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A novel biocompatible hyaluronic acid–chitosan hybrid hydrogel for osteoarthrosis therapy

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ABSTRACT

A conventional therapy for the treatment of osteoarthrosis is intra-articular injection of hyaluronic acid, which requires repeated, frequent injections. To extend the viscosupplementation effect of hyaluronic acid, we propose to associate it with another biopolymer in the form of a hybrid hydrogel. Chitosan was chosen because of its structural similarity to synovial glycosaminoglycans, its anti-inflammatory effects and its ability to promote cartilage growth.

To avoid polyelectrolyte aggregation and obtain transparent, homogeneous gels, chitosan was reacetylated to a 50% degree, and different salts and formulation buffers were investigated. The biocompatibility of the hybrid gels was tested *in vitro* on human arthrosic synoviocytes, and *in vivo* assessments were made 1 week after subcutaneous injection in rats and 1 month after intra-articular injection in rabbits.

Hyaluronic acid–chitosan polyelectrolyte complexes were prevented by cationic complexation of the negative charges of hyaluronic acid. The different salts tested were found to alter the viscosity and thermal degradation of the gels. Good biocompatibility was observed in rats, although the calcium-containing formulation induced calcium deposits after 1 week. The sodium chloride formulation was further tested in rabbits and did not show acute clinical signs of pain or inflammation. Hybrid HA–Cs hydrogels may be a valuable alternative viscosupplementation agent.

1. Introduction

According to the World Health Organization, 10% of men and 18% of women over 60 years of age have symptomatic osteoarthrosis (OA) in the knees, hips, fingers and lower spine (Symmons et al., 2000; Woolf and Pfleger, 2003). It is well established that OA is associated with aging and joint wear, but the molecular mechanisms of OA are still not clearly understood. Nonetheless, studies have shown that the degradation of hyaluronic acid (HA) naturally contained in the articular structures is related to inflammatory factors, enzymes, immune cells and oxidants present in the OA articulation (Hinton et al., 2002). One current therapy for the treatment of osteoarthrosis is intra-articular injection of hyaluronic acid (HA), which is known as viscosupplementation (Bellamy et al., 2006; Strauss et al., 2009). Although the mechanism of action of HA is not completely understood, it is known that HA downregulates inflammatory factors and restores the rheological properties of the synovial fluid (SF) (Jackson and Simon, 2006; Moreland, 2003; Takahashi et al., 1999). One reference commercial product is Ostenil®, which contains 1% HA and has to be injected once a week for a total of 3–5 weeks to achieve an effect that lasts for approximately six months. The risks related to the injection procedure and the need to improve patient compliance have spurred the search for long-lasting treatments.

The aim of the strategy developed in this paper is to improve the efficacy of HA gel within the articulation compared to available formulations using a potentially chondroprotective additive. The approach consists of associating HA with a second biopolymer, chitosan (Cs). Cs is biodegradable, and its *N*-acetyl-glucosamine and glucosamine monomers are natural components of cartilage that have been proven to be chondroprotective and to enhance the synthesis of HA as well as other cartilage components (Kumar et al., 2004; Lu et al., 1999; Shikhman et al., 2004). Cs itself has also been

Abbreviations: HA, hyaluronic acid; Cs, chitosan; PEC, polyelectrolyte complex; OA, osteoarthrosis; FLS, fibroblast-like synoviocyte; SF, synovial fluid; VS, viscosupplementation.

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found to increase chondrocyte proliferation when injected intra-articularly in rats (Lu et al., 1999). Its effects on cartilage as well as on osteoinduction and in wound healing are thought to be related to macrophage, fibroblast and polymorphonuclear cell activation, mediated by interleukine-1/8, (IL) tumor necrosis factor-α/β (TNF), fibroblast growth factor, nitric oxide and peroxide production (Kumar et al., 2004). Indeed, it seems that under OA conditions, when those factors are also upregulated, chitosan mediates them to a basal level to help resolve the inflammation and protect chondrocytes from apoptosis through the protection of the mitochondrial function (Chen et al., 2006; Suh and Matthew, 2000). The activation of macrophages is a major triggering effect of Cs when used for immunomodulation in vaccines, but it may also be a limitation because this effect causes a primary inflammation. Indeed, this pro-inflammatory activity is dependent on the source, purity and chemical modification of Cs but has been found to be resolved in the body without leading to a foreign body reaction (Barbosa et al., 2010; Chenite et al., 2000; Patois et al., 2009; Peluso et al., 1994; Usami et al., 1994a,b; VandeVord et al., 2002). Overall, chitosan is considered to be safe, and its LD_{50} is 10 g/kg for subcutaneous injection in mice, although severe pneumonia was found in dogs (Kumar et al., 2004; Minami et al., 1996; Usami et al., 1998). Cs is also an effective viscosifier, has been reported to have intrinsic antioxidant activity, and its enzymatic degradation can be decreased by changing the deacetylation ratio (Chen et al., 2006; Feng et al., 2007; Kumar et al., 2004; Lee et al., 1995; Tomida et al., 2009; Zhang and Neau, 2001).

The combination of HA and Cs generally leads to PEC formation due to their opposite charges. Thus, the so-called hybrid hydrogels produced by simply mixing these two polymers lack the properties of homogeneity, transparency and short-term stability, which are required for an injectable formulation (Denuziere et al., 1996, 1998, 2000). More complex systems have been proposed to circumvent these limitations, in which HA and Cs are chemically linked through a crosslinker moiety or Cs is crosslinked in presence of HA (Fang et al., 2007; Park et al., 2013; Tan et al., 2009). Patchornik et al. also described a thermoresponsive HA–Cs hybrid colloidal gel composed of HA and Cs oligomers in which HA is crosslinked (Chi2Gel®) (Patchornik et al., 2012). In this study, we chose to stabilize unmodified HA and reacetylated Cs through the addition of accepted additives to facilitate straightforward translation to clinical use. Reacetylated chitosan, with an acetylation degree of approximately 50%, possesses increased solubility at neutral pH, allowing the development of homogeneous and transparent gels compatible with intra-articular use (Berger et al., 2005; Luca et al., 2010; Patois et al., 2009; Sashiwa et al., 2002). This paper presents the development of a novel transparent and stable sterile formulation containing HA and Cs of very high molecular weight (MW), with an emphasis on their formulation and biocompatibility performances.

2. Materials and methods

2.1. Material

The HA used was a Good Manufacturing Practice (GMP) grade sodium hyaluronate from *Streptococcus* origin (HTL, La Boitardière, France; MW was 1300–2200 kDa measured by size exclusion chromatography – multi angle light scattering – refractive index; polydispersity index (PI) of 1.3–3.3). The Cs used was GMP grade KIOmedine-CsU® from *Agaricus bisporus* origin (Kitozyme, Herstal, Belgium; MW measured to be 127.5 kDa with a PI of 2.0; after reacetylation, the MW was 161 kDa and the PI was 1.3). Chitosan of shrimp origin from Sigma–Aldrich (Saint-Louis, USA) was also used. Commercial HA formulation was used as reference: Ostenil® (1% HA, 0.85% NaCl, 0.06% $Na_2HPO_4{\cdot}12H_2O$, 0.005% $NaH_2PO_4{\cdot}2H_2O$ and water for injection, pH 7.0, 350 mOsmol and a measured viscosity of 1.1 Pa·s).

2.2. Methods

2.2.1. Chitosan reacetylation

To enhance the solubility of the chitosan, it was reacetylated from a degree of deacetylation (DDA) of 85–48%. The method used was modified from that of Berger et al. (2005) and was as follows: 8.0 g of chitosan were dissolved in 400 ml of an acetic acid (10% (w/v))/methanol mix (50/50), and 162 ml of an acetic anhydride/methanol mix (2 ml anhydride in 160 ml methanol, depending on the starting DDA) was added under vigorous mechanical stirring with a paddle (33 mm diameter) in an ice bath. The solution was then dialyzed (MW cut off of 6–8000 Da, Spectra/Por Rancho Dominguez, USA) for one week, changing the water daily. The polymer was then precipitated with 160 ml of an ammonia (6.25% (w/v))/methanol (50/50) solution and methanol was added to a final volume of 1 l. The mass was then collected by filtration, re-dispersed in 1.5 l of methanol and left overnight. One liter of methanol was subsequently added, and the precipitate was passed through 5 μm nylon mesh filter. The filtrate was re-precipitated three times with 500 ml of methanol, filtered on fritted glass and finally dried in a desiccator at ambient temperature for one week. The DDA was measured by ^{1}H NMR (Gemini 300 MHz, Varian, Grenoble, France) in acidified water according to the method of Lavertu et al. (2003).

2.2.2. Molecular weight measurement

The molecular weight distribution of the HA and Cs used was measured using an HPLC system (Waters Alliance HPLC system) coupled to the following size exclusion chromatography columns: Waters Ultrahydrogel 2000, 1000, 250 and 120 columns in series for HA and an Ultrahydrogel linear column for chitosan (Milford, USA). A refractive index detector (Schambeck, Bad Honnef, Germany) and a multi-angle light scattering detector (MiniDawn, Wyatt, USA) (SEC–MALLS–RI) were used in series, and the data processing was performed using ASTRA V 5.1.9.1 analysis software. For HA analysis, the column temperature was 35 °C, and the mobile phase consisted of 50 mM Na_2HPO_4, 150 mM NaCl, and 0.05% NaN_3 at a pH of 7.0. The flow rate was 0.7 ml/min, the analysis time was 75 min, the injected volume was 50 μl and the sample concentration 1 mg/ml. The dn/dc used was 0.153, and A2 of 2.3e−3 was used, according to Baggenstoss and Weigel (2006). For the analysis of Cs, the mobile phase consisted of 0.15 M acetic acid, 0.1 M sodium acetate and 0.05% NaN_3 with a pH of 4.0. The flow rate was 0.4 ml/min, the analysis time was 40 min and the volume injected was 50 μl. The dn/dc used was 0.192 ml/g, according to Nguyen et al. (2009).

2.2.3. Hydrogel preparation

For hydrogel preparation, a batch size of 5 ml was prepared at ambient temperature. A Cs stock gel at 2.5% in 0.1 M hydrochloric acid was prepared, and the pH was adjusted to 6.5 by drop-wise addition of 1 M sodium hydroxide. The salts, water and Cs were mixed and, finally, HA powder was added. The formulation was left at rest at 4 °C overnight, leading to complete polymer hydration. The obtained hydrated gel was mixed with a helical stirrer for 10 min at 300 rpm. The obtained hydrogel was then autoclaved for 15 min at 121 °C (Certoklav, Austria), with a total cycle time of 35 min. All percentages in the formulations refer to % (w/v).

2.2.4. Hydrogel characterization

Aggregation and homogeneity of the formulations were observed by visual inspection under daylight in a spectroscopic plastic vial