International Journal of Biological Macromolecules 115 (2018) 176–184

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Cross-linked enzyme aggregates of alginate lyase: A systematic engineered approach to controlled degradation of alginate hydrogel

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ARTICLE INFO

Article history:
Received 20 January 2018
Received in revised form 19 March 2018
Accepted 20 March 2018
Available online 22 March 2018

Keywords:
Enzyme immobilization
Alginate lyase
Cross-linked enzyme aggregate
Alginate hydrogel
Enzyme-assisted degradation

ABSTRACT

An enzyme aggregate of alginate lyase (EC 4.2.2.3) from *flavobactierium* was prepared using ammonium sulfate. The resultant aggregates upon cross-linking with glutaraldehyde produced insoluble and catalytically active cross-linked enzyme aggregate (CLEA) enzyme. The catalytic activity and stability of the cross-linked enzyme aggregate of alginate lyase (CLEA-AL) was studied in the presence of various pH, temperatures and organic solvents. Reusability, storage stability and surface morphology of the CLEA-AL were also studied. The native enzyme and CLEA-AL exhibited maximum enzyme activity at pH of 6.3 and at a temperature of 40 °C. The CLEA-AL has good stability in nonpolar organic solvents and is thermally stable up to 50 °C over a period of 8 h. By encapsulating CLEA-AL into alginate hydrogel, we demonstrate that alginate hydrogels can be enzymatically degraded in a controlled fashion. The results also showed that degradation of alginate hydrogel with CLEA-AL incorporated beads is slower than native enzyme and therefore, CLEA-AL can be used for controlled degradation and release of various biologics from the degrading gel.

1. Introduction

Hydrogels of biopolymers are attractive vehicles for proteins, drugs and cells delivery due to their excellent biocompatibility along with the possibilities of tailoring the release characteristics [1]. In recent years, much attention has been drawn to new and promising applications of naturally derived polymers such as alginates in pharmacy and medicine, e.g. in drug or protein delivery, cell encapsulation, tissue regeneration, surgery and wound management. Besides, for drug delivery and tissue engineering applications, (injectable) alginate hydrogel is of great interest due to their good hydrophilicity and mechanical properties. However, the slow and uncontrollable degradation of alginate are major roadblocks for its successful clinical applications in regenerative medicine [2]. Bio-degradation is often a critical property of materials utilized in biomedical applications. The degradation kinetics of alginate hydrogels have been so far controlled by varying the molecular weight, size of cross-linked junctions, oxidation, photo cross-linking and enzymatic hydrolysis of alginate [3–10]. Enzymatic degradation of alginate gels using alginate lyase is fast evolving as an increasingly popular alternative to dissolution of gels, and lyases have been effectively applied in a variety of studies [8,9]. Alginate lyase, is an endo-hydrolase enzyme that can cleave the glycosidic bond of alginate though the β-elimination mechanism, generating a saturated uronate at the new reducing end and an unsaturated 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid at the new non-reducing ends [11]. Attempts were already made to degrade the alginate hydrogel by incorporation of alginate lyase entrapped PLGA microspheres within hydrogels [10,12,13]. The limitations of microsphere entrapped studies for alginate degradation are the following: First, the amount of entrapped PLGA micro particles in the beads will be low and second, the amount of released enzyme is insufficient to degrade the alginate completely. Cross-linked enzyme aggregates (CLEA) have therefore been proposed as an alternative to conventional immobilization on pre-existing solid supports [14–16]. Cross-linked enzymes are referred as 'carrier-free biocatalysts' and express very high catalytic activity per unit volume thereby maximizing the volumetric productivity and space-time yields over enzymes immobilized on substrates. CLEAs have been developed with a wide variety of enzymes such as laccases, lipase, galactose oxidases, phenylalanine ammonia lyase, and subtilisin [17–21]. In all cases, the enzyme activity was not only retained but also enhanced, highlighting CLEAs as versatile and reusable biocatalysts. During the course of the present study, Castro et al. reported on the preparation

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https://doi.org/10.1016/j.ijbiomac.2018.03.110
0141-8130/

of CLEA alginate lyase by ammonium sulfate and co-precipitation with biopolymers of alginate and different pectins, followed by crosslinking with bifunctional reagents for the use of oral treatment of cystic fibrosis by viscosity reduction of alginate aqueous solution [22]. In this paper, however, we report purely an encapsulated CLEA-AL based enzymatic approach to degrade the alginate hydrogel beads. The utility of the CLEAs as a biocatalyst was further evaluated by studying its enzyme activities under different conditions. Available literature reports that the pore size of alginate hydrogel microcapsules vary from 5 nm to 40 μm depending on the alginate and $CaCl_2$ concentrations and an increase of their concentrations is known to lead to smaller pore sizes. The CLEA-AL developed in this work, possess pore size diameters, which are larger than the usual pore sizes of the hydrogels but are not expected however, to cause leakage of the aggregated CLEA particles from the matrix under normal conditions. Therefore, it can be hypothesized that the encapsulation of the micron-sized CLEA in a non-toxic, hydrophilic, alginate matrix to form hydrogel beads can result in much controlled degradation of the matrices. The encapsulated enzyme aggregate with alginate hydrogels were further studied to explore their in-vitro degradation response.

2. Experimental section

Sodium alginate (Medium viscosity, Mw 80,000–120,000 Da, 61% mannuronic and 39% guluronic acid or M/G ratio of 1.56), alginate lyase produced by *flavo bactierium*, ammonium sulfate (enzyme grade), poly ethylene glycol, glutaraldehyde and calcium chloride were purchased from Sigma, St. Louis, USA. All the other reagents used were of analytical grade. Micro BCA protein assay kit was purchased from Pierce (IL, USA). Phosphate buffer solution was purchased from Lonza Biologics.

2.1. Alginate lyase enzyme assay

The activity of Alginate lyase was assayed using the 3,5-dinitrosalicylic acid (DNS) method of reducing sugar determination [23]. For native AL enzyme, an enzyme assay mixture containing 125 μL of alginate (1%) and 125 μL of alginate lyase in phosphate buffer (pH 7.0) was incubated at 37 °C for 30 min. The reaction was terminated by adding 500 μL of DNS solution (v/v), followed by boiling for 5 min. 500 μL of deionized water was added to the reaction mixture and mixed well. The absorbance of the reaction mixture was measured at 575 nm. The calibration curve for reducing sugar measurement was based on mannose, and therefore, the reducing sugar concentration is expressed as mannose equivalent. For the immobilized AL enzyme, 25 mg of CLEA-AL was added into 125 μL of alginate solution and incubated at 37 °C for 30 min. The reaction was also terminated by adding 500 μL of DNS solution (v/v), followed by boiling for 5 min. 500 μL of deionized water added to reaction mixture and mixed well. The absorbance of the reaction mixture was measured at 575 nm. The calibration curve for reducing sugar measurement was based on mannose, and therefore the reducing sugar concentration is expressed as mannose. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of reducing sugar end per minute at 37 °C and pH 7.0. The enzyme protein concentration was estimated by the bicinchoninic acid assay (BCA) method using bovine serum albumin as the standard. 1 U of native and CLEA-AL were used for all the experiments as described throughout the manuscript.

2.2. Preparation of CLEA-AL

Aggregation of Alginate lyase enzyme was achieved using ammonium sulfate, tert-butyl alcohol or with PEG. CLEA-AL was prepared using the crude alginate lyase enzyme solution alone at a concentration of 10 mg/mL (~100 U) in 0.1 M phosphate buffer at pH 7.4. Different concentrations of ammonium sulfate were added stepwise into the alginate lyase enzyme solution at regular intervals with stirring at 4 ± 1 °C. Following this, the super saturated solution was kept at this temperature undisturbed for 24–48 h. The aggregates formed were then separated by centrifugation at 5000 rpm for 10 min. Enzyme activity of the precipitate was measured at all the ammonium sulfate concentrations. Cross-linked aggregates of alginate lyase (CLEA-AL) were prepared by adding cross-linking solution of glutaraldehyde to an aggregate solution under stirring at room temperature. After the crosslinking step, the mixture was centrifuged at 5000 rpm for 10 min. The resulting CLEA-AL was washed with phosphate buffer (pH 7.0) and centrifuged at 5000 rpm for 10 min.

2.3. Determination of optimum temperature and pH of enzyme activity

Optimum temperature and pH were determined by changing each of the parameters by keeping all the other conditions constant and the enzyme activity was assayed. The effect of temperature on the alginate lyase activity was determined by assessing the enzyme activity at different temperatures (30–80 °C). The activity profile was studied in different pH ranges of 6.0–8.0 using phosphate buffers of varying pH.

2.4. Thermal stability of native and CLEA-AL

Thermal stability of CLEA-AL at different temperature (37, 40, and 50 °C) is compared to that of the native alginate lyase at different time intervals at pH 6.3 by measuring the enzyme activity.

2.5. Reusability of CLEA-AL

To determine the reusability of the CLEA-AL, the CLEA was washed with phosphate buffer (pH 6.3) after each usage and then suspended again in a fresh reaction mixture to measure the enzyme activity. The procedure was repeated until the enzyme lost 50% of its original activity (half-life).

2.6. Stability of CLEA-AL in different organic solvents

Stability of the CLEA-AL in organic solvent was assessed by incubating a specific amount of CLEA in various organic solvents for 72 h. The organic solvents were chosen according to the hydrophobicity, dielectric constants and log P (The partition coefficient, abbreviated P, is defined as a particular ratio of the concentrations of a solute between the two solvents (a biphasic state of liquid phases); specifically, for un-ionized solutes, and the logarithm of the ratio is thus log P values). After the incubation period, the enzyme assay of CLEA-AL was executed to assess the enzyme activity following the procedure mentioned earlier. The activity was compared with the original activity and the activity retention was accordingly calculated.

2.7. Storage stability of native and CLEA-AL

The native and CLEA-AL were incubated at 4 °C. The enzyme activity was determined at regular time intervals up to 50 days. The practical usefulness of an enzyme is often decided by its storage and operational stability.

2.8. Scanning electron microscopy

The surface morphology of the CLEA-AL was analyzed using scanning electron microscopy (SEM) with a Philips-XL30 FEG. All the samples used for SEM analysis were mounted on metal stubs using double-sided adhesive and sputtered with Pd using a sputter coater system. The micrographs were then taken at an accelerating voltage of 10 kV and at various magnifications.

2.9. Preparation and characterization of alginate hydrogel morphology by scanning electron microscopy

Sodium alginate was dissolved in deionized water at a concentration of 20 mg/mL. The hydrogel beads used for this study were prepared by extrusion of the alginate solution through a syringe having a diameter of 0.1 mm (24G). Beads were made in 0.5 M $CaCl_2$ solution and stirred at 250 rpm, cured for 30 min to 1 h in the same solution and removed by filtration followed by washing with deionized water to remove the excess of $CaCl_2$. To prepare the alginate hydrogels containing native or CLEA-AL, 1 U of enzyme was added to the sodium alginate solution, which was then extruded into a solution containing calcium chloride (0.5 M) as described above. The morphology of 2% alginate hydrogel beads (control), 2% alginate hydrogel beads containing both native and CLEA-AL were observed using scanning electron microscope after freeze drying the samples. For freeze drying, the wet beads were frozen at −80 °C first followed by freeze drying at −50 °C. To obtain accurate cross section of hydrogel beads, samples were initially frozen in liquid N_2 to allow the samples to be cut appropriately to obtain cross sections for suitable imaging using a sharp scalpel and then subjected to freeze drying following the same regular freeze-drying process.

2.10. Alginate hydrogel degradation studies

2.10.1. Stability of CLEA-AL protein

To test the stability of the AL enzyme protein released from the CLEA micro particles at different time intervals, 1 U enzyme activity of CLEA-AL particles were suspended in 2 mL of phosphate-buffered saline (PBS) for 21 days at 37 °C. Every alternate day, the entire buffer was taken out from the samples to measure the protein content using a Micro BCA Protein Assay Kit and fresh PBS was replaced into each sample to keep the incubation volume constant at 2 mL. The amount of protein released was measured at 540 nm using an ELISA microplate reader.

2.10.2. Degradation of alginate hydrogels

2 g alginate beads incorporated with 1 U amount of native enzyme and CLEA were individually placed in a glass vial with 2.0 mL of buffer (pH 7.4) and incubated at 37 °C. The buffer was taken and replaced with fresh buffer at regular time intervals. The reducing sugars in the supernatant of samples were then measured by the DNS method described earlier.

2.11. Statistical analysis

The mean values and standard deviation of three experiments were calculated and presented in each of the figures as error bars.

3. Results and discussion

Controlled degradation of hydrogels in particular, naturally derived polymers is still a major challenge and prevents their wide spread use in the biomedical field. This work also aims to provide a suitable approach for enzyme immobilization of alginate lyase and its use in enzymatic hydrolysis of alginate hydrogels. The present work is aimed at developing a method of preparation of catalytically active and stable cross-linked biocatalyst of a lyase group enzyme, which can catalyze the hydrolysis of alginate to small molecules and therefore, can control the degradation of the hydrogel. Alginate lyase was particularly chosen because of its usefulness as a biocatalyst for the degradation of alginate [10,12,22,24,25].

3.1. Selection of precipitants & effect of glutaraldehyde concentration

Usually, the procedure of CLEA preparation is composed of two steps: enzyme precipitation and subsequent cross-linking of the protein aggregates with a bi-functional agent such as glutaraldehyde. The as prepared solid aggregates are held together by non-covalent bonding and readily collapse and re-dissolve when dispersed in an aqueous medium. It has been suggested that the chemical cross-linking of these physical aggregates produces cross-linked enzyme aggregates in which the reorganized superstructure of the aggregates maintain their activity [15].

Precipitation, by the addition of salts, organic solvents or nonionic polymers to aqueous solutions of proteins, is a commonly used method for enzyme aggregation [16]. The resulting physical aggregates of enzyme molecules are of supramolecular structures that are held together by non-covalent bonding and can be easily re-dissolved in water. Initially we studied the effects of various precipitants and their concentration on the enzyme activity in the precipitated aggregates. During enzyme aggregate preparation, the soluble alginate lyase solution was precipitated using, polyethylene glycol 6000 (PEG), tert-butyl alcohol (TBA) and, ammonium sulfate under different conditions. No aggregates were obtained even after 48 h at 0 °C when PEG of various concentrations (50 to 75%) was used as a precipitant. The efficiency of tert-butyl alcohol as a precipitant was performed at room temperature due to the crystallization of the alcohol at low temperatures. The precipitation carried out with different concentrations of tert-butyl alcohol resulted in the formation of a precipitate with only 90% tert-butyl alcohol (v/v), which showed very poor enzyme activity and therefore, was not considered for further studies. Different concentrations of ammonium sulfate from 50 to 70% were also added to the enzyme solution at 4 °C to form precipitates. A maximum enzyme activity was obtained using 60% (w/v) of ammonium sulfate as precipitant and the results for which are shown in Fig. 1a.

Based on the above results, 60% ammonium sulfate was selected as the optimized precipitant for further investigation. Previous studies on CLEA also reported the aggregation of alginate lyase with ammonium sulfate but however, the CLEA showed no enzyme inactivation when the precipitation was combined with different biopolymers used as co-precipitants [22]. The present approach reported herein differs from the method described by Islan et al. [22] in that there is no co-precipitants used during aggregation. Due to the different biochemical and structural properties of enzymes, the best precipitant and cross-linker can vary from one enzyme to another [26].

Glutaraldehyde is a common cross-linking agent because of its versatility and cost effectiveness for CLEA preparation. The aldehyde group of glutaraldehyde can react with the amino group of the enzyme protein generating intermolecular and intramolecular covalent binding [27]. Optimization of the CLEA procedure also involves optimization of the glutaraldehyde/enzyme ratio. If too little cross-linker is used the enzyme molecule may still be too flexible and can re-dissolve in the solvent, while too much cross-linker can result in a loss of the minimum flexibility needed for the activity of enzyme [28]. Earlier work on CLEA-AL reported the cross linking of alginate lyase with glutaraldehyde-BSA combination to avoid the denaturation of the enzyme during cross linking [22]. It was also shown that the enzyme activity was lost when incubated with glutaraldehyde alone. Based on this result, attempts were carried out to confirm the acceptable amount of glutaraldehyde concentration on the activity recovery. Different concentrations of glutaraldehyde (0.1–2.0%) were used to determine the optimum quantity required for stable cross-linking of alginate lyase and the results are shown in Fig. 1b. After crosslinking, the formed CLEA particles are generally insoluble in both water and organic solvents, and show catalytic activity in severe conditions. At lower concentrations of glutaraldehyde, very little insoluble aggregates could be obtained; whereas at higher concentrations, the amount of insoluble aggregates increased, but no significant activity was detected.

The highest activity recovery could be obtained with 1% glutaraldehyde solution and 1 to 2 h of cross-linking time (Fig. 1b). Glutaraldehyde concentration of 1% gave maximum activity recovery and aggregation yield and hence, was used for all further experiments. These results, therefore demonstrate a simple way of aggregation and

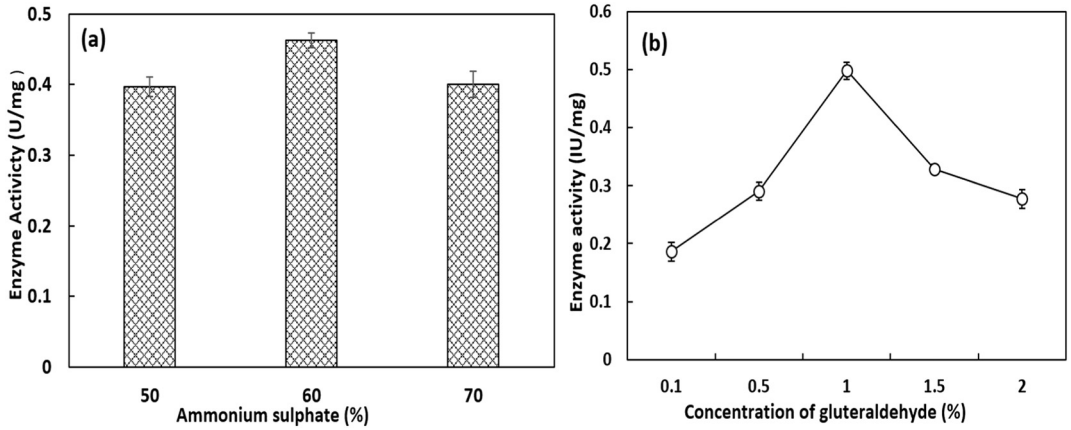

Fig. 1. (a). Effect of ammonium sulfate concentrations on enzyme activity during enzyme aggregation at 4 °C; (b) effect of glutaraldehyde concentration on the enzyme activity of CLEA-AL.

crosslinking method without the use of any co-precipitants or protective agents during the CLEA–AL preparation. The results also assert that the CLEA-AL exhibit the best enzyme activity under optimized conditions such as lower amount of glutaraldehyde as well as shorter crosslinking time. Fig. 2 shows the SEM micrographs of CLEA-AL with (10 mg/mL) 60% ammonium sulfate as the precipitant and 1% glutaraldehyde used as the cross linker. The image shows that the CLEA-AL enzyme aggregates into agglomerated particles with an average particle size of 50 ± 10 μm as estimated from SEM data.

3.2. Effect of pH and temperature on the activity of native and CLEA-AL

The catalytic potential of enzymes is affected by several environmental factors among which pH and temperature are the most significant. The effect of pH on the activity of CLEA-AL and native enzyme were investigated by carrying out the enzyme assay in the pH range of 6–8 at 37 °C, and the results are shown in Fig. 3a. The maximum activity was observed at pH 6.3 for CLEA-AL, and the activity is seen to decrease with increase in pH. The activity of native enzyme with changes in pH also shows a trend similar to CLEA-AL. Fig. 3a also indicates that crosslinking did not alter the influence of pH on enzyme activity of the alginate lyase in the pH range of 7.4 to 8. However, the effect of pH on the activity of CLEA-AL was more significant compared with that on native alginate lyase. The appearance of these high activities of CLEA-AL at initial pH could be associated with a change in the microenvironment surrounding the enzyme associated with their aggregation and crosslinking [28]. The goal of this work is to demonstrate potential application of CLEA-AL for biological applications in near future. Hence, the goal accordingly is to apply these CLEA-AL entrapped alginate microbeads for physiological applications. Depending on the in-vivo site of implantation and applications, the local pH over time can vary drastically. Hence, the stability and activity of CLEA-AL in this study were conducted under various pH ranges. For example, it is known that the during osteoclast mediated resorption of bone the local pH can drop well below physiological pH [29,30] and the pH value during skin wound healing process can also change from alkaline to acidic range [31,32].Therefore, understanding the influence of pH on CLEA-AL activity and stability is of immense importance. As the pH changes, the ionization of groups both at the enzyme's active site and on the substrate can alter, influencing the rate of binding of the substrate to the active site. These effects are often reversible. It is known that the optimum pH for an immobilized enzyme shifting to a higher or lower pH depends on the type of immobilization [33], ionic interaction between the enzyme and cross-linking agent [34]. The shift in optimum pH towards a less acidic pH value upon immobilization may be due to the difference in hydrogen ion formation of glutaraldehyde during the cross-linking process [35].

The activity of enzyme is also strongly dependent on the temperature. The influence of temperature on the activity was studied from 30 to 80 °C. Both native and CLEA alginate lyase showed that the enzyme activity increased with increase in temperature to reach a maximum before decreasing slightly. The native and CLEA-AL both show a maximum enzyme activity at 40 °C (Fig. 3b). However, at higher temperature, the CLEA-AL showed greater activity, which indicates that, the CLEA-AL displays better heat resistance than the native alginate lyase.

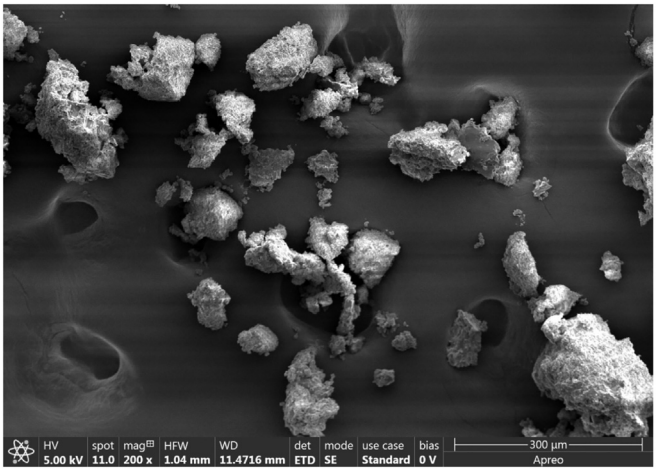

Fig. 2. SEM image of CLEA-AL with 60% ammonium sulfate.

3.3. Thermal stability of native and CLEA-AL

The aim of this study is not only to use the CLEA-AL entrapped alginate beads for biomedical application under physiological conditions, but also to fundamentally understand the stability of the CLEA-AL at various temperatures different from physiologically relevant temperatures. Fig. 4 shows the thermal stability of native and CLEA-AL at different temperatures with various time intervals. As indicated in Fig. 4 the thermal stability of CLEA-AL was significantly higher than the stability of native AL although as expected, the enzyme activity of both the CLEA and native AL decrease linearly as the incubation time is increased. Moreover, CLEA can be seen to be thermostable at 37 °C while above this temperature the activity decreases only marginally with increase in temperature.

This increased thermal stability may be due to the ordered arrangement of the molecules by virtue of the presence of inter and intramolecular cross-linkages that are observed within and between the aggregates, giving the rigidity of the three-dimensional arrangement of the molecules in the CLEA [36]. This increased thermal stability of CLEA-AL is indeed a useful property essential for organic reactions to

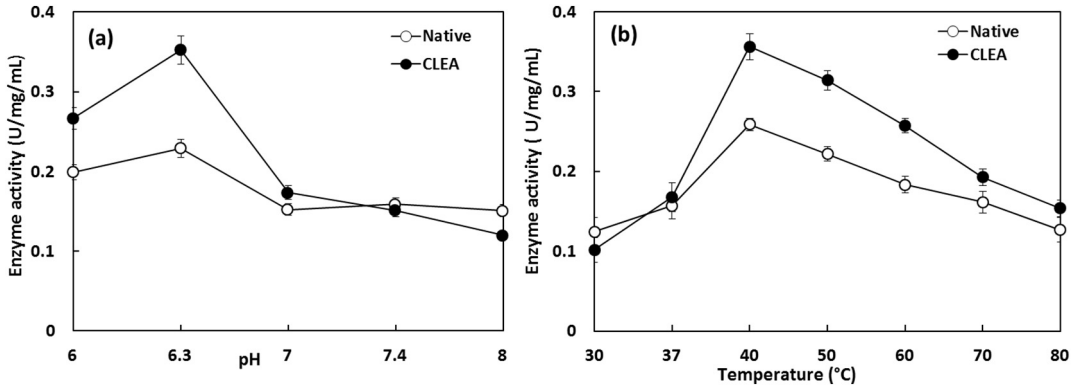

Fig. 3. (a). pH profile of native alginate lyase (1 U) and CLEA-AL (1 U) in phosphate buffer (pH 6–8); (b) temperature profile of native (1 U) and CLEA–AL (1 U) in phosphate buffer (temperature 30–80 °C).

be conducted at an elevated temperature. The previous reports on AL-CLEA also showed higher enzymatic residual activity than native enzyme at higher temperatures after incubation of 1 h [22]. The resistance of the immobilized enzyme to undergo any changes at higher temperature changes is considered as a significant and a major advantage in the multiple practical applications of enzymes in related technologies other than biomedical. It is known that the thermal stability of the enzyme will change after immobilization [28]. Most always, thermal stability of the immobilized enzyme significantly improves compared to native enzyme. This implies that the immobilization of the enzyme by cross-linking could increase the resistance to denaturation of the enzyme to maintain high catalytic activity within a broader temperature range, and this property is crucial in practical applications such as biotransformation reactions. At elevated temperatures, many enzymes tend to become (partly) denatured or inactivated, as a result of which they are no longer active and functional to perform the desired tasks.

3.4. Reusability of the CLEA-AL

The reusability of the immobilized enzyme is essential for cost-effective use of the enzyme in repeated batch or in continuous industrial processes as well as in drug delivery devices under various environmental conditions. Reusability is essentially defined as the number of times the enzyme can be recycled without much change in its activity [36].

The main advantage of cross-linked enzyme aggregates is that it leads to a reusable enzyme preparation. CLEAs can thus be reused either by filtration or by centrifugation. The reusability of CLEAs was studied up to 8 cycles. After each cycle, the CLEA was separated from the reaction mixture by centrifugation, washed and used again in the next cycle. As shown in Fig. 5, the activity of CLEA-AL decreased to approximately 20% of its original activity in each cycle and retains only 20% of its initial activity after eight cycles. This observed enzyme activity reduction can be explained by the leakage of unbound enzyme produced from the previous cycle as well as the reaction products attached to the CLEA surface which cannot diffuse out of the matrix similar to what has been observed before and reported [22].

3.5. Stability of CLEA-AL in organic solvents

Aqueous medium is generally known to be the natural environment for evaluating and assessing enzyme action. However, it is often advantageous to shift to a non-aqueous reaction environment when employing enzymes as practical biocatalysts in synthetic chemistry or biotechnology related fields. The enzyme activity in organic solvents is intimately related to the water content, size and morphology of the catalyst particle and to the enzyme microenvironment [37]. The most important factor in the balance between stabilization and inactivation, due to organic phase, is the solvent polarity. Solvents of lower polarity (i.e. greater hydrophobicity) are less effective in disrupting the structure of the necessary tightly bound water molecules in the cross-linked aggregates compared to a highly polar solvent [38]. Therefore, the measurement of the solvent polarity is important to understand the role of polarity on enzyme activity. The stability of CLEA-AL in different

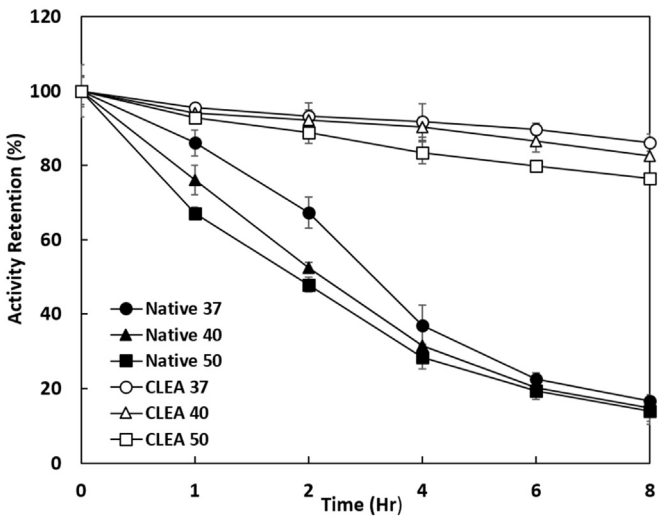

Fig. 4. Thermal stability of native (1 U) and CLEA-AL (1 U) at 37°, 40°, and 50 °C with time.

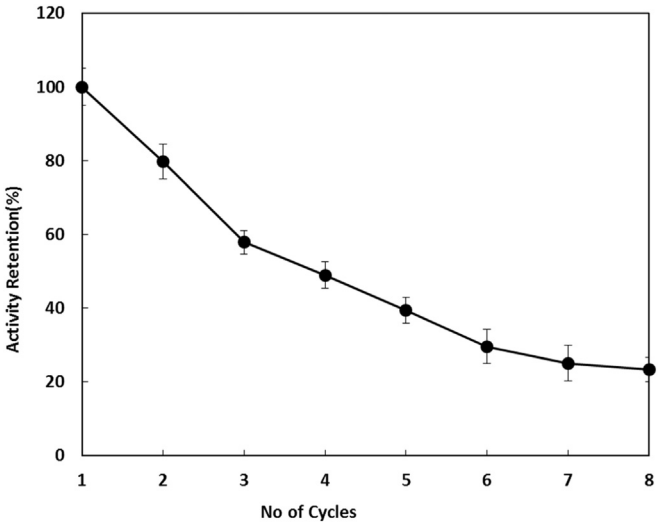

Fig. 5. Reusability of CLEA–AL (1 U).

solvents was accordingly studied and is shown in Table 1. The best measure of polarity is the logarithm of the partition coefficient (log P) in a standard octanol/water biphasic system, which is considered to be an indicator of the hydrophobicity coefficient of substances [39]. Elevated bio catalytic activity was found in solvents having log P value between 2 and 4. The enzyme activity is generally low in solvents with log P <2, medium in solvents with log P between 2 and 4, and high in non-polar solvents with log P >4 [21,40]. CLEA-AL has high activity in nonpolar solvents such as hexane and cyclohexane due to their low dielectric constants and higher log P values. CLEA-AL exhibited medium activity in organic solvents such as, isopropanol and ethyl acetate. Their log P values are in between 0.73 and −0.24 and the dielectric constant between 4.8 and 20.7.

The decrease in enzyme activity in these polar solvents is due to the stripping of water from the surface of the enzyme by the polar solvent molecule [38,39]. Removal of the water molecules leads to a decrease in the rate of the enzymatically catalyzed hydrolysis process and hence, reduced catalytic activity of alginate lyase. Solvents such as DMSO and DMF thus were accordingly, observed to display very low enzyme activity due to their higher dielectric constant. The relatively high activity retention of CLEA-ALs in the presence of organic non-polar solvents can be attributed to the rigidity of the enzyme. Multiple covalent linkages may exert a significant stabilizing effect on the enzyme, and these more rigid enzyme molecules are expected to be more resistant to the conformational alterations induced by the solvents. The previously published CLEA-AL result on solvent stability also exhibited reduced residual activity in ethanol and acetone [22].

3.6. Storage stability of native and CLEA-AL

Storage stability for the immobilized enzyme is one of the significant indexes to evaluate the properties of enzyme, which can make the immobilized enzyme more advantageous than that of the free counterpart. In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced [41].

The native and CLEA-AL were stored at 4 °C and the activity was measured at various time points up to 50 days to determine the storage stability of cross linked enzyme aggregate (Fig. 6). The residual activity of the immobilized enzyme was 90% after 50 days of storage compared to 65% of the native enzyme. The result clearly indicates that the CLEA-AL exhibits an improved stability compared to native soluble enzyme stored over a period of time.

3.7. Incorporation of native enzyme and CLEA-AL in alginate hydrogel

Encapsulation technologies are employed in medicine, pharmaceutics, agriculture, and the cosmetic industries for the development of controlled-release delivery systems [42,43] of biopolymers. Encapsulation or entrapment of soluble enzymes in various polymer matrices, although has been executed in the past is known to encounter serious problems owing to the enzyme permeating out of the matrix due to the larger pore size of the matrices of natural polymer derived materials [21]. This uncontrolled diffusion of the enzymes leads to unpredictable degradation of the gel and hence, undesired release of any drugs or biofactors from the hydrogel scaffolds. In this study, our focus was to encapsulate the native and CLEA-AL in alginate hydrogel beads and evaluate the effect both native and cross-linked enzyme on the degradation of the hydrogel. It is known that sodium alginate and $CaCl_2$ concentrations are the critical parameters for enzyme encapsulation in alginate gel beads due to gelation kinetics leading to cross-linking between alginate and Ca^{2+} ions. The influence of concentration of alginate, calcium chloride and several other parameters on the formation of hydrogel beads is well documented and has been widely reported by various researchers [44,45]. In order to prepare the hydrogel beads in this study, different alginate (1, 2 & 3%) and calcium chloride (0.1 to 1 M) concentrations were chosen. The formation of alginate beads with native enzyme was also assessed starting with different concentrations of enzyme in the alginate solutions. Hydrogel beads were formed instantaneously by ionotropic gelation in which inter molecular cross-links were formed between the divalent calcium ions and the negatively charged carboxyl groups of the alginate molecules. We noticed that the use of a low sodium alginate concentration (<1%, w/v) resulted in the production of mechanically weak and deformed hydrogel beads. On the other hand, use of a high alginate concentration (>3%, w/v) prevented the extrusion of the highly viscous solution through the needle. Depending on the uniform size and distribution of the hydrogel beads formed, the optimal alginate and cross-linking concentrations was identified to be 2% and 0.5 M respectively, and hence, these concentrations were selected for further experiments.

The morphology of control with no enzyme, native enzyme of concentration and CLEA-AL of enzyme concentration loaded alginate hydrogel beads were observed via optical dissection microscope and SEM. The optical dissection microscopic images (images were not given) of control hydrogel beads in the wet state were observed as spherical shape in appearance with a smooth surface. The native enzyme loaded beads showed varying spherical shapes due to the change in viscosity of the enzyme containing alginate solution. During enzyme concentration optimization process, it was found that with increasing the amount of enzyme to alginate solution, the shapes of the beads were lost due the instant hydrolysis of the alginate in addition to the low viscosity of the enzyme containing solution. The appearance and shape of CLEA-AL incorporated hydrogel beads were found to be similar to the control. SEM was further used to characterize the microstructure of the beads. Freeze dried CLEA loaded alginate hydrogel beads was

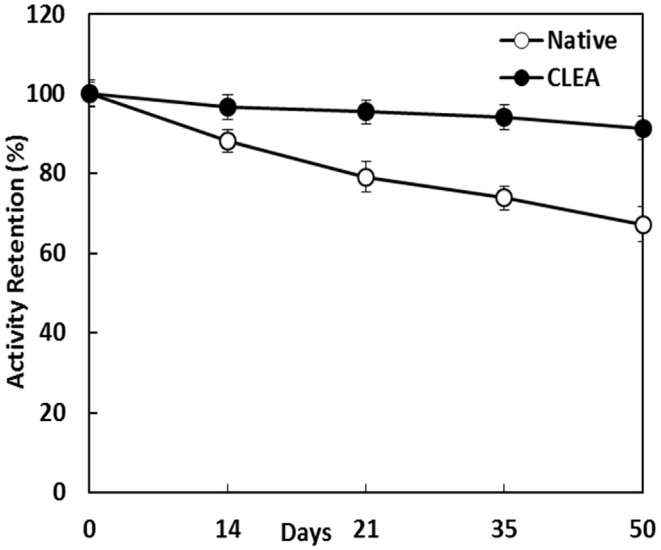

Fig. 6. Storage stability of free and CLEA-AL (1 U) at 4 °C.

Table 1
Stability of CLEA alginate lyase (1 U) in different solvents.

Solvent	Polarity	Dielectric constant ε	Log P	Activity retention (%)
Hexane	Nonpolar	1.9	4.0	68.0 ± 9
Cyclohexane	Nonpolar	2.0	3.44	58.5 ± 6
Water	Polar aprotic	80.2	1.0	46.4 ± 3
Isopropanol	Polar aprotic	19.9	0.05	43.9 ± 3
Acetone	Polar aprotic	20.7	0.2	37.8 ± 2
Ethanol	Polar aprotic	24.6	1.0	34.8 ± 4
Methanol	Polar aprotic	32.7	0.05	29.4 ± 4
Ethyl acetate	Dipolar aprotic	6.0	0.73	41.9 ± 1
DMF	Dipolar aprotic	38	−1.0	14.6 ± 1
DMSO	Dipolar aprotic	47	−1.3	22.4 ± 1

similar in appearance as of control alginate hydrogel beads (Fig. 7a and c). The beads were approximately spherical in shape and had a rough surface. In the case of native enzyme loaded alginate beads, the alginate appears to shrink and collapse slightly (Fig. 7b). The cross-sectional image of the control hydrogel bead (Fig. 7d) showed a homogeneous interconnected structure with numerous pores throughout the hydrogel bulk. The cross sectioned control hydrogel beads showed an average pore diameter of 35 ± 10 μm as estimated from SEM data. The native enzyme incorporated hydrogel bead (Fig. 7e) indicated presence of large pores (110 ± μm) and internal cavity with evenly distributed layers inside the beads. The change in the surface morphology can be attributed to the partial collapse of the polymer network due to the burst hydrolysis of gel via the native enzyme incorporation. However, in the case of the CLEA-AL loaded hydrogel beads the porous network structure appears to have disappeared (Fig. 7f), which might be due to the integration and filling up of the CLEA–AL particles into the pores of the alginate hydrogel. Further, it should be noted that the cross-linked aggregates structural integrity was fully preserved thus, confirming the encapsulation of the aggregates into the hydrogel.

3.8. Controlled release of alginate lyase CLEA protein and degradation of hydrogel beads

There are few immobilization methods of alginate lyase that are reported for controlling the rate of degradation of alginate hydrogel utilized for different application in the biomedical fields [10,12]. The different approaches reported primarily involve use of low concentration of native enzyme and direct entrapment in the alginate gel [8], the encapsulation of AL in the poly (lactide-*co*-glycolide) (PLGA) microspheres and subsequent entrapment of these microspheres in alginate gel [10,12]. These microsphere immobilization methods reported indicate efforts that have been partially successful by controlling the amount of enzyme incorporated in microspheres and its release from microspheres for shorter periods. However, due to the low enzyme loading efficiency in the PLGA microspheres, the amount of enzyme was insufficient to degrade the alginate hydrogel [12]. The degradation of alginate hydrogel beads occurs through alginate lyase dual mechanisms mainly comprising enzymatic hydrolysis and ion exchange. During the initial period of degradation, hydrolysis mainly occurs due to the enzymatic cleavage of the glycosidic bonds between the monomers of basic repeating M units of alginate [10]. Depending on the enzymatic hydrolysis of the glycosidic linkages, the cross-linked Ca^{2+} ions become more sensitive to ion exchange. The alginate structure thus disintegrates as a result of the out flux of Ca^{2+} ions, which are replaced by monovalent ions resulting in decreased crosslinking density and, thus, a weakened hydrogel structure results associated with an increase in pore size. The pore size of an encapsulation material is critical to both encapsulation and release kinetics. Larger pore size will allow for the leakage of the contents and the small pore size hinder timely release [46,47]. Alginate pore size has been extensively researched through various techniques, mainly via imaging and diffusivity measurements [46,48]. The CLEA-AL developed in this work, has an aggregate size of 50 ± 10 μm (Fig. 2) as indicated in Section 3.1, which is larger than the pore size of the control hydrogels beads (35 ± 10 μm, Fig. 7d). It can thus be construed that CLEA-AL developed in this work is not expected to cause leakage of the enzyme from the aggregates under normal conditions. However, it should be noted that the CLEA-AL encapsulated hydrogels showed no porosity (Fig. 7f). Hence, as we hypothesized, this encapsulation of the micron-sized CLEA-AL in the alginate hydrogel can help in controlled degradation of the matrices and therefore, can lead to the desired release of clinically relevant drugs, biologics or cells entrapped in the hydrogels. To verify this hypothesis, the degradation rate of the native and CLEA-AL entrapped alginate hydrogels beads were carried out in PBS at 37 °C. We investigated the activity of the released protein from the CLEA by studying the degradation of the CLEA encapsulated alginate hydrogel beads. Same amount of alginate lyase (1 U) either in native form or as a CLEA was entrapped inside the hydrogel beads and the degradation of the alginate beads were assessed by measuring the amount of reducing sugars released.

In order to check the stability of CLEA-AL, we monitored the release of alginate lyase enzyme protein from the CLEA at different time intervals. As shown in Fig. 8a, cumulative release of alginate lyase (AL) protein from CLEA increases with time and the amount of enzyme protein released from CLEA increased with time. It should be noted that one unit of CLEA–AL contains 1691 μg/mL of proteins as was measured by BCA method and therefore, it can be seen that all the protein was completely released from the CLEA within 28 days. The figure also indicates sustained release of the AL over a period of 28 days although the

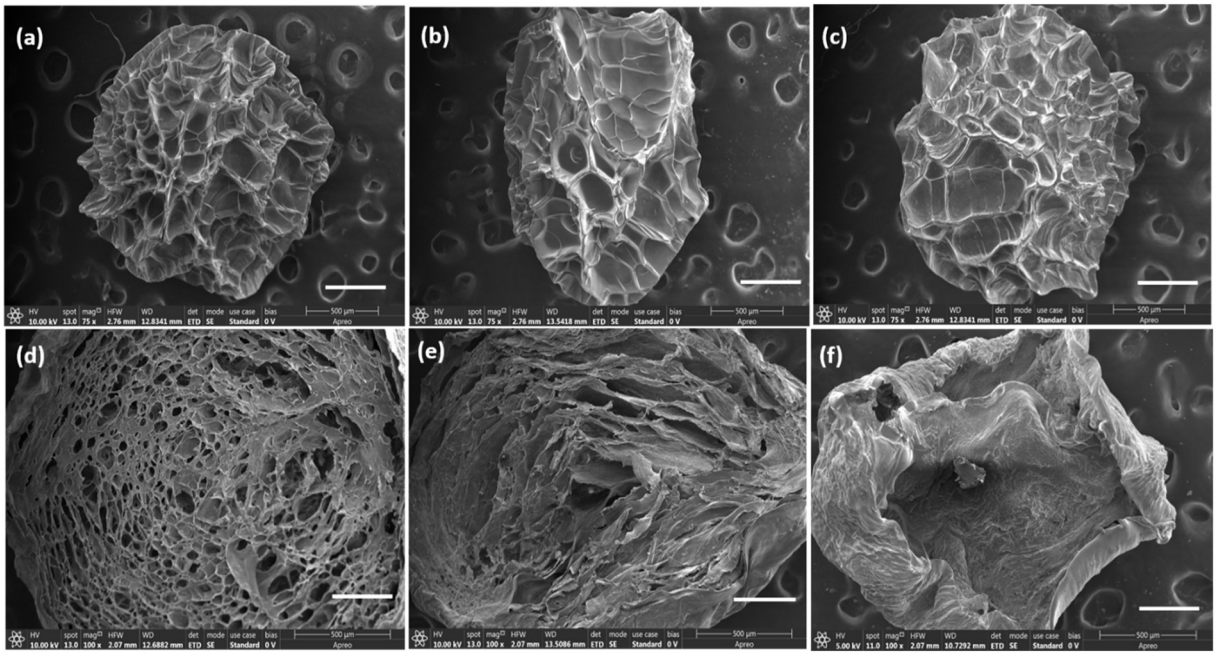

Fig. 7. SEM images of hydrogel beads with (a) control hydrogel (0 U); (b) native enzyme (1 U) and (c) CLEA-AL (1 U) incorporated; (d) cross sectioned control; (e) cross sectioned native and (f) cross sectioned CLEA-AL incorporated (scale bar 500 μm).

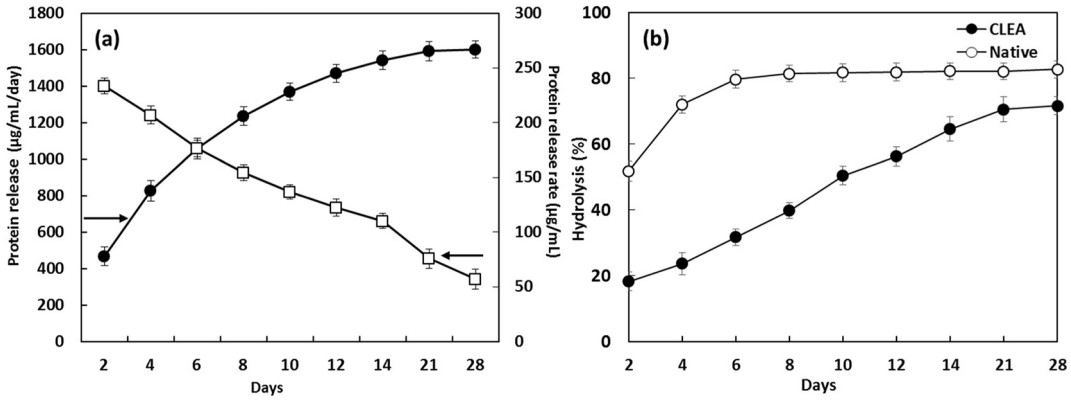

Fig. 8. (a). Cumulative release of AL enzyme protein from CLEA (left y axis) and protein release rate (right y axis) with time; (b) degradation of 2% alginate hydrogel beads with native and CLEA-AL enzyme at 37 °C.

rate of release decreases with time. This controlled release capability of CLEA-AL can be applied to degrade hydrogels in a controlled way by encapsulating them in hydrogels.

Fig. 8b on the other hand, shows that the hydrolysis of alginate hydrogel beads using native enzyme was rapid and 50% degradation was obtained within 48 h. Subsequently de-polymerization occurs from day 2 to day 6. Further increase in hydrolysis time shows no effect on the production of reducing sugar and therefore, it can be assumed that complete degradation of the hydrogel occurs within 6 days. On the other hand, CLEA-AL encapsulated hydrogel beads showed a slow release of the reducing sugar with the alginate lyase CLEA incorporated in the hydrogel beads showing a sustained degradation with time and 70% of the original hydrogel beads were hydrolyzed by day 21.

These results show that the hydrogel degradation with CLEA-AL encapsulated beads are slower compared to native enzyme incorporated hydrogel beads. This also indicates that the formation of a stronger aggregate was obtained with 1% glutaraldehyde and 60% ammonium sulfate and therefore, slower release of AL from CLEA can thus be achieved to control the degradation of the alginate hydrogel. Moreover, the size of the aggregates, especially, under a dynamic body fluid flow conditions can also be very important. If the fluid flow is high and sizes of the CLEA are small compared to the pore size of the hydrogel, the CLEA may diffuse out of the gel before it can start releasing the enzyme to degrade the hydrogel. As discussed earlier, the size of the CLEA particles obtained in this study (50 µm) is larger than the average pore diameter of the alginate hydrogel (35 µm). Therefore, it can be assumed that the likelihood of the CLEA particles diffusing out from the hydrogel is minimum and most of the CLEA particles remain entrapped inside the hydrogel network throughout the current period of study. It, therefore, can be concluded that the slow release of the enzyme from CLEA particles (Fig. 8a) entrapped inside the hydrogel causes the slow and controlled hydrolysis of the gel with time (Fig. 8b). This study also clearly shows the importance of the chemical nature of the cross-linked enzyme present in CLEA as well as the size of the CLEA aggregates on the controlled degradation of the alginate gel and hence, enabling a programmed release of drugs/cells/biomolecules from the hydrogel. Future studies will focus on use of this CLEA platform for studying control release of drugs/cell/s as well as biomolecules.

4. Conclusions

A simplified procedure for the preparation of immobilized alginate lyase using aggregation and cross linking is described. The obtained results demonstrated that the developed CLEA retains their enzyme activity under various pH, temperature, solvents, long storage period conditions, and can also be reused. Moreover, the degree of aggregation and size of the aggregate of the alginate lyase are the major factors to control the degradation of chemically cross linked alginate hydrogel. The degree of aggregation control the release of the native enzyme from the aggregate and larger size of the CLEA prevents the escape of the aggregated particles from the hydrogel. This method of entrapping CLEA along with various growth factors, drugs and cells in the hydrogel can therefore be used as a platform to achieve controlled release of biologics and cells from the alginate hydrogel that can also enhance the regeneration of various tissues.

Acknowledgements

The authors gratefully acknowledge the financial support of NSF-ERC, "Revolutionizing Metallic Biomaterials", Grant # EEC-0812348, and NSF-CBET 0933153. PNK also acknowledges the support of the Edward R. Weidlien Chair Professorship funds, University of Pittsburgh and the Center for Complex Engineered Multifunctional Materials (CCEMM), University of Pittsburgh for partial support of this research.

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