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Deciphering the interactions of fish gelatine and hyaluronic acid in aqueous solutions

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ABSTRACT

The interactions of fish gelatine (FG) with hyaluronic acid (HA) are studied in an aqueous environment at 25 °C by turbidimetric titration, confocal scanning laser microscopy, dynamic light scattering, zeta potentiometry, spectrophotometry with methylene blue, and construction of state diagrams. FG forms soluble complexes with HA above a boundary pH ($pH_{\varphi 1}$), where both biopolymers are net-negatively charged, but develop insoluble complexes as liquid-state complex coacervates below $pH_{\varphi 1}$, where the two biopolymers are oppositely charged. The insoluble complexes are continuously aggregated with further acid titration, followed by immediate visible phase-separation when another boundary pH (pH_p) is reached. The complex formation is mainly driven by electrostatic attractions rather than hydrogen bonding or hydrophobic interactions. The complex formation is promoted by increasing FG-to-HA weight ratio or total biopolymer concentration, or at a low ionic strength, but significantly suppressed in the presence of high ionic strength.

1. Introduction

Protein-polysaccharide interactions have attracted significant attention in the pharmaceutical, cosmetic, and food industries, because they play a key role in developing novel encapsulation devices, stabilising foams and emulsions, recovering proteins from industrial by-products, and engineering smart structures and textures for biocompatible materials, fat replacement, and meat mimesis [1–4]. These interactions also provide valuable insight into a number of relevant biological processes, such as cytoplasm organisation, elastogenesis, DNA/histone collapse, gene replication, antigen-antibody reactions, and enzymatic channelling [2,3]. When proteins and polysaccharides are mixed together in an aqueous environment, their interactions are either attractive or repulsive, depending on intrinsic factors such as molecular weight, molecular structure, net charge, and the flexibility of chains, as well as extrinsic factors such as pH, mixing ratio, total biopolymer concentration, ionic strength, temperature, time, and shearing [5–7]. Attractive interactions can mostly be attributed to electrostatic interactions between two oppositely charged biopolymers, and cause the formation of either soluble or insoluble biopolymer complexes [4]. These insoluble complexes may appear as liquids, *i.e.* complex coacervates, or in the solid state as fractal aggregates or electrostatic gel, *i.e.* precipitates [3,7].

Fish gelatine (FG) is considered a promising alternative to mammalian gelatine because it is devoid of socio-cultural and health-related consumer concerns, such as bovine spongiform encephalopathy, and is also a relatively cheap and widely available by-product of the fish processing industry [4,8]. Recently, our group reported aqueous interactions between FG and gum arabic at 40 °C and between FG and sodium alginate at 25 °C [3,4]. It was found that FG formed liquid-state complex coacervates with gum arabic, but solid-state precipitates with sodium alginate, mainly by electrostatic attractions; these interactions were strongly influenced by pH, the biopolymer mixing ratio, total biopolymer concentration, and ionic strength. Soluble complexes were formed even when both biopolymers were net-negatively charged above the isoelectric point (IEP) of FG, probably due to the existence of positively charged regions on the FG molecules. The formation and dissociation of

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FG-sodium alginate precipitates were found to be pH-dependently reversible, and the electrostatic attractions between FG and sodium alginate were strong enough to develop a coupled gel network during static incubation.

Hyaluronic acid (HA) is an anionic linear polysaccharide composed of β-1,4-linked repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid connected by β-1,3 glycosidic bonds [9]. HA is abundantly present in mammalian tissues as a common component of synovial fluid and extracellular matrix [10]. HA is considered a promising building block of novel biomaterials, not only for skin therapy but also for encapsulation and tissue engineering applications, due to its excellent unctuosity, hydrophilicity, biodegradability, and biocompatibility [11,12]. HA was reported to form soluble complexes or insoluble complexes as complex coacervates or precipitates with different types of proteins, such as bovine serum albumin, silk fibroin, and lysozyme, depending on pH, mixing ratio, and total biopolymer concentration [10,12–14]. Such soluble or insoluble HA-protein complexes could be used as basic structures in the development of novel HA-based biomaterials [11,12]. To our knowledge, however, the interactions of HA with FG or mammalian gelatines, which also have wide applications in pharmaceutical, cosmetic, and food industries, have not been investigated.

The objective of this study was to decipher the interactions between FG and HA in aqueous solutions at 25 °C using several complimentary techniques such as turbidimetric titration, confocal scanning laser microscopy, dynamic light scattering, zeta potentiometry, and spectrophotometry with methylene blue (MB). Firstly, the influence of pH and the FG-to-HA weight ratio (FG:HA) on the interactions, as well as the contribution of non-electrostatic interactions (*e.g.* hydrogen bonding or hydrophobic interactions) were investigated at a low total biopolymer concentration (C_T = 0.05%, w/v). Then, the effect of C_T on the interactions was examined at a fixed FG:HA, followed by evaluation of the impact of ionic strength on the interactions at a fixed FG:HA and C_T. The phase boundary pH values obtained were plotted against FG:HA, C_T, or ionic strength to generate state diagrams.

2. Materials and methods

2.1. Materials

Fish gelatine (FG, 58 kDa) from cold water fish skin and hyaluronic acid (HA, 1.6 MDa) from *Streptococcus equi* were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium azide (NaN_3) and methylene blue (MB) dihydrate ($C_{16}H_{18}ClN_3S{\cdot}2H_2O$) were obtained from Daejung Chemicals & Metals (Siheung, Korea) and Showa Chemicals Inc. (Tokyo, Japan), respectively. All other reagents were of analytical-grade purity.

2.2. Preparation of biopolymer mixtures

FG and HA were dissolved separately in distilled water with 0.02% (w/v) of sodium azide as a preservative and kept in a shaking water bath at 100 rpm and 40 °C overnight. The aqueous solutions of FG and AL were cooled to 25 °C and mixed together to make up a volume of 50 mL. The pH of the FG-HA mixtures was adjusted to about 8.0 using 0.1 M NaOH before the turbidimetric acid titration. The influence of FG-to-HA weight ratio (FG:HA) on the interactions between FG and HA molecules was examined by varying FG:HA from 0:100 to 100:0 at a total biopolymer concentration (C_T) of 0.05% (w/v). The contribution of non-electrostatic interactions to FG-HA interactions was explored by adding urea at a concentration of 200 mM at FG:HA = 70:30 and C_T = 0.05%. The effect of C_T on FG-HA interactions was examined by varying C_T from 0.05 to 0.60% at FG:HA = 70:30. The impact of ionic strength on FG-HA interactions was evaluated by adding NaCl at a concentration of 0–100 mM at FG:HA = 70:30 and C_T = 0.05%.

2.3. Turbidimetric acid titration

The appearance of light-scattering insoluble FG-HA complexes was examined during turbidimetric acid titration of the FG-HA mixtures at 25 °C. A T-50 titrator equipped with a DP5 phototrode at 590 nm (Mettler Toledo, Schwerzenbach, Switzerland) was used. The titration was performed to about pH 2 by adding dropwise acetic acid solutions, which were prepared at a series of concentrations (5, 10, 30, 50, and 100%, v/v) and used for the titration in a pH region of 5.0–8.0, 4.5–5.0, 4.0–4.5, 3.5–4.0, and 2.0–3.5, respectively, to minimise the dilution effect. Turbidity (τ, cm^{-1}), defined as follows, was recorded as a function of pH during the titration.

$$\tau = -\left(\frac{1}{L}\right)\ln\left(\frac{I}{I_0}\right) \quad (1)$$

where L = light path length (2 cm), I = intensity of transmitted light, and I_0 = intensity of incident light. All measurements were done at least in triplicate.

2.4. Zeta potential measurement

The zeta potential (ζ, mV) of the FG-HA mixtures was determined by measuring the electrophoretic mobility (μ_E, $m^2/V\,s$) at 25 °C by phase analysis light scattering-assisted laser Doppler electrophoresis (Zetasizer Nano ZS; Malvern Instruments, Worcestershire, UK), followed by conversion of the mobility to the zeta potential according to the Helmholtz-Smoluchowski equation:

$$\zeta\left(\times 10^3\right) = \frac{\eta\mu_E}{\varepsilon_r\varepsilon_0} \quad (2)$$

where η = the dynamic viscosity of the mixtures (8.937×10^{-4} Pa s for water at 25 °C), ε_r = the relative permittivity of the mixtures (78.2 for water at 25 °C), and ε_0 = the permittivity of vacuum (8.854×10^{-12} C/V m). All measurements were done at least in triplicate.

2.5. Particle size measurement

The volume-weighted mean diameter ($d_{4,3}$, nm) of the biopolymers and their complexes in the FG-HA mixtures was determined by measuring the diffusion coefficient (D, m^2/s) at 25 °C by dynamic light scattering using the Malvern Zetasizer, followed by transformation of the D value to the hydrodynamic diameter (d_H, nm) using the Stokes-Einstein equation:

$$d_H\left(\times 10^9\right) = \frac{kT}{3\pi\eta D} \quad (3)$$

where k = Boltzmann's constant ($1.38 \times 10^{-23}\,J\,K^{-1}$) and T = absolute temperature (K). All measurements were conducted at least in triplicate.

2.6. Methylene blue spectrophotometric analysis

The formation of soluble FG-HA complexes was examined at 25 °C by analysing the spectrophotometric response of FG-HA mixtures in the presence of MB, a cationic dye [4]. An aqueous MB solution shows a maximum absorption at 664 nm (A_{664}) and a shoulder peak at 615 nm (A_{615}). Upon the addition of an anionic polysaccharide to the MB solution, the A_{664} decreases and the A_{615} increases due to the electrostatic interactions of MB with the polysaccharide. Upon the subsequent addition of a net-positively charged protein, the increase in A_{664} and decrease in A_{615} occur