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Hyaluronan-based hydrogels particles prepared by crosslinking with trisodium trimetaphosphate. Synthesis and characterization

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Abstract

Hyaluronan in an emulsion was crosslinked with trisodium trimetaphosphate to synthesise hydrogel particles. The reaction was carried out to different crosslinking densities in the presence of sodium hydroxide and sodium chloride. Swelling degrees of the particles were measured in water and in sodium chloride at different concentrations to study the effect of the crosslinking density and the effect of the ionic strength on swelling. Finally, absorption of methylene blue in the particles allowed us to show a linear relationship between crosslinking density and methylene blue absorption.

Keywords: Hyaluronan; Trisodium trimetaphosphate; Crosslinking; Hydrogel; Swelling degree

1. Introduction

Hydrogels can be defined as water-soluble, crosslinked polymeric structures able to retain large quantities of water without dissolution or loss of their three-dimensional structure. The high water content is responsible for high diffusivity of small molecules. Biodegradable and/or biocompatible hydrogels can be used as wound covers, adhesives or drug carriers. Biopolymers as polysaccharides are largely used for the preparation of biodegradable hydrogels. For example such hydrogels have already been prepared using dextran (Kim, Won, & Chu, 1999), hyaluronan (Vandamme & Stoetaert, 1995), pullulan (Nishikawa, Akiyoshi, & Sunamoto, 1994) or other polysaccharide derivatives. Many polysaccharides possess chemically active functional groups, which can be used for protein drug delivery (Chen, Jo, & Park, 1995). In this work, the polysaccharide used is hyaluronan (HA) (Fig. 1), a linear polysaccharide consisting of alternating β-1,4-linked units of β-1,3-linked glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc). HA is one of several glucosaminoglucan components of the extracellular matrix, the synovial fluid of joints and the scaffolding comprising cartilage. The immunoneutrality of HA makes it an excellent material for tissue engineering and drug delivery systems. HA has found many biomedical applications in drug delivery and surgery. For example, HA is used in ophthalmic or in viscosurgery fields (Luo, Kirker, & Prestwich, 2000). Gels containing hyaluronan can be used for wound dressing, coating prostheses, orthopaedic applications, artificial tears in ophthalmology or moisturising agents in cosmetics (Jagur-Grodzinski, 1999).

Crosslinked polysaccharide microparticles are usually obtained by using epichlorohydrin as the crosslinker. Here, we have used triSodium TriMetaPhosphate (STMP) (Fig. 2) to synthesise polysaccharide-based hydrogels.

STMP has been reported to be an effective crosslinking agent for starches (Kasemsuwan, Bailey, & Jane, 1998; Muhammad, Hussin, Ghazali, & Kennedy, 2000; Woo & Seib, 1997). STMP is a salt of low toxicity having no adverse effects on humans. In the USA, this agent may be used legally to crosslink food grade starches. Guar gum was also crosslinked using STMP for applications in colon-specific drug delivery (Gliko-Kabir, Yagen, Penhasi, & Rubinstein, 2000). The crosslinking reaction occurred through the hydroxyl groups of the polysaccharide and led to ester linkages. It is one of the reasons we chose this interesting coupling agent because it does not react with carboxylic groups, which are free for eventual further modifications. Indeed, the usual coupling agents for crosslinking HA, such as adipic dihydrazide (ADH)

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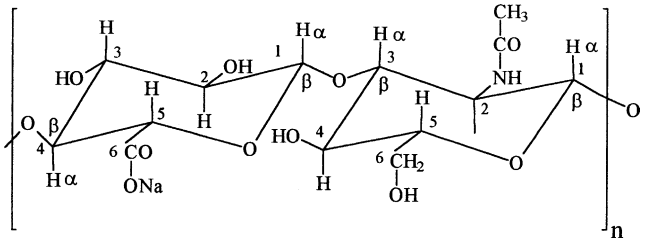

Fig. 1. Structure of hyaluronan (HA).

(Luo et al., 2000; Pouyani, Harbison, & Prestwich, 1994; Prestwich, Marecak, Marecek, Verkruyse, & Ziebell, 1998) react with carboxylic groups leading to bisdihydrazide crosslinks. The reaction with STMP introduces anionic charges through the phosphate groups (Fig. 3).

The aim of this work was to synthesise hydrogel microparticles of HA by a new method. The crosslinking reactions were carried out in an emulsion (the water-soluble polymer and crosslinking agent were dispersed in a non-solvent) to give microparticles of polysaccharides. Physico-chemical properties of such systems have been studied as a function to their crosslinking density or their degree of swelling. Scanning Electronic Microscopy (SEM) was used to estimate the size of the microparticles. The swelling behaviour of the hydrogels, which is already related to the crosslinking density, is important for understanding the diffusion of physiologically fluids in materials in view of controlled drug delivery applications, and was studied in different solvents such as water or NaCl solutions.

2. Materials and method

2.1. Materials

Hyaluronan (HA) (obtained by fermentation from a streptococci family) was purchased from Acros Organics, trisodium trimetaphosphate (STMP) from Sigma-Aldrich, cellulose acetate butyrate, (butyryl content 35–39%) from Acros Organics, sodium hydroxide, sodium chlorate and 1,2-dichloroethane from Prolabo (Merck Eurolab, France).

All compounds were used without further purification.

2.2. Methods

2.2.1. Crosslinking procedure

Solutions of STMP, sodium hydroxide, sodium chloride and HA in water (milli-Q) were prepared at a desired concentration. The solution of HA and sodium chloride

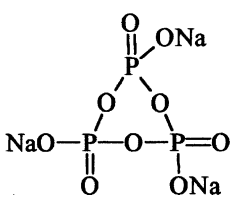

Fig. 2. Structure of trisodium trimetaphosphate (STMP).

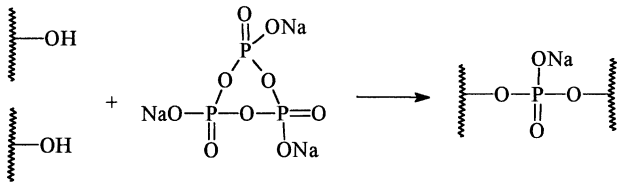

Fig. 3. Crosslinking reaction of HA with STMP (Gliko-Kabir, Yagen, Penhasi, & Rubinstein, 2000).

($2\ \mathrm{mol\ l^{-1}}$) was then mixed with the solution of sodium hydroxide and left under stirring for 1 h. Sodium chloride screened the carboxylate charges and as a result brought macromolecular chains closer. Sodium hydroxide is needed to form the necessary alkalinity for the crosslinking reaction with STMP. During this time, the organic solvent (1,2-dichloroethane) required for the emulsion was introduced into the reactor (about one volume of water for three volumes of organic solvent) then the stabilizer (cellulose acetate butyrate) was added at 5% w/V. The mixture was stirring during 1 h at ambient temperature to ensure the complete dissolution of the stabilizer.

Thereafter STMP was added under vigorous stirring to the emulsion. The system was stirred at 350 rpm at 50 °C during 3 h.

At the end of the reaction, the reaction mixture was filtered and washed with acetone to remove the stabilizer and to recover the particles of crosslinked polymer. The particles were then added to a large volume of water and the pH was adjusted to 7 by addition of HCl ($0.1\ \mathrm{mol\ l^{-1}}$). The particles were purified by dialysis against water (milli-Q) for several days, precipitated in ethanol and dried at 40 °C.

2.2.2. Degree of swelling

The measurements of the swelling degrees (Q) were made according to Eq. (1) where m is the weight of the swollen particles and m_0, the weight of the dried particles.

$$Q = \frac{m - m_o}{m_o} \tag{1}$$

The particles were added to a large volume of water or NaCl solutions (10^{-3}–$1\ \mathrm{mol\ l^{-1}}$) then filtered and weighted before and after drying until no variation in weight were observed.

In the case of the swelling measurements in NaCl solutions, the particles were rinsed before drying to remove the salt.

2.2.3. Crosslinking density measurement

Methylene Blue (MB) is a cationic molecule with a high affinity to negatively charged solids, therefore the absorption of MB in the particles can be related to the experimental crosslinking density. The amount of MB absorbed in the particles is proportional to the quantity of anionic groups, and therefore to the crosslinking density (since the crosslinking reaction leads to the formation of phosphate bridges in the polymer). The number of phosphate groups increased with the level of crosslinking.

The particles were placed in dialysis bags (M_w cut off 12–14000, Polylabo, France) and then immersed in 100 ml of methylene blue solution (10^{-5} mol l^{-1}) under stirring. Disappearance of methylene blue was measured by spectrophotometry (Spectrometer UV/VIS Lambda 2, Perkin Elmer) at $\lambda = 665$ nm until no variation in methylene blue absorbance was observed. Then, the crosslinking density was estimated by determination of the Relative amount of Methylene Blue bound to the particles using Eq. (2) (Gliko-Kabir, Yagen, Penhasi & Rubinstein, 2000).

$$\mathrm{RMB}_{\mathrm{absorbed}} = \frac{A_o - A}{A_o - A_{HA}} \quad (2)$$

where A_o is the initial absorbance of methylene blue before any adsorption occurred, A is the absorbance of the methylene blue solution containing hydrogel particles of HA and A_{HA} is the absorbance of methylene blue solution containing only native HA (before the crosslinking reaction).

2.2.4. Molecular weight

The determination of molecular weight of HA was performed by coupling on-line a size exclusion chromatography (SEC), a multi-angle laser light scattering photometer (MALLS) and a differential refractive index detector (RI). The eluent was $LiNO_3$ 0.1 mol l^{-1}.

The SEC columns used were composed of a guard column (PWH) as a protection and two serial TSK G4000PW and G6000PW columns (Toyo Soda) in polyether gel, rich in hydroxyl groups, specially designed for the separation of polysaccharides.

The MALLS photometer, a DAWN-F from Wyatt technology Incorporation (Santa Barbara, CA, USA) fitted with a K5 flow cell and a He–Ne laser ($\lambda = 632.8$ nm) was installed on-line between the columns and the RI detector (Shimadzu RID-6A). The principle of this technique is explained in previous works (Charpentier et al., 1997; Picton, Mocanu, Mihai, Carpov, & Muller, 1995).

3. Result and discussion

3.1. Crosslinking of HA

Differents hydrogels particles of HA have been prepared at different crosslinking densities by varying the STMP concentration (Table 1) at constant NaOH over HA molar ratio and at constant polymer concentration ([NaOH]/[HA] = 1; $C_{HA} = 90$ g l^{-1}). Volumes of organic and aqueous phases are, respectively, 20 and 4.4 ml. The duration of the crosslinking reaction is 3 h at 50 °C with a stirring speed of 300 rpm.

SEC/MALLS measurements indicated that no degradation of HA with NaOH 0.24 mol l^{-1} has occurred during one day (Fig. 4). Neither a molecular weight decrease nor a broadening of the polydispersity was observed on the chromatogram. The weight molecular weight ($\bar{M}_w$) was about 1,200,000 g mol^{-1} with a low polydispersity index ($\bar{M}_w/\bar{M}_n = 1.4$).

Table 1
Sample codes and sizes of the particles

Sample Code	[STMP]/[HA][a]	Size of dried particles (μm)[b]
HA-1	1	5–100
HA-1.5	1.5	5–150
HA-2	2	5–200
HA-3	3	40–200

[a] Molar ratio of STMP to HA.
[b] Size of dried particles determined by SEM.

These first tests allowed us to understand the importance of parameters such as the polymer concentration, the sodium hydroxide concentration and the smallest crosslinking density required to perform the reaction.

For low concentrations of HA (15 g l^{-1}) no crosslinking reaction occurred. The best results were obtained with a HA concentration of about 90 g l^{-1}, i.e. at a concentration where the density of entanglement was high.

The minimal molar ratio of STMP to HA required to crosslink hyaluronan was equal to 1. Below this value, no crosslinking reaction occurred.

The particles were observed by scanning electronic microscopy (SEM) to estimate their size at the dried state (Fig. 5a–d and Table 1). Two facts are to be mentioned: firstly the lower the crosslinking degree is, the smaller the size of the particles is and secondly the particles are more regular and less porous when the crosslinking degree is lower. In general, the size of the particles was very heterogeneous (about 5–200 μm). This could be attributed to the synthesis method in which, the step of incorporation of the viscous solution of HA into the reactor was not very regular because of the high viscosity of the solution. The stirring speed, the viscosity of organic phase, and the choice of stirring geometry could also explain the heterogeneity.

3.2. Degree of swelling

The degree of swelling of HA hydrogel particles has been studied in water and in NaCl solutions as a function of the molar ratio [STMP]/[HA]. As we worked with a constant concentration of HA, this ratio represents the crosslinking density. It could be expected that the swelling degree decreases when the crosslinking density increases but in the same time, one can imagine an increase of swelling as a result of the contribution of the negative charged functions brought by the STMP. Both effects may explain the swelling behavior of the four hydrogels at different ionic strengths (Fig. 6).

The swelling shows a maximum at a crosslink density of ([STMP]/[HA] = 2). It is greatest for pure water and decrease when the ionic strength increases. This result agrees with the expected behavior. As the crosslinking density increases to this maximum swelling, the swelling

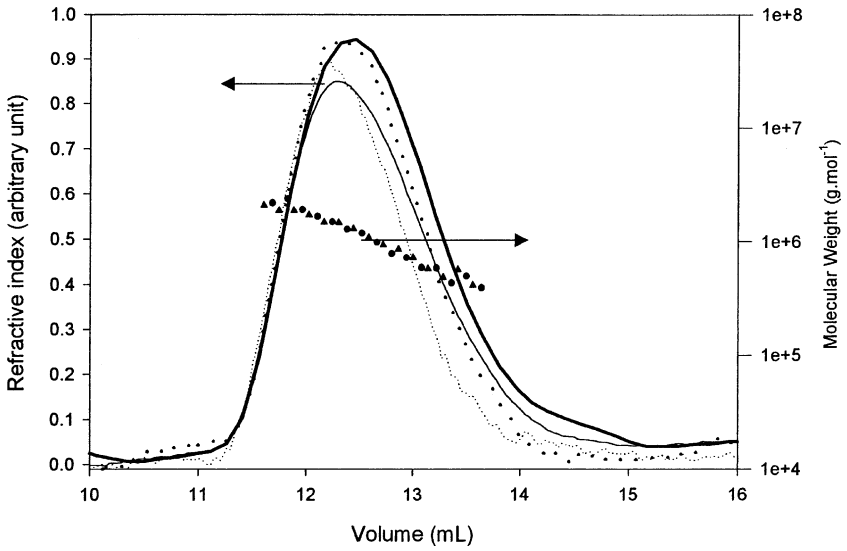

Fig. 4. Elution profiles (refractive index (full lines) and light scattering (dotted lines)) and molecular weight versus elution volume of HA before (bold) and after (fine) NaOH treatment.

of hydrogel is mainly influenced by negative charge repulsions. After the maximum the contribution of the crosslinking density becomes predominant. Increasing ionic strength minimize the repulsion effect by screening the negative charges. The maximum of swelling decreases noticeably with increasing salt concentration to its quasi minimum value for 1 mol l^{-1} NaCl. At this high ionic strength the complete screening of the charges results in a degree of swelling independent of the crosslinked quantity.

With the greatest quantity of STMP, the crosslinking density is sufficient to reduce the macromolecular chain mobility and the degree of swelling is lower. Consequently the swelling degree versus the ratio [STMP]/[HA] during the crosslinking reaction exhibits a bell shaped dependency.

This behavior had also been reported with other polysaccharides and with synthetic polymers such as poly(2-hydroxyethyl methacrylate-co-methacrylic acid), poly(2-hydroxyethyl methacrylate-co-acrylic acid) (Khare

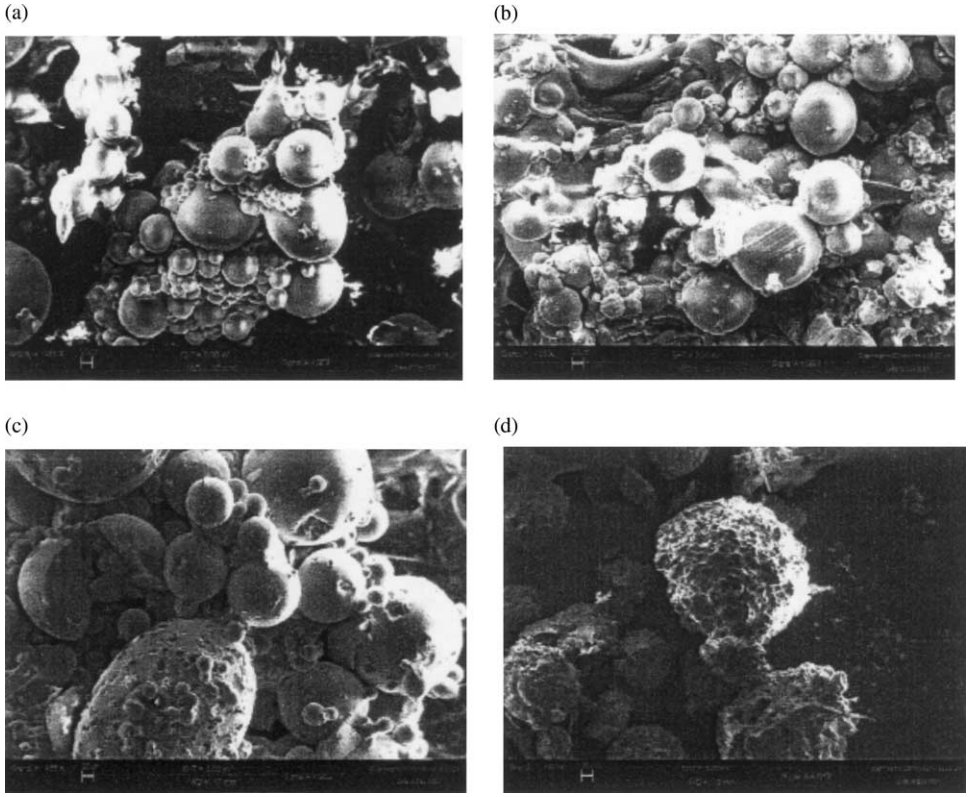

Fig. 5. Particles of HA obtained by SEM (× 455); (a) HA-1; (b) HA-1.5; (c) HA-2; (d) HA-3.

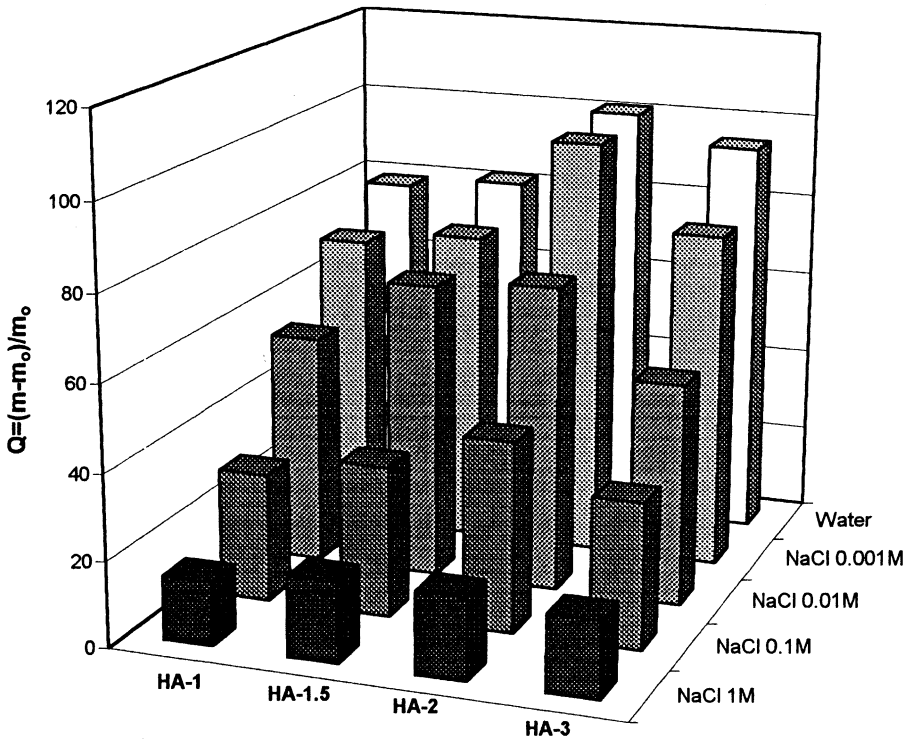

Fig. 6. Influence of crosslinking density and salinity on swelling degree of particles HA-1, HA-l.5, HA-2, HA-3.

& Peppas, 1995) and poly(organophosphazene) (Allcock & Ambrosio, 1996).

3.3. Estimation of crosslinking density from methylene blue binding measurements

An absorption kinetic study was carried out to determine the equilibrium time after which no further reduction in MB concentration could be detected. As shown in Fig. 7, the equilibrium was reached in about 24 h for hydrogels particles and native polymer. Therefore, this time was chosen to measure the absorption of native HA, HA-1, HA-1.5, HA-2 and HA-3 at equilibrium.

The plots of the relative amount of MB (RMB) bound to the particles as a function of the theoretical crosslinking density (Fig. 8) can be interpreted in two different ways. First, we can suppose that the graph shows a linear relationship between these two data when the theoretical

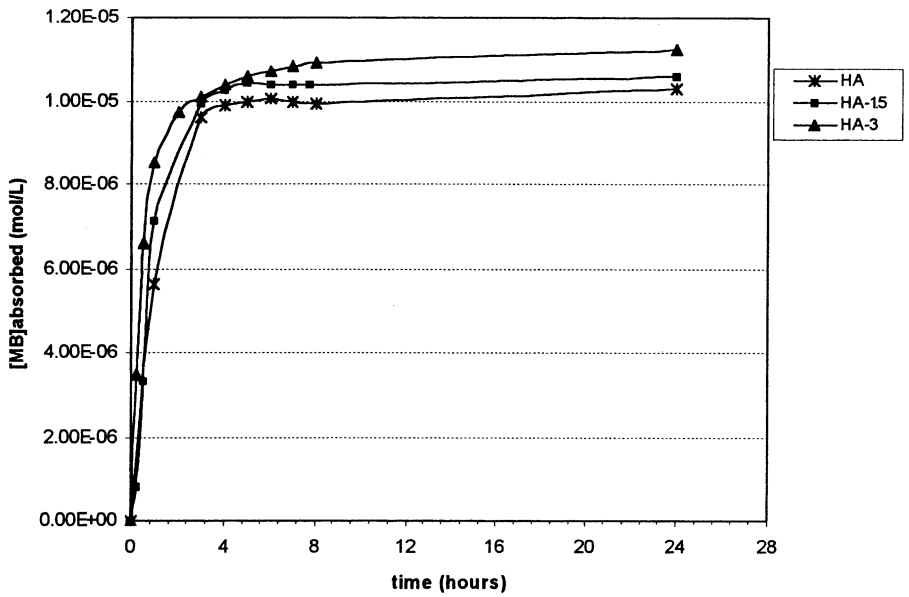

Fig. 7. Absorption kinetics of methylene blue on HA particles and native HA; Concentration in MB absorbed (at 665 nm) against time.

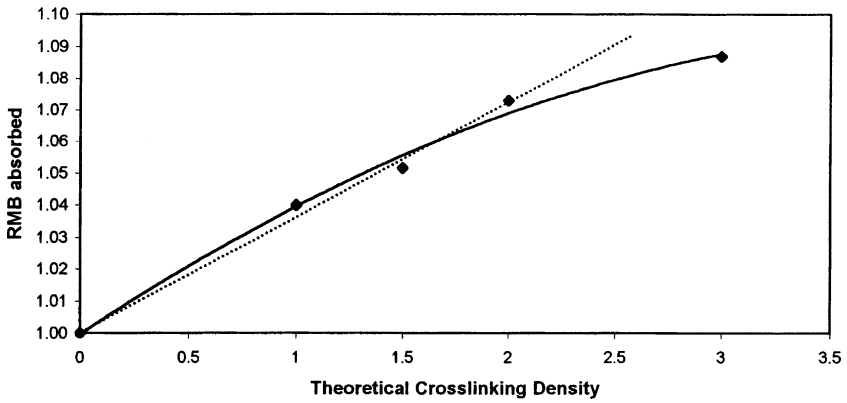

Fig. 8. Relative methylene blue absorbed on HA particles against theoretical crosslinking density.

crosslinking density is below 3 (dotted line) after that the amount of MB molecules absorbed in the particles reaches a limit even if the crosslinking density is increased. Second, we can suppose that the RMB absorbed in the particles follows a curve as a function of the theoretical crosslinking density (full line). In these two cases, we can conclude that the experimental values of the crosslinking densities (related to the RMB absorbed) are in agreement with the theoretical values until a limit after which the amount of absorbed MB does not change. We can explain this behavior by a maximum in the yield of the crosslinking reaction. The determination of the yield of the reaction and of the amount of phosphate groups present in the different hydrogels is currently being studied in our laboratory.

4. Conclusion

In the present study, we developed a new method to crosslink hyaluronan. Trisodium trimetaphosphate is a nontoxic agent leading to a crosslinking reaction in emulsion droplets of hyaluronan in the presence of sodium hydroxide. Hydrogels particles had been obtained and their swelling properties in different solvents studied. The swelling degree decreases with an increase of the ionic strength due to anionic charges in the hydrogel and show a bell-shaped dependency with the ratio [STMP]/[HA] as a result of the competition between the crosslinking density and the anionic charges brought by STMP. Absorption of methylene blue in the particles showed a dependency between the relative amount of methylene blue bound to the particles and the theoretical crosslinking density.

These hydrogel particles of hyaluronan could be specially used as cells vector in the field of cancer research.

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References

Allcock, H. R., & Ambrosio, A. M. A. (1996). Synthesis and characterization of pH-sensitive poly(organophosphazene) hydrogels. *Biomaterials*, *17*(23), 2295–2302.

Charpentier, D., Mocanu, G., Carpov, A., Chapelle, S., Merle, L., & Muller, G. (1997). New hydrophobically modified carboxymethylcellulose derivatives. *Carbohydrate Polymers*, *33*(2-3), 177–186.

Chen, J., Jo, S., & Park, K. (1995). Polysaccharide hydrogels for protein drug delivery. *Carbohydrate Polymers*, *28*(1), 69–76.

Gliko-Kabir, I., Yagen, B., Penhasi, A., & Rubinstein, A. (2000). Phosphated crosslinking guar for colon-specific drug delivery I. Preparation and physical characterization. *Journal of Controlled Release*, *63*(1-2), 121–127.

Jagur-Grodzinski, J. (1999). Biomedical application of functional polymers. *Reactive and Functional polymers*, *39*(2), 99–138.

Kasemsuwan, T., Bailey, T., & Jane, J. (1998). Preparation of clear noodles with mixtures of tapioca and high-amylose starches. *Carbohydrate Polymers*, *32*(3-4), 301–312.

Khare, A. R., & Peppas, N. A. (1995). Swelling/deswelling of anionic copolymer gels. *Biomaterials*, *16*(7), 559–567.

Kim, S. H., Won, C. Y., & Chu, C. C. (1999). Synthesis and characterisation of dextran-based hydrogel prepared by photocrosslinking. *Carbohydrate Polymers*, *40*(3), 183–190.

Luo, Y., Kirker, K. R., & Prestwich, G. D. (2000). Crosslinked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release*, *69*(1), 169–184.

Muhammad, K., Hussin, F., Ghazali, Y. C., & Kennedy, J. F. (2000). Effect of pH on phosphorylation of sago starch. *Carbohydrate Polymers*, *42*(1), 85–90.

Nishikawa, T., Akiyoshi, K., & Sunamoto, J. (1994). Supramolecular assembly between nanoparticles of hydrophobized polysaccharides and soluble protein complexation between the self-aggregate of cholesterol-bearing pullulan and α-chymotrypsine. *Macromolecules*, *27*(26), 7654–7659.

Picton, L., Mocanu, G., Mihai, D., Carpov, A., & Muller, G. (1995). Chemically modified exopolysaccharide pullulans: physico-chemical characteristics of ionic derivatives. *Carbohydrate Polymers*, *28*(2), 131–136.

Pouyani, T., Harbison, G. S., & Prestwich, G. D. (1994). Novel hydrogels of hyaluronic acid: Synthesis, surface morphology and solid-state NMR. *Journal of American Chemical Society*, *116*(17), 7515–7522.

Prestwich, G. D., Marecak, D. M., Marecek, J. F., Verkruyse, K. P., & Ziebell, M. R. (1998). Controlled chemical modification of hyaluronic acid: synthesis, applications and biodegradation of hydrazide derivatives. *Journal of Controlled Release*, *53*(1–3), 93–103.

Vandamme, E. J., & Stoetaert, W. (1995). Biochemical modification of carbohydrates. *FEMS Microbiology Reviews*, *16*(2-3), 163–186.

Woo, K., & Seib, P. A. (1997). Crosslinking of wheat starch and hydroxypropylated wheat starch in alkaline slurry with sodium trimetaphosphate. *Carbohydrate Polymers*, *33*(4), 263–271.