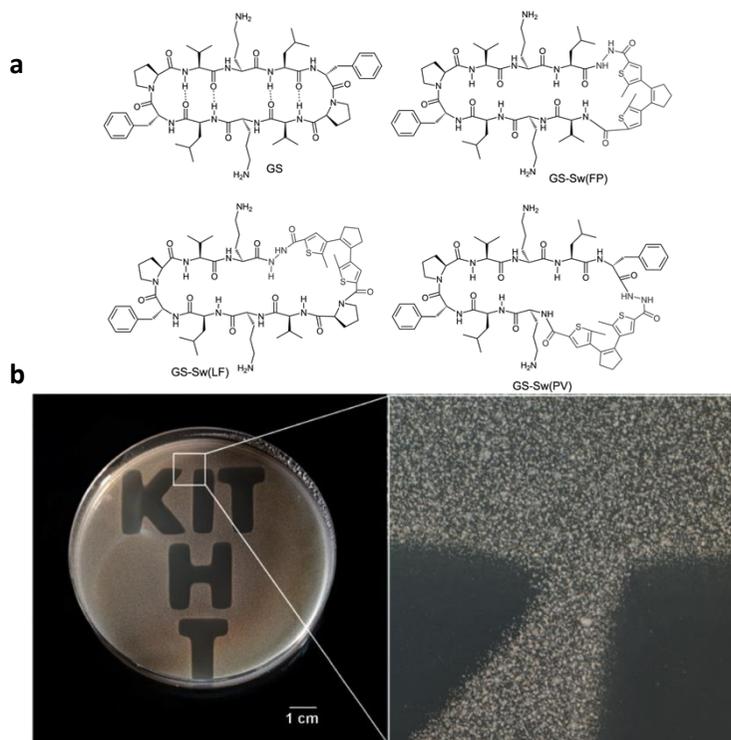


Figure 12: a) Gramicidin S (GS) and its photoresponsive analogues. b) Differences in the antimicrobial activities, against *S. xylosus*, of the “closed” form, which is the background and the “open” form, which is within the letters. Adapted with permission from ref. 59. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA.

1.3.2.4 Photoswitchable peptides for supramolecular assembly

Peptides that can self-assemble are well known and the process of the formation of their supramolecular architectures is studied intensively.⁶⁰ These peptides can be used to synthesize new materials, to obtain delivery systems or simply to study the formation of secondary and tertiary structures in proteins and enzymes. Therefore attempts towards the external control of the self-assembled peptides have been made.⁶⁰ Here we report illustrative examples of external control of the higher-order supramolecular structures formed by peptides.

Diphenylalanine is the shortest peptide that promotes self-assembly, due to the possibility of π - π stacking. Two research groups have been inspired by this dipeptide and the prospects of controlling its assembly with an azobenzene switch. Using a non-covalent approach, the group of Li mixed diphenylalanine with 4-[(4-ethoxy)phenylazo]benzenesulfonic acid (Figure 13a)⁶¹ and they found that the *trans*

form of the switch promotes interconnected, elongated nanoplates and helical-nanobelts (Figure 13b), while the *cis* form induces the formation of vesicle-like structures (Figure 13c).⁶¹ The photoswitching and the connected structural changes are reversible over many cycles.

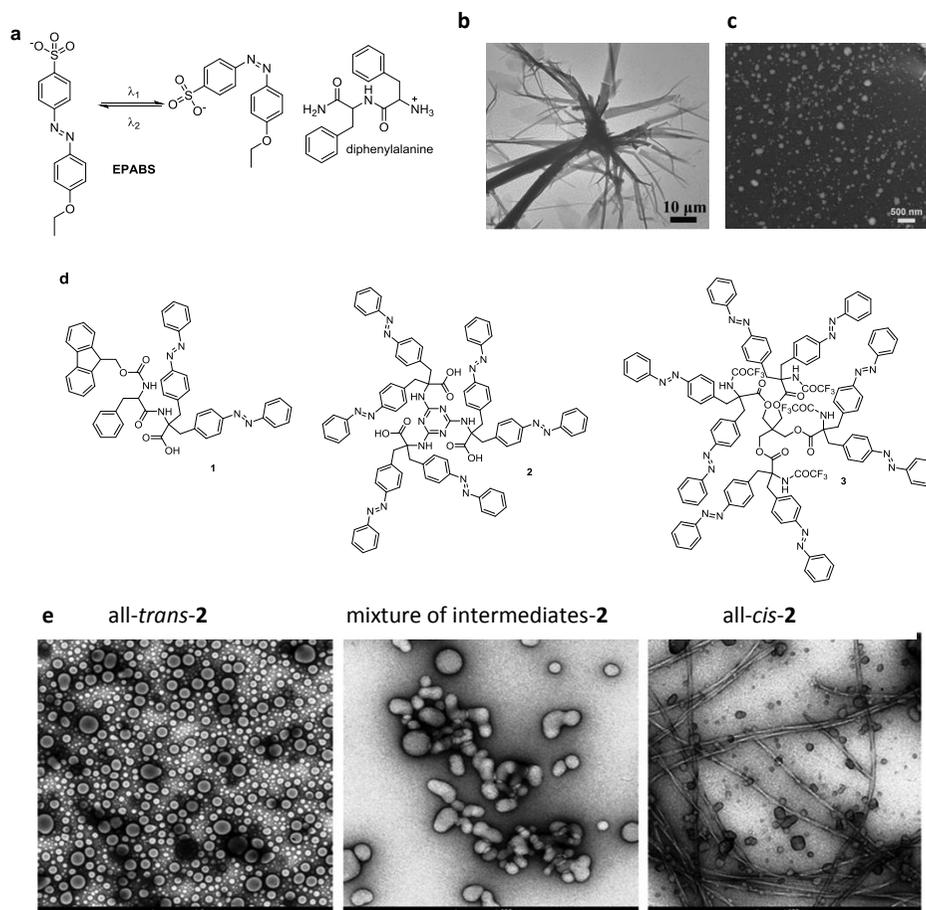


Figure 13: a) Structure of EPABS. b) TEM images of co-assembly of diphenylalanine peptide and EPABS under visible light. c) TEM images of co-assembly of diphenylalanine peptide and EPABS under UV light. d) Chemical structures of **1**, **2**, **3**. e) TEM images of **2** showing the morphological transition of this system in DMSO/H₂O suspension. Adapted with permission from ref. 61 and ref. 63. Copyright 2014 and 2013 Wiley-VCH Verlag GmbH & Co. KGaA.

The group of Moretto synthesized a surrogate of the Fmoc-protected diphenylalanine, which contains a bis[*p*-(phenylazo)benzyl]glycine (*pazoDbg*)⁶² instead of phenylalanine unit (compound **1**, Figure 13d).⁶³ The *trans* isomer forms vesicles that are disrupted upon irradiation. Then they increased the complexity of the system by coupling three *pazoDbg* to 1,3,5-trichlorotriazine and four *pazoDbg* to pentaerythritol (compounds **2**, **3**, Figure 13d).⁶³ In both cases, the *trans* isomer forms spherical

structures. Upon increasing the content of *cis* form by UV irradiation, the vesicles deform to finally form fibers (Figure 13e).⁶³

Using MD simulations, the group of Pellarin and Caflisch⁶⁴ has identified the smallest peptide that can give fibers similar to the one of amyloid aggregate.

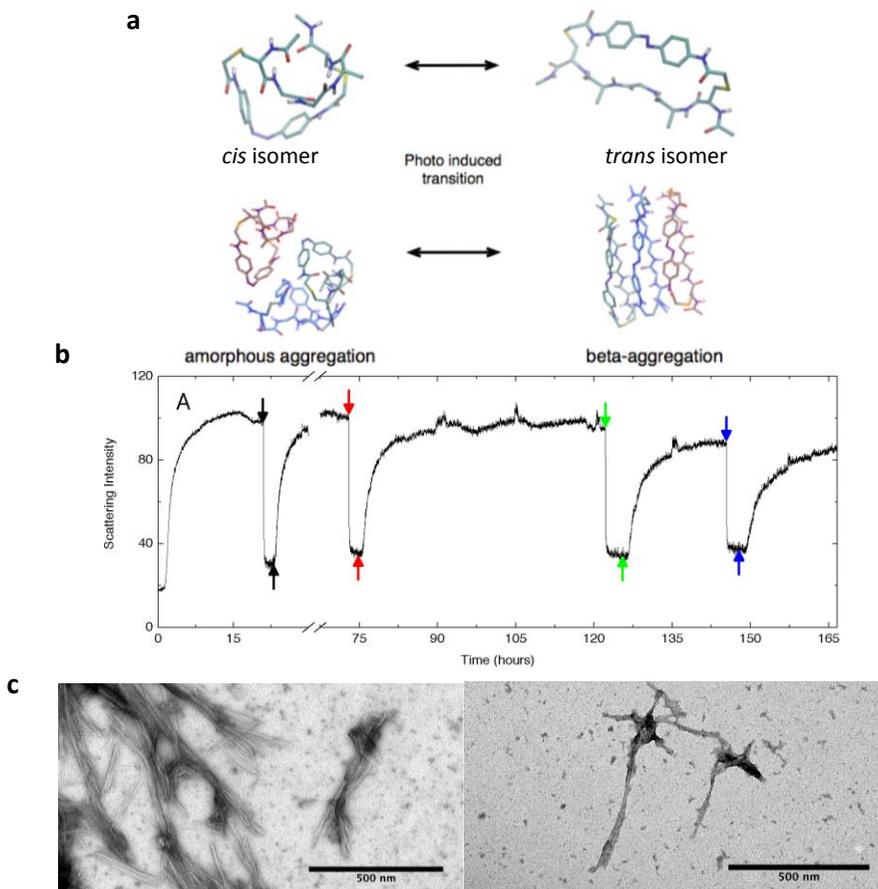


Figure 14: The photoswitching behavior of cross-linked peptide. a) Structure of the peptide and photocontrolled aggregation. b) A full scattering trajectory showing the initial lag-phase, aggregation, and stable scattering maximum by 15 h and then four sequential photocontrolled disassociation and aggregation events. Downward arrows represent UV irradiation and upward arrows represent white light illumination. c) TEM image of amyloid-like fibrils formed by the peptide in the *trans* conformation (left) and *cis* conformation (right). Adapted with permission from 64. Copyright (2012) American Chemical Society.

In fact, the simulation revealed that the photoswitch permits cross- β contacts in the peptide when the cross-linker was in the *trans* state, whereas in the *cis* state they self-assemble into amorphous aggregates (Figure 14a). A chosen peptide, Ac-Cys-His-Gly-Gln-Cys-Lys-NH₂, cross-linked at the two Cys residues with azobenzene, has been synthesized to verify the findings of the simulation. Indeed, the *trans* isomer induced

the formation of fibers, while UV irradiation leads to disaggregation (Figure 14b,c). The reversibility of the process was also proved, either with light scattering experiment (Figure 14b), or by TEM microscopy (Figure 14c).

The group of Zhang⁶⁵ attempted to use a peptide to interconvert between spherical and fiber architectures. A symmetrical Gemini peptide, linked by an azobenzene unit (Figure 15), was synthesized. This peptide adopts α -helical structure and forms different aggregates depending on the pH and the isomeric state of the azobenzene moiety. In acid media, the switching process promotes a change in architecture between nanofibers and nanospheres, whereas in basic media the switching interconverts between nanospheres and vesicles.

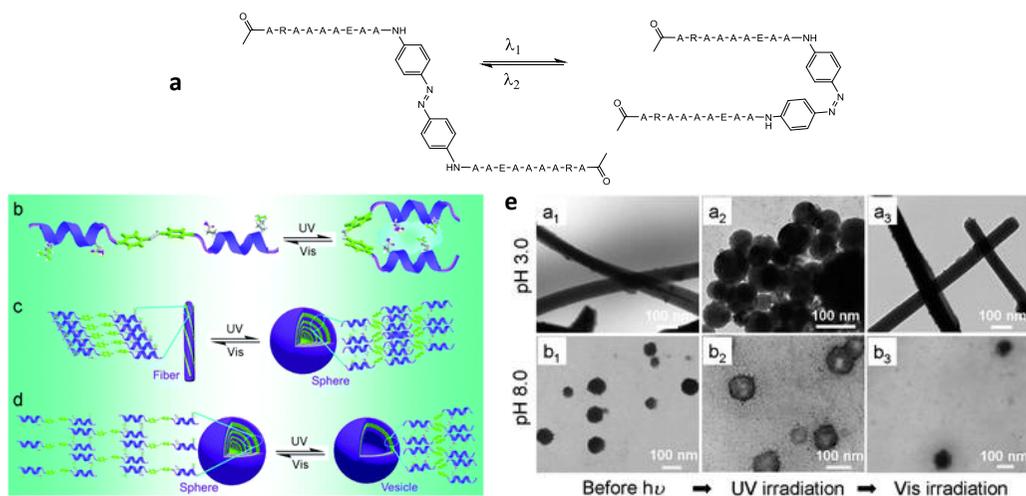


Figure 15: a) Structure of symmetrical Gemini peptide linked by an azobenzene unit. b) Model of the isomerization of the Gemini α -helical peptide. c) Light-switched self-assembly behavior of the Gemini α -helical peptide in acid medium. d) Light-switched self-assembly behavior of the Gemini α -helical peptide in basic medium. e) TEM images of the system under acid or basic media and different stages of irradiation. Reproduced with permission from ref. 65. Copyright 2013, The Royal Society of Chemistry.

1.4 Photoswitchable β -hairpins

β -Hairpins are small secondary structure elements, which play a key role in many biological processes: they are involved in protein-protein interactions,⁶⁶ in amyloidogenic diseases⁶⁷ and they are believed to be the nucleation sites for protein folding.⁶⁸ They are formed by two strands connected by a turn region (Figure 16a). In these strands, hydrophobic or highly branched amino acids are present, which interact

with each other forming a hydrophobic cluster that stabilizes the structure of the β -hairpin (Figure 16a).

The control of the secondary structure of the β -hairpin is possible by inserting a photoswitchable unit in the backbone of the β -hairpin.⁷⁰⁻⁷⁸ The photoswitches can be inserted in the turn region (Figure 16b)^{70-73,75-78} or they can substitute the amino acids responsible to form the hydrophobic cluster (Figure 16c).⁷⁴ In both cases (Figure 16 b,c), only one isomer of the photoswitch favors the formation of the secondary structure of the β -hairpin, therefore by irradiation the β -hairpin can be disassembled.

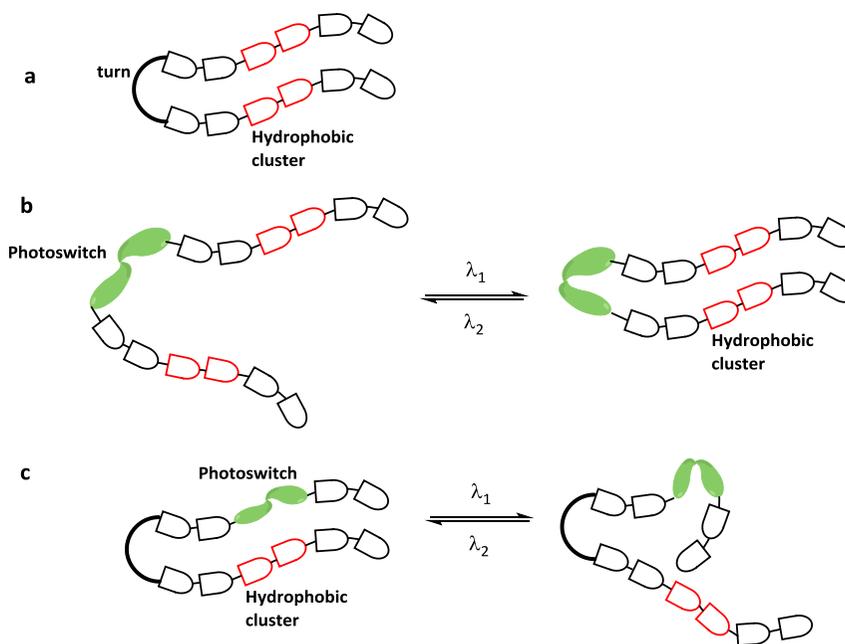


Figure 16: Secondary structure of β -hairpins and the strategies used to modulate the secondary structure by the insertion of photoswitches. a) Secondary structure of non-modified β -hairpin. b) Insertion of photoswitch into the turn region of the β -hairpin. c) Insertion of photoswitch into the hydrophobic cluster of the β -hairpin.

Photoswitchable β -hairpins, reported in literature, are mainly modified with an azobenzene linker (Figure 17b, Table 1, Entry 3-11).⁷³⁻⁷⁸ There is only one report, to the best of our knowledge, that describes β -hairpins modified with a stilbene linker (Table 1, Entry 1,2).⁷⁰ In this specific case, the stilbene occupies the turn region. The *trans*-isomer of the cyclic peptide (Table 1, Entry 2) adopts a disordered structure but the *cis*-form behaves like a β -hairpin.⁷⁰ However, with each photoisomer, the acyclic peptide (Table 1, Entry 1) forms a β -hairpin.⁷⁰

Azobenzene remains the most used switch for photocontrolling β -hairpin. In general, when the azobenzene occupies the turn-region, the *cis*-isomer can induce β -

hairpin formation; on the other hand, the *trans*-azobenzene-modified peptide doesn't adopt a unique structure. The substitution of the amino acids with azobenzene destabilizes the structure of the β -hairpin. The groups of Hilvert⁷¹ and Renner⁷² reported the first examples of azobenzene-modified β -hairpin peptide in the turn-region. Hilvert and co-workers used a peptide (Table 1, Entry 3), which was described by Gellman and co-workers to be monomeric in aqueous solution.⁶⁹

Entry	Sequence of β -hairpin peptide	Ref.
1	(CH ₃ CH ₂) ₂ CH ₂ CH ₂ CO-Leu-Ala-Thr-Thr- stilbene -Ile-Val-Leu-Leu-NH ₂	70
2	c-(-Leu-Ala-Thr-Thr- stilbene -Ile-Val-Leu-Leu- ^D Pro-Gly-)	70
3	H-Arg-Trp-Gln-Tyr-Val- AMPP -Lys-Phe-Thr-Val-Gln-NH ₂	71
4	H-Ser-Trp-Thr-Trp-Glu- AMPP -Lys-Trp-Thr-Trp-Lys-NH ₂	72
5	H-Gly-Tyr-Asp-Pro- AMPP -Gly-Thr-Trp-Gly-OH	73
6	Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu- AMPB1 -Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala	74
7	Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val- AMPP -Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala	75
8	Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly- AMPP -Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-	75
9	c-(AMPB1 -Leu-Glu-Thr-Thr-Phe-Thr-Gly-Asp-Gly-Thr-Pro-Lys-Thr-Ile-Arg-)	76
10	Ac-Ser-Trp-Thr-Trp-Glu- AMPP -Lys-Trp-Thr-Trp-Lys-NH ₂	77
11	Ac-(Arg-Ala-Asp-Ala) ₄ - AMPP -(Arg-Ala-Asp-Ala) ₄ -NH ₂	78

Table 1: Photoswitchable β -hairpins. For structure of switching units, see Figure 17.

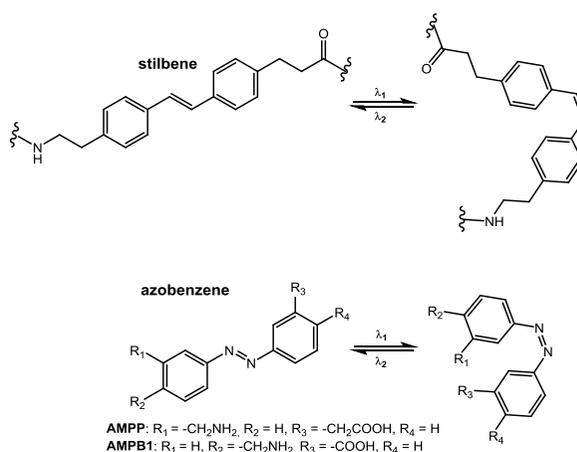


Figure 17: Photoswitches used in β -hairpins.

Supported by molecular dynamics calculations,⁷⁹ they found that AMPP in *cis* form can mimic the turn ^DPro-Gly, but this photolinker forms a more mobile turn than the conventional one (type I' and type II' turns): the strong constrain, which derives from H-bonding between the first and fourth amino acid of the turn, is lacking for an azobenzene linker. Moreover, the azobenzene linker favors a different H-bonding pattern of β -hairpin than the ^DPro-Gly, but still leaves intact the hydrophobic cluster of the amino acids Trp, Tyr, Phe, Val, proposed to be important for the stability of the β -hairpin.⁶⁹

Renner and coworkers used a Trp-zipper (Table 1, Entry 4), which is a β -hairpin stabilized by π - π stacking of the tryptophans.⁷² In this case, the content of Trp-zipper is estimated in buffer to be 50% at 5 °C for the *cis*-isomer; the irradiation at $\lambda = 430$ nm, which enables *cis-trans* isomerization, reduces the content to 18%. Due to the presence of fluorescent tryptophans in the peptide, a more detailed spectroscopic study was possible on the dynamics of the conformational change (Figure 18),⁸⁰ to study the folding dynamics and the influence that the stimulus has on this process. Two initiation stimuli were used to start the folding process of the Trp-zipper: temperature jump and isomerization of the azobenzene in the turn region. For both stimuli, the folding in β -hairpin structure occurs with a time constant of 16 μ s (Figure 18).

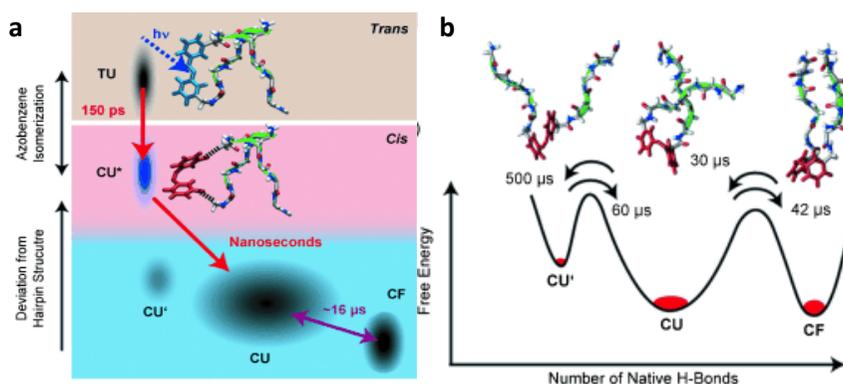


Figure 18: Dynamics of the conformational changes induced by two different stimuli, photoisomerization and temperature jump. a) *Trans* isomer permits a broad distribution of unfolded structures (TU). *Trans-cis* isomerization doesn't provoke significant structural changes. These transient unfolded structures (CU*) evolve on the nanosecond timescale into the unfolded conformations CU and partially to CU'. Formation of the folded structure CF in the isomerization-induced experiment is determined by a transition state between CU and CF. Temperature influence as well the equilibrium of populations of CF, CU, and CU*. After heating, a higher population of CU and CU* is observed. b) Free-energy profile of CF, CU, and CU*, the population in thermal equilibrium (red), and the corresponding structures. Adapted with permission from ref. 80. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA.

The free-energy profile that fits the obtained results is a transition state reaction-scheme with an energetic barrier of 48 kJ mol^{-1} from unfolded to folded structure. This energetic barrier is probably generated by the formation of the hydrophobic cluster (Figure 18).⁸⁰

As already mentioned, β -hairpins are important in the development of pathological diseases, especially of amyloid related diseases.⁶⁷ The role of the amyloidogenic peptides and the amyloid fibers in the pathology of the Alzheimer's disease is still under investigation. In this respect, the amyloidogenic peptide involved in Alzheimer's disease, A β 42 (Figure 19a), which is known to adopt a β -hairpin secondary structure, is studied intensely. The modification of peptides, derived from A β 42, with photoswitches has been used as a tool to obtain more information on the formation and toxicity of these peptides. Two different research groups were working at the same time on the modification of A β 42 with azobenzene unit (Table 1, Entry 6, 7, 8).^{74,75} Nilsson and coworkers substituted two or three amino acids in the turn region of A β 42 with the AMPP photoswitch (Figure 17 and Table 1, Entry 7, 8),⁷⁵ whereas Beyermann and coworkers used AMPB1 (Figure 17) to replace the hydrophobic region of the peptide, the VFFA motif, responsible for the transition from α -helix to β -sheet and therefore formation of cytotoxic fibrils (Table 1, Entry 6).⁷⁴

a **A β 42:** Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala

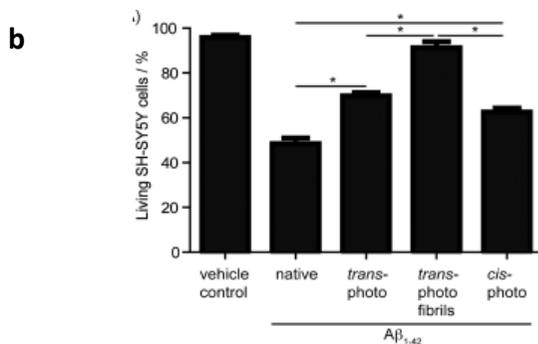


Figure 19: a) Native peptide A β 42. b) Toxicity of the oligomers of *trans*- and *cis*-A β 42, investigated using MTT assay with human neuroblastoma SH-SY5Y cells. Native A β 42, *cis*-A β 42 and freshly prepared *trans*-A β 42 are toxic to neuroblastoma cells, whereas mature fibrils formed by the *trans* form are nontoxic. Toxicity is described as percentage of cell death relative to vehicle control treated cells ($n=15-45$). A one-way ANOVA with use of Dunnett's multiple comparison test comparing peptides with untreated control was performed ($*p<0.01$). Adapted with permission from ref. 74. Copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA.

In the latter case (Table 1, Entry 6), the freshly prepared solution of *trans*-form of A β 42 is cytotoxic (\approx 70% living cells compared to 95% for vehicle control) but, after standing for two days, non-toxic fibrils are formed (91% living cells compared to 95% for vehicle control) (Figure 19); the illumination of these fibrils lead to the formation of the *cis*-isomer of A β 42, which shows higher toxicity (\approx 63% living cells compared to 49% for native A β 42) against SH-SY5Y cells (Figure 19). The oligomers formed from disassembly of the fibrils are tetra- to hexamers, which are believed to be the transition state between monomers and fibrils. This example supports the hypothesis that toxic oligomers are formed from plaque-deposited material.⁷⁴

Nilsson recognized that the turn region perturbs amyloid β -self-assemblies and therefore their cytotoxicity.⁸¹ When the *trans*-azobenzene occupies this region (Table 1, Entry 7, 8), modified A β 42 forms fibrils that are similar to the wild type A β 42, including morphology and cytotoxicity (Figure 20a-b).⁷⁵ *Trans*-azobenzene favors the structure of β -arc turn (Figure 20f), which is known to lead to the formation of cross β -fibrils. On the other hand, the *cis*-azobenzene, which is a type I' hairpin mimetic, leads

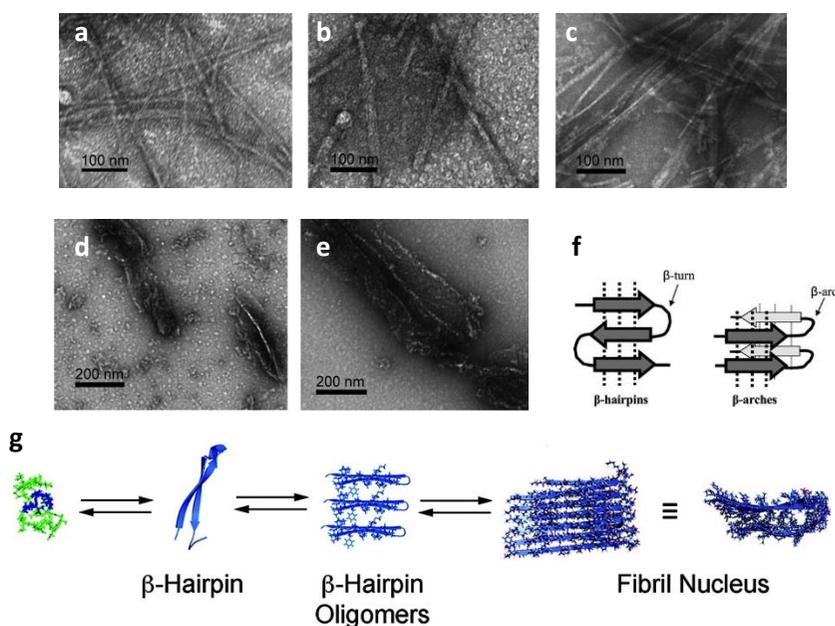


Figure 20: a) Transmission electron micrographs of fibrils derived from A β 42. b) TEM of *trans*-A β 42 (Table 1, Entry 7). c) TEM of *trans*-A β 42 (Table 1, Entry 8). d) TEM of *cis*-A β 42 (Table 1, Entry 7). e) TEM of *cis*-A β 42 (Table 1, Entry 8). f) β -arches and β -hairpins. The arrows indicate the β -strands and the dotted lines indicate the H-bonds.⁸² g) Proposed mechanism of formation of A β 42 fibrils depicting major folding events as nascent A β monomer self-associates into a fibril nucleus. Figures a, b, c, d, e, g were adapted with permission from (*ACS Chem. Neurosci.* 2012, **3**, 211). Copyright (2012) American Chemical Society.

to the formation of highly stable β -hairpin structure (Figure 20f). The amorphous and sedimentable aggregates (Figure 20d, e) are, in this case, not cytotoxic. This discovery brings new insights in the study of nucleation of the self-assemble toxic fibrils and the importance of the turn region in this process,⁷⁵ namely that the well-established idea that an initial nucleation into β -hairpin followed by a transition from β -turn to β -arc is necessary in the process, might be wrong (Figure 20g).⁷⁵

β -Hairpins are also involved in protein-protein interactions.⁶⁶ A photoswitchable, biologically relevant β -hairpin, based on the LETTF sequence, was synthesized by Beyermann and coworkers (Table 1, Entry 9).⁷⁶ LETTF is the recognition binding motif of nNOS β -finger and allows the binding of nNOS β -finger to syntrophin, which is involved in muscular contraction. Using plasmon resonance spectroscopy, it was found that *trans*-form of the peptide adopts a disordered structure and doesn't bind syntropin, whereas the *cis*-form adopts β -sheet structure and binds syntropin with a $K_D = 10.6 \mu\text{M}$, similar to the native peptide.⁷⁶

The well-studied sequence azo-TrpZip2 (Table 1, Entry 10, modification of Table 1, Entry 4) was modified with an acetyl group in the N-terminus.⁷⁷ This provokes amyloid-like aggregation when the azobenzene linker is in *trans* form. The illumination, which leads to the formation of the *cis*-form, causes defects on the structure and therefore loss of the aggregation; in the case of long exposition time of irradiation, larger precipitates are formed.

Lately, the external control over the self-assembly of small peptides is becoming more and more interesting for the synthesis of new nanobiomaterials.⁸³ A photoswitchable β -hairpin used for nanobiomaterial formation, was proposed by Nilsson,⁷⁸ who described the reversible photocontrol of the viscoelasticity of peptide-based hydrogel (Figure 21).⁷⁸ This was achieved by incorporating AMPP into (RADA)₄ (Table 1, Entry 11), a peptide that can adopt a β -hairpin structure.⁸⁴ It was observed that for both *cis*- and *trans*- isomers, the peptide form fibrils in saline solution, but the *trans*-isomer facilitates the formation of cross linked fibrils, which build up a stiff, self supporting hydrogel. The nature of the fibrils is believed to be different than the one observed for A β 42:⁷⁸ The *cis* isomer promotes β -hairpin structure whereas the *trans*-isomer adopts a β -arc conformation and the process is reversible (Figure 21c).⁷⁸

In conclusion, photoswitchable β -hairpins have been synthesized and well characterized and the examples in this chapter show that they can provide a powerful tool to understand processes like the folding of proteins and the formation of the assemblies involved in amyloidogenic diseases. The understanding of these processes also lead to the use of photoswitchable β -hairpins in photocontrolled nanomaterials.

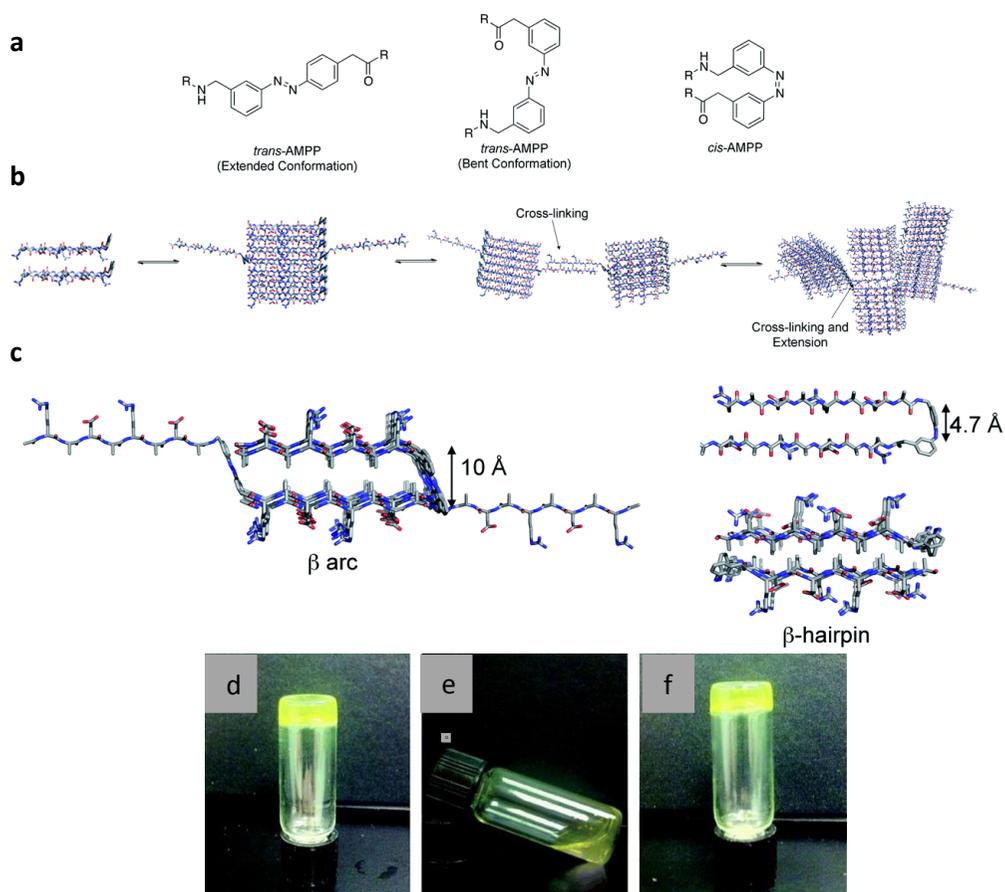


Figure 21: a) Chemical structures of the AMPP photoswitch in the extended-*trans*, bent-*trans*, and *cis* forms. b) AMPP-containing (RADA)₄ self-assembly scheme; extended β -sheets nucleate interfibril interactions at branching points, leading to crosslinking and stabilization of the fibril hydrogel network. c) Model of the peptide (Table 1, Entry 11) folding in the *trans* (left) and *cis* (right) forms. When azobenzene is in the *trans* form, the peptide adopts a strand-turn-strand motif resulting in a β -arc. The flexible methylene units proximal to the azobenzene chromophore permit strand-swapping between pre-formed fibrils. In the *cis* form, the β -hairpin is obtained, (upper right) that forms fibrils that are a bilayer of β -hairpin filaments (lower right). d) Digital photograph of 0.5 wt% self-supporting hydrogel of self-assembled *trans*-peptide (Table 1, Entry 11). e) Digital photograph of *cis*-peptide (Table 1, Entry 11) networks after irradiation for 15 min at 365 nm showing loss of rigidity. f) Digital photograph of 0.5 wt% solution of *trans*-peptide (Table 1, Entry 11) after irradiation of *cis*-peptide (Table 1, Entry 11) at 430 nm for 15 min exhibiting re-emergence of hydrogel stiffness. Reproduced with permission from ref. 78. Copyright 2014, The Royal Society of Chemistry.

1.5 Photocontrolled zinc fingers

Cys₂His₂-zinc fingers play an important role in gene expression because they are located in transcription factors and they are responsible for their interaction with DNA.⁸⁵ Cys₂His₂-zinc fingers are formed by two β -strands and an α -helix (Figure 22a). The secondary structure is stable in presence of a Zn²⁺ ion that coordinates two His and two Cys side chains present in the β -strands and α -helix.⁸⁵ The α -helix is responsible for the binding to the major groove of the DNA. Every zinc finger recognizes three base pairs of the DNA and therefore, in general, several zinc fingers are connected to ensure the sequence-specificity of the binding.⁸⁵ Due to this specificity, these peptides are interesting targets to control gene expression.

Controlling the binding to DNA or, in general, gene expression, by an external stimulus might open new possibilities in genetics, gene therapy and developmental biology. There are in literature two examples that use light as a stimulus to control peptides or proteins bearing zinc finger domains.^{86,87}

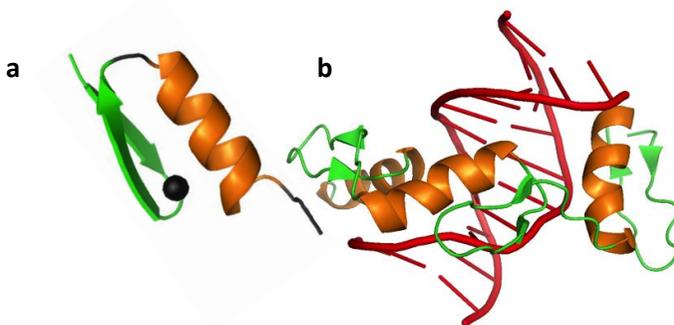


Figure 22: C₂H₂-type zinc finger. a) Tertiary structure of C₂H₂-type zinc finger. The figure was created from PDB 52FN.⁸⁸ b) C₂H₂-type zinc fingers bound to DNA. The figure was created from C₂H₂-type zinc finger, PDB 1A1L.⁸⁹

In the first example⁸⁶ the photoisomerisation process doesn't affect directly the zinc finger domain but a second domain attached to it. The coupling of a zinc finger domain to nuclease domain (ZFN) is a widely established tool for genetic modification of cells to investigate gene function and treat genetic disorders.⁹⁰ A nuclease domain can provoke a double strand break when it dimerizes with another nuclease domain.⁹⁰ Deiters et al. introduced a photocaged Tyr into the active site of the nuclease Fok1.⁸⁶ The photocaged ZFN was expressed and was found to be inactive, but upon irradiation the amino acid is uncaged and the ZFN is activated and it causes double strand break (Figure 23a).⁸⁶ The caged ZFN was efficient also when transfected into cells (Figure 23b).⁸⁶

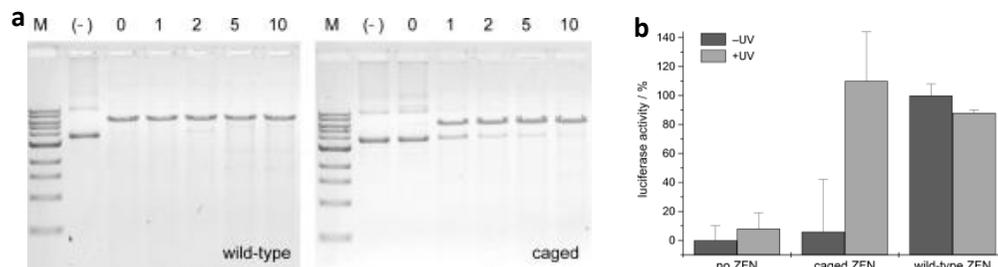


Figure 23: Light-induced uncaging and ZFN activation. a) Ethidium bromide-stained agarose gels for wild-type (WT)-ZFN and photocaged ZFN. DNA is digested using photocaged ZFN subjected to UV irradiation (0, 1, 2, 5, 10 min, $\lambda = 365$ nm). (-): no ZFN enzyme. b) Light-activation of homologous recombination and gene activation in mammalian cells by ZFN-mediated DSB and subsequent repair of the luciferase reporter gene. The error bars represent standard deviations from three independent experiments. Dark gray bars represent samples kept in the dark and light grey bars represent irradiated samples. Adapted with permission from ref. 86. Copyright 2011 Wiley-VCH Verlag GmbH & Co. KGaA.

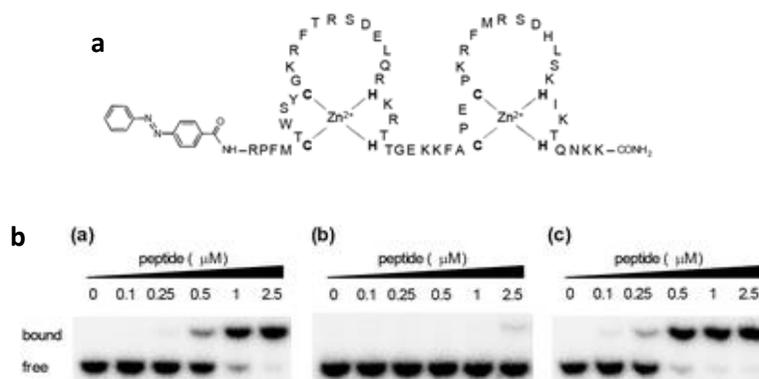


Figure 24: Photoswitchable zinc finger. a) Amino acid sequence of the zinc complex of the synthesized azobenzene-modified Sp1 peptide. b) Gel mobility shift assay of ^{32}P -labeled target duplex DNA containing a GC box region in the presence of Zn-ASP. (a) *trans*-Zn-ASP. (b) *cis*-Zn-ASP, obtained by $\lambda = 365$ nm light irradiation of *trans*-Zn-ASP for 0.5 h at 4 °C. (c) Unmodified peptide. The samples were measured in a solution of 20 mM Tris-HCl (pH = 8) containing 100 mM NaCl, 100 μ M $ZnCl_2$, 1 mM TCEP, 0.05% Nonidet P-40, 5% glycerol, 40 ng μ L $^{-1}$ BSA, and 100 ng μ L $^{-1}$ poly(dI-dC) at 4 °C. Reproduced with permission from ref. 87. Copyright 2009, The Royal Society of Chemistry.

The second example is a chemical approach to photocontrol directly the binding of zinc fingers to DNA.⁸⁷ Two coupled zinc finger domains, derived from Sp1 motif,⁹¹ were modified at N-terminus with an azobenzene unit (Figure 24a).⁸⁷ This modification doesn't affect the secondary structure of the zinc finger domain but affect the binding

to DNA.⁸⁷ *Cis*- and *trans*-forms of zinc finger have different affinity for DNA (Figure 24b).⁸⁷ This approach permits a reversible photocontrol of DNA-binding and therefore if introduced as a genetic tool, might lead to external control of gene expression.

1.6 Aim and outline of the thesis

The aim of this thesis is to use designed photoswitches and to explore their applicability in the control of peptides and enzymes. The resulted photo-sensitive peptides are studied to understand the influence of the photoswitches on the function or activity of the system and, as well, to obtain more information on biological processes in which the peptides and proteins are involved.

The thesis can be divided in two interconnected parts: the first one focuses on methodological aspects of the incorporation of photoswitches into peptides and proteins and the second is dedicated to the synthesis of photoswitchable peptides and, especially, the study of the influence of the photoswitch on the systems.

Chapter 2 and **Chapter 3** describe two methodological approaches to anchor photoswitches to enzymes or peptides, using copper-catalysed azide-alkyne cycloaddition and Staudinger-Bertozzi ligation. In **Chapter 2**, the immobilization of the enzyme lipase on quartz surface is described using a photoswitchable linker. Consequently the influence of the different isomers of the photoswitch on the activity of the enzyme is studied. In **Chapter 3**, new Staudinger-Bertozzi tags are described with an azobenzene unit present in their core. The applicability of these photoswitchable Staudinger-Bertozzi tags is explored on surfaces and with peptides.

The second part of the thesis describes, for the first time, the use of a well-known photoswitch, an overcrowded alkene, for controlling the properties of peptides. In **Chapter 4** the synthesis of an overcrowded alkene switch for solid phase peptide synthesis (SPPS) is described. In **Chapter 5** this overcrowded alkene switch is inserted into a β -hairpin peptide and the effects that the photoswitch induces are studied with different techniques, including circular dichroism spectroscopy, 2D-NMR spectroscopy and TEM microscopy. The final chapter, **Chapter 6**, describes the insertion of an azobenzene unit in a zinc-finger domain. For the first time, the influence of a photoswitch on the zinc binding of the peptide is presented.

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