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Lipase catalyzed inclusion of gastrodigenin for the evolution of blue light emitting peptide nanofibers†

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We report lipase catalysed regioselective inclusion of gastrodigenin (*p*-hydroxybenzyl alcohol: HBA) to a peptide Nmoc-Leu-Trp-OH at physiological pH 7.4. The resultant Nmoc-Leu-Trp-HBA from a reaction cycle of dissipative self-assembly evolves into blue light emitting peptide nanofibers.

Self-assembly[1] is a spontaneous process in which a number of components form an organized structure. Inspired from natural systems, several synthetic systems such as low molecular weight organic molecules have been adapted to develop nanoscale architectures.[2] In general, these self-assembling systems remain in a local thermodynamic minimal state due to minimization of energy or continuous influx of chemical energy. To date, various self-assembled architectures have been reported which are under thermodynamic equilibrium.[3] Recently, there has been growing interest to develop dissipative self-assembly (DSA) systems, which have potential to adapt themselves. These DSA systems consist of non-assembling entities, which can form different nanostructures depending on external stimuli through activation of an energy source. Despite a constant source of energy, the non-equilibrium counterpart of the DSA system leads to dis-assembly due to deactivation of the building blocks caused by energy dissipation. So far, few examples of dissipative self-assembly[4] have been reported. van Esch *et al.* reported an excellent example of dissipative self-assembly of a molecular gelator by using a chemical fuel.[5] Herein, we have used the enzyme lipase as an energy source to meet the requirement of dissipative self-assembly.

Ajayaghosh *et al.* exploited controlled donor self-assembly and energy transfer to form a white light emitting organogel.[6] However, biocatalytic evolution of blue light emitting biomaterials[7] in aqueous media is not yet reported in the literature. Xu and Ulijn are the pioneers of enzyme-catalysed peptide self-assembly.[8] The enzyme lipase is known to hydrolyze carboxylic acid ester[9] in aqueous medium but it also undergoes esterification[10] or trans-esterification[11] reactions in organic solvents. The simple visual colorimetric assay that responds to enzymatic reactions is the area of biosensing applications.[12] Enzymatic conjugation of fluorophores with enhanced emission properties in aqueous medium is more promising for bioassay and imaging applications.[13]

In this paper, we report lipase from *Candida rugosa* (CRL) catalyzed incorporation of gastrodigenin *p*-hydroxybenzyl alcohol (HBA) in Nmoc-protected peptides (Nmoc = naphthalene-2-methoxy carbonyl) followed by evolution of a dissipative blue light emitting hydrogel (Fig. 1). A very recent study shows the use of HBA as a promising candidate for the establishment of anti-angiogenic treatment strategies in cancer therapy.[14] Herein, we exploit CRL for the inclusion of HBA regioselectively

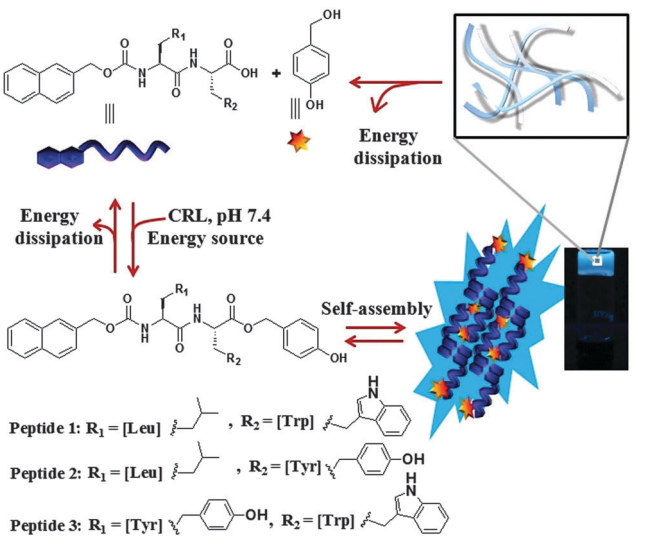

Fig. 1 Schematic representation of lipase (CRL) catalysed regioselective inclusion of HBA to Nmoc-peptides in aqueous medium. Lipase is used as a source of energy for the reaction cycle of the dissipative system. The corresponding ester evolves into a blue light emitting material upon the self-assembly process at pH 7.4.

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in the Nmoc based peptides *via* ester bond formation. Supramolecular chemistry presents the ability to drive the chemical processes reversibly through covalent and non-covalent interactions that result in more stable entities under the pressure of self-organization.[15] Keeping this idea in mind, we forced reverse ester hydrolysis of Nmoc-dipeptide with HBA using CRL in aqueous medium at physiological pH 7.4.

We have successfully synthesized three dipeptides bearing an N-terminal hydrophobic aromatic naphthalene-2-methoxy carbonyl moiety. The peptide Nmoc-LW **1** (20 mmol L^{-1}, 20.04 mg) (L: leucine, W = tryptophan) and *p*-hydroxybenzyl alcohol (HBA, 80 mmol L^{-1}, 19.84 mg) were dissolved in 2 mL of water at higher pH by adding 0.5 M sodium hydroxide. The pH was adjusted to 7.4 by adding 0.1 M hydrochloric acid followed by 0.5 mg mL^{-1} CRL. The resulting colorless solution was incubated at 37 °C for 24 h. The HPLC analysis (Fig. S1, ESI†) showed 24% conversion of Nmoc-LW-HBA ester at 24 h upon enzymatic activation. However, the colorless solution turned light pink, which later turned into a dark pink self-supporting hydrogel when the conversion reached 57% after 8 days (Fig. 2B). The self-assembly of small peptides bearing N-terminal hydrophobic moieties has been reported by several groups.[16] The HPLC kinetics showed that the initial rate of esterification was faster which was relatively slow after 8 days of enzyme reaction.

The 91% conversion was observed after 28 days of enzyme reaction. This activated product self-assembles to form a self-supporting hydrogel at pH 7.4. Two other dipeptides Nmoc-LY **2** (L: leucine, Y = tyrosine) and Nmoc-YW **3** (Y: tyrosine, W = tryptophan) were unable to form self-supporting hydrogels (Table S1, ESI†). Moreover, the HPLC analysis showed poor conversion of Nmoc-LY-HBA (12%) and Nmoc-YW-HBA (20%) (Fig. S2 and S3, ESI†). In general, lipase favours ester hydrolysis reactions instead of esterification reactions in aqueous medium. This may be the reason for the poor yield of esterification reactions of HBA with non-assembling peptides Nmoc-LY **2** and Nmoc-YW **3**. The exceptional activity and higher yield by CRL towards regioselective incorporation of HBA into Nmoc-LW **1** in aqueous medium is driven by supramolecular ordering of peptides, which is influenced by hydrogen bonding, π–π stacking and hydrophobic interactions of peptides. The synthesized Nmoc-LW-HBA leads to dis-assembly upon addition of OH^-. The increased concentration of OH^- hydrolysed Nmoc-LW-HBA to its parent Nmoc-LW peptide. The dissipation of energy deactivated the building blocks to its dis-assembled state despite a constant source of energy which is the enzyme lipase herein. We have synthesized Nmoc-LW-HBA by a chemical method to study the self-assembly behaviour in aqueous medium. The chemical incorporation of HBA to Nmoc-peptides is quite difficult due to the presence of two hydroxyl groups on HBA which leads to the mixture of two products (Fig. S4, ESI†). Moreover, chemical incorporation of HBA with Nmoc-protected dipeptide having hydrophobic amino acids gives direct esterified products which are poorly soluble in aqueous medium (Fig. S5, ESI†). The chemically synthesized Nmoc-LW-HBA was unable to self-assemble in aqueous medium. Hence, the biocatalytic method is more promising and highly selective. The CRL is an ideal biocatalyst that allows regioselective incorporation of HBA to the Nmoc-LW **1** peptide in aqueous medium. The benzylic hydroxyl group of HBA is linked to the carboxylic acid of Nmoc-LW **1** *via* an esterification reaction upon treatment with CRL. The CRL catalyzed regioselective ester product was confirmed by ^{1}H NMR, which is further supported by ESI-MS and HPLC (Fig. 2C, Fig. S1 and S6, ESI†).

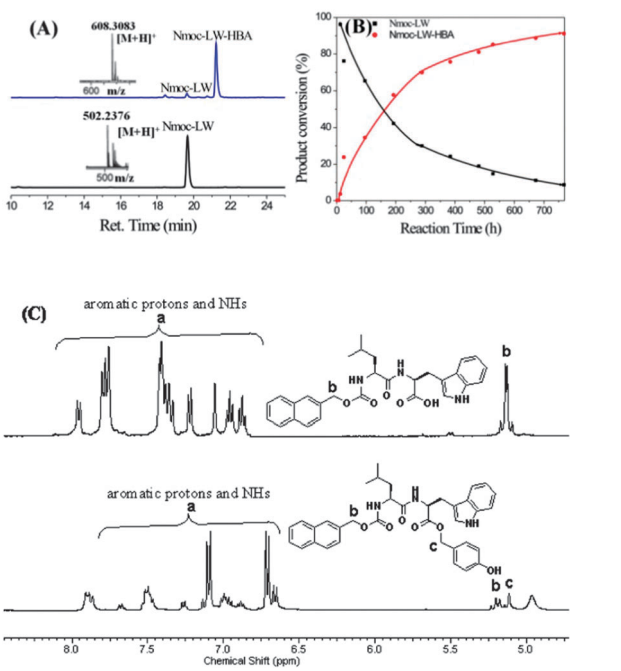

Fig. 2 (A) HPLC trace analysis of enzyme catalyzed esterification of Nmoc-LW **1** to Nmoc-LW-HBA with its corresponding ESI-MS. (B) Real time HPLC analysis for the formation of Nmoc-LW-HBA. (C) Partial ^{1}H NMR of Nmoc-LW **1** and its corresponding dried Nmoc-LW-HBA hydrogel synthesized *via* a CRL catalyzed esterification reaction in DMSO-d_6.

The flow behaviour and rigidity of the self-supporting hydrogel formed by Nmoc-LW-HBA ester was investigated by its rheological properties (Fig. S7 and S8, ESI†). To evaluate the storage modulus (G') and loss modulus (G''), a typical frequency sweep experiment was carried out. The frequency sweep data show that G' is higher than G″, which is an indication of the viscoelastic nature of the hydrogel. The viscoelastic nature of hydrogel **1** can be ascribed to the supramolecular ordering of peptide molecules in aqueous medium.

The supramolecular ordering of peptides is driven by various non-covalent interactions including hydrogen bonding and π–π stacking interactions.[17] Circular dichroism spectra of the solution of Nmoc-LW **1** and HBA (Fig. 4D) and the corresponding gel Nmoc-LW-HBA help to evaluate the supramolecular ordering of hydrogel **1**. The CD spectrum of the Nmoc-LW-HBA ester hydrogel shows a negative band near 193 nm and a positive band near 216 nm which are attributed to π → π* and n → π* transition of the CONH groups of the peptide backbone (Fig. 4D). The intense negative peak at 193 nm indicates the existence of a turn-type β-sheet conformation in the hydrogel state.[18] The UV-vis and fluorescence studies were performed to evaluate the supramolecular ordering of the self-assembled hydrogel. The UV-vis

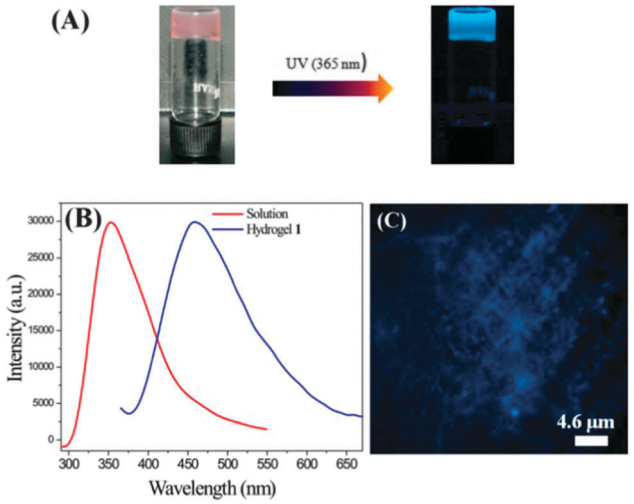

Fig. 3 (A) Optical images of hydrogel Nmoc-LW-HBA **1** under daylight and under UV light (365 nm). (B) Normalized emission spectra of solution of Nmoc-LW **1** with HBA (λ_{ex} = 280 nm) and the hydrogel formed by Nmoc-LW-HBA (λ_{ex} = 365 nm) and (C) the fluorescence microscopy image of gel at 2 mmol L^{-1} concentration.

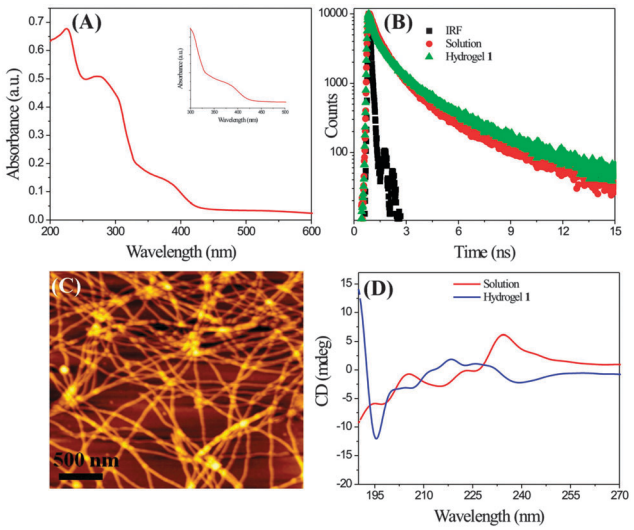

Fig. 4 (A) UV-vis absorption spectrum of hydrogel **1** at 2 mmol L^{-1} concentration. (B) Emission decay curves of solution of **1** and HBA and the corresponding self-assembled hydrogel **1** monitored at 455 nm (IRF: instrument response function). (C) AFM image of Nmoc-LW-HBA hydrogel indicating dense nanofibrillar networks in the gel phase medium and (D) CD spectra of a solution of Nmoc-LW **1** with HBA and the hydrogel of Nmoc-LW-HBA.

spectra of HBA and Nmoc-LW **1** in aqueous solutions were recorded at 2 mmol L^{-1} concentration. Both the solutions exhibit UV-vis absorption spectra in the range of 250 to 300 nm (Fig. S9, ESI†). However, the UV-vis absorption spectrum of Nmoc-LW-HBA exhibits a broad peak at 372 nm along with two peaks near 220–300 nm (Fig. 4A). The absorption peak at 372 nm for hydrogel Nmoc-LW-HBA was further confirmed by fluorescence excitation spectra at two different emission wavelengths (Fig. S10 and S11, ESI†). The higher red shift in the UV-vis spectrum is due to the self-organization of gelator molecules in the hydrogel state. The self-supporting Nmoc-LW-HBA hydrogel emits blue fluorescence upon exposure to UV light (illuminated at 365 nm) (Fig. 3A).

The self-assembly of Nmoc-LW-HBA was observed upon gradual conversion of Nmoc-LW **1** to Nmoc-LW-HBA *via* a CRL catalyzed esterification reaction. The self-assembled dark pink hydrogel under day light showed bright blue fluorescence under UV light which is further examined by steady state fluorescence spectroscopy (Fig. 3B). The self-assembled Nmoc-LW-HBA peptide is composed of three fluorophore moieties. In general, the assembled naphthalene double ring emits at 320 nm to 350 nm upon excitation at 280 nm.[19] However, the tryptophan moiety emits in the range of 300 nm to 350 nm, which depends upon the microenvironments. In this case, emission spectra were recorded upon excitation at 280 nm based on UV-vis absorption spectra. The emission maxima exhibit at 350 nm for the solution of Nmoc-LW **1** with HBA, a characteristic peak of tryptophan and naphthalene chromophores. To further confirm the individual emission of HBA and Nmoc-LW **1** solutions, emission spectra were recorded upon excitation at 280 nm. HBA gives emission at 305 nm whereas Nmoc-LW **1** exhibits emission at 354 nm (Fig. S12, ESI†). However, upon excitation at 280 nm, the self-assembled Nmoc-LW-HBA hydrogel shows a strong emission at 455 nm. Upon excitation at 365 nm, the hydrogel gives strong emission at 470 nm (Fig. 3B). The solution of Nmoc-LW and HBA gives weak emission in the range of 400–500 nm upon excitation at 365 nm (Fig. S13, ESI†). This happens due to strong π–π stacking interactions of the aromatic fluorophores in the gel phase. The observed significant red shift of emission maxima is attributed to the strong supramolecular ordering of peptide molecules in the gel phase. Moreover, the emission peak appearing at 455–470 nm corresponds to blue light in the visible region of the electromagnetic spectrum.

The time resolved fluorescence study was acquired using our time correlated single photon counting (TCSPC) setup (Fig. 4B). The decay curve of the Nmoc-LW-HBA hydrogel was recorded to investigate the higher order aggregates in the gel phase medium (Table S2, ESI†). The average lifetime of the peptide hydrogel was exhibited as 1.39 ns with lifetime components of 0.84 ns (81%) and 3.77 ns (19%). However, the average lifetime of the solution of Nmoc-LW and HBA is 1.25 ns, which shows fast biexponential decay with lifetime components of 0.90 ns (90%) and 4.44 ns (10%). Such difference in decay lifetime is evidence of non-radiative energy transfer[20] in solution. However, such processes are hampered in the self-assembled hydrogel state due to the rigid and close molecular packing through hydrogen bonding and π–π stacking interactions, which results in a higher lifetime.

Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to reveal the nanostructural morphology of the blue light emitting self-assembled hydrogel. As shown in the AFM image (Fig. 4C), the hydrogel has an interwoven nanofibrous network with an average height of 7 nm. The transmission electron

microscopic study also exhibits the formation of nanofibrillar networks with an average diameter of 90 nm (Fig. S14, ESI†). SEM images reveal morphological transformation of the Nmoc-LW-HBA hydrogel at different time scales (Fig. S15, ESI†). SEM images of the isolated products Nmoc-YW-HBA and Nmoc-LY-HBA show entangled nanofibers and long fibrillar structures respectively (Fig. S16, ESI†). The microscopic studies demonstrate the supramolecular ordering governed by formation of nanofibrillar networks in the hydrogel state. Fluorescence microscopic study was carried out to directly visualize the fluorescent properties of self-assembled nanofibers (Fig. 3C). The fluorescence microscopy image reveals that the fluorescent hydrogel is composed of blue light emitting entangled self-assembled nanofibers.[21]

In summary, a blue light emitting hydrogel upon bicatalytic incorporation of gastrodigenin (*p*-hydroxybenzyl alcohol, HBA) to an Nmoc-protected dipeptide has been discovered. Here, the enzyme lipase is used as a source of energy to exploit dissipative self-assembly. The resultant ester formation of a reaction cycle of dissipative self-assembly is an ideal example to achieve complex nanostructures in their self-assembled state. Besides, the lipase propensity towards the hydrolysis of ester bonds in aqueous medium, its catalytic activity is exploited in the development of fluorescent peptide nanostructures *via* an esterification reaction. The blue light emitting hydrogel could be an ideal biomaterial for bioimaging and bioanalysis of various cell processes.

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