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Preparation, Characterization, and Release Properties of Hydrogels Based on Hyaluronan for Pharmaceutical and Biomedical Use

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ABSTRACT: Hyaluronic acid (HA) is a natural polysaccharide that is widely distributed in the human body. Its physicochemical properties and high biocompatibility make it a good candidate for biomedical and pharmaceutical uses. In the present work, we report HA-based hydrogels that could be applied as drug delivery systems or as implants for the treatment of joint diseases. We use butanediol diglycidyl ether as a chemical crosslinker to obtain HA hydrogels. Using a new dissolution tester and ketoprofen (KP) as a model drug, we study the release properties of the hydrogels. We obtain homogeneous and transparent hydrogels with high strength and elasticity. The swelling ratio (SR) depends on the crosslinker concentration and pH of the medium. We also reveal differences between the release profile of KP from swollen and unswollen hydrogels. The characteristics and differences in KP release profiles depending on the SR suggest the possibility of obtaining controlled release from HA-based hydrogels. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 1377–1382, 2013

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INTRODUCTION

Hyaluronic acid (HA), also called hyaluronan, is a polysaccharide formed of repeated disaccharide units. It belongs to the family of glycosaminoglycans that consists of repeated disaccharides with the general formula: (sugar acid–amino sugar)$_n$. HA consists of *N*-acetyl-D-glucosamine as the amino sugar and D-glucuronic acid as the sugar acid, linked by β[1-4] glycosidic bonds. The disaccharides are linked by β[1-3] bonds to form the HA chain. This polymer is an important component of the extracellular matrix of connective tissue and is found in human skin, cartilage, vitreous humor, and intra-articular joint fluid. It also plays an important role in cartilage matrix stabilization, cell proliferation, control of morphogenesis, cancer metastases, inflammation processes, and wound healing.[1,2] HA molecules are not recognized as foreign by the immune system, and their administration does not cause inflammatory or toxic reactions. Besides its biocompatibility, the physicochemical properties of HA make it a good candidate for drug delivery as well as tissue engineering and viscosupplementation in treating osteoarthritis.[3–7] Therefore, it is an excellent candidate for different biomedical and pharmaceutical uses.[8,9]

It is well documented that under normal physiological conditions, HA pervades the surface of particular tissues and diffuses into the synovial space to lubricate joints and to prevent mechanical damage due to its shock-absorbing properties. Thanks to those properties, HA may benefit patients with arthritis by supplementing the lubricating characteristics of their synovial fluid. However, HA can be degraded *in vivo* by enzymes (such as hyaluronidase) which are present in human tissues. A useful approach to avoiding such degradation is the preparation of hydrogels based on chemically crosslinked HA which is responsible for an increase in its resistance to hyaluronidase.[10,11]

Hydrogels are crosslinked networks of hydrophilic polymers. They can be classified according to a variety of characteristics such as the nature of the side group, their physical structure, their method of preparation, and their responsiveness to physiologic environmental stimuli such as pH or temperature.[12] Hydrogels are very similar to biological tissues in terms of their physical properties, due to their high water content and soft consistency. Furthermore, the capacity of hydrogels to contain molecules of different sizes enables us to use them as drug delivery systems via several administration routes.[13] The most common chemical crosslinking agents of HA are diglycidyl ethers[11,14] and divinylsulfone.[15,16]

In this work, we use butanediol diglycidyl ether (BDDE) as a crosslinking agent, because it is already used in commercialized

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formulations of HA as dermal filler.[17] The epoxy groups of BDDE react with the OH groups present in the HA.[18] HA hydrogels was used as controlled drug delivery systems, and we identified the main factors influencing the release of a model drug [ketoprofen (KP)]. The biocompatibility and biodegradability properties of HA,[1] together with the tunable KP release profiles of HA hydrogels obtained as a function of swelling ratio (SR), suggest new approaches to the design of drug delivery systems for lipophilic drugs, via several administration routes.

EXPERIMENTAL

Materials

HA sodium salt from *Streptococcus equi* with a molecular weight of approximately two million daltons and a purity of 97% was obtained from Sigma-Aldrich. The chemical crosslinker BDDE with a molecular weight of 202.25 g mol^{-1} and a purity of 95% was obtained from Sigma-Aldrich. Hyaluronidase from bovine testes at a concentration of 801 U mg^{-1} was obtained from Sigma-Aldrich. KP, used as a model drug, with a purity of 99.8% was purchased from Fagron Iberica. Phosphate buffer solution pH 7.4 (PBS) was prepared from KH_2PO_4 from Fagron Iberica, Na_2HPO_4 from Probus SA, NaCl from Acofarma, and Milli-Q® deionized water. Cellulose tubular membrane was purchased from Orange Scientific. Its properties are a 12,000–14,000 nominal molecular weight cut-off (MWCO) and 20 μm wall thickness. The mobile phase for high-performance liquid chromatography (HPLC) (pH 3.0) was prepared from 45% of aqueous phase, comprising 99.5% pure citric acid from Acofarma, NaCl from Acofarma, NaOH from Acofarma, and Milli-Q® deionized water; and 55% organic phase, which was 99.9% pure acetonitrile obtained from Carlo Erba. The commercial gel used as a reference contains 2.5% of KP, and the excipients are carbomer, ethanol (96%), diethanolamine, methyl parahydroxybenzoate, propylparahydroxybenzoate, essence, and water. To study the release of KP, a dissolution tester Elite 8™ manufactured by Hanson Research Corporation was used. To determine the concentration of KP, a Shimadzu HPLC equipped with a Kromasil® 100-5C18 column purchased from Akzo Nobel and a UV detector were used.

Methods

Preparation of Chemically Crosslinked Hydrogels. For the preparation of HA hydrogels, 50 mg of sodium hyaluronate was introduced into 12 × 75 mm test tubes, to which 500 μL of crosslinking solution, consisting of BDDE in alkaline media (0.2M NaOH), was added. Then, the HA and the crosslinking solution was stirred with a vortex till a homogeneous mixing. The resulting mix was incubated for 8 h, and the HA crosslinked hydrogels were obtained. The epoxy groups of BDDE react with the OH groups present in the HA polymer as indicated in Figure 1.

Additionally, HA hydrogels loaded with 2.5% (w/w) of KP were prepared. KP was added to the crosslinking solution just before hydrogel formation.

Hydrogel SR Measurements. The hydrogels were swollen in ultrapure water (pH 5.5) at room temperature for 1 week. Later, the hydrogels were carefully weighed, and the SR was calculated according to eq. (1):

$$\mathrm{SR} = \frac{W_S - W_d}{W_d} \quad (1)$$

where W_S (mg) represents the weight of the swollen hydrogel, and W_d (mg) is the weight of the dry hydrogel.

Also, we studied the SR as a function of time at different pH values. The swelling medium used was Britton–Robinson buffer. This consists of a mixture of 0.04M H_3BO_3, 0.04M H_3PO_4, and 0.04M CH_3COOH that has been titrated to the desired pH with 0.2M NaOH. The pH values selected for the SR study were 1.5, 3.0, 7.0, and 12.0.

Rheological Measurements. We studied the rheological properties of the HA hydrogels. We determined the elastic modulus (G') and the viscous modulus (G'') as a function of frequency.

Dynamic viscoelastic measurements were performed using a Thermo Haake RheoStress 300 in parallel serrated plate mode with a diameter of 35 mm. The parallel plate gap was 1.0 mm. The samples were swollen in water, and all measurements were performed at 25°C.

Determination of KP Solubility in PBS and Crosslinking Solution. Determination of the maximum solubility of KP in PBS is required to ensure sink conditions in the release experiments. The solubility of KP in the crosslinking solution is required to ensure that 2.5% (w/w) of KP can be added to the hydrogels (it is the concentration of KP in commercial formulations).

The maximum solubility of KP in PBS and in the crosslinking solution was determined by adding an excess of the drug to a small amount of solution, maintained at 25°C. The samples were thoroughly stirred and sonicated. They were then left to stabilize overnight in a water bath at 25°C. The supernatant was analyzed for KP quantification.

HA Hydrogels Degradation by Hyaluronidase. To determine the amount of KP in the hydrogels, we degraded the hydrogels by treating them with 1000 U mL^{-1} hyaluronidase at 37°C in PBS. Degradation was assessed by complete weight loss from the hydrogels after 48 h. Then, the KP in the recovered solution was analyzed. To test the strength of the hydrogels against hyaluronidase, these were immersed in a solution of hyaluronidase, 1000 U mL^{-1} PBS, at 37°C. We determined the degradation profile by representing the weight (%) of the hydrogels remaining as a function of time at different crosslinker concentrations.

Determination of KP Concentration by HPLC. KP was analyzed by HPLC. The chromatographic system consisted of Shimadzu equipment with a Kromasil® 100-5C18 column and a UV detector set at 233 nm for KP determination.

Separation was carried out at room temperature using 55% acetonitrile and 45% aqueous phase (pH 3.0), as the mobile phase, with a flow rate of 1 mL min^{-1} and an injection volume of 20 μL. The KP retention time was approximately 7 min.

Release Studies of KP from Hydrogels. *In vitro* release studies were carried out in a dissolution tester. Dissolution testing is a

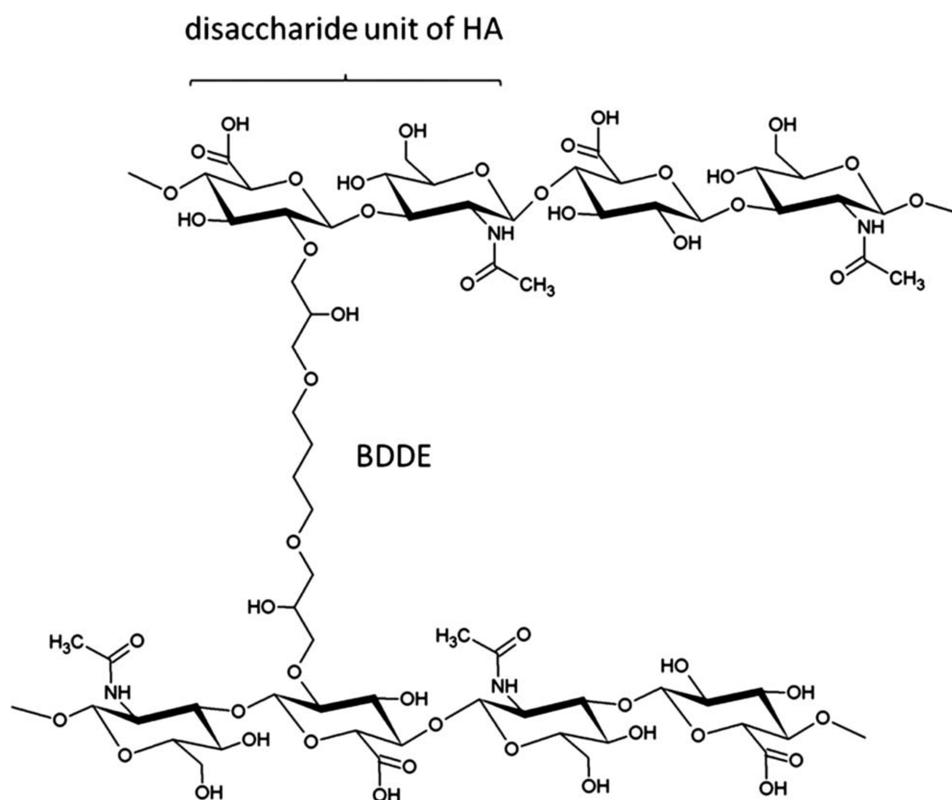

Figure 1. Reaction between the epoxy groups of BDDE and hydroxyls presents in HA.

well-established and standardized test in Pharmacopoeias for evaluating solid and semisolid dosage forms. Dissolution testing allows one to examine the dissolution behavior of pharmaceutical dosage forms *in vitro*, in order to differentiate formulation types and to estimate dissolution behavior *in vivo*.[19,20]

The dissolution apparatus used was an Elite 8™ dissolution tester from Hanson Research Corporation. It consists of eight dissolution vessels immersed in a thermostatized bath. Each dissolution vessel consisted of a 150 mL glass vessel with a setup for semisolid formulations called an ointment cell, as shown in Figure 2. A cellulose membrane was placed in each ointment cell to separate the hydrogel from the receptor solution. The receptor solution consisted of 150 mL of PBS (pH 7.4). The temperature of the receptor solution was 37°C. The stirring speed of the paddles in each dissolution vessel was 25 rpm.

Approximately 500 mg of hydrogel was placed in each ointment cell. Then, 150 mL of receptor solution (PBS) was placed in the glass vessel. In order to determine the amount of drug released as a function of time, 0.5 mL of receptor solution was removed for analysis, and the same amount of virgin PBS solution was replaced. The release study lasted for 24 h. The first sample was withdrawn 10 min after the beginning of this study; subsequent samples were obtained in intervals of 15 min for a total period of 2 h. Then samples were obtained at intervals of 1 h until a total elapsed time of 8 h was reached. The last samples were obtained 22, 23, and 24 h after the beginning of this study; at which point this study was concluded.

RESULTS

HA Hydrogel Formation

We obtained transparent and stiff HA hydrogels with different degrees of crosslinking using different concentrations of BDDE as the chemical crosslinker as indicated in Table I. We also obtained HA hydrogels containing 2.5% (w/w) of KP.

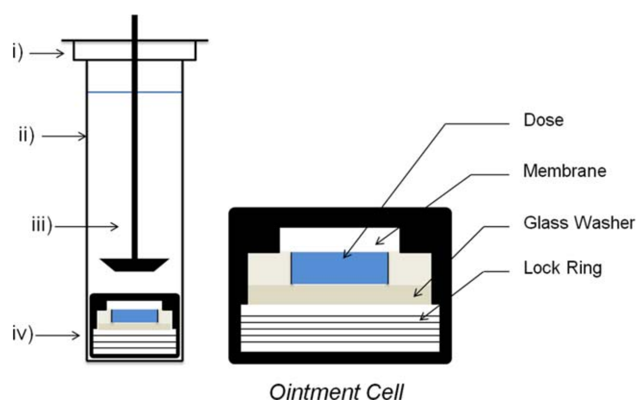

Figure 2. Schematic diagram of the dissolution vessel. (i) Adapter, (ii) dissolution vessels (150 mL glass vessel flat-bottom), (iii) paddle, and (iv) ointment cell. (right) Different parts of ointment cell are represented in detail. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Crosslinking Solution with Different Concentrations of BDDE

BDDE[a] (%, v/v)	Molar ratio between BDDE and HA[b]
1.25	0.2
2.5	0.4
3.5	0.6
5.0	0.9
10.0	1.7
20.0	3.6

[a] Concentration of BDDE in crosslinking solution (%, v/v).
[b] Molar ratio between BDDE and disaccharide unit of HA.

Characterization of HA Hydrogels

Crosslinked HA hydrogels exhibited the capacity to absorb a large amount of water and swell (Figure 3). We determined the SR as a function of crosslinker concentration and incubation temperature. As shown in Figure 4, the capacity to absorb water depends on crosslinker concentration and incubation temperature. The crosslinking reaction is more efficient at 40°C, because, for a given crosslinker concentration, the swelling of the hydrogels was lower at 40°C than at room temperature, especially at BDDE concentrations lower than 5%. For higher concentrations, we did not observe significant differences. We also studied the dependency of SR on the pH of the medium. Figure 5 shows that the SR for HA hydrogels is higher for a value of pH above the pK_a value of the polymer (pK_a = 3.0). To determine the hydrogel stiffness, we perform the rheological characterization of the chemically crosslinked hydrogels. The rheological measurements were carried out on swollen hydrogels. The HA hydrogels with a crosslinker concentration of 2.5% exhibited an elastic modulus of approximately 1500 Pa and a viscous modulus of approximately 300 Pa (Figure 6).

Degradation of HA Hydrogels with Hyaluronidase

We showed that the resistance against hyaluronidase increases with increasing concentration of the crosslinker (Figure 7). For a crosslinker concentration of 2.5%, complete degradation occurred after 48 h. However, for a concentration of 5%, the hydrogels were not completely degraded after 48 h. This method was also used to ensure a homogeneous concentration of the drug in the hydrogels, as required for the release study.

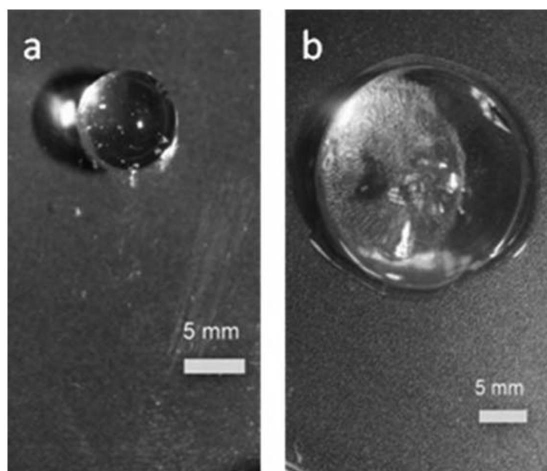

Figure 3. (a) Unswollen hydrogel with a crosslinker concentration of 2.5% BDDE and (b) swollen hydrogel after 48 h immersed in water.

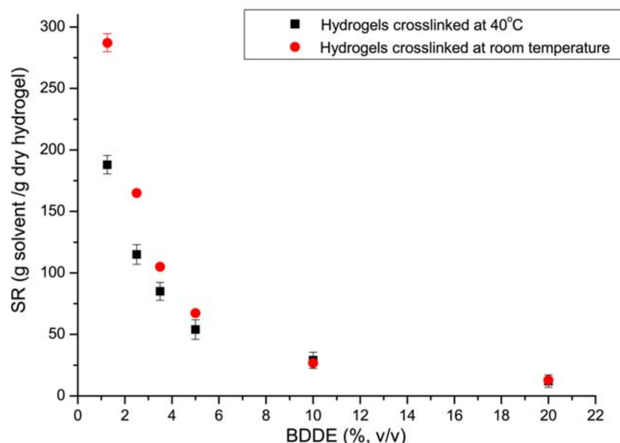

Figure 4. The ability to absorb water depends on the crosslinker concentration and incubation temperature. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Release Studies of KP from HA Hydrogels

Figure 8 shows that the release profiles of KP from unswollen hydrogels, swollen hydrogels, and commercial gel (2.5% KP) took place with no lag-time, but it revealed slight differences. Both unswollen and swollen hydrogels showed a fast initial release which slowed down progressively after several hours, but the maximum KP released was approximately 100% for unswollen hydrogels and 85% for swollen hydrogels. In order to determine the influence of the cellulose membrane on the release profile, we performed a release study without a membrane. The release profile was compared to those obtained with a membrane (Figure 9). This study was only performed for unswollen hydrogels, as we could not guarantee the integrity of the swollen hydrogels during the experiment.

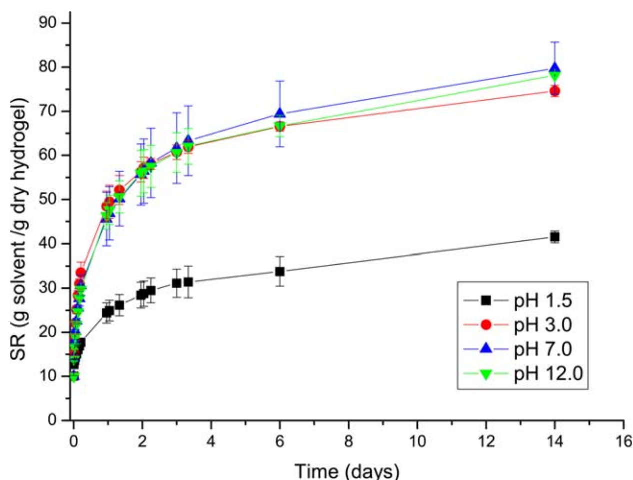

Figure 5. SR for hydrogels with 2.5% BDDE versus time at different pH values of the medium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

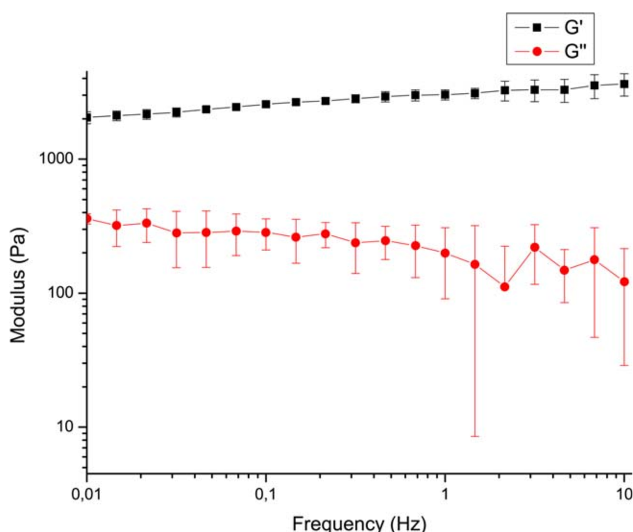

Figure 6. Viscoelasticity of the HA hydrogels chemically crosslinked with 2.5% BDDE. The elastic modulus (G') and the viscous modulus (G'') are plotted as a function of frequency. Data points are the mean ± standard deviation of four measurements. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

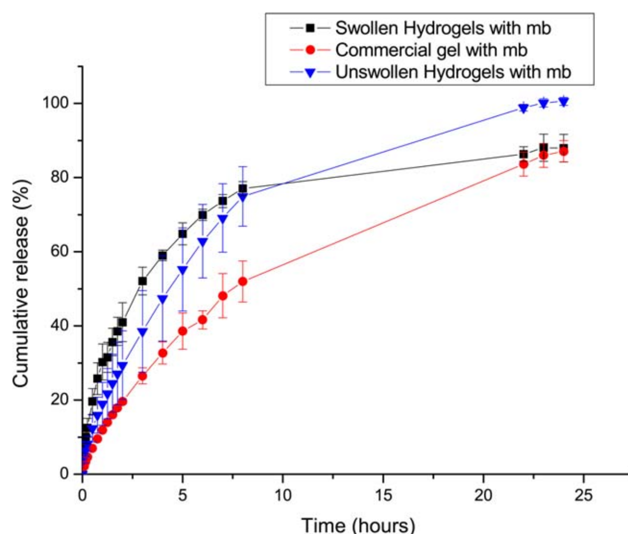

Figure 8. Comparative profile of cumulative release of KP as a function of time from unswollen hydrogels, swollen hydrogels, and commercial gel. These results have been obtained by using a cellulose membrane (mb) in the ointment cell. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

In this work, we develop hydrogels based on HA. We also identify several key parameters in the crosslinking process, such as BDDE concentration, temperature, and incubation time. By visual observation of the appearance of phase separation and/or fluency at room temperature, we determined the stability of the hydrogels. It is important to remark that the stirring process was carried out immediately after the mixing of the crosslinker solution and HA. Otherwise, the jellification would not have been homogeneous. Such jellification happened in different phases, and a slightly viscous fluid was obtained after incubation in the oven. If the mixture of HA with the crosslinking solution was kept in the oven for longer or at a higher temperature than the previously indicated, the result was a highly fluid yellow solution that could not be considered a hydrogel. This is due to the increased exposure to heat destroying the chemical crosslink.

In order to determine the equilibrium SR of the hydrogels, they were immersed in a large amount of water in a Petri dish. Their capacity to absorb water is attributed to the presence of hydrophilic groups such as OH. The observed decrease in hydrogel swelling as a function of crosslinker concentration may be due to the stiffness of the hydrogel and the presence of unreacted OH groups of HA. Higher concentrations of crosslinker result in the presence of fewer unreacted OH groups of HA due to the reaction of the epoxy groups of BDDE with OH. This means there are fewer hydrogen bridges between OH of HA and water, and therefore, there is less swelling. The SR also depends on the

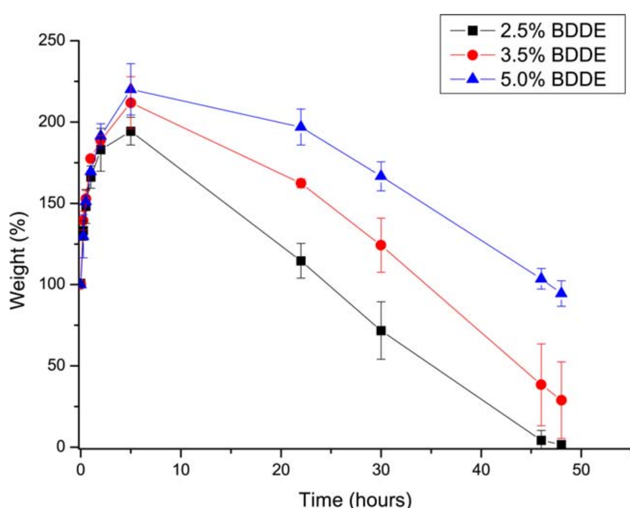

Figure 7. Degradation of HA hydrogels with different concentrations of crosslinker as a function of time. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

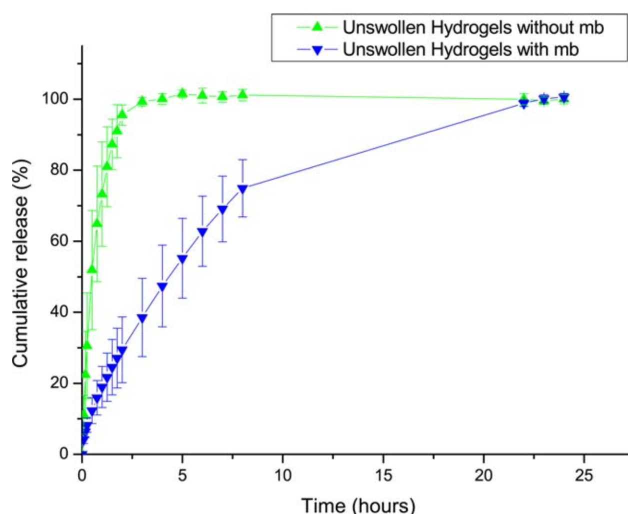

Figure 9. Cumulative release of KP as a function of time from unswollen hydrogels with and without membrane (mb). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pH of the medium. When the pH is above the pK_a value of HA, the latter is ionized. Under such circumstances, the stiffness of the polymer network is lower so water can penetrate it more easily; therefore, there is increased swelling.

It is interesting to note that once the hydrogel has been formed, its shape is maintained as it swells. This may be of interest in tissue engineering applications, as smaller dehydrated prostheses could be implanted in the body via small incisions and made to swell *in vivo* until they reach desired size of the defective tissue. The swollen hydrogels exhibit high stability. After sealing them in a Petri dish for more than 2 years, their appearance remains unchanged.

To understand the factors influencing the release behavior of KP incorporated into HA hydrogels, we studied the release profile in both unswollen and swollen hydrogels. We prepared unswollen HA hydrogels with 2.5% (w/w) of KP. The amount of KP to be incorporated into the hydrogels was chosen taking into account the solubility of the drug in the crosslinking solution, therapeutic dosages, and also the requirement of sink conditions in the receptor solution during the release experiments. Some of the HA hydrogels with 2.5% (w/w) of KP were immersed in water to obtain swollen hydrogels. The concentration of KP in the swollen hydrogels (0.2%, w/w) was then lower than that in the unswollen hydrogels. To determine the amount of KP in the samples, we used an HPLC method. The original setup used to study drug release, designed specifically for semisolid formulations, allowed us to determine the amount of KP that could be released from the HA hydrogels into a PBS receptor solution. We found that the release from unswollen and swollen hydrogels shows some differences; while approximately 100% of the drug can be released from unswollen hydrogels after 24 h, only approximately 85% was released from swollen hydrogels. It seems that for a swollen hydrogel, a fraction of the drug is retained within the hydrogel after 24 h, while the tendency of unswollen hydrogels to absorb water may facilitate the complete release of KP from them. By using hyaluronidase to degrade the hydrogels, we determine the amount of KP present in the hydrogels. The concentration of hyaluronidase employed was higher than the concentration of this enzyme present under physiological conditions.[21]

A dialysis membrane can influence the release behavior of molecules[22,23]; however, in this study, no effect would be expected due to the cellulose membrane, as the MWCO of the membrane used in far above the molecular weight of KP. However, the passage of the molecule through the membrane seems to be a rate-limiting step, for both unswollen and swollen hydrogels. The effect of the membrane was to delay KP release. So, while the unswollen hydrogels reached maximum release after 3 h without membrane, this only occurred after 20 h with membrane. Overestimation of the concentration of KP was considered, as some of the receptor solution was absorbed by the hydrogel in swelling.

CONCLUSIONS

We developed hydrogels based on HA and identified several key parameters of the polymerization process, such as HA and BDDE concentrations, temperature, and incubation time.

The SR of hydrogels depends on the crosslinker concentration and the pH of the medium. After swelling, the hydrogels remained in their original shape. This fact and the long stability of the hydrogels make them promising candidates for implants.

The biocompatibility and biodegradability properties of HA, together with the tunable KP release profiles of HA hydrogels obtained as a function of the SR, suggest new approaches to the design and development of drug delivery systems based on HA hydrogels for lipophilic drugs via several administration routes.

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