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Extended Abstract

# Hyaluronic acid hydrogel particles obtained using liposomes as templates†

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**Abstract:** Hydrogels (HG) are 3D network of hydrophilic macromolecules linked by different "cross-linking points", which have as main advantage their capacity for the adsorption of large amounts of water without any apparent dissolution. This allows hydrogels to undergo reversible swelling-shrinking processes upon the modification of the environmental conditions (pH, ionic strength or temperature). This stimuli-responsiveness and their ability for entrapping in their interior different types of molecules makes hydrogels suitable platforms for drug delivery applications. Furthermore, HGs exhibit certain similarities to the extracellular tissue matrix and can be used as a support for cell proliferation and migration.

**Keywords:** hydrogels; hyaluronic acid; swelling; shrinkage; liposomes; template-assisted.

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## 1. Introduction

Template-assisted methodologies have been successfully applied on the preparation of particles of alginate, agarose, milk protein or whey protein with a well-defined shape and size [1-4]. The particles obtained by templating techniques can be exploited for the encapsulation of different actives with interest in different industries and technological fields [3-5]. Among the used templates, the droplets of water in oil (W/O) emulsions are probably accounted as the most extended. However, the use of emulsions present an important drawback related with the presence of an organic solvent, which can remain partially trapped in the particle matrix. This may alter the safety of the obtained particles, especially when they are intended for applications involving the interaction between particles and biological systems.

The use of liposomes as template instead of emulsion droplets for liposomes emerges as a very important alternative for reducing the use of organic compounds, and improving the toxicological profile of the obtained particles. Liposomes are defined as spherical structures consisting on a lipid bilayer surrounding a hydrophilic core, commonly filled by water, which can be a very suitable environment for preparing hydrophilic polymeric particles [6,7].

This work is focused on the preparation of hydrogels particles of hyaluronic acid. Polymer hydrogels, or simply hydrogels (HG), are three-dimensional macromolecular network, formed by hydrophilic polymer chains linked via physical or chemical interactions through different "crossing points", i.e. they form cross-linked structures. The branched structures of the HGs allow them to absorb large amounts of water, while the cross-linking of the networks prevents their dissolution (see Figure 1 for a sketch). In contact with water, these materials have the ability to swell and form elastic, soft and flexible materials, while also retain significantly amount of solvent within their structure. These properties provide to HGs certain similarities to the extracellular tissue matrix,

allowing their use as substrates for cell proliferation and migration or to control drug release [8].

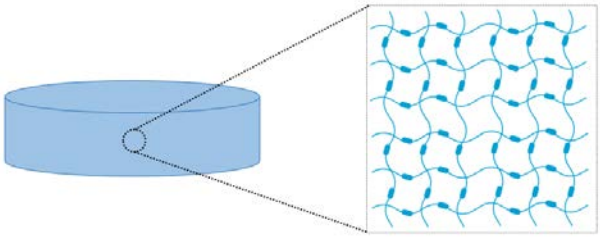

**Figure 1.** Sketch of the typical cross-linked structure of a polymeric hydrogel.

Hyaluronic acid (HA) is a polysaccharide frequently used in different biotechnological applications due to its natural, biodegradable, and nontoxic character which enables the formation of inert nanoparticles. In recent years, an extensive research on polysaccharide nanoparticles for several applications has been developed [9,10]. HA hydrogels have currently a large number of applications in biomaterials, including their use in tissue regeneration because of their high biocompatibility, or as drug delivery systems because their ability to retain liquids and bioactive compounds [11].

The aim of this work the fabrication of agarose nanoparticles using liposomes as templates for substituting the commonly use emulsions. The use of liposomes as templates is preferred due to their stability, simplicity of preparation and the absence of organic solvents. Furthermore, nanosized liposomes provide a suitable environment for the fabrication of nanosized hydrophilic particles.

## 2. Experimental Section

### *2.1. Chemicals*

L-α-Phosphatidylcholine (PC, 2-linoleoyl-1-palmitoyl-sn-glycero-3-phosphocholine) with a purity higher than the 95% and a molecular weight of 782.08 g/mol was supplied for Alfa Aesar (*Haverhill, MA,* USA). Sodium hyaluronate (HANa) with molecular weight in the range 1.5-1.8 x $10^3$ kDa was purchased from Sigma-Aldrich (Saint-Louis, MO, USA). PC and HANa were used as received without any further purification.

Hydrochloride acid (HCl, aqueous solution at 35 wt%) for fixing the pH, glucose (purity 99%) and diethyl ether (CHROMASOLV™, for High Performance Liquid Chromatography, purity 99.9%) were supplied for Sigma-Aldrich (Saint Louis, MO, USA).

Ultrapure deionized water used for cleaning and solution preparation was obtained by a multicartridge purification system aquaMAX™-Ultra 370 Series (Young Lin Instrument, Co., Anyang, Korea). The water used had a resistivity higher than 18 MΩ·cm, and a total organic content lower than 6 ppm.

### *2.2. Preparation of liposomes loaded with Hyaluronic acid*

Liposomes were prepared following a procedure adapted from that commonly followed in the reverse phase evaporation method [12,13]. This technique relies on the formation of reverse micelles, which are latter transformed in liposomes. For this purpose, an organic phase composed of phosphatidylcholine dissolved diethyl ether and an aqueous phase corresponding containing the substance to be encapsulated, i.e. hyaluronic acid, at a concentration of 0.2 g/L are mixed in 1:1 volume ratio. These mixtures is left for equilibration during 30 minutes, and then is centrifuged for 30 minutes at 400 rpm, which evidences a clear separation of phases between the aqueous and organic ones. This allows removing the aqueous fraction containing the excess of non-encapsulated hyaluronic acid. Afterwards, the organic fraction containing reverse micelles loaded with hyaluronic acid is mixed with water in 1:1 volume ratio, which is followed by the addition of volume

similar to that added of water of an aqueous solution containing 5 wt% glucose solution. The above mixture is placed in an ultrasonic bath for 5 minutes, and then the organic solvent is removed using a rotary evaporator, which results in an aqueous dispersion of liposomes loaded with hyaluronic acid. It should be noted that the preparation of bare liposomes, without hyaluronic acid, were performed following a similar approach without adding hyaluronic acid to the aqueous phase.

*2.3. Preparation of hyaluronic acid hydrogels*

Hyaluronic acid has the ability to undergo self-crosslinking process in acid medium, i.e. it can form ester bonds with another hyaluronic acid molecules. This requires to reduce the pH of the dispersion of liposomes loaded with hyaluronic acid by adding HCl down to a value of 1.5 [14]. Thus, it is possible to form inter- and intra-chain ester bonds, which leads to the formation of hydrogels particles adopting the form of the environment containing the polymer chains. Figure 2 shows a sketch representing the formation on an ester bonds between two hyaluronic acid monomers.

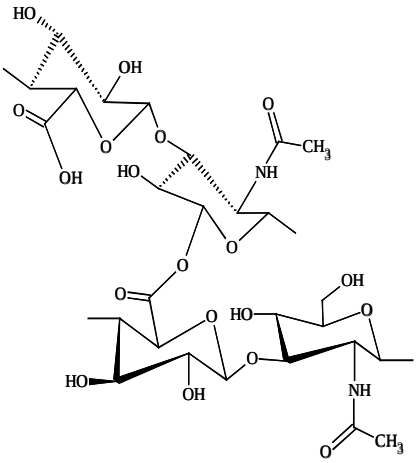


**Figura 2.** Sketch of the cross-linking between hyaluronic acid molecules.

*2.4. Characterization techniques*

Dynamic Light Scattering (DLS) experiments for characterizing the size of bare liposomes, hyaluronic acid loaded liposomes and hyaluronic acid hydrogels were performed by using a Zetasizer Nano ZS device (Malvern Instrument, Ltd., Malvern, UK). Thus, it is possible to perform the evaluation of the size of the object dispersed in the aqueous medium in terms of the apparent hydrodynamic diameter (in the following diameter, *d*).

The cross-linking of hyaluronic acid was confirmed by measuring the changes in the infrared spectrum by using Spectrophotometer FT-IR Nicolet iS50 (Thermo Fisher Scientific, Waltham, MA, USA).

## 3. Results and discussion

*3.1. Verification of the formation of liposomes loaded with hyaluronic acid using the reverse phase technique*

The use of DLS has provide information about the formation of liposomes by using the reverse phase technique, and the monodisperse character of the obtained liposomes. Figure 3 shows the size distribution obtained for the liposomes contained in a dispersion obtained following the above describe procedure. From the results, it is clear that the used methodology allows obtaining liposomes with sizes contained in a very narrow diameter distribution (monodisperse dispersions), and an average diameter of 206±2 nm.

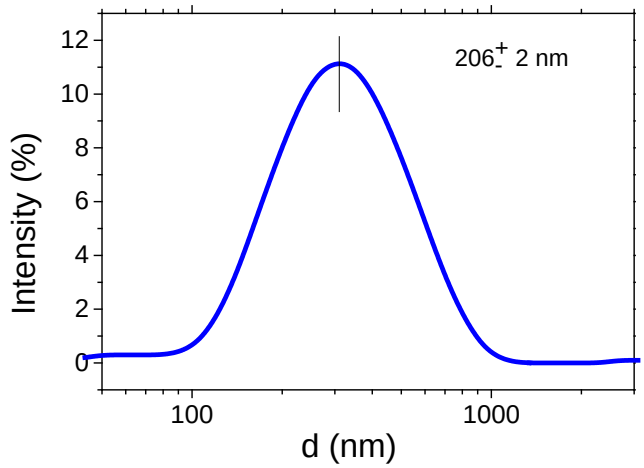


**Figure 3.** Distribution of diameters obtained by DLS for liposomes loaded with hyaluronic acid.

The possibility to obtain monodisperse liposomes is key for their use as templates for obtaining hydrogels with controlled sizes and shapes.

*3.2. Hydrogel formation*

It was stated above the ability of hyaluronic acid for undergoing a self-crosslinking process under acidic conditions. This allows tuning the degree of crosslinking of the hydrogels by varying the HCl concentration added to the aqueous medium. The effect of the degree of crosslinking as function of the added HCl concentration is reflected in changes on the average diameter of the liposomes loaded with hyaluronic acid as is shown in Figure 4. The increase of HCl concentration causes a swelling of the liposomes, which can be rationalized in terms of changes on the crosslinking of the HA encapsulate. This leads to the formation of hydrogel particles with different rigidity and swelling degree, which push the liposomes walls, resulting in an increase of the average thickness of the liposomes. Therefore, by modifying the pH of the medium it is possible to obtain liposomes with hyaluronic acid hydrogels having different degrees of crosslinking.

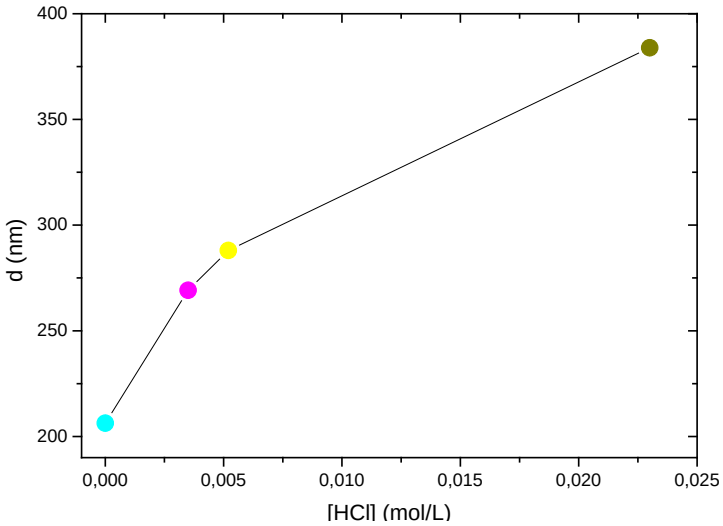


**Figura 4.** Average diameter for liposomes loaded with hyaluronic acid as function of the HCl in the aqueous medium.

It should be noted that an excessive increase of the HCl concentration in the medium results in the degradation of the liposomes, which results in a release of the encapsulated hydrogel particles followed by their aggregation as evidence the images shown in Figure 5.


**Figure 5.** Images showing a dispersion of liposomes loaded with hyaluronic acid at physiological pH **(a)** and a dispersion of liposomes and aggregate hydrogel particles at pH 2.03 **(b)**.

*3.3. Evaluation of the cross-linking of the obtained hydrogels*

The crosslinking of hyaluronic acid hydrogel particles upon exposure at acid medium was confirmed by using infrared spectroscopy. For this purpose, the IR spectra of hyaluronic acid particles encapsulated in the liposomes and bare hyaluronic acid were analyzed by infrared spectroscopy, and the results were compared (see Figure 6). As can be observed, the sample of crosslinked hyaluronic acid presents an adsorption band at 1730 $cm^{-1}$, associated with the C-O tension of ester groups, which confirms the esterification process and consequently provides evidences of the formation of the hyaluronic acid hydrogels by a self-crosslinking process in acid medium.

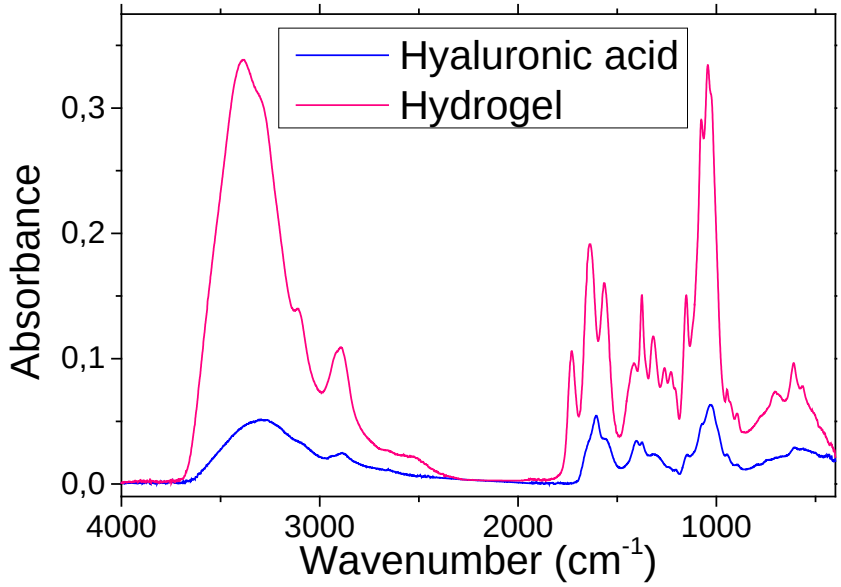


**Figure 6.** Infrared spectra for bare hyaluronic acid (blue line) and hyaluronic acid hydrogel (red line).

## 4. Conclusions

This work reports a new route for preparation of stable and monodisperse nanosized hyaluronic hydrogel nanoparticles. This was possible by a pH-triggered gelation of the aqueous interior of the liposomes used as a template for controlling the size and morphology of the nanoparticles. Such nanoparticles can be transferred to aqueous medium to obtain a dispersion of nanoparticles which present a physico-chemical behavior reminiscent of a chemically cross-linked gel which can undergo a reversible swelling-shrinking process. This open new avenues for design system for controlled loading and release of active molecules.

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