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A Biomimetic Approach to Active Self-Microencapsulation of Proteins in PLGA

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Abstract

A biomimetic approach to organic solvent-free microencapsulation of proteins based on the self-healing capacity of poly (DL)-lactic-co-glycolic acid (PLGA) microspheres containing glycosaminoglycan-like biopolymers (BPs), was examined. To screen BPs, aqueous solutions of BP [high molecular weight dextran sulfate (HDS), low molecular weight dextran sulfate (LDS), chondroitin sulfate (CS), heparin (HP), hyaluronic acid (HA), chitosan (CH)] and model protein lysozyme (LYZ) were combined in different molar and mass ratios, at 37 °C and pH 7. The BP-PLGA microspheres (20–63 μm) were prepared by a double water-oil-water emulsion method with a range of BP content, and trehalose and MgCO₃ to control microclimate pH and to create percolating pores for protein. Biomimetic active self-encapsulation (ASE) of proteins [LYZ, vascular endothelial growth factor165 (VEGF) and fibroblast growth factor (FgF-20)] was accomplished by incubating blank BP-PLGA microspheres in low concentration protein solutions at ~24 °C, for 48 h. Pore closure was induced at 42.5 °C under mild agitation for 42 h. Formulation parameters of BP-PLGA microspheres and loading conditions were studied to optimize protein loading and subsequent release. LDS and HP were found to bind >95% LYZ at BP:LYZ >0.125 w/w, whereas HDS and CS bound > 80% LYZ at BP:LYZ of 0.25–1 and < 0.33, respectively. HA-PLGA microspheres were found to be not ideal for obtaining high protein loading (>2% w/w of LYZ). Sulfated BP-PLGA microspheres were capable of loading LYZ (~2–7 % w/w), VEGF (~ 4% w/w), and FgF-20 (~2% w/w) with high efficiency. Protein loading was found to be dependent on the loading solution concentration, with higher protein loading obtained at higher loading solution concentration within the range investigated. Loading also increased with content of sulfated BP in microspheres. Release kinetics of proteins was evaluated *in-vitro* with complete release media replacement. Rate and extent of release were found to depend upon volume of release (with non-sink conditions observed < 5ml release volume for ~18mg loaded BP-

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PLGA microspheres), ionic strength of release media and loading solution concentration. HDS-PLGA formulations were identified as having ideal loading and release characteristics. These optimal microspheres released ~ 73–80 % of the encapsulated LYZ over 60 days, with > 90 % of protein being enzymatically active. Nearly 72% of immunoreactive VEGF was similarly released over 42 days, without significant losses in heparin binding affinity in the release medium.

1. INTRODUCTION

The rapidly emerging classes of bio-pharmaceutical products present increasingly complex challenges to the traditional pharmaceutical formulation paradigms. New delivery strategies and technologies have been developed to address these needs, although a vast majority of the new approaches have yet to produce significant number of commercially viable products. Numerous local/regional and targeting modalities/strategies have also been used in conjunction with these systems to enhance delivery, efficacy and reduce costs, but better drug delivery methodologies need to be developed to help increase the use of biologics [1, 2].

Biodegradable microspheres composed of natural or synthetic polymers have emerged as suitable delivery systems for controlled release of proteins, peptides, growth factors, small molecules and chemotherapeutic agents [3, 4]. Poly(lactic-co-glycolic acid) (PLGA)-based polymers possess highly desirable qualities such as biodegradability and biocompatibility, when employed to fabricate systems for drug delivery[5]. PLGA has been incorporated in numerous products approved by the United States Food and Drug Administration, and this established use makes it an attractive polymer for developing new delivery systems. One major drawback of the polymer is the commonly observed acidic microenvironment, due to the build-up of degradation products in the polymer [6, 7]. However, this issue has largely been overcome by incorporation of poorly soluble basic additives and other pH-modifying species into the polymer [8, 9]. Additional considerations for protein encapsulation in microspheres are exposure to the organic/aqueous interface, shear, air/liquid interface, organic solvents, and high temperatures [3, 10, 11]. A number of formulation methods have been explored to improve drug stability, loading, release characteristics, and processing [4, 12, 13]. These factors combine to make protein encapsulation one of the few major issues in the development of controlled-release formulations for protein drugs, along with instability during *in-vivo* release, needle size, residual organic solvents, and scale-up to large-scale manufacture. [6, 14, 15].

Recently our group has developed a novel aqueous-based encapsulation method, which is capable of microencapsulating proteins safely with high efficiency by simple mixing of porous PLGA and the protein of interest and healing the pores at near physiological temperatures [16, 17]. The active self-healing method employs the incorporation of a trapping agent in the PLGA pores, which strongly binds the protein (e.g., alum absorption of vaccine antigens) before the PLGA pores are healed as temperature is raised above the hydrated glass transition temperature of the polymer (T_g). Active self-encapsulation (ASE) is favorable for protein stability during encapsulation owing to the absence of organic solvents, high shear, and other protein-denaturing stresses. Although temperatures as high as

43 °C are commonly used, the heat denaturing stress is commonly acceptable to most proteins for modest times, particularly if protein-stabilizing excipients are placed in the loading solution during healing [17]. Plasticizers have also been added to PLGA to reduce the healing temperature to 37 °C [17]. ASE can be highly efficient (>97%), achieving elevated loading (>1 % w/w) from low protein concentrations (< 1 mg/ml), and providing long-term controlled release with excellent protein stability [17]. However, until now protein drugs have not been encapsulated by this approach at high loading and encapsulation efficiency.

In this paper, we build upon our active encapsulation paradigm by introducing a biomimetic approach with incorporation of biopolymers (BPs) in the microspheres, which are known to actively bind, sequester, and stabilize growth factors (GFs) *in-vivo* [18–27]. BPs can also potentially enhance the biological effect of the protein by acting as co-factors [19, 21, 26]. Incorporation of glycosaminoglycans into the pores of the self-healing PLGA microspheres would presumably enhance the protein loading efficiency as well as its stability during the encapsulation and subsequent release, by protein immobilization. Note that the biomimetic complexation of human growth hormone with Zn^{2+} in the Nutropin Depot formulation, as occurs naturally in the pituitary, has been used successfully to achieve excellent stability of the encapsulated protein during spray-congealing encapsulation and long-term release [28, 29]. Several BPs were selected in this work as trapping agents for biomimetic ASE, namely, hyaluronic acid, chondroitin sulfate, heparin, chitosan and dextran sulfate. These BPs are members of the glycosaminoglycan (GAG) family or have similar structural moieties (e.g, dextran sulfate and chitosan).

To develop the biomimetic self-healing strategy, we studied the binding of these BPs (total of 6) to a model basic enzyme with positive net charge at neutral pH, lysozyme (LYZ), and used this binding to encapsulate protein (by ASE) in PLGA microspheres at high loading and efficiency, and with useful controlled release. Subsequently, we tested the approach for two therapeutic growth factors, fibroblast growth factor 20 (FgF-20) and vascular endothelial growth factor (VEGF165). LYZ was used as a model protein as it has been very well characterized, is a basic highly soluble protein, and has heparin binding activity[30], similar to VEGF and FgF-20. LYZ and VEGF have reported pI values of 11.35[31] and 8–8.5[32] ; whereas FgF-20 has a theoretically calculated pI of 8.9[33]. We demonstrate here the feasibility of achieving high protein loading with very high encapsulation efficiency in the absence of organic solvents, by incorporating BPs in PLGA microspheres. In addition, slow and continuous release of proteins with a high level of stability was similarly achieved.

2. EXPERIMENTAL METHODS

2.1 Materials

PLGA with an inherent viscosity (i.v.) of 0.57 dLg^{-1} (50:50, PLGA DL LOW IV, lot # A11-071, lauryl ester end group, 51 kDa) was purchased from Lakeshore Biomaterials (Birmingham, Alabama). High molecular weight (~ 500 kDa) dextran sulfate (HDS), low molecular weight (~15.5 kDa) dextran sulfate (LDS), chondroitin sulfate (~ 63 kDa, shark cartilage) (CS), porcine heparin (~18 kDa) (HP) sodium salts were purchased from Sigma-

Aldrich. Chitosan (~ 61 kDa) was also from Sigma-Aldrich. Sodium hyaluronate (HA) powders and VEGF165 were obtained as a gift from Lifecore Biomedical (MN, USA) and Genentech, respectively. Lysozyme (chicken egg white) and magnesium carbonate were purchased from Sigma-Aldrich. FgF-20 was obtained from the National Cancer Institute-Biometric Research Branch (NCI-BRB) preclinical repository. Human VEGF standard ELISA development kit was purchased from Peprotech (NJ, USA). Cyanine-5 labelled lysozyme was bought from Nanocs (NY, USA). All other reagents, common solvents, and supplies were obtained from Sigma-Aldrich, unless otherwise specified.

2.2 BP Screen for LYZ Binding

Aqueous solutions of BPs (HDS, LDS, CS, HP, HA of ~66 kDa, and CH) and LYZ, were combined at a total BP + LYZ concentration of 1 mg/ml in different mass ratios (0.09, 0.11, 0.14, 0.20, 0.33, 1, 2, 3, 5 w/w), at pH 7 and 24 °C. After 3 h of incubation, the free LYZ was quantified by UV spectroscopy at 282 nm and size exclusion chromatography (SE-HPLC), as described below.

2.3 Analysis of LYZ-BP Complexes

LYZ and BP solutions were combined to form BP-LYZ complexes with initial w/w BP:LYZ ratios (0.33, 0.14, 0.11 and 0.7). The amount of bound LYZ was evaluated by SE-HPLC. Release kinetics of complexes in the absence of PLGA was determined in 1 ml PBS at pH 7.4 with complete replacement of release media at 37 °C, and LYZ quantification via SE-HPLC. Similarly, release kinetics and complex stability in special cases were analyzed in PBS + 0.3M NaCl, PBS + 0.6 M NaCl and PBS + 0.9 M NaCl at pH 7.4.

2.4 Preparation of Biomimetic Active Self-encapsulating BP-PLGA Microspheres

Porous active self-microencapsulating (SM) microspheres with BP for protein absorption in the PLGA pores, MgCO₃ as a pH modulator and porosigen [3], and trehalose [34] to similarly enhance the percolating pore structure of the microspheres, were prepared by double water-oil-water (W/O/W) emulsion. The first emulsion was created by homogenizing 1 ml of 250 mg/ml PLGA and MgCO₃ in CH₂Cl₂ with an inner water phase of varying amount of BPs, at 18000 rpm for 60s over an ice bath, using the Tempest IQ² (Virtis, USA). Two ml of 5% PVA solution was added to the resultant emulsion and the second emulsion was created by vortexing at 10000 rpm for 60s. The w/o/w emulsion was added to 100 ml of 0.5% PVA solution, and allowed to harden at room temperature for 3 h. Hardened microspheres (20–63 µm) were collected using sieves, washed with double-distilled water and immediately lyophilized.

2.5 Scanning Electron Microscopy

Scanning electron microscopy (SEM) images were obtained using a Hitachi S3200N scanning electron microscope (Hitachi, Japan). Briefly, lyophilized microspheres were fixed on double-sided adhesive carbon tape. Samples were coated with a thin layer of gold (~ 5 nm) under vacuum and images were taken at 10–15 kV excitation voltage. EDAX® software was used to obtain the final image.

2.6 ASE of proteins with BP-PLGA microspheres

ASE is a two-step process consisting of a loading phase followed by pore closure. Studies were carried out to determine the lowest temperature and duration at which a majority of surface pores closed while encapsulating the maximum amount of protein from the loading solution (*data not shown*). Biomimetic ASE of protein was achieved by incubating blank BP-PLGA microspheres in protein solution for 48 h at 24 °C. LYZ loading solutions were prepared in 10 mM phosphate buffer (pH 7) and FgF-20 loading solutions were prepared in 0.5 M arginine, 0.05 M sodium phosphate and 0.08% polysorbate 20 (pH 7). VEGF loading solutions were prepared in 5 mM succinate buffer, 275 mM trehalose and 0.01% polysorbate 20 (pH 5).

Pore closure was induced at 42.5 °C under mild agitation for 42 h. These parameters were determined by quantifying effect of temperature and duration of pore closure on LYZ loading (*data not shown*). After pore closure, BP-PLGA microspheres were removed from the loading solution, washed with double-distilled water, and immediately lyophilized. To freeze-dry, microspheres were flash frozen in liquid nitrogen and lyophilized using a FreeZone 2.5 (Labconco, USA) at < 0.080 mbar and – 42 °C, for 24 h. SEM images were taken to check if a majority of surface pores had closed.

2.7 Determination of loading and encapsulation efficiency

Loading of BP-PLGA microspheres was determined by a) protein content in loading solution after ASE, as described in section 2.10 and/or b) direct hydrolysis of ASE BP-PLGA microspheres followed by amino acid analysis (AAA), as described in section 2.11. ASE of LYZ by HA-PLGA microspheres was quantified by AAA and SE-HPLC. ASE of VEGF was determined by SE-UPLC and Coomassie Plus assay, whereas FgF-20 was quantified by SE-UPLC. Percentage w/w loading was quantified as

$$\left(\frac{\text{mass of protein encapsulated in microspheres}}{\text{total mass of microspheres in loading solution}} \right) \times 100. \text{ Percentage encapsulation efficiency was calculated as } \left(\frac{\text{mass of protein encapsulated in microspheres}}{\text{total mass of protein in loading solution}} \right) \times 100.$$

2.8 Fluorescent confocal microscopy

HDS-PLGA microspheres were prepared as described in section 2.4. The microspheres were loaded from Cyanine-5 (Cy5) labeled LYZ at 1 mg/ml concentration in 10 mM phosphate buffer (pH 7), as described above for unlabelled LYZ. After ASE, the microspheres were washed and lyophilized. Volumetric images of HDS-PLGA microspheres encapsulating Cy5-LYZ were generated by taking images at different focal lengths with a step size of 2 µm (Nikon A1R/A1 confocal microscope). NIS-Elements® software was used to combine these images into volumetric images.

2.9 Evaluation of release kinetics

The release kinetics of LYZ was determined by incubating loaded BP-PLGA microspheres at 37 °C in the specified volume of PBS or PBST (PBS with 0.02% Tween 80) with complete replacement of release media. The amount of protein released was assayed by size-

exclusion (SE) chromatography, as described below. The activity of released LYZ was quantified by Enzchek®, described below. Release kinetics of VEGF was determined in PBST+10 mg/mL BSA, also with complete media replacement at each time point by ELISA, as described below.

2.10 Quantification of Protein

2.10.1 Size Exclusion (SE) Chromatography—SE chromatography was performed using high performance liquid chromatography (HPLC) and ultra performance chromatography (UPLC) systems (Waters, USA). The mobile phase consisted of 0.1 M sodium phosphate with 0.3 M sodium sulfate at pH 6.7 at the rate of 0.8 ml/min and 0.4 ml/min for SE-HPLC and SE-UPLC, respectively. Samples and standards were injected onto TSKgel G2000SWxl (Tosoh Bioscience, USA) and Acquity BEH200 (Waters, USA) on HPLC and UPLC systems, respectively. Protein detection by UV was done at 214 and 282 nm. Retention times of roughly 11 and 5 min were obtained for LYZ during SE-HPLC and SE-UPLC, respectively. FgF-20 and VEGF both had retention times of roughly 5 min during SE-UPLC. The UPLC was preferentially used in quantifying protein release kinetics in 5 ml release media, as it had higher sensitivity when compared to SE-HPLC for the conditions studied.

2.10.2 UV Spectroscopy—Quantification of protein was carried out with Synergy 2 microplate reader (Biotek, USA) at 214 and 282 nm with appropriate standards and controls, using 96 well plates (Corning, USA).

2.10.3 Coomassie Plus protein assay—Quantification was carried out using appropriate standards and controls at 595 nm using the microplate reader, as per protocol provided by manufacturer (Coomassie Plus, Pierce). The assay was used to quantify VEGF loading along with SE-UPLC.

2.11 Amino Acid Analysis

Amino Acid Analysis (AAA) was performed to determine the total content of LYZ loaded in BP-PLGA microspheres (~ 5 mg), soluble protein solutions and standards were weighed into clear glass ampules in a total volume of 1.5 ml 6 N HCl. Ampules were sealed under light vacuum, and incubated at 110 °C for 24 h. Following incubation, each vial was completely emptied into microcentrifuge tubes, and the solution was evaporated under vacuum at room temperature. One ml of 1.0 M sodium bicarbonate buffer (pH 9.5) was added to each tube to neutralize the remaining acid. For individual amino acid analysis, a weighed amount of 350 µl of hydrolyzed protein solution and 350 µl of o-phthaldialdehyde reagent solution were mixed and immediately injected onto a C18 (Waters, USA) column fitted with a guard column [35, 36]. Each sample had a run time of 50 min, with a mobile phase at 1.4 ml/min of A) methanol-water (65:35) and B) methanol-THF-50 mM phosphoric acid (titrated to pH 7.5 with 10 N NaOH (20:20:960)). The run started with 40% A for 0.5 min, 17 min gradient to 50% A, 15 min gradient to 100% A, a 5 min isocratic elution with 100% A, 7.5 min linear gradient to 40% A, and isocratic 40% A for 5 min. The fluorescence (excitation at 350 nm; emission at 455 nm) detection was used for quantification. Protein

and standards were quantified using the average of the 3 individual amino acids alanine, phenylalanine, and lysine[16].

2.12 LYZ Activity Assay

The activity of LYZ in solution was determined by Enzc hek® lysozyme assay (Life Technologies, USA) as per the protocol provided. As CS was found to interfere with the enzyme assay, activity data for CS containing formulations are not reported.

2.13 VEGF enzyme linked immunosorbent assay

VEGF enzyme linked immunosorbent assay (ELISA) was carried out as per the manufacturer's protocol. Briefly, 96 well microplates were incubated overnight with 100 µl of 0.5 µg/ml capture antibody in PBS at room temperature. After washing with PBS containing 0.05% Tween 20, 100 µl of standards and samples in diluent (PBS with 0.05% Tween 20 and 0.1% BSA) were added in triplicate and incubated for 2 h at room temperature. After washing, 100 µl of 0.25µg/ml biotinylated secondary antibody in diluent was added to each well and allowed to incubate for 2 h at room temperature. After another wash, 100 µl of diluted (1:2000) avidin-HPR conjugate was incubated in each well for 30 min. After a final wash, 100 µl of ABTS substrate was added to each well for detection. Activity was determined after 25 min by monitoring plate optical density at 405 nm with wavelength correction set at 650 nm and fitting the data using the Gen5 software (Biotek, USA).

2.14 Heparin-affinity chromatography

Heparin-affinity chromatography was performed using an Alliance HPLC system (Waters, USA). Each sample had a run time of 15 min, with a mobile phase at 1.0 ml/min of A) 50 mM phosphate buffer (pH 7) and B) 50 mM phosphate buffer with 3M NaCl (pH 7). Samples and standards were injected onto POROS® Heparin 50 µm column (Applied Biosystems®, USA). The run started with 100% A for 5 min, 2 min gradient to 0% A, 5 min gradient to 100% A, and an isocratic 100% A for 3 min. Heparin binding VEGF was detected by UV at 214 and 282 nm with a retention time of ~ 10 min. The large BSA peak from the release media samples was baseline separated from the VEGF peak and had a retention time of ~ 3.5 min. Percentage heparin binding VEGF was quantified as

$$\left(\frac{\text{mass of heparin binding VEGF}}{\text{mass of immunoreactive VEGF}} \right) \times 100.$$

3. RESULTS AND DISCUSSION

3.1 Selection of BPs and Protein-BP interactions

The BPs used in this study are members of the glycosaminoglycan (GAG) family or have similar structural moieties (e.g., dextran sulfate and chitosan). GAGs are polysaccharides constituting a major portion of the extra cellular matrix. They are known to bind and regulate a number of distinct proteins, including chemokines, cytokines, growth factors, morphogens, adhesion molecules and enzymes [18, 20, 26]. They are linear, generally sulfated, negatively charged polysaccharides with molecular weights in 10–100 kDa range [18, 27]. Non-sulfated GAGs include hyaluronic acid (HA), whereas the sulfated include

chondriton sulfate (CS), dermatan sulfate (DS), ketran sulfate (KS), heparin and heparin sulfate (HP).

HA is a poly-anionic polysaccharide consisting of N-acetyl-D-glucosamine and β -glucuronic acid. It is found in synovial fluid, vitreous humor, cartilage, blood vessels, skin, and the umbilical cord [37]. Acting as a kind of extracellular glue, it is believed to play a vital role in the homeostasis, tissue development, cell signaling, regeneration, wound healing and tumor invasion [38, 39]. Its unique viscoelastic nature along with its biocompatibility and non-immunogenicity has led to its use in a number of cosmetic, medical, and pharmaceutical applications. Among the sulfated BPs, heparin [(1 $\rightarrow$ 4) pyranosyluronic acid 2-amino-2-deoxyglucopyranose] has been widely studied due to its well understood role in anti-coagulation pathway [20]. Chondroitin sulfate, polysaccharide chain of alternating units of N-acetylgalactosamine and glucuronic acid [β -glucuronic acid-(1 $\rightarrow$ 3)N-acetyl- β -galactosamine] is the most abundant GAG in the body. Found in the cartilage, tendon, ligament, and aorta it binds to proteins and forms proteoglycan aggregates [18, 27]. A number of growth factors bind to the ECM of target tissues by forming tight complexes with sulfated-glycosaminoglycans [40].

Chitin, poly[h(1 α 4)-2-acetoamido-2-deoxy-D-glucopyranose], is one of the most abundant natural polysaccharides and is present in crustaceans, insects, fungi, and yeasts. Deacetylation of chitin by alkali produces CH. The molecular structure of CH is believed to be a copolymer of N-acetyl-glucosamine and glucosamine; usually the glucosamine content is more than 90% [27]. Dextran sulfates are derived from dextran (linear backbone of α -linked d-glucopyranosyl repeating units) via sulfation. The long history of the safety of dextrans has allowed them to be used as additives to food and chemicals, and in pharmaceutical and cosmetics manufacturing [41].

Ionic interactions are known to govern the specificity of GAG-protein interactions [26]. The carboxylate and sulfate groups of GAG's interact with the basic amino acid residues on the protein to create optimal structural fits of binding sites. Thus, the distribution and topology of the basic residues dictates the specificity of interactions and location of binding sites on the protein [20, 23]. For strong binding affinity, oligosaccharide sequences on the GAG have to provide optimal charge (orientation of sulfate groups) and surface (van der Waals contact) [18, 20]. Typically, GAGs interact with amino acid residues that are prominently exposed on the surface of proteins, with the three-dimensional structure and conformation of GAGs playing a crucial role. Arginine, lysine and, to a lesser extent, histidine are involved in the ionic interactions with highly acidic sulfate groups present on the GAG chains [23].

3.2 Screening BPs for ability to bind LYZ

Biopolymers were screened for their ability to act as protein trapping agents in order to enhance efficiency of ASE in PLGA. Ideal characteristics for such BPs include the ability to a) absorb protein from aqueous solution and ideally provide a synergistic effect along with the protein, b) stabilize the bound protein and release the bound protein when PLGA pores open, and c) not alter self-healing characteristics when formulated with PLGA.

BPs were quantified for their ability to bind to LYZ (Fig 1). HP and LDS were found to bind LYZ at high efficiency (> 95%) across the entire range of ratios investigated. In contrast, HDS and CS displayed an optimum LYZ binding efficiency at a BP:LYZ of ≈ 0.1 . The highest binding efficiency was observed at low BP:LYZ, and decreased as the ratio increased. In case of HA, the binding efficiency was found to be the highest at the lowest HA:LYZ and decreased as the ratio was increased to 10. The efficiency increased to $\approx 18\%$ at around a HA:LYZ ratio of 49. A similar trend was seen in the case of CH as the binding efficiency decreased from 68% to 28% at around a BP:LYZ ratio of 1 and then increased to $\approx 100\%$ as the ratio increased to 25. CH also had very poor solubility (< 3 mg/ml) in 10 mM phosphate buffer solution at pH 7. Thus, because of poor solubility CH was not a suitable candidate for developing BP-PLGA formulations with high w/w CH content.

It is noted that HA formed gels when combined with LYZ solutions, whereas the sulfated BPs formed visible insoluble precipitates. Thus, BP-PLGA microspheres were expected to sequester proteins into the microspheres from the aqueous protein solution during microencapsulation. Based upon the binding efficiencies, LDS and HP were expected to perform well in BP-PLGA microspheres (see below) as they have excellent binding at BP:LYZ ratios of > 0.13, whereas HDS and CS formulations would have to be optimized to ensure high loading and encapsulation efficiency of protein uptake from aqueous solution during ASE.

After screening for the ability to bind LYZ, the next step was to check the stability of sulfated BP-LYZ complexes and evaluate the release kinetics of the bound LYZ. BP:LYZ complexes were formed by combining BP and LYZ solutions at different mass ratios. The complexes were incubated in PBS, at pH 7.4 and 37 °C. Release kinetics were evaluated with complete release media replacement and quantified by SE-HPLC (Fig 2).

The extent of LYZ released across complexes of a given BP formed at different BP:LYZ, were found to be very similar after 100 h. HDS-LYZ complexes had the lowest rate and extent of release at $\sim 55\text{--}60\%$ over 100 h, making them an excellent candidate for developing long-acting release formulations. LDS-LYZ complexes had comparatively a higher rate and extent of release of $\sim 75\text{--}80\%$. LDS-LYZ and HDS-LYZ complexes formed at a BP:LYZ ratio of 0.07 had significantly faster rate and extent of release compared to complexes formed at higher ratios. By contrast, CS-LYZ complexes formed at all CS:LYZ ratios were found to have similar release kinetics, with $\sim 78\text{--}90\%$ LYZ released over 100 h. HP-LYZ complexes formed at lower HP:LYZ ratios had faster LYZ release over 100 h when compared to the complex formed at higher HP:LYZ. Across all BPs, complexes formed at lower BP:LYZ ratios had the highest burst release.

3.3 Development of HA-PLGA microsphere formulations

Excipients were included in the organic/water phase to control pore structure and emulsion parameters in order to obtain a percolating pore network. The percolating pore network is required for allowing the biomacromolecule to enter deep within the microsphere during ASE and diffuse out during subsequent controlled release. On the other hand, the pores should not be too large as they could lead to incomplete self-healing and/or poor loading efficiency, and higher burst release. The pore network is usually obtained by incorporating

simple osmotic agents like a small sugar (e.g., trehalose) or alternative porosigen (e.g., MgCO_3). MgCO_3 reacts with acidic residues in the polymer to form salts and/or gaseous CO_2 , helping to protect the encapsulated proteins from an acidic microenvironment.

To obtain well-formed BP-PLGA microspheres with a uniform pore structure, a number of formulation parameters were investigated with HA as the BP in the inner water phase of the double emulsion. The addition of the BP in the inner water phase adds another variable, which affects the pore structure, relative to passive self-encapsulation [16]. The M_w of BP, volume of inner water phase, polymer concentration, microspheres size, trehalose loading, MgCO_3 loading were manipulated to obtain well-formed microspheres (*data not shown*).

Upon analyzing SEM images of the microspheres, it was decided to use 250 mg/ml PLGA and 200 μl of inner phase volume for formulating ASE BP-PLGA microspheres. First and second emulsions were obtained at 18,000 rpm for 60 sec and 10,000 rpm for 60 sec, respectively. Higher speeds were used to ensure that small primary emulsions were created with the viscous BP containing inner water phase. The effect of M_w of HA, and HA and MgCO_3 contents on ASE of LYZ were investigated, as described below.

3.3.1 Effect of M_w and content of HA on formulation of HA-PLGA

microspheres—HA of M_w of 66, 356 and 1010 kDa were used to formulate 2, 4, 8 and 13 % w/w HA-PLGA microspheres. SEM images were used to check if microspheres obtained were well formed and had a visibly uniform pore structure. A major issue with the formulation of the HA-PLGA microspheres was the viscosity of the HA solution constituting the inner water phase. Increasing viscosity associated with higher M_w and w/w content of HA in the microspheres led to poorly formed primary emulsions, which in turn, led to deformed microspheres with non-uniform pore structure. In some cases, no microspheres were obtained. More work would be needed to understand the role played by viscosity of inner phase and its effect on the quality of emulsions, and subsequent microsphere formation if microspheres with high M_w HA at the elevated content were desired. By contrast, we were able to obtain well-formed HA-PLGA microspheres with 66 kDa HA at 2 – 13 % w/w. With 1010 and 357 kDa HA, we observed well-formed microspheres only for 2%, and 2 and 4 % w/w HA, respectively.

3.3.2 Effect of M_w and HA content on ASE of LYZ

—HA-PLGA microspheres were prepared with 66 (4 and 12 % w/w), 356 (2 and 4 % w/w) and 1010 (2% w/w) kDa HA. These microspheres (~20 mg) were loaded in 0.5 ml of 1 mg/ml LYZ solution and the results are reported in Table 1. As seen in the table, active encapsulation of LYZ increased with increasing M_w . A similar trend was seen with increasing HA content in the HA-PLGA microspheres. Higher M_w HA was expected to improve LYZ loading of HA-PLGA microspheres as the longer HA polymer chains were expected to be retained in the PLGA pores to a higher extent along with the bound LYZ. By contrast, the lower M_w HA was expected to diffuse out of HA-PLGA during the loading process. More work is needed to understand the role of molecular chain length, viscosity, and polymer dynamics on protein binding and retention.

Increased HA content provides more HA for LYZ binding, which should theoretically increase the LYZ loading. Such an effect was observed, but was less pronounced than expected (Table 1). Upon the addition of 3% w/w MgCO_3 , the effect of varying M_w and HA content on the active self-encapsulation of LYZ was greatly reduced. This trend could be due to the increased osmotic pressure created by presence of MgCO_3 , which could counteract the effect of higher M_w and HA content. In addition, the difference in the loading of LYZ by AAA and SE-HPLC could be possibly due to the high water uptake and gel-forming nature of the HA. The uptake of the protein solution by the HA-PLGA microspheres could lead to a decrease in the loading solution volume and thus underestimate the loss of protein from solution, as measured by the SE-HPLC.

3.3.3 Effect of M_w and HA content in PLGA on the Release Kinetics of

Biomimetically Encapsulated LYZ—The size and solubility of the HA-LYZ complex governs the amount of free LYZ available to percolate through the microsphere pore structure and diffuse out into the release media. Higher M_w HA was expected to slow the release of LYZ, as LYZ bound to higher M_w HA would be expected to facilitate retention in the PLGA matrix. HA-PLGA formulations released ~40–44% of the encapsulated LYZ over 16 days and the release plateaued beyond that (Fig 4). However, no statistical differences were seen in the release kinetics of LYZ over 28 days in 1 ml PBST at pH of 7.4 among the HA-PLGA formulations. Similarly, no significant differences were found among HA-PLGA formulations of the same M_w when compared across different HA content or when HA content was compared across different M_w . Our data suggested that HA-PLGA microspheres were not suitable for obtaining high protein loading (>2% w/w of LYZ) and had poor release kinetics. Thus, we decided to investigate sulfated BP-PLGA microsphere formulations.

3.4 Sulfated BP-PLGA Formulations

BP-PLGA microspheres were prepared with HDS, LDS, CS, and HP, based on parameters used for formulating optimum HA-PLGA microspheres. The role of the type of BP, content of BP in PLGA and concentration of loading solution on ASE of protein were investigated. Factors governing protein release kinetics from the BP-PLGA microspheres were also investigated. The effect of BP type, BP content in PLGA microspheres, volume of release and ionic strength of release media were studied as described below.

3.4.1 Effect of Loading Solution Concentration on Biomimetic ASE of LYZ—

Based on the binding curves and formulation issues associated with high BP content, 4% w/w BP-PLGA (~20 mg) microspheres were loaded from 0.5, 1, and 1.5 mg/ml LYZ solutions (Table 2). BP-PLGA ASE microspheres exhibited an excellent active protein loading capabilities and efficiency. LDS formulations had the lowest loading efficiencies (and corresponding LYZ loading) among the BPs tested. HDS, CS and HP formulations had greater than 90% efficiency across all loading concentrations investigated, and HDS-PLGA microspheres were found to have the highest loading efficiency. Most of the LYZ partitioned into the BP containing PLGA pores when LYZ concentration in the loading solution increased from 0.5 mg/ml to 1 mg/ml and 1.5 mg/ml (Table 2). This high loading capacity (~7 wt %) and efficiency (94–96%) was achieved at a lower BP:LYZ ratio in the

loading solution. The loading was found to increase with the concentration of the loading solution in the range investigated (Fig. 5). Thus, the loading solution concentration could be used to obtain ASE formulations with desired protein content. It is important not to draw conclusions regarding the exact BP:LYZ ratio for high efficiency loading, as the data does not quantify the BP:LYZ in the integral pores of BP-PLGA microspheres.

3.4.2 Effect of BP content on ASE of LYZ—To study the effect of % w/w BP, ~10 mg of HDS-PLGA (2, 5.1 and 12.6 %w/w HDS) microspheres were loaded from 1 ml of 3 mg/ml and 0.5 ml of 6 mg/ml LYZ loading solutions (Table 3). A smaller amount of microspheres (~ 10 mg), were loaded with an excess of LYZ (3 mg) to further investigate the role of loading solution concentration. Loading was found to be higher at 6 mg/ml than at 3 mg/ml. The theoretical amount of LYZ bound to 1 mg of HDS increased with loading concentration. The data indicates that the maximum ASE capacity of HDS-PLGA microspheres increases with loading concentration of LYZ. Higher self-encapsulation was also seen with increasing HDS content, when loaded with an identical LYZ concentration. Overall, the low loading and encapsulation efficiency could be attributed to the HDS-PLGA having exceeded the protein binding capacity, as a smaller mass of microspheres was loaded with higher amount of protein. Thus, the significance of loading solution concentration was verified at constant volume of LYZ loading solution and constant amount of LYZ in the loading solution.

3.4.3 Distribution of Biomimetically ASE Cy5-LYZ in HDS-PLGA microspheres—Cy5-LYZ was encapsulated by ASE to visualize the distribution of LYZ in the HDS-PLGA microsphere, after ASE (Fig 6). Volumetric images were generated by combining multiple images obtained at different focal lengths. The image shows that LYZ was able to enter the porous microsphere extensively, with greater distribution in the outer areas of the particle. This was expected, as during the loading process LYZ was diffusing into the microsphere from liquid-microsphere interface via the surface pores. Red domains indicate areas of high LYZ concentration near the surface of the HDS-PLGA microsphere. Ideally, uniform distribution of LYZ in the microsphere would allow for a long acting release formulation with slow continuous release, without a large burst release. This suggests that improving the distribution of the biomimetically ASE Cy5-LYZ could potentially improve first-order type release kinetics, as described below.

3.4.4 Release Kinetics of sulfated BP-PLGA Microspheres—The release kinetics of biomimetically encapsulated LYZ from sulfated BP-PLGA microspheres was initially investigated in 1 ml PBS at pH 7.4. PBS was used to overcome initial complications associated with LYZ quantification, due to overlap of Tween 80 and LYZ peaks during SE-HPLC analysis.

Across all formulations, the cumulative release plateaued at around day 7 and increased after 28 days of release (Fig 7). The first stage of this two-phase release would be expected to be driven by diffusion of the LYZ, which was either poorly encapsulated or released from the microsphere prior to healing of newly formed pores created during drying or initial hydration of the microspheres. After this initial phase, continuous release could be sustained by the presence of the MgCO_3 excipient, which reacts with the degradation products to

create osmotic pressure and new pores in the polymer matrix. The kinetics is also likely governed by the BP-LYZ interactions and the movement of complexes and soluble LYZ, through the pore network.

BP-PLGA microspheres loaded with 1.5 mg/ml LYZ had the highest rate of release and extent ($\approx 80\%$) of the self-encapsulated LYZ. LDS-PLGA formulations also exhibited a higher rate and extent of release when compared to HDS loaded at 1.5 mg/ml LYZ. CS-PLGA formulations had the highest burst release ($> 40\%$) across the sulfated BP-PLGA microspheres. Overall, HDS-PLGA microspheres had some of the lowest burst release, as well as rate and extent of long-term release.

Upon examining [LYZ released/Mass of BP-PLGA microspheres] versus time, no significant differences were seen in release kinetics of BP-PLGA formulations loaded with 0.5, 1 and 1.5 mg/ml LYZ loading solutions, except for HDS-PLGA (supplementary data, Fig S1). HDS-PLGA formulations exhibited increased rate of LYZ release when loaded with higher LYZ loading solution (0.5, 1, 1.5 mg/ml), potentially providing a method to modulate release kinetics of encapsulated protein for this BP trapping agent.

3.4.5 Effect of Ionic Strength on Release Kinetics—To evaluate the role of BP:LYZ interactions on release kinetics, the ionic strength of the release media was varied. *In-vitro* release of LYZ loaded BP-PLGA microspheres were quantified in 1ml of PBS+0.3 M NaCl and PBS+0.6 M NaCl (supplementary data, Fig S1). Due to the electrostatic component of the BP-Protein interaction, the rate and extent of LYZ released was expected to increase with ionic strength of the release media. This was verified, as the rate and extent of LYZ release was found to increase across all ASE formulations, with increasing ionic strength. Similar results were also reported by Park *et al.* on LYZ release kinetics *in-vitro*, suggesting that the NaCl disrupts the ionic interactions between BP-LYZ [42]. Lee *et al.* have also reported similar effects of ionic strength on release of BSA and histone [43]. Upon examining [LYZ released/mass of BP-PLGA microspheres] versus time, the amount and rate of LYZ release increased with ionic strength across all ASE formulations. No significant differences were seen in the rate of release amongst the LDS-PLGA, HP-PLGA and CS-PLGA microspheres loaded with different concentrations of LYZ in PBS, PBS +0.3M NaCl and PBS+0.6M NaCl. In PBS, clear differences were seen in the rate of LYZ released amongst the HDS-PLGA microspheres loaded with different LYZ concentrations (supplementary data, S1). The rate decreases with the decreasing LYZ loading solution concentration, suggesting that the HDS:LYZ ratio during loading is indeed an important variable governing LYZ release kinetics.

3.4.6 Effect of Volume of Release on Release Kinetics—To study the effect of volume on kinetics of release, LDS-PLGA microspheres loaded with $\approx 2\%$ w/w LYZ solution were incubated in PBS at pH 7.4 over a range of volumes (0.5 – 20 ml), with the LYZ release being quantified by SE-UPLC. The rate and extent of LYZ release from LDS-PLGA was found to increase as the volume of release was increased from 0.5 ml to 5ml (Fig 8). LYZ release in 10, 15 and 20 ml was similarly analyzed up to 3 days, beyond which the LYZ concentration in the release media could not be detected by the analytical method. Upon comparing the LYZ release in 5, 10, 15 and 20 ml, no significant differences were

seen, suggesting that sink conditions were attained at and above 5 ml of PBS for $\approx$ 18 mg of LYZ loaded LDS-PLGA microspheres. This effect of volume should be considered while estimating release kinetics during *in-vitro* experiments.

3.4.7 Release Kinetics in Optimized Release Media Volume—From our data, we have identified HDS-PLGA formulations to be ideal for evaluating controlled release formulations as they consistently have amongst the highest loading (and encapsulation efficiency) and the lowest burst release of all formulations investigated. The HDS-LYZ complexes also had shown excellent release characteristics to be used for developing long-acting-release formulations. The data further suggests that the rate of release could be adjusted by changing the HDS:protein ratio during ASE. In addition, the high M_w of HDS should minimize any potential leaching and complications during ASE of proteins.

Based upon the release data above, $\sim$ 18 mg of HDS-PLGA (loaded with 1.5 mg/ml LYZ) formulations were incubated in 5 ml PBST to evaluate LYZ release kinetics and activity of released LYZ (Fig 9). It was not feasible to use a higher release volume as released LYZ concentrations would be too low to be quantified using SE-UPLC beyond 3 days of release. HDS-PLGA formulations were found to have good release kinetics with $\sim$ 60% released over 21 days. The remaining protein was gradually released over the period monitored, leading to $\sim$ 73–80% of encapsulated protein being released over 55 days. The average activity of the released LYZ was found to be $> 90\%$ during the entire course of release experiment.

3.5 Biomimetic ASE of FgF-20 and VEGF

Based upon the success of encapsulating LYZ in sulfated BP-PLGA microspheres via ASE, the feasibility of the biomimetic paradigm was verified by the encapsulation of recombinant human FgF-20 and VEGF165 (Table 4). Loading efficiencies of $>80\%$ with $\sim 2\%$ (w/w) were obtained for FgF-20, across all the BP formulations analyzed. These results are very encouraging as encapsulation was achieved in presence of 0.5 M Arg in loading solution to overcome solubility and stability issues associated with FgF-20. Upon biomimetic ASE of VEGF by HDS-PLGA microspheres, $\sim 4\%$ (w/w) loading was obtained with nearly 75% efficiency. This was achieved in presence of 275 mM trehalose, to improve stability of the VEGF during the loading incubation.

The release kinetics of VEGF loaded HDS-PLGA was evaluated in 5 ml PBST + 1% BSA, with full media replacement (Fig 10). BSA was added in the release media to improve stability of released VEGF and prevent non-specific adsorption to the release vessel. Over 42 days, nearly 72 % of encapsulated VEGF was released in an immunoreactive form. The released VEGF retained its ability to bind to heparin ($> 92\%$ relative to immunoreactive VEGF over 23 days), consistent with retaining a very high fraction of the growth factor's native structure during long-term release. The release kinetics were found to be slightly faster when compared to LYZ, which would have resulted from the presence of 275 mM trehalose and 10 mg/ml BSA in the external loading solution, which reasonably could affect the VEGF-HDS interaction. Release kinetics of FgF-20 was not characterized because of the lack of a suitable release assay for a viable release media.

4. Potential advantages of Biomimetic ASE for delivery of VEGF and other proteins

The biomimetic approach provides significant advantages over traditional encapsulation paradigms. For example, a) outstanding protein stability during encapsulation is observed owing to the absence of organic solvents, high shear, and other protein-denaturing processes; b) high protein loading (~5% w/w) and efficiency (>90%) of encapsulation is achievable suitable for pharmaceutical protein controlled release by simple mixing of blank BP-PLGA microspheres with aqueous protein solution and heating; c) appropriately chosen BPs can act as potential co-factors for the protein being delivered, greatly enhancing therapeutic effect, and reducing protein dosage and costs; d) there is a potential for controlled release of active protein over 4–8 weeks; and e) a potential to sterilize ASE microsphere formulations by gamma irradiation, allowing for bulk manufacturing for multiple growth factors or other compatible proteins [44].

The biomimetic paradigm provided excellent LYZ, VEGF165 and FgF-20 loading, encapsulation efficiency, and potential for long-term release. The absorption data also support the use of this approach as a new facile method to microencapsulate off-the-shelf therapeutic proteins with SM polymer excipients, suggesting that suitably selected BPs could be incorporated in materials of any shape and form, e.g., eluting stents, sutures, biodegradable implants, etc., for protein delivery. This work builds upon the previous work done by our group [16, 17], by expanding our self-encapsulation paradigm for therapeutic proteins at low protein concentrations making it possible to quickly develop and evaluate controlled release formulations. Recently, 4–10 % w/w LYZ loading was reported via passive self-encapsulation using 250 mg/ml LYZ [45], but the prohibitive cost of this approach is not very feasible for encapsulation of recombinant therapeutic proteins. In contrast, ASE of pharmaceutical proteins using the approach described here could be brought about by simply mixing microsphere formulations with low concentration protein solutions (<10 mg/ml), to reduce losses of expensive recombinant proteins during formulation.

The ~ 4 % w/w loading of VEGF achieved by ASE is significantly higher [46–48], than the literature where the VEGF was included in the first emulsion, during the preparation of PLGA particles. The encapsulation efficiency is also higher than reported in most cases [49–51]. This is significant as our methodology allows the protein to be loaded after the formation of the PLGA microparticle, protecting it from harsh conditions and processes associated with manufacturing. The release kinetics reported here is comparable to the literature with ≈ 70 % of immunoreactive VEGF released over 30 days with excellent stability without significant losses to heparin binding affinity, making it a promising delivery system. LYZ release from PLGA has been studied extensively, and it is reported to suffer from stability issues when encapsulated with traditional paradigms [11, 42, 52, 53]. In our work, we were able to demonstrate high LYZ loading and encapsulation efficiency with excellent release profile and stability over 60 days. Thus, our data demonstrates the potential for using biomimetic ASE for developing long-acting-release products for protein delivery.

Future work in our group is being directed towards understanding the nature of the stabilizing interactions between the BPs and proteins. Our efforts are also being directed towards investigating the effect of BP and its distribution on the pore structure of the PLGA polymer. For example, we wish to understand how to control the co-distribution of BPs and encapsulated protein, which would be important for developing formulations with optimal release kinetics and minimal protein residue in the polymer matrix. This information would be crucial in developing even better formulations, which have desirable release characteristics and high protein stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

BP-LYZ binding curves at 24 °C after 3 h incubation in 10 mM phosphate buffer (pH 7).

Solubility of BP limited the range of BP:LYZ investigated. Values are expressed as mean $\pm$ SE, $n = 3$.

Figure 2.

LYZ release profiles in 1ml PBS (pH 7.4) at 37 °C from BP-LYZ complexes formed at BP:LYZ of 0.33 (●), 0.14 (○), 0.11 (▼) and 0.07 (△) quantified by SE-HPLC at 282 nm. The values are expressed as mean ± SE; n=3.

Figure 3. SEM images of ASE BP-PLGA microspheres as a function of trapping agent. *Top (left to right):* PLGA with CS, HDS, and LDS trapping agents before ASE. *Bottom (left to right):* PLGA with HA and HP trapping agents, before ASE. Theoretical loading of BPs was 4% w/w.

Figure 4. LYZ release profiles in 1 ml PBS (pH 7.4) at 37 °C quantified by SE-HPLC at 282 nm. ASE HA-PLGA microspheres were loaded from 1 mg/ml LYZ in 10 mM phosphate buffer (pH 7). The values are expressed as mean $\pm$ SE; n=3; total microsphere mass in release media was ~ 18 mg.

Figure 5.

A fit of the % w/w LYZ loading of optimal ASE BP-PLGA microspheres as a function of the LYZ loading concentration. Theoretical content of BPs, trehalose and MgCO_3 was ~ 4, 3 and 3 % w/w, respectively, in the formulations.

Figure 6. Distribution of ASE Cy5-LYZ in a HDS-PLGA microsphere recorded by fluorescence confocal microscopy. Volumetric image was obtained by combining images taken at different heights. Red regions indicate regions of very high concentration of Cy5-LYZ.

Figure 7.

LYZ release profiles in 1 ml PBS (pH 7.4) at 37 °C quantified by SE-HPLC at 282 nm. ASE BP-PLGA microspheres were loaded from LYZ in 10 mM phosphate buffer at concentrations of 0.5(—●—), 1(—○—) and 1.5(—▼—) mg/ml. The values are expressed as mean $\pm$ SD; n=3; total microsphere mass in release media was $\sim$ 18 mg.

Figure 8.

LYZ release profiles from LDS-PLGA microspheres in PBS (pH 7.4) at 37 °C, quantified by SE-UPLC at 282 nm, as a function of release media volume. Microspheres were loaded from 1 mg/ml LYZ in 10 mM phosphate buffer (pH 7). The values are expressed as mean $\pm$ SE; $n=3$; microsphere mass in release media was $\sim$ 18 mg. Insert - Release in high volume was not feasible beyond 3 days due to poor quantification of protein at very low concentration via SE-UPLC.

Figure 9.

LYZ release profiles in PBST (PBS with 0.02% Tween 80) quantified by SE-UPLC at 282 nm. ASE HDS-PLGA microspheres were loaded from 1.5 mg/ml LYZ in 10 mM phosphate buffer (pH 7). The values are expressed as mean $\pm$ SE; $n = 3$; microsphere mass in solution was ~ 18 mg.

Figure 10.

VEGF release profile from HDS-PLGA microspheres in 5 ml PBST (pH 7.4 with 1% BSA), were quantified using ELISA. ASE HDS-PLGA microspheres were loaded from 1 ml of 1.0 mg/ml VEGF solution (5 mM succinate buffer, 275 mM trehalose and 0.01% polysorbate 20, pH 7). The values are expressed as mean $\pm$ SE; $n = 3$, and total microsphere mass in release solution was ~ 18 mg. Insert - Heparin binding capacity of released VEGF was quantified using heparin-affinity chromatography (it was not feasible to accurately quantify heparin binding VEGF beyond day 23). Heparin-binding VEGF was normalized by immunoreactive VEGF.

Table 1

Self-microencapsulation capacity and efficiency of optimal ASE HA-PLGA microspheres loaded from 0.5 ml of 1 mg/ml LYZ loading solution in 10 mM sodium phosphate (pH 7)¹.

HA content		MgCO ₃ content		LYZ Encapsulation	
M _w	% (w/w)	% (w/w)	by SE (% w/w) ^a	by AAA (% w/w) ^b	Efficiency (%) ^a
66	4	NA	0.34 ± 0.01	0.33 ± 0.11	18 ± 1
66	12	NA	1.37 ± 0.07	1.5 ± 0.30	68 ± 2
357	2	NA	0.42 ± 0.01	0.41 ± 0.07	21 ± 0.4
357	4	NA	1.34 ± 0.01	1.33 ± 0.07	71 ± 5
1010	2	NA	1.61 ± 0.02	1.57 ± 0.21	73 ± 7
66	4	3	0.73 ± 0.05	NA	37 ± 1
66	12	3	0.73 ± 0.03	NA	36 ± 2
357	2	3	0.83 ± 0.09	NA	41 ± 4
357	4	3	0.90 ± 0.07	NA	44 ± 1
1010	2	3	0.78 ± 0.06	NA	38 ± 1

¹Theoretical content of trehalose was ~3 % w/w in the formulations. Data reported as mean ± SE, *n* = 3 for (a) SE and (b) AAA, respectively; total microsphere mass in loading solution was ~20 mg

Table 2

Self-microencapsulation capacity and efficiency of optimal ASE BP-PLGA microspheres loaded from 1 ml LYZ solutions in 10 mM phosphate buffer (pH 7)².

BP	Loading Solution Conc. (mg/ml)	LYZ Encapsulation	
		Loading (% w/w)	Efficiency (%)
HDS	0.5	2.2 ± 0.1	90 ± 0.1
HDS	1	4.6 ± 0.1	95 ± 0.2
HDS	1.5	7.0 ± 0.1	97 ± 0.1
LDS	0.5	1.5 ± 0.1	63 ± 2
LDS	1	4.1 ± 0.1	83 ± 2
LDS	1.5	6.8 ± 0.2	91 ± 1
CS	0.5	2.1 ± 0.1	89 ± 0.1
CS	1	4.5 ± 0.1	95 ± 0.3
CS	1.5	7.4 ± 0.1	97 ± 0.2
HP	0.5	2.2 ± 0.1	90 ± 0.3
HP	1	4.7 ± 0.2	94 ± 1
HP	1.5	7.3 ± 0.1	97 ± 0.1

²Theoretical content of MgCO₃, trehalose and BP was ~3, 3 and 4 % w/w in the formulations, respectively. ASE of LYZ quantified by SE-HPLC and UV spectroscopy. Data reported as mean ± SE, *n* = 3; total microsphere mass in loading solution was ~20 mg.

Table 3

Self-microencapsulation capacity and efficiency of a small amount of ASE HDS-PLGA microspheres loaded with excess of LYZ from 10 mM phosphate buffer³.

BP	HDS (% w/w)	Loading Solution		LYZ Encapsulation		LYZ bound (mg/mg HDS)
		Vol.(ml)	Conc.(mg/ml)	Loading (% w/w)	Efficiency (%)	
HDS	2	1	3	1.3 ± 0.1	4.5 ± 0.1	0.66 ± 0.01
HDS	5.1	1	3	3.4 ± 0.4	13.5 ± 0.4	0.67 ± 0.03
HDS	12.6	1	3	3.4 ± 0.3	13.1 ± 0.2	0.28 ± 0.02
HDS	2	0.5	6	4.1 ± 0.01	13.7 ± 0.1	2.03 ± 0.04
HDS	5.1	0.5	6	6.2 ± 0.3	20.9 ± 0.5	1.26 ± 0.02
HDS	12.6	0.5	6	8.1 ± 0.2	27.1 ± 0.3	0.67 ± 0.03

³Theoretical content of MgCO₃ and trehalose was ~3 % w/w in the formulations. ASE of LYZ quantified by SE-HPLC and UV spectroscopy. LYZ binding capacity was calculated using theoretical amount of HDS and LYZ in the loading solution. Data reported as mean ± SE, *n* = 3; total microsphere mass in loading solution was ~10 mg.

Table 4Biomimetic ASE of growth factors in BP-PLGA microspheres⁴.

BP	Loading Solution	Loading (% w/w)	Encapsulation Efficiency (%)
HDS	0.5 mg/ml FgF-20	2.3 ± 0.1	87 ± 1
LDS	0.5 mg/ml FgF-20	2.1 ± 0.1	84 ± 1
CS	0.5 mg/ml FgF-20	1.9 ± 0.2	85 ± 2
HP	0.5 mg/ml FgF-20	2.1 ± 0.1	81 ± 1
HDS	1 mg/ml VEGF	4.1 ± 0.4	73 ± 6

⁴Theoretical content of MgCO₃, trehalose and BP was ~3, 3 and 4 % w/w in the formulations, respectively. ASE of FgF-20 was from 1ml of 0.5 mg/ml FGF-20 loading solution (0.5M arginine, 0.05 M sodium phosphate and 0.08% polysorbate). ASE of VEGF in HDS-PLGA microspheres was from 1ml of 1 mg/ml VEGF loading solution (5 mM succinate buffer, 275 mM trehalose and 0.01% polysorbate 20). ASE quantified by SE-UPLC and/or coomassie plus assay. Mean ± SE, *n* = 3; total microsphere mass in loading solution was ~20 mg.