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Research Paper

New Core-Shell Nanoparticules for the Intravenous Delivery of siRNA to Experimental Thyroid Papillary Carcinoma

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Purpose. Development of efficient *in vivo* delivery nanodevices remains a major challenge to achieve clinical application of siRNA. The present study refers to the conception of core-shell nanoparticles aiming to make possible intravenous administration of chemically unmodified siRNA oriented towards the junction oncogene of the papillary thyroid carcinoma.
Methods. Nanoparticles were prepared by redox radical emulsion polymerization of isobutylcyanoacrylate and isohexylcyanoacrylate with chitosan. The loading of the nanoparticles with siRNA was achieved by adsorption. The biological activity of the siRNA-loaded nanoparticles was assessed on mice bearing a papillary thyroid carcinoma after intratumoral and intravenous administration.
Results. Chitosan-coated nanoparticles with a diameter of 60 nm were obtained by adding 3% pluronic in the preparation medium. siRNA were associated with the nanoparticles by surface adsorption. *In vivo*, the antisense siRNA associated with the nanoparticles lead to a strong antitumoral activity. The tumor growth was almost stopped after intravenous injection of the antisense siRNA-loaded nanoparticles, while in all control experiments, the tumor size was increased by at least 10 times.
Conclusion. This work showed that poly(alkylcyanoacrylate) nanoparticles coated with chitosan are suitable carriers to achieve *in vivo* delivery of active siRNA to tumor including after systemic administration.

KEY WORDS: nanoparticles; thyroid papilloma carcinoma; poly(alkylcyanoacrylate); siRNA.

I\NTRODUCTION

Chromosomal aberrations play a crucial role in many cancers, especially in leukemia and solid tumors. New potential therapeutic methods aim to down-regulate the expression of the resulting altered genes. Those based on the use of small interfering RNA (siRNA) appear very promising (1–4).

siRNAs consist of a 21 to 23 double-stranded RNA with two 3′ overhang nucleotides. They are incorporated in the nucleoproteic complex RISC involving Argonaut family proteins and subsequently induce degradation of the targeted mRNA (5,6). These highly specific and double-stranded nucleic acids were already shown to be more efficient as compared to antisense oligonucleotides both *in vitro* and *in vivo* (7). However, as all molecules of polyelectrolyte nature, siRNAs raise many challenges to be administered *in vivo* with the aim to reach their intracellular mRNA target. Their high susceptibility to nuclease degradation make these compounds poorly stable in biological media, and their association with a drug carrier is required to protect them from degradation. Their hydrophilicity hampered their intracellular penetration, while their biological activity is sustained from intracellular penetration. Therefore, their association with a drug carrier is also required to improve their intracellular uptake (1,3,4,8–11).

Although a couple of works have considered the use of liposomes (12,13) and of polyelectrolyte complexes (14,15), the *in vivo* delivery of short fragments of nucleic acids including siRNA was also explored using polymer nanoparticles (4,16–21). More specifically, chitosan-coated poly(isohexylcyanoacrylate) (PIHCA) and poly(isobutylcyanoacrylate) nanoparticles prepared by anionic emulsion polymerization were found suitable to deliver active antisense phosphodiester oligonucleotides in an experimental model of Ewing sarcoma in mice (18). These nanoparticles were able to promote a specific antitumoral growth inhibition by a vectorized antisense oligonucleotide, while the free antisense oligonucleodide stimulated tumor growth due to a non-specific polyanionic effect (22). In a preliminary study, we have recently shown that a new type of chitosan-coated poly(isobutylcyanoacrylate) (PIBCA) nanoparticle was able to deliver active siRNA *in vivo* by intratumoral administration (23). The antitumoral effect was relevant to the antisense activity of the siRNA. One key to this success can be attributed to the protection of the siRNA against enzymatic degradation when they were injected in the tumor under the form of nanoparticles for at least 48 h. The nanoparticles were synthesized by a redox radical emulsion polymerization of alkylcyanoacrylate monomers, which is a very different route of synthesis than the one used for the synthesis of poly (alkylcyanoacrylate) (PACA) nanoparticles tested in previous works (18). The main difference between the two types of nanoparticles is subtle, including a different spatial arrangement

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of the polysaccharide chains at the nanoparticle surface (24,25). As a consequence, the nanoparticles showed opposite capacity to activate the complement system (25). Therefore, it can be expected that the new type of nanoparticles, which does not activate the complement system, will remained in the blood stream and will be able to deliver the active siRNA to a tumor from an intravenous administration (26,27). From recent published reports, it can also be expected that the siRNA delivered to the tumor under the form of nanoparticles will promote a specific siRNA-mediated tumor growth inhibitory effect (22,23).

The aim of the present work was to design chitosan-decorated nanoparticles with a diameter smaller than 100 nm to achieve *in vivo* targeting of a phosphodiester siRNA in a tumor from an intravenous administration thanks to the enhancing permeation effect (EPR effect). The small size of the nanoparticles is mandatory to exploit the EPR effect for targeting the nanoparticles to the tumor (28). The siRNA used in this work was designed to target the junction oncogene of thyroid papilloma carcinoma (23). The intravenous route is clinically relevant for the administration of anticancer drugs, and it was used to give proof of concept of the new methods of *in vivo* delivery of siRNA developed in this work. It was also used to validate the hypothesis that the new type of chitosan-coated nanoparticles is a suitable carrier for systemic administration of challenging drugs. Intratumoral administrations were used to select the more promising nanoparticle formulations to be injected intravenously.

MATERIALS AND METHODS

Material

Chitosan (medium molecular weight) was supplied by Fluka (ref 22742) and oxidized by the method previously used by Bertholon *et al.* (25) with concentrations of $NaNO_2$ of 8%, 7% and 6%, to prepare chitosan with the respective molecular weight of 20 kDa, 35 kDa and 70 kDa, respectively. Isobutylcyanoacrylate and isohexylcyanoacrylate were kindly provided as a gift by Henkel Biomedical (Ireland). Pluronic (Lutrol F68) was provided by BASF (Germany). siRNAs were obtained from Eurogentec (Belgium). 2′-deoxynucleotides residues were used as the 3′ overhangs in both the sense and antisense strands. One sequence was selected complementary to the junction of the ret/PTC1 fusion oncogene (sequence noted As): 5′-CGUUACCAUCGAGGAUCCAdAdA-3′ and 5′-UGGAUCCUCGAUGGUAACGdCdU-3′. This active sequence was tested against a relevant control sequence (sequence noted Ct): 5′-GCCAGUGUCACCGUCAAGG dAdG-3′ and 5′-CCUUGACGGUGACACUGGCdTdT-3′ (23). The siRNA As has a molecular weight of 13,315 g.mol^{-1}.

Synthesis of Nanoparticles by Redox Radical Emulsion Polymerization

The hydrolyzed chitosan (0.138 g) was dissolved in an aqueous solution of nitric acid (8 mL, 0.2 N) in a glass tube at 40°C under gentle stirring and argon bubbling. After 10 min, 0 % to 3 % of pluronic (Lutrol F68) were added, and argon bubbling was slowed down. After complete dissolution of the solid compounds, a solution of Cerium (IV) ammonium nitrate (2 mL, 8×10^{-2} M in 0.2 N nitric acid) was added. Then 0.5 mL of the monomer, isobutylcyanoacrylate (IBCA) or isohexycyanoacrylate (IHCA) was immediately added under vigorous stirring, and gentle argon bubbling was maintained for 10 min. The reaction was allowed to continue at 40°C for 40 min. After cooling at room temperature, the pH of the solution was increased to 5.0 with NaOH (1 N). The nanoparticle suspension was then purified by dialysis (Spectra/Por membranes, MWCO: 100,000, Biovalley) against water. To obtain fluorescent labeled nanoparticles, 2 ml of a rhodamine-labelled monomer, PolyFluor® 570 (methacryloxyethyl thiocarbamoyl rhodamine B (N-[9-(2-carboxy-x-methacryloxy-ethylthiolcarbamoyl-phenyl)-6-diethylamino-3H-xanthen-3-ylidene]-N- ethyl-ethanaminium chloride, Polyscience, BioValley, France) solution dissolved in acetonitrile (4 mg/ml) was added to the polymerization medium 2 min after the addition of the monomer (IBCA). This monomer was able to copolymerize with IBCA allowing a labelling of the core of the nanoparticles (29).

Nanoparticle Characterization

Particle Size

The hydrodynamic diameter of the nanoparticles was measured at 20°C by quasi-elastic light scattering using a Nanosizer N4 PLUS (Beckman-Coulter, France), operating at 90°. 60 µL of each sample was diluted in 2 mL of MilliQ water. The temperature was allowed to equilibrate 5 min before measurement. The results gave the mean hydrodynamic diameter of the dispersed particles obtained from three determinations. The standard deviation and polydispersity index were also determined.

Zeta Potential

Zeta potential was measured using a Zetasizer 4 (Malvern Instrument Ltd, France). Dilution of the suspensions (1:33 (*v/v*)) was performed in NaCl 1 mM.

Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was performed using a Philips EM208 with a large format CCD camera AMT at the CCME Orsay (Centre Commun de Microscopie Electronique) France. The samples were diluted in MilliQ water 1:100 and deposited on a preionized Formvar-Carbon-coated Electron Microscope Grid. After 5 min, the excess was removed and the sample stained with neutral 1% aqueous phosphotungstic acid for 30 s.

siRNA Radiolabelling

siRNA were labelled at the 5′-end by T4 polynucleotide kinase (New England Biolabs) and [γ-^{33}P]-ATP (Amersham Bioscience) in 50 µl of the reaction mixture. Thus, 5 µl of siRNA stock solution (20 mM), RNase-free water (30 µl), 10× kinase buffer (5 µl; New England Biolabs), 5 µl of T4 polynucleotide kinase (10 U/µl), and 5 µl of [γ-^{33}P]-ATP (>5000 Ci/mmol) were incubated at 37°C for 1 h. The enzyme was inactivated by heating at 65°C for 20 min. The reaction mixture was placed in the upper chamber of MicroCon YM3

(cutoff value Mr 3,000; Millipore). After adding 200 µl of RNase-free water, it was centrifuged (4,000 g). The procedure was repeated four times. Material remaining in the upper chamber was regarded as ^{33}P –labelled siRNA. The labeled siRNA formation was performed by raising the temperature to 65°C for 5 min and heating for 1 h at 37°C to let the two strands of the siRNA reassemble properly.

siRNA Adsorption on Nanoparticles

Nanoparticle (NP) suspensions were prepared at different mass ratios in nanoparticles/siRNA: 0; 10; 20; 25; 30; 40; 45; 50; 100 and 300 using [γ-^{33}P]-labeled siRNA. The final siRNA concentration was 0.5 µM in a total volume of 300 µL. Liquid paraffin was then added to equilibrate the tubes for ultracentrifugation (total volume 1.2 mL). After 10 min of incubation, the suspension was centrifuged in a 70.1 Ti rotor at 45,000 rpm (140,000 g) for 1 h at 4°C. The radioactivity of 100 µL of the supernatant was measured to determine the amount of siRNA which were not associated with the nanoparticles. The amount of siRNA adsorbed on the nanoparticles was then deduced from the difference between the initial amount of siRNA incubated with the nanoparticles and the amount of siRNA found in the supernatant.

Transfection of Cells for Expressing the ret/PTC1 Fusion Oncogene

The RP1 cells consist of NIH/3 T3 cells stably transformed by the pBABE-puro plasmid expressing the ret/PTC1 oncogene under the LTL promoter. The plasmid was kindly provided by Dr. A. Fusco (University Federico II, Naples, Italy). RP1 cells were grown in DMEM medium (Gibco) containing 10% heat-inactivated newborn calf serum (Gibco) and a blend of antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, Gibco) at 37°C, 5% CO_2, in a moist atmosphere.

Cytotoxicity Studies

Cytotoxicity of the nanoparticles was evaluated on the RP1 cells by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method (30). The cells (2.10^5 cells per well) were seeded in 6-well plates and incubated with 1 mL of Dulbecco's Modified Eagle Medium (D-MEM) with glutamax (Gibco) containing 10% of heat-inactivated newborn calf serum (Gibco) and a blend of antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, Gibco) at 37°C, 5% CO_2, in a moist atmosphere. After 24 h, the cell culture medium was replaced by 900 µL of fresh culture medium and 100 µL of nanoparticles diluted in the cell culture medium. The cells were further incubated for 24 h. Then, 100 µL of a dimethylthiazol diphenyl tetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added in each well. After 3 h of incubation at 37°C, the medium was removed, and the cells were lysed with 1 mL of a freshly prepared 10 mM hydrochloric acid solution containing 10% sodium dodecyl sulfate. After 18 h of incubation at 37°C, 200 µL of the medium contained in each well were transferred in a 96-well plate, and the optical density was measured at 570 nm using a microplate reader (MRXII, Dynex Technology). Non-treated cells were used as a control. Each nanoparticle concentration was evaluated three times, and each measurement was made in triplicate. The cytotoxicity was expressed as the concentration required to inhibit 50% of cell proliferation (IC_{50}).

Visualization of the Uptake of Nanoparticles by Cells Using Fluorescent Confocal Microscopy

The cells were seeded in 6-well plates containing a cover glass (1.5×10^5 cells/well). The cells were grown for 3 h in the cell culture medium containing fluorescent labelled nanoparticles in the same conditions as those explained above. The medium was removed, and the cells were washed with PBS and then fixed with formaldehyde 4% in PBS for 20 min at 4°C. The slides were then observed with a confocal microscope (Zeiss LSM 510/ Axiovert 200 M with a 63_/1.4 oil immersion objective) after Mowiol (Calbiochem, Bad Soden, Germany) mounting as an antifading agent.

Animal Experiment

All animal experiments were carried out according to the French Laws of animal welfare and were approved by the Ethics Commission of the official veterinary authorities.

Preparation of Tumor-Bearing Mice

The tumorigenicity tests were performed on nude mice produced by the laboratory. The mice were pre-irradiated by 5 Gy one day before tumor implantation. Living RP1 cells (10^6) were injected subcutaneously into the right flank of nude mice. The size of the tumors was evaluated by measuring their diameter with a digital slide caliper, and the tumor volume in mm^3 was calculated by the formula: volume = $(\text{width})^2 \times \text{length}/2$. Data were presented as mean ± standard error. Treatments were started after 1 week, when the tumors reached a volume of 43± 9 mm^3 (n=30). Animals were divided in groups of 5 animals each and injected intratumorally or intravenously.

Intratumoral Treatments

In a first series of experiments, the tumor-bearing nude mice (~20 g weight) were treated by intratumoral injections of different formulations of the siRNA As or Ct. Two cumulative doses of siRNA were tested: 1 mg/kg and 5 mg/kg. The nanoparticulate formulations of siRNA tested in these experiments included both PIBCA and PIHCA core nanoparticles with a shell of chitosan 20 kDa. The nanoparticles were dispersed in a 0.9% saline solution.

To achieve a cumulative dose of siRNA of 1 mg/kg in 5 injections, the solution prepared for each injection contained 4 µg of siRNA associated with 200 µg of nanoparticles dispersed in a final volume of 100 µL of 0.9% saline solution. The injections were performed at day 0, 2, 4, 7 and 10. The preparation of the siRNA solution to be administered at the cumulative dose of 5 mg/kg was obtained by mixing 20 µg of siRNA with 800 µg of nanoparticles dispersed in a final volume of 100 µL 0.9% saline solution. To reach the cumulative dose of 5 mg/kg, the 100 µL solution was administered 5 times at day 0, 2, 4, 7 and 10.

Control experiments were performed using physiological serum (0.9% saline solution), as well as free siRNA at the same doses (1 mg/kg and 5 mg/kg, expressed as cumulative doses) or empty nanoparticles dispersed in physiological serum at con-

centrations used in the experiments performed with the siRNA-loaded nanoparticles (200 µg nanoparticles/100 µl dispersion and 800 µg nanoparticles/100 µl).

In all experiments, each animal received five intratumoral injections of 100 µL each as indicated above. After the first injection, the size and the volume of the tumors were evaluated every day as explained above over a period of 14 days. Results were expressed as the relative tumor volume given by the ratio of the tumor volume measured at each day (volume of the tumor at day x) on the initial tumor volume (volume of the tumor at day 0).

Systemic Treatment by Intravenous Injections

In a second series of experiments, the tumor-bearing nude mice were treated by intravenous injections of siRNA associated or not with chitosan-coated PIBCA or PIHCA nanoparticles. The nanoparticles considered in these experiments were prepared with chitosan 20 kDa in the presence of 3% pluronic. The cumulative dose of siRNA administered to the mice was fixed at 5 mg/kg and was given in 5 intravenous injections of 100 µl each at day 0, 2, 5, 7, and 9. Each of the 5 intravenous injections was prepared with 20 µg of siRNA associated with 800 µg of nanoparticles and dispersed in a volume of 100 µL of 0.9% saline solution.

Control experiments were performed with physiological serum (0.9% saline solution) and with saline solutions of the relevant siRNA, i.e. siRNA As and siRNA Ct (cumulative dose 5 mg/kg).

Intravenous injections were performed in the retro-orbital vein according to the schedule indicated above. After the first injection, the size of the tumor was evaluated on a daily basis over a period of 10 days as explained above.

Calculation of the number of injected nanoparticles, N_P, was obtained from the total weight of injected nanoparticles, m, (250 mg) and the mass of one particle, M_p, calculated from the volume of one particle, V_P and the density, d, of the particles (d_{PIBCA} nanoparticles = 1.13, d_{PIHCA} nanoparticles = 1.15 (31).

$$N_P = \frac{m}{M_P} = \frac{m}{V_P.d} = \frac{m}{4/3.\pi.R^3.d}$$

with R being the radius of the nanoparticles.

The number of injected siRNA, N_{siRNA}, was calculated from the mass of injected siRNA, m_{siRNA} (5 mg), the Avogadro number, N_a, and the molecular weight of the siRNA (MM_{siRNA} = 13,315 g.mol^{-1}).

$$N_{siRNA} = \frac{N_a.m_{siRNA}}{MM_{siRNA}}$$

Q PCR

The tumoral tissues were excised from the animals at the end of the experiments, and total RNA were recovered using TRIzol reagent (Invitrogen, France) according to the manufacturer instructions. Total RNA was quantified using the ND-1000 spectrophotometer (Nanodrop, USA). Superscript II Reverse Transcriptase kit (Invitrogen) was used for reverse transcription. The primers used for the amplification of ret/PTC1 were forward 5′-AGATAGAGCTGGAGACCTAC-3′ and reverse 5′ CTGCTTCAGGACGTTGAA 3′. Taq Universal PCR Master Mix (Apleira) was used as fluorescent dye. Samples were amplified for 50 PCR cycles with continuous monitoring of fluorescence. 18 S gene was used as a PCR control using Human 18 S rRNA MGB kit (Apleira). The amplification was monitored on ABI prism 7700 real-time PCR apparatus (Perkin-Elmer).

RESULTS

Optimizing Small Size Chitosan-Coated Nanoparticles and Characterization

PIBCA and PIHCA nanoparticles were prepared using chitosan of three different molecular weights. In agreement with previous study, small particles were obtained with polysaccharides of low molecular weight (Fig. 1A and B) (24). The diameter of the PIHCA nanoparticles was slightly above those of the PIBCA nanoparticles.

The diameters of the smallest nanoparticles were much above the limit of 100 nm expected to suit with requirements for *in vivo* delivery of siRNA to the tumor by passive targeting thanks to EPR effect after intravenous administration. To further reduce size of the nanoparticles, new preparations were carried out by adding pluronic in the polymerization medium. The rationale behind this choice was that as pluronic is a surfactant, it may reduce size of nanoparticles produced by emulsion polymerization. The results showed that addition of increasing amounts of pluronic in the polymerization medium led to a dramatic decrease in the nanoparticle size. Practically, by the addition of 3% of the surfactant, the size of the PIBCA and PIHCA nanoparticles could be reduced to 62 nm and 94 nm respectively (Fig. 1A and B). For instance, it decreased from 230 nm to 94 nm for PIHCA nanoparticles prepared with chitosan 20 kDa and 3% pluronic. Noteworthy, the addition of Pluronic after completion of nanoparticle preparation did not induce any size reduction and, on the contrary, the size of the nanoparticles even tended to increase (Fig. 1C). This effect was more marked with PIBCA nanoparticles than with PIHCA nanoparticles.

As indicated by the results of zeta potential measurements, all nanoparticles coated with chitosan were positively charged (Fig. 1 D, E). This was in agreement with the fact that the nanoparticles prepared by this technique were coated by the polysaccharide possessing amine function with a pKa around 6.2, which is therefore partially protonated at neutral pH. Thus, nanoparticles prepared in a pluronic-free polymerization medium showed a zeta potential ranging between +30 and +36 mV, except for the chitosan 35 kDa, which gave nanoparticles with a lower zeta potential (+26 mV) (Fig. 1D). Regardless of the chitosan molecular weight and the nature of the polymer's core (either PIBCA or PIHCA), the increase of the pluronic concentration in the polymerization medium induced a decrease of the zeta potential of the corresponding nanoparticles. It can be suggested that pluronic residues remained attached to the chitosan-coated nanoparticle surface, which subsequently partially masked the positive charges of the chitosan chains stranded on the nanoparticle surface.

Morphology of the Nanoparticles

Both chitosan (20 kDa) PIHCA and PIBCA nanoparticles were observed by TEM. The shape of the nanoparticles was spherical. The addition of pluronic during the polymerization

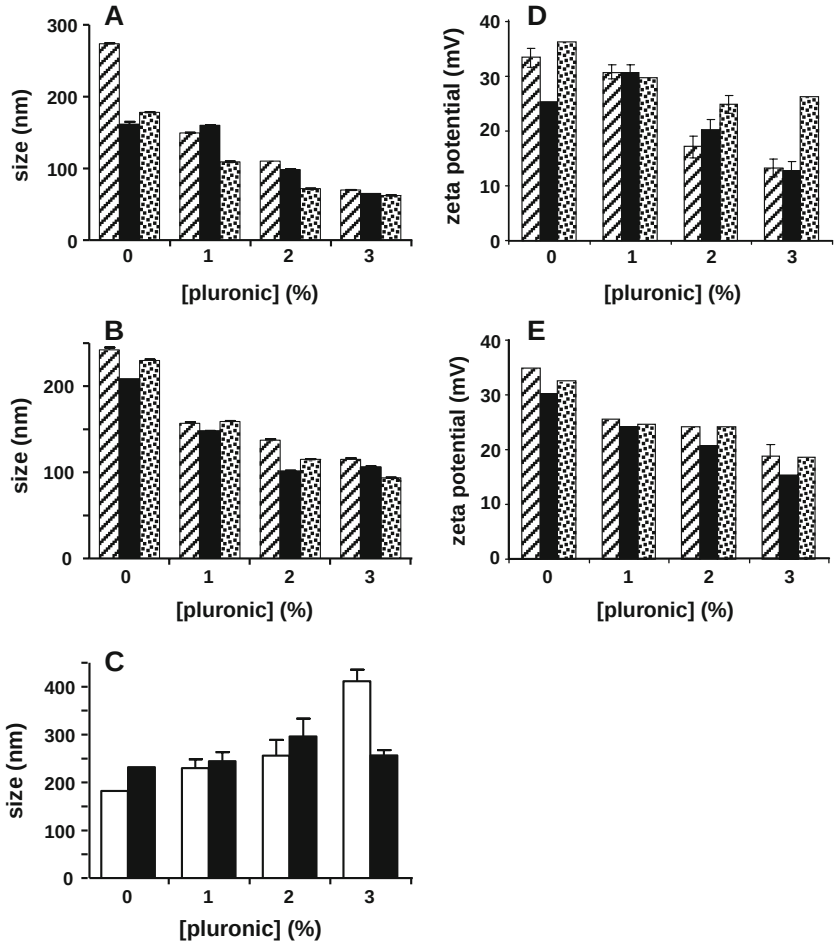

Fig. 1. Influence of pluronic on the nanoparticle size. A, B, C and zeta potential D, E. A, B, D and E show the results from PIBCA (**A**, **D**) and PIHCA (**B**, **E**) nanoparticles prepared with different concentrations in pluronic and with chitosan 70 kDa (hashed bars), 35 kDa (black bars) and 20 kDa (dotted bars). **C** shows the diameter of the nanoparticles obtained by addition of pluronic after preparation of the nanoparticles with chitosan 20 kDa and without pluronic. PIBCA: white bars, PIHCA: black bars.

led to a dramatic decrease of nanoparticle diameters (Fig. 2). It is noteworthy that the nanoparticle diameters measured by TEM were smaller than those determined by quasi-elastic light scattering. This discrepancy may, however, be explained by the strong dehydration of the samples before TEM analysis.

Adsorption of siRNA onto the Nanoparticles

Association of siRNA with chitosan-coated nanoparticles was obtained by single mixture of siRNA with nanoparticles. Fig. 3 shows adsorption isotherms of siRNA on nanoparticles prepared with different core polymers (i.e. PIBCA or PIHCA), in the absence and in the presence of 3% of pluronic.

Isotherms obtained with the different nanoparticles were almost identical, although the nanoparticle size was different. For all preparations, a mass ratio of NP/siRNA of 50 or of 1/50 if expressed as siRNA/nanoparticles yielded a loading of 90% of the initial amount of siRNA. Noteworthy, the association of siRNA with the nanoparticles did not influence the hydrodynamic diameter of the particles (Fig. 4A).

For all types of nanoparticles, the adsorption of siRNA led to a reduction of the zeta potential which confirmed that siRNA could be loaded on chitosan-coated PACA nanoparticles thanks to ionic interactions.

The zeta potential of PIBCA nanoparticles prepared with pluronic was reduced from a highly positive value of +26.4 mV without siRNA to a negative value of −17.8 mV for the nanoparticles with a mass ratio siRNA/nanoparticles of 1/25 (Fig. 4B). With the corresponding PIHCA nanoparticles, the zeta potential was reduced to a lower extent from +17.4 mV to −2.5 mV (Fig. 4B).

The same chitosan molecular weight was used in all preparations of nanoparticules. No influence of the nature of the surface can explain such a difference of zeta potential evolution found between the nanoparticles having a PIBCA and a PIHCA core. By comparing the evolution of the nanoparticle size and zeta potential during siRNA adsorption, it can be pointed out that a correlation seems to exist between these two parameters. The smaller the size of the nanoparticle was, the higher the zeta potential was influenced by the adsorption of the siRNA.

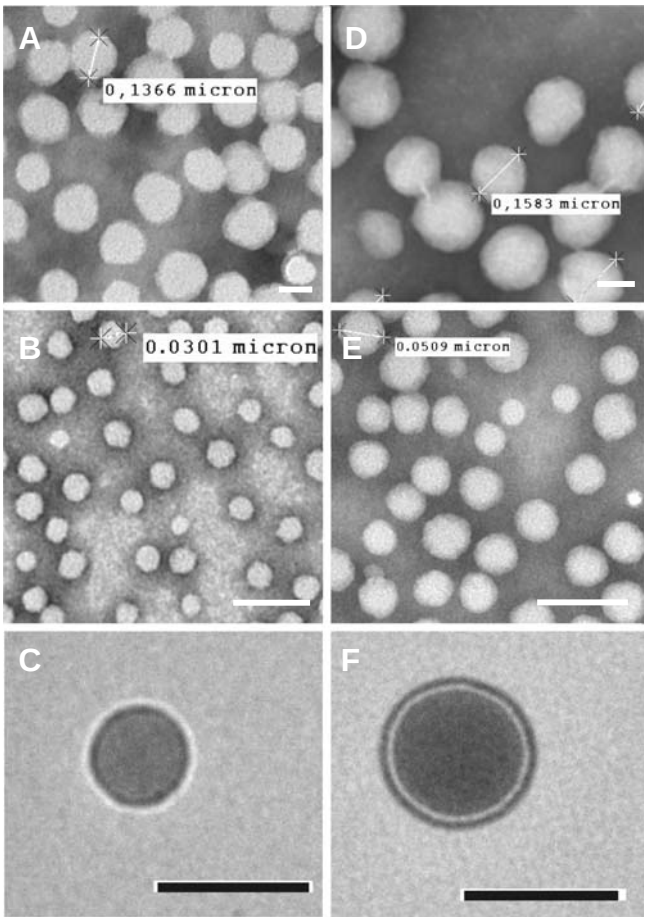

Fig. 2. TEM pictures of the nanoparticles stained with 1% aqueous phosphotungstic acid. PIBCA nanoparticles prepared with chitosan 20 kDa and 0 (*A*) and 3% (*B*,*C*) pluronic. PIHCA nanoparticles prepared with chitosan 20 kDa and 0 (*D*) and 3% (*E*,*F*) pluronic. The scale bar represents 100 nm.

In Vitro Cytotoxicity of the Nanoparticles on the RP1 Cells and Visualization of Nanoparticle Uptake by Cells

Fig. 5A shows the percentage of survival cells after incubation with the chitosan-coated PIBCA nanoparticles. From the survival curve, it can be deduced that the concentration in nanoparticles inducing 50% of cell growth inhibition was 750 μg/mL.

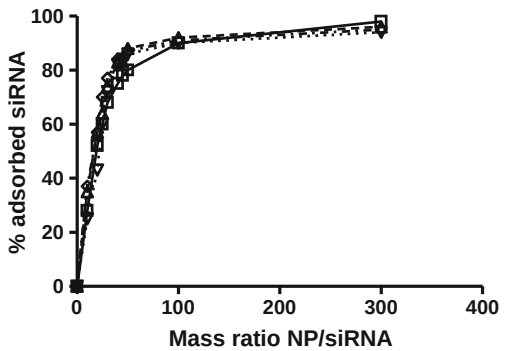

Fig. 3. siRNA adsorption isotherm on PIBCA or PIHCA nanoparticles prepared with chitosan 20 kDa in the absence (PIBCA (□), PIHCA (△) or in the presence of 3% pluronic (PIBCA 3% pluronic (▽), PIHCA 3% pluronic (◇). In this experiment, the concentration in siRNA was fixed at 0.5 μM.

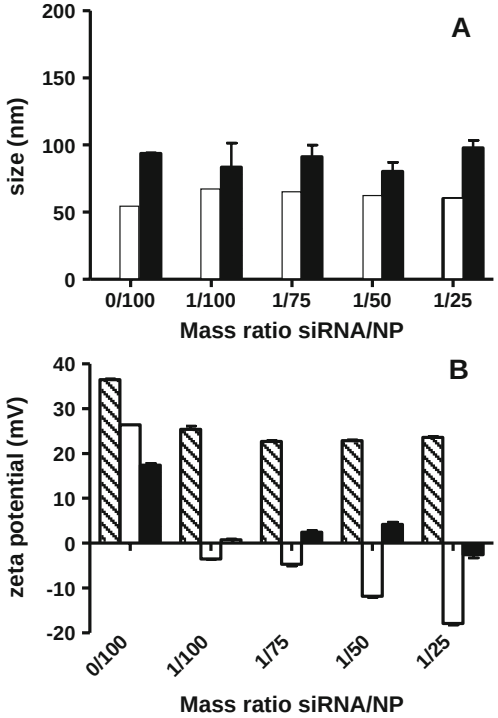

Fig. 4. Influence of siRNA on nanoparticle properties. Different mass ratio siRNA/nanoparticles were tested, keeping constant the concentration in siRNA to 0.5 μM. The highest nanoparticle concentration is on the left of the graphs. **A** Nanoparticle size. **B** Nanoparticle zeta potential. Nanoparticles of PIBCA, chitosan 20 kDa without pluronic (Hashed bars); nanoparticles of PIBCA, chitosan 20 kDa, and 3% pluronic (white bars); nanoparticles of PIHCA, chitosan 20 kDa, and 3% pluronic (black bars). In this experiment, pictures pluronic was added in the polymerization medium before starting the polymerization process (see materials and methods).

Confocal fluorescent microscopy was used to visualize the intracellular uptake of the siRNA-loaded nanoparticles by the RP1 cells. For this experiment, the nanoparticles were labeled with rhodamine, while non-labelled siRNA was adsorbed on the nanoparticle surface as described above. Fig. 5B shows that fluorescence due to the nanoparticles was concentrated in intracellular compartments located in the cytoplasm of the RP1 cells, indicating that the nanoparticles were taken up by the cells.

In Vivo Treatment of a RP1-Derived Tumor in Mice by ret/PTC1 siRNA As-Loaded Nanoparticles

Intratumoral Treatment

The graphs presented in Fig. 6 give the relative tumor volume measured over a period of 14 days after the beginning of the treatment by intratumoral administration of siRNA As-loaded nanoparticles. High values meant an important increase of the tumor volume. In contrast, a value of relative tumor volume remaining close to zero meant that the volume of the tumor did not increase during the time of the experiment. A reduction of the relative tumor volume compared to the control experiments performed by injection

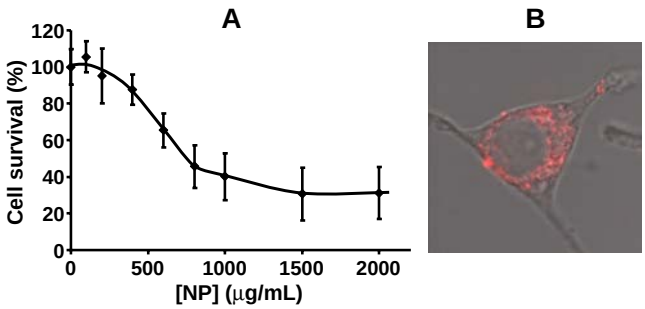

Fig. 5. Cell survival curve in the presence of increasing concentration in PIBCA nanoparticles prepared with chitosan 20 kDa and 3% pluronic (**A**) and intracellular penetration of siRNA As-loaded fluorescently labeled nanoparticles in RP1 cells (**B**).

of saline solution to mice meant that the treatment induced an inhibition of the tumor growth.

The curves presenting the evolution of the relative tumor volume with time showed that the increase of the volume of the tumor beard by mice which received the treatment of 1 mg/kg siRNA As associated with the nanoparticles was much lower than the increase of the tumor volume shown by control mice (Fig. 6A and B). This indicated that a significant inhibition of the tumor growth was observed in the animals treated by intratumoral injection of the formulation of siRNA As-loaded nanoparticles (cumulative siRNA dose of 1 mg/kg, i.e., 50 mg nanoparticles/kg). All other treatments failed to inhibit tumor growth (comparatively to the placebo injection of NaCl 0.9%), including intratumoral injection of free siRNA As at the same dose and of siRNA Ct-loaded nanoparticles. Fourteen days after the beginning of the treatment, siRNA As associated with PIBCA and PIHCA nanoparticles were able to inhibit the tumor growth by 64% and by 59%, respectively (P value <0.05 for each), in comparison with the untreated mice (Fig. 6C). Noteworthy, treatments of mice with a cumulative dose of 50 mg/kg of nanoparticles were well-tolerated as indicated by the behaviors of animals.

Results were very different considering the intratumoral administration of a higher cumulative dose of siRNA (5 mg/kg). Indeed, in this case, all curves showing the relative tumor volume were lower than the corresponding curve obtained for the untreated mice (Fig. 6D and E). At this dosage, empty nanoparticles used at a cumulative dose of 250 mg/kg also induced a reduction of the tumor volume compared with the placebo treatment (NaCl 0.9%). After 14 days, no difference was clearly found comparing the mice treated with the siRNA As or with the siRNA Ct, whether they were administered under the free form or associated with the nanoparticles (Fig. 6F). Moreover, the small difference observed by comparing the results was not statistically different.

Intravenous Treatment

The evolution of the relative tumor volume obtained after intravenous injection of different formulations of siRNA at a dose of 5 mg/Kg (i.e., 250 mg nanoparticles/kg) is presented in Fig. 7A. The relative tumor volume increased identically in all mice treated by systemic injections of the different control formulations, including physiological serum, free siRNA As, free siRNA Ct and nanoparticles loaded with the siRNA Ct. In contrast, a significant inhibition of the tumor growth was only observed after treatment performed with siRNA As loaded on PIBCA nanoparticles.

Fig. 7B and C highlight the differences observed on day 10. While tumor size of the control groups increased 10 times, tumors of the group treated with the PIBCA nanoparticles loaded with the siRNA As increased only 2 times ($P<0.01$). The Q-PCR analysis of the mRNA extracted from the tumor showed a reduction of the amount of the targeted mRNA in the experiment performed with the siRNA As associated with the chitosan-coated PIBCA nanoparticles (Fig. 8). The final relative tumor volume observed in the group of mice treated with the siRNA As loaded on PIHCA nanoparticles was lower than the relative tumor volume evaluated for the other control groups. However, the statistical analysis revealed that the difference found for this group was not different enough ($p>0.05$) compared with control experiments. These results indicated that only the siRNA As associated with the PIBCA nanoparticles were clearly able to treat the tumor after administration to mice by the intravenous route.

DISCUSSION

Physico-chemical Characterization of the Nanoparticles

The reduction in size of the nanoparticles observed by adding pluronic in the polymerization medium was in agreement with what could be expected by the addition of a surfactant in the emulsion polymerization medium of a monomer. Indeed, in general, addition of a surfactant in emulsion polymerization media improved the stability of smaller monomer droplets and of the nanoparticles which formed during polymerization. In the present system, size reduction of the nanoparticles may also be explained by the formation of a collapsed gel-like structure on the nanoparticle surface which may result from the interactions of chitosan with pluronic at the nanoparticle surface (32). Experiments showing that addition of pluronic on preformed nanoparticles did not induce dramatic reduction of the nanoparticle diameter indicated that there was no contribution of such an effect on the size reduction of the nanoparticles when they were synthesized in the presence of pluronic. On the contrary, it seems that the addition of pluronic after polymerization reduced the electrostatic repulsions occurring between the nanoparticles and induced aggregation thanks to Van der Waals attraction forces. Thus, the reduction in size of the nanoparticles observed when nanoparticles were synthesized in the presence of pluronic can be attributed to a higher stabilization effect of the emulsion template and of the formed nanoparticles by pluronic.

As indicated by results of zeta potential measurements and observations of the nanoparticle morphology by TEM, nanoparticles obtained in the presence of pluronic showed similar core-shell structures than previous nanoparticles prepared in the absence of pluronic. As the zeta potential of the nanoparticles prepared in the presence of pluronic remained mainly positive, it indicated that the nanoparticles were coated with chitosan. The small decrease in zeta potential observed for the nanoparticles prepared in the presence of pluronic can be attributed to the incorporation of some pluronic in the nanoparticle corona which remained

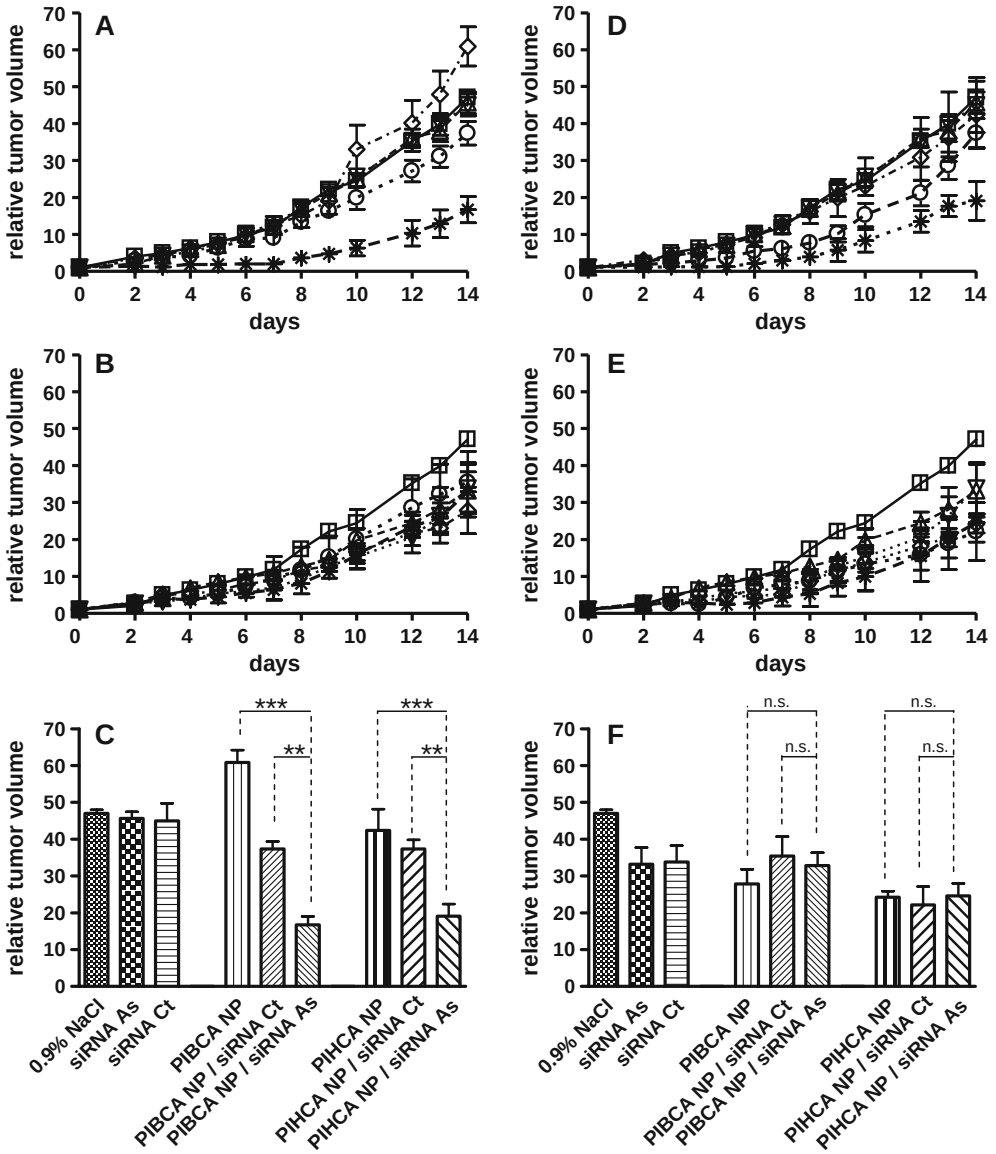

Fig. 6. Tumor growth in mice treated by intratumoral injection of siRNA As-loaded PIBCA (A, D) and PIHCA (B, E) nanoparticles. Either the control sequence Ct or the relevant sequence As were tested, associated or not with nanoparticles. The free nanoparticles corresponded to an injection of the same nanoparticle dose used for the complex siRNA/nanoparticles. □: NaCl 0.9 %, △: siRNA As, ▽: siRNA Ct, ◇: free nanoparticles, ○: siRNA Ct-loaded nanoparticles, *: siRNA As-loaded nanoparticles. **A** Relative tumor volume evolution after intratumoral treatment with PIBCA-based nanoparticles, at a cumulative dose of siRNA of 1 mg/kg. **B** Relative tumor volume evolution after intratumoral treatment with PIHCA-based nanoparticles, at a cumulative dose of siRNA of 1 mg/kg. **C** Relative tumor volume evolution after intratumoral treatment with PIBCA-based nanoparticles, at a cumulative dose of siRNA of 5 mg/kg. **D** Relative tumor volume evolution after intratumoral treatment with PIHCA-based nanoparticles, at a cumulative dose of siRNA of 5 mg/kg. C and F: Relative tumor volume obtained on day 14, after the five intratumoral injections showing the statistical analysis. **C** corresponds to the administration of a cumulative dose of siRNA of 1 mg/kg, and **F**, to the administration of a cumulative dose of 5 mg/kg. n.s. = no statistical difference; **: $p<0.01$; ***: $p<0.001$.

strongly associated with the nanoparticle surface even after purification by dialysis.

Adsorption of siRNA on the Nanoparticles

As indicated by the adsorption isotherms, siRNA can adsorb on chitosan-coated PIBCA and PIHCA nanoparticles prepared in the presence or in the absence of pluronic. The fact that the zeta potential of the nanoparticles is reduced after association of siRNA is in agreement with a mechanism of association of the siRNA with the nanoparticles via ion-pair formation. Ion-pairs can form between the negatively charged phosphate groups of the siRNA and the positively charged amino groups of chitosan which are accessible at the surface of the nanoparticles. This led to a progressive neutralization of the positive charges of the chitosan when the concentration of siRNA increased. The charge

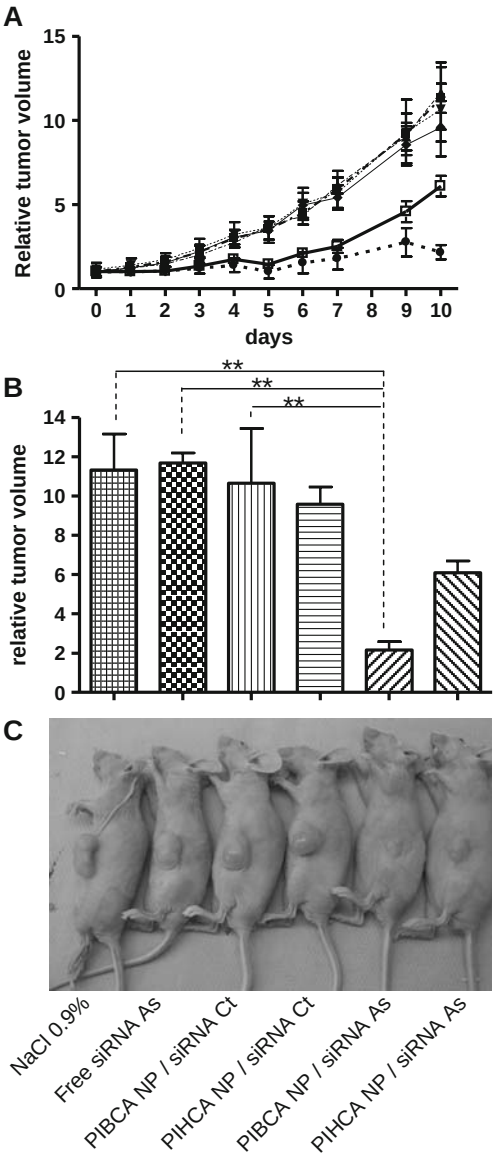

Fig. 7. Intravenous treatment of mice with nanoparticle formulations of siRNA. NP=nanoparticles. **A** Relative tumor size growth during treatment. The mice received a cumulative dose of siRNA of 5 mg/kg. The NaCl 0.9% (■), the free siRNA As (▲) and the siRNA associated with either PIBCA (□: siRNA As, ▼: siRNA Ct) or PIHCA (◆: siRNA As, ●: siRNA Ct) nanoparticles were tested. **B** Relative tumor growth obtained on day 10, after the end of the treatment. $^{**}p<0.01$. **C** Pictures of the mice at day 10, after the end of treatment. Note the small tumor size observed after treatment of the mice by the siRNA As-loaded PIBCA nanoparticles.

annealing phenomenon due to the association of the siRNA was amplified when the nanoparticles were prepared with pluronic.

This may be due to a location of the siRNA much on the surface of the chitosan corona when the nanoparticles were prepared with pluronic. This effect seemed to be further enhanced when the size of the nanoparticles was small, i.e. PIBCA nanoparticles.

The fact that all adsorption isotherms were superimposed was unexpected. This result suggests that the apparent surface area available for siRNA adsorption was the same for all nanoparticles. However, it is in contradiction with the fact that the different nanoparticles had a different diameter. Thus, nanoparticles of the smallest diameter should display a higher specific surface area—hence a much higher siRNA adsorption capacity. Two effects may contribute to the observed results. First, it can be considered that the adsorption of siRNA on the nanoparticle surface did not take place on a smooth surface but in the volume of the chitosan or of the chitosan-pluronic outmost. Second, pluronic may hinder the accessibility of siRNA with the positive charges of chitosan, especially those located close to the nanoparticle core. Since the smaller nanoparticles are prepared with pluronic, the hindrance effect can be the more pronounced on these nanoparticles. Additionally, this effect, which can compromise interactions of siRNA with chitosan of the whole volume of the nanoparticle corona, can dramatically limit the amount of siRNA which can be loaded on the nanoparticles. This effect is in agreement with the results of zeta potential, which suggested that siRNA were interacting with the positive charges of chitosan located at the surface of the outmost of the nanoparticles when they were prepared with pluronic.

Cytotoxicity and Intracellular Uptake

As shown by the confocal microscopy, the nanoparticles could penetrate in the RP1 cells without causing major problem of cytotoxicity. Indeed, compared with other works, the cytotoxicity found for the PIBCA nanoparticles developed in the present study was low (33,34). It is noteworthy that the negative charge of the siRNA-loaded nanoparticle surface did not compromise the intracellular uptake of the nanoparticles by the RP1. Although contradictory to what could be expected with nanoparticles and cells bearing surface charges of same sign, this result was not surprising because such a behavior was already reported with other types of nanoparticles and cells (21,35). The fact that the nanoparticles can penetrate in the RP1 cells suggests that they can deliver the siRNA inside the cells. This was not

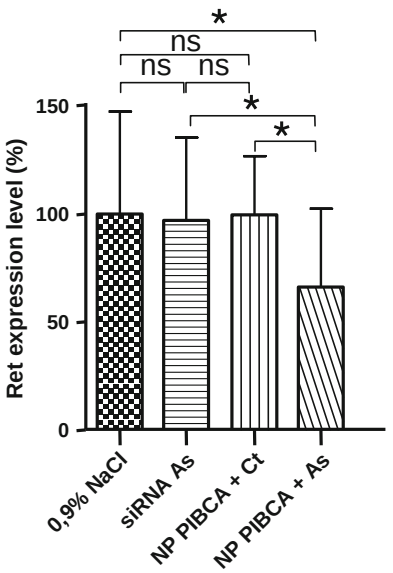

Fig. 8. Q-PCR analysis of the expression of the mRNA targeted by the siRNA As after intravenous administration to mice. Results were expressed as a percentage of the expression of RET mRNA against 18 S RNA. ns: non significatif, * significative difference with $p<0.5$.

directly demonstrated in the experiment because the siRNA was not labeled. However, an indirect proof was given by an experiment performed with a fluoresceine-labeled antisense oligonucleotide loaded on the same type of nanoparticles. After incubation of the double-labeled formulation with the RP1 cells, the fluorescence due to the nanoparticles was found in the intracellular compartments as in the previous experiment, while the fluorescence associated to the antisense oligonucleotide appeared in the cell nucleus (data not shown). This clearly indicated that the chitosan-coated nanoparticles developed in this work can deliver a short fragment of nucleic acid inside the RP1 cells.

In Vivo Delivery of siRNA Associated with Nanoparticles

The activity of siRNA designed against the mRNA of the ret/PTC1 fusion oncogene was evaluated *in vivo* after association with chitosan-coated nanoparticles on a model of tumor implanted in nude mice. The experiments were performed with the smaller nanoparticles including PIBCA and PIHCA nanoparticles prepared with chitosan 20 kDa and 3% pluronic. Both types of nanoparticles showed similar surface properties regarding their siRNA adsorption. Tumor-bearing mice were treated first by intratumoral injection of siRNA formulated as nanoparticles to verify that the siRNA can be delivered under its active form in the tumor. The main interest of intratumoral injections is that the total dose of the injected formulation reaches the tumor tissue. Indeed, this method avoids dilution of the drug formulation outside the tumor and bypasses all kinds of barriers that can hinder the delivery of the active compound in the target tissue. It is noteworthy that it is not a relevant route of administration for drug in anticancer clinics. Thus, another series of experiments considering the administration of the siRNA by intravenous injection was also considered in this study.

Intratumoral Treatment

siRNA As associated with PIBCA and PIHCA nanoparticles and administered at the cumulative dose of 1 mg/kg were the only formulations which were able to induce a significant inhibition of the tumor growth. As shown in a preliminary work, this effect was specific to the antisense activity of the siRNA As because it was accompanied by a high level of down regulation of the expression of the ret/PTC1 gene (23). Both types of nanoparticles led to the same level of inhibition of the tumor growth, indicating that there was no effect of the nature of the polymer constituting the core of the carrier on their capacity to deliver the active siRNA in the tumor cells.

Treatments considering a higher cumulative dose of siRNA led to unexpected results. All formulations, including both the siRNA associated with the nanoparticles, the nanoparticles alone and the free siRNA showed the same reduction of tumor growth compared with the animals which received injections of a sodium chloride solution. Moreover, there was no difference in the inhibition of the tumor growth between the siRNA As and the siRNA Ct associated with the nanoparticles. This indicated that the reduction of relative tumor volume monitored in this experiment was not specific to the siRNA As in contrast to what was observed at a lower cumulative dose of injected siRNA. The fact that the free siRNAs and the control empty nanoparticles also produced an inhibition of the tumor growth indicated that the reduction observed with this injected cumulative dose was due to toxic effects. These toxic effects may be due to the fact that a quite large concentration of nanoparticles (250 mg/kg) and siRNA (5 mg/kg) were directly injected in the tumor.

Systemic Treatment

After intravenous injection, the injected formulation can distribute in the whole body of the mice. To counterbalance this effect, which undeniably induced a dilution of the formulation, a cumulative dose of 5 mg/kg (siRNA) was injected in the retroorbital vein of the mice. The results showed that the intravenous injection of the free siRNA As was not able to reduce tumor growth. This was in agreement with the fact that the siRNA As did not show any activity when it was administered directly in the tumor under the free form. This result also agreed with the known difficulties of the *in vivo* delivery of nucleic acids making their development as therapeutic agents challenging (23).

The only formulation which induced a clear and significant reduction of the tumor growth corresponded to the siRNA As-loaded PIBCA nanoparticles. Results from the analysis of the mRNA extracted from the tumoral tissue by Q-PCR indicated that the tumor growth reduction was accompanied with a reduction of the amount of the mRNA targeted by the siRNA As (Fig. 8). This result revealed that the observed tumor growth reduction occurred thanks to a specific action of the siRNA As. Also interesting was that the PIBCA nanoparticles were able to transport the active siRNA As from the intravenous administration site (retro-orbital vein) down to the tumoral tissue implanted in the right flank of the mice. The results also showed that the nanoparticles were able to deliver the active siRNA As in the tumor at a concentration reaching the therapeutic level and that they allowed the siRNA to reach the intracellular target after being administered by an intravenous route.

Intravenous treatment performed with the siRNA As formulated in the PIHCA nanoparticles was less efficient than the treatment considering the siRNA As loaded on the PIBCA nanoparticles. Indeed, it did not induce a significant reduction of tumor growth compared with the control experiments. Several hypotheses can be raised to explain the superiority of the PIBCA nanoparticles to deliver active siRNA to the tumor after intravenous injection. The diameter of PIBCA nanoparticles (62 nm) was much lower than that of PIHCA nanoparticles (94 nm). In solid tumors, the enhanced permeability and retention effect is a key mechanism involved in the passive targeting of drug carriers to tumor (36). The increased permeability of blood vessels in tumor tissue is believed to allow passage of small particles with the maximum diameter of about 100 to 300 nm (28). However, the maximum size of the particles retained by tumors thanks to the enhancing permeability and retention effect can be affected by many parameters, including the type of the tumor, its age and the site of implantation. Thus, it can be suggested that the diameter of the PIHCA nanoparticles, which was close to 100 nm, was too large to allow these nanoparticles to escape the blood compartment and to distribute in the model

tumor, we have used in this work. In contrast, PIBCA nanoparticles which had a much lower diameter could cross the blood epithelium and accumulate more easily in the tumor, delivering the siRNA As in the diseased tissue (Fig. 9). Because of the difference in size between the two types of nanoparticles, the total number of injected particles was 2.3 times higher in the case of the PIBCA nanoparticles than the total number of nanoparticles injected in the case of the PIHCA nanoparticles (Table I). Thus, statistically, a much larger number of PIBCA nanoparticles may be distributed in the tumor compared to the number of PIHCA nanoparticles. The size of PIBCA nanoparticles and the number of injected nanoparticles are two factors which can contribute together to explain the superiority of the *in vivo* performance of the PIBCA nanoparticles to deliver the siRNA to the tumor after intravenous administration (Fig. 9). The amount of siRNA delivered by one nanoparticle to the tumor may be another factor which can explain the difference in efficacy observed between the two types of nanoparticles. Indeed, it can be calculated that the loading in number of siRNA per PIHCA nanoparticle is 2.3 times larger than the loading of the PIBCA nanoparticles (Table I). From this calculation, it can be expected that PIHCA should be a more interesting carrier regarding the payload. However, the factors of differences between nanoparticle size and loading in siRNA compensated each other, and because PIBCA nanoparticles appeared superior, this superiority can better be attributed to a size effect than to the payload of a single particle.

From another point of view, the pharmacokinetic and the biodistribution of nanoparticles are generally greatly influenced by the physico-chemical properties of the nanoparticle surface. In the present case, the zeta potential of the siRNA-loaded PIBCA nanoparticles, which was negative, was different from the zeta potential of the corresponding PIHCA nanoparticles, which was slightly positive. It can be expected that this parameter may impose very different pharmacokinetics and biodistribution between the two types of nanoparticles. The very small size and the negative zeta potential of the PIBCA nanoparticles are favorable parameters for these nanoparticles to escape uptake by macrophages of the mononuclear phagocyte system and to remain in the systemic circulation without being recognized by the defense mechanisms of the host organism. Therefore, they can reach tumoral tissue and accumulate there thanks to the retention permeation effect.

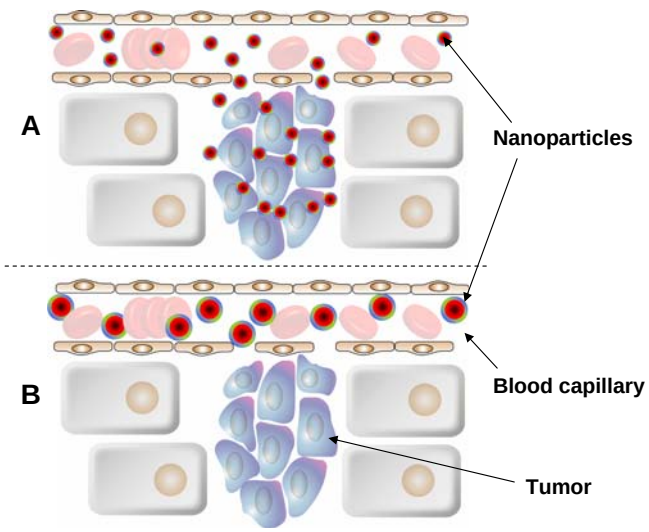

Fig. 9. Scheme illustrating the uptake of the nanoparticles in the tumoral tissue from blood capillaries thanks to the enhancing permeability and retention effect. (**A**) siRNA-loaded PIBCA nanoparticles, (**B**) siRNA-loaded PIHCA nanoparticles.

Table I. Comparison of the Properties of the PIBCA and PIHCA Nanoparticles Loaded with siRNA Which were Administered to Mice by Intravenous Administration

Parameter	PIBCA nanoparticles	PIHCA nanoparticles
Diameter (nm)	60.9±0.5	82±11
Zeta potential (mV)	−11.8±0.5	+ 4.3±0.9
Number of injected nanoparticles	18.8 10^{14}	8.0 10^{14}
Number of injected siRNA molecules	2.26 10^{17}	2.26 10^{17}
Number siRNA/nanoparticle	120	280

Thus, it can be proposed that the most probable mechanisms which can explain the superior efficacy of the PIBCA nanoparticles to deliver siRNA to the tumor after intravenous administration compared with PIHCA nanoparticles is linked to both their capacity to escape macrophage uptake and their small size, which allow them to leave the blood compartment at the tumor level where they can accumulate in the tumor tissue.

CONCLUSION

In this work, we have developed a new carrier for siRNA delivery, and we have demonstrated its efficacy to deliver active siRNA to tumor after intravenous administration to ret/PTC1 tumor-bearing mice model.

The formulation of this new carrier made of poly(alkylcyanoacrylate) nanoparticles coated with chitosan was optimized in order to provide nanoparticles of a very small size (down to 60 nm in diameter). The nanoparticles were loaded with siRNA through interactions with the chitosan corona located at the nanoparticle surface. Only the formulations of the siRNA As loaded on the PIBCA and PIHCA nanoparticles were able to induce a clear reduction of the tumor growth after intratumoral administration. This demonstrated the requirement of the drug carrier to access a successful *in vivo* delivery of the siRNA. Increasing the injected dose by intratumoral administration led to the appearance of a non-specific reduction of tumor growth, which was attributed to toxic effect due to either the nanoparticles or the siRNA. A very important result brought by this study was that the PIBCA nanoparticles were also able to deliver a therapeutic dose of a biologically active siRNA in the tumor after intravenous administration. This demonstrated that the PIBCA nanoparticles can carry active siRNA and deliver active dose of siRNA to tumor nodules from the systemic circulation. Results obtained from the intratumoral administration did not allow for anticipating the fact that the PIBCA nanoparticles were the more efficient carrier for the siRNA delivery to the tumor after intravenous administration. This result emphasized the fact that experiments considering administration of formulated siRNA by the intravenous routes are

required to validate proof of concept for *in vivo* delivery strategies of nucleic acids.

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