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Amphiphilic ionic complexes of hyaluronic acid with organophosphonium compounds and their antimicrobial activity

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ABSTRACT

Amphiphilic ionic complexes of hyaluronic acid and alkyltrimethylphosphonium soaps with alkyl chains containing even numbers of carbons from 12 to 22 have been produced. The complexes have a nearly stoichiometric composition, are non-water soluble, and are stable to heat up to temperatures above 200 °C. These complexes are amphiphilic and able to adopt a biphasic structure with the paraffinic and polysaccharide phases ordered arranged with a periodicity ranging between 3 and 5 nm depending on *n*. The paraffinic phase in complexes with $n \geq 18$ was crystallized and showed melting at temperatures between 58 and 70 °C depending on the *n* value. The complexes decomposed upon incubation in water under physiological conditions, and undergone extensive biodegradation by the action of hyaluronidases. Biocide assays carried out in both solid and liquid media demonstrated a high antimicrobial activity of the complexes against Gram-positive *S. aureus* but moderate against Gram-negative *E. coli* and *C. albicans* fungi.

1. Introduction

Hyaluronic acid (HyA) is a linear polysaccharide which is ubiquitous in the human body. Its high viscoelasticity and great capacity for holding water, as well as its inherent biocompatibility and biodegradability, are properties that make HyA an outstanding biomaterial for medical and pharmaceutical applications [1,2]. Furthermore hyaluronic acid is also important in the regulation of injury associated reactions because it promotes early inflammation, which is a critical step for starting wound healing [3,4]. A number of strategies for the chemical modification of HyA addressed to improve its physicochemical properties, most of them based on the reaction of carboxyl or hydroxyl groups, have been developed [1,5,6]. A valid alternative approach for the modification of HyA is its coupling with cationic surfactants. It has been widely reported that this type of modification carried out on polyelectrolytes (either polypeptides or polyuronic acids) gives rise to stable comb-like ionically-linked systems able to self-assemble in amphiphilic structures [7,8]. These structures consist of two phases, one paraffinic and the other made of the biopolymer, that alternate in layers with a periodicity of several nanometers. Interestingly, the nanometric dimensions of such structures are reversibly responsive to thermal effects. Consequently, these derivatives are potentially useful to design medical devices with temperature-depending transport activity [9]. Additionally, the convenience of nanostructured polymeric systems as antimicrobial materials has been recently featured [10].

According to what has been noticed in a recent investigation, HyA is able to exert bacteriostatic effects and also to display antimicrobial and antiviral properties [11,12]. However, as it has been reported by a number of authors, the biocide effect of this polysaccharide is not clear, and it seems to critically depend on its molecular weight and its concentration in solution. It is therefore advisable that HyA is used in combination with well recognized antimicrobial agents in order to avoid such dependences and ensure its bacteriostatic effect. Nisin, silver and polyhexanide are among others, active compounds that have been added to HyA to make it an efficient antimicrobial system [13–15].

In this study tetraalkylphosphonium surfactants have been coupled to HyA to generate stable ionic complexes with comb-like architecture and antimicrobial properties. The biocide activity of quaternary-onium salts has been widely demonstrated [16], and organophosphonium salts are particularly efficient in this regard [17]. In fact, these compounds have been extensively used in the preparation of polymeric materials with remarkable antimicrobial activity [18–21]. Furthermore organo-phosphonium compounds are known to be much more resistant to temperature than their organo-ammonium analogs [22]. In this work, alkyltrimethylphosphonium bromide soaps, abbreviated as *n*ATMP·Br, with *n* standing for the number of carbon atoms contained in the linear alkyl chain and taking even values from 12 to 22, have been coupled with HyA to give the ionic *n*ATMP·HyA complexes. The study is parallel to those recently published by us on the ionic complexes of HyA with

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alkyltrimethylammonium bromide salts (*n*ATMA·Br) [8], and it has been conceived with the purpose of evaluating the advantages and disadvantages that derive from the replacement of organoammonium by organophosphonium surfactant concerning the structure and properties of the complexes. Firstly a detailed chemical characterization of *n*ATMP·HyA including their thermal stability was performed. Then the nanostructure adopted by the complexes and their thermal behavior were examined in detail since significant differences between the two complexes series should be expected in these regards from the larger size of the phosphorous atom respect to the nitrogen one. Finally the antimicrobial activity against bacteria, including Gram-negative and Gram-positive species, as well as against a fungus, was evaluated to ascertain to what extent the biocide activity of *n*ATMP surfactants is retained once that ionic complexes are formed.

2. Experimental

2.1. Materials

The sodium salt of hyaluronic acid (Na·HyA) with a weight-average molecular weight of about 50,000 Da used in this work was purchased from Enze Chemicals. Alkyltrimethylphosphonium surfactants (*n*ATMP·Br with *n* values from 12 to 22) were synthetized by a procedure previously described by us [23] based on the reaction between the corresponding 1-bromoalkane and a trimethylphosphine solution in toluene.

2.2. Measurements

FTIR spectra were recorded within the 4000–600 cm^{-1} interval on a FTIR Perkin Elmer Frontier spectrophotometer provided with a universal ATR sampling accessory for the examination of solid samples. ^1H NMR spectra were recorded on a Bruker AMX-300 NMR instrument operating at 300.1 MHz and using TMS as internal reference. Deuterated methanol and water were used as solvents. Thermogravimetric analyses (TGA) were performed on a Mettler-Toledo TGA/DSC 1 Star System over the 30 to 600 °C interval at a heating rate of 10 °C·min^{-1} under an inert atmosphere. Sample weights of 10–15 mg were used for TGA analysis. Calorimetric measurements (DSC) were carried out on a Perkin-Elmer DSC 8000 instrument calibrated with indium and zinc. Heating-cooling cycles at a rate of 10 °C·min^{-1} within the −30 to 120 °C temperature range under a nitrogen atmosphere were applied in these assays using sample weights of 2–5 mg. Real time X-ray diffraction studies were performed using X-ray synchrotron radiation at the BL11 beamline (NCD, Non-Crystalline Diffraction) of ALBA synchrotron in Cerdanyola del Vallès, Barcelona. Simultaneous SAXS and WAXS were taken from powder samples subjected to heating-cooling cycles at a rate of 10 °C·min^{-1}. The radiation energy employed corresponded to a 0.10 nm wavelength, and spectra were calibrated with silver behenate (AgBh) and Cr_2O_3 for SAXS and WAXS, respectively. The nanostructure morphology of *n*ATMP·HyA complexes was visualized using a Philips TECNAI 10 transmission electron microscope operating at 100 kV. Specimens were prepared by casting a solution of *n*ATMP·HyA complexes in MeOH:BuOH (4:1) over a water surface. Pieces of the floating film were picked up with carbon coated grids and stained with phosphotungstic acid (PTA) previous to observation.

2.3. Complexes formation and films preparation

The procedure initially reported by Ponomarenko et al. [24] for the synthesis of complexes of poly(α-amino acids) and ionic surfactants has been used in this work. The Ponomarenko's methodology with some minor modifications has been extensively used in our group for coupling either PGGA (poly-γ-glutamic acid) or polyuronic acids with quaternary ammonium salts bearing long linear alkyl chains [8, 25–27] and also for the preparation of *n*ATMP·PGGA complexes [28]. The methodology essentially consists of adding dropwise an aqueous solution of the *n*ATMP·Br salt to an aqueous solution of Na·HyA at a temperature between 25 and 65 °C (depending on the water solubility of the surfactant), and keeping the mixture under stirring for several hours. The complex is recovered as a white precipitate, which is isolated by centrifugation, repeatedly washed with water, and finally dried under vacuum for at least 24 h.

Films preparation was carried out by casting a solution of the *n*ATMP·HyA complex in methanol on 3 × 3 cm^2 Petri plates. After drying at room temperature, films were cut as either 1 × 1 cm^2 squares or 5 mm-diameter discs, and further dried under vacuum for 24 h. Film thickness were estimated to be 115 ± 10 μm as measured by a Mitutoyo micrometer (Osaka, Japan).

2.4. Hydrolytic degradation and release of nATMP surfactant

For degradation studies, discs of *n*ATMP·HyA complexes with n = 16 and 22 were placed in vials to which 5 mL of the corresponding buffer solution were added. Phosphate buffers pH 7.4, either with or without enzymes added, and pH 5.5 were the solutions used for incubation. Sealed vials were stored at 37 °C in a heat chamber and discs were taken out at scheduled times, washed with distilled water, dried under vacuum at room temperature, and finally weighted. Chemical modifications involved in degradation were followed by NMR analysis of both, the remaining disk and the products released to the degradation medium.

For measuring the amount of *n*ATMP released from complexes upon incubation in aqueous medium, discs were placed into cellulose membrane tubes (2000 Da cut-off) which were then introduced in vials containing 10 mL of buffer solution and subjected to gentle stirring for 24 h. Assays were carried out at pH 7.4 (at 25 and 37 °C) and 5.5 at 25 °C. The amount of released *n*ATMP was determined by measuring the absorbance of the dialysate at 208 nm at scheduled times.

2.5. Antimicrobial activity of complexes

The antimicrobial activity of *n*ATMP·HyA complexes was tested *in vivo* against *Staphylococcus aureus* CECT 6538 and *Escherichia coli* ATCC 9001 as Gram-positive and Gram-negative bacteria, respectively, and against *Candida albicans* CECT 1392 as yeast. All the microorganisms were tested in both solid and liquid culture media following the experimental procedure described by Muriel-Galet et al. [29]. Bacterial cultures were incubated in TSA (Triptic Soy Agar, Difco Laboratories) for 24 h at 37 °C and then transferred to a TSB (Triptic Soy Broth) medium where they were left to grow for 18 h at 37 °C to assess their exponential growth. Cultures were then diluted to obtain the desired concentration which was fixed at $7.2 \cdot 10^7$ and $1.59 \cdot 10^7$ CFU·mL^{-1} (OD_{600} = 0.9, in the solid medium) and at $1.37 \cdot 10^5$ and $4.67 \cdot 10^5$ CFU·mL^{-1} (OD_{600} = 0.2, in the liquid medium) for *S. aureus* and *E. coli*, respectively. *C. albicans* cells were left to grow for 18 h at 30 °C in Potato Dextrose Broth (PDB) medium. Cultures were then diluted to obtain $1.33 \cdot 10^5$ (OD_{600} = 1.06) and $1.26 \cdot 10^3$ (OD_{600} = 0.2) concentration for solid and liquid media, respectively.

The antimicrobial activity on solid media was tested using the agar diffusion method which consists of spreading 100 μL of microorganism solution over a prepared TSA or TSB (for *C. albicans*) surface, and placing the 5 mm-diameter film disk at the center of a Petri dish. Plates were incubated at 37 °C for 24 h and the diameter of the resulting inhibition zone was measured directly after the incubation period. HyA was used as negative control and all measurements were made in triplicate.

The antimicrobial activity in liquid media was tested immersing 1 × 1 cm^2 film squares of *n*ATMP·HyA complexes in 10 mL of TSB (PDB for *C. albicans*) containing 100 μL of each microorganism for incubation at 22 °C during 24 h. For quantification, 100 μL aliquots were removed from the suspension at scheduled times, diluted with peptone buffer solution and plated in triplicate in Petri dishes in a TSA (or PDA for

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