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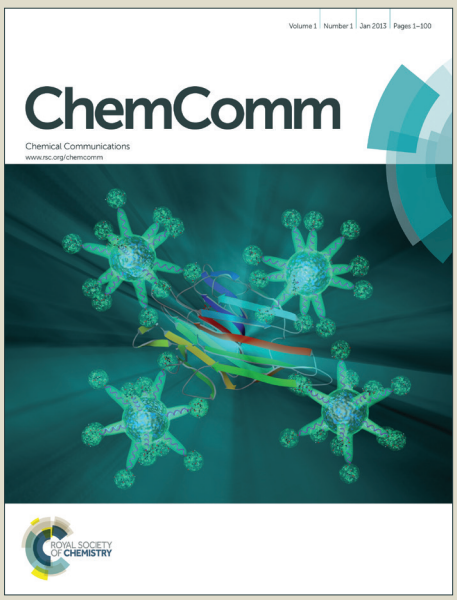

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ARTICLE TYPE

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*-hydroxy benzyl alcohol: HBA) to a peptide Nmoc-Leu-Trp-OH at physiological pH 7.4. The resultant Nmoc-Leu-Trp-HBA of a reaction cycle of dissipative self-assembly evolves 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. Till now, various self-assembled architectures are reported which are under thermodynamic equilibrium.[3] Recently, there is growing interest to develop dissipative self-assembly (DSA) system, 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 energy source. Despite constant source of energy, non-equilibrium counterpart of DSA system leads to dis-assemble 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] In here, we have used an enzyme lipase as energy source to meet the requirement of dissipative self-assembly.

Ajayaghosh *et al*. exploited controlled donor self-assembly and energy transfer to form 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 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 dissipative blue light emitting hydrogel (Fig. 1). 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 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 evolve 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.

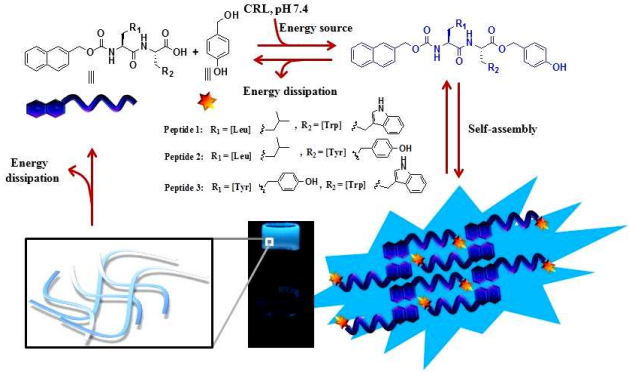

Fig. 1 Schematic representation describes lipase (CRL) catalysed regioselective inclusion of HBA to Nmoc-peptides in aqueous medium. Lipase is used as source of energy for the reaction cycle of dissipative system. The corresponding ester evolves blue light emitting material upon self-assembly process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

We have successfully synthesized three dipeptides bearing 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) showed 24% conversion of Nmoc-LW-HBA ester at 24 h upon enzymatic activation. However, the colorless solution turned to light pink, which later turned to dark pink self-supporting hydrogel when the conversion reached to 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 initial rate of esterification was faster which was relatively slow after 8 days of enzyme reaction.

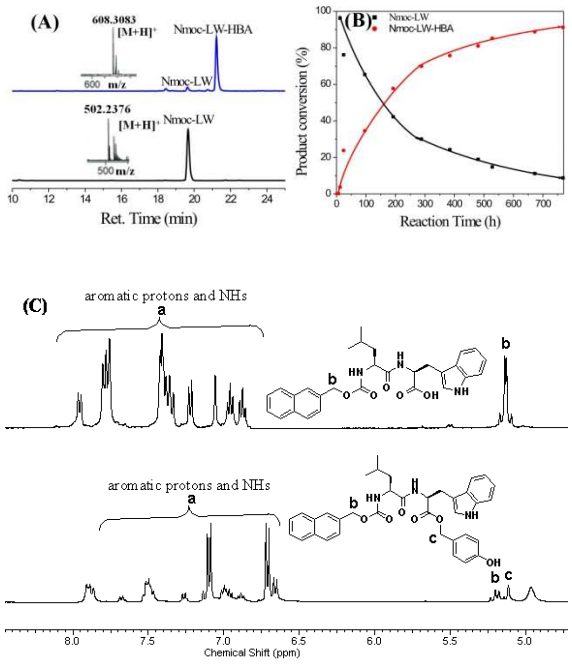

Fig. 2 (A) HPLC trace analysis of enzyme catalyzed esterification of Nmoc-LW **1** to Nmoc-LW-HBA with its corresponding ESI-MS. (B) Real time HPLC analysis for the formation of Nmoc-LW-HBA. (C) Partial ^{1}H NMR of Nmoc-LW **1** and its corresponding dried Nmoc-LW-HBA hydrogel synthesized via CRL catalyzed esterification reaction in DMSO-d6.

The 91% conversion was observed after 28 days of enzyme reaction. This activated product self-assembles to form 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 hydrogel (Table S1). Moreover, the HPLC analysis showed poor conversion of Nmoc-LY-HBA (12%) and Nmoc-YW-HBA (20%) (Fig. S2 and S3). In general, lipase favours ester hydrolysis reactions instead of esterification reactions in aqueous medium. This may be the reason for poor yielding 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 to 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-assemble upon addition of OH^-. The increase concentration of OH^- hydrolysed Nmoc-LW-HBA to its parent Nmoc-LW peptide. The dissipation of energy deactivated the building blocks to its dis-assemble state despite constant source of energy which is an enzyme lipase in here. We have synthesized Nmoc-LW-HBA by chemical method to study the self-assembly behaviour in aqueous condition. The chemically 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). Moreover, chemical incorporation of HBA with Nmoc-protected dipeptide having hydrophobic amino acids gives direct esterifies products which are poor soluble in aqueous medium (Fig. S5). The chemically synthesized Nmoc-LW-HBA was unable to self-assemble in aqueous condition. Hence, the biocatalytic method is more promising and highly selective. The CRL is an ideal biocatalyst that allows regioselective incorporation of HBA to Nmoc-LW **1** peptide in aqueous medium. The benzylic hydroxyl group of HBA is linked to carboxylic acid of Nmoc-LW **1** *via* 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, S1 and S6).

The flow behaviour and rigidity of self-supporting hydrogel formed by Nmoc-LW-HBA ester was investigated by their rheological properties (Fig. S7 and S8). To evaluate the storage modulus (G') and loss modulus (G''), a typical frequency sweep experiment was carried out. The frequency sweep data shows that G' is higher than that of G'', which is an indication of viscoelastic nature of hydrogel. The viscoelastic nature of hydrogel **1** can be ascribed from 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 corresponding gel Nmoc-LW-HBA help to evaluate the supramolecular ordering of hydrogel **1**. The CD spectrum of Nmoc-LW-HBA ester hydrogel shows negative band near 193 nm and a positive band near 216 nm which are attributed from $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transition of the CONH groups of the peptide backbone (Fig. 4D). The intense negative peak at 193 nm indicates the existence turn-type β-sheet conformation in hydrogel state.[18] The UV-vis and fluorescence study were performed to evaluate the supramolecular ordering of self-assembled hydrogel. The UV-vis 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). However, 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, S11). The higher red shift in UV-vis spectrum is due to the self-organization of gelator molecules in hydrogel state. The self-supporting Nmoc-LW-HBA hydrogel emits blue fluorescence upon exposure to UV light (illuminated at 365 nm) (Fig. 3A).

Self-assembly of Nmoc-LW-HBA was observed upon gradual conversion of Nmoc-LW **1** to Nmoc-LW-HBA via 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 characteristics 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). However, upon excitation at 280 nm, the self-assembled Nmoc-LW-HBA hydrogel shows a strong emission at 455 nm. While 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). This happens due to strong π-π stacking interactions of the aromatic fluorophores in gel phase. The observed significant red shift of emission maxima is attributed from the strong supramolecular ordering of peptide molecules in gel phase. Moreover, the emission peak appeared at 455-470 nm corresponding to blue light in the visible region of electromagnetic spectrum.

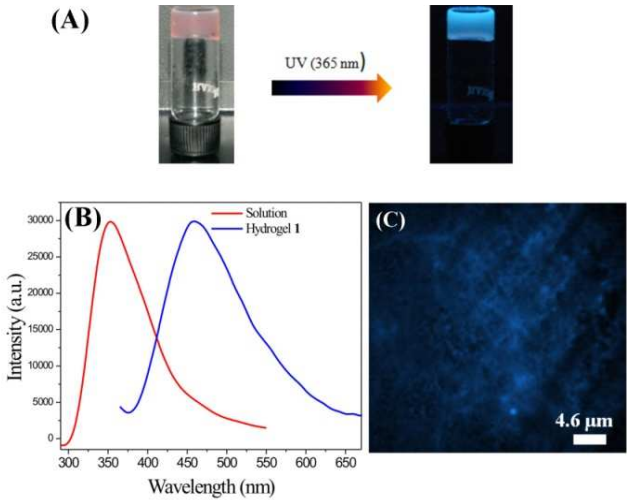

Fig. 3 (A) Optical images of hydrogel Nmoc-LW-HBA **1** under daylight and under UV light (365 nm). (B) Normalized emission spectra of solution of Nmoc-LW **1** with HBA (λ_{ex} = 280 nm) and hydrogel formed by Nmoc-LW-HBA (λ_{ex} = 365 nm) and (C) fluorescence microscopy image of gel at 2 mmol L^{-1}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

The time resolved fluorescence study was acquired using our time correlated single photon counting (TCSPC) setup (Fig. 4B). The decay curve of Nmoc-LW-HBA hydrogel was recorded to investigate the higher order aggregates in gel phase medium (Table S2). The average lifetime of 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 an evidence of non-radiative energy transfer[20] in solution. However, such processes are hampered in self-assembled hydrogel state due to the rigid and close molecular packing through hydrogen bonding and π-π stacking interactions, which results in higher lifetime.

Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to reveal nanostructural morphology of blue light emitting self-assembled hydrogel. As shown in AFM image (Fig. 4C), the hydrogel gives interwoven nanofibrous network with average height 7 nm. The transmission electron microscopic study also exhibits the formation of nanofibrillar networks with average diameter 90 nm (Fig. S14). SEM images reveal morphological transformation of Nmoc-LW-HBA hydrogel at different time scales (Fig. S15). SEM images of isolated products Nmoc-YW-HBA and Nmoc-LY-HBA show entangled nanofibers and long fibrillar structures respectively (Fig. S16). The microscopic studies demonstrate that supramolecular ordering governed by formation of nanofibrillar networks in hydrogel state. Fluorescence microscopic study was carried out to directly visualize the fluorescent properties of self-assembled nanofibers (Fig. 3C). Fluorescence microscopy image reveals that fluorescent hydrogel is composed of blue light emitting entangled self-assembled nanofibers.[21]

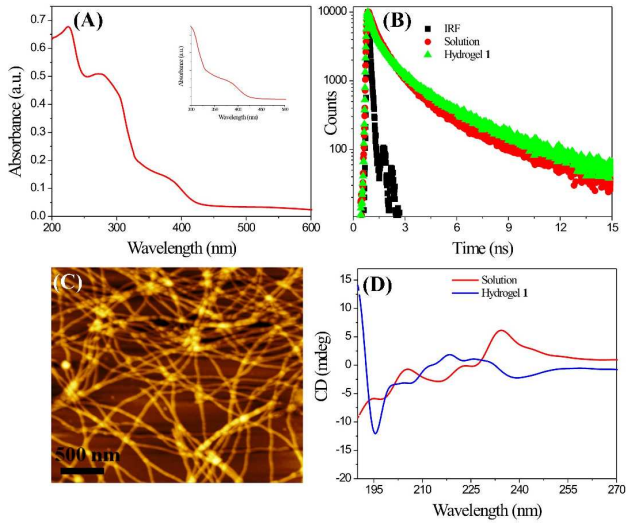

Fig. 4 (A) UV-vis absorption spectra of hydrogel **1** at 20 mmol concentration. (B) Emission decay curves of solution of **1** and HBA and corresponding self-assembled hydrogel **1** monitored at 455 nm (IRF: instrument response function). (C) AFM image of Nmoc-LW-HBA hydrogel indicating dense nanofibrillar network in gel phase medium and (D) CD spectrum of solution of Nmoc-LW **1** with HBA and hydrogel of Nmoc-LW-HBA.

In summary, blue light emitting hydrogel upon bicatalytic incorporation of gastrodigenin (*p*-hydroxybenzyl alcohol, HBA) to an Nmoc-protected dipeptide has been discovered. Here, an enzyme lipase is used as 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 its 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 esterification reaction. The blue light emitting hydrogel could be an ideal biomaterial for bioimaging and bioanalysis of various cell processes.

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Notes and references

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