On the Synthesis and Characterization of Biodegradable Dextran Nanogels with Tunable Degradation Properties

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ABSTRACT: Hydrogels are widely investigated as carriers in drug delivery. To be suitable carriers for intracellular drug delivery, hydrogels should be small enough to be able to enter cells. This paper reports on the synthesis and characterization of both lipid coated and naked biodegradable hydroxyethyl methacrylated dextran (dex-HEMA) nanogels. Dynamic light scattering (DLS), atomic force microscopy, and transmission electron microscopy showed that lipid coated nanogels could be obtained by polymerization of an aqueous dex-HEMA solution entrapped in SOPC/DOTAP liposomes (SOPC and DOTAP, respectively, being 1-stearoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleolyl-3-trimethylamonium propane chloride). Naked dex-HEMA nanogels were prepared by removing the lipid coating by Triton X100. DLS measurements on dex-HEMA nanogels stored in buffer at 37 °C revealed that the degradation time depended on the cross-link density of the nanogels: dex-HEMA nanogel prepared from dextran lowly substituted with HEMA degraded quickly, while it took days to weeks for nanogels prepared from highly substituted dextran. Furthermore, confocal laser scanning microscopy showed that SOPC/DOTAP coated dex-HEMA nanogels can be taken up by VERO-1 cells.

Introduction

Many molecules that are under development as therapeutic agents have targets located intracellularly. Examples are certain proteins, oligonucleotides, plasmid DNA, and interference RNA. However, most of these molecules show a poor cellular uptake and become easily degraded in extra- and intracellular media. Appropriate nanosized delivery systems that protect and deliver drug molecules intracellularly are therefore required.[1] While intracellular drug release has been shown to occur from many types of nanoparticles, which parameters govern the drug release process once the nanoparticles enter the cell remain unknown. This is partly due to difficulties in characterizing quantitatively the biophysical behavior of nanoparticulate matter in living cells. As a consequence, it remains also very challenging to design drug loaded nanoparticles with optimized intracellular release properties. Indeed, while micron sized particles (which remain extracellular after injection) with tunable release properties have been well-studied,[2−8] little attention has been paid so far to nanoparticles that could slowly release the drug in the cell after uptake by the cells.

In previous work, we investigated the physicochemical and release properties of biodegradable dextran-hydroxyethyl methacrylate (dex-HEMA, Figure 1A) hydrogels and microgels.[3,9−13] Dex-HEMA gels are biocompatible[14] and degrade spontaneously under physiological conditions.[12] Drug molecules can be entrapped in a dex-HEMA network, and controlled degradation of the network can result in sustained drug release during several days. The degradation rate of dex-HEMA hydrogels and dex-HEMA microgels is dependent on their cross-link density, which can be varied by altering the degree of substitution of the dextran (DS, i.e., the numbers of HEMA groups per 100 glucose units) and the dex-HEMA concentration. Recently, we became interested in dex-HEMA nanogels for sustained intracellular drug delivery. This paper deals with the first step in this research: it describes the preparation of dex-HEMA nanogels and tuning of their degradation rate by changing their composition.

Several methods have been reported to obtain nanoscopic hydrogel particles. A commonly used method is emulsion polymerization:[15−17] a stable emulsion is formed, and next the droplets of the aqueous polymer solution are polymerized to form the nanogels while the surrounding organic solvent is removed by evaporation[15] or extraction and dialysis.[17] Major drawbacks of this method are the use of organic solvents and the need of energy (e.g., sonication) to form the emulsion, which may inactivate the entrapped therapeutic molecules. Also, nanogels obtained by emulsion polymerization are often strongly polydisperse in size. An interesting approach, without the use of organic solvents, has been recently described by the group of Levon:[18,19] nanogels were made by UV polymerization of a solution of acrylamide and *N*,*N*′-methylenebis(acrylamide) entrapped in liposomes. After the formation of these nanogels, the surrounding lipid layer was removed by the addition of a detergent. Besides the absence of organic solvents, another advantage of this method is that the size and polydispersity of the nanogels can be easily controlled since the liposomes, which act as a container in which the nanogels are composed, are formed by multiple extrusion through a membrane with a well-defined pore size. Another benefit of this method is that both noncoated (naked) nanogels and lipid coated nanogels can be made, which may be attractive for

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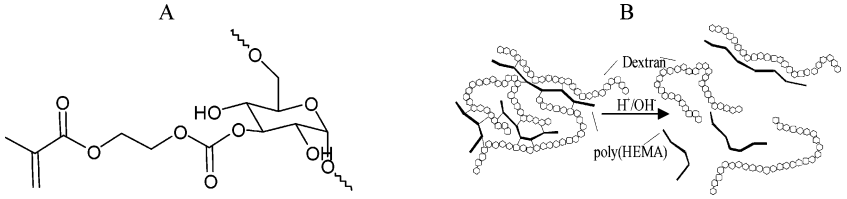

Figure 1. (A) Chemical structure of the monomer in dex-HEMA (i.e., glucopyranose substituted with HEMA). (B) Schematic representation of the dex-HEMA polymer network before (left) and after (right) degradation. The HEMA cross-links degrade through hydrolysis of the carbonate esters, resulting in dextran chains and poly(HEMA).

certain drug delivery applications. Recently, Patton et al. also made use of liposomes as nanoreactors for making artificial oxygen carriers as blood substitutes:[20] bovine hemoglobin was entrapped in poly(acrylamide)/poly(*N*-isopropylamide) nanogels prepared in liposomes. Another recent study reported on actin nanogels prepared by polymerizing actin monomers to filamentous structures in phosphatidyl choline based liposomes.[21,22] These lipid coated actin nanogels were aimed at revealing how the cytoskeleton, coupled to the surrounding cell membrane, determines the cell shape during dynamic processes.

The aim of this paper is three-fold. First, we wonder whether both lipid coated and naked dex-HEMA nanogels could be obtained by using liposomes as a nanoreactor. Second, we investigate whether in this way dex-HEMA nanogels could be obtained that differ in degradation properties. Third, we are interested in knowing whether the obtained dex-HEMA nanogels could be taken up by cells.

Materials and Methods

Dex-HEMA Preparation and Characterization. Dex-HEMA batches were prepared and characterized according to a method described elsewhere.[23] Dextran (Fluka, from *Leuconostoc ssp.*) with a number average molecular weight of 19 000 g/mol was used. The degree of substitution (DS, i.e., the number of HEMA groups per 100 glucopyranose residues of dextran) was determined by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) in D_2O with a Gemini 300 spectrometer (Varian). The DS of the dex-HEMA used in this study were 2.5, 5.4, and 8.9, respectively. In the atomic force microscopy and transmission electron microscopy experiments, we used dex-HEMA with a DS of 18.9.

Preparation of dex-HEMA Nanogels. As outlined in the Introduction, dex-HEMA nanogels were prepared using liposomes as a nanoscaled reactor. A conventional procedure to prepare the liposomes was used. A 5 mg lipid film of SOPC/DOTAP (9:1) (SOPC and DOTAP, respectively, being 1-stearoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleolyl-3-trimethylammonium propane chloride, Avanti Polar Lipids) was made by dissolving the lipids in chloroform and drying this solution under a nitrogen flow while spinning the vial gently. This resulted in a thin lipid film on the bottom of the vial. The vial was placed under vacuum for at least 4 h to remove all remaining chloroform. Subsequently, this dry lipid film was hydrated with 1 mL of a dex-HEMA solution (i.e., 20% dex-HEMA in 50 mM phosphate buffer (PB) at pH 7.0) that contained 0.05% Irgacure 2959 (Ciba Specialty Chemicals) as a photoinitiator. The resulting dispersion was placed for 30 min at 25 °C while vortexing every 5 min. This dispersion of large vesicles was aged overnight. Next, the dispersion was extruded with a hand-held syringe fitted with a standard filter holder (Avanti Polar Lipids) provided with a 450 nm polycarbonate membrane (Schleicher and Schuell). After 11 back-and-forth passages of the dispersion through the extrusion membrane, the liposome dispersion was diluted 10 times with PB. This dilution was necessary as not all the dex-HEMA was entrapped in the liposomes. If not diluted, this free dex-HEMA would form a gel in the polymerization step, thereby enclosing the liposomes in a polymer matrix. The dispersion was subsequently exposed to UV light (365 nm from a Bluepoint 2.1 UV source, Honle UV Technology) at 25 °C during 450 s, which cross-linked the dex-HEMA solution in the liposomes with the formation of lipid coated dex-HEMA nanogels. To obtain naked dex-HEMA nanogels, the lipid layer was removed by addition of 20 μL of a 100 mM solution of the detergent Triton X 100 (TX100, Merck) to 1 mL of the liposome dispersion.

Dynamic Light Scattering (DLS) Analysis on dex-HEMA Nanogels. Dynamic light scattering measurements were done with a Malvern Autosizer 4700 at a fixed angle of 90°. A He−Ne laser (633 nm, 25 mW) was used as a light source. PB, used to dilute the dispersions of the nanogels for DLS measurements, was filtered through a 0.1 μm Millipore Durapore filter. Polystyrene nanospheres (220 ± 6 nm; Duke Scientific Corporation) were used to check the performance of the DLS instrument. The mean hydrodynamic diameter of the particles (d_h) was computed from the intensity of the scattered light using the Malvern software package based on the theory of Brownian motion and the Stokes−Einstein equation

$$D = \frac{kT}{3\pi\eta d_h} \tag{1}$$

where D is the diffusion coefficient (the primary parameter obtained from DLS measurements), k is the Boltzmann constant, T is the temperature (298 K), and η is the viscosity of the solvent (0.96 cP).

To study the degradation of the nanogels in a function of (degradation) time, a cuvette was filled with 1.2 mL of the nanogel dispersions and sealed with Parafilm to avoid contamination with dust particles. The cuvette was placed at 37 °C, and DLS measurements on the degrading nanogels were performed at regular times.

Atomic Force Microscopy (AFM) on dex-HEMA Nanogels. Atomic force microscopy images of the nanogels were recorded in air at ambient temperature with an Autoprobe CP (Thermomicroscopes) using a 5 μm scanner. The images were recorded in the intermittent-contact (tapping) mode. Point-probe Low Frequency Noncontact Sensors (Nanosensors) were used. These cantilevers have a resonance frequency around 150−170 kHz, a typical spring constant of about 50 N/m, and an integrated Si tip with a radius of curvature at the apex around 10 nm. To minimize particle deformation due to tip indentation, all the images were acquired in the so-called soft-tapping conditions with a ratio between the set-point amplitude and the free amplitude of vibration larger than 0.9.

The nanogels made for AFM measurements were prepared from dex-HEMA and photoinitiator in distilled water (instead of PB). After polymerization, the free dex-HEMA, initiator, and mixed micelles in the water surrounding the lipid coated dex-HEMA nanogels were removed by dialysis using SpectraPore 50 kDa membrane tubes (dialysis during one week, against distilled water, changing the water once a day) at 4 °C (to avoid degradation of the dex-HEMA nanogels[12]). Using water to dissolve the dex-HEMA (instead of PB), intensive dialysis of the nanogel dispersion was necessary to avoid the deposition of salts and micelles on the mica surface, which would complicate the AFM imaging of the nanogel.

Negative Stain Transmission Electron Microscopy (TEM) on dex-HEMA Nanogels. Lipid coated and naked

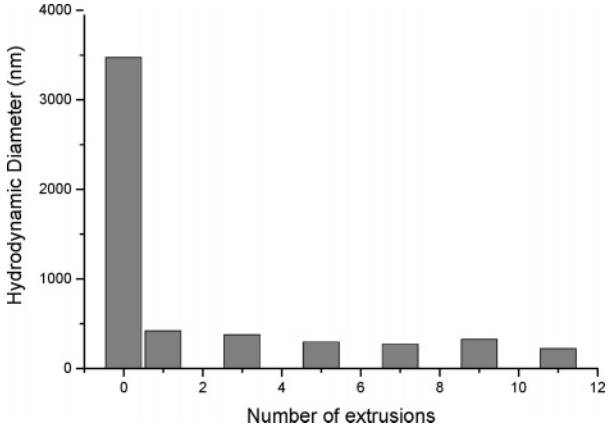

Figure 2. DLS measurements on liposomes (SOPC/DOTAP (9:1)) loaded with dex-HEMA before polymerization by UV light. The lipid film was hydrated with a 20% dex-HEMA (DS 5.4) solution. The *x*-axis indicates how many times the liposome dispersion was extruded through the polycarbonate membrane. The polydispersity of the particles before extrusion and after one extrusion was >0.7. After that, polydispersity never exceeded 0.3.

dex-HEMA nanogels were adsorbed on glow discharged Formvar-carbon-coated copper grids, subsequently washed twice in hepes buffered saline solution, and negatively stained with 2% (w/v) uranyl acetate. The ultrastructure was analyzed with a Tecnai 10 electron microscope (FEI Company, Eindhoven, The Netherlands) at a 100 kV acceleration voltage.

Cellular Uptake of dex-HEMA Nanogels. African green monkey kidney (VERO-1) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycine. Cells were prophylactically treated against mycoplasma with Plasmocin (Invivogen). The cells were seeded onto sterile glassbottomed culture disks (MatTek Corporation) and were allowed to grow and adhere for 1 day (at 37 °C in a humidified atmosphere containing 5% CO_2) before the nanogel dispersions were applied.

For evaluation of the cellular uptake of the dex-HEMA nanogels, the nanogels were fluorescently labeled with Texas Red labeled dextran (70 kDa, Molecular Probes). Therefore, 60 μL of a Texas Red dextran solution (25 mg/mL) was added to the 1 mL dex-HEMA/photoinitiator solution used to hydrate the lipid film. To remove the Texas Red dextran, which was not entrapped in the liposomes, we used Microcon centrifugal filter devices (molecular weight cutoff 100 kDa, Millipore Corporation). After centrifugation (at 14 000*g* for 24 min), the fluorescent dex-HEMA filled liposomes were recovered by inverted spin and diluted 10 times with DMEM before UV polymerization. DLS measurements showed no remarkable change in size after centrifugational filtration.

A total of 200 μL of lipid coated nanogels (containing Texas Red labeled dextran) was added to the cells in one well and incubated for 1 h at 37 °C. The cells were washed three times with PB before imaging by confocal laser scanning microscopy (CSLM, Bio-Rad MRC 1024) using a 60 × water immersion objective and a krypton/argon laser (568 nm) for the excitation of the Texas Red labeled nanogels.

Results and Discussion

Loading Liposomes with dex-HEMA. The first step in the preparation of dex-HEMA nanogels was the loading of the liposomes with dex-HEMA. It is well-known that liposomes can be formed upon hydration of a thin lipid film: the hydrated lipid film detaches during agitation and self-closes to form large multilamellar liposomes.[24] Figure 2 shows DLS measurements on the dispersions obtained by hydrating a SOPC/DOTAP lipid film with a dex-HEMA/photoinitiator solution, respectively, before and after extruding the dispersion through a 450 nm polycarbonate membrane. It is clear that before extrusion strongly polydisperse, micron sized, badly defined aggregates exist. Upon extrusion through the polycarbonate membrane, the aggregates turn into (less polydisperse) vesicles of about 400 nm in diameter, approximately being the pore size of the used polycarbonate membrane. Overall, the DLS data in Figure 2 show that liposomes are indeed formed after hydrating the SOPC/DOTAP film with (the rather viscous) dex-HEMA/photoinitiator solution followed by extrusion through a 450 nm membrane. Extrusion of the liposome dispersion through 100 nm membranes was not feasible, probably due to the high viscosity of the dispersion. We also tried to reduce the size of the liposomes obtained after hydrating the lipid film with dex-HEMA/photoinitiator solution by sonication (using a tipsonicator), according to the method described by Kazakov et al.[18] However, small metal particles were coming off the tip sonicator and interfered strongly with the DLS measurements, which made it impossible to accurately characterize the obtained liposomes by DLS.

Evidence for dex-HEMA Nanogels from DLS Measurements. In the next step, the liposomes, as characterized in Figure 2, were exposed to UV light to polymerize the dex-HEMA in the liposomes. Figure 3A shows that the average size of the nanoparticles did not change upon polymerization. Subsequently, TX100 was added to the dispersions to solubilize the lipid coating. Particles of about 400 nm in size could still be detected (Figure 3A), a first evidence that polymerization of the dex-HEMA in the liposomes occurred. However, also much smaller particles (~12 nm) were detected (Figure 4). These were probably mixed micelles formed by TX100, SOPC, and DOTAP. They indicated that TX100 was able to remove the lipid layer from the particles resulting in a mixture of naked dex-HEMA nanogels and micelles. Another indication for the removal of the lipid layer was the significant drop of the intensity of the light scattered by the dispersion after adding TX100 (data not shown). The removal of the lipid layer lowers the difference in refractive index between the nanogels and the dispersion solvent, which explains the lower intensity of the scattered light. We would like to emphasize that, although most of the TX100 was removed from the naked nanogel dispersion by one week dialysis at 4 °C (to avoid degradation of the nanogels), the naked dex-HEMA nanogels still may have some TX100 at their surface.

Although the DLS measurements described prviously indicated the formation of naked dex-HEMA nanogels, we tried to confirm that the 400 nm particles in Figures 3A and 4 were indeed naked dex-HEMA nanogels. Therefore, 20 μL of a Dextranase solution (10 U/mL) was added to 1 mL of a dex-HEMA nanogel dispersion. Figure 3B shows that no nanoparticles were detected anymore after addition of Dextranase. Clearly, this was attributed to the degradation of the nanogels. However, when Dextranase was added to the (still) lipid coated dex-HEMA nanogels, nanoparticles remained present (Figure 3B). This was explained by the fact that the enzyme could not pass through the lipid coating. These observations all together indicate that naked dex-HEMA nanogels were indeed obtained after UV polymerization of dex-HEMA filled liposomes followed by TX100 treatment.

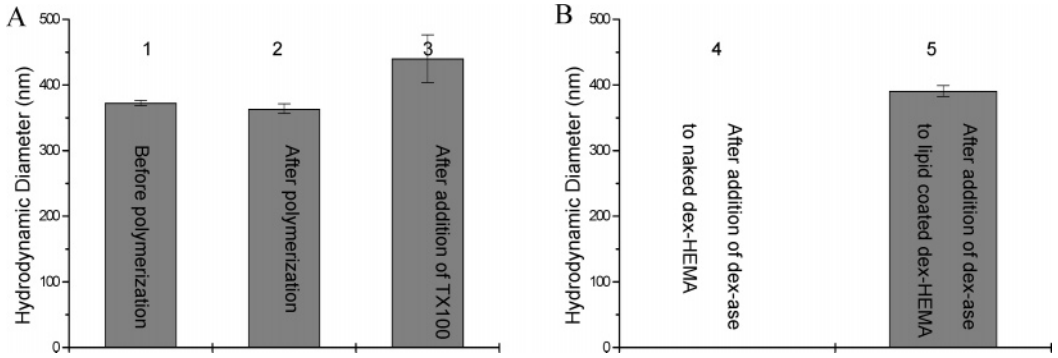

Figure 3. (A) Bar 1 shows the hydrodynamic diameter of the dex-HEMA containing liposomes described in Figure 2 before UV polymerization. Bar 2 represents the hydrodynamic diameter of the corresponding particles after UV treatment. Bar 3 shows the hydrodynamic diameter of the particles after addition of TX100. (B) Bar 4: DLS on naked dex-HEMA nanogels treated with Dextranase revealed that the nanogels were not present anymore. Bar 5 shows the DLS data obtained after adding Dextranase to lipid coated dex-HEMA particles.

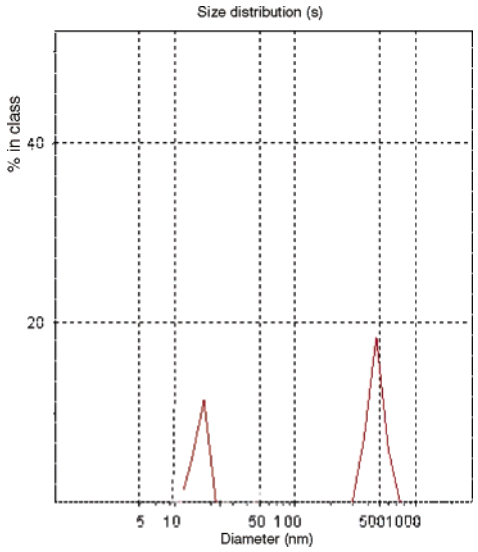

Figure 4. Typical outcome of a DLS experiment on lipid coated dex-HEMA nanogels treated with TX100. The *x*-axis shows the hydrodynamic diameter of the observed particles. The SOPC/DOTAP (9:1) lipid film was hydrated with a 20% dex-HEMA (DS 5.4) solution. The peak with an average size of 12 nm is attributed to micelles; the one with an average size of 450 nm comes from naked dex-HEMA nanogels.

Microscopic Characterization of the dex-HEMA Nanogels. Confocal (fluorescence) microscopy is limited in imaging of particles of a few hundred nanometers. Therefore, we preferred to further characterize the nanogels by AFM and negative stain EM.

While most of the nanogels investigated in this work were prepared from dex-HEMA with a DS of 5.4, we preferred to do AFM imaging on nanogels synthesized from dex-HEMA with a high DS of 18.9. A major reason being that less rigid nanogels (which are obtained when dex-HEMA with lower DS is used) stuck to the cantilever and therefore interfered with the AFM imaging, which was done in tapping mode. Figure 5A shows an AFM image of SOPC/DOTAP coated dex-HEMA nanogels: the lipid coating is clearly visible at the surface of the dex-HEMA nanogels as a rough ring around the particle. The average size of the four lipid coated nanogels with DS 18.9 seen by AFM in Figure 5A is 356 nm. The average is in good agreement with an average diameter of 327 nm as obtained by DLS. One should take into account that flattening of the nanogels, during drying (necessary for AFM analysis), might influence the size of the nanogels as obtained by AFM. Figure 5B is an AFM image of nanogels treated with TX100: as compared to Figure 5A, a more smooth line confines the nanoparticles, suggesting that the lipid coating has indeed been removed.

EM on the nanogels further confirmed the AFM and DLS data: the surface of the lipid coated dex-HEMA nanogels seems different from that obtained after the addition of TX100. As Figure 6A shows, the lipid surface of the nanogels was clearly visible on EM images. The gel core in Figure 6A seems very dark, which may be explained by uranyl acetate (the staining) entering the gel through the lipid coating, which seemed partially ruptured probably attributed to the drying process upon EM imaging. Figure 6B shows that the lipid coating disappeared after treating the lipid coated nanogels with TX100. The halo-like effect at the surface of the nanogels may be attributed to a flattening of the nanogels under vacuum, applied in the EM experiments, resulting in less dense dex-HEMA regions at the surface of the nanogels.

Monitoring the Degradation of dex-HEMA Nanogels. As schematically shown in Figure 1B, dex-HEMA hydrogels consist of dextran chains that are mutually cross-linked by hydroxyethyl methacrylates. Our group, as well as the Hennink group, have documented[3,12,25,26] that the HEMA cross-links in dex-HEMA hydrogels spontaneously degrade by hydrolysis of the carbonate ester links formed between the hydroxyethyl methacrylate groups and the dextran chains (Figure 1A). Especially, the cross-link density (determined by both the dex-HEMA concentration in the gel and the DS of the dex-HEMA) determines the degradation rate of dex-HEMA hydrogels. It was shown experimentally that, the higher the cross-link density, the longer it takes for dex-HEMA gel slabs[3,12,26] and dex-HEMA microgels[3,25] to degrade. We wondered whether also dex-HEMA nanogels could be obtained, which show different degradation times. Tunable degradation properties could be of interest to regulate the drug release from the nanogels after uptake by cells.

Lipid coated and naked dex-HEMA nanogels were dispersed in PB (pH 7.0) at 37 °C, and their size was measured by DLS at different times. The behavior of lipid coated nanogels in PB is depicted in Figure 7, while Figure 8 shows the behavior of the naked nanogels. Figure 7A shows that the size of empty SOPC/DOTAP liposomes (i.e., without dex-HEMA) in PB (at 37 °C) does not change drastically in 2 weeks time. Figure 7B−D reveals that nanoparticles exist for at least 2 weeks

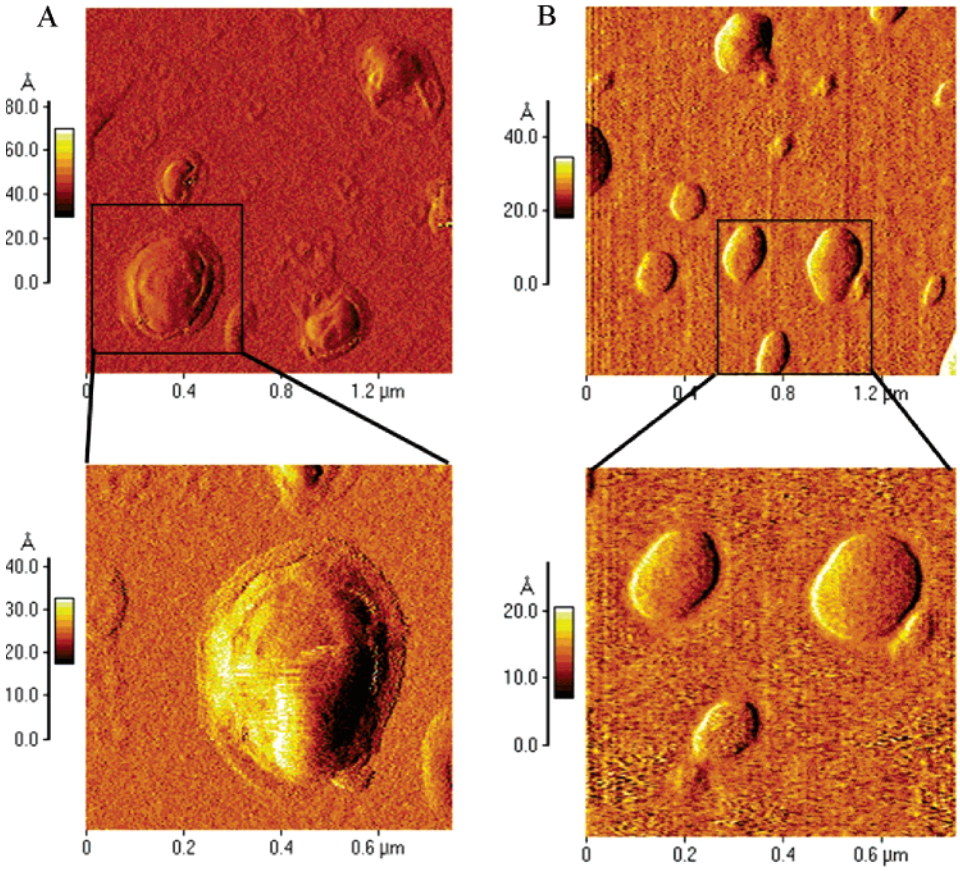

Figure 5. (A) AFM image of SOPC/DOTAP coated dex-HEMA nanogels. The SOPC/DOTAP (9:1) lipid film was hydrated with a 20% dex-HEMA (DS 18.9) solution. (B) AFM image of naked dex-HEMA nanogels obtained by adding TX100 to the dispersion imaged in panel A.

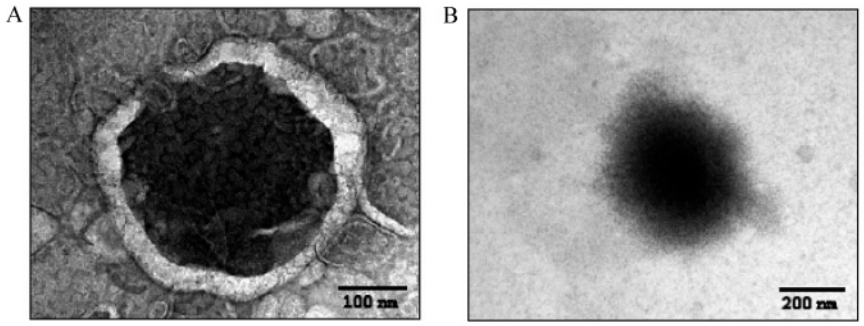

Figure 6. (A) Negative stain EM image of SOPC/DOTAP coated dex-HEMA nanogels. The SOPC/DOTAP (9:1) lipid film was hydrated with a 20% dex-HEMA (DS 5.4) solution. (B) Negative stain EM image of naked dex-HEMA nanogels obtained by adding TX100 to the dispersion imaged in panel A.

when lipid coated dex-HEMA nanogels are dispersed at 37 °C in PB (independent of the DS of the dex-HEMA). At first sight, one could conclude that the dex-HEMA nanogels in the liposomes do not degrade. However, another explanation could be that hydrolysis of the dex-HEMA nanogels in the liposomes does occur but that the degradation products of the nanogels (being dextran chains and poly(HEMA),[12] Figure 1B) remain in the liposomes as these are stable for weeks (see Figure 7A). The latter hypothesis was confirmed by the following experiments: lipid coated nanogels (DS 2.5, 5.4, and 8.9) were allowed to degrade for, respectively, 5, 14, and 18 days. After that period, TX100 was added. In this way, only particles of about 12 nm were still detected. This proved that the nanogels in the liposomes were indeed completely degraded into a dextran and poly(HEMA) solution. Theoretically, one could argue that the turnover from the gels into the solution of degradation products would increase the osmotic pressure inside the liposomes, which should rupture this lipid layer, especially as the lipid membranes are not permeable for the large dextran chains (19 000 g/mol) used to synthesize the dex-HEMA in this study. Indeed, we showed previously that the osmotic pressure of a dex-HEMA gel increases upon degradation, which also explains the swelling of degrading dex-HEMA gels.[26,27] However, the following considerations point out that the increase in osmotic pressure is probably too low to osmotically destroy the liposomes. Following the method of Mui et al.,[28] the tensile strength (τ) of a SOPC/DOTAP (9:1) film was determined and turned out to be 0.075 N/m. From Laplace's law (eq 1)

$$\tau = \frac{\Delta P r}{2} \qquad (2)$$

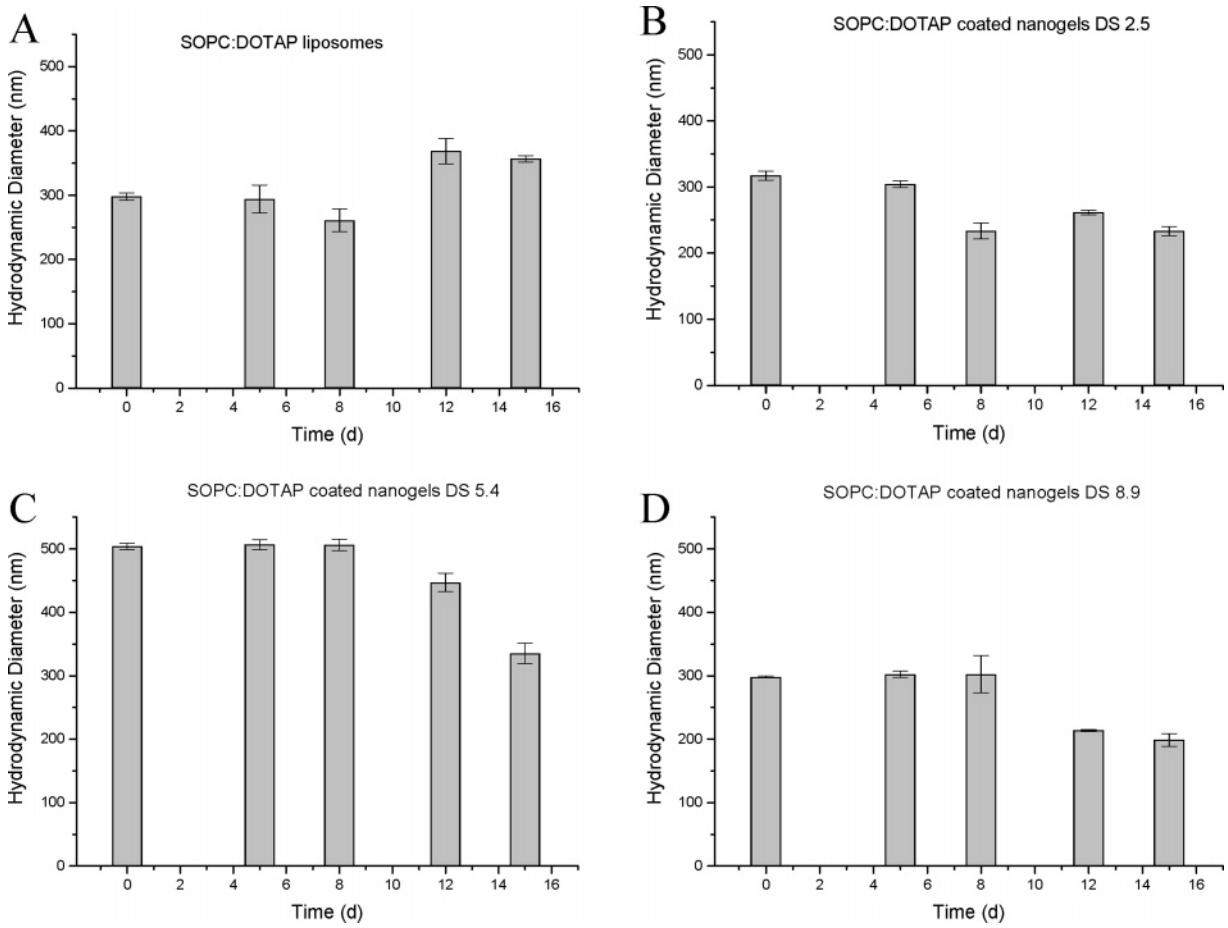

Figure 7. DLS measurements on SOPC/DOTAP (9:1) liposomes (A) and SOPC/DOTAP (9:1) coated nanogels (B, C, and D) stored in PB at 37 °C. To prepare the dex-HEMA nanogels, the SOPC/DOTAP (9:1) lipid film was hydrated with a 20% dex-HEMA solution. The DS of the dex-HEMA was, respectively, 2.5 (B), 5.4 (C), and 8.9 (D). At this moment, it is not clear why the DS 5.4 nanogels were larger than the DS 2.5 and 8.9 nanogels. Data shown are the result of one set of experiments. The values are the mean of five size measurements on the same sample, but repeated experiments revealed the same results.

in which ΔP (in N/m^2) stands for the pressure difference over the lipid membrane, τ (in N/m) being the tensile strength and r (in m) the radius of the liposome, one can calculate that for a 450 nm SOPC/DOTAP (9:1) liposome, the pressure increase needed to overcome the tensile strength (i.e., to rupture the membrane) should exceed 666 kPa. We measured previously the osmotic pressure of completely degraded dex-HEMA hydrogels[26,27] and could estimate that the osmotic pressure of the solutions obtained by degradation of the dex-HEMA nanogels used in Figure 7B−D (assuming the dex-HEMA concentration of these gels is 20%) is only 120 kPa and thus insufficient to rupture the liposomal membrane. This supports the idea that the SOPC/DOTAP lipid coated dex-HEMA nanogels do degrade yielding SOPC/DOTAP liposomes filled with a dextran/poly(HEMA) solution and that the degradation products remain in the liposomal vesicles.

Figure 8 (panels B−D) shows DLS data obtained on naked dex-HEMA nanogels stored in PB at 37 °C. As Figure 4 already illustrated, DLS measurements on naked dex-HEMA nanogels indicated the presence of both small micelles and larger nanogels. The y-axis in Figure 8 plots the average size of the nanogels. Clearly, the average size of the nanogels in the dispersions decreases (rather slowly) in time, indicating that they indeed degrade. Especially, as seen earlier for dex-HEMA hydrogel slabs and dex-HEMA microgels,[3,12,25,26] the degree of cross-linking influences the degradation rate of the dex-HEMA nanogels: the higher the DS of the dex-HEMA (i.e., the higher the cross-link density of the dex-HEMA nanogels), the longer nanogels can be detected in the dispersions (compare Figure 8B−D).

Considering the gradual decrease in diameter of the degrading nanogels (Figure 8), at a first sight one could argue that the nanogels degrade through surface erosion. However, as dex-HEMA nanogels degrade through hydrolysis of the carbonate ester link (Figure 1) and as water is present all over the nanogels, we hypothesize that bulk degradation occurs. Possibly, the gradual decrease in size as seen in Figure 8 should be explained by bulk erosion of the nanogels accompanied by fragmentation of the nanogels resulting in smaller gel particles. Note that before degradation starts (i.e., at $t = 0$ in Figure 8), dex-HEMA nanogels DS 2.5 are smaller than the nanogels DS 5.4 and 8.9. This is possibly due to the fact that the dex-HEMA nanogels DS 2.5 were already partially degraded at the time of the first DLS measurement (as dex-HEMA nanogels DS 2.5 degrade very fast due to the low cross-link density).

Uptake of dex-HEMA Nanogels by VERO-1 Cells. We studied the cellular uptake of the lipid coated dex-HEMA nanogels. As explained in the Materials and Methods, for monitoring the cellular uptake, the nanogels were fluorescently labeled with Texas Red dextran. To remove free Texas Red dextran (i.e., Texas Red dextran not encapsulated in the nanogels), the nanogel dispersions were filtrated by centrifugational filtration.

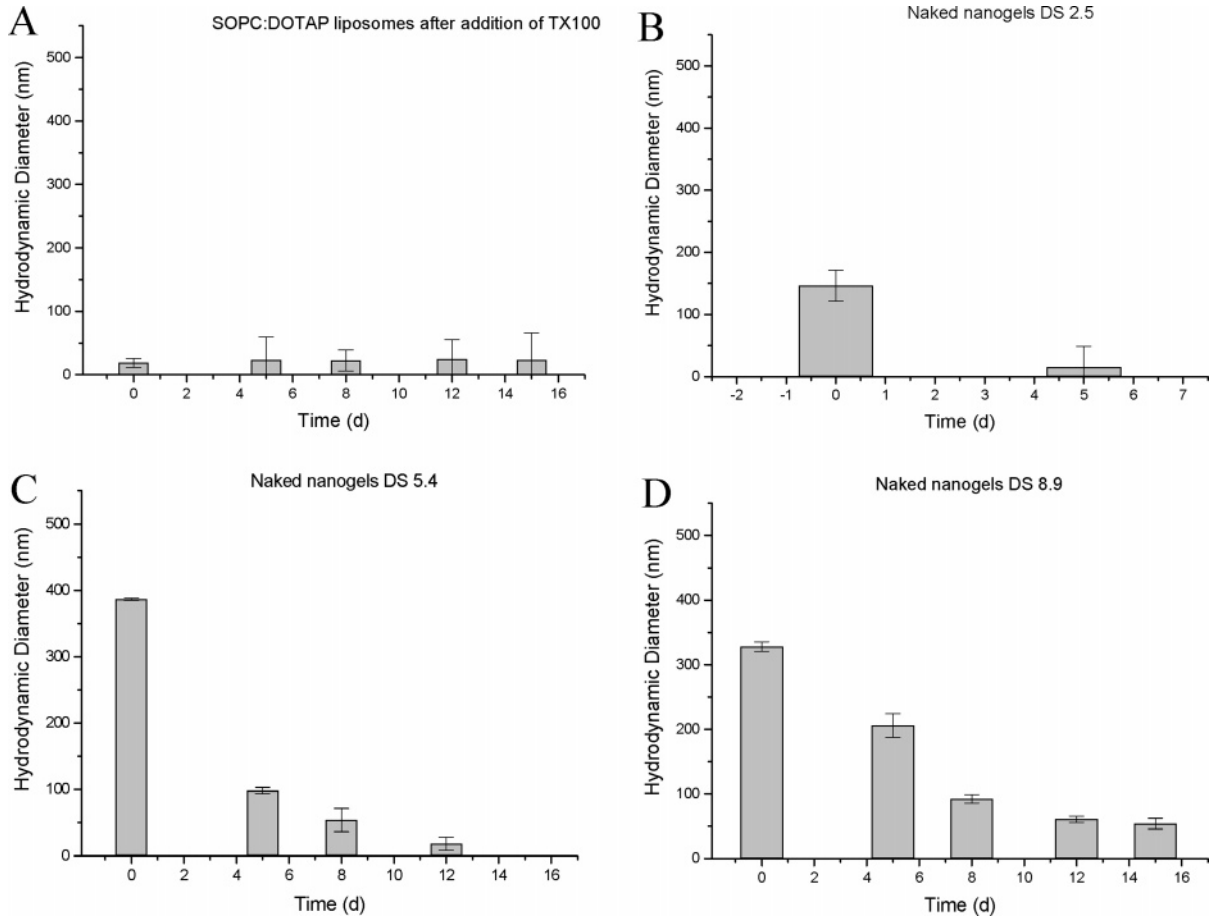

Figure 8. DLS measurements on SOPC/DOTAP (9:1) liposomes exposed to TX100 (A) and naked dex-HEMA nanogels (B, C, and D) stored in PB at 37 °C. To prepare the naked dex-HEMA nanogels, the SOPC/DOTAP (9:1) lipid film was hydrated with a 20% dex-HEMA solution. The DS of the dex-HEMA was, respectively, 2.5 (B), 5.4 (C), and 8.9 (D). Subsequently, the lipid coating was removed by adding TX100. The values are the mean of five measurements on the same sample. Repeated experiments revealed the same results.

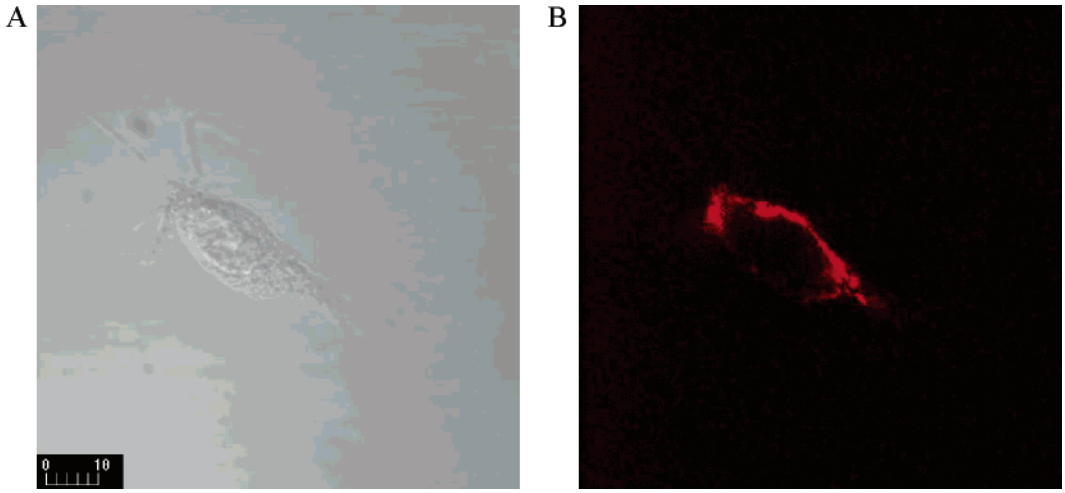

Figure 9. (A) Transmission image and (B) CLSM image of a VERO-1 cell incubated with lipid coated dex-HEMA nanogels (containing Texas Red labeled dextran). To prepare the dex-HEMA nanogels, the SOPC/DOTAP (9:1) lipid film was hydrated with a 20% dex-HEMA solution (DS 5.4).

Separate fluorescence measurements showed that the 70 kDa Texas Red dextran chains could freely pass through the used membrane (molecular weight cutoff 100 kDa), while the nanogels (a few hundred nanometers in size) were prevented from passing the membrane, as expected.

Figure 9 shows confocal microscopy images of a VERO-1 cell 1 h after incubation with lipid coated dex-HEMA nanogels: internalization (most likely by endocytosis) of the nanogels clearly occurred as highly fluorescent punctuations were observed in the cytoplasm. Also, the dex-HEMA nanogels seemed to be excluded from the nucleus. This can be expected considering that the pores in the nuclear membrane are by far too small to allow the passage of the (intact/degrading) nanogels.[29] Importantly, when VERO-1 cells

were incubated with naked dex-HEMA nanogels, we observed a significant number of dead cells. This is probably attributed to the incomplete removal of TX100, which possibly remains, to a certain extent, adsorbed to the surface of the nanogels.

Conclusions

This paper shows that lipid coated dex-HEMA nanogels can be prepared by UV polymerization of dex-HEMA containing liposomes that were obtained by hydrating a SOPC/DOTAP lipid film with a dex-HEMA solution. DLS measurements indicated that the lipid coated dex-HEMA nanogels thus obtained were around 350 nm in size, as could be expected considering the 450 nm pores of the membranes used to extrude the liposome dispersions. DLS measurements also clearly showed that TX100 efficiently removed the lipid coating as the intensity of the light scattered by the dispersions significantly dropped upon adding TX100. On its turn, this was attributed to a lowering of the difference in refractive index between the nanogels and the solvent upon removing the lipid coating. AFM by EM imaging confirmed the existence of the particles and showed that both lipid coated and naked dex-HEMA nanogels could be obtained.

Compared to, for example, the emulsion polymerization method, the use of liposomes to prepare nanogels comprehends several advantages. The size of the nanogels can be controlled by using membranes with the appropriate pore size in the preparation of the liposomes. Also, organic solvents are not used, which is a clear benefit when the nanogels are considered for pharmaceutical purposes (e.g., drug inactivation may occur upon contact with organic solvents). Moreover, and especially, the liposome based method allows the design of both naked nanogels as well as lipid coated nanogels. The lipid coating may offer additional interesting features to the nanogels. For example, it may prevent the burst release of drug molecules, which may easily leave the nanogels by simple diffusion. As another example, the use of pH sensitive lipids[30−32] may improve the escape of the nanogels from endosomes, which is attractive if the nanogels have to be delivered in the cytosol. Also, in analogy with immunoliposomes, which are liposomes bearing antibodies at their surface, immunogels may be easily designed by grafting the antibodies at the lipid surface of the lipid coated nanogels, making use of well-known chemistry.

A major question in this work was whether nanogels could be obtained that degrade over different times. DLS measurements on naked dex-HEMA nanogels stored in PB at 37 °C revealed that slowly degrading dex-HEMA nanogels could indeed be realized by increasing the cross-link density of the dex-HEMA nanogels (through the use of dextran highly substituted with HEMA): nanogels prepared with dex-HEMA DS 2.5 were completely degraded in a few days, while nanogels synthesized from dex-HEMA DS 8.9 existed, roughly spoken, during 2 weeks. Our experiments turned out that in lipid coated dex-HEMA nanogels stored over days in buffer at 37 °C, the dex-HEMA gel also degraded. This resulted in a dextran/poly(HEMA) solution that remained entrapped in the liposomes.

As this work showed that the dex-HEMA nanogels could be taken up by endocytosis in VERO-1 cells, future research will explore the potentials of the dex-HEMA nanogels for intracellular delivery of antisense therapeutics, plasmid DNA, and proteins. Drug loading and release will be focused on as well as the behavior of the nanogels in biological media like serum, which may induce aggregation of the nanogels through, for example, the adsorption of proteins at their surface.

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