

## Research paper

# Extended release of high $pI$ proteins from alginate microspheres via a novel encapsulation technique

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**Abstract**

Alginate has potential as a matrix for controlled delivery of protein-based drugs that require site-specific long-term delivery. In the current work albumin, lysozyme and chymotrypsin were encapsulated into alginate microspheres using a novel method that involved soaking the microspheres in a protein-containing NaCl solution. This was followed by recrosslinking with calcium chloride. High  $pI$  proteins also appeared to physically crosslink the sodium alginate which resulted in more sustained release. Release was affected by the nature of the releasate solution. In TRIS buffered saline, the high  $pI$  proteins chymotrypsin and lysozyme showed sustained release lasting over 150 h. Release into 0.15% NaCl led to relatively constant release of lysozyme and chymotrypsin over more than 2000 h; reduction of the releasate volume lengthened the lysozyme release to greater than 8 months. Released lysozyme was shown to remain active for at least 16 days, in some cases with activity greater than 100% of the active control. This encapsulation technique can therefore be used to rapidly load alginate microspheres with proteins, with high isoelectric point proteins showing particular promise. Furthermore, the interactions between the high  $pI$  proteins and the alginate gel could potentially be exploited to generate new protein delivery systems.

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**Keywords:** Alginate; Crosslinking; Protein delivery; Isoelectric point; Lysozyme; Chymotrypsin; Albumin

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**1. Introduction**

Biopharmaceutical development has led to the production of innovative protein products with the potential to cure disease and rejuvenate tissue. However, delivery of these proteins to specific sites of action in an active form, at an appropriate concentration and for an appropriate duration still remains a challenge especially since the labile structures of proteins are prone to hydrolytic and enzymatic degradation [1,2]. Various modalities have been used to facilitate the delivery of active proteins to the site of action. One promising method of delivery involves the use of microspheres, which can be used to target localized delivery sites [3,4]. Hydrogel microspheres may protect proteins from degradation while

releasing them continuously over a prolonged period of time. Ideally, a controlled release profile with no burst will be followed by device degradation into bio-inert products [5]. By delivering active protein at a designed rate to a localized site, efficacious treatment of a variety of diseases may be achieved [6].

Alginate is a naturally occurring, heteropolysaccharide hydrogel of  $\beta$ -D-mannuronate and  $\alpha$ -L-guluronate, which is physically crosslinked with divalent ions such as calcium to form an anionic hydrogel [7,9]. Alginate hydrogels are ideal for protein delivery applications since the mild crosslinking conditions used for the preparation of these gels do not require the use of solvents that may interfere with protein activity and *in vivo* response to the gel. Since the bonds in calcium-crosslinked alginate are reversible, the gel is prone to rapid bulk degradation in the presence of chelating or monovalent ions. As a result, burst release profiles have been observed, particularly with electronegative proteins such as albumin [10,11]. However, recent studies involving the encapsulation and release of electropositive

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proteins have shown encouraging lengthened release profiles of active protein. Proteins such as vascular endothelial growth factor (VEGF) have shown high encapsulation efficiency in alginate followed by lengthy and controlled release past a period of 3 weeks [10,12]. The sometimes accompanying increased protein-activity relative to controls was suggested to be the result of gel–protein interactions [13]. There is clearly a need to better establish the relationship between protein charge and release profile from alginate gels to better determine whether these gels are suitable for protein delivery.

Loading of protein into alginate microspheres is commonly performed during their synthesis by the addition of the protein to the sodium alginate solution prior to crosslinking [7]. However, this may result in protein loss during gelation due to diffusion from the concentrated gel to a less concentrated large volume crosslinking solution. Methods involving the immersion of gelled alginate in protein solutions have also previously been employed [8]. A quick encapsulation method performed post-synthesis would be more effective, minimizing protein loss and increasing encapsulation efficiency. In addition, such a procedure would facilitate large-scale synthesis allowing high amounts of blank microspheres to be synthesized and subsequently loaded for delivery various proteins in many different applications.

In the current work, a novel post-synthesis encapsulation method that involves the partial degradation and recrosslinking of alginate microspheres was developed and has been investigated as a more efficient soaking encapsulation method. A comparison between electronegative and electropositive proteins was performed. Further exploration of the potential of the electropositive proteins in controlling release from the spheres was examined with comparison to the well-established release of the protein bovine serum albumin (BSA). Finally, the effect of different release solutions was examined to determine their influence on *in vitro* release profiles, with correlation to potential applications for these delivery systems.

## 2. Materials and methods

### 2.1. Materials

Sodium alginate produced by the kelp *Macrocystis pyrifera* was purchased from Sigma–Aldrich (Oakville, ON). This alginate has a molecular weight ranging from 12,000 to 80,000 and contains 61% mannuronic acid and 39% guluronic acid making it a low viscosity alginate. BSA, lysozyme and chymotrypsin were also obtained from Sigma–Aldrich (Oakville, ON). Coomassie Brilliant Blue G-250 was purchased from Fluka Chemicals (Switzerland). The *Micrococcus lysodeikticus* cells were from Worthington Biochemical Corporation (Lakewood, NJ). Other reagents were purchased from Sigma–Aldrich (Oakville, ON) and EM Science (Gibbstown, NJ).

### 2.2. Microsphere synthesis

Dropwise addition of a 3% sodium alginate solution into a 4 °C crosslinking bath containing 0.1 M calcium chloride was used to produce calcium alginate microspheres similar to previous literature [14–16]. Using a syringe pump, droplets were extruded at 5 mL/h through a flat 25G5/8 sterile needle. Airflow of 16.86 L/min through 4.5 mm tubing was applied downwards over the needle to reduce droplet size. Observations suggested that the droplets were spherical as they entered the crosslinking bath. Newly formed microspheres were stirred in the calcium chloride bath for 30 min. The resulting microspheres had minimal defects and a diameter of  $700 \pm 120 \mu\text{m}$  as determined by light microscopy and light scattering.

### 2.3. Protein encapsulation

Lysozyme and chymotrypsin as model protein drugs were investigated for encapsulation and release using calcium alginate microspheres and the results compared with those obtained for BSA as the release of BSA from alginate has been well documented in the literature. Relevant protein properties are shown in Table 1.

Typically for loading, protein is added to a sodium alginate solution followed by crosslinking of the alginate into spheres [7]. Alternatively, protein may be loaded by immersing the spheres in an aqueous protein-containing solution [8]. BSA was loaded by dissolving it in the aqueous sodium alginate solution, up to a concentration of 15 mg/mL, and subsequently crosslinking during microsphere synthesis. Electropositive proteins have also been previously added to alginate using both methods [10,12,17–19]. However, the interaction of both lysozyme and chymotrypsin with alginate was strong enough to result in precipitation. Thus a soaking method was required for loading these proteins into the matrix.

The encapsulation technique developed involves the production of blank microspheres that are then soaked in a low concentration aqueous protein solution containing sodium chloride (NaCl) (0.15%), which partially degrades the alginate, allowing for the uptake of the protein. This was followed by immersion in calcium chloride solution (1 M) for 2 min to ensure complete recrosslinking of the alginate. Approximately 1 mL of protein solution, ranging in concentration from 1 to 20 mg/mL, was used per 10 mg of freeze-dried or 0.5 g of swollen spheres. Freeze-dried microspheres were investigated as this allows for long-term storage and they may potentially take up more of the protein as they swell in the concentrated protein-containing

Table 1  
Properties of BSA, lysozyme and chymotrypsin

Protein	BSA	Lysozyme	Chymotrypsin
Molecular weight	66,400 Da	14,400 Da	25,000 Da
Isoelectric point	4.7	11.0	9.1

solution. A protein soaking solution containing no NaCl served as a control. Following recrosslinking the microspheres were rinsed with water to remove residual calcium chloride.

Encapsulation studies were performed to determine the time required for the protein in the soaking solution to equilibrate within the spheres. Loaded microspheres were dissolved in 5% sodium citrate and the resultant solution tested for protein content. The loading was calculated as the  $\mu\text{mol}$  of protein per gram of alginate in the microspheres. Comparisons were made between the novel method and the typical method of protein addition prior to crosslinking.

#### 2.4. Protein release studies

Release studies were carried out in three different media: phosphate buffered saline (PBS) (pH 7.4), TRIS buffered saline (TBS) (pH 7.4) or 0.15% sodium chloride. BSA-containing spheres were generally prepared by adding the protein to the alginate solution followed by crosslinking as this was found to result in the highest levels of loading, although the degradative-soaking loading method was used for comparison in TBS release. Conversely, release studies were performed on lysozyme and chymotrypsin-loaded microspheres prepared using the novel soaking method. Protein concentrations of 150 mg/mL for BSA and 1 mg/mL for lysozyme and chymotrypsin in alginate were used for the novel soaking encapsulation. Note that higher concentration soaking solutions were required for isonegative BSA in order to obtain comparable loadings to the isopositive lysozyme and chymotrypsin. For analysis, two repeats were performed for each release study; this is considered representative since each sample contained of the order of hundreds of microspheres. Release medium (approximately 20 mL/g of swollen spheres or 0.6 mL/mg of dried spheres) was added to the normal and freeze-dried microspheres, respectively, and the studies were performed in milk powder-blocked centrifuge tubes that were placed in a shaking water bath held at 37 °C and 40 rpm. Samples were taken at regular intervals and replaced with fresh medium. Sample volumes were reduced over time to accommodate the increasing volume of microspheres, which had a tendency to swell in the release medium. Once the spheres had swollen substantially, release study containers were centrifuged at 600–3000 rpm for 4–5 min. Release was carried out until no more protein was detected and microspheres were completely degraded, as noted through visual inspection with a light microscope.

#### 2.5. Protein detection

Protein was detected using a variation of the well-established Bradford assay adapted for use in a microplate reader [20]. Briefly, 100–200  $\mu\text{L}$  of Coomassie Brilliant Blue dye

reagent containing 0.01% Coomassie Brilliant Blue G-250, 4.5% w/v ethanol and 8.5% w/v *O*-phosphoric acid prepared in de-ionized water was added to the protein samples. The absorbance was read at 595 nm and the concentration determined from comparison with known standards.

#### 2.6. Activity of released lysozyme

Lysozyme bioactivity can be measured by the break up of *Micrococcus lysodeikticus* cell walls [21]. To detect the activity of protein released from alginate, microspheres containing lysozyme were soaked in sodium chloride solutions. After periods of time, microspheres were removed, rinsed with water, and then soaked in PBS for 2 h to ensure high concentrations of protein were available for testing. The PBS-release samples were then tested for activity and compared against fresh, active controls of the same concentration. The initial rate of turbidity reduction between release samples and their positive controls were compared to determine the percent bioactivity of the released lysozyme.

### 3. Results

#### 3.1. Protein encapsulation

The standard encapsulation method of adding protein prior to microsphere crosslinking was successful for BSA loading. However, it was found that when the sodium alginate (3.75% w/v) was directly mixed with 1 mL of 30 mg/mL solution of lysozyme or chymotrypsin, the mixture gelled. This even occurred following the removal of any salt present in the lysozyme solution by dialysis. The degradative-soaking encapsulation method, however, allowed for the incorporation of significant amounts of isopositive protein that would otherwise crosslink the alginate. More than twice as much lysozyme could be incorporated into the spheres when a 10 mg/mL protein loading solution contained degradative NaCl (0.15%) (8  $\mu\text{mol}$  lysozyme per gram of alginate) instead of water alone (3.4  $\mu\text{mol}$  of lysozyme per gram of alginate). Loading efficiencies are summarized in Fig. 1. Clearly, higher loading efficiencies were observed with lysozyme and chymotrypsin when the novel soaking encapsulation was used. Fig. 1 shows the encapsulation of BSA, lysozyme and chymotrypsin over time for both normal spheres and freeze-dried spheres. While approximately 30 min of soaking was found to result in the loading of consistent amounts of lysozyme and BSA, the spheres prepared for release studies were soaked for 1 h to ensure complete protein loading and equilibration within the sphere. BSA soaking concentrations were increased to 150 mg/mL to allow comparable protein encapsulation amounts for the release studies. While a loading time of 1 h was also used for chymotrypsin, Fig. 1 suggests that longer loading times may be necessary, presumably due to the slightly larger size

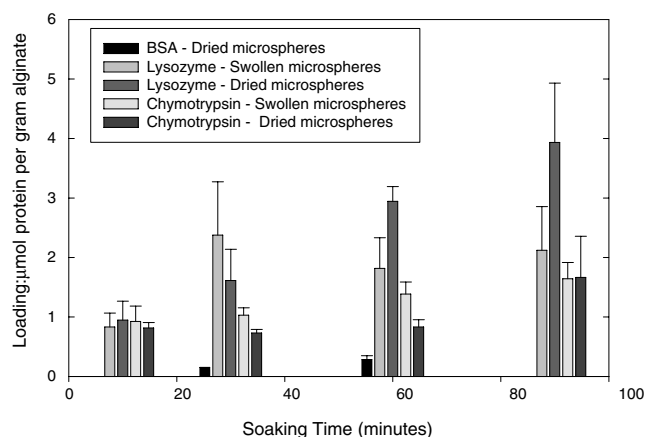


Fig. 1. Loading of proteins using the soaking method  $\pm$  standard error. BSA soaking solutions contained 20 mg/mL of protein in 0.15% NaCl. Lysozyme and chymotrypsin soaking solutions contained 1 mg/mL of protein in 0.15% NaCl. Note that no detectable amount of BSA was incorporated at 10 min. Similar amounts of lysozyme could be incorporated at 90 and 120 min (data not shown), suggesting that an equilibrium had been reached.

of chymotrypsin inhibiting its diffusion as seen with larger molecules ( $>20,000$  Da) in past alginate diffusion studies [11].

### 3.2. Protein release

As shown in Fig. 2, release of BSA and lysozyme into PBS showed a burst and relatively rapid release over a period of 3 h, consistent with the literature [22,23]. Furthermore, and more importantly, it can be seen from Fig. 2 that the release profiles for the two proteins into PBS were similar despite differences in size, charge and loading method.

Protein release into TBS, summarized in Fig. 3, occurred over much more sustained time frames compared to PBS. All proteins were encapsulated by the new soaking technique and all spheres contained similar amounts of protein

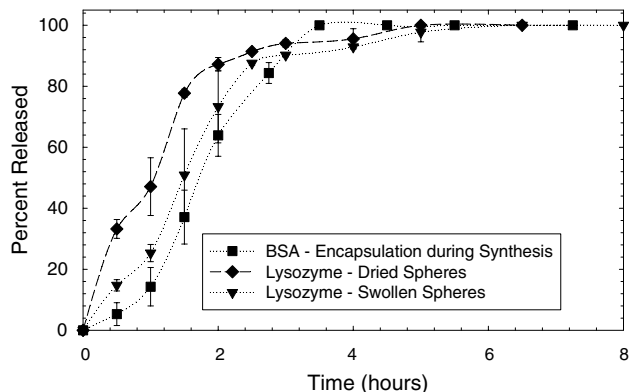


Fig. 2. Protein release from calcium alginate microspheres (diameter = 700  $\mu$ m) into PBS  $\pm$  SEM. BSA encapsulated during synthesis and lysozyme encapsulated through soaking were released into PBS at 37  $^{\circ}$ C and 40 rpm. All releases resulted in bursting profiles.

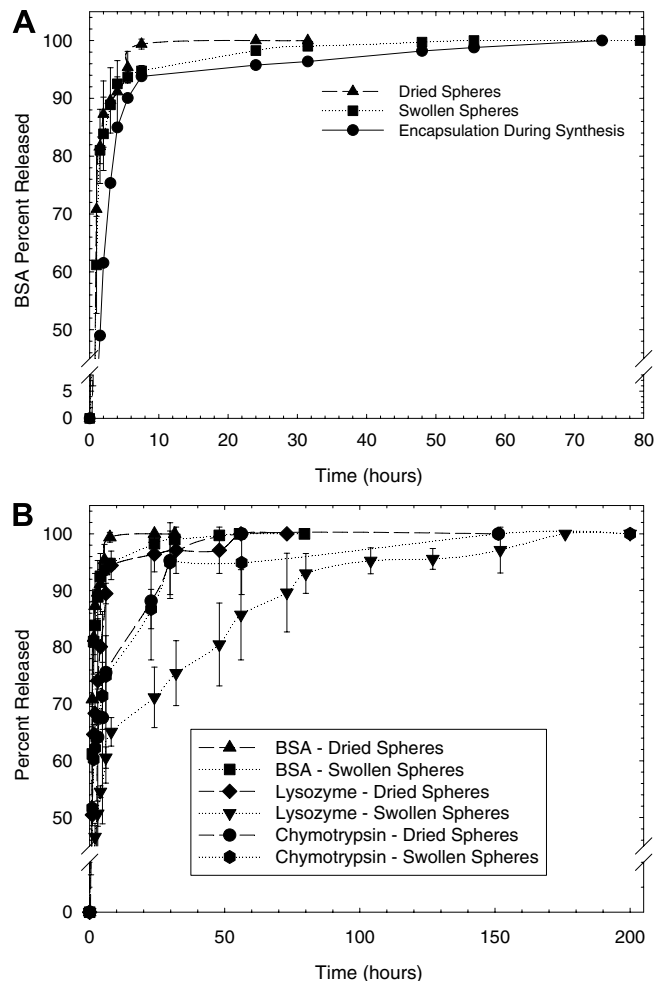


Fig. 3. Protein release from calcium alginate microspheres (diameter = 700  $\mu$ m) into TBS  $\pm$  SEM. (A) A comparison of the release from different loading methods of BSA released at 37  $^{\circ}$ C and 40 rpm, note their similarities. (B) BSA, lysozyme and chymotrypsin were all encapsulated through soaking and were released at 37  $^{\circ}$ C and 40 rpm. Bursting profiles were obtained for BSA and some sustained profiles were obtained for lysozyme and chymotrypsin.

to enable direct comparison. Release of BSA loaded using the standard encapsulation method with addition prior to crosslinking is included for comparison.

Release of the proteins into a 0.15% NaCl solution showed remarkably different profiles for electronegative BSA compared with electropositive lysozyme and chymotrypsin, as shown in Fig. 4. By using a low concentration NaCl solution as the release medium, BSA release was lengthened to approximately 120 h compared from only 3.5 h for PBS and 56 h for TBS. The lower ion concentration in this NaCl solution presumably reduced alginate bulk degradation allowing for examination of the effects of protein–alginate interactions and their influence on release rates. Release of lysozyme from dried spheres was found to increase to approximately 300 h. However, the release of lysozyme from swollen spheres and chymotrypsin from swollen and dried spheres that had been loaded through the soaking method was extremely controlled,



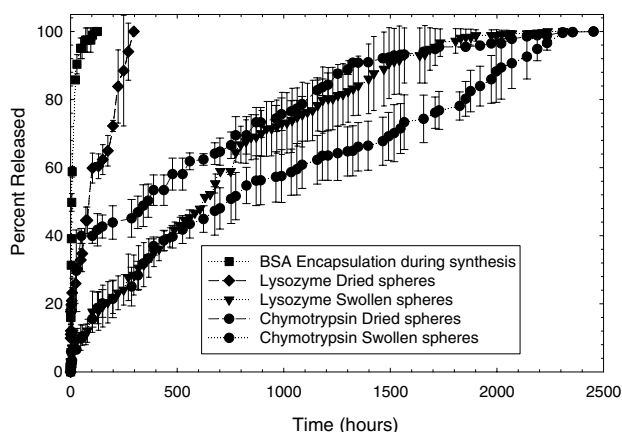


Fig. 4. Protein release from calcium alginate microspheres (diameter = 700  $\mu\text{m}$ ) into 0.15% NaCl ( $n = 2$ )  $\pm$  SEM. BSA encapsulated during synthesis and lysozyme and chymotrypsin encapsulated through soaking were released at 37  $^{\circ}\text{C}$  and 40 rpm. A lengthened profile was noted for lysozyme release from swollen microspheres and for chymotrypsin release from dried and swollen microspheres.

with seemingly zero order release kinetics over a period of more than 90 days. As shown in Fig. 5, decreasing the volume of the release medium to microsphere mass ratio from on average 22 mL/g of spheres to 8 mL/g of spheres resulted in a further prolongation of the release of lysozyme from swelled spheres to over 8 months.

### 3.3. Protein activity

Lysozyme activity was measured for 16 days of release to determine the effect of the alginate on the protein activity. Activities of between 50% and 150% of the control were consistently observed for 16 days, suggesting that the interaction between the alginate and lysozyme has a protective effect reducing protein hydrolysis.

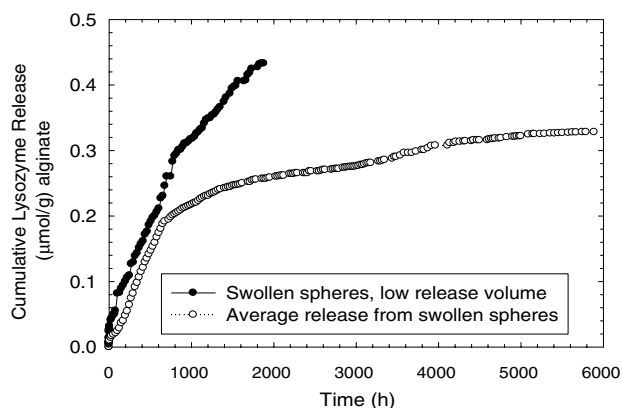


Fig. 5. A comparison on release volume effect on the cumulative release of encapsulated lysozyme from swollen calcium alginate microspheres into 0.15% NaCl at 37  $^{\circ}\text{C}$  and 40 rpm. The sample with a low volume to sphere ratio contains 8 mL of 0.15% NaCl per gram of spheres versus the average 22 mL of 0.15% NaCl per gram of sphere used in most studies. Note that the release conditions were maintained throughout the entire 8 months (37  $^{\circ}\text{C}$  and 40 rpm).

## 4. Discussion

A novel encapsulation technique was developed that allowed for the efficient encapsulation of electropositive positive proteins into alginate microspheres. This was necessary as these proteins showed high affinity for electronegative alginate sufficient to result in its crosslinking and subsequent precipitation. Similar crosslinking phenomena have been observed in other studies between lysozyme and alginate [24]. This new technique allowed for loading to occur post-crosslinking, while still generating robust materials containing large amounts of the proteins. The degradative-soaking mechanism resulted in rapid infusion of electropositive proteins by opening the pore structure through calcium removal by sodium ions. Subsequent “recrosslinking” re-established a tight matrix with sufficiently small pores to trap the protein within the matrix. It was found that twice as much protein could be loaded into the alginate microspheres using 0.15% NaCl versus water imbibition over time periods of the order of an hour or less. These rapid loading times are particularly important as they reduce the potential for hydrolytic degradation of the proteins in solution. Using a soaking solution also eliminates diffusional protein loss that occurs when the protein-containing microspheres sit in a large crosslinking bath. While there is statistical error noted in the encapsulation amounts, refinement of the method over time will likely yield more consistent results. The loadings detected after encapsulation suggest that the protein was loaded within the alginate matrix and was not solely adsorbed to the outer edge of the microspheres. Calculations based on the hydrodynamic radius of the proteins [25,26] and the size and mass of the alginate microspheres indicate that monolayer protein adsorptions would result in  $1.6 \times 10^{-2}$   $\mu\text{mol}$  of lysozyme and  $3.17 \times 10^{-3}$   $\mu\text{mol}$  of BSA per gram of alginate. A substantially higher amount of protein was detected after encapsulation at over 0.5  $\mu\text{mol/g}$  of alginate (Fig. 1) confirming that, in addition to protein adsorption to the surface of the spheres, there is a significant amount of protein loaded into the bulk microsphere structure. This new encapsulation method is of particular relevance to electropositive proteins; similar loadings of electronegative BSA were observed regardless of the method used to load it into the spheres, although the proposed method was found to be considerably simpler and more repeatable. Recent publications loading fibre-based alginate scaffolds with a simultaneous NaCl/CaCl<sub>2</sub> soaking followed by immersion in protein solutions have shown similar potential for quick loading times [27].

Chymotrypsin and lysozyme, with similar electrostatic charge but different molecular weights, were investigated and the release results compared with those obtained for BSA. Into PBS, all three proteins showed a burst release due to the high concentration of degradative phosphate ions within the medium. Lysozyme and chymotrypsin showed more sustained release into TBS and 0.15% NaCl, with chymotrypsin also showing a sustained release from

dried microspheres. The bursting profiles of lysozyme from dried spheres are likely attributable to damage sustained during freeze-drying, with the high loadings presumably due to increased protein adsorption to the higher surface area of these materials from the incurred damage allowing them to release quickly (ESEM photos, not shown).

The positive nature of lysozyme and chymotrypsin resulted in an isoelectric attraction with the anionic alginate, promoting a hindered outwards diffusion that was seemingly independent of molecular weight. In TBS, the release profile of BSA was lengthier but similar to that observed in PBS, which may suggest that the release of BSA from these materials is purely diffusional and is not dominated by alginate degradation or electrostatic interactions. Therefore, based on these results and those of others which suggest that molecules with molecular weights of greater than 20,000 Da diffuse more slowly through alginate [11,28], it would be predicted that lysozyme, with a molecular weight less than 20,000 Da and chymotrypsin with a molecular weight significantly less than BSA, would release faster. However, the sustained release of lysozyme and chymotrypsin suggests that electrostatic interactions of proteins with alginate play a more dominant role in the release kinetics. These interactions appear to affect the diffusive properties of the protein in the matrix, with negative proteins diffusing more quickly and positive proteins diffusing more slowly. Furthermore, positive proteins, particularly when encapsulated using the method developed in this work, seem to act as physical crosslinks which are less susceptible to degradation by the presence of monovalent ions and which therefore are presumably stronger than those due to calcium. Evidence of this is not only shown through the lengthier degradation rates that occur with the microspheres during release studies using isopositive proteins, but also with the observed crosslinking reaction that occurs when these same proteins are added to sodium alginate in solution.

Importantly, the activity of lysozyme interacting with alginate was maintained and in fact this interaction appeared to have a protective effect. Past studies with alginate have shown it to interact in a protective or destructive manner on different isopositive proteins. For example, alginate has been shown to negatively affect the activity of transforming growth factor  $\beta$  [29] but has been shown to protect the activity of vascular endothelial growth factor [13]. In this case, it appears that alginate may actually enhance the activity of lysozyme.

The differences in the kinetics of protein release from alginate microspheres into PBS, TBS and 0.15% NaCl clearly demonstrate the important role that release medium plays in the degradative release of proteins. The ion content is a critical factor as it is also observed that the phosphate content of PBS rapidly degrades the alginate matrix whereas TBS has a reduced degradation due to the lack of phosphate ions in the TRIS buffer system. Further decreasing the sodium content of the release solution slows alginate degradation to a large extent as

observed by Wang and Spencer [30]. It is stressed that the release medium must be designed to be representative of the eventual application since alginate may have been discarded as a release material in past studies due to an inappropriate release medium causing an *in vitro* bursting release. Furthermore, the volume of exposure also plays a vital role; decreases in release medium volume to sphere mass ratios appear to dramatically increase the release time from 2238 h (around 3 months) to over 8 months in the current work. It is hypothesized that the absolute amount of ions present to effect removal of the calcium is more critical to degradation than the specific concentration of the surrounding medium. This may provide an explanation for differences in release results for the same protein from different studies, for example VEGF showed release that varied from 8 days [17] up to over 3 weeks [10] likely due to different media volumes, concentrations and times between media changes. This is important to consider since many *in vitro* release studies do not take into account ion availability as an important factor influencing the release results. *In vivo*, ion availability may vary depending on the properties of the delivery site. For example hyaluronan, a common matrix glycosaminoglycan found in osmotic, swelled tissues such as cartilage [31] and the vitreous humour [33], is anionic and may likely also sequester the positive degradative ions such as the sodium, an ion commonly found in tissues. This research suggests that this will result in lengthened release rates from alginate *in vivo*. Low ion availability may also be representative of stagnant drug delivery target tissues such as the vitreous humour [32] at the back of the eye. In addition, delivery into areas that contain isopositive proteins such as lysozyme in tear fluid may alter the release kinetics in similar ways by the isopositive protein acting to reduce alginate degradation as has been observed in previous studies on pilocarpine delivery [34].

## 5. Conclusions

Alginate is a viable material for the sustained delivery of isopositive proteins. The novel soaking encapsulation technique developed in the current work is able to result in the entrapment of significant amounts of high isoelectric point proteins such as lysozyme and chymotrypsin. This technique is more appropriate for protein encapsulation since short encapsulation times are possible resulting in minimal diffusional loss such as would occur by crosslinking protein-containing spheres in a large calcium chloride bath. Taken together, mechanisms of protein release from alginate microspheres include diffusion, bulk degradation (hydrolysis), swelling and charge interactions, dependent in all cases on the protein selected. The ion content and availability of the release medium used for alginate *in vitro* release studies is clearly crucial and has a significant influence on the release profile. Care must be taken to ensure it mimics relevant *in vivo* conditions.

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