Long-Circulating Nanoparticles Bearing Heparin or Dextran Covalently Bound to Poly(Methyl Methacrylate)

Catherine Passirani,[1] Gillian Barratt,[1] Jean-Philippe Devissaguet,[1] and Denis Labarre[1,2]

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Purpose. In a biomimetic approach to the development of drug carriers escaping early capture by phagocytes, nanoparticles made of amphiphilic copolymers of either heparin or dextran and methyl methacrylate were evaluated relative to their *in vivo* blood circulation time. They were compared to bare PMMA nanoparticles.

Methods. Owing to the fluorescent properties of the covalently attached N-vinyl carbazole, the particles could be detected directly in mouse plasma. Samples were drawn at different time intervals and fluorescence was recorded.

Results. After an initial phase of elimination from the blood with a half-life of 5 h, the remaining heparin nanoparticles circulated for more than 48 h and were still detectable in the plasma at 72 h. Dextran nanoparticles were also eliminated very slowly over 48 h. Bare poly (methyl methacrylate) nanoparticles were found to have a half-life of only 3 min.

Conclusions. Both types of nanoparticles proved to be long-circulating. The potent capacity for opsonisation of the poly(methyl methacrylate) core were hidden by the protective effect of either polysaccharide, probably due to a dense brush-like structure. In the case of heparin nanoparticles, the "stealth" effect was probably increased by its inhibiting properties against complement activation.

KEY WORDS: nanoparticles; heparin; dextran; poly(methyl methacrylate); blood elimination; long-circulating.

INTRODUCTION

The development of colloidal systems as efficient drug carriers is limited by their distribution within the organism. It is now well established that after intravenous administration nanoparticles are rapidly opsonized by plasma proteins. These absorbed proteins or bound protein fragments promote recognition and uptake by cells of the mononuclear phagocyte system (MPS), particularly the Kupffer cells of the liver (1). The activation of complement, especially by the alternative pathway, plays an important, but probably not exclusive, role in the processes of opsonization and phagocytosis.

Two approaches to the problem of reducing opsonization have been envisaged. The first one is to create a steric barrier around the particles, able to repel the proteins, providing that the density of the "brush" is sufficient. The theoretical conditions for obtaining such an effect have been modelized by Jeon, *et al.* (2) and studied experimentally over the last years by several authors in the case of liposomes (3) or nanoparticles (4–7). For example, Tröster, *et al.* (8) studied the body distribution of surfactant-coated and uncoated poly(methyl methacrylate) (PMMA) nanoparticles. All surfactants (poloxamers / poloxamines, polysorbates and polyoxyethylene lauryl ether) reduced the liver accumulation and increased the uptake in other parts of the body. Some of them were especially effective in reducing the liver accumulation and maintaining significantly higher blood levels. The adsorption of plasma proteins has also been studied as a function of the steric barrier (9). The second approach can be described as biomimetic because it seeks to imitate cells and pathogens which avoid phagocytosis by reducing or inhibiting the activation of complement. One example is the development of liposomes with the ganglioside GM1 (10) incorporated into the membrane, which have shown long-circulating properties.

Other compounds are able to modulate the action of complement. For example, heparin, known as an anticoagulant, has also been shown to inhibit complement activation at different stages (11–14). Binding of heparin to surfaces has been suggested to be a way of improving blood-compatibility of surfaces by increasing the local activity of inhibitory proteins controlling coagulation and complement activation (15–19). Dextran, another polysaccharide, has different effects depending on its conformation. Indeed, some fractions of this neutral sugar, located in the middle range of molecular weight, are used as a plasma expander. However, when crosslinked (Sephadex®), dextran has been shown to be a strong activator of the alternative pathway of complement (20) and this activation was found to be modulated by anti-dextran antibodies whose presence varied between individual donors (21,22).

In a previous paper (23), nanoparticles (Nanohep) made from copolymers of heparin covalently bound to PMMA have been shown to retain on their surface at least some of the complement-inhibiting properties of soluble heparin. Nanoparticles (Nanodex) made from dextran-PMMA copolymers have been shown to be weak activators of complement and to behave more like soluble dextran than like crosslinked dextran, i.e. Sephadex®. For instance, 50% consumption of complement occured in the *in vitro* test system in the presence of 1200 cm^2 of Nanodex particles in contrast to 50 cm^2 of crushed G25 Sephadex® beads. In the Nanohep and Nanodex particles, the potent activating properties of the PMMA core are hidden by the protective effect of both polysaccharides, which is hypothesized to be due to the presence of a dense brush-like layer on the surface of both types of particles. In the case of Nanohep, this effect is probably increased by the inhibiting properties of the bound heparin.

In the present work, the *in vivo* behavior of these two kinds of nanoparticles has been investigated and compared with that of bare PMMA nanoparticles. In order to detect the particles in blood samples, fluorescent particles have been prepared. The fact that PMMA is a non-biodegradable polymer is an advantage for following the fate of the particles *in vivo*. So, a fluorescent label which could be covalently linked to the core of the nanoparticles was incorporated. N-Vinyl carbazole (NVC), a powerful charge donating monomer, is known to copolymerize with methyl methacrylate (MMA) by a free radical chain reaction

[1] Laboratoire de Physico-Chimie, Pharmacotechnie et Biopharmacie, URA CNRS 1218, Faculté de Pharmacie, Université Paris XI, 5 rue J.B. Clément, 92296 Châtenay-Malabry, France.

[2] To whom correspondence should be addressed.

ABBREVIATIONS: MMA, methyl methacrylate; PMMA, poly (methyl methacrylate); NVC, N-vinyl carbazole; s.d., standard deviation.

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in different solvents (24, 25). The synthesis was adapted to an aqueous medium and the fluorescent nanoparticles were injected into mice to determine their rate of elimination.

MATERIALS AND METHODS

Preparation and Characterization of the Fluorescent Nanoparticles

The detailed synthesis of Heparin-PMMA-NVC (Nanohep*) and Dextran-PMMA-NVC (Nanodex*) nanoparticles will be described elsewhere. Briefly, amphiphilic copolymers were synthesized in 32 ml of 0.2 M nitric acid aqueous medium at pH = 1 by copolymerization of a mixture of MMA (2 ml) (Fluka) and NVC (Fluka) (5% in weight/MMA) on either heparin (0.55 g) (heparin sodium salt from porcine intestinal mucosa, average molecular weight 16,000–17,000, Sigma) or dextran (0.55 g) (produced by *leuconostoc mesenteroides*, average molecular weight 70,000, Sigma).

The incorporation of NVC in the three kinds of nanoparticles suspended in water was first evaluated by recording their excitation and emission fluorescence spectra. For each suspension, we obtained the maxima of excitation and emission at λ_{ex} = 335 nm and λ_{em} = 363 nm respectively. No peak was found at 420 nm, indicating no excimer formation and, therefore, the presence of isolated NVC units. During the synthesis of Nanohep* particles with increasing proportions of added NVC, increasing amounts of precipitate were formed. Thus, it was decided to use for the present study the suspensions obtained from the copolymerization of monomer mixtures containing 5% of NVC (Nanohep*, Nanodex* and PMMA*), which gave a low level of precipitation and sufficient sensitivity in fluorimetric measurements.

Polymerization was initiated by polysaccharide radicals produced in the presence of cerium IV ions (8 ml at 8.10^{-2} M in 0.2 M HNO_3). At these concentrations, the copolymers spontaneously formed submicronic particles, which were stable in the absence of added surfactant. The particle suspensions were dialysed four to six times against water, in the presence of sodium citrate to remove cerium ions, unreacted monomers and polysaccharide fragments, in Spectra/Por 4 dialysis bags (MWCO 12 to 14,000). Under these conditions, the molecular weight of heparin and dextran incorporated into nanoparticles were respectively 7,100 and 28,500. They were then filtered on 0.45 μm filters (Millipore), in order to eliminate any precipitated material, which occurred mainly with Nanodex*. PMMA-NVC (PMMA*) nanoparticles were synthesized in aqueous suspension (26). After the addition of the monomer mixed with NVC (5% in weight/MMA), the polymerization was initiated by addition of potassium peroxymonosulfate (Sigma) and heating to 70°C. Submicronic particles, stable in the absence of added surfactant, were also spontaneously formed. The yield of nanoparticles was determined by measuring the dry weight of an aliquot of the suspension after dialysis. The nanoparticles were stored at 4°C without freeze-drying. Freshly prepared nanoparticles were used for *in vivo* experiments.

The mean diameter of the particles was determined by quasi-elastic light scattering using a Nanosizer N4MD (Coultronics, Margency, France). The zeta potential of the particles dispersed in 20 mM phosphate buffer, pH 7.0, was measured using a Zetasizer 4 (Malvern Instruments, Orsay, France).

Measurements of Fluorescence

The spectral characteristics of the different nanoparticles suspended at increasing concentrations (0–100 mg/l), either in water or in diluted mouse plasma, were determined using a SPEX 1681 Fluorolog spectrofluorimeter in the ranges 250–350 nm and 340–440 nm respectively for excitation and emission spectra. The intensities at the maxima of excitation and emission were recorded at 335 nm and 363 nm respectively. The plasma samples were prepared as follows: 120 μl of blood were added to 1.680 ml of isotonic mixtures of saline and nanoparticles and centrifuged to remove cells (10 min, 2500 rpm at 6°C, in a Jouan-Paris K-63F centrifuge); 1.5 ml of the supernatant were used for analysis.

The range of concentrations of the particles were chosen to correspond with the possible range of concentrations of the fluorescent particles that might be obtained after injection to mice and blood sampling: it was intended to inject into mice weighing 20–22 g, with a blood volume of approximately 2 ml, a maximum dose of 150 mg/kg (0.2 ml of a suspension at 15 mg/ml). Each blood sample would have a volume of 120 μl. After addition to 1.680 ml of normal saline, the maximum concentration that could be obtained would be about 100 mg/l.

Determination of the *In Vivo* Blood Circulation Time

Female CD1 mice, weighing 20–22 g were obtained from Charles River, France. The research adhered to the "Principles of Laboratory Animal Care." Suspensions containing the fluorescent nanoparticles at concentrations 15 and 5 mg/ml were prepared extemporaneously in isotonic glucose solution. Under anaesthesia, 0.2 ml of each suspension was injected into the tail vein of the mice (respective approximate doses 150 or 50 mg/kg). Blood samples (120 μl) were drawn from the retro-orbital sinus at various time intervals with an heparinized Pasteur pipette and immediately diluted in 1.680 ml of normal saline. Since the blood samples taken were small, each mouse could be used for a range of time points. After centrifugation, excitation at 335 nm, and emission at 363 nm was measured in 1.5 ml of the supernatant. As shown in the previous section, the fluorescence associated with the nanoparticles could be determined in the diluted plasma samples without any extraction step. The values reported on the graphs are the mean of 3 to 5 animals.

RESULTS AND DISCUSSION

Physico-Chemical Characteristics of the Suspensions

Copolymers of heparin or dextran with PMMA and NVC, a fluorescent monomer, were prepared. These amphiphilic copolymers were able to self-assemble into nanoparticles in the absence of surfactant. Table I shows the properties of the different suspensions. In contrast to the particles prepared in the absence of NVC, the three kinds of nanoparticles prepared in the presence of NVC were slightly colored. They were monodisperse in size and remained stable in suspension for several weeks. However, Nanodex* was less stable than the other two and started to precipitate within approximately one month. The results of zeta potential measurements and the stability of the suspensions suggested that Nanohep* and Nanodex* were organized with the polysaccharidic moieties on the surface and the

Table I. Properties of Nanoparticle Suspensions

Name of nanoparticles	Polymer composition	Mean diameter (nm ± s.d.[a])	Zeta potential (mV ± s.d.)
Nanohep*	Heparin-PMMA-NVC	160 ± 10	−51.2 ± 1.3
Nanodex*	Dextran-PMMA-NVC	240 ± 15	−8.1 ± 0.7
PMMA*	PMMA-NVC	160 ± 10	−68.9 ± 1.3

[a] s.d.: standard deviation.

more hydrophobic block (PMMA-co-NVC) in the core. The very negative zeta potential of PMMA* nanoparticles showed that the sulfate groups resulting from decomposition of the initiator were on the surface.

Firstly, the incorporation of the fluorescent compound into the nanoparticles was verified. Many pieces of evidence demonstrate this fact: the particles were colored and fluorescent, with maxima of excitation and emission respectively at 2_{ex} = 335 nm and 2_{em} = 363 nm; they were larger than the non-labeled particles and, at least in the case of Nanodex*, were less stable than the non-labeled particles. The variation of the fluorescence of Nanohep*, Nanodex*, and PMMA* was first studied as a function of the concentration of the nanoparticles in water (between 0 and 100 mg/l). The maximum intensities measured in emission and excitation spectra were similar for each preparation and varied in a linear relationship with concentration. However, the slopes were very different between the three suspensions: about $8.2.10^3$, $4.8.10^3$, and $1.2.10^3$ cps.mg^{-1}.l for Nanohep*, PMMA* and Nanodex* respectively.

Then, the fluorescence of the nanoparticles were studied in the same range of concentration in diluted mouse plasma. Linear relationships were again obtained, but the slopes were lower than those obtained for the same batch in water: respectively, $5.8.10^3$, $3.3.10^3$, $0.7.10^3$ cps.mg^{-1}.l for Nanohep*, PMMA*, and Nanodex*. Moreover, fluorescence emission spectra were found to be contaminated by light scattering, inducing a slight deformation of the spectra. To avoid this drawback and because excitation and emission spectra gave the same results in terms of quantification of NVC, it was chosen to use only the intensity maximum of the excitation spectrum for the *in vivo* study. Under these conditions, diluted plasma without nanoparticles gave an intensity of 3000 cps.

These results showed that the concentration of the particle suspensions could be quantified directly without extraction, both in water and in diluted blood plasma, at concentrations corresponding to those expected *in vivo*. They demonstrated the differences in excitation and emission intensity between the three kinds of suspensions and as a function of the medium examined (water or diluted plasma). It is noteworthy that Nanohep* and Nanodex* were synthesized in the same way, but gave very different signals intensities; Nanohep* being much more fluorescent. PMMA* gave results which fell between the other two in terms of intensity. This could mean that the presence of a polysaccharide significantly modified the copolymerization of MMA/NVC and that heparin and dextran did not act in the same way.

Another result was that the signal obtained in plasma was much lower than that recorded in water because of the influence of the various components of the plasma. In conclusion to the spectral studies, we have shown that the fluorescence intensities in diluted plasma were proportional to the concentration of the nanoparticles in a given batch but, while Nanohep* and PMMA* gave high signals even at low concentrations, the limits of detection (under 3000 cps) would rapidly be obtained with Nanodex*. Significant variations of fluorescence observed between different batches allowed us to express the results only in terms of counts per second and not in terms of amounts.

Blood Circulation Time

The hydrophobic core made of a non-biodegradable and fluorescent copolymer allowed us to follow the *in vivo* fate of these nanoparticles after intravenous injection into mice. Firstly, the three kinds of suspension were injected into mice at a dose of 150 mg/kg and blood samples were drawn at 2, 30, 60, 120 and 240 min after injection in the case of Nanohep* and Nanodex* and at 0.5, 2, 4, 8, 12 min for PMMA*. The mice did not bleed abnormally during or after the experiments, even with Nanohep* containing heparin which is a potent anticoagulant. The results are shown in Fig. 1. A dramatic difference of behavior between PMMA* and the two other formulations was demonstrated. No PMMA* could be detected 30 minutes after injection, while a substantial proportion of Nanohep* and Nanodex* was still circulating at 4 h. The half-life calculated for PMMA* was 3 min. They represented an appropriate control for Nanohep* and Nanodex* since they had the same hydrophobic core and fluorescent marker and were of a similar size. The difference in intensity levels between Nanohep* and Nanodex* was simply due to the lower fluorescence intensity of the latter suspension. A slight and continuous decrease was found for Nanohep*, whereas a small increase of intensity up to one hour followed by a decrease similar to Nanohep* was found for Nanodex*. These results led us to examine what happened at longer times for Nanohep* and Nanodex*.

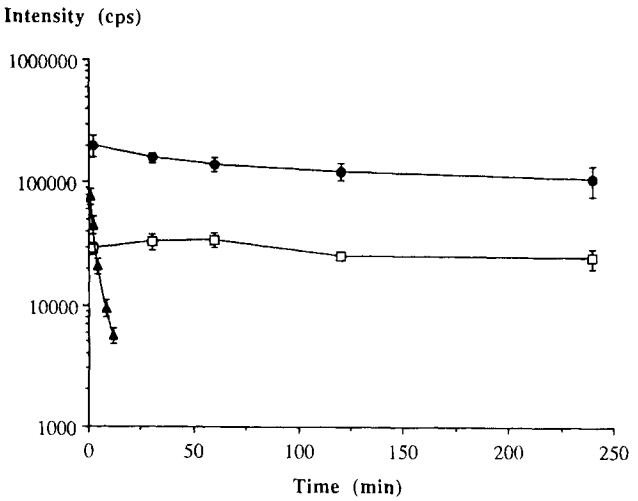

Fig. 1. Plasmatic concentration versus time profile at short times. 0.2 ml of suspensions in saline of the three kinds of nanoparticles (●: Nanohep*; □: Nanodex*; ▲: PMMA*) were injected into the tail vein of Swiss mice, weighing 20–22 g, at the dose of 150 mg/kg. Blood samples were drawn at various intervals and processed as described in Materials and Methods. The results are the mean of 3 to 5 mice.

Fig. 2 shows the plasma concentration versus time profiles of Nanohep* and Nanodex*, at a dose of 150 mg/kg, up to 48 h. After a sharp decrease for Nanohep* and a small increase followed by a decrease for Nanodex* during the first 5 h, both types of particles presented a very slow rate of elimination. Their presence could still be quantified in the blood 48 h after their injection and a significant amount of Nanohep* was still detected at 72 h.

The fast and complete disappearance of PMMA* injected at 150 mg/kg showed that the MPS was not saturated by this dose. Therefore, the long-circulating properties of Nanohep* and Nanodex* at this dose were not due to saturation of the MPS. To determine whether the initial sharp elimination of Nanohep* followed by a slow elimination could be due to uptake and saturation of another cellular compartment, these particles were also injected at a dose of 50 mg/kg. This experiment was impossible with either PMMA*, because their uptake was too fast even at 150 mg/kg, or Nanodex*, because the initial fluorescence intensity was already too low at 50 mg/kg. In order to compare the profiles of elimination from the blood of Nanohep* at 50 and 150 mg/kg easily, the results of intensity were normalized by taking the intensity measured at 4h as 100 in both cases. It can be seen in Fig. 3 that during an initial period of 8 h, the rate of elimination of the two doses was identical. In the case of 50 mg/kg, the same elimination rate was maintained for 24 h, after which time the particles could not be quantified. A logarithmic regression analysis allowed us to calculate a half-life of 5 h for the lower dose. This is much longer than the half-lives reported in the literature for unmodified polymeric nanoparticles, for example, polyalkylcyanoacrylate (27) or polystyrene (28), and very similar to that observed for nanoparticles prepared from poly(lactic acid)–poly(ethylene glycol) (29). In the case of 150 mg/kg, the elimination rate was reduced after these first 8 h.

When a larger dose of Nanohep* was given, the initial uptake compartment seemed to be saturated and the remaining particles disappeared extremely slowly. We do not yet know the nature of the compartment responsible for this elimination. The half-life of 5 h and the results obtained with the PMMA* nanoparticles suggest that it is not the MPS. It is known that

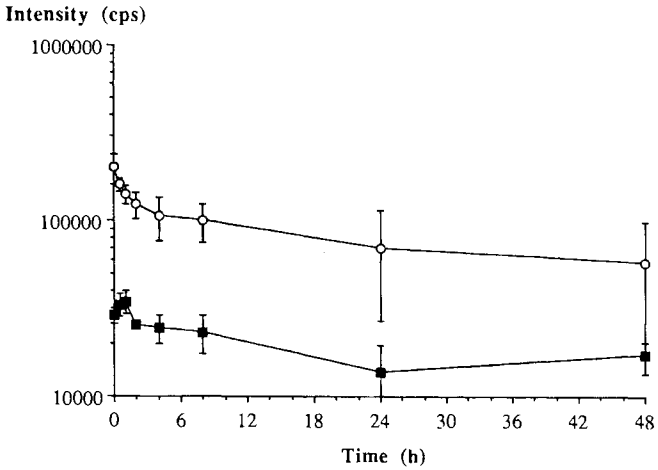

Fig. 2. Plasmatic concentration versus time profile at long times. The two kinds of nanoparticles (○: Nanohep*; ■: Nanodex*) were injected and treated in the same experimental conditions as in Fig. 1.

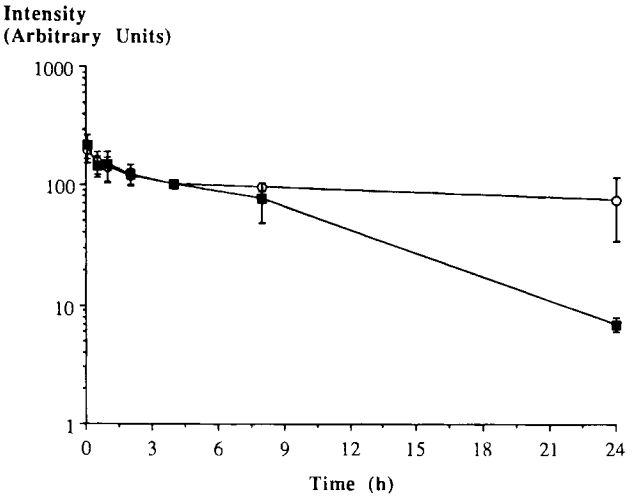

Fig. 3. Effect of dose on plasma elimination of Nanohep* injected at doses of 150 mg/kg (○) and 50 mg/kg (■). The results were normalized by taking the intensity measured at 4h as 100 in both cases. Same treatment as in Fig. 1.

vascular endothelial cells express receptors for heparin (30), so it is tempting to hypothesize that such cells could bind and perhaps internalize Nanohep*, but this remains to be investigated experimentally.

The slow disappearance of Nanodex*, depicted in Fig. 1 and 2, is somewhat unexpected. Our previous *in vitro* experiments indicated that these particles were activators of complement and would therefore be expected to disappear from the circulation rapidly. However, Nanodex was a much weaker activator of complement than Sephadex® at equivalent surface area—i.e. about 24 times—and behaved more like soluble dextran. In addition, despite having a surface charge close to neutrality, Nanodex* remained in suspension without aggregation for a few weeks. Both facts suggested a brush-like organization of the polysaccharide chains on the surface, providing a steric barrier analogous to that of poly(ethylene glycol). The behavior during the first hour (increase in fluorescence followed by a decrease) was also surprising but very reproducible. Our hypothesis is that it could be due to a reversible aggregation or capture and release after injection.

CONCLUSIONS

In conclusion, the aim of this work was to examine the *in vivo* elimination from the blood of a new type of nanoparticles bearing heparin or dextran covalently bound to random copolymers of MMA and NVC. It has been previously demonstrated that such particles were able to limit complement consumption *in vitro*, in contrast to bare PMMA nanoparticles (23), suggesting that the potent complement-activating properties of the PMMA core were hidden by the protective effect of either polysaccharide. In the case of Nanohep*, this effect is probably increased by the inhibiting properties of bound heparin versus complement activation. Therefore, opsonization by complement, which is one of the most important limitations to the development of "stealth" particles, would be reduced or inhibited, and long-circulating properties could be expected *in vivo*.

The particles could be detected directly in blood plasma owing to the fluorescent properties of the covalently attached

NVC. Due to the self-organizing properties of the copolymers in aqueous medium, the P(MMA-co-NVC) core of the nanoparticles was covered by a layer made up of the polysaccharides, probably with a dense brush-like structure. This external structure was able to dramatically increase their half-life of circulation in blood (from 3 min for bare PMMA* to more than 5 h for Nanohep* and Nanodex*). These results emphasize the importance of the external structure for polysaccharide-bearing particles. Taken together, the results presented in this paper and in a previous one (23) show the potential of heparin-bearing particles to prepare active targeting drug carriers able to inhibit opsonization processes on their surface.

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