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Gelatin-alginate coacervates for circumventing proteolysis and probing intermolecular interactions by SPR

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ABSTRACT

Complex coacervates based on natural biopolymers have been explored as a protein delivery system to provide controlled release of loaded protein and circumvent the proteolytic degradation in chronic wounds. The coacervates composed of gelatin A (GA) and sodium alginate (SA) were optimized with respect to turbidity, size, zeta potential, and polydispersity index. Bovine serum albumin (BSA), a model protein, was effectively encapsulated in the coacervates, resulting in protection from trypsin digestion and controlled release. In contrast, EGF was not encapsulated in the same coacervates. Striking difference in the encapsulation efficiencies of BSA and EGF, despite their similar net charges, was attributed to their different levels of binding to GA based on the surface plasmon resonance (SPR) biosensor analysis. In conclusion, GA and SA coacervates can protect the encapsulated protein from proteolytic degradation, demonstrating its potential as a delivery system in the chronic wounds. SPR biosensor is proposed as an analytical tool to study the interactions between polymers and proteins in association with encapsulation efficiency in complex coacervation. The results of EGF studies suggested that GA was not a suitable polymer for EGF encapsulation and therefore, further investigation would be needed to find suitable polymer systems for improved encapsulation efficiency.

1. Introduction

In chronic wounds such as diabetic foot ulcer (DFU), high levels of inflammatory cells lead to the elevated levels of proteases that appear to degrade extracellular components, growth factors, and other proteins. Elevated levels of proteases in the chronic wound environment can also lead to degradation of the growth factors topically applied for wound healing purposes [1,2]. Hence, treatment options with growth factors for chronic wounds would exhibit limited efficacy unless the drug delivery system is designed to circumvent protease degradation in the wound bed. In addition to improved efficacy, ease of administration and prolonged storage stability at ambient conditions would benefit both patients and care givers. Maintaining the integrity of the protein as its native form is vital to retain functional activity of the protein before being delivered to the patients [3]. An advanced topical delivery system satisfying above mentioned features for chronic wounds is yet to be developed.

In light with the limitations of topical delivery system for chronic wound healing, a number of complex coacervate systems have been studied as a topical delivery platform with the hypothesis that coacervate would reduce the degradation of encapsulated protein and maximize the release efficiency at wound beds. For example, a synthetic positively charged polymer, polyethylene adipate, and heparin were used as polyelectrolytes complex to carry the desired growth factors such as epidermal growth factor (EGF) and vascular endothelial growth factors (VEGF) [4–10]. Complex coacervate is a liquid-liquid separation system held together mainly by electrostatic interactions between polyelectrolytes [11–14]. It often appears in the form of droplets in spherical shape and resembles the form of emulsion of dispersant and the size varies from micro to nanometers. Upon formation, the complex will appear in two phases, dilute and dense phases. In the past, complex coacervate has been often applied in food industry and recently, coacervate in drug delivery system (DDS) has been reported for application in the DFU, inflammation, and even in the cardiovascular diseases [8,10,15,16]. Synthetic biodegradable polymers such as polypeptides, poly (L-lysine), and poly (D/L-glutamic acid) were used to encapsulate BSA as potential DDS in biomedical application [17]. However, information on the coacervate using natural biopolymers in biomedical science is still limited. Hence, further investigation on the use of natural biopolymer based coacervate can potentially provide an alternative platform for topical wound treatment.

In the present study, we aimed to develop coacervate composed of natural biopolymers, sodium alginate (SA) and gelatin A (GA), to circumvent encapsulated proteins from protease in the wound bed. SA and GA were selected as polyelectrolytes for coacervate system because of their well-known wound healing effect [4,18–20]. GA is also known to carry anti-inflammatory properties, which can accelerate wound

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healing activity [21,22]. The coacervates composed of sodium alginate and gelatin A (GA/SA) were optimized with respect to the physical properties such as turbidity, size, zeta potential, and polydispersity index (PDI). In addition, BSA was selected as a model protein to evaluate the encapsulation efficiency in the present GA/SA coacervate. Encapsulation of EGF was then measured to evaluate the feasibility of the present GA/SA coacervate as a drug delivery system to chronic wounds. Despite the previous reports on the use of coacervate system in biomedical research, science behind the system remains poorly understood. Another aim of the present study was to use SPR biosensor to analyze the interactions between proteins and biopolymers by determining their specificity and kinetics in real-time, in particular, to better understand the encapsulation efficiency of different target proteins, BSA and EGF. To date, there is no report on the use of SPR biosensor to study the GA/SA coacervate encapsulating BSA or EGF.

2. Materials and methods

2.1. Materials

Sodium alginate was from Daejung (Gyeonggi-do, Korea), Korea. Gelatin A (Gel strength 300, and 90–110) was obtained from Sigma-Aldrich (St. Louis, MO, USA), Fluorescein isothiocyanate (FITC) was purchased from TCI (Tokyo, Japan). Phosphate buffer solution (PBS) buffer was obtained from Gibco, USA. Bovine serum albumin (BSA) was obtained from Milipore, (Bedford, MA, USA). Epidermal growth factor (EGF) was a gift from Daewoong Pharmaceutics Company, South Korea. Sephadex™ G-50 Fine, CM5 sensor chip, amine coupling kit, 10 mM sodium acetate buffer at different pH, 50 mM NaOH, ethanolamine were obtained from GE Healthcare, Bio-Sciences AB, (Uppsala, Sweden).

2.2. Formulation and characterization of coacervate

2.2.1. Coacervate formulation based on gelatin and sodium alginate to encapsulate BSA and EGF as model drugs

0.5% (w/v) of gelatin A (GA), 0.5% (w/v) of sodium alginate (SA), and 1 mg/mL of BSA were prepared as a stock solution for fabrication of BSA coacervate. To optimize the formation of BSA coacervate, GA:SA mixture at different volume ratios (3:1, 4:1, 5:1, 6:1) v/v and BSA solution of 100 μg/mL were added sequentially into a 1.5 mL tube to a final volume of 1 mL. The pH of each formulation was adjusted to determine the efficiency of coacervation. Optimal coacervate was chosen based on turbidity and minimal formation of precipitates among various pH conditions. Each vial of formulation was incubated at room temperature for 30 min for equilibrium. Coacervate was concentrated when necessary, via centrifugation at 14,000 rpm for 10 min in a bench top centrifuge (Labogene, Seoul, Korea) for collecting the pellet. GA:SA with the highest encapsulation efficiency was used in the subsequent studies. EGF coacervate was prepared using the same formulation developed for BSA coacervate. EGF solution of 100 μg/mL was used to prepare the EGF coacervate with GA and SA at several ratios (3:1, 4:1, 5:1, and 6:1).

2.2.2. Physical characterization of protein coacervate

The zeta potentials of proteins and optimized coacervate formulation were measured using Zetasizer Nano-ZS (Malvern Instruments, Worcester, UK). The measurement was based on electrophoretic light scattering method. In brief, 1 mL of 100 μg/mL sample in distilled water was transferred into a folded capillary cell (Malvern Instrument, DTS 1060C) using a disposable syringe. Three runs were done with each run consisting of 10 single measurements. The size distribution and polydispersity (PDI) of the BSA coacervate were measured using dynamic light scattering (DLS) on Zetasizer Nano-S (Malvern Instruments, Worcester, UK). All measurements were repeated at least twice. Turbidity of the BSA and EGF coacervates (pH 4–8) at 600 nm was measured in 1 mL cuvette using SpectraMax 384 microplate reader (Molecular Devices, Sunnyvale, USA). All the measurements were done in triplicate.

2.2.3. Preparation of FITC labelled BSA

Fluorescein isothiocyanate (FITC) labelled BSA (FITC-BSA) was prepared according to the manufacturer's protocol. The FITC-BSA was then filtered through a Sephadex™ G-50 Fine gel matrix with an exclusion limit of 20,000–50,000 to collect the FITC-BSA. The FITC-BSA was then used for further quantification of encapsulation, release studies, and proteolysis activity.

2.2.4. Encapsulation and loading efficiency

For qualitative estimation of encapsulation efficiency, the supernatant and pellet of the coacervate after centrifugation were analyzed using sodium dodecyl sulfate polyacryclamide gel (SDS-PAGE). In brief, concentrated complex coacervate was centrifuged at 14,000 rpm for 10 min (Labogen, Seoul, Korea). After collecting the supernatant, pellets were dissolved in PBS. A total of 20 μL each of pellet and supernatant were heated at 100 °C for 10 min and loaded onto 15% SDS-PAGE. Silver stain was carried out to determine the encapsulated and free protein in pellet and supernatant respectively.

For quantitative measurement of the encapsulation efficiency, the percentage of FITC-BSA encapsulated in the coacervate droplet was calculated as follows:

$$\%\text{Encapsulation efficiency} = \left[\left(\text{Total BSA} - \text{Free "unencapsulated BSA"/Total BSA}\right)\right] * 100$$

Free "unencapulated BSA" was quantified by measuring the fluorescence intensity of FITC-BSA in the supernatant after centrifugation of the coacervation reaction solution at 14,000 rpm for 10 min (Labogene, Seoul, Korea). The fluorescence intensity was measured using Multimode Microplate Reader (Molecular Devices, Sunnyvale, USA) with excitation at 495 nm and emission at 595 nm.

Loading efficiency was calculated based on the BSA encapsulated and the total dried weight of the complex. Encapsulated BSA was determined from the free "unencapsulated BSA" quantified above.

$$\%\text{Loading efficiency} = [\text{Encapsulated BSA/polymer complex dried weight}] * 100$$

Different volume ratios of GA:SA were used to determine the encapsulation efficiency and loading efficacy of BSA coacervate.

2.2.5. Proteolytic digestion of BSA in the coacervate relative to free BSA

BSA coacervate and physical mixture were prepared at GA:SA ratio 4: 1 and 100 μg/mL BSA as described in Section 2.2.1. BSA-coacervate, physical mixtures of BSA and free BSA were prepared with GA:SA ratio 4:1 without and with 0.5 M acetic acid 5 μL, respectively. Samples were incubated at 37 °C with 100 rpm for 2 h in the presence of trypsin (weight ratio of BSA: trypsin, 1:1 and 1:4) to determine the protection of BSA in the coacervate from degradation compared to free BSA and physical mixture. To adjust the pH of BSA-coacervate samples which can impact the trypsin activity, 50 mM NaOH 7 μL was added in the sample tube. All samples were analyzed using 12% SDS-PAGE to determine the protection ability of BSA-coacervate.

2.2.6. Freeze-drying of coacervate

Concentrated BSA coacervate obtained by centrifugation of the coacervation reaction solution in 1.5 mL tubes followed by removal of the supernatant were first frozen at −80 °C overnight and then dried using a freeze dryer (Gperon, Korea).