Stimuli-responsive, protein hydrogels for potential applications in enzymology and drug delivery[§]

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Abstract. Enhancing the stability of enzymes for sensing or biocatalysis applications is still an unmet challenge. Ordinary paper is a very attractive support for anchoring enzymes but enzyme attachment to cellulose without surface activation is still another challenge. To make progress toward these goals, we developed a simple method to prepare highly active and stable enzyme-hydrogels within the mesh of the cellulose fibers of paper. A mixture of the desired enzyme, bovine serum albumin (BSA) and arginine were reacted with carbodiimide to form stable hydrogels. A set of critical concentrations ($\text{BSA}([\text{BSA}]_0) \geq 1 \text{ mM}$, $[\text{carbodiimide}]_0 \geq 100 \text{ mM}$ and $[\text{amino acid}] \geq 100 \text{ mM}$) were required to form transparent hydrogels. The thermal reversibility of gelation proved that the gels are stabilized by non-covalent bonding interactions between the BSA oligomers that were formed *via* covalent interactions. Both dynamic light scattering and SDS-PAGE studies, under pre-gelation conditions, support idea that one BSA oligomeric unit contained 40–70 protein molecules. Scanning electron micrographs, thermogravimetry and swelling studies suggest that the formation of water cavities inside the cross-linked gel matrix, where the water mass was 7–8 times higher than that of the protein and the free amino acid used as a linker/spacer. Due to the higher water content and benign gelation conditions, active enzymes could be incorporated into the gel structure during the synthesis. Hydrogels, thus, embedded with glucose oxidase (GOx) and horseradish peroxidase (HRP) showed catalytic activity towards glucose, where efficient channeling of hydrogen peroxide from GOx to HRP was observed (70% efficiency in initial rate compared to free enzymes in solution). Moreover, the enzymes retained their activity after pasting the hydrogel onto ordinary paper, which was demonstrated as a glucose sensing platform with a detection limit of 5 mM glucose. Trypsin embedded in the gel showed temperature dependent self-degradation by utilizing optimum protease activity at 37 °C. The temperature-triggered degradation of the gel can be used as a drug delivery vehicle, which was demonstrated using a reporter dye. The hydrogel made of a completely proteinaceous material that releases drugs at body temperature but bound to the matrix at room temperature (25 °C) is useful for noninvasive drug delivery platforms. The biocompatibility and non-thermal synthetic route for the hydrogel makes it a superior material for incorporation of temperature sensitive enzymes, drug molecules or nucleic acids, for a diverse set of applications.

Keywords. Bovine serum albumin; glucose oxidase; dynamic light scattering; SDS PAGE; rhodamine B; glucose detection; paper; guaiacol.

1. Introduction

Biomaterials at gel phase have been used for several biomedical applications due to their high solvent

retention and biocompatibility.¹ Polymeric and biomolecule-based hydrogels are demonstrated as a platform for drug delivery, regenerative medicine and wound healing applications.^{2,3} Hydrogels can be derived from synthetic polymers, carbohydrates, proteins, DNA or small molecules.⁴ Intermolecular covalent and non-covalent bonds and high solvent affinity stabilizes gel systems from complete dissolution and phase

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[§]Dedicated to Professor M V George on the occasion of his 90th Birth Anniversary.

segregation. Stable hydrogels are prepared by thermal annealing or by chemical crosslinking of hydrated pro-gel materials.^{5,6} Heat treatment forms hydrogels by physical entanglement of the peptide chains of proteins and strong non-covalent interactions among the units of the pro-gel matrix. Chemically cross-linked gels are stabilized by single or multiple covalent bonds between the pro-gel precursors formed under specific conditions. The formation of hydrogels in the above cases is very specific to the reaction conditions such as concentrations of the precursors, temperature, pH and solubility.

Protein-based hydrogels feature biocompatibility, ease of preparation and they are cost-effective.⁷ Gelatin, collagen and albumin-based thermal and chemical hydrogels are well characterized and used for numerous applications in biomedicine.⁸ Gelatin is an inexpensive byproduct of the meat industry which is readily insoluble in water at normal temperatures, but quickly soluble above 60 °C. Upon cooling the solution back to normal temperature, gelatin solutions form hydrogels. Similarly, gels can be prepared from albumin and collagen under specific conditions. One drawback with these thermal gels is the heating step required, where the incorporation of a temperature sensitive drug or a delicate therapeutic protein is challenging. Another problem is the insolubility of gelatin in water at room temperature, which makes it difficult to purify.

Hydrogels formed *via* covalent networks can be prepared under biologically feasible conditions. Protein-based gels are usually prepared by crosslinking of amines and/or carboxylic groups. For example, a bifunctional compound such as glutaraldehyde was used to crosslink the amine groups through the imine bond formation but these linkages are sensitive to hydrolysis.⁹ The dynamic nature of imine bonds is used for stimuli-responsive properties of the gels. In another case, peptide coupling reagents were used to tether proteins by linking the amines and carboxylic groups of different protein molecules, which are expected to be more stable to hydrolysis than imines.¹⁰ So amide bonds are preferred when strong connections between the components are required. We have previously shown that bovine serum albumin (BSA) gels can be prepared by chemical peptide crosslinking by carbodiimide chemistry.¹¹ The resulting materials were used as biodegradable platforms for biosensing and bioelectronics.^{11,12}

The choice of BSA for the hydrogel synthesis is justified by a number of its attributes. It is the major serum protein of mammals and a waste product from the meat industry. BSA is non-toxic,¹³ readily digested by proteases,^{14,15} and has been approved by the Food

Scheme 1. Formation of BSA oligomers by crosslinking individual protein molecules using Arginine under carbodiimide coupling conditions, and subsequent formation of hydrogel matrix by noncovalent interactions of the oligomeric units. Photographs show the transition from protein solution to transparent, stable gel as demonstrated by the gel inversion test (Gelation Conditions: 75 mg/mL BSA, 100 mM Arginine, and 100 mM EDC).

and Drug Administration (US-FDA) for medicinal applications, making it an excellent material to be used for biological nanotechnologies. BSA is a natural drug-delivery vehicle that has the ability to bind a number of small molecules, dyes, peptides, hormones, and drugs.¹⁶ BSA has ~150 reactive functional groups on its surface (99 COOH groups from glutamate and aspartate residues, and 59 NH₂ groups from lysine residues), which are available for covalent linking to form particles of any desired size, depending on the reaction conditions.

Here, we report a protein hydrogel derived by the crosslinking of BSA in the presence of an amino acid using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the condensing agent. The formation of the gel depended on [BSA], [Arginine], [EDC] and pH. The gel formed without any heating/cooling step, composed of 90% water, which is a much higher water content than other protein gels reported (Scheme 1).⁸ Arginine (ARG) was used for the gel formation, not only to retain excess water but also to provide a specific site for proteases such as trypsin to recognize and cleave the gel, when desired. We show that albumin binding small molecules and enzymes could be incorporated into the gel matrix, during the synthesis step, without affecting the gelation, under benign conditions without any heating. We demonstrate that active enzymes can be embedded inside the gel matrix with excellent activity retention which may be used for biomedical or biocatalytic applications. Our results are presented below.

2. Experimental

2.1 Materials

Bovine serum albumin was obtained from EquitechBio (Kerville TX) and used without further purification. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from TCI American (Portland, OR). Arginine, Rhodamine B (RhB), Bovine Trypsin, Glucose oxidase (*Aspergillus niger*), Horseradish Peroxidase, Guaiacol, Glucose and hydrogen peroxide were purchased from Sigma Aldrich (Saint Louis, MO).

2.2 Methods

2.2a Synthesis of BSA gel: BSA (375 mg) was dissolved in distilled water (5 mL) at room temperature ($\sim 25^\circ\text{C}$) and ARG was then added to the BSA solution to get a final concentration of 100 mM ARG. The pH of this solution was adjusted to 4.5 using dilute hydrochloric acid (~ 0.5 mL, 1 M) followed by the addition of 78 mg of solid EDC to achieve 100 mM total concentration. For kinetic experiments [BSA], [ARG] and [EDC] were varied accordingly and the gelation was followed by monitoring the absorbance of Rhodamine B (RhB, 550 nm), which was added to the pre-gel mixture. Gel formation was checked by the inversion test. No buffers were used during gelation because common buffers at pH 4–5 are carboxylic acids which may react with EDC. The role of ionic strength in gel formation is not investigated in this work.

2.2b SDS-PAGE: Molecular weight increase of BSA crosslinked products prior to hydrogel formation was monitored by polyacrylamide gel electrophoresis. Bovine serum albumin, 75 mg/mL in DI, was added to 100 mM arginine and allowed to equilibrate before EDC cross-linker was added to the solution. Samples containing 10, 20, 30, 35 and 40 mM EDC were synthesized and the crosslinked products separated by size on a 7% resolving acrylamide gel with a 4% stacking gel. Each lane was loaded with 8 μg of protein per lane. Gels were run at 200 V for 35 min in buffer (24.8 mM Tris, 191.8 mM Glycine pH 8.3). Protein bands were imaged by staining the gel in 10% v/v acetic acid, 10% v/v isopropanol, 0.02% Coomassie Blue overnight. Stain-I was removed from the gel and replaced with stain II solution (10% v/v acetic acid, 0.02% Coomassie Blue) overnight and then destained by being placed in 10% v/v acetic acid overnight. The gel was scanned with a Cannon desktop scanner.

2.2c Dynamic light scattering (DLS) measurements: Crosslinking of protein in solution was monitored via dynamic light scattering as a function of crosslinker concentration prior to hydrogel formation. Solutions containing 75 mg/mL BSA with 100 mM Arginine were stirred and either 10, 20, 30, 35, or 40 mM EDC were allowed to equilibrate before being filtered through a 0.22 μm PDVF 13 mm syringe filter. The solution was transferred to a clean plastic $1 \times 1 \text{ cm}^2$ cuvette and monitored on a Precision Detectors

(Varian Inc.), CoolBatch+ dynamic light scattering apparatus with a 658 nm excitation laser source at 90° geometry between the excitation and monitoring light beams. Data collection was done at room temperature, for 1 s, 3 repetitions with 200 accumulations. Precision Ellucitate v 1.1.0.9 and Precision Deconvolve v 5.5 were used to collect and analyze the data, respectively.

2.2d Scanning electron microscopy (SEM) studies: SEM images of the vacuum dried hydrogels were taken on a JEOL FESM 6335 operated at 5 kV accelerating voltage. Before imaging, the specimen was coated with Au–Pd in Edwards E306A Coating System.

2.2e Swelling studies: Hydrogels with different compositions of BSA and EDC were prepared for swelling studies.¹⁷ About 1 g of the hydrogel was then dried at 90°C in an oven and dry weight was taken. The dried gel then incubated in 10 mL DI water and the weight of the gel was followed over time. Water on the gel surface was wiped using Kim wipes.

2.2f Thermogravimetric (TGA) studies: Thermogravimetric analysis was carried out using a TGA Q500 Thermogravimetric Analyzer, TA Instruments, Milford MA. Experiments were non-isothermal in the range of 25 – 600°C during ramping at a heating rate of $0.5^\circ\text{C}/\text{min}$. Samples were then equilibrated at 100°C . Initial sample mass was 12.8 mg and the N_2 flow rate was 60 mL/min. TGA data were analyzed using Origin software.

2.2g Enzyme activity: Glucose oxidase (GOx) and HRP were embedded in the BSA gel matrix by dissolving the enzymes (GOx to HRP mole ratio 1:2) in the pre-gel solution. The enzyme-containing gels were washed in DI water to remove any loosely bound enzyme. Oxidase activity of GOx was measured using D-glucose as a substrate by a known colorimetric method.¹⁸

2.2h Self-degrading gels: BSA/ARG (75 mg/mL/100 mM) was prepared, as mentioned above, with the exception that trypsin (0.33 and 1 mg/mL) was dissolved in the BSA solution before gelation. Degradation of the gel by proteases with respect to temperature was measured by incubating the gel matrix in the buffer at 37°C , in a water bath. The absorbance of the released RhB was measured by taking aliquots from the mixture at specific intervals.

3. Results and Discussion

Stimuli-responsive protein-based hydrogels were prepared by extensive covalent conjugation of BSA and arginine, as model systems. The gel formation and gel behavior showed interesting structural features and novel applications, as described below.

3.1 Hydrogel formation

Protein gels were prepared by EDC coupling of carboxyl and amine groups on the protein as well as on the amino acid (AA). Both reactants are capable of self-crosslinking or cross-linking with each other or in a combination, therefore, thus forming multiple covalent links between two discrete units which can result in protein-AA networks. Rapid formation (<5 min) of the gel from the continued growth of the initial, smaller units into larger networks was observed under specific conditions, BSA (75 mg/mL, ~ 1 mM) and arginine (ARG, 100 mM) and 100 mM EDC at pH 4.5. This is a general composition for efficient gel formation which was identified by varying the concentrations of the three individual components, the type of amino acid and the reaction pH. We found that stable gels are formed when the initial concentration of BSA ($[BSA]_0$) exceeds 75 mg/mL (~ 1 mM), verified by simple inversion tests, at 100 mM ARG and 100 mM EDC. The growth was followed by monitoring the absorbance of Rhodamine B (RhB) as a reporter molecule, added to the reaction mixture. The absorption maximum of the dye at 550 nm increased upon the hydrogel formation which is probably due to the differences in its microenvironment as a function of the reaction progress. The apparent increase in absorbance with respect to time, monitored at 550 nm, is taken as a measure of the rate of gelation. Initial rate ($\Delta A/\Delta t$) represents the change in absorbance per second over the first 200 s, calculated from the slope of the linear fit of the kinetics plot.

The effect of BSA concentration on the rate of gel formation was studied, first by keeping the initial concentrations of arginine and EDC constant ($[ARG]_0$ and $[EDC]_0$ constant at pH 4.5). As shown in Figure 1A, and the largest rate was noted at 75 mg/mL $[BSA]_0$ and the rate decreased at 100, 150 and 200 mg/mL concentrations of BSA, although, stable gels were formed at these other conditions, confirmed by the inversion test, after longer timescales. At $[BSA]_0$ of 50 mg/mL,

a high viscous solution was observed but it failed the inversion test, and below that concentration, no significant changes were noted in the absorbance. Thus, there was a direct correlation between the likelihood of gel formation and absorption changes, except that below the threshold concentration of BSA (75 mg/mL) there was no gel formation, even after several hours. But above this threshold, the formation was slow but gels did form. The slow rate of gelation at high BSA concentration may be explained by the diffusional limitation of the crosslinker (EDC) in highly viscous BSA solutions. Also, when the concentration of BSA is high, protein-protein crosslinks are expected to dominate rather than the formation of protein-ARG crosslinks. We show that the hydrogel formation is controlled by the EDC concentration, as described below.

The increase in EDC concentration had a direct effect on the rate of gelation as demonstrated in Figure 1B. There was an initial lag phase followed by a steep rise in the rate, which suggests that the hydrogel formation is directly proportional to the $[EDC]_0$ over certain concentration range, and we could not test higher concentrations of EDC (> 200 mM) due to practical reasons of solubility and mixing highly viscous solutions, because gelation kinetics were faster than dissolution of EDC in the cuvette. But our inversion tests showed that no gels were formed below 50 mM $[EDC]_0$, but the viscous nature of the solution increased with the increase in the EDC concentration. Stable hydrogels were formed within 10 min by using > 100 mM $[EDC]_0$, however, stable gels were formed when $[EDC]_0 = 75$ mM, over a long period of time (> 4 h). But no gels were formed below 75 mg/mL even with the long incubation period. Figure 1B shows that 75 mM EDC is the point when the initial rate starts to increase, suggesting the formation of BSA oligomers. Next, we examined the effect of $[ARG]_0$ on the rate of gelation because its inclusion in the synthesis is primarily to provide strongly hydrated side chains which may be essential for the increased stability of the hydrogels.

Figure 1. Initial rate of gelation as a function of (A) $[BSA]_0$, (B) $[EDC]_0$ and (C) $[ARG]_0$. Change in the absorbance of Rhodamine B at 550 nm during the reaction time is used as a reporter. (single measurements, estimated error $< \pm 2\%$).

Increase in concentration of $[\text{ARG}]_0$, as expected, increased the rate of gelation (Figure 1C) rapidly, until its concentration reached 100 mM, and then the rate was slightly decreased at 150 mM. Gel formation was noted when $[\text{ARG}]_0$ was 75 mM within 10 min, and with 50 mM ARG gelation occurred over longer periods. We note that even in the absence of arginine, when $[\text{BSA}]_0 > 75 \text{ mg/mL}$, hydrogels formed after treatment with 100 mM EDC. But more rapid gel formation was noted as the $[\text{ARG}]$ increased, suggesting that the formation of $(-\text{BSA-ARG-BSA-})_n$ junctions enhanced the gel formation. We also noted that at high $[\text{ARG}]_0$, the diffusion of ARG could be slower in the viscous medium and may have contributed to an underestimation of rates at high $[\text{ARG}]$. Thus, the above kinetic data are only qualitative but from these kinetic assessments, it is clear that the gels were formed by the reaction between BSA, ARG and EDC because all three reagents controlled the rate of gel formation to a significant extent. The transition of the initial protein crosslinks forming the oligomeric BSA into hydrogels, especially at low BSA concentrations, was intriguing. Next, we tried to follow the gelation process using SDS PAGE and DLS to analyze the size of the aggregates formed and their size change before the gels are formed or at the pre-gelation stage.

3.2 SDS-PAGE and DLS studies

Molecular weights of the crosslinked oligomers formed were followed by SDS-PAGE, at lower EDC concentrations (10–40 mM). In general, we found that the molecular weight of the oligomers increased, which are completely soluble, with increase in $[\text{EDC}]$ (Figure 2). At very low $[\text{EDC}]_0$ (lane 3), the protein cross-linked sample completely ran through the well, and prominent bands appeared around 130 kDa, 250 kDa and

higher multiples of 65 kDa (molecular weight of BSA, lane 2). When the $[\text{EDC}]_0$ was raised to 50 mM (lane 7), we still could see free BSA at 65 kDa, but a significant portion of the gel was stuck in the well or just below it, which is indicative of the presence of very high molecular weight oligomers. To further substantiate these data, we carried out dynamic light scattering (DLS) studies and followed the change in size with respect to $[\text{EDC}]_0$.

An exponential increase in size with respect to $[\text{EDC}]_0$ was noted at pre-gelation conditions of BSA. At low EDC concentration (10 mM), the average hydrodynamic radii of the precursors were $\sim 20 \text{ nm}$, which corresponds to 2–4 BSA molecules linked together. As the EDC concentration increased, the crosslinking increased the particle size, and at 40 mM EDC, 350 nm particles were formed, which may contain a large number of BSA molecules, in a single particle. DLS measurements were not possible above this $[\text{EDC}]_0$ because of high viscosity. The SDS-PAGE and DLS data together confirm the presence of higher order BSA oligomers in the mixture, rather than a monolith formation. These oligomers then self-assemble to form the gel, which is noted under the inversion tests.

3.3 Scanning electron microscopy (SEM) studies

Morphology of dried hydrogels ('xerogel') showed changes, as the gels formed due to BSA cross-linking. The SEM images in Figure 3 show a rough protein surface with voids in them, where the majority of the pores must have collapsed during the drying. These micron-sized pores, still seen in the SEM images, maybe the water channels present in the original network. The porous nature of the protein gels is further confirmed by swelling studies.

Figure 2. (A) SDS-PAGE shows molecular weight changes as a function of $[\text{EDC}]_0$ in the pre-gelation region. (B) Change in hydrodynamic radii of oligomers formed as a function of $[\text{EDC}]_0$.

Figure 3. SEM images show the collapsed structure of the dried hydrogel. Gelation Conditions: 75 mg/mL BSA, 100 mM Arginine and 100 mM EDC.

3.4 Swelling studies

Swelling of the gel in a solvent gives an idea about the extent of crosslinks present in the gel matrix. When the gel forms a highly cross-linked network, water penetration through the gel is limited, so is the extent of swelling. So, higher the swelling, lesser is the degree of crosslinking, in a qualitative manner.¹⁷ Swelling behavior of the hydrogels in water, therefore, was studied to examine the effect of $[EDC]_0$ and $[BSA]_0$.

Increase in EDC concentration, at 75 mg/mL BSA and 100 mM ARG (resulted in higher swelling in the gel from 400 to 800%, with respect to the initial mass of the dried gel (Figure 4A). This is surprising because the expected nature was the opposite, higher the $[EDC]$, higher will be the degree of crosslinking and less permeable or porous the gels are supposed to be. This increase in swelling with $[EDC]$ is possible only if all the protein is not incorporated into the network at lower $[EDC]_0$.

Another unexpected trend was noted for the swelling nature of gels at high $[BSA]_0$. At constant $[EDC]_0$ and $[ARG]_0$, increase in $[BSA]$ from 75 to 100 mg/mL resulted in an increase in swelling from 600 to 900%, but further increase caused decrease in swelling (200%,

Figure 4B). However, both studies taken together suggest that gels with tunable water uptake can be designed by manipulating the concentrations of protein as well as the crosslinking agent. The slight decrease in swelling ratio after the maxima was noted during some measurements, which may be an experimental error from material loss or loosely bound water.

3.5 Thermogravimetric (TGA) studies

Water composition in the hydrogels was characterized using TGA. A scan from 25 to 600 °C at a scan rate of 0.5 °C/min showed characteristic changes in each case. Hydrogel showed about 95% mass loss at a range from 100 to 170 °C corresponding to the dehydration of the gel (orange line, Figure 5). No such noticeable change was noted for either BSA (in red) or the gel that was dehydrated prior to the experiment (90 °C in an oven for 12 h). The hydrogel presented here contains >90% water, and it is comparable to other protein gels, which are usually about 70–80% water. This supports the swelling ratio of ~700% under the same gelation conditions. BSA gel decomposition (green curve) occurred at 250 °C, suggesting high stability when compared to BSA hydrogels synthesized by glutaraldehyde cross-linking¹⁹ or BSA hydrogels (orange line) reported here. Therefore, gel dehydration increased the stability, resulting in a decomposition curve similar to BSA (green line). In fact, the first derivative curves of BSA (red dashed line) and the BSA gel (green dashed line) peak at about 300 °C, indicating the point of most rapid decomposition. The first derivative curve of the BSA hydrogel (dashed orange line) peaks at about 120 °C, indicating reduced stability. Notably, the BSA decomposition curve (red) was comparable to previously reported

Figure 4. Swelling percentage of the dried gels as a function of $[EDC]_0$ and $[BSA]_0$, soaked in water (single measurements, estimated error $< \pm 8\%$).

Figure 5. Thermograms of BSA hydrogel (orange line) in comparison with that of BSA (red) and dehydrated gel (green). First derivatives of the thermograms are also shown in dotted lines. Gelation Conditions: 75 mg/mL BSA, 100 mM Arginine, and 100 mM EDC.

curves that have been obtained from thermogravimetric analysis of BSA.²⁰

3.6 Enzyme activity when embedded in the hydrogel

One major hypothesis verified in this paper is the possibility of enzymes being trapped in the gel matrix and using their enzymatic activities for biological applications. We tested the activity of three different enzymes, glucose oxidase (GOx), Horseradish Peroxidase (HRP) and Trypsin which are embedded in these hydrogels under benign conditions. The enzymes were incorporated within the gel matrix, during the synthesis, and the gel formation kinetics were nearly unaltered by the presence of these enzymes in the reaction mixture due to their low mole fractions when compared to the other gel constituents. This feature allowed us to tune the composition of the gel (% enzyme content) at the synthetic step itself.

Preserving the activity of the enzyme inside the gel matrix is challenging because of two reasons: 1) deactivation or denaturation of the enzyme under the gelation conditions, and 2) the diffusional limitation of the movement of the substrates across the gel matrix. To test for these two possibilities, we carried out the enzyme activity assay of GOx. Figure 6 shows the activity of GOx, under three different conditions. When both GOx and HRP were inside the gel, glucose was able to diffuse through the matrix and the produced H_2O_2 reached HRP. GOx showed 70% retention in activity when compared to 100% activity of the dissolved enzymes, under the

same solution conditions. Since the slowest step in this cascade reaction is glucose oxidation by oxygen, the rate is independent of HRP activity. But guaiacol is the chromogenic substrate for HRP activity, which is necessary for monitoring the reaction cascade.

The role of substrate diffusion was estimated by another experiment where the hydrogel was prepared with only GOx, and HRP was added to the solution during the course of activity assay. Under these conditions, interestingly, essentially no activity ($\sim 4\%$) was noted. This indicates two important facts: (1) H_2O_2 is not able to diffuse from the gel to reach HRP in the solution during the activity time scale (300 s), and (2) substrate channeling is driving the reaction when both GOx and HRP were present in the gel together. The latter is a significant observation and could be used for multi-step synthesis reactions using cascade biocatalysis. Next, the activity of the (GOx+HRP) hydrogel is used to develop paper-based glucose sensors, for exploring the utility of these gels for practical applications.

In situ trapping of hydrogels containing GOx and HRP was used to sense glucose colorimetrically. To prepare the sensing paper strips, BSA gel was prepared with 2 mg/mL GOx and 1 mg/mL HRP and the reaction mixture has been immediately transferred to a paper before gelation occurred. Thus, the gels are trapped in the micro-pores in the paper. To detect glucose, the paper was first immersed in guaiacol solution, which acts as a chromogenic reagent for GOx reaction, coupled with HRP (Figure 6A). The sensing strips prepared were able to sense glucose levels as low as 5 mM (Figure 7), which is in the range of the blood from healthy individuals. A quantitative measure showed that the color intensity on the paper is linearly depended on glucose concentration (Figure S1, Supplementary Information). Next, we explored the activity of proteases in the gel matrix for drug delivery applications.

3.7 Trypsin-encapsulated hydrogels for drug delivery

BSA hydrogels prepared with trypsin showed self-destruction property as a result of protease-mediated degradation of the peptide bonds of the gels, under specific conditions. This property can be used for the gradual release of small molecules or drugs from the gel by tuning the protease concentration or the gel temperature. The role of [trypsin] in the degradation of the gels was demonstrated by the release of RhB from the gel matrix (added during synthesis), as the degradation by trypsin occurs. The absorbance of the dye released into the solution was monitored over time to study the gel degradation (Figure 8A).

Figure 6. (A) Schematic of the activity assay for GOx. (B) Oxidase activity of GOx embedded in the gel showed 70% of activity (in green) in comparison with GOx in solution (red). When HRP was not inside the gel but added to the solution phase, GOx-gel showed negligible activity (blue).

Figure 7. Top: Ordinary paper loaded with BSA-GOx-HRP hydrogel, and the lettering developed when written with ink containing glucose and guaiacol. Bottom: Colorimetric detection of glucose using GOx/HRP trapped in the gel loaded onto regular printing paper. The lowest concentration of glucose detected by the naked eye was 5 mM (80 mg/dL). Healthy blood glucose levels in humans are 5–8 mM (80–126 mg/dL).

When there was no trypsin present in the gel, slow diffusion of RhB to the bulk was noted over 8 h, which was about 40% compared to the initial [RhB]. When there was about 0.3 mg/mL trypsin present, the rate was increased up to 80% release in 8 h. A complete degradation of the gel was noted at 1 mg/mL [trypsin]. After 8 h, the solution was clear, showing the complete degradation (Figure 8D) at room temperature. Next, we analyzed the role of temperature in gel degradation.

Trypsin activity is optimum at body temperature and this property was used to tune the release of RhB and

the degradation of the gel. The release of RhB at 4, 25 and 37 °C from gels containing 0.3 mg/mL trypsin is shown in Figure 8C. At the first two temperatures, slow release of the dye was noted over 20 h, due to poor trypsin activity at these temperatures. However, at 37 °C, the steady release of RhB occurred with trypsin and the gel has been completely broken down after 18 h (time also depends on the mass of the gel) while the sample lacking trypsin is mostly intact. This property of temperature dependence of release will help in tuning the drug release rate and storage conditions of the gel.

3.8 Discussion

Protein gels formed by the crosslinking of BSA with arginine and active enzymes are characterized and their potential biomedical applications demonstrated. Our gel did not require heat treatment for the formation, which is a significant advantage to incorporate heat-sensitive molecules such as enzymes into the matrix. Furthermore, the presence of additional molecules such as RhB or other enzymes did not affect the gel formation. The gel formation mechanism, followed by DLS and SDS PAGE gel show the formation of BSA oligomers in the matrix. We hypothesize that the gel formation is facilitated by the physical interaction between oligomer chains that are formed during the initial phase of the gelation. To support this hypothesis, we heated the gels to about 120 °C, where the gels meltdown to form a viscous liquid and upon cooling, re-gelation was noted. This is possible only if reversible non-covalent interactions are responsible for the gel formation from precursor oligomers.

The role of the charge–charge interaction between the oligomers to form the gel is also noted. Under pH conditions above 5.0, there was no gel formation, but

Figure 8. (A) Release of RhB into the solution from the gel at different trypsin concentrations which showed complete release in 8 h, at 1 mg/mL [trypsin] at room temperature. (B) Change in absorbance of the dye in solution from the gel with trypsin as a function of temperature. (C) Maximum release rate at 37 °C (body temperature) is shown. (D) Picture showing the self-destructing nature of the gel in 10 mM phosphate buffer at pH 7.4, 18 h, 37 °C. Gel with trypsin (1 mg/mL) completely degraded itself (left sample in each of the two photographs) when compared to hydrogel without trypsin (error <2%).

at pH 4 the gels formed readily. This could be due to the optimum EDC chemistry at acidic pHs. So, we altered the amino acid used for gel formation. Out of 17 soluble amino acids tested for gelation conditions, only Lysine and Arginine favor gelation, under these conditions within 5 min. This points out to the charge neutralization possibility of BSA oligomers with negatively charged BSA (pI 4.2).²¹ It is also interesting to note that the gelation is closer to the pI of BSA, supports the charge neutralization driven gelation mechanism.²²

The gelation concentrations of BSA and EDC also give an insight into the oligomerization. For 1 mole of BSA, >50 moles of EDC were required for gelation. One BSA molecule has 59 lysines, 40 aspartic acid and 59 glutamic acid units (crystal structure data).²³ So there are a total of 59 moles of potentially reactive amines per BSA molecule and 99 carboxylates to couple with them. So >50 moles EDC requirement suggests that most of the amines in the BSA are activated by EDC while assuming 100% consumption. However, due to geometrical restrictions, diffusional limitations and steric hindrance all the amines are unlikely to be coupled with carboxylates from neighboring BSA molecules. The BSA molecule has an average spherical surface area of 150 nm² (radius ~3.3 nm),²⁴ and a maximum footprint of ~34 nm². Thus, by geometrical considerations,

there must be a maximum of 5 other BSA molecules around a BSA, which may be connected by single or multiple peptide bonds. Addition of ARG during gelation reduces this geometrical constraint by bridging the BSA molecules, which facilitated the formation of BSA oligomers. However, we have not measured the amount of ARG completely incorporated into the matrix.

In order to preserve the enzyme activity inside the gel matrix, two conditions are required.²⁵ First is the diffusion of the substrate through the matrix to the enzyme active site. SEM images of the gel showed the presence of pores in the gel, and when hydrated, water channels might be present in those spaces, which can help the small molecules to diffuse through. This supports the 70% retention of GOx activity and its substrate channeling to HRP through the matrix. This is possible only if the gel is not a fully cross-linked monolith, which supports the gelation via association of BSA oligomers. The activity of GOx and HRP was used for sensing glucose after making the gel on a regular paper substrate. This dynamic nature of the gel matrix was much clearer in the case of trypsin-encapsulated gels.

The second requirement for enzyme activity retention is that the gel must have enough hydration to preserve the native or native-like structures of the enzymes. As we have shown from the swelling and TGA studies,

the gel had 80–90% of water. ARG played a major role in retaining water since guanidinium cations have a high affinity for water.²⁶ The highly hydrated gel matrix with channels for substrate diffusion is optimal for heterogeneous biocatalysis.

When gels contained trypsin, slow destruction of the gels occurred over 8 h (1 mg/mL trypsin). In order to break down the gel, trypsin has to break the peptide bonds holding the BSA molecules together, thereby forming short peptides or smaller oligomers. In a cross-linked monolith, trypsin will be completely trapped in the matrix losing its ability to digest these links. But in the dynamic gel matrix, trypsin was able to freely find the peptide bonds and degrade the gel. This self-destructing property was used to demonstrate, potential drug delivery applications in the intestine, where trypsin is externally present as well.²⁷ The selectivity of trypsin for arginine-containing peptide sequences²⁸ facilitated the dynamics of gel breakdown. Thus, the kinetics of degradation can be controlled by varying the [ARG].

The gels presented here can be prepared by different alterations of albumin, amino acids and coupling reagent, required by the target application. For example, if a protease is specific to a certain amino acid sequence that can be used for the crosslinking, the gels will break down more rapidly in the presence of these proteases. Furthermore, TGA analysis suggests that gel stability can be controlled by tuning the degree of hydration. Thus, gel structure can be varied to accommodate a wide variety of drugs. This can be used for targeted drug delivery purposes. Similarly, here we used GOx for sensing glucose, which can be substituted by another enzyme to sense different analytes. The versatile nature of the gel is demonstrated here, which opens up new possibilities for functional biomaterials.

4. Conclusions

Multifunctional hydrogel formation by protein chemical crosslinking and self-assembly is demonstrated here. The gel formation was driven by both covalent and non-covalent coupling of the monomeric units, so it is a thermally reversible process to a large extent and novel. Gelation under ambient conditions of temperature and pressure under aqueous conditions is suitable to derive biological materials from abundant and inexpensive starting materials such as BSA. Due to the benign nature of the gel and high water retention in the gel matrix, we showed that active enzymes can be inserted inside the gel. The catalytic activity of glucose oxidase, horseradish peroxidase and trypsin are studied as a proof-of-concept. The demonstrated applications

of enzyme embedded gels in biocatalysis and drug delivery vehicles are highly promising to diversify the uses of these hydrogels. For example, the trypsin embedded gels were self-destructible by temperature stimuli, and interestingly the switch happens at body temperature (37 °C). These gel systems can further be tuned by using different combinations of enzyme dependent stimuli such as pH, inhibitors, surfactants or ionic strength or each of them alone.

Supplementary Information (SI)

Figure S1 is available in Supplementary Information at www.ias.ac.in/chemsci.

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