Hyaluronate-Covered Nanoparticles for the Therapeutic Targeting of Cartilage

Hamed Laroui,[†,‡] Laurent Grossin,[‡] Michèle Léonard,[†] Jean-François Stoltz,[§] Pierre Gillet,[‡] Patrick Netter,[‡] and Edith Dellacherie*[,†]

Laboratoire de Chimie Physique Macromoléculaire, UMR CNRS-INPL 7568, BP 20451, 54001 Nancy Cedex, France Laboratoire de Physiopathologie et Pharmacologie Articulaires, UMR CNRS-UHP 7561, BP 184, 54505 Vandoeuvre-lès-Nancy Cedex, France Equipe de Mécanique et Ingénierie Cellulaire et Tissulaire, LEMTA, UMR CNRS-INPL-UHP 7563, BP 184, 54505 Vandoeuvre-lès-Nancy Cedex, France

Received July 30, 2007; Revised Manuscript Received October 3, 2007

Hyaluronic acid (HA) has a high affinity for the CD44 receptor present at the surface of articular cells, particularly of chondrocytes. HA-covered polylactide nanoparticles containing bioactive compounds such as HA and chondroitin sulfate (CS) were thus prepared in order to achieve a controlled delivery targeted to cartilage cells after injection near articular alterations/erosions. Such nanoparticles (diameter = 700 nm) were prepared by double emulsion/solvent evaporation, using amphiphilic derivatives of HA, as stabilizer of the secondary emulsion. These nanoparticles were incubated with articular cells, and several tests were carried out. First, they proved that the nanospheres provoked no decrease in cell viability, even after 72 h of contact. Second, a confocal microscopy analysis on fluorescent HA-covered particles showed that they were captured by articular cells, while with those covered with poly(vinyl alcohol), the uptake was far lower. Third, a scattering electron microscopy analysis proved that the HA-coated nanoparticles were localized in the cell intracytoplasmic area.

Introduction

The concept of targeted drugs is not new but dates back to 1906 when Ehrlich[1] first postulated the "magic bullet". The challenge was (i) to find a drug capable of treating a particular disease, (ii) to find a means of carrying the drug *in vivo* in a stable biocompatible form, and (iii) to find the proper target for the concerned disease and then to define components able to establish selective interactions with specific receptor sites of the target organ.

Among the various carriers proposed for drug delivery, polymeric nanoparticles have been studied for several decades. The most widely used polymers to engineer nanoparticles are certainly aliphatic polyesters such as polylactide (PLA), polyglycolide, and copolymers[2–4] whose biocompatibility and (bio) degradability are well-known and which are FDA approved.

To increase the therapeutic efficacy of the nanoparticle-encapsulated drugs, nanoparticles may be delivered to specific sites by passive or active targeting.[5] For passive targeting, one can play with the size of nanoparticles: thus, drug-loaded nanoparticles can be retained in arteries after local administration by virtue of their size.[6] It was also shown that rigid long-circulating carriers with a diameter larger than 200 nm might act as splenotropic particles.[7] Passive delivery may also be directed to the mononuclear phagocyte system and circulating macrophages which are specialized in eliminating invaders that have gained entry to tissue fluids.[5] On the other hand, the route of administration for nanoparticles can also have an important role in successful targeting. Thus, transdermal, pulmonary, or ocular delivery allows a great improvement in the therapeutic efficacy.

However, active targeting is the only means to get the greatest therapeutic efficacy and to reduce toxicity, as it greatly increases the probability of redirecting long-circulating particles to designated but accessible targets. Unlike passive targeting, ligands or homing devices that specifically bind to surface epitopes or receptors on the target sites are coupled to the surface of the carriers. A lot of effort has been devoted to achieving active targeting in order to deliver drugs to the right cells and tissues,[5,8] particularly for cancer therapy: thus, as the elevated expression of the folate receptor has frequently been observed in various types of human cancers, folic acid has been attached to liposomes and nanoparticles.[9] The folate nanoparticles were found to selectively target cancer cells and to improve the internalization of the encapsulated drugs.[10] Other endogenously occurring ligands and substances have also been attached to vesicles or particles, which include oligosaccharides, for example, sialyl Lewis X,[11] galactosyl,[12] and so forth, and antibodies.[13] Oligomers of hyaluronate (HA) were also used as ligands to target liposomes to the CD44 cell-surface marker overexpressed in certain tumor cells.[14] CD44 is a receptor that binds HA, a polysaccharide consisting of β-1,3-N-acetylglucosaminyl-β-1,4-glucuronide.

The CD44 receptor is also expressed at the surface of chondrocytes, the cartilage cells, and its presence provokes the internalization of HA.[15] Thus, nanoparticles covered with HA, containing molecules active in the treatment of cartilage pathologies, and directly injected in the joint cavity, could be both passively (by the effect of particle size) and actively targeted to the chondrocytes. This paper describes the synthesis and characterization of PLA nanoparticles covered with HA and containing water-soluble macromolecules such as chondroitin-sulfate (CS) and HA, both known for their positive effect on cartilage repair.[16,17]

* To whom correspondence should be addressed. E-mail: edith.dellacherie@ensic.inpl-nancy.fr.
† Laboratoire de Chimie Physique Macromoléculaire.
‡ Laboratoire de Physiopathologie et Pharmacologie Articulaires.
§ Equipe de Mécanique et Ingénierie Cellulaire et Tissulaire.

10.1021/bm700836y CCC: $37.00
Published on Web 11/27/2007

These nanospheres were then incubated with chondrocytes and synoviocytes in order to study the cytocompatibility of the particles and their capacity to be internalized into the cells.

Experimental Section

Preparation of Nanoparticles and Characterization. *Materials.* Sodium HA was obtained from Bioibérica S.A. (Barcelona, Spain). Its average molar masses were calculated from size exclusion chromatography coupled to multiangle laser light scattering (SEC-MALLS) and were found to be 366 000 and 625 000 g/mol for M_n and M_w, respectively.

SEC was performed using a Waters HPLC model 590 pump with a serial set of OH Pack SP 806, 805, and 804 HQ columns (Shodex) and an OH Pack SBG as the guard column. The injection volume was 200 μL. The eluent was a 0.1 M $NaNO_3$ solution containing NaN_3 as a bactericide (0.4 g/L) and was filtered (0.1 μm; Millex VC, Millipore, France) prior to use. The flow-rate was 0.7 mL/min. The polymer solutions were prepared by dissolution in the same eluent and were left under stirring for 24 h. They were filtered (0.45 μm; Millex GSWP, Millipore, France) right before injection, in order to remove possible aggregates. Elution was dually monitored by MALLS detection, using a Mini-Dawn (Wyatt Technology Corporation, USA) unit equipped with a He−Ne laser operating at 690 nm and by differential refractometry (Waters Model 410). dn/dc was measured in 0.1 N $NaNO_3$ using an Optilab 903 refractometer (Wyatt Technology Corporation, USA).

Poly(D,L-lactide) (PLA) was purchased from Sigma-Aldrich (France) and was characterized by a molar mass between 75 and 120 kg/mol. All of the reagents and solvents used for the polymer syntheses and the emulsions were obtained from the suppliers indicated by Pelletier et al.[18] and Chognot et al.,[19] respectively. The CS was a gift from Bioibérica SA (Barcelona, Spain). Bovine serum albumin (BSA), hyaluronan lyase (HA-lyase, from *Streptomyces hyaluroticus*), and chondroitin lyase ABC (CS-lyase, from *Proteus vulgaris*) were purchased from Sigma-Aldrich (France).

Synthesis of Amphiphilic HA and Characterization. The amphiphilic derivatives of HA (HA-Cn) were prepared by the fixation of aliphatic bromides (chains with 6 or 12 carbon atoms) according to the method already described.[18] Briefly, it consisted of the reaction, in a homogeneous medium (dimethylsulfoxide), of an alkyl halide (here, hexyl or dodecyl bromide) with the carboxylic groups of HA, preliminarily transformed into its tetrabutylammonium salt (HA-TBA). The alkyl chains are thus linked to the polysaccharide backbone via ester functions. The nomenclature used is HA-Cn-x, where Cn is the covalently bound long alkyl chain ($n = 6$ or 12) and x is the substitution ratio (mol of alkyl chain/100 mol of monosaccharide unit).

The content in aliphatic chains was determined as reported by Pelletier et al.[18] after hydrolyzing the ester function linking the Cn chain and the HA backbone. The dodecanol and hexanol thus released were extracted by toluene and quantified by gas chromatography. For hexanol, as it is slightly soluble in water, the content in C6 chains on HA-C6 was calculated by taking into account the partition coefficient between toluene and the aqueous solution resulting from the hydrolysis.

Surface tension measurements were carried out at 25 °C in 0.15 M NaCl using a TRACKER dynamic tensiometer (Model I.T. Concept, Longessaigne, France). All samples were equilibrated for a sufficient time to reach constant readings. Depending on the composition of HA derivatives, the time to reach equilibrium was between 30 min and 4 h. The measurements were made in triplicate, and the average value was then retained.

Preparation of Core–Shell Nanoparticles. The nanoparticles were produced by the double emulsion/solvent evaporation procedure derived from the procedure described by Chognot et al.[19] for the elaboration of MPEO-covered PLA nanoparticles. It consists of using an amphiphilic copolymer as the surfactant of the secondary emulsion, here, HA substituted by pendent alkyl chains (HA-Cn).

Typically, a primary water-in-oil (w/o) emulsion was prepared by mixing an organic phase (4 mL of dichloromethane) containing PLA (25 g/L) with an internal aqueous phase (200 μL) containing or not containing BSA (50 g/L). The mixture was stirred for 2 min with a Vortex mixer (Top Mix, Bioblock Scientific, France), then sonicated (2 min, power 5, 50% active cycle, in an ice bath) using a Vibracell model 600 W (Sonics & Materials, Danbury, USA). This primary emulsion was poured into a second aqueous phase (8 mL; external aqueous phase) containing an amphiphilic derivative of HA (HA-C6 or HA-C12; 0.5, 1, or 5 g/L). The w/o/w emulsion was then obtained by sonication (same conditions as those used for the primary emulsion). This double emulsion was then transferred into an aqueous 10^{-2} M $NaNO_3$ dispersing phase (40 mL) and stirred for 5 min. The organic solvent was evaporated under stirring in ambient air, and the collected solid nanospheres were resuspended in water, then centrifuged again in order to remove the excess HA-Cn. This purification procedure was repeated two times. The final suspension was then freeze-dried.

Nanoparticles covered with poly(vinyl alcohol) (PVA) were prepared by the same method but using 10 g/L of PVA (Aldrich, Milwaukee, WI; molar mass = 13 000–23 000 g/mol, hydroxyled at 87–89%) instead of HA-Cn in the external aqueous phase.

Nanoparticles covered with HA or PVA and containing fluorescent dextran-FITC (molar mass = 500 000 g/mol, Sigma-Aldrich, France) were prepared according to the procedure described above. Dextran-FITC was dissolved in the internal aqueous phase at a concentration of 2.5 g/L.

Experiments were also carried out by replacing the ultrasonic device with a microfluidizer under 2 bar of pressure (model 110S, Microfluidics Corporation, Newton, MA).

The size distribution of nanospheres (mean diameter and standard deviation) was determined by photon correlation spectroscopy (PCS) using Malvern equipment (Model 4600, Malvern Instruments, UK) in 10^{-3} M NaCl at 25 °C.

Scattering Electron Microscopy (SEM) of Nanoparticles. After plate deposition and drying of the nanoparticle aqueous suspensions (1 mg/mL), the plates were fixed with 2.5% glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.2, for one night at 4 °C. Afterward, the samples on the plug were rinsed and dehydrated by successive 2 min baths in a series of increasing concentrations of ethanol/water (30°, 50°, 70°, 80°, 90°, and 100°) and then finally treated with a 5 min bath of hexamethyldisilazane and dried under a range hood. The samples were then fixed on an aluminum support with a "carbon adhesive glue" and metallized with gold−palladium (Spotter Coater SC7640). The samples were observed with a Stereoscan S 240 (Cambridge Instruments UK).

Measurement of the Amount of HA Present at the Nanoparticle Surface. The principle consists of enzymatically hydrolyzing the HA present at the surface, by HA-lyase. A total of 300 mg of nanoparticles were dispersed in 10 mL of a 20 mM acetate buffer, pH 6, by sonication for 5 min. Then, 200 μL of this suspension was treated with 20 μL of a HA-lyase solution (100 units/mL, 20 mM acetate buffer pH = 6) for 72 h at 37 °C. The resulting polysaccharidic fragments were then analyzed and quantified by capillary electrophoresis according to a method described elsewhere.[20]

Preparation of HA-Coated Nanoparticles Loaded with a Glycosaminoglycan. HA-coated nanoparticles loaded with HA or CS were prepared according to the procedure described above. The glycosaminoglycan (GAG) was dissolved in the internal aqueous phase of the primary emulsion at a concentration of 5 g/L.

Measurement of the Amount of Encapsulated Glycosaminoglycan. Each sample of HA-coated GAG-loaded nanoparticles was treated as follows: one-half was dispersed in a 20 mM buffer acetate, pH 6, and subjected to HA-lyase as described above. This experiment allowed the quantification of the HA present at the nanoparticle surface. In fact, preliminarily, it had be shown that, in the case of bare nanoparticles loaded with HA and treated with HA-lyase, the enzyme could not penetrate inside the PLA matrix as no peaks of polysaccharidic fragments were observed in the capillary electophoregram.

The other half was treated at pH 10 with 1 M NaOH in order to degrade the particle PLA matrix.

For each HA determination, typically, 90 mg of nanoparticles were treated with 9 mL of 1 M NaOH for 2 h at 25 °C. Then, the solution pH was decreased down to 6 with concentrated acetic acid and diluted to 12 mL with a 20 mM acetate buffer, pH 6. A total of 200 μL of this solution was subjected to enzymatic hydrolysis with 20 μL of HA-lyase (100 units/mL). For the CS determination, the same process was used. The HA-lyase was just replaced by CS-lyase at the same concentration. All solutions were then filtered on an Ultrafree-MC (Millipore Corporation, Bedford, MA) of 10 000 nominal molecular weight limit.

The amounts of GAGs (at the surface or encapsulated) were calculated from calibration curves preliminarily determined with known concentrations of HA and CS.

The polysaccharidic fragments were quantified by capillary electrophoresis.[20] In the case of HA-coated, HA-loaded nanoparticles, the amount of encapsulated HA was calculated by the difference between the value obtained after nanoparticle degradation (total HA) and that obtained by the controlled enzymatic hydrolysis of surface HA.

In Vitro Biological Evaluation on Cartilage Cells. *Isolation and Culture of Rat Chondrocytes and Synoviocytes.* Animal experiments were conducted in accordance with institutional guidelines following the recommendations of the NIH for animal experimentation. Six-week-old male Wistar rats were kept in a 12/12 light/dark cycle (light-on period, 6:00 a.m. to 6.00 p.m.) in a controlled-temperature chamber (24 ± 1 °C).[21]

Normal articular cartilage was obtained from Wistar male rats (175–200 g, Charles River Laboratories, l'Arbresle, France) sacrificed under anesthesia [ketamine (50 mg/kg) and acepromazine (1.25 mg/kg)]. After joint surgery, articular cartilage pieces were aseptically dissected from femoral head caps, and chondrocytes were obtained by sequential digestion with Pronase (2 mg/mL, 2 h) and collagenase B (1.5 mg/mL, overnight) (Roche, Meyland, France). Synoviocytes were isolated from synovial membranes, digested overnight by a mix of collagenase and Dispase (respectively 0.1 units/mL and 0.8 units/mL, Roche). Then, cells were washed two times in PBS and cultured to confluence in 75 cm^3 flasks at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was DMEM/Ham's F-12 (Invitrogen, France) supplemented with L-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), and heat-inactivated fetal calf serum (10%) (Invitrogen, France) in an incubator at 37 °C and 5% CO_2.

Assessment of Cytotoxicity by MTT Assay. The MTT test was carried out following a protocol already described.[20] Cells were seeded in 96-well plates at a density of 5×10^4 cells per well, and they were exposed to several concentrations of HA- or PVA-coated nanoparticles loaded with dextran-FITC for several time periods (24, 48, and 72 h). The assay is based on the cleavage of the soluble yellow MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, France] by dehydrogenase present in intact mitochondria, forming insoluble purple formazn crystals. After being washed, the formazan crystals are solubilized in sodium dodecylsulfate/dimethylformamide (SDS/DMF), and the absorbance of the resulting colored solution is measured at 580 nm (Multiskan microplate reader, Labsystems, Montigny-le Bretonneux, France). Cells were incubated with 100 μL of DMEM/F12 and 25 μL of MTT solution (5 mg/mL) for 4 h. Then, the supernatant was removed and 100 μL of the SDS/DMF solution was added into each well. The plate was incubated overnight before measurement. Results were expressed as means ± standard deviation for at least three assays. Comparisons were made by ANOVA, followed by the Fisher protected least-squares difference posthoc test using the Statview 5.0 software (SAS Institute Inc.). A P value of less than 0.05 was considered significant.

Single-Photon Confocal Microscopy Analysis. Chondrocytes and synoviocytes were cultivated under classical conditions and exposed to nanoparticles (100 μg/mL) for various time periods (6, 12, and 24 h). Then, cells were fixed by a 4% paraformaldehyde solution, pH 7.4, for 15 min at room temperature, then washed three times (5 min each) with PBS. Slides were observed with a Leica TCS SP2 equipped with an Acousto-Optical Bind Splitter. Excitation for dextran-FITC (E_{emmax} = 518 nm, E_{exmax} = 492 nm) was achieved by the 488 nm line from an argon laser. All photomultipliers used were adjusted to the same sensitivity (gain/offset). A 1.32 numerical aperture oil immersion lense (Leica 63x, Planapo, Wetzlar, Germany) was used. Three-dimensional images of reconstituted tissues were compiled either into a single-view projection using LCS3D image processing software (Leica Microsystems, Mannheim, Germany) or into a 3D projection using Imaris reconstruction software (Bitplane AG, Suisse).

Scattering Electron Microscopy Analysis. Chondrocytes or synoviocytes were cultivated (90 000 cells for a 13-mm-diameter chamber, i.e., approximately 70 000 cells/cm^2) on culture chambers (Laboratory-Tek, Nalge Nunc International, Naperville, IL) and left to adhere for 48 h. The wells were washed in DMEM without red phenol for 15 min. Then, they were fixed with 2.5% glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.2, for one night at 4 °C. The rest of the experiment was performed as described above for SEM nanoparticle analysis.

Results and Discussion

Synthesis and Characterization of HA-Coated Nanoparticles. The method used for preparing the nanoparticles coated with HA and loaded with water-soluble macromolecules was derived from the method described by Chognot et al.[19] for the elaboration of MPEO-covered nanoparticles. As mentioned above, it consists of using the double emulsion/solvent evaporation procedure in which the surfactant of the secondary emulsion is an amphiphilic copolymer whose hydrophilic part is designed according to the expected properties of the nanoparticle shell. After solvent evaporation, the nanoparticles present this hydrophilic part at their surface, the hydrophobic one being anchored inside the PLA matrix. This property has already been proved for PLA nanoparticles prepared by simple emulsion in the presence of amphiphilic dextran[22] or water-soluble monomethoxypoly(ethylene oxide)-b-PLA copolymers (MPEO-b-PLA).[23] This method was also used for the preparation of PLA nanospheres by double emulsion in the presence of water-soluble MPEO-b-PLA copolymers.[19]

In this study, this principle was applied to HA-based polymeric surfactants, HA-Cn.

Properties of the Synthesized HA-Cn. Two amphiphilic derivatives of HA were prepared: HA-C6–14 (HA with 14 mol pendent C6 chains/100 mol monosaccharide units) and HA-C12–5.5 (HA with 5.5 mol C12 pendent chains/100 mol monosaccharide units). These polymers were found to be slightly surfactive as their tension at equilibrium was about 60 mN/m at 0.5 g/L for each sample while pure water presents an air/water tension of about 70 mN/m under the same conditions.

Preparation of HA-Covered Nanoparticles. Study of a Simple Oil-in-Water Emulsion. This study was realized in order to determine the influence of some emulsification parameters on the size of particles. The simple oil-in-water emulsion was prepared with 10 mL of water containing 0.5 g/L of HA-C6 and 1 mL of dichloromethane containing PLA at 25 g/L.

First of all, the influence of the emulsification method (microfluidizer or ultrasonic device) on the emulsion size was studied. The microfluidizer treatment consisted of several passes in the device (10, 20, or 30 passes). The sonication was applied for 2 min, at a power of 5, and using a 50% active cycle in an ice bath, after 2 min of Vortex mixing.

Figure 1 shows the results concerning the droplet diameter obtained with these two methods. One observes that, with microfluidizer, it decreases from 400 nm to about 200 nm, whe

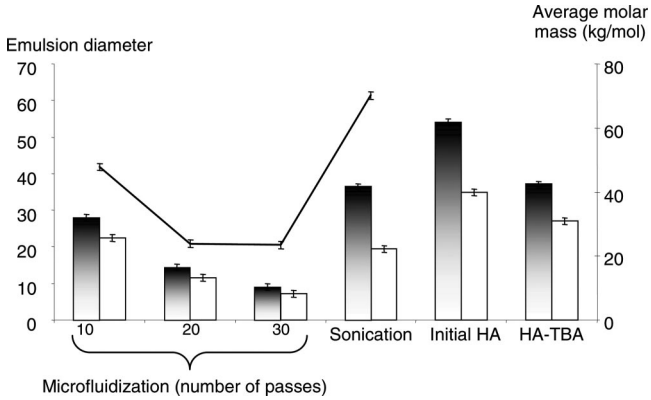

Figure 1. Influence of the emulsification method on the diameter of emulsion droplets and on the average molar masses of HA-TBA. Two simple emulsions were used as described in the text. The continuous line is related to the emulsion diameter, the histogram to the polymer average molar masses. "HA-TBA" and "initial HA" were not subjected to the emulsification process. Values expressed as means of three experiments.

the number of passes increases from 10 to 30. With sonication, the droplets are bigger (about 600 nm).

We have also controlled the influence of the two emulsification processes on the molar mass of HA. To this aim, the emulsion was constituted of 10 mL of HA-TBA (3 g/L in water) and 1 mL of dichloromethane. HA-Cn could not be used in these experiments as the amphiphilic derivatives could not be eluted on SEC columns because of their adsorption on the stationary phase. HA-TBA is the derivative of HA obtained during the synthesis of HA-Cn, just before the reaction with alkyl bromide. PLA was not added in the organic phase, in order to favor the demixion of the emulsion into two phases and to allow the measurement by SEC-MALLS of HA-TBA present in the aqueous phase.

Figure 1 gives the average molar masses of HA-TBA before the emulsification process and after. It is first seen that HA-TBA before emulsification possesses average molar masses slightly lower than those of unmodified HA. This has already been pointed out[18] and was attributed to the synthesis chemical treatment. The sonication process applied to HA-TBA does not greatly modify M_w but slightly decreases M_n. Several papers have already reported on the degradation of polysaccharides by ultrasounds[24] and particularly of HA,[25] and this degradation was attributed to cavitation, stress concentration, and radical attachment to the depolymerization of polysaccharides.[24]

On the other hand, when HA-TBA was subjected to the microfluidization process, a strong degradation of the HA backbone was observed as M_w and M_n were very low after 20 passages. Such a mechanical degradation by microfluidization has already been reported for polysaccharides and particularly for traganthan[26] and xanthan[27] and would be provoked by the high shearing stresses and the turbulent forces generated in the microfluidizer.

To explain the smaller droplet diameter obtained at 20 and 30 passes, one can thus assume that the low-molecular-weight HA-C6 fragments thus formed have different behavior from that of longer chains. In fact, these types of amphiphilic polysaccharides are well-known to exhibit associative properties in aqueous solutions:[18,28] the smaller the chains, the lower the aggregation strength. The short fragments would thus be less compact than the long chains and could better adsorb at the interfaces, thus providing a better stabilization of small droplets in the emulsion.

Table 1. Influence on the Size of Emulsion Droplets, of the HA-Cn Concentration in the Aqueous Phase of the Simple Emulsion[a]

polymer concentration (g/L)	nanoparticle average diameter (nm)		
	0.5	1	5
HA-C12	320 ± 30	350 ± 50	1630 ± 80
HA-C6	290 ± 30	380 ± 50	<5000

[a] Values expressed as means of three experiments.

Finally, taking into account the fact that bioactive polysaccharides had to also be encapsulated, the sonication method was chosen in order to limit the possible degradation of active macromolecules.

Concerning the effect of the HA-Cn concentration on the droplet size, several concentrations in the aqueous phase were studied, that is, 0.5, 1, and 5 g/L. The results are given in Table 1, and they show that, whatever the HA-Cn, the higher its concentration, the bigger the droplets. The intermolecular hydrophobic aggregation of HA-Cn in the aqueous phase at high concentrations could thus lead to the adsorption of aggregates at the organic surface, making it responsible for higher particle diameters. A HA-Cn concentration of 0.5 g/L was then used for all of the following experiments as it allowed the production of the smallest droplets.

Preparation of Nanoparticles by Double Emulsion/Solvent Evaporation. First, we studied the formation of the water-in-oil primary emulsion prepared with 200 μL of water and 4 mL of dichloromethane with 25 g/L of PLA. No stable emulsion could be formed as two phases were observed after 10 min. We had already shown that BSA was required to stabilize the primary emulsion in the case of MPEO-coated nanoparticles.[19] In this work, we found that a 50 g/L BSA concentration in the aqueous phase of the primary emulsion was sufficient to stabilize it.

Taking into account the results of the preliminary experiments, the retained conditions for the preparation of GAG-loaded HA-covered particles were thus as follows: BSA 50 g/L and GAG 5 g/L in the internal aqueous phase, HA-Cn 0.5 g/L in the external aqueous phase, 2 min Vortex, and 2 min sonication (power 5, 50% active cycle) for both emulsions. The other parameters were those described in the Experimental Section.

Figure 2 presents a SEM photo of nanoparticles prepared with HA-C12, before washing, centrifugation, and freeze-drying. It is seen that the nanospheres are spherical and that their size distribution is relatively homogeneous.

Table 2 gives the size of particles after washing, centrifugation, and freeze-drying, prepared with HA-C6 or HA-C12 as the surfactant and with or without HA as the active polysaccharide (AP). The experiments were carried out with or without BSA in the aqueous phase of the primary emulsion. In this table, by comparing the results obtained with BSA and AP with those obtained with BSA without AP, it is seen that AP has an effect more or less favorable to the particle size depending on the nature of HA-Cn. It is likely that HA in the internal aqueous phase can contribute to stabilizing the primary emulsion as poly(vinyl alcohol) can do it.[4] By comparing the results obtained with AP and BSA, with those obtained with AP without BSA, one observes that BSA contributes to the particle final size decrease. The influence of BSA on the stabilization of the primary emulsion has been already discussed by Chognot et al.[19]

On the other hand, if one compares the results concerning particle size in Figure 2 and Table 2, one observes that the average particle size evaluated by PCS is around 600–700 nm

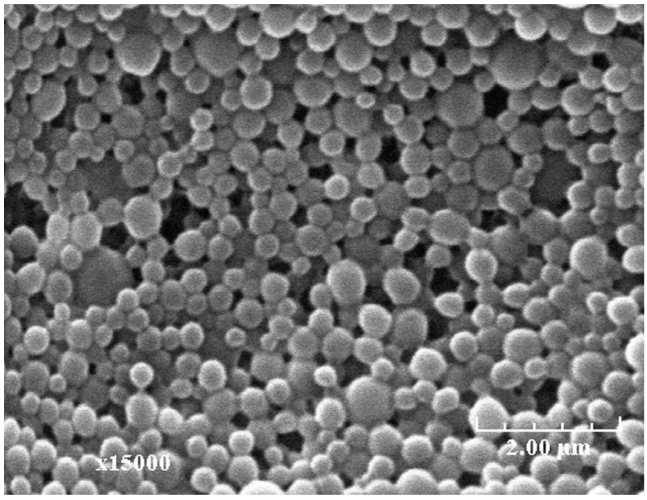

Figure 2. SEM photo of HA-covered nanoparticles loaded with HA. Nanoparticles prepared as described in the text with HA-C12 and without washing, centrifugation, and freeze-drying.

Table 2. Size of Nanoparticles (nm) Prepared under the Conditions Described in the text with HA as Active Polysaccharide (AP) in the Aqueous Internal phase [HA] = 5 g/L[a]

amphiphilic polymer	BSA,[b] without AP	AP, without BSA	BSA[b] and AP
HAC_6	450 ± 20	790 ± 40	650 ± 40
HAC_{12}	650 ± 35	895 ± 60	690 ± 40

[a] [HA-Cn] = 0.5 g/L in the aqueous external phase. [b] With BSA at 50 g/L in the aqueous internal phase. Diameters determined after the complete process of particle preparation, i.e., after washing, centrifugation, and freeze-drying.

Table 3. Characteristics of HA-Coated Nanoparticles Loaded with HA or CS[a]

encapsulated GAG	particle average diameter (nm)	HA at the surface (µg/mg of nanoparticles)	GAG loading (µg/mg of nanoparticles)
HA	650 ± 40	21 ± 4	13 ± 1
CS	690 ± 50	23 ± 5	26 ± 3

[a] Nanoparticles were prepared as described in the Experimental Section with: HA-C6 (0.5 g/L), BSA (50 g/L), and GAG (5 g/L). Results expressed as means of eight experiments.

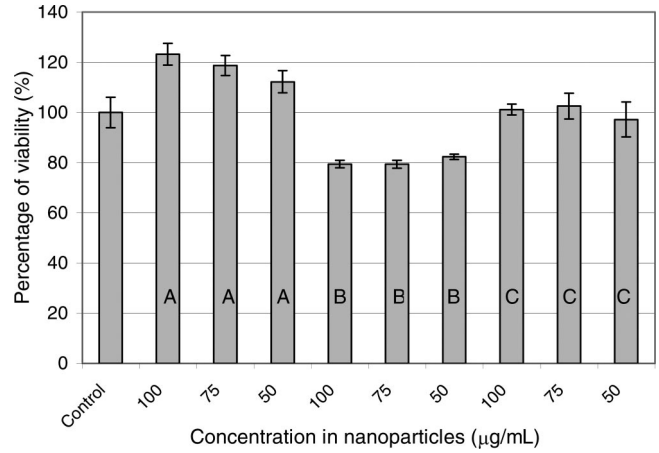

Figure 3. Cellular viability of synoviocytes in the presence of nanoparticles after 72 h of incubation and at different concentrations of nanospheres. (A) HA-covered nanoparticles loaded with dextran-FITC, (B) PVA-covered nanoparticles loaded with dextran-FITC, (C) HA-covered nanoparticles loaded with HA. (Control) Cells without nanoparticles.

while on the SEM photo, the average diameter is rather around 300 nm. This difference can be explained by various reasons: first, the particles of Figure 2 were studied just after the solvent evaporation before washing, centrifugation, and freeze-drying, while the size of the other ones was measured after these processes. Some aggregation may thus occur, particularly during freeze-drying, and could explain the bigger size seen in Table 2. A second reason is related to the technique of SEM that requires the study of the dry particles under a vacuum, which can provoke particle shrinkage. Finally, by PCS, one calculates an intensity average size, while by SEM, one evaluates a number average size which is generally lower than the other one.

Amount of HA Present at the Nanoparticle Surface. The HA present at the nanoparticle surface is a part of the HA-Cn used as the surfactant of the secondary emulsion in the nanoparticle preparation. We have thus verified that it could be correctly quantified after enzymatic hydrolysis by using the capillary electrophoresis method described for unmodified HA.[20] Defined amounts of HA-C6 and HA-C12 were treated by HA-lyase under the same conditions as for HA, and the results obtained for HA-Cn and unmodified HA after ECZ were found to be very close, which means that this method is valid for lowly substituted HA-Cn.

Table 3 shows the surface characteristics of the nanoparticles prepared.

Amount of Encapsulated GAG. The amounts of HA and CS encapsulated in the nanoparticles were enzymatically determined, after PLA matrix chemical degradation, as described in the Experimental Section.

The results are given in Table 3.

It is seen that HA and CS were really encapsulated but with ratios that are rather different, with 26 µg/mg of nanoparticles for CS and 13 µg/mg for HA. The greater amount found for encapsulated CS could be related to possible ionic interactions between the GAG and BSA in the internal aqueous phase of the primary emulsion. These interactions could be stronger for CS (strongly negative sulfate groups) than for HA (weak carboxylic/carboxylate groups) and hamper the GAG leak in the external aqueous phase during the second emulsification.

***In vitro* Biological Evaluation on Cartilage Cells.** The biological evaluation was carried out with nanoparticles of about 700 nm in diameter. According to the experiments to be carried out, some of them were prepared with HA-C6 and thus covered with HA (about 20 µg/mg nanoparticles), and other ones were coated with PVA. Some of them were loaded with HA as the bioactive molecule and other ones with dextran-FITC as the fluorescent marker.

Cytotoxycity Analysis. The MTT test was performed on synoviocytes and chondrocytes at three time points (24, 48, and 72 h) in the presence of nanoparticles covered with HA or PVA loaded with HA or dextran-FITC. This analysis allows the detection of mitochondrial activities that are the early changes observed in cells undergoing the death process. Three concentrations of nanoparticles (50, 75, and 100 µg/mL) were tested.

No significant differences were detected between control cells (no nanoparticles) and cells exposed to HA-covered nanoparticles. A slight decrease (20%) in cell viability was observed for cells incubated with PVA-coated nanoparticles at 72 h in both cell lines. None of the three concentrations of HA-coated nanoparticles affected cellular viability at any time or for either cell line. For example, Figure 3 shows the results obtained with synoviocytes.

The MTT analysis thus clearly indicates that the use of HA-covered nanoparticles has no deleterious effect on synoviocytes

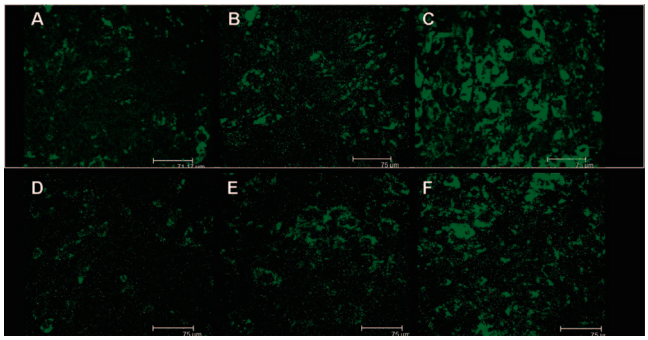

Figure 4. Kinetics of the uptake of HA-covered nanoparticles loaded with dextran-FITC by articular cells, as studied by confocal microscopy. Chondrocytes (A, B, C) and synoviocytes (D, E, F) at 6, 12, and 24 h of incubation, respectively.

and chondrocyte viability up to a nanoparticle concentration of 100 μg/mL and for a 72 h period of time. A slight decrease is observed with PVA-coated particles, but it was mainly balanced at 96 or 120 h.

Study of the Nanoparticle Uptake by Articular Cells. Confocal Microscopy Analysis. In order to study the uptake of nanoparticles by articular cells, experiments were performed in which cells were incubated for 6, 12, and 24 h with dextran-FITC-loaded nanoparticles covered with HA. Cells were fixed, and slides were observed on a Leica confocal microscope. The results are presented in Figure 4.

It can be seen that with HA-coated particles, the amount of fluorescence in chondrocytes was just detectable at 6 h (Figure 4A), but the intensity of the signal was increased at 12 h (Figure 4B) and above all at 24 h (Figure 4C). Concerning the synoviocytes, the signal of fluorescence was weaker than for chondrocytes at any time (Figure 4D, E, and F). On the other hand, only a weak signal in a few cells was observed with PVA-coated nanospheres for both cell lines (not shown).

Additional experiments were performed on chondrocytes and synoviocytes, in which cells were preincubated with an anti-CD44 antibody (monoclonal anti-CD44 antibody mouse, Sigma-Aldrich, France) for 2 h at 5 μg/mL, washed twice to remove antibody excess, and then incubated with HA-covered dextran-FITC loaded nanoparticles. The results of confocal microscopy are presented in Figure 5. It is shown that, for chondrocytes (Figure 5A,B) and synoviocytes (Figure 5C,D), a decrease in the amount of fluorescence was observed between cells without CD44 antibody pretreatment (Figure 5A,C) and preincubated cells (Figure 5B,D). Thus, this means that the HA-coated nanoparticles' uptake is at least partly mediated by the CD44 receptor as its inhibition decreases the particle internalization. The fact that the uptake is not completely canceled after CD44 antibody pretreatment is probably the result of a too-weak concentration in antibodies compared to the number of CD44 receptors present at the cell surface.

Scattering Electronic Microscopy Analysis. The fluorescence observed in both cell lines seems to have an intracytoplasmic localization, with many fluorescent vesicles observed. But we were not able to determine the exact localization with the confocal microscope, and to overcome this technical drawback, a SEM analysis was performed to clearly localize the nanoparticles inside the cells. The pictures taken on a Stereoscan S240 are presented in Figure 6. The fluorescent vesicles are quite visible, and these photos clearly demonstrate that they are localized inside the chondrocytes, and probably in the cytoplasm, and not at their surface.

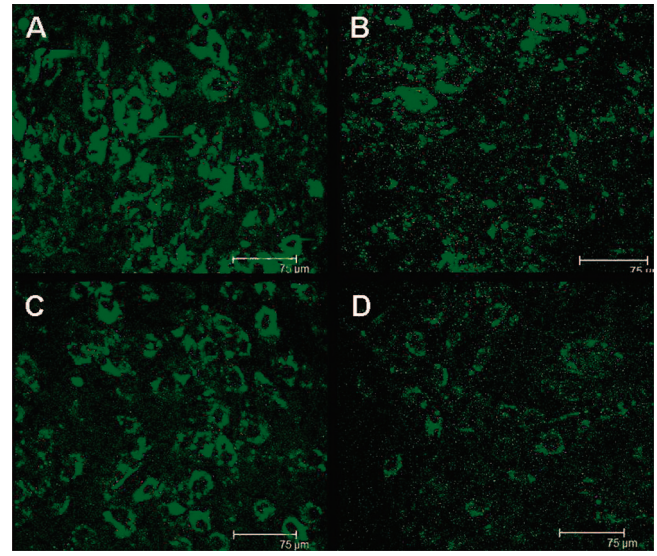

Figure 5. Influence of the cell treatment by CD44 antibody, on the uptake of HA-covered nanoparticles loaded with dextran-FITC by articular cells, as studied by confocal microscopy. The cells were incubated with particles for 24 h. (A and B) Chondrocytes without CD44 antibody preincubation (A) and after 2 h of incubation with CD44 antibody (B). (C and D) Synoviocytes without CD44 antibody preincubation (C) and after 2 h of incubation with CD44 antibody (D).

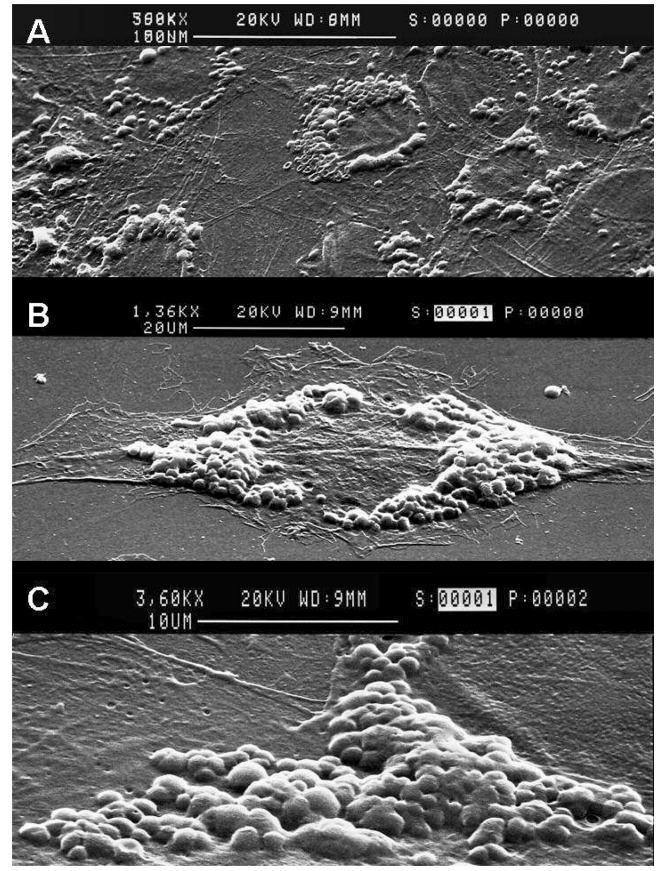

Figure 6. SEM pictures of chondrocytes after 24 h of incubation with HA-covered nanoparticles loaded with dextran-FITC. Photos at increasing manignification values. (A) ×465, (B) ×1750, (C) ×4600.

Conclusion

This paper has shown that PLA nanoparticles (around 700 nm) covered with HA could be prepared by the double emulsion

solvent evaporation procedure by using amphiphilic HA as the surfactant of the secondary emulsion. Polysaccharidic molecules active in cartilage treatment (HA, chondroitin sulfate) were then encapsulated, the objective being that, as HA specifically recognizes the CD44 receptor present at the chondrocyte surface, the encapsulated polysaccharides could be released inside the cells.

An *in vitro* biological evaluation on articular cells (chondrocytes and synoviocytes) was performed using HA-loaded nanoparticles covered with HA or PVA, and with HA- or PVA-covered particles loaded with dextran-FITC. The cytotoxicity analysis proved that none of the HA-coated nanospheres led to a decrease in cell viability. The analyses carried out with dextran-FITC-loaded particles in confocal microscopy and scanning electron microscopy showed that the HA-coated nanospheres were captured by chondrocytes and to a lesser extent by synoviocytes, while PVA-covered particles were not captured or were done so very weakly. This means that the uptake of HA-covered particles is mainly mediated by the presence of HA at the nanoparticle surface, the lower CD44 concentration at the synoviocyte surface being a possible reason for the slighter internalization. To confirm this hypothesis, some additional experiments should be performed, like the quantification of CD44 expression rate by chondrocytes and synoviocytes by, for example, a Western-Blot analysis. On the other hand, the very weak uptake of PVA-covered particles is probably due to a classical endocytosis mechanism and a lack of interactions between nanoparticles and articular cells. The SEM analysis confirmed the conclusion of a specific uptake by chondrocytes and showed that the localization inside the cells was cytoplasmic.

All these results prove that a target delivery of active molecules in articular cells could be achieved by this kind of HA-coated particles, but some other experiments are required, in particular, *in vivo* assays on rat cartilage defects, to verify the feasibility of this concept. Such *in vivo* experiments are now under investigation.

Acknowledgment. The authors thank Dominique Dumas and Luc Marchal for their helpful assistance in confocal microscopy and scattering electronic microscopy analyses. They acknowledge Région Lorraine and Communauté Urbaine du Grand Nancy for their financial support. Hamed Laroui was supported by a grant of the French Ministry in charge of research.

References and Notes

(1) Ehrlich P. *The collected papers of Paul Ehrlich*; Pergamon: London, 1960; Vol. 3.
(2) Langer, R.; Folkman, J. *Nature* **1976**, *263*, 797–800.
(3) Blanco, D.; Alonso, M. J. *Eur. J. Pharm. Biopharm.* **1997**, *43*, 287–294.
(4) Zambaux, M.-F.; Bonneaux, F.; Gref, R.; Dellacherie, E.; Vigneron, C. *J. Controlled Release* **1999**, *60*, 179–188.
(5) Moghimi, S. M.; Hunter, A. C.; Murray, J. C. *Pharmacol. Rev.* **2001**, *53*, 283–318.
(6) Guzman, L. A.; Labhasetwar, V.; Song, C.; Jang, Y.; Lincoff, A. M.; Levy, R.; Topol, E. J. *Circulation* **1996**, *94*, 1441–1448.
(7) Moghimi, S. M.; Porter, C. J. H.; Muir, I. S.; Illum, L.; David, S. S. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 861–866.
(8) Brigger, I.; Dubernet, C.; Couvreur, P. *Adv. Drug Delivery Rev.* **2002**, *54*, 631–651.
(9) Stella, B.; Arpicco, S.; Peracchia, M. T.; Desmaële, D.; Hoebeke, J.; Renoir, M.; D'Angelo, J.; Cattel, J. L.; Couvreur, P. *J. Pharm. Sci.* **2000**, *89*, 1452–1464.
(10) Stella, B.; Marsaud, V.; Couvreur, P.; Arpicco, S.; Peracchia, M. T.; Geraud, G.; Immordino, M. L.; Cattel, L.; Renoir, M. In *Proceedings of the Controlled Release of Bioactive Materials Congress*,San Diego, CA, 2001;No. 5200.
(11) DeFrees, S. A.; Phillips, L.; Guo, L.; and Zalipsky, S. *J. Am. Chem. Soc.* **1996**, *118*, 6101–6104.
(12) Liang, H. F.; Chen, C. T.; Chen, S. C.; Kulkarni, A. R.; Chiu, Y. L.; Chen, M. C.; Sung, H. W. *Biomaterials* **2006**, *27*, 2051–2059.
(13) Dinauera, N.; Balthasarb, S.; Weberb, C.; Kreuterb, J.; Langerb, K.; von Briesen, H. *Biomaterials* **2005**, *26*, 5898–5906.
(14) Eliaz, R. E.; Szoka, F. C., Jr. *Cancer Res.* **2001**, *61*, 2592–2601.
(15) Dean, J. A.; Knudson, W.; B.; Knudson, C. B. *Exp. Cell Res.* **1999**, *252*, 292–302.
(16) Bassleer, C. T.; Bougaret, S.; Malaise, M. *Osteoarthritis Cartilage* **1998**, *6*, 196–204.
(17) Noble, P. *Matrix Biol.* **2002**, *21*, 25–29.
(18) Pelletier, P.; Hubert, P.; Lapicque, F.; Payan, E.; Dellacherie, E. *Carbohydr. Polym.* **2000**, *43*, 343–349.
(19) Chognot, D.; Léonard, M.; Six, J.-L.; Dellacherie, E. *Colloid Surf., B* **2006**, *51*, 86–92.
(20) Gerard, C.; Catuogno, C.; Amargier-Huin, C.; Grossin, L.; Hubert, P.; Gillet, P.; Netter, P.; Dellacherie, E.; Payan, E. *J. Mater. Sci. Mater. Med.* **2005**, *16*, 541–551.
(21) Grossin, L.; Cournil-Henrionnet, C.; Mir, L. M.; Liagre, B.; Dumas, D.; Etienne, S.; Guingamp, C.; Netter, P.; Gillet, P. *FASEB J.* **2003**, *17*, 829–835.
(22) Rouzes, C.; Léonard, M.; Durand, A.; Dellacherie, E. *Colloid Surf., B* **2003**, *32*, 125–135.
(23) Chognot, D.; Six, J.-L.; Léonard, M.; Bonneaux, F.; Vigneron, C.; Dellacherie, E. *J. Colloid Interface Sci.* **2003**, *268*, 441–447.
(24) Lii, C. Y.; Chen, C. H.; Yeh, A. I.; Lai, V. M. *Food Hydrocolloids* **1999**, *13*, 477–481.
(25) Rehakova, M.; Bakos, D.; Soldan, M.; Vizarova, K. *Int. J. Biol. Macromol.* **1994**, *16*, 121–124.
(26) Silvestri, S.; Gabrielson, G. *Int. J. Pharm.* **1991**, *73*, 163–169.
(27) Lagoueyte, N.; Paquin, P. *Food Hydrocolloids* **1998**, *12*, 365–371.
(28) Sinquin, A.; Dellacherie, E.; Hubert; P., *Langmuir* **1993**, *9*, 3334–3337.
(29) Zambaux, M.-F.; Bonneaux, F.; Gref, R.; Dellacherie, E.; Vigneron, C. *J. Controlled Release* **1999**, *60*, 179–188.

BM700836Y