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Dissipation of Self-assemblies by Fusion of Complementary Gels: An Elegant Strategy for Programmed Enzymatic Reaction

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Cholesterol based phenylboronic acid and glucose tailored complementary gels were developed which underwent mutual self-destruction on mixing due to the formation of boronate-diol adduct. This dissipation of complementary gels was employed in programmed enzymatic reaction and pro-drug activation.

Molecular gels originating from self-assembled amphiphiles showed unique dynamic properties. In supramolecular gels, low molecular mass amphiphiles are held together by means of weak non-covalent interactions such as hydrogen bonding, π-π stacking, hydrophobic interactions and thus it can be easily disassembled in response to external stimuli.[1-3] Development of stimuli responsive functional gels like photo-responsive, pH-responsive, heat-sensitive, enzyme-responsive are finding notable importance in drug release, disease diagnosis, tissue engineering and so on.[4-7] Hamachi et al. developed phosphate-based hydrogelator that exhibited macroscopic gel to sol transition in response to multiple stimuli: temperature, pH, Ca^{2+}, and light.[8] In another work, Lutolf and co-workers had reported rational control in the degradation of polymeric gels via selection of suitable amino acid and consequent release of drug.[9] In this context, functionally sensitive molecules as well as enzyme/protein are known to be immobilized within gel matrix for better stabilization.[10] Design of stimuli responsive matrix that will entrap chemically active substances, variety of catalyst/biocatalyst without deactivation and will release them as when required through degradation of the matrix is in great demand.[11,12] With this aim, we intend to develop complementary gels that can serve as encapsulating agent of chemical/biochemical species and will degrade upon mixing. The gel dissolution upon mixing will be useful to program chemical or biochemical processes. Separately immobilized reactants in different gels will have the propensity to come in close proximity to facilitate the reaction if those matrix are mutually self-destructing upon mixing. Till date report on degradation of molecular gels in response to another self-assembled gels is really scarce. Thus, designing gelators with complementary binding functionality to each other that would undergo physicochemical change in response to one another would be of great importance. The present study delineates the development of complementary gels that underwent mutual self-destruction upon mixing. This dissolution of complementary gels was employed in programmed enzyme catalysis including pro-drug activation.

To develop such interactive gelators, complementary functional moieties need to be included in the molecular design so that one gelator will interact with other one either by covalently or non-covalently. In this regard, phenylboronic acid (PBA), a recognized saccharide receptor and glucose are well known for complementary binding functionalities that form boronate-diol adduct through reversible covalent bonding. Accordingly, we synthesized two gelators; (i) gelator-**1** was composed of hydrophobic cholesterol unit coupled with terminal PBA by C-12 long chain spacer and (ii) gelator-**2** was made of same cholesterol unit attached with D(+)-gluconic acid through C-6 long chain linker (Fig. 1a and Scheme S1, ESI).[13] The synthesized amphiphiles were characterized by ^{1}H-NMR, high resolution mass spectrometry (HRMS) (Fig. S1-S4, ESI). Gelation ability of these amphiphiles was tested in water, different organic, binary solvent mixtures and the minimum

Fig.1 (a) Structures of gelator-**1** and **2**; photographic images of (b) gel-**1** and **2** in DMF-water (2:1 v/v) and sol state after mixing of two gels; (c) dye entrapped gel-**1** (red) and gel-**2** (blue) and its mixed state (purple) after 6h incubation.

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†Electronic Supplementary Information (ESI) available: synthetic procedure, Mass and ^{1}H-NMR **1** and **2** and their adduct, TEM images, temperature dependent ^{1}H-NMR and FTIR spectra, UV-vis and fluorescence spectra of ANS in gelator solutions, complex viscosity vs. angular frequency plot. See DOI: 10.1039/x0xx00000x.

gelation concentration (MGC) was depicted in Table 1. Both **1** and **2** were unable to form gel in pure water while they remain soluble in dimethyl sulphoxide (DMSO), *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF). Amphiphile-**2** formed gel in chloroform, chlorobenzene, nitrobenzene and DMSO-water (4:1 v/v) with MGC of 5.0-7.9 mg/mL. Interestingly, both amphiphiles (**1** and **2**) showed gelation ability in DMF-water mixture (2:1 v/v) and in organic solvents like toluene, benzene, xylene.

Table.1 Minimum gelation concentration (MGC) of gelator-**1** and **2** in different solvents (S=soluble, Ins=Insoluble).

Solvents	MGC(mg/mL)		Solvents	MGC(mg/mL)	
	1	**2**		**1**	**2**
Water	Ins	S	n-Hexane	S	S
DMSO	S	S	Chloroform	S	7.9
DMF	S	S	Benzene	9.2	6.1
THF	S	S	Toluene	10.3	5.0
DMSO-water(4:1, v/v)	S	6.5	*o*-Xylene	7.8	4.8
DMF-water(2:1, v/v)	10.1	8.2	Chlorobenzene	S	5.4
THF-water	S	S	Nitrobenzene	S	5.0

Various intermolecular non-covalent interactions operating between the amphiphilic molecules during self-aggregation were investigated by FTIR and temperature dependent ^{1}H-NMR spectroscopy. FTIR spectra of **1** in non-self-assembled state (KBr pellet) showed sharp transmittance at 1511, 1652 and ~3329-3346 cm^{-1} due to amide $\delta_{N\text{-}H}$ (amide II, bending), $\nu_{C=O}$ (amide I, stretching) and coexistence of $\nu_{N\text{-}H}$ (amide A) with $\nu_{O\text{-}H}$ peaks, respectively (Fig. S5a, ESI). Interestingly, in DMF-D_2O (2:1 v/v), the transmittance signals were shifted to 1549, 1634 and ~3319-3516 cm^{-1} (broad band), respectively. Similar shifts were noticed for gelator-**2** upon transition form non-self-assembled to self-assembled state in DMF-D_2O (2:1 v/v, Fig. S5b, ESI). These shifts in stretching and bending frequencies indicate the participation of intermolecular hydrogen bonding between carbonyl (C=O) and amide N-H moieties during self-assembled gelation.[13] Temperature dependent ^{1}H-NMR study was carried out for both gels in benzene-d6. With steady increase in temperature up to 80-85 °C, the suppressed broad peaks owing to cholesteryl, alkyl chain spacer, phenyl ring (for **2**) and amide protons became more prominent with increased peak intensity (Fig. S6, ESI). Non-covalent forces involved in gelation got dissociated at higher temperature leading to gel to sol transition where the characteristic molecular ^{1}H-NMR signals were observed. Moreover, gradual increase in the emission intensity of 8-anilino-1-naphthalenesulfonic acid, ANS (λ_{ex} = 360 nm) upon successive increase in the concentration of both gelators ensured the involvement of hydrophobic interaction during gelation (Fig. S7, ESI).[14]

With the primary objective of the present study, we were curious to investigate whether the mixing of gel-**1** and gel-**2** leads to any kind of macroscopic and microscopic changes through interaction between complementary functional moieties. DMF-water (2:1 v/v) was used for the experiment because this is the only water containing solvent system where both amphiphiles exhibited gelation ability. Upon placing the gel-**1** over the gel-**2,** the physical state of the mixed gel slowly got changed. With time it gradually lost the solvent imbibing ability resulting in gel to sol transition. After 6h incubation, the mixture (gel-**1** + gel-**2**) completely lost the characteristic semisolid behaviour of gels and became free flowing viscous sol (Fig. 1b). We repeated the experiment by including two different dyes in each of the gel and placed one over another for comprehensible macroscopic observation. Upon incubation for 6h, the individual blue and red coloured gels became purple coloured viscous sol (Fig. 1c). Notably, in contrast to the gelation behaviour of individual amphiphile, the solid mixture of amphiphile-**1** and **2** could not exhibit any gelation ability in DMF-water (2:1 v/v) binary solvent mixture.

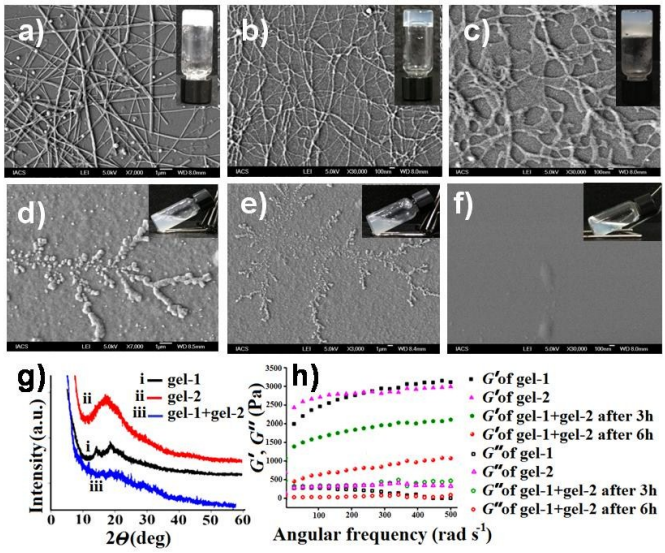

Fig. 2 FESEM images of (a) gel-**1**, (b) gel-**2** and (c) fused gels (**1**+**2**) after 1h, (d) 3h, (e) 5h, (f) 6h incubation; (g) XRD of gel-**1** and **2** and mixed gels; (h) rheology of gels **1** and **2** and its mixtures after varying incubation period.

This macroscopic physical change (gel-to-sol) caused by amalgamation of the two gels intrigued us to investigate the morphological transformation of self-aggregates upon mixing through microscopic techniques. According to field emission scanning electron microscopic (FESEM) and transmission electron microscopic (TEM) studies both gels showed the existence of entangled fibrillar network (Fig. 2a,b and Fig. S8, ESI). Gel-**1** has 40-50 nm thick fibre diameter with length of several micrometer while in gel-**2**, comparatively thinner fibre diameter of 20-30 nm was observed. Next, we have taken FESEM images of the incubated mixed gels at different time interval. After 1h incubation, the fused gel showed different characteristics. The mixed gel fibre diameters were larger compared to native gels and loosening of the fibrillar network was noticed (Fig. 2c). After 3h incubation the mixed gel lost its well-defined fibril structure and formed small irregular bulks (Fig. 2d). These irregular bulks were further divided into tiny fragments with increase in incubation period up to 5 h (Fig. 2e). Finally, no well-defined morphologies were observed after 6 h incubation (Fig. 2f), indicating the complete transition of the fused gel to sol. Mixing of the two complementary gels led to the gradual swelling of gel fibres presumably owing to the formation of boronate-diol adduct between two gelators and lost its compact entangled fibrillar network. X-ray diffraction (XRD) study further supported the destruction of self-assembled fibrillar network after mixing of gels (Fig. 2g). Dried

sample of gel-**1** in DMF-water (4:1 v/v) showed an XRD peak at $2\Theta \approx 14.13°$ and 18.66° corresponding to the spacing between intermolecular steroidal backbones and phenyl rings, respectively.[15] Gel-**2** showed peaks at $2\Theta = 17.02°$, that may be correlated to the spacing between intermolecular steroidal moieties. Interestingly, the incubated (6h) mixed gel did not show any distinguishable XRD peak, which indicates the loss of ordered molecular arrangement upon mixing of two gels. The dissolution of complementary gels due to fusion was further confirmed from rheological experiment. Herein, the storage modulus (*G*') represents the ability of a deformed material to restore its native form while the loss modulus (*G*'') refers to the flow behaviour of the material under applied stress. For viscoelastic materials like gels, *G*'>*G*'' and in the sol state *G*''>*G*' (*G*'$\approx\omega^2$ and *G*''$\approx\omega$, ω=angular frequency). In a typical oscillatory frequency sweep experiment of the native gel-**1** and gel-**2** at a fixed strain (0.01%), *G*' and *G*'' were recorded to be ~2760, 2690 and ~320, 360 Pa, respectively (Fig. 2h). When the gels were mixed and aged, the *G*' value of the fused gel dropped to ~1630 Pa after 3h incubation. After 6h, it was further decreased to ~640 Pa. This gradual drop in *G*' value of the fused gels with aging clearly indicates the decrease in mechanical stiffness of the resulting material owing to dissolution of mixed gels to viscous sol. In a different experiment, a strain controlled (strain fixed at 1%) frequency sweep was carried out for gel-**1**, gel-**2** and their mixture to measure complex viscosity (η*) as a function of angular frequency (10^{-3} to 10 rads^{-1}, Fig. S9, ESI). At low frequency range (angular frequency $\approx 10^{-3}$), η* value was found to be very high (~16200 and ~13500 Pa for gel-**1** and gel-**2**, respectively) probably due to well ordered molecular packing in the gel structure. However, after 3h and 6h incubation of both gels, η* value dropped to ~6900 and ~2000 Pa, respectively. This gradual drop in complex viscosity of the fused gels with aging suggested the decrease in compactness and stiffness of the resulting mixtures. Hence, the microscopic investigation, XRD and rheology analysis clearly delineate that the compact entangled fibrillar network of gel-**1** and gel-**2** gradually destroyed possibly through formation of boronate-diol adduct upon mixing of the two complementary gels.

At this instant, we investigated the change in molecular packing arrangement of complementary gels before and after mixing. Since the gelators are devoid of any chromophoric moiety, absorbance was recorded for ANS that was doped within the different solutions of both gelators and its mixture. ANS doped solution of **1** and **2** in the non-self-assembled state (in DMF) showed absorbance maxima at 371 nm (Fig. S10, ESI). Upon transition to the self-assembled state of the gelators in DMF-water (2:1 v/v), ANS showed red shifted UV-maxima at 375 and 378 nm for **1** and **2**, respectively. The bathochromic shift during the self-assembly of gelators indicates the *J*-type (head-to-tail) aggregation pattern of the amphiphiles (**1** and **2**).[16] ANS doped within the two gelator's solutions exhibited absorbance maxima at 376 nm and 373 nm within 1h and 3h of mixing, respectively (Fig. S10, ESI). After 6h aging, it showed UV maxima at 372 nm similar to that in native gelator's solutions (in DMF). It indicates that the microenvironment in vicinity of ANS within the dissolute gel mixture and in non-self-assembled solutions of individual gelator was almost comparable. ANS encapsulated within the hydrophobic domain of native gels got freed in the bulk domain when ordered molecular arrangement was self-destructed due to the mixing of gels. Thus, the spectroscopic observation further confirmed the dissipation of self-assemblies owing to the fusion of complementary gels.

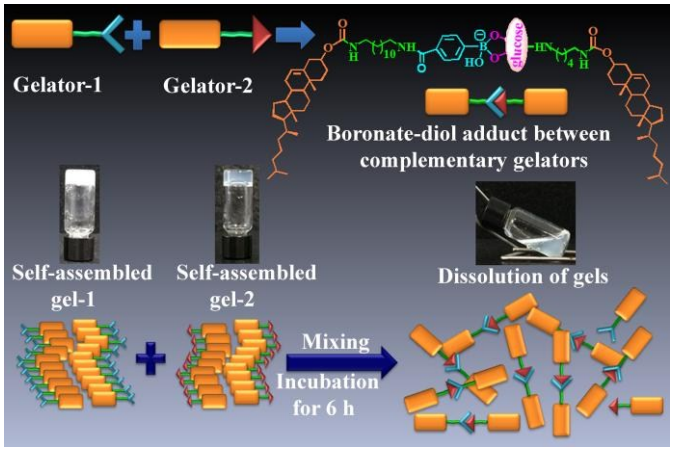

Fig. 3 Schematic representation of the dissolution of complementary gels upon mixing.

The question tickles at this point that what could be the reason behind dissolution of gels upon mixing. An optimum hydrophilic lipophilic balance (HLB) in a small molecule leads to successful gelation. Amphiphile **1** and **2** possibly maintained the suitable HLB for gelation in DMF-water (2:1 v/v). Mixing of gels yielded the formation of cyclic boronate-diol adduct (characterized by ^{1}H-NMR and mass spectra, Fig. S11, S12, ESI) via covalent linkage between complementary functional group of **1** (PBA) and **2** (gluconic acid) (Fig. 3). The adduct might not have the required HLB for gelation resulting in gel dissolution.

Herein, we intend to use this mixing induced dissolution of complementary gels in programmed enzymatic reactions, specially for pro-drug activation. Enzyme catalyzed ester hydrolysis within the mixed gels (**1**+**2**) was monitored as a representative bio-catalytic reaction (Fig. 4a). We entrapped hydrolytic enzyme *Chromobacterium Viscosum* (CV) lipase within gel-**1** and its substrate (*p*-nitrophenyl-*n*-octanoate) within gel-**2** without disconcerting the stability of the native gels. Lipase entrapped gel-**1** was placed over substrate included gel-**2** and the consequent change was monitored with time. A yellow colour was appeared at the junction of the two gels and slowly spread over the whole system. After 6h aging, complete gel dissolution took place with the formation of yellow colour free flowing viscous sol (Fig. 4b). Lipase entrapped in gel-**1** came in contact with the substrate entrapped in gel-**2** at the junction point and yellow coloured *p*-nitrophenol was liberated at the junction. With gradual dissolution of the complementary gels upon mixing, availability of substrate to enzyme steadily increased. Consequently more amount of *p*-nitrophenol was liberated resulting in the formation of yellow coloured viscous sol. The formation of *p*-nitrophenol was also monitored by UV-vis spectroscopy. Lipase

catalyzed hydrolysis was carried out within the mixture of self-assembled solution of amphiphiles at five times lower than MGCs to avoid turbidity related error. The substrate showed absorbance maxima at 303 nm while the hydrolysed product exhibited absorbance maxima at 316 nm, which confirmed the formation of *p*-nitrophenol in the mixed solutions (Fig. S13a, ESI).[17] A gradual increase in the absorbance of liberated *p*-nitrophenol with time was noted within the mixed solutions. Higher amount of product formation was observed owing to greater enzyme-substrate interaction within dissipated gel mixture.

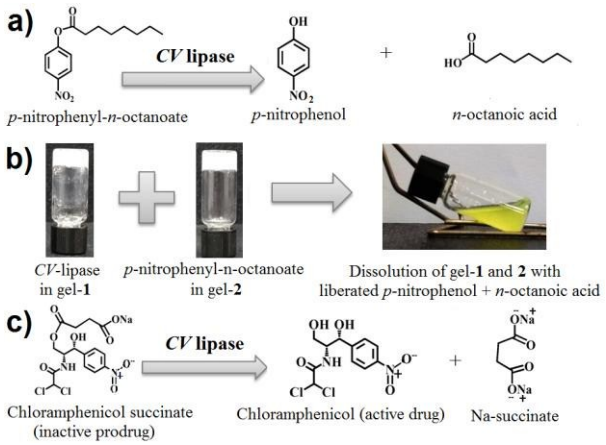

Fig. 4 (a) Lipase catalyzed hydrolysis of *p*-nitrophenyl-*n*-octanoate (substrate); (b) photographs of enzyme and substrate included gels and its dissolution upon mixing with liberated yellow coloured *p*-nitrophenol; (c) Lipase catalysed hydrolysis of a pro-drug (CPS) to form activated drug (CP).

The preceding observation encouraged us to employ this fusion triggered dissolution of complementary gels in pro-drug activation. A pro-drug is an inactive precursor of drug that is converted into a pharmacologically active drug by enzymatic or non-enzymatic reaction. Controlled degradation of the drug encapsulating agent assists in the sustained release of drug, thereby stirring up biological response over a prolonged time period. Herein, we tried to tune pro-drug activation through gradual dissolution of enzyme and pro-drug encapsulating gels. We used chloramphenicolsuccinate sodium salt (CPS) as a water soluble pro-drug, which upon enzymatic hydrolysis will produce antibiotic chloramphenicol (CP) (Fig. 4c).[18] Accordingly, CV lipase and CPS were separately encapsulated in gel-**1** and gel-**2**, respectively. Enzyme triggered hydrolysis of CPS was followed by measuring the absorbance of liberated CP. CPS showed UV absorbance maxima at 272 (Fig. S13b, ESI). Upon addition of lipase included gel-**1** to the CPS entrapped gel-**2**, initially the absorbance maxima red shifted to 278 nm possibly due to enzymatic hydrolysis of CPS to CP. The absorbance maxima of pure CP (as procured) was also found to be at 278 nm. Hence, the enzymatic hydrolysis of CPS resulted in the red shift in UV maxima due to the formation of CP. Interestingly, the absorbance value of liberated CP gradually enhanced with time possibly due to higher accessibility of the enzyme and CPS within dissipated gels resulting in the formation of higher amount of CP. In absence of lipase, neither *p*-nitrophenol nor CP formation was observed upon mixing of gels. Thus, dissipation of self-assemblies owing to the fusion of complementary binding gels can not only be used in the programmed pro-drug activation but will be also useful in the sustained release of drugs. Considering biological relevance of enzymatic reaction and pro-drug activation, further change in the amphiphile's structure is needed (by inclusion of more hydrophilic spacer) to make them pure hydrogelators and carry out the biocatalysis within pure aqueous domain.

In conclusion, we have developed two cholesterol based gelators bearing phenylboronic acid and glucose units as complementary binding sites in their motifs. Mixing of these two gels resulted in the gel to sol transition through gradual destruction of their fibrillar network. Dissipation in the self-assemblies took place due to the formation of cyclic boronate-diol adduct, which lacks the required HLB for gelation. The dissolution of complementary gels was judiciously employed in the programmed enzymatic reaction and pro-drug activation. The present report endows a new understanding of disassembly generated from mixed self-assembled systems as well as its prospect in controlled enzymatic reaction.

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