Effect of Complex Coacervation with Hyaluronic Acid on Protein Transition in a Subcutaneous Injection Site Model System

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The occurrence of complex coacervation in an aqueous mixture of proteins (lysozyme, albumin, immunoglobulin G) and hyaluronic acid and its effect on protein transition in a model system was studied to elucidate factors determining the bioavailability of subcutaneously injected therapeutic proteins. Mixing of hyaluronic acid and the model proteins induced complex coacervation at solution pH close to or below the isoelectric point of the proteins. In vitro dialysis using membranes with large pore size tube represented a limitation in the protein transition of the coacervation mixture. Thermal analysis suggested there was retention of the protein conformation in the polymer complex.

Key words subcutaneous injection; coacervation; bioavailability

Introduction

Subcutaneous (s.c.) injection of therapeutic protein formulations, including self-administration products, is increasing due to the advantages in reducing required patient time and healthcare professional resources compared to intravenous (i.v.) injection and infusion formulations.1–4 The subcutaneous administration, however, is often accompanied by a lower and more varied bioavailability of the active ingredient (e.g., 50–80%), which may affect the product’s efficacy and safety.5–7 Steric barriers and heteromolecular interactions are considered to slow transition of the injected protein molecules through the extracellular space of the subcutaneous tissue (hypodermis) toward the blood or lymphatic capillaries.1 Higher viscosity of many high concentration monoclonal antibody formulations may further perturb their transition. Possible catabolism of the retained proteins may also reduce the bioavailability. Longer retention of the protein in the interstitial matrix, consisting largely of collagen, glycosaminoglycans (GAGs), and proteoglycans, would allow their potential degradation by enzymes or provides greater possibility of being recognized as antigen that induces anti-drug antibodies (ADA).5,8 Hyaluronic acid, consisting of repeated units of N-acetylgalactosamine and glucuronic acid, is the most common GAG in the hypodermis.9 Thus the protein mobility in the region depends largely on electrostatic interactions with the negatively charged hyaluronic acid. Proteins with a positive charge under physiological conditions often show lower bioavailability following the subcutaneous injections.5 In order to study the transition of small and large active pharmaceutical ingredient (API) molecules in the subcutaneous environment, some systems including agarose gel layers9 or a combination of hyaluronic acid-rich media mimicking the interstitial matrix and dialysis membranes (e.g., Subcutaneous Injection Site Simulator9) have been proposed as novel in vitro models.

It has long been known that mixing some polysaccharide and protein solutions induces complex coacervation.10,11 Hyaluronic acid and some basic proteins (e.g., lysozyme) are typical combinations that show complex coacervation at a pH close to and/or below the isoelectric point (pI) of the proteins.12–17 The potential for occurrence and effects of coacervation in the hypodermis have not been well elucidated, partially due to insufficient information regarding the microenvironment. The purpose of this study was to elucidate the factors that affect the coacervation of a mixture of protein and hyaluronic acid and the effect of coacervation on the transition of protein in media using a large pore dialysis membrane model. The conformational stability of the coacervated proteins was also evaluated.

Experimental

Hen egg lysozyme (MW: 14.4 kDa, Seikagaku Co., Japan), recombinant human albumin (MW: approx. 67 kDa, expressed in rice, Sigma-Aldrich Co., U.S.A.), and bovine immunoglobulin G (MW: approximately 150 kDa, MP Biomedicals LLC., U.S.A.) were dialyzed against designated buffers before use. Hyaluronic acid sodium salt (MWCO: 3.5 kD) and a dialysis tube were purchased from Wako Pure Chemical Corporation (Japan) and Spectrum Laboratories, Inc. (U.S.A.), respectively. Changes in the appearance of aqueous polymer mixtures were studied by mixing solutions containing 5% protein and 1% sodium hyaluronate in sodium phosphate buffer (20 mM) at room temperature (total volume: 1 mL). The samples were centrifuged to separate the supernatant and semi-solid precipitate before measuring the amount of protein in the fractions using the Pierce BCA Protein Assay Kit. The amounts of protein in the solution phase of the mixed solutions or coacervate were derived from the volume and protein concentration of the supernatants and were reported as the ratio to those present in the initial solutions.

Large pore dialysis tubes (Float-A-Lyzer Dialysis Devices, MWCO: 1000 kD, Spectrum Lab. Inc.) and hyaluronic acid-rich solutions were used as model systems to study the transition of protein from the hyaluronic acid-rich solutions. Aqueous solutions or suspensions (5 mL) containing varied mass...
ratios of protein (lysozyme or albumin), hyaluronic acid, NaCl, and sodium phosphate buffer at varied mass ratio were put into the tube and dialyzed against a corresponding buffer (500mL) at room temperature for 16h. The amount of proteins in the solutions on both sides of the tube, as well as the inner precipitate were obtained. The precipitates were dissolved by adding NaCl solutions before measuring the amount of protein.

A differential scanning calorimeter (DSC, TA Instrument, DSC Q2000) and a differential thermal analysis (DTA) system (Rigaku, TG-DTA8122) equipped with a sample observation camera were used to record the changes in appearance of the mixture during the heating. Aliquots (20µL) of solutions or coacervated suspensions were deposited into aluminum cells and were subjected to thermal scan by the DSC starting at 10°C at a rate of 5°C/min. The changes in appearance of the coacervation precipitates (approximately 15mg), obtained by centrifuging the mixture, were recorded by heating the sample at 5°C/min in open aluminum cells using the DTA system.

**Results**

Mixing aqueous solutions containing a protein (hen egg lysozyme, recombinant human albumin, and bovine immunoglobulin G (IgG)) and hyaluronic acid at different mass ratios induced rapid clouding of the solution and dispersion of white semi-solids or precipitates, typical of complex coacervation of proteins, or remained in the single-phase solution, depending largely on the polymer combinations, mixing ratio, and pH of the solution (Table 1). The presence (lysozyme, pI: 10.7) or absence (albumin, pI: 4.7) of coacervation when mixed with hyaluronic acid at the neutral pH was in agreement with previous reports in the literature. Effect of NaCl (100mM) to prevent the phase separation in the lysozyme and hyaluronic acid mixture solution suggested the contribution of an electrostatic interaction on the coacervation phenomena. Mixtures of bovine IgG with hyaluronic acid showed a different propensity for coacervation depending on the pH of the solution and composition of the pH-modifier. The different profiles of supernatant protein concentration depending on the combination suggested that there was a variation in the mass ratios of the polymers forming the complex (Fig. 1).

The distribution of proteins found inside (solution, precipitate) and outside (solution) of the large pore membrane tubes after dialysis of the protein and hyaluronic acid mixtures are shown in Fig. 2. The large pore dialysis tube was used to find mobility difference between the free and complexed protein molecules. The majority of lysozyme molecules (approximately 85%) moved out of the dialysis tube presumably by diffusion through the large membrane pore in the absence of the polysaccharide. Mixing the protein with hyaluronic acid retained approximately 60% of the lysozyme in the coacervation precipitate inside of the tube. The addition of 100mM NaCl kept the lysozyme and hyaluronic acid mixture miscible and allowed the protein to transition through the membrane. Hyaluronic acid did not significantly alter the transition of albumin in the single-phase mixture. The larger size of the albumin molecule compared to that of the lysozyme molecule may explain the slightly higher ratio of albumin remaining inside of the tube in the absence of the hyaluronic acid.

The lysozyme solutions and coacervate precipitates were subjected to thermal analysis to study potential changes in protein conformation (Fig. 3). The thermal scan of the lysozyme solutions showed the presence of apparent endothermic denaturation at 73.9°C (2.5% (w/w) n = 3). The coacervation induced by the addition of hyaluronic acid (0.5% (w/w)) slightly lowered the peak temperature (1.1°C) and decreased the enthalpy change (approximately 10%) of the lysozyme denaturation. The results strongly suggested the complex coacervation had limited effects on the structural and thermal stability of lysozyme. Heating of the white coacervation precipitate reduced the turbidity for temperatures up to 65°C, and following changes to opaque at above the temperature (Supplementary Figure 1). The process suggested the protein denaturation following hydration and/or re-solubilization of the molecules. Minor differences in the denaturation tempera-

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**Table 1. Physical States of Aqueous Protein and Sodium Hyaluronate Mixtures**

| Protein/Na hyaluronate mass ratio | pH modifier | Sodium phosphate | Histidine HCl |
|---------------------------------|-------------|------------------|--------------|
| Protein/Na hyaluronate mass ratio | pH 7.0  | pH 7.0, 100mM NaCl | pH 7.0 | pH 6.0 | pH 7.0 | pH 6.0 | pH 7.0 |
| 0.55 | PCC | S | S | S | S | S | S |
| 1.23 | CC | S | S | PCC | S | PCC | S |
| 1.64 | CC | S | S |
| 2.10 | CC | S | S | CC | S | CC | S |
| 2.63 | CC | S | S |
| 3.24 | CC | S | S | CC | S | CC | S |
| 4.00 | CC | S | S |
| 4.89 | CC | S | S | CC | S | CC | S |
| 5.96 | CC | S | S | CC | S | CC | PCC |
| 7.32 | CC | S | S | CC | S | CC | CC |
| 9.04 | CC | S | S | CC | S | CC | CC |
| 11.34 | CC | S | S | CC | S | CC | CC |
| 14.54 | CC | S | S | CC | S | CC | CC |
| 19.45 | CC | S | S | CC | S | CC | CC |
| 27.55 | CC | S | S | CC | S | CC | CC |
| 43.81 | CC | S | S | CC | S | CC | CC |

S: Single phase, CC: Complex coacervate, PCC: Partial coacervate.
ture (approximately 78°C) of bovine IgG solution (pH 6.0) and its coacervated precipitate with 0.5% hyaluronic acid also suggested retention of the protein conformation in the coacervate (data not shown).

Discussion

The results indicated complex-coacervation that occurs in some protein and hyaluronic acid combinations, as well as its apparent effect on the transition rate. A slow transition may lead to slower absorption of the coacervated proteins into the blood circulation and/or lymph systems. The physical properties (e.g., size, molecular structure, isoelectric point, hydrophobicity) of the constituent proteins and environmental factors (e.g., mass ratio, pH, co-solute composition, ionic strength, temperature) greatly impacted on the physical changes. Furthermore, the retention of the protein conformation in the coacervate suggested there was a lower risk of increasing any irreversible aggregation and/or enzyme digestion in the hypodermis.19

This simple test system consisting of a combination of hyaluronic acid solution and the large pore dialysis tubes might provide valuable information on the behavior of protein mol-
ecules injected via the subcutaneous route into the hypodermis layer. Flexibility in the choice of dialysis tube and solute composition in both sides of the membrane are advantages of the system. Optimization of the components and of the solute properties to more closely mimic the morphological and compositional heterogeneity present under physiological conditions of the subcutaneous environment would further improve its applicability and to assess relevance of the phenomena. Potential contribution of the heteromolecular interactions on protein transition from the subcutaneous environment should be an intriguing topic for further study. The system may also be valuable for assessing the effect of possible insolubilization/precipitation of proteins in the hypodermis.

Understanding the protein, formulation, and host factors affecting protein behavior after subcutaneous injection should be inevitable to control amount and speed of the protein absorbed into the systemic circulation. The large effect of solution pH and solute composition on the occurrence of the coacervation in the hyaluronic acid and bovine IgG mixture, which have isoelectric points at weakly acidic to neutral pH range (4.6–6.5), suggested the relevance of the optimizing protein structure (e.g., surface charge) and formulation in product developments. The high isoelectric point of many therapeutic monoclonal antibodies (pI 7.5<) should provide greater possibility for strong electrostatic interactions with hyaluronic acid in physiological conditions. Other formulation factors such as use of hyaluronidase, which hydrolyzes polymer molecules to increase the injectable solution volume, would also have an effect on bioavailability.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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