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Short Communication

Nanometer- and Submicrometer-Sized Hollow Spheres of Chondroitin Sulfate as a Potential Formulation Strategy for Anti-inflammatory Encapsulation

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Purpose. The synthesis of nanometer and submicrometer hollow particles could be a motivating way to imprint new therapeutic properties into a chondroitin sulfate-based hydrogel formulation. The use of hollowed polymer structures as a formulation strategy is expected to have an impact in the effective therapy in the treatment of rheumatoid arthritis.
Methods. Chemical modification of the chondroitin sulfate with glycidyl methacrylate (GMA) was performed in water under thermal and acid stimuli. The hydrogel spheres were formed upon cross-linking reaction of modified chondroitin sulfate (CSM) in a water-in-benzyl alcohol nano-droplet emulsion.
Results. ^{1}H NMR and ^{13}C NMR spectra showed that the carbon–carbon π-bonds coming from the GMA were incorporated onto backbones of CS. ^{13}C-CP/MAS NMR spectra revealed that the formation of the CSM hydrogel spheres during the dispersion stage occurred by way of carbon–carbon π-bonds on the CSM structure. The spherical shapes of the particles with diameters in the range of 20 μm to 500 nm were very clearly verified by SEM images where the dark center and edge of the hollow spheres could be identified easily.
Conclusions. Nanometer- and submicrometer-sized hydrogel spheres with hollow interior were produced from chondroitin sulfate by using a new strategy of hydrogel synthesis.

KEY WORDS: chondroitin sulfate; free-radical polymerization; hollow spheres nanotechnology; hydrogel.

INTRODUCTION

Chondroitin sulfate (CS), a mucus polysaccharide, is composed of sequences of alternately sulfated residues of D-glucuronic acid and *N*-acetyl-D-galactosamine linked to each other by β1–4 and β1–3 bonds (1). The varied amount of sulfate groups found in CS-forming polysaccharide chains has been associated to origin of the tissue, its age, and the species (2). The CS has successfully been used in a series of pharmaceutical formulations such as tablets, ophthalmic solutions, liquid preparations, soft and hard capsules, and so forth (3–17). The biodegradation of the CS by enzymes enables it to be used as a potential carrier for drug delivery (18, 19). Release systems that can be used to transport required amount of drugs up to specific local have many advantages over the conventional systems (which can provide undesirable side effects), for instance, capsule and tablets, because they can reduce the administrated dose of the drug (20–27). The synthesis of nanometer- and submicrometer-sized particles with a specific structure (28–30), for example, hollow spheres, could be a motivating way to imprint new therapeutic properties into the CS-based hydrogel formulation. The use of such a structure is expected to have an impact in the effective therapy in the treatment of rheumatoid arthritis, for the reason that a supplemental amount of drug (or encapsulation at a micro- or a nanoscale) could be loaded onto the device concerned. Upon loading with a given anti-inflammatory, this type of spheres would be directly injected into join articulations of the patient (31). The loaded particles could infiltrate deep inward tissues by way of fine capillaries, and then the anti-inflammatory agent targets into the specific site of action. After having been injected the spheres of CS hydrogel, the level release of the drug may be controlled by a specific kinetic mechanism of the polymer device. The drug release is the resulted of interactions and/or physical–chemical affinities of the drug between the polymer device and the solvent (32). In other words, the drug release from a polymer network is an effect of both the diffusion and the partition. Furthermore, the spheres that are indeed constituted of CS, upon association with synovial liquid, will be able to have a lubricant action on join articulations, thus reducing the friction between them.

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However, there is still no any efficient way to produce a hydrogel from the original CS, thus suggesting the need for a new methodology to synthesis of spheres based on sufficiently stable CS hydrogels. The chemical modification of the CS with glycidyl methacrylate (GMA), which consists of coupling carbon–carbon π-bonds coming from GMA onto polysaccharide structure (33–38), could be a promising route to overwhelm such a limitation. A water-in-benzyl alcohol nano-droplet emulsion, which has not yet been tested for the synthesis of hollow structure, was prepared in an attempt to control both the shape and the size of the particles. The formation of hydrogel particles could be then processed by way of cross-linking reaction of the CSM for the duration of the dispersion stage.

MATERIALS AND METHODS

Chondroitin Sulfate (CS) was kindly supplied by Company-Solabia, Maringá, Brazil. The other reactants were obtained from the following Companies: Glycidyl Methacrylate (Acros Organics), Sodium Persulfate (Sigma), *N,N,N',N'*-tetramethylethylenediamine TEMED (Sigma), 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt, TSP-d_4 (Sigma), dimethyl sulfoxide-d_6, DMSO-d_6 (Sigma), tetramethylsilane, TMS (Sigma). High retention seamless cellulose tubes (D-0530, lot 103H0525) with average flat width 32 mm (1.3 in.), MWCO 12400, 99.99% retention for dialysis were purchased from Sigma.

Methods

Spheres Preparation

Chemical Modification of Chondroitin Sulfate. The CS-modifying solution was prepared upon addition of 22.5 g CS and 4.6 g GMA into 150-ml aqueous solution. The pH of the solution was adjusted to 3.5 by dropping concentrated HCl solution. The resultant mixture was then stirred for 24 h at 50°C. After that, the product was precipitated in ethanol for three times to remove any residues of impurities. The modified material was separated by filtration under reduced pressure and dialyzed in Milli-Q® water at 5°C. The water was renewed every 12 h for a total period of 72 h.

Synthesis of CSM Hydrogel Spheres in Nano-droplet Emulsion. An aqueous phase consisting of 15% CSM and 0.1 mM sodium persulfate (SP) was mixed into benzyl alcohol by stirring at 6,000 rpm under nitrogen bubbling for 5 min. The water/benzyl alcohol mixture composition was used in the 1:4 proportion (water:benzyl alcohol, the volume of benzyl alcohol corresponded to four times the volume of water). The final mixture was then stirred vigorously by a high shear lab mixing (Quimis®, Dispersor Extratur®) with speed of 12,000 rpm. The gelling process was started off with the introduction of 0.127 mMol TEMED as the catalytic agent. Then, 300 ml of a hydro-alcoholic solution in the 1:1 proportion were introduced to form particle aggregates for further vacuum filtration. After having been completely purified with ethanol and water, the spheres-containing suspension was spray-dried in the following conditions: pump speed 6 ml min^{-1}, inlet air temperature 110±5°C, outlet air temperature 70±5°C, aspirator setting level 80%, atomization pressure 5 bar, and air flow rate 800 l h^{-1}. A drawing for a better understanding of the formation process of nano- and submicrospheres was illustrated in Fig. 1.

Spheres Characterization

Nuclear Magnetic Resonance Spectroscopies. Nuclear magnetic resonance spectra were recorded on a Varian spectrometer, model Mercury Plus BB 300 MHz, by applying frequencies of 300.059 MHz and 75.457 MHz for the nuclei of ^{1}H and ^{13}C, respectively. D_2O solutions consisting of 150 mg l^{-1} of CS, 150 mg l^{-1} of CSM and 0.05% 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4), as the reference line, were prepared to acquire the spectra. The angle pulse of 90° with a relaxation delay of 30 s was used for ^{1}H NMR spectra. The signal associated with water was suppressed by irradiation during the relaxation. For the ^{13}C NMR, a pulse angle of 30° was used with a relaxation delay of 1 s. Dimethyl sulfoxide-d_6 (DMSO-d_6), as the solvent, and tetramethylsilane (TMS) as the reference line, were used for recording the GMA spectra. DEPT, HMQC and COSY spectra were also performed for helping on ascriptions of the chemical shifts of both the ^{1}H and the ^{13}C nuclei. The chemical shift was reported as δ values (ppm).

Solid-State ^{13}C-CP/MAS NMR Analysis

Solid-state ^{13}C-CP/MAS NMR spectra were obtained on Varian spectrometer model Oxford 300 by applying a frequency of 74.475 MHz. The samples were placed into a 4-mm rotor; other important parameters were adjusted as follows: pulse angle θ=37°, spinning rate of 12 kHz, contact time of 3 ms, and relaxation time of 3 s.

Scanning Electron Microscopy for Sphere Morphologies

The morphologies of the samples were studied on an emission scanning electron microscope (Shimadzu, model SS 550). The hydrogel samples swollen to equilibrium at 37°C

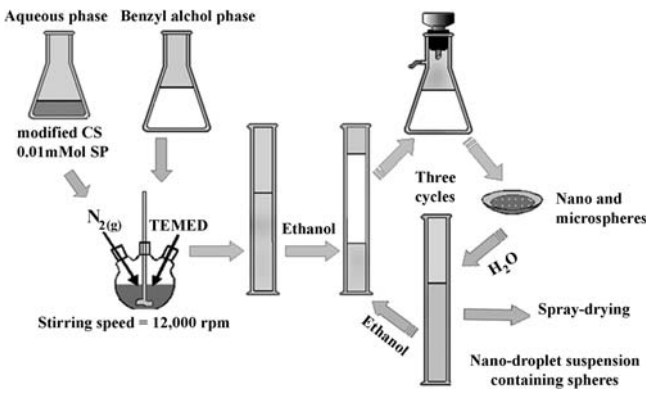

Fig. 1. Schematic illustration of the formation process of nano and submicrospheres. *CS* chondroitin sulfate, *SP* sodium persulfate, *TEMED N,N,N',N'*-tetramethylethylenediamine.

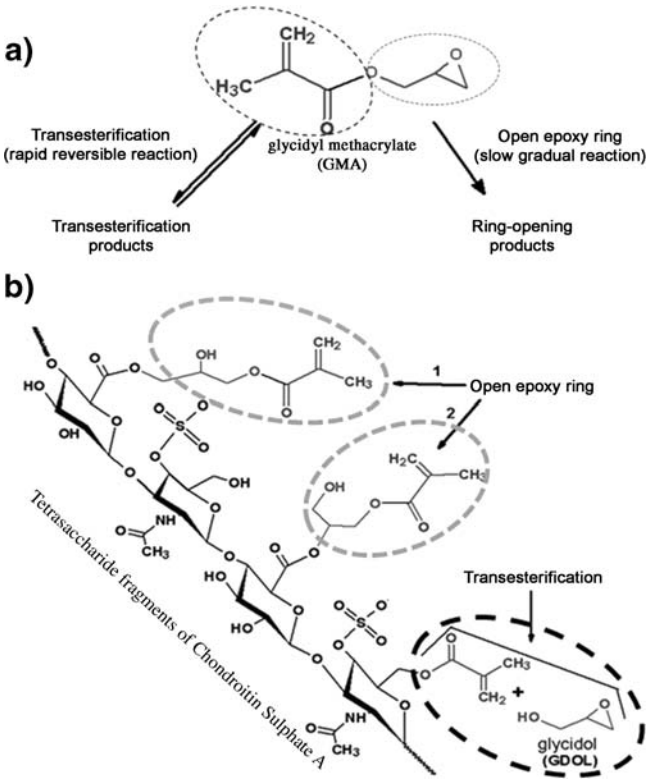

Fig. 2. **a** Two different reaction routes for the glycidyl methacrylate (*GMA*): epoxy ring opening and transesterification mechanisms, and **b** a drawing of the epoxy ring opening and transesterification routes for chemical modification of the CS with GMA.

were firstly frozen in nitrogen liquid and then lyophilized by a freeze dried for 24 h. After that, as-prepared samples were sputter-coated with a thin layer of gold on their surface for visualization by SEM images. The micrographs were taken by applying an accelerating voltage of 12 keV.

RESULTS AND DISCUSSION

Theory and Factors Affecting the Polysaccharide Modification

There are two reaction routes explaining the chemical modifications of polysaccharides with GMA that have been reported in the literature (33–38): namely, transesterification and epoxy ring-opening mechanisms. Fig. 2a shows a simplified schema drawing of both the reaction routes. The reaction mechanisms between the CS and the GMA have been already discussed by Li *et al.* (37). In view of this, our investigations have not been focused on demonstrating what the reaction mechanism is predominant, whether transesterification and/or epoxy ring-opening reactions occur. It is known that both the reaction mechanisms could or could not take place simultaneously, depending on chemical nature of the solvent utilized. The transesterification mechanism, classified as a rapid and reversible reaction via, is verified when an aprotonated solvent, as the DMSO, is used as a reaction medium. By making the assumption that the transesterification reaction takes places, methacrylate groups-carrying polysaccharide is formed as the main product, and glycidol, as a by-product. The epoxy ring-opening mechanism, a slow and irreversible reaction route, is expected to occur when an aqueous environment is used to dissolve the polysaccharide of interest.

It is important to reassert that we describe an optional method to insert the vinyl groups (C = C) from GMA onto CS structure for a further polysaccharide-gelling process. The idea is to produce a covalently cross-linked hydrogel from a polysaccharide, keeping in mind the main focus of developing either nano- or submicroscale materials with a specific structure. This process is expected to occur in the water phase where the CSM is able to be cross-linked/polymerized, because of its high solubility in aqueous media. Fig. 2b shows a drawing of the epoxy ring opening and transesterification routes for chemical modification of the CS with the GMA.

Sphere Characterization

Spectroscopic Analyses

Fig. 3 shows the nuclear magnetic resonance reference spectra of the GMA dissolved in DMSO-d_6 for the nuclei of

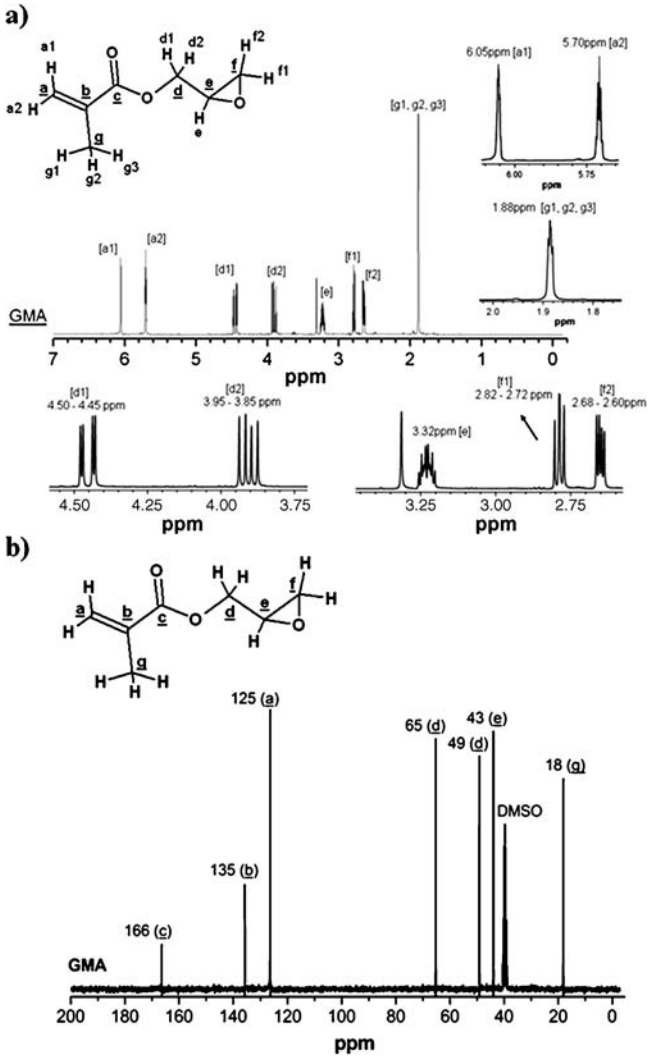

Fig. 3. Nuclear magnetic resonance reference spectra of pure GMA dissolved in DMSO-d_6: **a** ^{1}H NMR, and **b** ^{13}C NMR.

(a) ^{1}H, and (b) ^{13}C. All the peaks appearing in both the NMR spectra were identified and associated to corresponding ^{1}H and ^{13}C nuclei on the GMA molecule, which is represented in inset. These signals were marked to demonstrate the similarity to the vinyl moiety of the MCS.

Fig. 4 shows the ^{1}H NMR spectra of CS and MCS in spectral range of 7.0 to 0 ppm. The signals at δ 6.17 and δ 5.57 ppm were attributed to the hydrogen linked to a vinyl carbon (see 2a- and 2b-numbered hydrogen). The appearance of a high-intensity signal at δ 1.95 ppm, in same spectrum, was associated to the three hydrogen atoms (see 3a, 3b and 3c) of the methyl group which is linked to a vinyl carbon. The signals in ^{1}H NMR data are resulted of the incorporation of vinyl groups coming GMA onto the polysaccharide, indicative of occurrence of the chemical modification of CS.

Fig. 5 shows the ^{13}C NMR spectra of MCS and CS in the spectral range of 200 to 0 ppm. The signal at δ 172 ppm in spectrum of MCS was ascribed to carbonyl resonance of the $-CO-(H_3C)C = CH_2$ molecule; identified as four-numbered carbon in inset. The signals at δ 138 and δ 130 ppm were attributed to the one- and two-numbered carbons, respectively, and signal at δ 20 ppm was associated to the three-numbered carbon. These findings confirm the incorporation of vinyl groups coming GMA onto the CS structure, thus corroborating with the ^{1}H NMR spectra.

Fig. 6 shows solid-state ^{13}C-CP/MAS NMR spectra of CS, MCS and hydrogel spheres. This analysis gives an overview of CSM-gelling process by way of ^{13}C-resonances of the methacrylate-conjugation. The signals at spectral range of 140–120 ppm in hydrogel spectrum, assigned to the one- and two-numbered carbons, were shifted to δ 46.02 and δ 40.61 ppm after the cross-linking reaction. The carbon–carbon π-bonds on the CSM were converted to form carbon–carbon σ-bonds, causing a spectral shift of nearly 94–80 ppm. The signals at δ 170 and δ 19.18 ppm in MCS spectrum, attributed to the four- and three-numbered carbons, were marked to associate with the loss of carbon–carbon π-bonds. Note that there was a complete disappearance of these peaks in the same spectral region of hydrogel spectrum. This correlation also implied the consumption of carbon–carbon π-bonds during the gelling process.

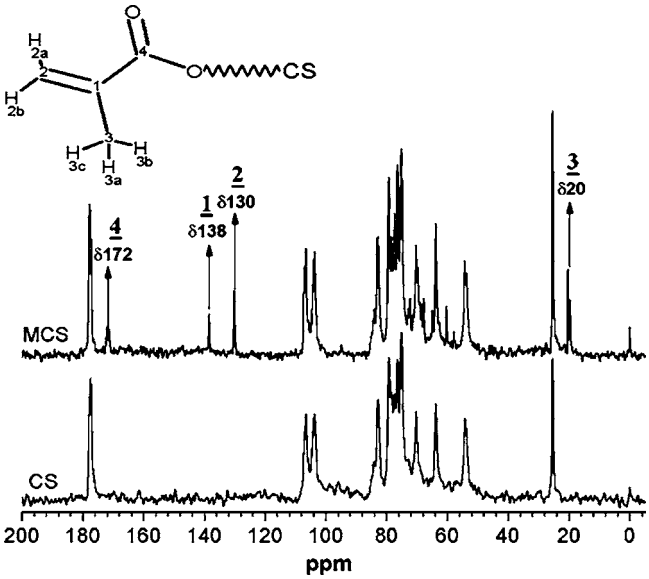

Fig. 5. ^{13}C NMR spectra of CS and MCS dissolved in D_2O. The signals at δ 172, δ 138, δ 130 and δ 20 ppm are indicative of the incorporation of $-CO-(H_3C)C = CH_2$ onto CS structure.

Microscopic analyses

Figs. 7a, b show typical SEM images of spray-dried CSM hydrogel spheres. The CSM hydrogel spheres exhibited

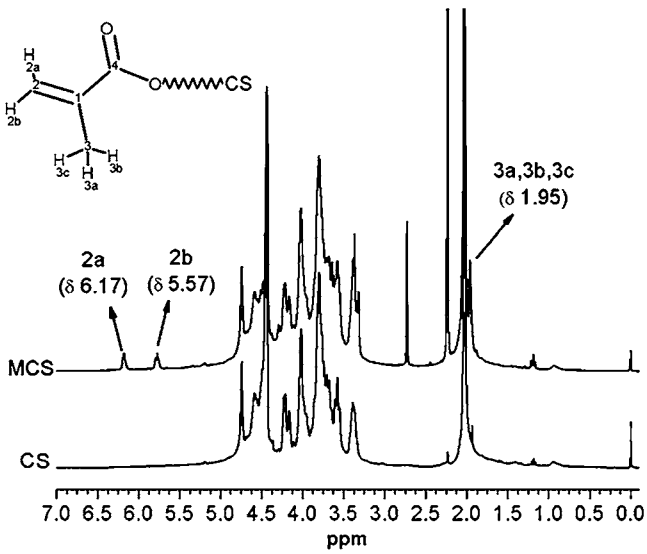

Fig. 4. ^{1}H NMR spectra of CS and MCS dissolved in D_2O. The signals at δ 6.17, δ 5.57 and δ 1.95 ppm indicate the incorporation of $-CO-(H_3C)C = CH_2$ groups onto CS structure.

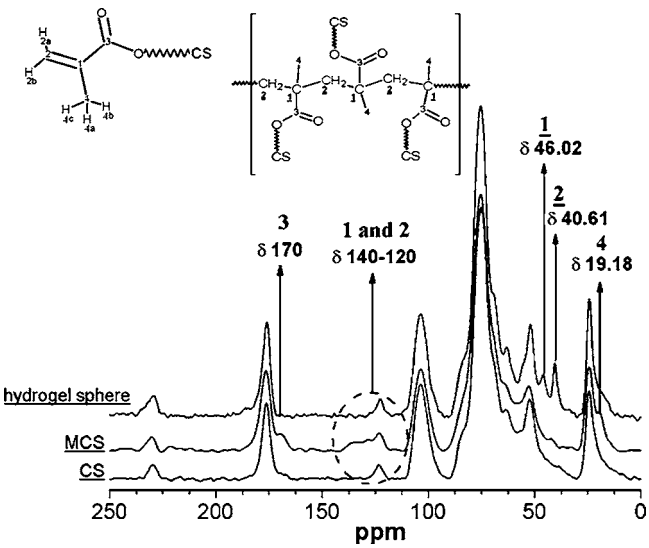

Fig. 6. CP/MAS ^{13}C NMR spectra of CS, MCS and hydrogel. *Inset* shows the structure fragment of the product resulting from the hydrogel formation.

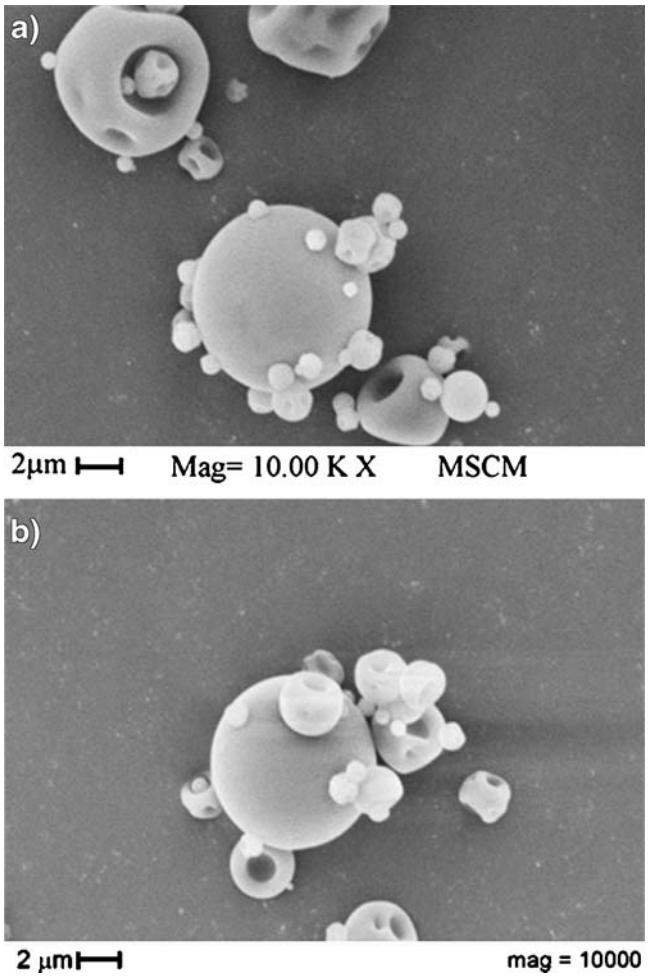

Fig. 7. Scanning electron microscopy (SEM) photomicrographs from nano and submicroscale hollow spheres taken at ×10,000 magnification after having been spray-dried.

hollow structure with diameters in the range of 20 μm up to 500 nm. Both the images illustrate very clearly the center and edge of the spheres. The formation of nanometer or submicrometer hollow polymer structure with an approximately spherical shape has been supposed to be dependent on two causes. The first cause, considered the most important, was associated to vigorous stirring which was applied to keep the suspended particles in the water-in-alcohol benzyl mixture. At high stirring speed, there is a random movement of benzyl alcohol droplets inward the water phase. In the most elementary form, the alcohol molecules diffuse into the water droplets that form new alcohol driblet inside water droplets, resulting in a multiple emulsion with a water–benzyl alcohol–water configuration. The imprisonment of a phase in a two-phase microemulsion has discussed by Zhang *et al.* (39). There will not be then cross-linking reaction at droplet center because the CSM has limited solubility in the benzyl alcohol phase. Fig. 8 shows a drawing of the formation process CSM hydrogel spheres with hollowed structure. A solid sphere was also drawn for a mere comparison. Either nano- or microencapsulation of an anti-inflammatory agent onto hollow of the sphere could be processed upon addition of a benzyl alcohol-miscible pharmaceutical solute for the duration of the dispersion stage. After being dissolved, the anti-inflammatory agent is then carried in the benzyl alcohol flow in the direction of droplet center. The second cause was related to high water content within the CSM hydrogel, because it has sufficient ability to swell upon contact with the water phase, and therefore its polymer networks are significantly expanded. After having been spray-dried, the particle-suspended emulsion is atomized to finer droplets in which the water of the hydrogel is rapidly evaporated in a warm air current without its polymer chains being relaxed. The movement of water toward the outside would make a hollow shell inside the particle. It could be then assumed that the original formation of the polymer chains in swollen state is preserved practically.

CONCLUSIONS

The chemical modification of the CS with GMA was processed in water under thermal and acid stimuli. Incorporation of carbon–carbon π-bonds coming from the GMA onto backbone of the CS was checked by both the ^{1}H NMR and the ^{13}C NMR spectra. ^{13}C-CP/MAS NMR spectra revealed that the formation of the CSM hydrogel spheres occurred by way of carbon–carbon π-bonds on CSM. Nanometer- and submicrometer-sized hollow spheres were produced from cross-linking reaction of chemically modified CS in a water-in-benzyl alcohol nano-droplet emulsion. The CSM hydrogel spheres exhibited a hollow shell inside particle and diameters in the range of 20 μm to 500 nm. The CSM hydrogel spheres exhibited a specific structure enabling them to be used as a potential strategy for anti-inflammatory encapsulation in the treatment of rheumatoid arthritis via intramuscular administration or subcutaneous route.

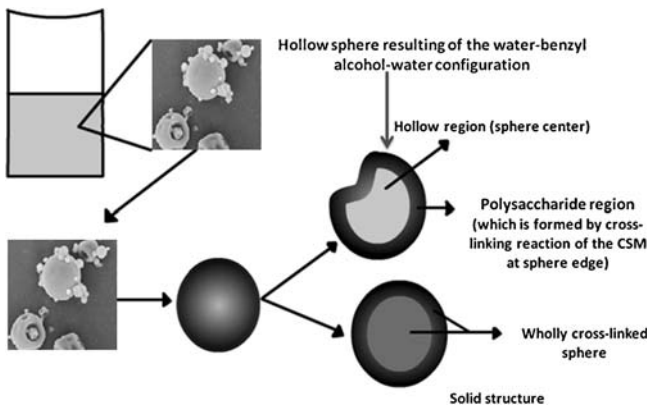

Fig. 8. A drawing of the formation process CSM hydrogel spheres with hollowed structure. A *solid sphere* was also drawn for a mere comparison. For this type of structure, it is assumed that the cross-linking reaction of the CSM occurs on whole particle.

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