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Rheological study of in-situ crosslinkable hydrogels based on hyaluronanic acid, collagen and sericin

Raluca Vulpe [a,c], Didier Le Cerf [b,c,d], Virginie Dulong [b,c,d], Marcel Popa [a,e,f], Catalina Peptu [a], Liliana Verestiuc [g], Luc Picton [b,c,d,*]

[a] "Gheorghe Asachi" Technical University, Faculty of Chemical Engineering and Environmental Protection, Department of Natural and Synthetic Polymers, 73 Prof. Dr. docent Dimitrie Mangeron Street, 700050 Iasi, Romania
[b] Normandie Université, France
[c] Université de Rouen, Laboratoire Polymères Biopolymères Surfaces, F-76821 Mont Saint Aignan, France
[d] CNRS UMR 6270 & FR3038, F-76821 Mont Saint Aignan, France
[e] Academy of Romanian Scientists, Splaiul Independentei, 54, Sector 5, 050094, Bucuresti, Romania
[f] "Apollonia" University of Iaşi, Faculty of Dental Medicine, Muzicii Avenue, 2, 700399, Iasi, Romania
[g] "Grigore T. Popa" University of Medicine and Pharmacy, Faculty of Medical Bioengineering, Department of Biological Sciences, 9-13 Kogalniceanu Street, 700454 Iasi, Romania

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ABSTRACT

The elaboration of chemically crosslinked hydrogels based on collagen (C), hyaluronanic acid (HA) and sericin (S) with different polymer ratios was investigated by in-situ rheology. This reaction was performed via amide or ester bond reaction activated by carbodiimide, in pure water. Prior to molecule crosslinking, the rheological behaviour of the biopolymers (alone or in mixture) was characterized in a semi-dilute concentration regime. Both flow and dynamic measurements showed that uncrosslinked collagen alone appears to be rather elastic with yield stress properties, whereas uncrosslinked HA alone appears to be rather shear thinning and viscoelastic in agreement with entangled polymer behaviour. Sericin exhibited Newtonian low viscosity behaviour according to its very low molar mass. Before crosslinking, HA exhibited viscoelastic behaviour at concentrations above the critical entangled concentration (C^*) in the mixtures, thus HA shows promise as a matrix for future crosslinked networks, whereas sericin did not significantly modify the rheology. During the reaction, followed by rheology, the kinetics were slower for pure HA systems compared with the mixtures (i.e., with added collagen and/or to a lesser extent sericin). At the same time, the final network of hydrogels (i.e., the elastic modulus) was more structured in the mixture based systems. This result is explained by ester bonds (the only possibility for pure HA systems), which are less favourable and reactive than amide bonds (possible with sericin and collagen). The presence of collagen in the HA matrix reinforced the hydrogel network. SEM studies confirmed the structure of the hydrogels, and *in vitro* degradability was globally consistent with the effect of the selected enzyme according to the hydrogel composition. All the elaborated hydrogels were non-cytotoxic in vitro.

1. Introduction

For many years, increased interest in tissue engineering techniques has led to substantial progress in many applications such as wound healing, cell culture and biological implants. Ideally, matrixes based on biopolymers are preferred, notably for their biocompatibility. Collagen and hyaluronan are the major constituents of the extracellular matrix and are good candidates for use in biomedical materials. Collagen is an extracellular matrix protein with strong mechanical properties that play important structural roles in specific tissues [1]. Hyaluronic acid (HA), a glycosaminoglycan, is a mucopolysaccharide that is found in various types of tissues. Its immune neutrality makes it an excellent biopolymer, and it is largely used as a block scaffold for biomaterials that are employed in tissue engineering, cell growth or drug delivery systems [2]. Sericin is a glue like glycoprotein from the cocoons of the silk worm *Bombyx mori* [3]. Its presence in the composition of collagen scaffolds has been correlated with stimulatory potential in cellular collagen production [4]. Sericin has been used to augment biomaterial scaffolds, thereby enhancing biological performance [5–7]. The biological functions and physicochemical properties of these polysaccharides and proteins are important features in various organisms, and have been correlated to molar mass, molecular dynamics and rheological behaviour under various conditions [8,9]. Thus, conducting a reliable characterization of these rheological parameters (molecular dynamics) as a function of the biopolymer concentration and ratio before and/or during the crosslinking reaction (kinetics of gelation and the gelling time) is of

* Corresponding author at: Université de Rouen, Laboratoire Polymères Biopolymères Surfaces, F-76821 Mont Saint Aignan, France.
E-mail address: luc.picton@univ-rouen.fr (L. Picton).

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interest for a better understanding of their influence in both physicochemical and biological fields [10–14].

Rheological studies of collagen have been performed for a long time making it one of the most investigated natural polymers used as a tissue restoration and drug delivery substrate [15–19]. The viscoelastic properties of hyaluronan generally agree with the Maxwell model (entangled polymer solution), and its flow behaviour is often correlated to shear thinning fluids [20–22]. In contrast, silk sericin has not been as widely investigated as the other two macromolecules, as the main protein of interest from silks is silk fibroin [23–25]. The rheological behaviour of different solutions is directly dependent on the polymer concentration, especially for high molar mass macromolecules (e.g., collagen and hyaluronan). In this matter, polymer content and ratio become important characteristics that may influence hydrogel structure [11]. Rheological measurements could reveal the details of gelation processes, providing information on the hydrogel structure and mechanical properties during gelation. The integration of collagen, hyaluronan and sericin in the hydrogel network, could be a key factor for the physicochemical properties of materials, i.e., the mesh pore-size, water uptake and degradability. Crosslinking collagen, hyaluronan and sericin by glutaraldehyde [26–28] or genipin [29,30] has been reported in the literature. Nevertheless, to limit cytotoxicity, the use of both ester- and amide-activating agents such as carbodiimide has been investigated, leading to interesting crosslinked hydrogels [31–33]. In this context, we recently proposed a study relating the elaboration of crosslinked hydrogels synthesized for the first time by covalent linking of three biopolymers (collagen, hyaluronic acid and sericin) previously lyophilized and immersed in a 40% v/v ethanol/water in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) [34]. We produced hydrogels with a macroporous structure, high swelling degree and *in vitro* enzymatic resistance compared to uncrosslinked collagen. It was observed that the addition of sericin improved the biocompatibility of the hydrogels. In this previous work, no information was obtained about the influence of the biopolymer composition or the influence of the dynamic rheological properties of the biopolymers on the chemical crosslinking kinetics or on the final mechanical properties. In the current work, the protocol for hydrogel preparation was adjusted in pure water to evaluate the crosslinking gelling reaction in-situ by monitoring the rheological behaviour. In a first step, we developed a rheological map (flow and dynamic mode) of the biopolymers alone or in mixture in various amounts. In a second step we monitored both viscous and elastic moduli (respectively G″ and G′) as a function of time during the crosslinking reaction to attempt to determine the kinetics and final mechanical properties of the in-situ-formed gel. The aim of this study was to propose an overview of the molecular dynamics of biopolymer solutions (alone or in mixtures at various ratios) and then to correlate these results with the rheological data collected during the crosslinking process. The elaborated hydrogels were also characterized in terms of morphology (SEM images), swelling properties, biological degradation under the influence of specific hydrolysing enzymes and biocompatibility.

2. Materials and methods

2.1. Materials

Collagen solution type I + III in H_2O (acid-soluble, bovine provenience) was supplied by Lohmann & Rauscher, Newied, Germany. Hyaluronan from *Streptococcus equi* and sericin from *Bombyx mori* were purchased from Sigma-Aldrich (France). The other reagents: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, Sigma-Aldrich), *N*-hydroxysuccinimide (NHS, Sigma-Aldrich), phosphate buffered saline (PBS, Sigma-Aldrich), collagenase (*Clostridium histolyticum*, type I, 220 units/mg solid, Gibco Life Technologies) and hyaluronidase (bovine, type I-S, 400–1000 units/mg solid, Sigma-Aldrich), were used as received. The physicochemical characteristics of the three biopolymers were determined in our previous study by size exclusion chromatography coupled on line with multi angle light scattering, differential refractive index and viscometry (SEC/MALS/DRI/Visco) [34]. These results are listed in Table 1:

2.2. Preparation of the hydrogels

Hydrogels were obtained in aqueous solution by preparing different mixtures of collagen, hyaluronan and sericin (see Table 2). The mixtures were left on a magnetic stirring plate for 24 h, after which EDAC and NHS were added according to COOH:EDAC:NHS = 1:10:2 (molar ratio). These conditions were fixed based on our previous experience with the reaction with the carbodiimide/NHS activator [35]. The carboxylic group content for collagen was calculated as Olde Damink et.al [31] stipulated. The carboxylic group content of hyaluronan was calculated and reported for each monomer unit. The crosslinking process was monitored through rheological measurements. After synthesis, the hydrogels were purified in Milli-Q water for 1 week, and then lyophilized. The reaction yield (Y) was calculated with Eq. (1), where C_p represents practical quantity (g) and C_t is the theoretical (initial) quantity of biopolymers (g).

$$Y\,(\%) = \frac{C_p}{C_t} \times 100 \quad (1)$$

2.3. Rheological measurements

Rheological measurements were performed in aqueous media (Milli-Q water) with different biopolymer concentrations, using a Discovery Hybrid Rheometer (HR2 from TA Instrument (U.K.)) with standard-size double concentric cylinder geometry and plan-cone geometry (for viscous solutions and hydrogel crosslinking, respectively). Flow experiments were performed for between 0.01 and 1000 s^{-1} at 25 °C. The oscillation procedures were performed from 0.1 to 10 Hz for the frequency sweep. The linearity domain of the biopolymer solutions was checked by performing a stress sweep procedure, at 1 Hz between 0.01 and 100 Pa at 25 °C. Crosslinking reactions of hydrogels were performed by recording both elastic modulus (G′) and viscous modulus (G″), time dependent, at 1 Hz with 3% strain (the conditions were particularized for the hydrogels). All the experiments were performed at 25 °C and reproduced twice with good accuracy. The results were analysed with TRIOS V 3.1.0.3538 rheology software. To dispose of comparative data, we chose to note the G′ at 150 min and the characteristic time for gel (t_{gel}) when G′ reaches the plateau or at least a linear evolution as a function of time.

2.4. Scanning electron microscopy (SEM)

The surface morphology of hydrogels was studied by scanning electron microscopy (SU1510 Scanning Electron Microscope, HITACHI, Japan). Samples were gold sputter coated before analysis.

2.5. Water uptake of hydrogels

Swelling kinetics conducted on similar hydrogels were studied in a previous work. We showed that all of the systems reach the equilibrium

Table 1
Molar masses and intrinsic viscosities of biopolymers.

	Collagen[a]	Hyaluronic acid	Sericin
Mw (g·mol-1)	290,000	1,525,000	8400
Mn (g·mol-1)	200,000	1,150,000	7000
[η]w (mL·g-1)	250	1875	9

[a] Note that collagen shows an aggregative behaviour in aqueous solution and the following data concerns the water soluble fraction.