International Journal of Biological Macromolecules 63 (2014) 29–37

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Surface patch binding and mesophase separation in biopolymeric polyelectrolyte–polyampholyte solutions

Jyotsana Pathak[a,1], Kamla Rawat[b,1], H.B. Bohidar[a,b,*]

[a] Polymer and Biophysics Laboratory, School of Physical Sciences, Jawaharlal Nehru University, New Delhi 110067, India
[b] Special Center for Nanosciences, Jawaharlal Nehru University, New Delhi 110067, India

ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form 15 October 2013
Accepted 15 October 2013
Available online 23 October 2013

Keywords:
Surface patch binding
Complex coacervation
Interaction potential
Phase diagram

ABSTRACT

Surface patch binding (SPB) induced mesophase separation causing complex coacervation between biopolymers: gelatin A–gelatin B, chitosan–gelatin A, chitosan–gelatin B, and, agar–gelatin B was investigated with and without salt (I = 0–0.3 M NaCl). SPB was induced by pH change and three characteristic pHs identified transitions in a turbidity plot: intermolecular interactions ensued at pH_c, coacervation transition occurred at pH_Φ and phase separation was noticed at pH_{prep}. Associative interactions lead to formation of soluble complexes at pH_c exclusively through SPB whereas the coacervation transition was driven by electrostatic binding (EB). Neither pH_c nor pH_Φ displayed discernible ionic strength (till 50 mM) or temperature dependence, but coacervate yield reduced with increase in ionic strength. Coacervation was completely suppressed beyond 50 mM NaCl. Linear combination of attractive and repulsive parts operating between a polyelectrolyte (charged rod) with a polyampholyte (dipole or point charge) was used to model the interaction potential as function of ionic strength. Relative strength of SPB *vis a vis* EB was used as SPB index to establish a linear relationship with zeta potential ratio of binding partners. Different phase diagrams could be constructed which clearly identified distinct interaction regimes encountered in solutions undergoing coacervation transition.

1. Introduction

Coacervation is a thermodynamic transition which allows a homogeneous solution of charged macroions to undergo liquid–liquid phase separation, giving rise to a polymer-rich dense phase coexisting with its supernatant. These two liquid phases are immiscible but are thermodynamically compatible. The polymer-rich dense phase is often called the coacervate. Structurally it lies between the crystalline and liquid phases. Thus, it can bear intermediate range structural order and can be referred to as a mesophase. Coacervation has been mostly studied in aqueous solutions of charged synthetic or biological macromolecules in the past. In particular, protein–polysaccharide and protein–protein coacervates have attracted much attention because of their inherent potential in generating new biomaterials. In addition, such studies provide basic understanding of specific and non-specific interactions operating between complementary polyelectrolytes [1–7] or polyelectrolyte–polyampholyte [8–16] pairs. Normally, polysaccharides are strong polyelectrolytes whereas proteins, in addition, can be polyampholytes. Hence, the association problem reduces to that of the general study of interaction between polyelectrolyte (PE) and polyampholyte (PA) molecules [6,7]. A recent review encapsulates many of the anomalous as well as the salient features of protein–polyelectrolyte interactions [1]. The phenomenon of protein based coacervates, formed of strong electrostatic interactions, has been reported for β-lactoglobulin–gum Arabic [8,9], whey protein–gum Arabic [10,11], gelatin–chitosan [12], gelatin–agar [13,14], gelatin–gelatin [15], gelatine–DNA [16] and β-lactoglobulin–pectin systems [17]. The diversity of material properties associated with coacervates can be gauged from the fact that β-lactoglobulin–gum arabic coacervates were found to be associated with vescicular to sponge-like internal structure whereas whey protein–gum arabic coacervate was observed to be a highly concentrated (melt-like) phase. In contrast, β-lactoglobulin–pectin coacervates were found to be a heterogeneous phase comprising of pectin networks with protein domains forming the junction points [17]. It has been shown that a polyelectrolyte, DNA and a polyampholyte, gelatin can undergo associative interactions and form complex coacervates with interesting thermal properties [16]. Further, it has been realized that in a class of systems coacervation transition is governed by surface selective patch binding even though both the polyions carry similar net

* Corresponding author at: Polymer and Biophysics Laboratory, School of Physical Sciences, Jawaharlal Nehru University, New Delhi 110067, India.
Tel.: +91 11 2670 4637; fax: +91 11 2674 1837.
E-mail addresses: bohi0700@mail.jnu.ac.in, bohidar@hotmail.com (H.B. Bohidar).
[1] Both authors have contributed equally to this work.

0141-8130/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijbiomac.2013.10.020

charge [12,18,19]. Recently, it was reported that the surface patch binding (protein charge anisotropy) influences the binding and, hence the selective protein purification [20,21]. In particular, in SPB interactions complementary polyions (normally a PA–PE pair) seek oppositely charged patches to bind overcoming the repulsion occurring between similarly charged surface patches. This is often referred to as *binding on the wrong side of pH*.

The aim of the present work was to investigate the dependence of ionic strength on surface selective patch binding interactions operating between a pair of polyions where one partner is preferentially a polyelectrolyte and other is a polyampholyte. This study is important for the better understanding of mechanism involved in formation of bimolecular complexes which has its bearing on the partitioning and purification of biopolymers in general. To meet this objective, we choose four biopolymer pairs: gelatin A–gelatin B (GA–GB), chitosan–gelatin A (C–GA), chitosan–gelatin B (C–GB) and agar–gelatin B (A–GB) that caused intermolecular association explicitly through surface patch binding and explore their ionic strength dependence by subjecting these samples to controlled experiments. In addition, we show that SPB index is linearly related to zeta potential ratio of the constituent biopolymers.

The following provides a brief structural introduction to various biomolecules used in the present study. As per Merck index, gelatin, a polyampholyte obtained from denatured collagen, is a polypeptide with the chemical composition of this biopolymer given as follows: Glycine constitutes 26%, alanine and arginine are in 1:1 ratio together constitute ≈20%, proline is ≈14%, glutamic acid and hydroxyproline are in 1:1 ratio constituting ≈22%, aspartic acid ≈6%, lysine ≈5%, valine, leucine and serine constitute ≈2.0% each, rest 1% is comprised of isoleucine and threonine etc. Depending on the process of recovery the gelatin molecules bear different physical characteristics. Type-A gelatin is acid processed, has an isoelectric pH, pI ≈ 9 whereas the alkali processed type-B gelatin has pI ≈ 5.

Chitosan (poly [β-(1–4)-2-amino-2-deoxy-D-glucopyranose]) is a biodegradable cationic polysaccharide produced by partial deacetylation of chitin derived from naturally occurring crustacean shells. The polymer is comprised of copolymers of glucosamine and N-acetyl glucosamine. Chitosan has an apparent p*K*a value between 5.5 and 6.5 and upon dissolution in acid media the amino groups of the polymer are protonated rendering the molecule positively charged. At neutral and alkaline pH, most chitosan molecules lose their charge and precipitate from solution [22].

The biopolymer, agar comprises mainly of alternating β-(1–4)-D and α-(1–4)-L linked galactose residues in a way that most of α-(1–4) residues are modified by the presence of a 3,6 anhydro bridge [23]. Other modifications commonly observed are mainly substitutes of sulphate, pyruvate, urinate or methoxyl groups. The gelation temperature of agar is primarily decided by the methoxy content of the material. Agar sols form thermo-reversible physical gels with the constituent unit being anti-symmetric double helices [24,25].

2. Materials and methods

In this study gelatin A (procine skin extract, bloom = 300 and molecular weight 100 kDa), gelatin B (bovine skin extract, bloom = 75 and molecular weight 100 kDa), chitosan (molecular weight 150 kDa) were in powder form were bought from Sigma–Aldrich chemical company (USA). Powdered agar (*Gracilaria dura*, molecular weight 300 kDa [25,26]) was supplied by Central Salt and Marine Research Institute (CSMRI), Bhavnagar, India. The chemicals used were of analytical grade and were brought from Thomas Baker, India. All the proteins had nominal impurities and were used as received. These preparations were devoid of any *Escherichia coli* and liquefier presence. It needs to be mentioned that the proteins were not subjected to further purification by dialysis which would have made these salt free. All concentrations mentioned are in the units of w/v except for salt which is expressed in mM.

We prepared gelatin A (GA) and gelatin B (GB) solution by dissolving known amount of the protein in double distilled deionized water at 40 °C using a magnetic stirrer for almost 1–1.5 h. Agar (A) solution was prepared by dissolving agar power in the solvent using an autoclave. Chitosan (C) solution was prepared by dissolving the powder in acidified (1% acetic acid) double distilled deionized water at 50 °C and stirred well for an hour to get a homogenous solution.

All the solutions appeared optically clear and transparent after preparation except GB and chitosan which appeared pale yellow in colour. Final samples for experiments were prepared by mixing the biopolymer pair solutions from their stock and appropriate amount of salt was added. Equal volumes of the constituents were mixed to generate the reacting solutions. Specifically, the following compositions were used: (i) for GA–GB, the concentrations used was 0.42% GA and 0.28% GB, (ii) for C–GA, this was maintained at 0.1% C and 0.1% GA, (iii) for C–GB, it was fixed at 0.1% C and 0.1% GB and (iv) for A–GB, the same was kept at 0.05% A and 0.1% GB. The salt concentration was varied from 0 to 50 mM. The pH of the stock solution (6.0 ± 0.5) remained invariant of polyion or salt concentration except for Chitosan (3.0 ± 0.5).

These samples were stored in air tight borosilicate glass bottles for further analysis which, in all instances, did not exceed more than 12 h after preparation. All Ionic strength dependent investigations were carried out at room temperature, 20 °C and relative humidity in the room was less than 50%.

The extent of intermolecular interactions can be easily monitored through quantitative measurement of solution turbidity which is a function of size, mass and concentration of the scattering moiety. The change in transmittance (%T) of the solution was monitored continuously using a turbidity metre (Brinkmann-910, Brinkmann Instruments, and USA) operating at 450 nm with 1 cm path length probe and calibrated to 100% transmittance with water. The transmittance and pH change of the mixture were noted throughout by titrating with 0.1 M NaOH or 0.1 M HCl as required with gentle magnetic stirring. Solution turbidity is given by (100-%T) which was systematically measured as function of solution pH and ionic strength for all interacting solutions to generate coacervation transition profile.

The zeta potential measurements were performed on an electrophoresis instrument (Model: ZC-2000, Microtec, Japan). The individual biopolymer and coacervating solutions were diluted 10-times in order to know the surface charge of streaming particles. In case of the interacting solutions if one uses the zeta potential (ζ) as an approximation of the surface potential φ of a uniformly charged sphere, the theory gives [27]

$$\zeta \cong \varphi = 4\pi \left(\frac{\sigma}{\varepsilon \kappa} \right) \quad (1)$$

where σ is the surface charge density of the particle, and ε and κ are the dielectric constant and Debye–Hückel parameter of the solution, respectively. It has been shown that to a very good approximation the surface potential can be determined from the potential existing at the hydrodynamic slip plane which is called the zeta potential. The relationship between the mobility (μ) and the zeta potential is $Z = 4\pi(\mu\eta/\varepsilon)$. Then, μ can be written as $\mu = \sigma/\eta\kappa$ where η is the viscosity of the solution.

3. Results and discussion

Interaction among a set of four common biopolymers, chitosan (C), agar (A), gelatin-A (GA), and gelatin-B (GB), were examined in