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Biocatalytic amide condensation and gelation controlled by light†

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We report on a supramolecular self-assembly system that displays coupled light switching, biocatalytic condensation/hydrolysis and gelation. The equilibrium state of this system can be regulated by light, favouring *in situ* formation, by protease catalysed peptide synthesis, of self-assembling *trans*-Azo-YF-NH_2 in ambient light; however, irradiation with UV light gives rise to the *cis*-isomer, which readily hydrolyzes to its amino acid derivatives (*cis*-Azo-Y + F-NH_2) with consequent gel dissolution.

Molecular self-assembly[1] provides a methodology for the fabrication of functional nanostructures for next generation healthcare and energy related technologies.[2] Peptide based systems are attractive in this regard, as they combine chemical versatility with ease of synthesis and biological relevance.[3] Aromatic peptide amphiphiles, comprising di- or tripeptides incorporating (non-biological) aromatic residues are being increasingly studied due to their simplicity and versatility.[4] The interplay of kinetics (self-assembly route)[5] in addition to thermodynamics (optimised supramolecular interactions) contributes to the properties of the assembled material. Methods to control the self-assembly process are therefore extensively researched.

Light has been used to control molecular assembly, typically by *cis*-to-*trans* isomerisation and consequent differences in stacking of the required light sensitive aromatic residues (typically derivatives of azobenzene). Using light in this context is attractive due to its non-invasiveness, wavelength selectivity and the possibility of patterning using photo-masks. A number of light responsive peptide self-assembly systems have been reported including an azo-dipeptide.[6]

Alternatively, biocatalytic control of molecular self-assembly,[7,8] *i.e.* enzymatic conversion of non-assembling precursors into self-assembling molecules, provides an attractive approach, combining biological selectivity, a level of space-time control of nucleation and structure growth and amplification associated with catalysis with both equilibrium[8] and non-equilibrium[9] approaches demonstrated. Bing Xu's group recently demonstrated a system that combined enzymatic and light responsiveness, where an enzymatic reaction was performed to generate a hydrogel that showed light responsive assembly and disassembly.[10]

Responsiveness in biological systems is commonly achieved by molecular systems that are able to respond to applied stimuli by a redistribution of components in coupled (networked) reactions. A similar adaptive behaviour may be achieved in synthetic systems, by coupling reactions. Conceptually, this idea fits with the objectives of systems chemistry[11] to make synthetic networked molecular systems that are adaptive.

Herein, we demonstrate the coupling of light switching with biocatalytic amide condensation/hydrolysis. The equilibrium position of the amide reaction is controlled by the self-assembly propensity of the azo-peptide *vs.* its amino acid precursors,[8] which in turn is regulated by the input of energy (light), thus indirectly affecting the equilibrium situation of the system (Scheme 1). The free energy change associated with molecular self-assembly and gelation is sufficiently thermodynamically favourable to overcome the bias for peptide hydrolysis normally observed in aqueous systems to facilitate condensation. This has been shown before for Fmoc-,[8a] naphthoxy[8b] peptides as well as purely peptidic[12] systems but has not yet been demonstrated for peptides linked to light responsive aromatic moieties. This reversible biocatalytic condensation is combined with the well-known *cis*-to-*trans* isomerisation for azobenzene, which has been used successfully in a range of light switchable systems.[13] Xu's system focuses on the enzymatic activation of the system which in itself is irreversible. Our system is different in that the enzyme action and self-assembly is coupled and reversibly influenced by light.

Azobenzene functionalised tyrosine was prepared *via* a four step synthetic procedure (Scheme S1, ESI†) to form **Azo-Y** (compound **5** in Scheme S1, ESI†). First, we investigated, the enzymatic formation (using thermolysin from *Bacillus thermoproteolyticus rokko*) of different **Azo-YX-NH_2** derivatives (X = F-, L-, V-, represented as **YX**) in order to select a system most suited to demonstrate

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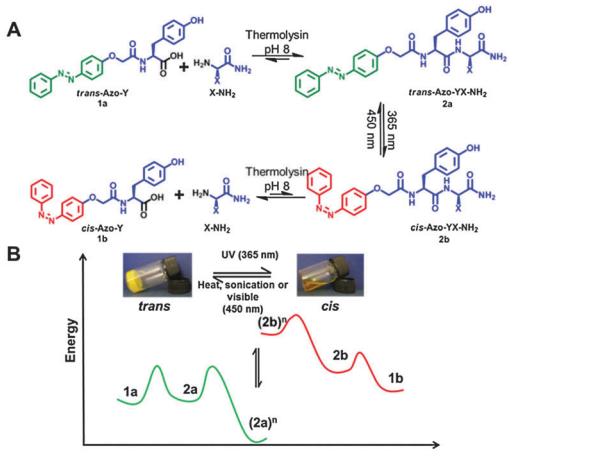

Scheme 1 (A) *trans*-Azobenzene substituted tyrosine derivatives (*trans*-**Azo-Y**) upon enzymatic condensation with amide derivatives of amino acids (F-, L-, V-NH_2) generate the corresponding dipeptide hydrogelators (**Azo-YX-NH_2**, X = F, CH_2–C_6H_5; L, CH_2–CH–$(CH_3)_2$; V, CH–$(CH_3)_2$) in presence of thermolysin at pH 8 in phosphate buffer under ambient conditions. Exposure to UV-light induces Azo switching, resulting in disassembly and hydrolysis of the gelator. (B) Proposed energy diagrams for condensation and self-assembly of azo-peptide hydrogel for the *trans*- and *cis*-isomer. Left: energy difference for self-assembly exceeds that of hydrolysis, resulting in overall favourable condensation and self-assembly. Right: self-assembly is not favoured resulting in hydrolysis, rather than condensation of the azo-peptide.

the concept. The molecular self-assembly of building blocks upon the addition of thermolysin, was macroscopically observed by transformation from a translucent solution to a self-supporting (yellow coloured) hydrogel. Of the systems tested, **YF** gelled rapidly (within 2–5 min after the addition of enzyme) while **YL** and **YV** gelled within periods of 15 and 30 min, respectively. The percentage conversion to the dipeptide derivatives was determined using reverse-phase high performance liquid chromatography (HPLC). As shown in Fig. 1A, the dipeptide derivatives formed in good yields of **YF** (84%), **YL** (59%) and **YV** (63%) at 24 h after enzyme addition. While **YL** and **YF** appeared to have reached an equilibrium conversion after 4 hours, the **YV** conversion was slower and still

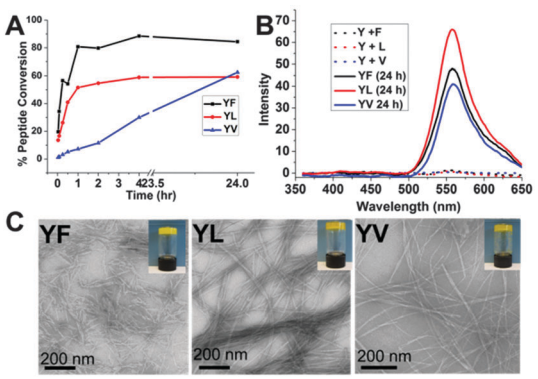

Fig. 1 (A) HPLC data, showing the percentage peptide conversion of different dipeptide derivatives over time (λ = 340 nm). (A) Fluorescence spectroscopy of azobenzene based dipeptide hydrogels (**Azo-YX-NH_2**, X = F, L, V) before and 24 h after thermolysin addition ($\lambda_{excitation}$ = 343 nm). (C) TEM images of the dipeptide derivatives.

increased substantially between 4 and 24 h. These results indicate that the gelation-driven self-assembly is sufficiently thermodynamically favourable to reverse peptide hydrolysis substantially to condensation. The contributing interactions are π–π stacking between *trans*-azobenzene moieties, as well as the aromatic amino acids, combined with and hydrogen bonding interactions between dipeptide units. The higher percentage conversion obtained for **YF** is likely a result of its more favourable self-assembly, due to additional aromatic stacking contributions in **F** compared to **V** and **L**. Competitive sequence selection for self-assembly under thermodynamic control for naphthalene-peptides also showed **YF** as a preferred sequence.[8b]

Next, fluorescence spectroscopy was used to monitor any changes in the fluorophore environment upon assembly. The fluorescence emission peak of the starting mixture (**Azo-Y** + **X-NH_2**) exhibits a weak emission peak at 552 nm. The addition of thermolysin to this mixture induces a red-shift (558 nm) accompanied by a substantial increase in the relative emission intensity peak (Fig. 1B). This aggregation-induced emission (AIE)[14] behaviour indicates that self-assembly of the dipeptide derivatives induces dramatic changes in the electronic properties, most likely due to changes in π-stacking interactions. In addition to spectroscopy, investigations into their nanoscale architecture were carried out using transmission electron microscopy (TEM). The starting materials, before enzyme addition, showed micellar structure while after enzyme addition, the presence of entangled nanofibres was observed for the three dipeptide sequences investigated (Fig. 1C). The diameter of the nanofibres was 20–30 nm and the length of the fibres was up to several micrometers, in line with nanostructures observed for other aromatic peptide amphiphiles.[4] Fibres formed by **YF** appeared to be significantly shorter compared to those found for **YL** and **YV**.

The gel-like behaviour of the self-supporting hydrogels was confirmed by oscillatory rheology (Fig. S2, ESI†). For the three dipeptide derivatives, the elastic modulus (G') is over 10 times greater than the viscosity modulus (G'') confirming the existence of a strong hydrogel network for each system with similar moduli observed for each system. The data suggest that **YV** has more stiffness (higher G') than **YL** and **YF** derivatives.

Among the three dipeptide derivatives tested, **YF** showed a high conversion and rapid response time, and so was used to investigate its light-responsive properties. When the hydrogel (10 mM, 24 h after enzyme addition) was irradiated using a UV lamp (365 nm), the hydrogel disintegrated and dissolved after 48–72 h of exposure.[15] This gel–sol transition of **YF** is expected due to the conformational change of azobenzene from planar *trans*-(*E*) to non-planar (bent) *cis*-(*Z*) form, with the *cis*-isomer prohibiting effective hydrophobic association and π–π stacking between azobenzene chromophores.[10] This unfavourable self-assembly further results in a switch from favourable condensation in the self-assembling system (Scheme 1) to a situation where the enzymatic peptide hydrolysis takes over (Fig. S5, ESI†), resulting in degradation of the fibrous network. (HPLC yields confirm the peptide hydrolysis, as discussed below.) Upon heating and sonication for 60 s, the *cis*-isomer (sol), reverts back to the *trans*-isomer after 48–72 h and gelation is observed. This gel–sol–gel transition of the system upon UV

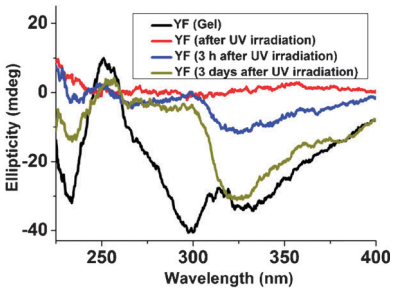

Fig. 2 Circular dichroism (CD) spectra of **Azo-YF-NH$_2$** (10 mM) before (gel) and after (sol) UV irradiation. Corresponding HT (high tension) voltage (Fig. S3, ESI†).

irradiation was further characterised by TEM. As shown in Fig. S4 (ESI†), in the gel-phase **YF** arranges itself into a three dimensional network of nanofibres which, upon UV irradiation, converts to micellar aggregates. Under ambient light, nanofibres reform over time (48–72 h).

CD analysis reveals insights into changes in the chiral supramolecular arrangement during the photoisomerisation process as previously shown for azo-dipeptide.[6b] As shown in Fig. 2, for *trans*-**Azo-YF-NH$_2$** the origination of a broad CD signal at 300–360 nm is observed, due to the π–π* transition of the azobenzene chromophore.[13,16] After UV induced gel dissolution, the system becomes CD silent, which is in line with expectation for the formation of a micellar aggregate system. 3 h after UV treatment, we observe supramolecular chirality, which confirms that the isomerisation of the *cis*- into *trans*-form favours the reformation of chiral nanofibres. After 3 days, the CD signal reverts back to similar intensity, albeit, with a change in the spectral shape, suggesting a slight difference in the supramolecular arrangement compared to the initial gel formed. HPLC analysis (Fig. S5, ESI†) was performed to assess the influence of light on the condensation/hydrolysis equilibrium, demonstrating that the peptide (**2a**) upon UV irradiation hydrolysed back to **1a** and **1b** (Fig. S5, ESI†).

To further confirm that UV irradiation not only switches the self-assembly properties but also enables hydrolysis, the system was started under UV illumination (Scheme 1B, right). To this end, the starting solution (**Azo-Y** + **F-NH$_2$**; 10 mM) was isomerised under UV-light in solution state before addition of the enzyme. The conversion of *trans*- to *cis*-isomer could be monitored by UV-vis spectroscopy (Fig. S6, ESI†). The enzyme was subsequently added and the biocatalytic self-assembly of the dipeptide derivative monitored using HPLC, giving rise to a 7.6% conversion after 24 h (Fig. S7, ESI†) (instead of 84% for the *trans* isomer), which indicates that the *cis*-isomer dis-favours condensation and gelation.

In summary, we demonstrated the ability to control and direct enzymatic amide condensation using light. This is achieved by using a coupled system, involving biocatalytic condensation, gelation and light switching. Influencing of biological pathways by using light may provide important tools for the development of adaptive nanotechnology, with possible therapeutic implications, *e.g.* in the photo-modulation of cellular environments.[17]

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