Composite Nanoparticles Based on Hyaluronic Acid Chemically Cross-Linked with α,β-Polyaspartylhydrazide

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In this paper, new composite nanoparticles based on hyaluronic acid (HA) chemically cross-linked with α,β-polyaspartylhydrazide (PAHy) were prepared by the use of a reversed-phase microemulsion technique. HA−PAHy nanoparticles were characterized by FT-IR spectroscopy, confirming the occurrence of the chemical cross-linking, dimensional analysis, and transmission electron micrography, showing a sub-micrometer size and spherical shape. Zeta potential measurements demonstrated the presence of HA on the nanoparticle surface. A remarkable affinity of the obtained nanoparticles toward aqueous media that simulate some biological fluids was found. Stability studies showed the absence of chemical degradation in various media, while in the presence of hyaluronidase, a partial degradation occurred. Cell compatibility was evaluated by performing in vitro assays on human chronic myelogenous leukaemia cells (K-562) chosen as a model cell line and a haemolytic test. HA−PAHy nanoparticles were also able to entrap 5-fluorouracil, chosen as a model drug, and release it in a simulated physiological fluid and in human plasma with a mechanism essentially controlled by a Fickian diffusion.

Introduction

Over the past years, the development of nanoparticles as effective drug delivery systems has been the common interest of several researchers. These systems have unique advantages belonging to their sub-cellular and sub-micrometer size, such as possible administration by all the routes and a high capability to cross various physiological barriers, such as the intestinal mucosa and the blood−brain barrier (BBB).[1−4]

Nanoparticles have also many advantages in comparison with other colloidal drug delivery systems, such as drug protection against degradation, the capability to generate a controlled release, and cell internalization, as well as the ability to reverse the multidrug resistance of tumor cells.[3] However, nanoparticles are rapidly sequestrated by the mononuclear phagocyte system (MPS); this is an advantage if the final target is the MPS, but this is unfavorable if other tissues or organs should be reached. Hydrophilic polymers are widely used to coat the surface of nanoparticles for preventing protein adsorption and the MPS sequestration.[5]

Surface modified nanoparticles with hydrophilic materials, such as poly(ethylene glycol), have a prolonged blood circulation time as compared to uncoated nanoparticles that could permit the passive targeting to tumors or inflammatory tissues due to an increased vascular permeability.[6] Polysaccharides have been considered as interesting surface coating materials due to their biocompatibility, biodegradability, and mucoadhesive properties as well as to the possibility of achieving an active targeting toward tissues and organs. In fact, they are involved in the cell surface properties including transport mechanisms, they have specific receptors in certain cells or tissues, and several mucosal surfaces are good targets for polysaccharide recognition.

Various drug delivery systems have been decorated with polysaccharides, such as liposomes, polymeric micelles,[7,8] and self-assembled nanoparticles of hydrophobically modified polysaccharide.[9]

Among polysaccharides, hyaluronic acid (HA) is very attractive; it is a bioactive glycosaminoglycan copolymer of D-glucuronic acid and *N*-acetyl-D-glucosamine, found as the major extracellular component of connective tissues, a constituent of the synovial fluid and the vitreous humor of all vertebrates, and associated with several cellular processes, including angiogenesis and the regulation of inflammation.[10] HA controls important functions regulating cellular proliferation and differentiation. Cellular interactions with HA occur through cell surface receptors (CD44, RHAMM, and ICAM-l) and influence processes such as morphogenesis, wound repair, and metastasis.[11] CD44 receptors are overexpressed in the tumor cell surface, and they play a role in tumor cell migration.[12]

HA has been successfully utilized for biomedical applications as hydrogels for viscosurgery and viscosupplementation,[13] scaffolds for wound healing applications,[14] and hydrophilic coatings for medical devices.[15] HA also has been proposed as a macromolecular carrier for anti-tumoral drugs and for preparing microspheres,[16,17] but there are only a few examples of nanoparticles containing HA, which is just adsorbed onto the surface.[18,19]

Therefore, taking into account the biological and physicochemical properties of HA, it is reasonable to suppose that HA-containing nanoparticles could improve drug administration and delivery by active targeting to tumoral cells. For this reason, in this paper, we report the preparation and characterization of new biodegradable composite nanoparticles based on HA chemically cross-linked with α,β-polyaspartylhydrazide (PAHy) as potential carriers for anti-tumoral drugs. PAHy is a non-toxic, non-antigenic, non-teratogenic, water-soluble polymer with a protein-like structure, already proposed as a plasma expander,[20] carrier for macromolecular conjugates,[20] starting polymer for preparing

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hydrogels,[21] and polycation for gene delivery.[22] The combination of a natural biocompatible polysaccharide such as HA with a synthetic polyaminoacid such as PAHy, which possesses advantages such as chemical versatility, easy processing, and low production costs, seems a useful strategy to obtain nanoparticles with suitable physicochemical properties and a reduced rate of degradation by hyaluronidase.

The preparation of these nanoparticles has been performed by using a reversed-phase microemulsion technique recently developed in our laboratory.[23] After purification, HA−PAHy nanoparticles have been characterized by FT-IR spectrophotometry, transmission electron microscopy, size distribution analysis, and zeta potential measurements. Swelling studies have been performed to determine their affinity toward various aqueous media, and chemical and enzymatic hydrolysis studies also have been performed to evaluate their stability or degradability. Cell compatibility has been assayed in vitro on human chronic myelogenous leukaemia cells (K-562) chosen as a model cell line. The hemeolytic activity was also investigated. Moreover, the potential of HA−PAHy nanoparticles as drug delivery systems has been evaluated by using 5-fluorouracil (5-FU) as a model drug.

Materials and Methods

Chemicals. All reagents were of analytical grade, unless otherwise stated. DL-Aspartic acid, hydrazine hydrate, *N*,*N*′-dimethylformamide (DMF), *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide sodium salt (NHSS), and 5-FU were obtained from Fluka. Hyaluronidase (HAase) from bovine testes (type IV−-S, 1320 units/mg) was obtained from Sigma. Propylene carbonate (PC), ethyl acetate (EtOAc), and sorbitan trioleate (SPAN 85) were purchased from Aldrich Chemical Co. Human chronic myelogenous leukaemia cell line (K-562) was purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Umbertini". The Spectra/Por microdialysis membrane (M_w CO 10 000 Da) was purchased from Prodotti Gianni SpA.

PAHy was prepared via polysuccinimide (PSI) by polycondensation of DL-aspartic acid in the presence of H_3PO_4 followed by reaction with hydrazine hydrate in DMF solution. PAHy was isolated by filtration and purified as reported elsewhere.[20] Analytical and spectral data of PAHy were in agreement with literature values.[20] PAHy weight-average molecular weight determined by light scattering was 23 kDa (M_w/M_n = 1.78).

HA sodium salt, M_w 1500 kDa, was a generous gift from SIFI. HA with a low weight-average molecular weight (employed in our experiments) was prepared by acidic degradation as reported by Shu et al.[24]

Briefly, a 1% w/v solution of hyaluronic acid sodium salt was degraded in HCl solution (pH 0.5) at 37 °C for 24 h. After this time, the pH was corrected to 7.0, and the solution was subjected to extensive dialysis by using Spectra/Por tubing with a molecular cutoff of 3500 Da. After dialysis, the solution was freeze-dried, and then the weight-average molecular weight of HA was determined by SEC analysis and resulted to be 174 kDa (M_w/M_n 1.75).

Apparatus. The weight-average molecular weight of PAHy was determined by a SEC system equipped with a pump system, two Phenogel columns from Phenomenex (5 μm particle size, 10^3 and 10^4 Å pore size), and a 410 differential refractometer (DRI) as a concentration detector, all from Waters. The molecular weight was determined by using PEO/PEG as standards (range 4−300 kDa) and a phosphate buffer solution pH 7.8 + 0.1 N $NaNO_3$ as a mobile phase, at 37 ± 0.1 °C and a flow rate of 0.8 mL/min.

Weight-average molecular weights of HA were determined by the same SEC system previously reported but using a Universal column from Waters (particle size 5 μm). The molecular weight was evaluated by using HA standards (range of 100−800 kDa) from Hyalose and 200 mM phosphate buffer (pH 6.5)/MeOH 90:10 (v/v) as a mobile phase, at 36 ± 0.1 °C and a flow rate of 0.6 mL/min.

The cross-linking reaction was carried out by mixing the microemulsion with an Ultra-Turrax T 25, Janke and Kunkel Ika Labortechnik. Centrifugations were performed with an International Equipment Company Centra MP4R equipped with an 854 rotor and a temperature control. FT-IR spectra were obtained, after 100 scans, with a PerkinElmer 1720 Fourier transform spectrophotometer in the range of 4000−400 cm^{-1} with a 1 cm^{-1} resolution. Samples were in KBr pellets. The size and zeta potential measurements were determined by a Zetasizer Nano ZS (Malvern Instruments). Transmission electron micrography (TEM) images were recorded with a Philips EM420 electron microscope at an accelerating voltage of 100 kV. UV spectra were obtained with a Jasco instrument. HPLC analyses were carried out using a Varian 9012 liquid chromatograph equipped with a Rheodyne Injector 7125 (fitted with a 10 μL loop), a Kontron HPLC Detector 432, and a Hewlett-Packard 3394 integrator. For the analyses, a reversed-phase C_{18} column (Luna; 5 μm 250 mm × 4.6 mm i.d., obtained from Phenomenex) was used.

Preparation of HA−PAHy Nanoparticles. Solutions of HA (12.5 mg/mL) or PAHy (16 mg/mL) both were prepared in twice-distilled water, and the pH was adjusted to 7.5. Aliquots of each solution were mixed together with a molar ratio between PAHy repeating units and HA repeating units equal to 4. Two hundred microliters of an EDC solution (120 mg/mL) at pH 7.5 was added to the polymeric solution to obtain a ratio between mol of EDC and mol of HA repeating units equal to 2.5. Then, the solution was vigorously mixed for 30 min.

Sixty milliliters of the organic phase, prepared by mixing 45.6 g of EtOAc (ρ = 0.902) with 39.7 g of PC (ρ = 1.189) and saturated with twice-distilled water before use (0.65 g/mL),[23] were placed in a round-bottomed cylindrical glass reaction vessel fitted with an Ultraturrax and then treated with the aqueous phase containing polymers. The ratio (v/v) between the aqueous and the organic phase was 1:15. The density of the organic phase was adjusted so that the density was close to that of the aqueous phase. Then, the mixture was treated with 200 μL of SPAN 85 and stirred at 8000 rpm for 5 h. During this time, 200 μL of a NHSS solution at pH 7.5 (130 mg/mL) was added to obtain a ratio between mol of NHSS and mol of HA repeating units equal to 2.5.

After the reaction time, the sample was centrifuged at 15 000 rpm for 30 min at 4 °C to separate nanoparticles from the organic phase. Nanoparticles were purified by washing with twice-distilled water (15 mL) 5 times and then were centrifuged from time to time at 15 000 rpm at 4 °C for 1 h. Then, after further washing with acetone (15 mL), the powder was dried at 10^{-1} mmHg in presence of P_2O_5 until its weight remained constant.

Acetone used for washing was analyzed by HPLC in a reversed-phase C_{18} column, MeOH/H_3PO_4 0.1 vol % (50:50, v/v) at a flow of 1.0 mL/min; the eluate was monitored at 265 nm and a retention time of SPAN = 15.1 min to evaluate the eventual residues of SPAN 85 that were absent.

HA−PAHy nanoparticles were characterized by FT-IR analysis. The principal FT-IR peaks were as follows: a broad band centered at 3400 cm^{-1} (ν_{as} OH + ν_{as} NH + ν_s NH of HA and ν_{as} NH + ν_s NH + ν_s NH_2 of PAHy) and bands at 1738 (ν_{as} COO ester), 1654 (amide I of HA and PAHy), 1541 (amide II of PAHy), 1417 (ν_s COO^- of HA), 1152, 1082, and 1045 (C−O alcohol and ether stretching of HA) cm^{-1}.

Particle Size and Zeta Potential Measurements. The average diameter, width of distribution (polydispersity index (PDI)), and zeta potential of HA−PAHy nanoparticles were determined by photon correlation spectroscopy (PCS) at a fixed angle of 90° at a temperature of 25 °C and using twice-distilled water and an aqueous solution of NaCl (0.9% w/v) or phosphate buffer solution 0.15 M (NaCl, Na_2HPO_4, and KH_2PO_4) at pH 7.4 as the suspending media. Each suspension was properly diluted and was kept in a cuvette and analyzed. Particle size distribution was determined by using an algorithm based on the non-negative least-squares (NNLS) method.

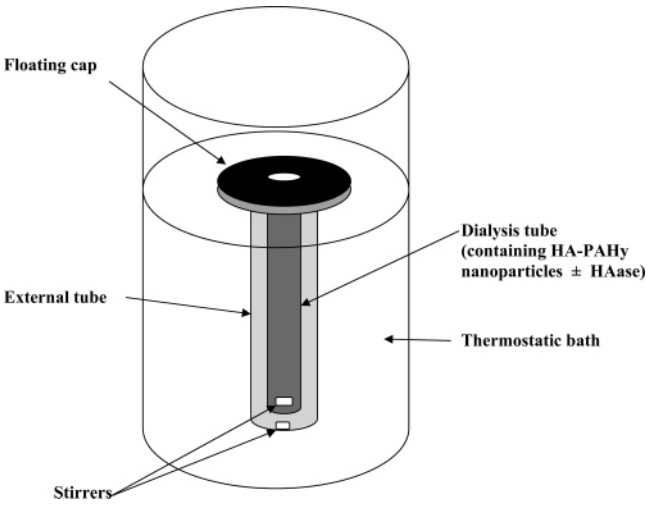


Figure 1. Schematic system used to determine the degradation percentage of HA-PAHy nanoparticles.

TEM. A dried nanoparticle sample was dispersed directly into twice-distilled water, and then a copper grid coated with a carbon film was put into the previous suspension several times. After being stained by 1 wt % ammonium molybdate solution and dried at room temperature, the sample was ready for TEM investigation. Observation was performed at an accelerating voltage of 100 kV.

Swelling Studies. The swelling ability of HA−PAHy nanoparticles was determined at 37 ± 0.1 °C in twice-distilled water, a phosphate buffer solution 0.15 M (NaCl, Na_2HPO_4, and KH_2PO_4) at pH 6.8 or 7.4, and HCl 0.1 N (pH 1.0). In particular, exactly weighed aliquots of the dried sample were kept in contact with the liquid medium for 2 h, then each swollen sample was filtered, plugged by blotting paper, and weighed. The weight swelling ratio was calculated as follows:

$$q = W_s/W_d$$

where W_s and W_d are the weights of swollen and dry sample, respectively.

Chemical Hydrolysis. Chemical hydrolysis was investigated in HCl 0.1 N (pH 1.0) for 2 h and phosphate buffer solution 0.15 M (NaCl, Na_2HPO_4, and KH_2PO_4) pH 6.8 or 7.4 for 24 h. Samples (15 mg) were dispersed in 10 mL of liquid medium, then kept in a water bath at 37 ± 0.1 °C with continuous stirring (100 rpm). After the incubation time, the samples were neutralized (when necessary) and centrifuged at 15 000 rpm at 4 °C for 15 min, and the supernatant was separated. For each sample, the remaining nanoparticles were washed 5 times with twice-distilled water (15 mL) under continuous stirring at 37 ± 0.1 °C for 1 h to extract some soluble polymer chains and electrolytes entrapped in the network. Finally, the sample was washed with acetone (15 mL) and centrifuged at 15 000 rpm at 4 °C for 15 min. The recovered solid residue was dried, weighed, and characterized by swelling studies in twice-distilled water.

Enzymatic Hydrolysis. A new method was developed to analyze the degradation of HA−PAHy nanoparticles, using the system illustrated in Figure 1.

Ten milligrams of HA−PAHy powder was suspended in 3 mL of citrate buffer pH 6.3 in the absence or in the presence of hyaluronidase (75 U/mL) and transferred inside of a Spectra/Por microdialysis membrane (M_w CO 10 000 Da, internal volume 3 mL). This dialysis membrane was placed inside a tube containing 7 mL of citrate buffer pH 6.3. The system was placed in a thermostatic bath at 37 ± 0.1 °C, and the mixing was performed by inserting a small stirrer inside the dialysis tube.

Aliquots of 100 μL of solution were taken from the outside of the dialysis membrane at different intervals of time, mixed with 1 mL of sulfuric acid, and analyzed for uronic acid content according to the carbazole assay.[25] The percent degradation of HA−PAHy nanoparticles taken from the outside of the dialysis membrane was calculated by comparing the absorbance at λ 520 nm of the solutions after the incubation with or without HAase with the absorbance value obtained when the HA−PAHy nanoparticles were completely degraded at the same conditions.

Cell Viability Assay. Cell compatibility of HA−PAHy nanoparticles was tested in vitro by using human chronic myelogenous leukaemia cells (K-562) and the Trypan blue exclusion assay. Cells were suspended at a density of 1×10^5 cells mL^{-1} in RPMI-1640 medium (supplemented with 10 vol % of fetal calf serum, 2 mmol/l L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) (Sigma Aldrich), transferred to a 24-well plate (1 mL per well), and incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2, in the presence of HA−PAHy nanoparticles at three different concentrations (0.25, 0.5, and 1.0 mg/mL). After incubation time, 100 μL of each suspension (containing cells and nanoparticles) was diluted (1:1) and colored with Trypan blue solution (0.4 wt %). The viable cell number was calculated in a hemeatocytometer by dye exclusion, and viability was expressed as a percentage of the uncolored cells in comparison with total cells. In the control experiment, cells were incubated in RPMI-1640 medium in the absence of nanoparticles.

Haemolytic Test. Human erythrocytes isolated from fresh citrated-treated blood were collected by centrifugation at 2200 rpm for 10 min at 4 °C; the pellet was washed 4 times with an isotonic phosphate buffer solution (PBS) at pH 7.4 by centrifugation at 2200 rpm for 10 min at 4 °C and resuspended in the same buffer.

The erythrocyte pellet was diluted in PBS at pH 7.4 to a final concentration of 4% erythrocytes. This stock dispersion was always freshly prepared and used within 24 h after preparation.

Nanoparticle dispersions at different concentrations (0.25, 0.5, and 1 mg/mL), also prepared in PBS, were added to the erythrocyte suspension and were incubated for 60 min at 37 °C under constant shaking. After centrifugation at 2200 rpm for 10 min, at 4 °C, the release of haemoglobin was determined by photometric analysis of the supernatant at 540 nm. Complete haemolysis was achieved by using an aqueous solution of Triton X-100 (1 wt %) (100% control value). Each experiment was performed in triplicate and repeated twice. The percentage erythrocyte lysis was calculated according to the following formula:

$$\%\ \text{lysis} = [(A_{\text{nanoparticles}} - A_{\text{blank}})/(A_{100\%\text{lysis}} - A_{\text{blank}})]100$$

where $A_{\text{nanoparticles}}$ is the absorbance value of the haemoglobin released from erythrocytes treated with nanoparticle dispersion; A_{blank} is the absorbance value of the haemoglobin released from erythrocytes treated with PBS buffer, and $A_{100\%\text{lysis}}$ is the absorbance value of the haemoglobin released from erythrocytes treated with 1% Triton X-100 solution.

Drug Loading Procedure. HA−PAHy nanoparticles were immersed in a concentrated aqueous solution of 5-FU and left to soak for 3 days at room temperature. After this period, the nanoparticles were rapidly washed with ethanol and dried to a constant weight. The amount of drug loaded into the nanoparticles was determined by extensive extraction with water at room temperature. The amount of drug extracted was determined by HPLC performed on a reversed-phase C_{18} column with MeOH/H_3PO_4 0.05 vol % (2:98 v/v) as a mobile phase at 1.0 mL/min, and the eluate was monitored at 270 nm. The percentage of encapsulation efficiency (% EE) of the sample was calculated as follows:

$$\%\ \text{EE} = \frac{\text{weight of drug (mg)} \times 100}{\text{total weight (nanoparticles + drug) (mg)}}$$

Drug Release from 5-FU Loading Nanoparticles at pH 7.4 or in Human Plasma. Various aliquots of 5-FU loading nanoparticles (20 mg) were dispersed in 2 mL of phosphate buffer solution 0.15 M (NaCl, Na_2HPO_4, and KH_2PO_4) pH 7.4 or 1.5 mL of preheated human plasma and kept at 37 ± 0.1 °C by a water bath under stirring. Sink conditions

were maintained during the experiments (drug concentration was always below 10% of drug solubility). At suitable time intervals, samples dispersed in phosphate buffer were filtered through 0.2 μm regenerated cellulose (RC) membrane filters and analyzed by HPLC, while samples dispersed in human plasma were centrifuged at 12 000 rpm for 15 min at 4 °C. Methanol (1 mL) was added to 1 mL of supernatant to deproteinize the blood plasma, and the obtained suspension was centrifuged at 12 000 rpm for 5 min at 4 °C. Then supernatant was filtered through 0.2 μm RC membrane filters and assayed by HPLC. The HPLC analysis was performed on a reversed-phase C_{18} column with MeOH/H_3PO_4 0.05 vol % (2:98 v/v) as a mobile phase at 1.0 mL/min, and the eluate was monitored at 270 nm.

Results and Discussion

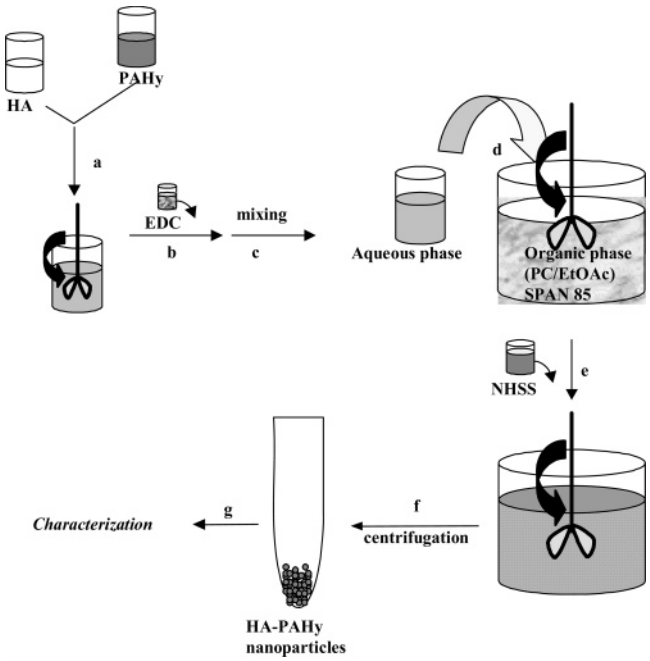
Preparation of HA−PAHy Nanoparticles. In a previous study, the chemical cross-linking between HA and a polyhydrazide polymer, such as PAHy, has been performed to prepare novel biomaterials, shaped as scaffolds and proposed as candidates for tissue enginnering. These systems show remarkable hydrolitical and mechanical resistances and a suitable degradation rate by hyaluronidase.[26] By exploiting the reactivity of HA toward PAHy, the aim of the present work has been the preparation of nanoparticles based on HA chemically cross-linked with PAHy and proposed as novel anticancer drug delivery systems.

Obviously, for obtaining nanoparticles, suitable experimental conditions have been employed. The cross-linking reaction has been carried out in a water/oil microemulsion in which the nanodroplets of the aqueous phase (containing both these polymers) act as nanoreactors where chemical cross-linking occurs. This procedure is easy to carry out, gives products with a high yield, and avoids the use of toxic chlorinated solvents. The complete process of nanoparticle preparation is schematically depicted in Scheme 1.

In particular, an inverse microemulsion has been prepared by using an aqueous phase obtained by mixing proper amounts of two preformed solutions of PAHy and HA, both at pH 7.5, to have a molar ratio between PAHy repeating units and HA repeating units equal to 4 and an organic phase formed by a mixture between PC and EtOAc, saturated with twice-distilled water. Span 85 has been employed as a surfactant, and *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)/*N*-hydroxysulfosuccinimide sodium salt (NHSS) chemistry has been employed to obtain an efficient cross-link between the carboxylic groups of hyaluronic acid and the hydrazide groups of PAHy (see Materials and Methods).

The reaction was performed at pH 7.5. In a preliminary study, we tried to cross-link HA and PAHy by using EDC alone at pH 4.75. This pH was chosen since it provides the opportunity for optimum coupling of HA with EDC as reported elsewhere.[35] In these conditions, the formation of an *O*-acylureic compound due to the reaction between HA and EDC occurs, and then the hydrazide groups of PAHy react with activated carboxylic groups of HA to give a di-amide linkage HA−PAHy with the formation of a polymeric network. However, in these conditions, the speed of cross-linking is too high (a few seconds); therefore, to slow up the gel formation and to increase the cross-linking efficiency, we thought to combine EDC with NHSS as reported by other authors and to perform the reaction at pH 7.5.[36] Actually, at pH 7.5, the speed of activation of HA by EDC is slower than that observed at pH 4.75, and the nucleophilic coupling of NHSS on EDC activated carboxylic groups of HA avoids the rearrangement of the unstable *O*-acylisourea derivative to the most stable and non-reactive *N*-acylurea derivative.[37]

Scheme 1. Schematic Representation of the HA−PAHy Nanoparticle Preparation through the Method of an Inverse Microemulsion[a]



[a] (a) Mixing of the HA and PAHy aqueous solutions at pH 7.5; (b) addition of the EDCI solution to the aqueous phase; (c) mixing of the obtained solution by a vortex for 30 min; (d) formation of the inverse microemulsion; (e) addition of the NHSS solution and stirring; (f) nanoparticle collection; and (g) purification and drying of the sample.

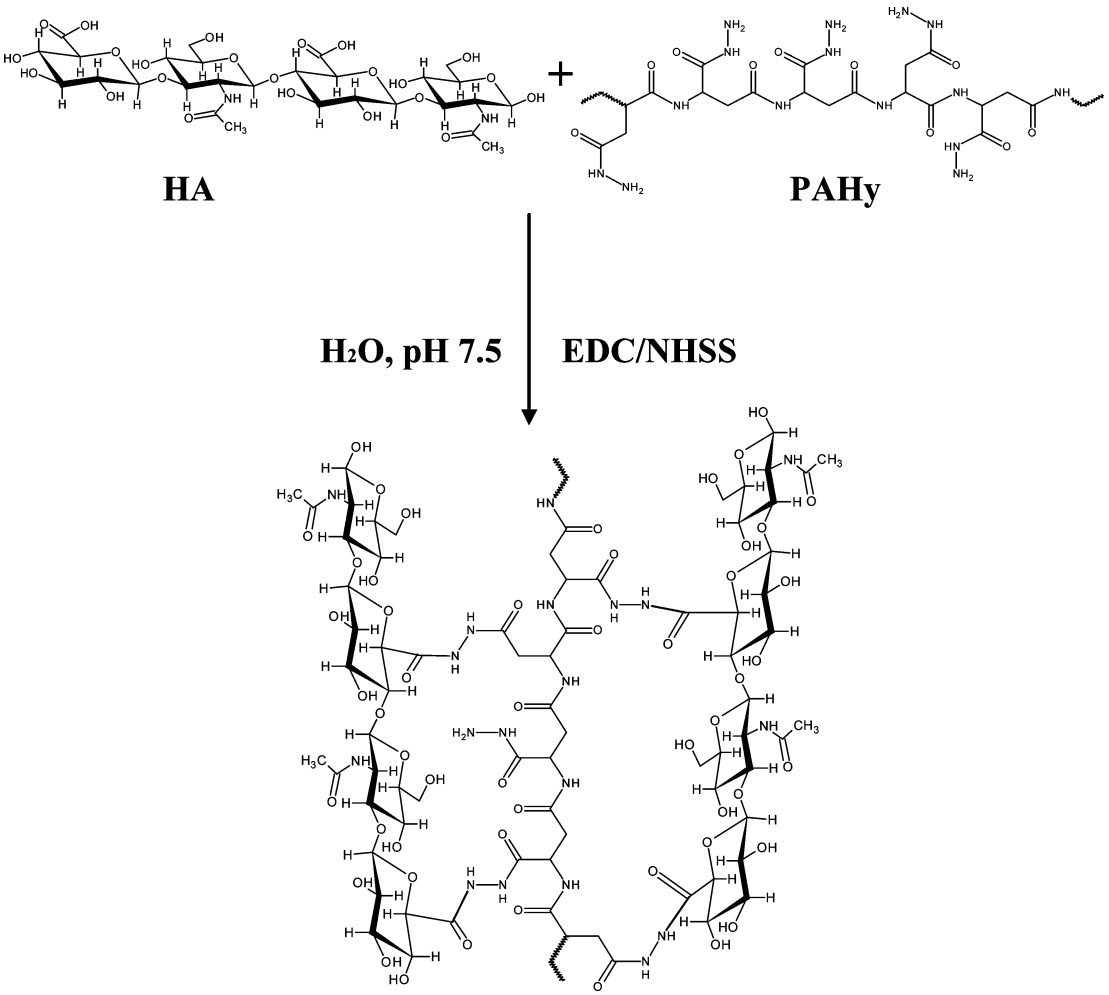
Therefore, the lower speed of activation at pH 7.5 and the presence of a two phase mechanism of activation of carboxylic groups of HA retards the point gel and allows a more efficient cross-linking of the obtained nanoparticles.

The yield of the sample recovered after purification and further drying resulted in being equal to 94 wt % based on the total weight of polymers dissolved in the aqueous phase. The absence of residues of SPAN 85 in the acetone used for the purification of nanoparticles has been established by HPLC analysis.[23] The schematic representation of the cross-linked structure of HA−PAHy nanoparticles is reported in Scheme 2.

Characterization of HA−PAHy Nanoparticles. HA−PAHy nanoparticles are insoluble in water and in common organic solvents, such as dichloromethane, acetone, ethanol, dimethyl sulfoxide, dimethylacetamide, and dimethylformamide. This result suggests that HA and PAHy are cross-linked through covalent linkages; this has been confirmed by FT-IR analysis. In particular, the spectrum of HA−PAHy nanoparticles as compared to that of HA and PAHy alone (see Figure 2) shows a broad band centered at 3400 (ν_{as} OH + ν_{as} NH + ν_s NH of HA and ν_{as} NH + ν_s NH + ν_s NH_2 of PAHy), two bands at 1654 and 1541 cm^{-1} corresponding to amide I of PAHy and HA and amide II of PAHy, respectively, and bands at 1152, 1082, and 1045 cm^{-1} attributable to the stretching of the C−O group derived from alcohols and ethers of HA. A reduction of the band at 1417 cm^{-1} of COO^- symmetric stretching is evidenced in the HA−PAHy nanoparticle spectrum as compared to that of HA, attributable to the reduction of carboxylate groups responsible for the cross-linking reaction with PAHy. A new band at 1738 cm^{-1} also appears due to the formation of ester linkages between carboxylic and hydroxyl groups of HA.

In Table 1, the mean diameter, PDI, and zeta potential values of HA−PAHy nanoparticles in various aqueous media are reported.

Scheme 2. Synthetic Scheme of Reaction between HA and PAHy



It is evident that the diameter of nanoparticles is affected by the ionic strength of the external medium (with similar pH values); in fact, this value increases from 215.5 nm, found in twice-distilled water, to 285.1 nm in phosphate buffer at pH 7.4. At pH 1, the nanoparticle diameter is lower than that found in other media due to the presence of carboxylic groups in the polymeric matrix, which are undissociated at this pH value. The low value of the PDI in all the investigated media indicates a rather homogeneous particle size distribution.

The zeta potential value results in being negative probably due to the presence of HA also on the nanoparticle surface. This value increases from −40.88 mV in twice-distilled water to −15.91 mV in phosphate buffer at pH 7.4 according to the known observation that the zeta potential of nanoparticles depends on the kind and concentration of ions present in the aqueous medium, and it is strongly affected by the size and localization of ions in the diffusive layer of each nanoparticle.[27]

Morphological Characterization of HA−PAHy Nanoparticles. Figure 3 shows a TEM photograph of HA-PAHy nanoparticles. As can be observed, these nanoparticles have a spherical shape and a homogeneous size distribution in accordance with the low PDI reported in Table 1. The spherical shape of the HA−PAHy nanoparticles confirms that the reaction occurs inside of the nanodroplets of the microemulsion.

Swelling Studies. To investigate the affinity of these nanoparticles toward an aqueous medium, swelling measurements have been carried out in twice-distilled water at 37 °C for 2 h. It was demonstrated that the equilibrium of swelling was reached after few minutes of contact between the sample and the liquid medium (data not shown). Swelling measurements have been also performed in media at different pH values to simulate some biological fluids (simulated gastric, intestinal, and extracellular fluids at pH 1.0, 6.8, and 7.4, respectively). Table 2 reports the values of weight swelling ratio (q) determined in the investigated media.

As can be seen, HA−PAHy nanoparticles show a great affinity toward aqueous media. Besides, due to the presence of carboxylic groups in the polymeric matrix (belonging to HA), this swelling is dependent on the pH of the medium. In particular, at pH 1.0, there is a considerable lowering of the q value due to the presence of pendant acidic groups, undissociated at this pH value. When the pH increases until 7.4, the presence of anionic carboxylic groups leads to an electrostatic repulsion among the polymer chains that causes an increase in the interchain distances and degree of swelling. The swelling in aqueous media of HA−PAHy nanoparticles is an important property for their biocompatibility, and it increases the possibility to prevent protein adsorption and to avoid MPS sequestration. In addition, it is interesting to underline that even if HA−PAHy nanoparticles show a good affinity toward the aqueous medium, the swollen particles, as evidenced in Table 1, maintain their sub-micrometer dimension.

Chemical Hydrolysis. Taking into consideration the presence of potentially degradable bonds in the chemical structure of HA−PAHy nanoparticles, chemical hydrolysis studies have been performed under simulated physiological conditions, supposing a potential parenteral or oral administration. In particular, the sample was treated at 37.0 ± 0.1 °C at pH 1.0 for 2 h (to

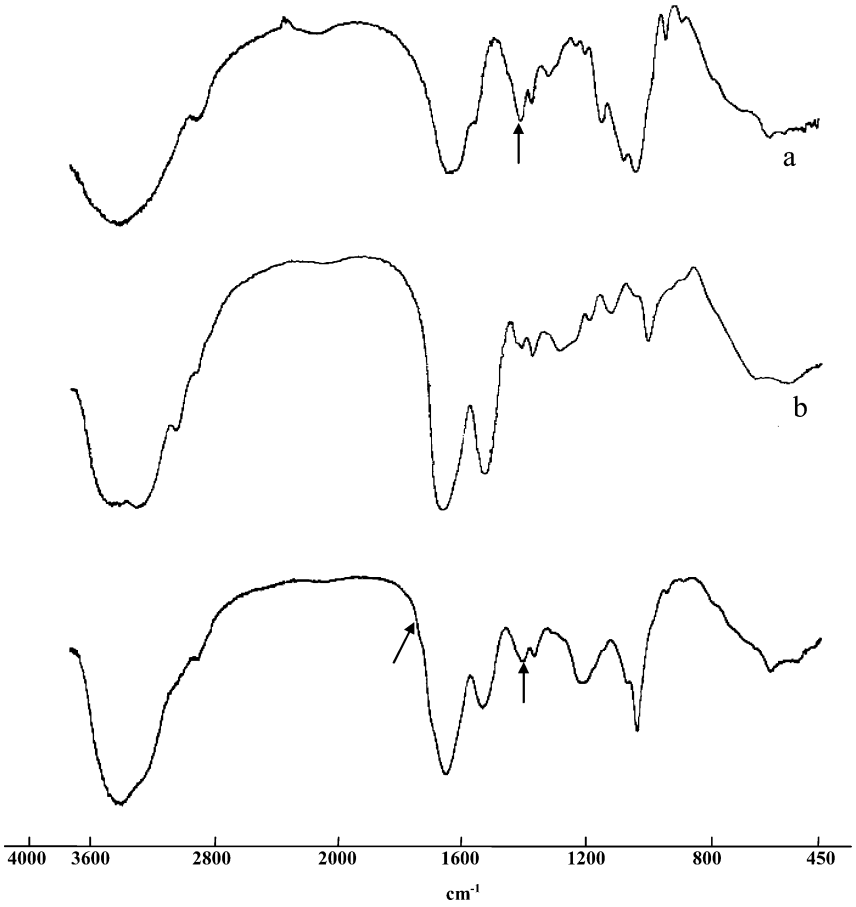


Figure 2. FT-IR spectra of (a) HA, (b) PAHy, and (c) HA−PAHy nanoparticles.

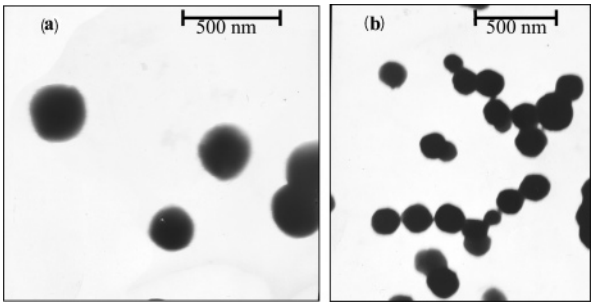


Figure 3. Transmission electron micrographs of HA−PAHy nanoparticles at a calibrated magnification of (a) 36 000× and (b) 30 000×.

Table 1. Mean Diameter, PDI, and Zeta Potential Values of HA−PAHy Nanoparticles in Various Aqueous Media[a]

medium	mean diameter (nm)	PDI	zeta potential (mV)
twice-distilled water (pH 7.0)	215.5	0.24	−40.88 ± 1.91
HCl 0.1 N (pH 1)	201.8	0.28	−31.78 ± 1.54
0.9% NaCl (pH 6.6)	237.9	0.27	−24.47 ± 0.93
PBS (pH 6.8)	264.6	0.27	−17.44 ± 0.70
PBS (pH 7.4)	285.1	0.25	−15.91 ± 0.80

[a] Values are means ± SD ($n = 3$). The average size of nanoparticles was calculated in the range between 5 and 95% for each sample.

simulate gastric fluid) and at pH 6.8 and 7.4 for 24 h (to simulate intestinal fluid or extracellular fluid). After this time, the percent residual weight has been determined, and the recovered sample has been characterized by swelling studies in twice-distilled water. These results are reported in Table 3.

Table 2. Weight Swelling Ratio (q) of HA−PAHy Nanoparticles in Various Aqueous Media[a]

medium	q
twice-distilled water (pH 7.0)	25.7 ± 0.3
HCl 0.1 N (pH 1.0)	15.4 ± 0.3
PBS (pH 6.8)	23.2 ± 0.2
PBS (pH 7.4)	32.7 ± 0.6

[a] Values are means ± SD ($n = 3$).

Table 3. Yield (wt %) and Swelling Degree (Expressed as q) in Twice-Distilled Water of HA−PAHy Nanoparticles after Chemical Hydrolysis at pH 1.0 for 2 h and pH 6.8 or 7.4 for 24 h at 37.0 ± 0.1 °C[a]

hydrolysis medium	yield (wt %) after chemical hydrolysis	q in twice-distilled water
HCl 0.1 N (pH 1.0)	90.3 ± 2.6	28.9 ± 0.5
PBS (pH 6.8)	97.3 ± 2.5	26.1 ± 0.4
PBS (pH 7.4)	98.0 ± 2.7	26.3 ± 0.4

[a] Values are means ± SD ($n = 3$).

The yield values of the sample recovered after these treatments as well as the slight increase in swelling suggest that HA−PAHy nanoparticles do not undergo a significant degradation under the investigated conditions.

Enzymatic Hydrolysis. Hyaluronidase (HAase) is the enzyme responsible for HA degradation in the human body; it is found in several fluids and tissues (skin, blood, tear liquid, etc.).[28,29] The digestion of HA by HAase results in the cleavage of the β-*N*-acetyl-hexosamine (1 → 4) glycosidic bonds, the products comprising a series of oligosaccharides with *N*-acetylglucosamine at the reducing terminus. Upon exhaustive

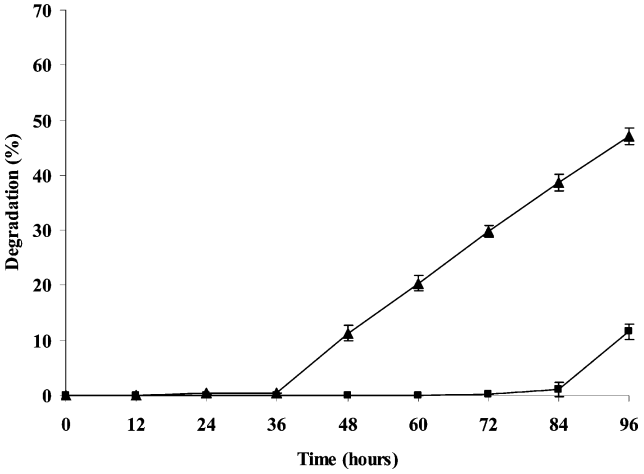


Figure 4. Degradation percentage of HA−PAHy nanoparticles in citrate buffer pH 6.3 in the absence (■) and in the presence of HAase (75 U/mL) (▲) as function of the incubation time. Values are means ± SD ($n = 3$).

digestion, the tetrasaccharide is the major product, closely followed by the hexasaccharide.[28]

A dialysis method has been applied to analyze the rate of degradation of HA−PAHy nanoparticles by HAase, as described in the Materials and Methods and Figure 1. A membrane for dialysis with a M_w CO of 10 000 Da has been chosen to ensure the free diffusion of HA oligofragments after degradation of nanoparticles. The percentage of degradation has been expressed by comparing the absorbance values found at different time intervals for each sample incubated in the absence and in the presence (75 U/mL) of enzyme with the absorbance values corresponding to the complete degradation of HA−PAHy nanoparticles. In Figure 4, the degradation percentage (%) of HA−PAHy nanoparticles is reported.

As it can be seen from the Figure 4, in the absence of hyaluronidase, HA−PAHy nanoparticles are resistant to degradation for almost 72 h, but they begin to lose small fragments of oligosaccharides only after 96 h (11% of degradation). As expected, the degradation of HA in HA−PAHy nanoparticles is faster in the presence of enzyme: in effect, there is an 11% degradation already after 48 h and almost the 50% after 96 h.

Cell Viability Assay. To investigate the effect of HA−PAHy nanoparticles on cell viability, various concentrations of nanoparticles (0.25, 0.5, and 1 mg/mL) have been kept in contact with the K-562 cell line (human chronic myelogenous leukaemia cells). In particular, cell viability has been evaluated by the Trypan blue exclusion assay, considering that cells with damaged membranes after the contact with HA−PAHy nanoparticles will be colored by the dye. In Figure 5, cell viability as a function of the investigated concentrations of HA−PAHy nanoparticles is reported. Data suggest that no cytotoxic effect on cells occurs for all the concentrations used in our experiments.

Haemolytic Activity. The potential nanoparticle interaction with erythrocyte membranes was investigated by haemolysis experiments. The release of haemoglobin was used to quantify the membrane-damaging properties of the nanoparticles. For obtaining the values corresponding to 100 and 0% of lysis, the erythrocytes were treated with 1% Triton X-100 and phosphate buffer (PBS), respectively. Erythrocytes were incubated with three different nanoparticle concentrations in the range of 0.25−1.0 mg/mL for 1 h. Under these conditions and for all investigated samples, nanoparticles showed no haemolytic effects, indicating no detectable interaction with the red blood

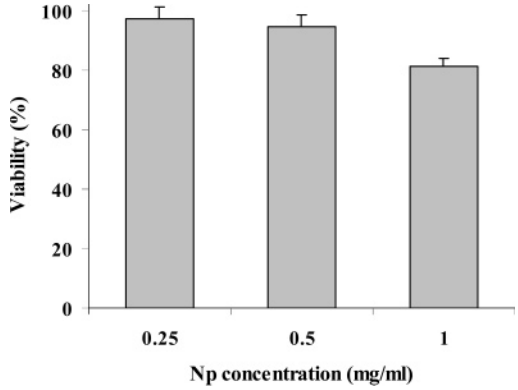


Figure 5. Viability (%) of K-562 cells after 48 h of incubation in the presence of HA−PAHy nanoparticles as a function of nanoparticle (Np) concentration. Viability of cells in the culture medium alone was considered 100%. Values are means ± SD ($n = 3$).

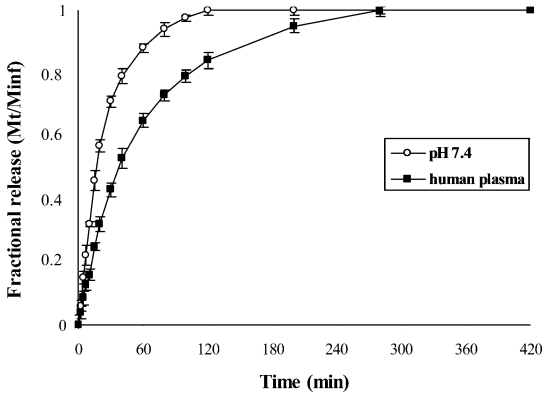


Figure 6. In vitro release of 5-FU from HA−PAHy nanoparticles at pH 7.4 and in human plasma at 37.0 ± 0.1 °C. Values are means ± SD ($n = 3$).

cell membranes. In fact, the percentage of haemolysis was always less than 0.3%, data comparable to those of the blank. Moreover, no aggregation of erythrocytes was observed by a microscope after incubation with nanoparticles (data not shown). Taking into account these results and the biocompatibility study data, HA−PAHy nanoparticles show good properties for an in vivo endovenous administration as a drug carrier.

In Vitro Release Studies of 5-FU. To estimate the ability of these nanoparticles to incorporate drug molecules and release them in a physiological medium, 5-FU was chosen as a water-soluble model drug with a low molecular weight, and it was loaded into nanoparticles by a soaking procedure. The percent encapsulation efficiency has been evaluated by HPLC analysis and resulted in being 14.7 wt %. The evaluation of drug release has been performed in simulated extracellular fluid (pH 7.4) and in human plasma at 37 °C. The release profiles, reported in Figure 6, show a complete drug delivery within 5 h in both media.

The drug release can be affected by two phenomena (i.e., drug diffusion into swollen polymers and swelling owing to the penetrating medium). The kinetics of 5-FU release was analyzed using the empirical exponential equation[30]

$$\frac{M_t}{M_\infty} = Kt^n \qquad (1)$$

with $M_t/M_\infty \leq 0.6$. In eq 1, M_t/M_∞ is the drug fraction released at time t, and K and n are the constant and the kinetic exponent of drug release, respectively. Although the use of this equation requires detailed statistical analysis,[31] the calculated exponent

Table 4. Fitting of Release Data with Eqs 1 and 2

$M_t/M = Kt^n$			
medium	$K \times 10^3$ (min^{-n})	exponent n	R
PBS (pH 7.4)	129.5	0.45	0.955
Plasma	876.0	0.46	0.950

$M_t/M = K_1t^{1/2} + K_2t$			
medium	$K_1 \times 10^3$ (min$^{-1/2}$)	$K_2 \times 10^3$ (min^{-1})	R
PBS (pH 7.4)	130.9	−3.12	0.956
Plasma	876.6	−1.38	0.964

n gives an indication of the release kinetics (for spherical matrices, it ranges from Fickian ($n = 0.43$) to case II transport ($n = 0.85$), while it is anomalous for intermediate values).[32] The n values obtained are 0.45 at pH 7.4 and 0.46 in plasma indicating that the kinetics of 5-FU release is anomalous in both media, being slightly higher than the ideal behavior.

A more informative analysis can be obtained by fitting the data with the model proposed by Peppas and Sahlin.[33] The equation for this model is

$$\frac{M_t}{M_\infty} = K_1 t^{1/2} + K_2 t \qquad (2)$$

with $M_t/M_\infty \leq 0.95$. In this equation, the first term is the Fickian contribution, and the second term is the case II relaxational contribution. Table 4 reports K_1 and K_2 values calculated according to eq 2. The term $K_1t^{1/2}$ is greater than the term K_2t in both the experiments, thus indicating that the predominant mechanism for 5-FU release is the Fickian diffusion through the swollen nanoparticles that is in agreement with the fast swelling observed.

Finally, to calculate the apparent diffusion coefficient, D_i, the final portion of the release profiles ($0.6 \leq M_t/M_\infty < 1$) was analyzed by means of the approximate diffusion equation for spherical matrices[34]

$$1 - \frac{M_t}{M_\infty} = \left(\frac{6}{\pi^2}\right) \exp - \left(\frac{\pi^2 D_i t}{r^2}\right) \qquad (3)$$

r being the mean radius of the swollen particles. The calculated D_i values resulted in being 3.82×10^{-14} cm^2 s^{-1} in PBS at pH 7.4 and 1.48×10^{-14} cm^2 s^{-1} in human plasma in agreement with the obtained release profiles.

Conclusion

In this paper, the preparation and characterization of novel polysaccharide−polyaminoacid nanoparticles based on HA and PAHy are reported. To obtain HA−PAHy nanoparticles, an inverse microemulsion was employed where aqueous nanodroplets acted as nanoreactors in which the cross-linking reaction between both the polymers occurs. Obtained nanoparticles showed a spherical shape with a size in the nanometric scale and a negative surface charge. A remarkable affinity toward aqueous media, affected by the pH of the penetrating medium, has been observed. Stability studies in media at various pH values have shown a non-significant chemical degradation, but in the presence of hyaluronidase, HA−PAHy nanoparticles are partially degraded as a function of incubation time. In vitro cytotoxicity assays have also demonstrated that these nanoparticles do not affect negatively the viability of the K-562 cell line and do not possess haemolytic activity. A model drug, such as 5-FU, has been loaded into HA−PAHy nanoparticles, and it has been released in simulated extracellular fluid and in human plasma by a mechanism controlled essentially by diffusion. The obtained results make HA−PAHy nanoparticles attractive candidates as drug carriers for parenteral administration.

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