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Insight into the ionotropic gelation of chitosan using tripolyphosphate and pyrophosphate as cross-linkers

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ABSTRACT

Ionotropic gelation of chitosan by means of opposite charged ions represents an efficient alternative to covalent reticulation because of milder condition of use and, in general, higher biocompatibility of the resulting systems. In this work 90° light scattering (turbidimetry), circular dichroism (CD) and ^{1}H NMR measurements have been performed to study the interactions between the biopolymer and ionic cross-linkers tripolyphosphate (TPP) and pyrophosphate (PPi) in dilute solutions. Thereafter, a dialysis-based technique was exploited to fabricate tridimensional chitosan hydrogels based on both polyanions. Resulting matrices showed a different mechanical behavior because of their peculiar mesh-texture at micro/nano-scale: in the present contribution we demonstrate that TPP and PPi favor the formation of homogeneous and inhomogeneous systems, respectively. The different texture of networks could be exploited in future for the preparation of systems for the controlled release of molecules.

1. Introduction

Ionotropic gelation of biopolymers has all along gained appeal due to its ability to favor the fabrication of biocompatible systems to be used in biomedical field. This method exploits the electrostatic interaction that occurs between polyions and oppositely charged specific cross-linkers under defined range(s) of concentration and/or pH. Ionic cross-linking requires mild conditions of use; however, fine control of the gelation rate is a challenging task, because of the very fast self-assembly between polymer and cross-linker.

Among various biopolymers amenable to ionic cross-linking, chitosan represents a noteworthy example [1]. This polymer is mostly obtained from the alkaline deacetylation of chitin, one of the most abundant natural polysaccharide on earth [2,3], but other sources can directly provide chitosan as such (*e.g.* yeasts). With respect to the parent polymer, chitosan is soluble in water under acidic condition thanks to the protonation of its amino groups (pKa values range from 6.2 to 7 depending on the type of chitosan and conditions of measurement) [2] by favoring the solvation of polymer chains. In these conditions, chitosan behaves as a polycation and, consequently, may favor electrostatic interactions.

On the other hand, tripolyphosphate (TPP) is by far the most employed cross-linker to ionically reticulate chitosan due to its high net negative charges (ranging from one to five depending on pH) per monomeric unit. For instance, TPP was successfully exploited to obtain chitosan nanoparticles and nano/micro-gels [4–7]. It has been demonstrated that the colloidal stability of these systems is deeply affected by many parameters like polymer and cross-linker concentration, pH, ionic strength, temperature and others [8]. In the recent years, the molecular interactions between chitosan and TPP have been investigated by several authors. For instance Koukaras et al. identified primary-interaction ionic cross-linking configurations defined as H-link, T-link, and M-link, and quantified the corresponding interaction energies [9]. Lapitsky et al. demonstrated the pivotal role of the monovalent salt sodium chloride (NaCl) to weaken and to slow down the otherwise too strong polycation-TPP interactions, thus ensuring the colloidal stability of resulting micro-gels [7,10]. Moreover, they also proved that not only TPP but also pyrophosphate (PPi) was able to boost the ionotropic gelation of chitosan to the extent of obtaining stable suspensions of nanoparticles [11,12]. On the other hand, Shu and Zhu used both polyanions to cross-link chitosan films upon soaking in TPP/PPi solutions [13].

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Although much work has been done by evaluating such interactions in dilute solutions, the formation of macroscopic hydrogels chitosan-based was by far less investigated with respect to negatively charged polysaccharides, *e.g.* alginate. An interesting attempt was provide by Khong et al. who mixed at a pH of about 7 an intermediate acetylated chitosan (F_A = 0.4) with mannuronate oligomers and subsequently lowered the pH by using GDL, thus ensuring the protonation of chitosan and the consequent interactions with oligomers [14]. Simultaneously, in our previous contributions a dialysis-based technique (named "slow ion diffusion technique") was devised, which proved to control the ionotropic gelation of a confined chitosan solution enabling the fabrication of cylindrical hydrogels and dried membranes for biomedical application purposes [15,16].

In the first part of the present contribution the investigation upon TPP-chitosan interactions in dilute solutions is reported. A comparison with PPi is presented in order to spot the different contribution of polyanions to polymer binding. Switching from dilute to concentrated solutions, the rationale for the second part of work is to study the different contribution of the two cross-linkers to the formation of chitosan-based hydrogels obtained by means of the slow ion diffusion technique. To the best of our knowledge, in this work we report for the first time the ability of the less popular cross-binder PPi giving rise to the formation of cylindrical macroscopic networks, distinguished by lower mechanical properties and with an inhomogeneous polymer profile with respect to TPP-hydrogels.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan (fraction of acetylated units, F_A = 0.23 determined by ^{1}H NMR), was purchased from Sigma-Aldrich and purified by precipitation with isopropanol, followed by a dialysis against deionized water. The molecular weight of chitosan was determined by viscosity measurements (see below). Sodium perchlorate was from Carlo Erba, Italy. Sodium tripolyphosphate pentabasic – $Na_5P_3O_{10}$ – (TPP ≥ 98.0%), sodium pyrophosphate tetrabasic – $Na_4P_2O_7$ – (PPi ≥ 95%), sodium chloride and glycerol (*ReagentPlus*® ≥99.0%) were all purchased from Sigma-Aldrich Chemical Co.

2.2. Viscosity measurements

The intrinsic viscosity [η] of chitosan was measured at 25 °C by means of a Schott-GeräteAVS/G automatic measuring apparatus and a Schott capillary viscometer. Chitosan was solubilized in deionized water (final concentration = 2 g L^{-1}) and pH was adjusted to 4.5 by HCl 0.5 M. After overnight stirring, an equal volume of buffer AcOH/AcNa (40 mM) – NaCl (0.2 M), pH = 4.5, was added. The polymer was filtered through 0.45 μm Millipore filters prior the measurements. Intrinsic viscosity was calculated by analyzing the polymer concentration dependence of the reduced specific viscosity η_{sp}/c and of the reduced logarithm of the relative viscosity $\ln(\eta_{rel})/c$ by use of the Huggins (Eq. (1)) and Kraemer (Eq. (2)) equations, respectively:

$$\frac{\eta_{sp}}{c} = [\eta] + k[\eta]^2 c \quad (1)$$

$$\frac{\ln \eta_{rel}}{c} = [\eta] - k'[\eta]^2 c \quad (2)$$

where k and k' are the Huggins and Kraemer constants, respectively. The intrinsic viscosity values were averaged and the resulting [η] was found to be 836 mL g^{-1}. The molecular weight of chitosan was calculated in agreement to the Mark-Houwink equation reported in Berth *et al.* [17] and it was found to be 270000.

2.3. Light scattering (Turbidimetry)

A Perkin-Elmer LS50 B spectrofluorimeter was used to record the intensity of the light scattered (90°) by dilute chitosan solutions (0.05% w/v and 2 mL as final volume), upon irradiation with a 550 nm incident light (T = 25 °C). Chitosan was solubilized in acidified deionized water (pH = 2.6) and resulting solutions were titrated using either TPP or PPi solutions (6 μL injections) to gradually increase the molar ratio (r) between the cross-linker and the monomeric unit of chitosan (r = [crosslinker]/[monomeric unit]$_{ru}$). Each injection increased r by 0.02 units. Before analysis, solutions were allowed to equilibrate for one minute. Considering the contribution of *N*-acetylglucosamine unit, the molecular mass of chitosan monomeric unit resulted 171 g mol^{-1}.

2.4. Circular dichroism (CD)

CD spectra of dilute chitosan solutions (0.05% w/v) were recorded in acidified deionized water (pH = 2.6) with a Jasco J-700 spectropolarimeter. The chitosan solution was titrated using either TPP or PPi solutions (3 μL injections) to gradually increase r. Each injection increased r by 0.02 units. For all measurements, the volume of each injection was considered negligible with respect to that of chitosan solution (1 mL): in fact, the volume increase (*i.e.* chitosan dilution) was about 13% at r = 1, while r never exceeded 0.24, corresponding to a dilution of less than 4%. Before analysis, solutions were allowed to equilibrate for one minute. Ellipticity of titrated solutions (θ_r) was normalized by that of chitosan (θ), with no correction for dilution. Data are expressed as values of reduced specific ellipticity in agreement to the equation

$$\Delta\theta = (\theta_r - \theta)/|\theta|$$

A quartz cell of 1 cm optical path length was used, always using the following setup: bandwidth 1 nm, time constant 2 s, scan rate 20 nm min^{-1}, wavelength range 250–208 nm. Three spectra were averaged for each measurement.

2.5. Nuclear magnetic resonance (^{1}NMR)

^{1}H NMR analyses were performed on chitosan solutions (0.2% w/v in 0.2 M acetic acid as a solvent, pH = 2.6), with addition of TPP or PPi with final value r = 0.16. The ^{1}H NMR spectra were recorded at 25 °C, on a Varian VNMRS (11.74 T) NMR spectrometer operating at 499.65 MHz for proton. Water suppression was accomplished by means of WET [18]. A total of 512 scans were accumulated with a spectral width of 6 kHz over 16384 complex data points. The data were multiplied by a decaying exponential function (broadening factor 0.5 Hz) and zero filled twice prior to Fourier transform. The chemical shifts are referred to the chemical shift of the proton of HOD at 4.645 ppm.

2.6. Chitosan hydrogels preparation

Wall-to-wall hydrogels were obtained by a slow diffusion technique [15]. Briefly, a solution composed by chitosan (3% w/v) and glycerol (5% v/v) was casted into a mold (diameter = 22 mm, thickness = 2.5 mm) closed by two dialysis membranes (average flat width 33 mm, Sigma Aldrich, Chemical Co.) and fixed by double circular stainless iron rings. The system was hermetically sealed and immersed into a gelling solution (final volume 50 mL) containing anions (TPP or PPi) – NaCl (150 mM) – glycerol (5% v/v). TPP and PPi concentrations were varied so that r spanned in the range from 0.3 to 7. Ion diffusion proceeded for 24 h under moderate stirring at room temperature allowing hydrogel formation.