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Protein Encapsulation via Polypeptide Complex Coacervation

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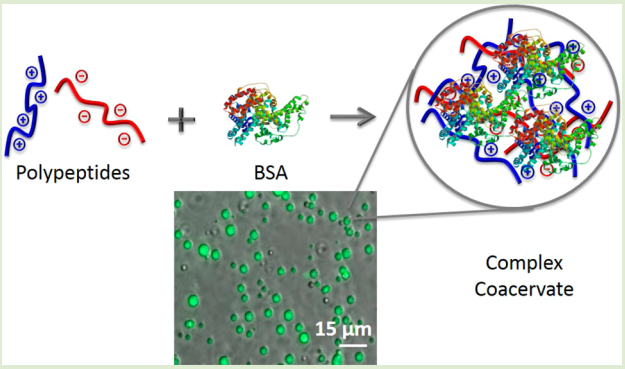
Supporting Information

ABSTRACT: Proteins have gained increasing success as therapeutic agents; however, challenges exist in effective and efficient delivery. In this work, we present a simple and versatile method for encapsulating proteins via complex coacervation with oppositely charged polypeptides, poly(L-lysine) (PLys) and poly(D/L-glutamic acid) (PGlu). A model protein system, bovine serum albumin (BSA), was incorporated efficiently into coacervate droplets via electrostatic interaction up to a maximum loading of one BSA per PLys/PGlu pair and could be released under conditions of decreasing pH. Additionally, encapsulation within complex coacervates did not alter the secondary structure of the protein. Lastly the complex coacervate system was shown to be biocompatible and interact well with cells in vitro. A simple, modular system for encapsulation such as the one presented here may be useful in a range of drug delivery applications.



Recent advances in molecular biology have enabled the discovery of a plethora of protein and peptide therapeutics. Protein therapeutics are now used to treat a variety of diseases, including diabetes[1] and cancer.[2] Though protein therapeutics enjoy specificity and high potency, effective and efficient delivery remains challenging. When delivered intravenously, protein drugs suffer from low bioavailability and are easily degraded. Proteins in the bloodstream can be degraded by proteases, denatured, or targeted by the mononuclear phagocyte system for removal, greatly limiting their efficacy.[3]

Various strategies have been developed to improve the delivery of proteins, including chemical modification with polyethylene glycol and encapsulation into liposomal or polymeric carriers.[3−5] One polymeric carrier system, poly(lactic-*co*-glycolic acid) (PLGA) has received a lot of attention due to its biocompatibility and ability to degrade by hydrolysis over time.[6] PLGA has been shown to effectively encapsulate insulin,[7] and is also approved by the FDA for use in human growth hormone delivery to treat deficiencies in pediatric patients.[8] PLGA particles are typically formed using a solvent emulsion or nanoprecipitation method. Although this system has had much success, the conditions under which the particles are formed, using harsh solvents, may cause denaturation and inactivity of the protein.[9]

An alternative method for polymeric encapsulation is the electrostatically driven phenomenon of complex coacervation. Complex coacervates are formed when oppositely charged polyelectrolytes are mixed in aqueous solution. Coacervation results in a liquid−liquid phase separation in which a dense polymer-rich phase (coacervate) separates from the dilute polymer-poor solution phase (aqueous phase). This process was first observed using natural polymers gelatin and gum Arabic.[10] More recently, systems of complex coacervation have been explored for drug delivery using such naturally occurring polymers as alginate, chitosan, and heparin.[11,12] Though this method shows promise, the process of encapsulation, tunable parameters, and biocompatibility are not well characterized for the application of protein delivery. Additionally there is a need to expand coacervate-based drug delivery platforms to include synthetic systems, so as to enable de novo design with enhanced functionality and precise molecular control.

Synthetic polypeptides offer a wide range of tunability and control of coacervate formation based on the diversity of amino acid sequences used. Synthetically produced polypeptides such as the ones used here, poly(L-lysine) and poly(D/L-glutamic acid), are biocompatible and have been used in biomaterial applications such as coatings and covalent drug modifications.[13] Previous work identified the conditions under which these polypeptides form complex coacervates in solution.[14−16] In

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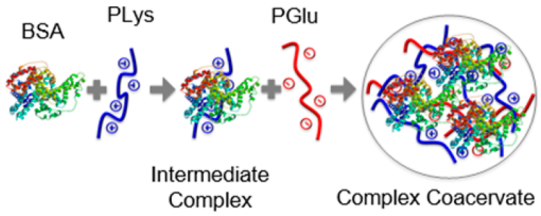
addition to polymer chemistry, salt concentration, pH, polycation/polyanion stoichiometry, total polymer concentration, and temperature are important system parameters that can be tuned to control coacervate formation.[16,17] Additionally, it was found that polypeptide complex coacervates exhibit low interfacial tension with water, which may be useful for encapsulation of charged materials.[18−20]

We present a versatile method to encapsulate proteins by complex coacervation using polypeptides. Incorporation efficiency was studied by separating the dense coacervate phase from the corresponding solution phase and quantifying the excess protein present in solution (and thus not incorporated). Preservation of secondary structure and conditions of release were also examined, as these present challenges to the field of protein delivery. Lastly, the interaction of the protein-loaded coacervates with cells was studied as an initial test for the feasibility of using this system clinically.

Charged proteins can be encapsulated via complex coacervation using benign, aqueous conditions (phosphate buffered saline (PBS), pH 7.4). First, the protein of interest, bovine serum albumin (BSA), and the polycation, poly(L-lysine) (PLys), were mixed to form an intermediate complex based on the electrostatic interaction of the positively charged PLys with BSA, which has a net negative charge at neutral pH. In order to visualize the encapsulated protein, a fluorescently tagged BSA protein was used. Subsequently, the polyanion, poly(D/L-glutamic acid) (PGlu), was added and protein-containing complex coacervates were formed (Scheme 1).

Scheme 1. Preparation of Polypeptide Complex Coacervates with Encapsulated Proteins



The successful encapsulation of protein can be confirmed visually using optical microscopy, based on the colocalization of the green fluorescence signal from the protein with the coacervate droplet (Figure 1, right). Image analysis was

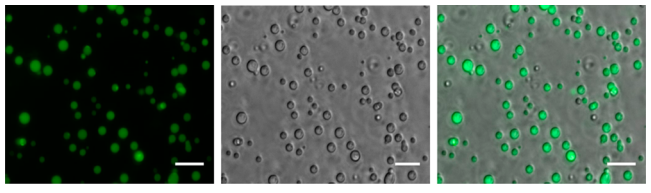


Figure 1. Optical micrograph of FITC-BSA encapsulation within coacervate droplets shown in fluorescence (left) and brightfield (center). Colocalization of fluorescence within the droplets confirms protein encapsulation (right). Scale bar represents 15 μm.

performed using ImageJ to determine the diameter of the droplets. Using conditions of 30 μM total polypeptide and 1.5 μM BSA coacervate droplets are formed measuring 4.3 ± 1.0 μm in diameter.

The efficiency of protein encapsulation was measured using a protein colorimetric assay (Bradford). Briefly, coacervates were formed as described above, keeping the overall concentration of PLys and PGlu constant while steadily increasing the amount of BSA (Supporting Information). Encapsulation was measured by separating the coacervates from the solution phase by centrifugation and then quantifying the amount of protein remaining in the solution phase (i.e., not encapsulated). Values were compared to a control with no polypeptides. At a ratio of 0.05 BSA per polypeptide (or 20 polypeptides per BSA molecule), 100% of the added BSA was encapsulated. As the ratio of BSA to polypeptide was increased, the total amount of BSA encapsulated increased up to a maximum of 0.31 BSA per polypeptide, representing 63% encapsulation efficiency (Figure 2). Depending on the application needs, a choice can be made

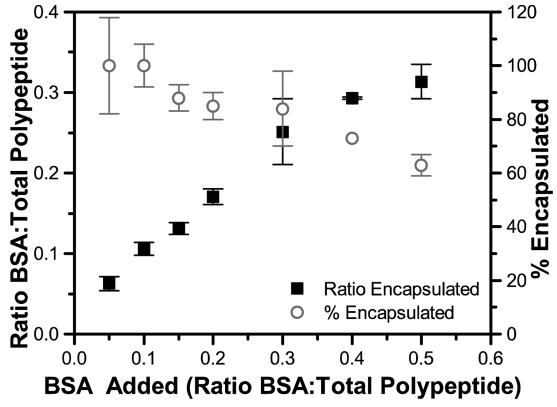


Figure 2. Encapsulation efficiency of polypeptide coacervate system. As the ratio of BSA to polypeptide was increased, more BSA was encapsulated, but the efficiency of encapsulation was decreased. Error bars represent standard deviation.

between efficiency and uptake, thus reducing either waste or the product loading. Where proteins are expensive to produce, the ability to control uptake efficiency is important for clinical success.[21]

Preservation of secondary structure is an important criterion for protein delivery, as structure can relate directly to activity and function. The potential for variations in protein structure was examined using circular dichroism. BSA is a primarily α-helical protein, as evidenced by the two characteristic minima at 208 and 222 nm.[22] A similar α-helical structure was observed for BSA encapsulated in coacervates (Figure 3). The observed decrease in intensity was likely the result of loss of signal due to scattering from the droplets.

Polypeptide-based complex coacervates form as a result of electrostatic associations between oppositely charged amino acid side chains and, thus, are sensitive to changes in pH. This pH responsiveness makes them ideal for drug delivery, because they can disassemble and trigger the release cargo upon entry into a low pH environment, such as in the endosome or lysosome of cells. Turbidity was used as a measure of total complex formation. Coacervate disassembly was determined by assuming that turbidity values at pH 7.4, the pH at which the coacervate phase is stable, correspond to 0% disassembled (eq 1) where $T_{7.4}$ is the turbidity reading at pH 7.4 and T_X corresponds to turbidity at a given pH X.

$$\%\text{dissassembled coac.} = \left[\frac{T_{7.4} - T_X}{T_{7.4}}\right] \times 100\% \quad (1)$$

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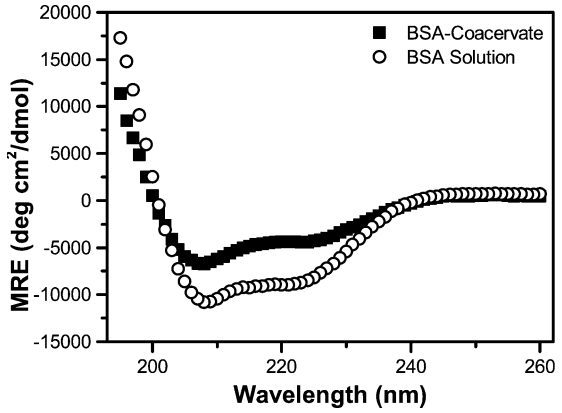

Figure 3. CD spectra of free (open) and encapsulated (solid) BSA showing characteristic dual minima of 208 and 222 indicative of α-helical secondary structure. Encapsulation was performed at a ratio of 0.05 BSA to polypeptide to ensure 100% encapsulation.

As pH was decreased, the complex coacervates disassembled and were fully disassembled by pH 2 (Figure 4). With decreasing pH, the PGlu carboxylic acid side chains (pK_a = 4.25) become increasingly protonated, thus weakening the electrostatic interaction between PGlu and PLys. Shorter polypeptides (N = 100) showed lower stability as a function of pH. An inflection point in the disassembly curve of polypeptides with N = 100 occurs at pH = 5, and for longer chains, N = 400 at pH = 4 (Figure 4). Polypeptide molecular weight thus provides an additional parameter for controlling protein release. Secondary structure was also measured following release at conditions of decreasing pH and it was found that at a pH of 5 and 4, released BSA retained α-helical structure (Figure S1).

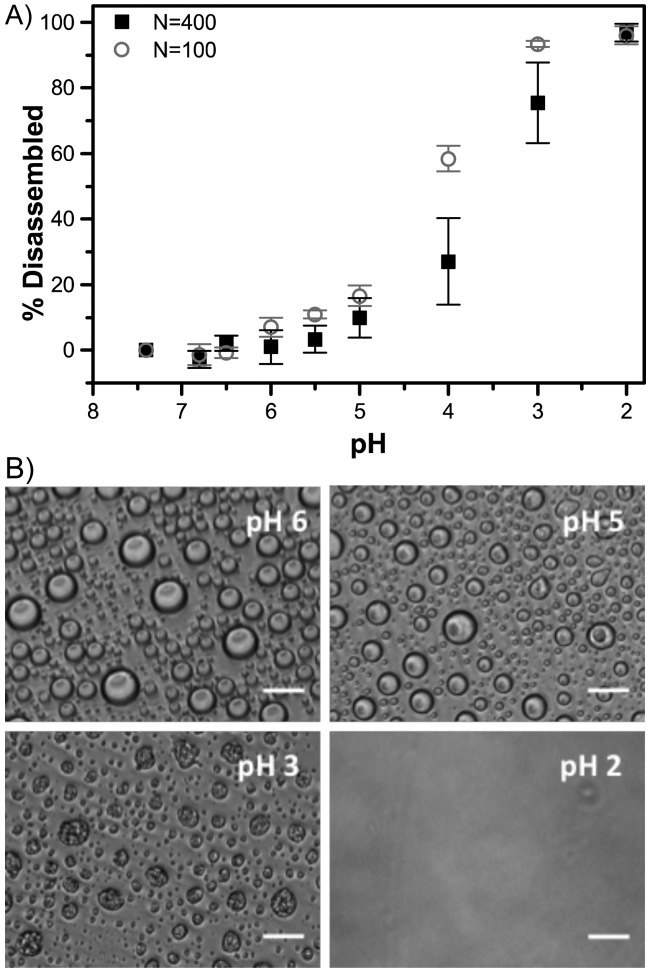

Figure 4. (A) In vitro release of FITC-BSA from coacervates (N = 400 and 100) with decreasing pH. (B) Representative images of coacervates as a function of decreasing pH (N = 400). Scale bars represent 25 μm.

The interaction of BSA loaded coacervates with NIH 3T3 cells was also investigated. Cell viability was measured after incubation with BSA loaded coacervates, BSA, polypeptide-only coacervates, PLys, PGlu, and an untreated control. PLys alone showed a slight toxicity, which was statistically significant compared to other treatment groups (ANOVA, Tukey p < 0.05; Figure 5A). This result was expected as positively charged polymers may coat or interact with the negatively charged membrane of cells, causing membrane disruption and cell death.[23] Due to the interest in polycations, such as PLys, for other applications, such as gene delivery, several strategies to improve biocompatibility have been suggested such as reducing the molecular weight and modifying the primary amine group.[24] Here we present cytotoxicity studies for the longest polypeptide (60 kDa) used in this work. Based on literature, decreasing the polypeptide length to 30 or 15 kDa would decrease the cytotoxicity. Importantly, no significant toxicity for BSA loaded coacervates, coacervates alone, BSA alone or PGlu alone was observed (Figure 5A).

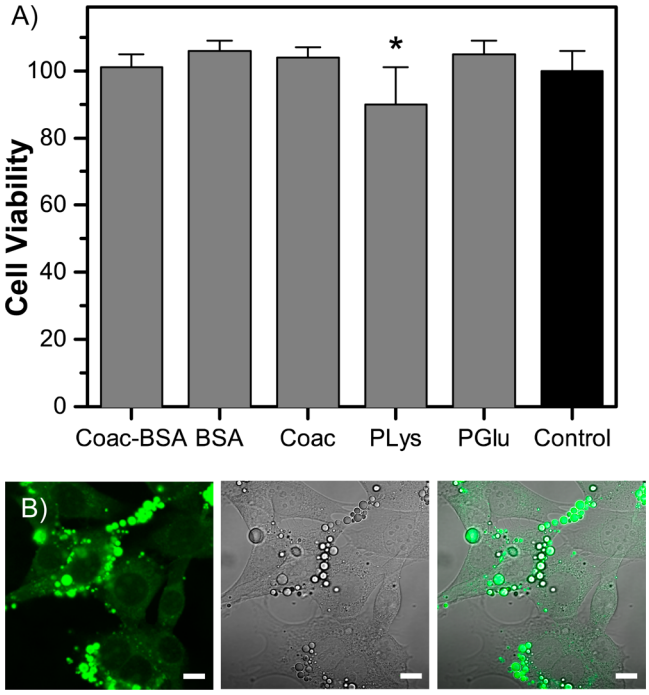

Figure 5. (A) Cell viability after 24 h incubation with BSA loaded coacervates (Coac-BSA), BSA alone, coacervates alone (Coac), PLys, or PGlu relative to a cell control. (B) Representative fluorescence (left), brightfield (middle), and overlay (right) images of cells incubated with FITC-BSA loaded coacervates. Scale bar represents 20 μm.

Direct inspection of cells incubated with FITC-BSA loaded coacervates for 24 h show cells that were well spread and have a

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healthy appearance. BSA loaded coacervates appear to associate strongly with cell membranes, remaining attached despite multiple washing steps prior to imaging (Figure 5B). Further studies are necessary to determine the mechanism of the observed interaction, as well as to quantify internalization. The viability and imaging studies presented here indicate a biocompatible and positive interaction with cells.

In conclusion, we present here a method for encapsulating proteins via the electrostatic association of charged polypeptides. Complex coacervates are formed via simple mixing in benign, aqueous conditions, making them advantageous for protein encapsulation. A model protein, BSA was encapsulated with a tunable efficiency ranging from 100 to 63% corresponding to a protein to polypeptide ratio of 0.05 to 0.3. The ability to tune loading of the protein and control efficiency of uptake is particularly important for the use of protein therapeutics, which can be expensive to produce. The encapsulation process did not affect the secondary structure of the protein, often an important condition for activity. Release of BSA was demonstrated under conditions of decreasing pH. Triggered release at low pH may be advantageous for delivering protein cargo once the assembly enters the cell. Lastly, protein-loaded coacervates were shown to be nontoxic in a cell viability assay.

The studies here present a simple and effective method for encapsulating proteins using polypeptide-based complex coacervates. The primary advantage that our complex coacervation technology has over existing products is that it is an organic solvent-free system. Prior polymer encapsulation such as PLGA particles require organic solvent emulsion or phase separation techniques where sensitive proteins may be susceptible to denaturation, oxidation, and cleavage at the aqueous−organic solvent interface resulting in loss of function to the protein.[25,26] Encapsulation of proteins via complex coacervation of polypeptides is a method driven by electrostatic interaction in aqueous buffer and therefore avoids the unwanted loss of function effect of organic solvents. Such benefits have recently been demonstrated a chitosan complex coacervation system.[27,28] Additionally, the use of an aqueous buffer system negates the need for filtration or separation steps to remove toxic organic solvents prior to use in medical or food applications.[29] A system such as this could solve the problems with other protein delivery systems specifically in terms of process compatibility and scale up. Future studies will seek to elucidate the location of the protein within the coacervate droplets as well as methods of stabilization to prevent droplet coalescence.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, methods, and CD spectra for released BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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■ REFERENCES

(1) DeWitt, D. E.; Hirsch, I. B. *J. Am. Med. Assoc.* **2003**, *289*, 2254−2264.
(2) Scott, A. M.; Wolchok, J. D.; Old, L. J. *Nat. Rev. Cancer* **2012**, *12*, 278−287.
(3) Brown, L. R. *Exp. Opin. Drug Delivery* **2005**, *2*, 29−42.
(4) Jevsevar, S.; Kunstelj, M.; Porekar, V. G. *Biotechnol. J.* **2010**, *5*, 113−128.
(5) Torchilin, V. P. *Nat. Rev. Drug Discovery* **2005**, *4*, 145−160.
(6) Danhier, F.; Ansorena, E.; Silva, J. M.; Coco, R.; Le Breton, A.; Préat, V. *J. Controlled Release* **2012**, *161*, 505−522.
(7) Kumar, P. S.; Ramakrishna, S.; Saini, T. R.; Diwan, P. V. *Pharmazie* **2006**, *61*, 613−617.
(8) Johnson, O. L.; Cleland, J. L.; Lee, H. J.; Charnis, M.; Duenas, E.; Jaworowicz, W.; Shepard, D.; Shahzamani, A.; Jones, A. J. S.; Putney, S. D. *Nat. Med.* **1996**, *2*, 795−799.
(9) Giteau, A.; Venier-Julienne, M.-C.; Marchal, S.; Courthaudon, J.-L.; Sergent, M.; Montero-Menei, C.; Verdier, J.-M.; Benoit, J.-P. *Eur. J. Pharm. Biopharm.* **2008**, *70*, 127−136.
(10) Bungenberg de Jong, H. B.; Kruyt, H. R. *Proc. Sect. Sci, Koninkijke Ned. Akad. van Wet.* **1929**, *32*, 849−856.
(11) Sarmento, B.; Ribeiro, a; Veiga, F.; Sampaio, P.; Neufeld, R.; Ferreira, D. *Pharm. Res.* **2007**, *24*, 2198−2206.
(12) Chu, H.; Gao, J.; Chen, C.-W.; Huard, J.; Wang, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 13444−13449.
(13) Shih, I.-L.; Van, Y.-T.; Shen, M.-H. *Mini-Rev. Med. Chem.* **2004**, *4*, 179−188.
(14) Priftis, D.; Tirrell, M. *Soft Matter* **2012**, *8*, 9396−9405.
(15) Priftis, D.; Megley, K.; Laugel, N.; Tirrell, M. *J. Colloid Interface Sci.* **2013**, *398*, 39−50.
(16) Perry, S.; Li, Y.; Priftis, D.; Leon, L.; Tirrell, M. *Polymers (Basel, Switz.)* **2014**, *6*, 1756−1772.
(17) Priftis, D.; Xia, X.; Margossian, K. O.; Perry, S. L.; Leon, L.; Qin, J.; de Pablo, J. J.; Tirrell, M. *Macromolecules* **2014**, *47*, 3076−3085.
(18) Spruijt, E.; Sprakel, J.; Cohen Stuart, M. a.; van der Gucht, J. *Soft Matter* **2010**, *6*, 172−178.
(19) Priftis, D.; Farina, R.; Tirrell, M. *Langmuir* **2012**, *28*, 8721−8729.
(20) Qin, J.; Priftis, D.; Farina, R.; Perry, S. *ACS Macro Lett.* **2014**, *3*, 565−568.
(21) Jain, R. a. *Biomaterials* **2000**, *21*, 2475−2490.
(22) Holzwarth, G.; Doty, P. *J. Am. Chem. Soc.* **1965**, *87*, 218−228.
(23) Kim, S. W. *Cold Spring Harbor Protoc.* **2012**, *2012*, 433−438.
(24) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. *Biomaterials* **2003**, *24*, 1121−1131.
(25) Pisal, D.; Kosloski, M.; Balu-lyer, S. *J. Pharm. Sci.* **2010**, *99*, 2557−2575.
(26) Burgess, D. J.; Ponsart, S. *J. Microencapsulation* **1998**, *15*, 569−579.
(27) Koppolu, B. P.; Smith, S. G.; Ravindranathan, S.; Jayanthi, S.; Suresh Kumar, T. K.; Zaharoff, D. a. *Biomaterials* **2014**, *35*, 4382−4389.
(28) Ozbas-Turan, S.; Aral, C.; Kabasakal, L.; Keyer-Uysal, M.; Akbuga, J. *J. Pharm. Pharm. Sci.* **2003**, *6*, 27−32.
(29) Schmitt, C.; Turgeon, S. L. *Adv. Colloid Interface Sci.* **2011**, *167*, 63−70.