# Spleen Capture of Nanoparticles: Influence of Animal Species and Surface Characteristics

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***Purpose.*** To investigate the influence of animal species and nanoparticle surface characteristics on the intrasplenic distribution of polystyrene nanoparticles.

***Methods.*** Two types of fluorescent polystyrene nanoparticles (Estapor® and Fluoresbrite®), plain or coated, were used in mice and rats. First, a fluorimetric method was developed for nanoparticle tissue quantification. Then, intrasplenic distribution of plain or coated nanoparticles was studied using histological examination and image analysis. Finally, the role of direct interactions between nanoparticles and spleen capturing cells was assessed by *in vitro* binding assays, using incubation of thick spleen slices with polystyrene nanoparticles.

***Results.*** The two types of polystyrene nanoparticles showed different levels of trapping: Fluoresbrite® nanoparticles were more efficiently trapped by the spleen than Estapor® nanoparticles, both in mice and rats. In mice, most of the injected nanoparticles were localized in the marginal zone of the spleen, involving a special population of capturing cells, while in rats, the predominant capture occured in the red pulp. In mice, coated nanoparticles were localized both in the marginal zone and in the red pulp, whereas the coating did not seem to change the intrasplenic distribution of the nanoparticles in rats.

***Conclusions.*** These complementary approaches showed different uptake pathways of nanoparticles, according to their surface characteristics and the rodent species used.

**KEY WORDS:** spleen; mouse; nanoparticles; quantification; targeting; surfactants.

## INTRODUCTION

Different particular carriers (i.e. liposomes or nanoparticles) might be used for targeting drugs to the spleen tissue (1,2,3,4). A prerequisite for their therapeutic utilization is a good understanding of the precise intrasplenic distribution and kinetics. Most of the previous studies have been performed only with one type of polystyrene nanoparticles, purchased from Polysciences (U.K.), in one murine species the rat (5,6,7). In this species, it has been shown an increased spleen trapping for nanoparticles coated with block-copolymer surfactants (poloxamer 407 or poloxamine 908) (5,7). On the contrary, studies with rabbits did not show an increase in the capture of coated nanoparticles by the spleen (8,9). In the same way, in mice, using poloxamine 908-coated nanoparticles, the spleen capture was not enhanced (10). The results obtained with different species were so discordant that one might doubt that the classical studies could be automatically extrapolated to other animals and other types of nanoparticles.

Moreover, the previously reported studies did neither precise the distribution of the carriers into the spleen nor evaluate the modification of this intrasplenic repartition according to the surface characteristics of the nanoparticles (6,7,8,9). Therefore, the aim of the present study was to compare the spleen capture of fluorescently labeled nanoparticles in two species of rodents, mouse and rat and to assess the effect of poloxamer 407 or poloxamine 908 coating on their intrasplenic distribution.

## MATERIALS AND METHODS

### Nanoparticles

The fluorescent polystyrene nanoparticles used in this study were Estapor® (FX-010, 185 nm in diameter) from Prolabo (France) and Fluoresbrite® Plain YG (260 nm in diameter, Polysciences, U.K.), previously used after radiolabeling, by others (7,11). The main difference between the two types of nanoparticles was the fluorescent labeling procedure: anthracene for Estapor® and fluoresceine for Fluoresbrite® nanoparticles. Poloxamer 407 was purchased from B.A.S.F. (Germany) and poloxamine 908 provided from I.C.I. Chemicals (U.K.).

### Nanoparticles Size and Zeta Potential Determinations

Nanoparticles size was determinated by a laser light-scattering method (N4MD, Coulter, France) and nanoparticles surface charge was measured by laser doppler anemometry (L.D.A.) using a Malvern Zetasizer II in connection with a Multi 8 Correlator, Series 7032 (Malvern Instruments, U.K.).

### Nanoparticles Coating with Poloxamer 407 or Poloxamine 908

The commercial suspension of plain nanoparticles was first washed by ultracentrifugation (200 000 g, 1 h, Beckmann L7-55) in order to remove any unknown tensioactive agents. The pellet was then washed twice in distilled water after ultracentrifugation (200 000 g, 30 min) and resuspended in Sorensen's phosphate buffer (pH 7.3). The coating was performed by mixing an equal volume of this nanoparticles suspension (1% w/v) and a solution of poloxamer 407 or poloxamine 908 (2% w/v), overnight, in the dark, at room temperature according to Müller and Wallis (12). Free surfactant was removed by ultracentrifugation as above-mentioned.

The adsorption of the surfactants onto the surface of the nanoparticles was confirmed by measuring the surface hydrophobicity and zeta potential of the nanoparticles with hydrophobic interaction chromatography (H.I.C.) and L.D.A. respectively.

For H.I.C. studies, an adaptation of the method of Moghimi *et al.* was performed (11). We used a 50 ml propylagarose column (Sigma, France) (equilibrated with Sorensen's phosphate buffer, pH 7.3, containing 0.082 M NaCl at 20°C). Briefly, 0.5 ml of nanoparticles suspension (1 mg/ml) were loaded onto the column and eluted with 30 ml of Sorensen's buffer at a

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flow rate of 0.8 ml/min. The elution pattern was read photometrically at 350 nm. The column was then washed using the same buffer with the addition of 1% (v/v) Triton X-100 until all the nanoparticles had been recovered (40 ml). Hydrophilized nanoparticles were eluted without interaction, whereas plain nanoparticles showed a retarded elution.

### Quantification of Nanoparticles Uptake by the Spleen

In order to quantify the spleen uptake of fluorescently labeled polystyrene nanoparticles, an original procedure was developed to extract fluorescence from polystyrene nanoparticles trapped in the spleen. Briefly, 0.3 ml of nanoparticles resuspended in Sorensen's phosphate buffer (pH 7.3) were intravenously injected (5 mg of polystyrene/kg) then, 3 h after administration to female $CD_1$ mice (25–30g) and Wistar rats (160 g), spleens were removed, crushed in aqueous solution and water was eliminated by lyophilization.

Then, the lyophilizates were incubated with 3 ml of chloroform in a shaking water bath (Rotatest, Bioblock Scientific, France) in the dark, at 30°C, for 24 h. The extraction was repeated 3 times in order to dissolve completely the polystyrene. The fluorescence intensity was analyzed spectrofluorimetrically (LS50B, Perkin Elmer, Beaconsfield, Buckinghamshire, U.K.).

All experiments were performed at least twice. The Mann and Whitney test was used, at the level of $p < 0.05$, to compare splenic trapping of plain and hydrophylized nanoparticles, in mice and rats. The number of animals per group was rather small and this distribution-free test seemed to be the most appropriate.

### Histological Studies of Splenic Distribution

The animals were injected with nanoparticles and histological studies were carried out as previously described (13). Briefly, 3 h after injection, the spleens were removed, frozen in OCT Compound (Miles, USA). Then, frozen sections were cut (5 μm thick), fixed and observed under an epifluorescence microscope (magnification: ×100, ×1000).

### Determination of Intrasplenic Distribution of Polystyrene Nanoparticles

This method allowed us to compare the distribution of nanoparticles in three areas of the spleen: red pulp, white pulp, marginal zone and to assess the variation of the distribution profile according to the animal species and the surface hydrophilicity of nanoparticles.

Spleens of five animals were removed 3 h after injection of the nanoparticles as previously reported (13). Briefly, five frozen sections representing different levels of each spleen were studied under the epifluorescence microscope (×100) and the capture was semi-quantitatively evaluated in the 3 zones of interest, using a Visiolab 1000 image analyzer (Biocom, France).

### Influence of the Intrasplenic Bloodstream on the Spleen Distribution of Nanoparticles

An *in vitro* methodology allowed us to study, in conditions close to *in vivo*, the uptake of nanoparticles without involving the intrasplenic bloodstream. Briefly, animal spleens were sliced (250 μm thick) with a vibratome (Leica, France). Then, sections containing living cells with all their phagocytozing abilities, were incubated in IMDM (Iscove Modified Dulbecco's Medium, Gibco, France), pH 7.4, supplemented with 10% Fœtal Calf Serum (Gibco, France), 3 h, at 37°C, 5% $CO_2$, under slow rotation. The slices were rinsed and observed under an epifluorescence microscope.

## RESULTS AND DISCUSSION

### Effect of Surfactant Coating on Nanoparticles Size, Zeta Potential, and Hydrophilicity

As shown in Table 1, an increase of nanoparticles size was observed after coating especially for Estapor® nanoparticles. The negative surface charge of nanoparticles dramatically decreased after coating, especially in the case of Fluoresbrite® nanoparticles (Table 1). The H.I.C profiles confirmed that fluorescent nanoparticles had a more hydrophilic surface after coating (Fig. 1). These variations of size, zeta potential and hydrophilicity suggested that coating was effective. It has been shown that the coating layers of poloxamer and poloxamine on polystyrene nanoparticles are extremely stable against desorption, even in presence of plasma proteins after intravenous injection (12).

### Quantitative Fluorimetric Determination of Nanoparticles Spleen Capture

The behavior of Estapor® and Fluoresbrite® nanoparticles was studied in mice and rats, which are representative of the two types of mammalian spleens: non-sinusoidal spleen for mice and sinusoidal spleen for rats. Non-sinusoidal and sinusoidal spleens have different microcirculatory pathways (4) and different mechanisms of blood clearance (14).

The quantitative study showed almost the same pattern for Estapor® and Fluoresbrite® nanoparticles: the coating stimulated their spleen uptake, both in mice and rats. However, the difference between the two species was not statistically different for coated Estapor® nanoparticles, whereas for Fluoresbrite® coated nanoparticles, the difference was statistically significant according to the Mann and Whitney test (Table 2).

Moreover, it was observed that the spleen uptake of Fluoresbrite® nanoparticles was higher than for Estapor® nanoparticles. This difference could probably be attributed to some

**Table 1.** Effect of Surfactant Coating on the Size and the Charge of Fluorescent Polystyrene Nanoparticles

| Nanoparticles | Size (nm) | Zeta potential (mV) |
|---|---|---|
| Estapor® | | |
| Plain nanoparticles | 145 ± 8 | −37.4 ± 0.5 |
| Poloxamer 407-coated nanoparticles | 183 ± 26 | −17.7 ± 0.2 |
| Poloxamine 908-coated nanoparticles | 178 ± 16 | −18.4 ± 0.5 |
| Fluoresbrite® | | |
| Plain nanoparticles | 196 ± 9 | −53.8 ± 6.7 |
| Poloxamer 407-coated nanoparticles | 216 ± 21 | −4.4 ± 0.4 |
| Poloxamine 908-coated nanoparticles | 226 ± 12 | −2.3 ± 0.4 |

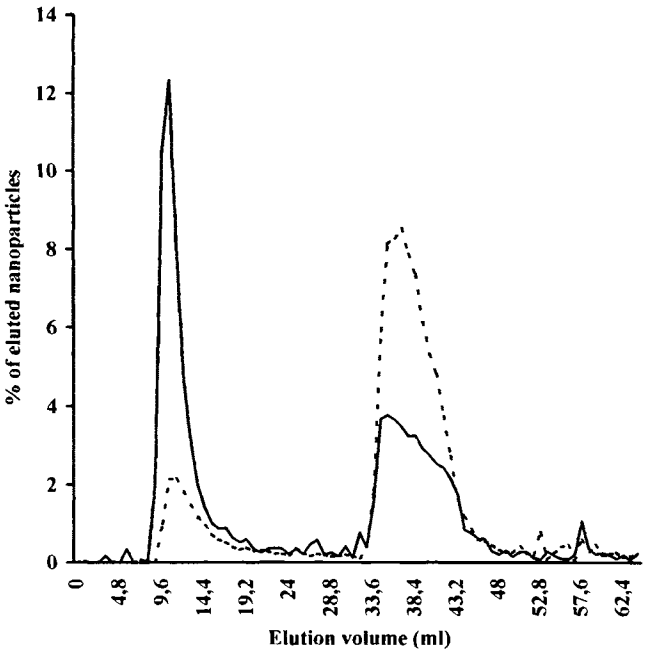


**Fig. 1.** H.I.C. profiles of Estapor® nanoparticles. First peak: nanoparticles eluted before washing, second peak: nanoparticles eluted after washing with Triton X-100. - - - - - uncoated nanoparticles; -------- poloxamer 407-coated nanoparticles (a similar profile was obtained for poloxamine 908-coated nanoparticles). Similar profiles were obtained for Fluoresbrite® nanoparticles.

differences in the surface characteristics of these nanoparticles. Indeed, according to zeta potential measurements, the surface properties before coating were different and we noticed a stronger decrease of zeta potential for Fluoresbrite® than for Estapor® nanoparticles after coating. It could also be supposed that the fluorescent marker has an influence on the surface properties of the nanoparticles, as shown by Müller *et al.* (15). These results suggested that data previously published concerning one type of polystyrene nanoparticles (polystyrene purchased from Polysciences) could not be automatically extrapolated to others.

**Table 2.** Spleen Uptake (% of the Injected Dose) 3 h After Administration to Mice and Rats of Polystyrene Nanoparticles (Estapor® and Fluoresbrite®)

| Nanoparticles | Mice | Rats |
|---|---|---|
| Estapor® | | |
| Plain nanoparticles | 2.2% ± 0.3 | 1.7% ± 0.6 |
| Poloxamer 407-coated nanoparticles | 4% ± 0.7 | 3.5% ± 0.9 |
| Poloxamine 908-coated nanoparticles | 4.7% ± 0.9 | 4.9% ± 1.2 |
| Fluoresbrite® | | |
| Plain nanoparticles | 12.1% ± 3.9 | 13.2% ± 0.7 |
| Poloxamer 407-coated nanoparticles | 18.4% ± 6.2[a] | 34.9% ± 1.9[a] |
| Poloxamine 908-coated nanoparticles | 22.2% ± 3.8[a] | 29.9% ± 2.4[a] |

[a] The uptake is stastistically different in mice and rats.

## Intrasplenic Distribution

Both histological (Figs. 2, 3) and image analysis (Fig. 4) studies showed differences concerning the spleen distribution of nanoparticles in mice and rats: in mice, the marginal zone retained most of the uncoated polystyrene nanoparticles, whereas red pulp appeared far less effective (Fig. 2a) (magnification: ×100). On the contrary, in rats, the main part of the nanoparticles was localized in the red pulp (Fig. 2b) (magnification: ×100).

Furthermore, at a higher magnification (×1000), in the marginal zone of mice spleen, we observed the cells responsible for the uptake of uncoated nanoparticles: they were large angular cells with long and thin processes (Fig. 3), F4/80 negative and esterase negative cells (13).

In mice, clear distribution modifications were observed after hydrophilization treatment: the capture appeared mainly in the red pulp of the spleen for Fluoresbrite® nanoparticles (Fig. 4a), whereas the distribution was not changed for Estapor® nanoparticles (Fig. 4b). In rats, the coating did not seem to modify the nanoparticles distribution (Fig. 4c).

More interesting was the observation that, when the nanoparticles were coated, they did not seem to be localized inside the phagocyting cells but were remaining on their surface, both in mice and rats. This result was in accordance with Moghimi's

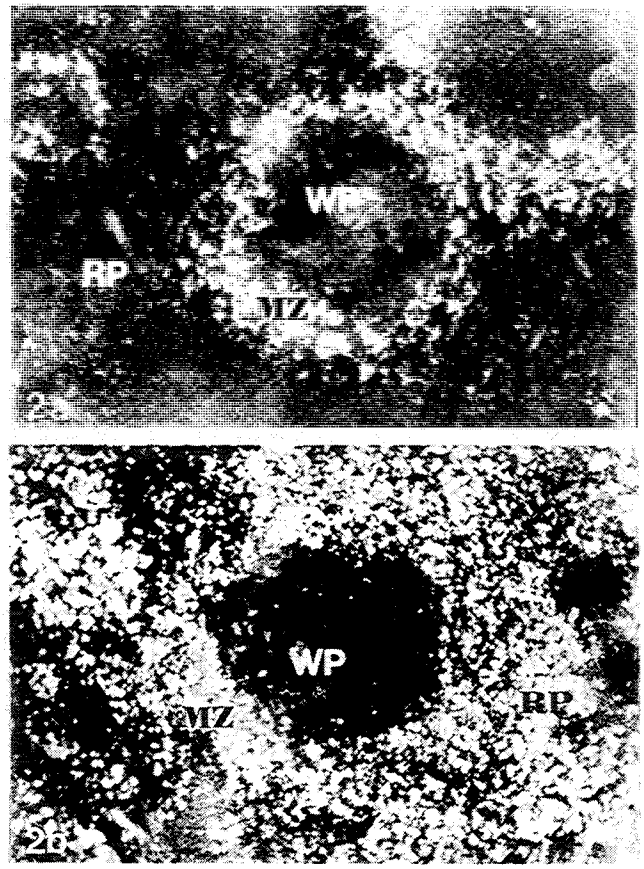


**Fig. 2.** Cryostat sections of spleen (5 μm), 3 h after I.V. administration of fluorescent polystyrene nanoparticles showing the concentration of nanoparticles in the marginal zone. (magnification: ×100; reduced at 57%) (WP: white pulp, RP: red pulp, MZ: marginal zone). (a) mouse spleen; (b) rat spleen.

**Fig. 3.** Cryostat section of mouse spleen (5 μm), 3 h after I.V. administration of fluorescent polystyrene nanoparticles showing a capturing cell of the marginal zone of the spleen. (magnification: ×1000; reduced at 57%)

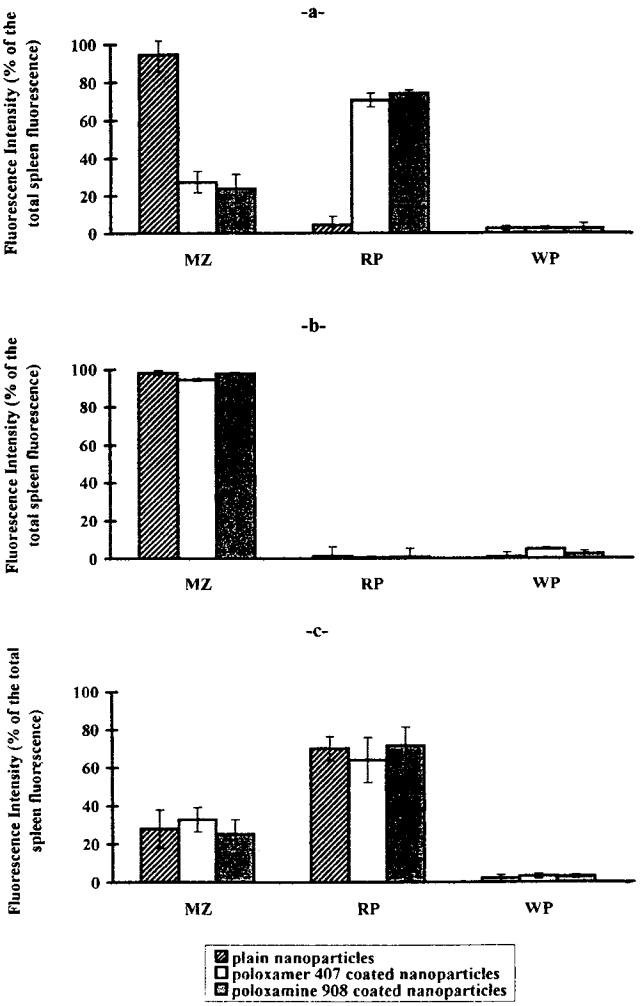


**Fig. 4.** Distribution of the nanoparticles evaluated by image analysis (3 h after I.V. injection). -a- mouse spleen (Fluoresbrite® nanoparticles); -b- mouse spleen (Estapor® nanoparticles); -c- rat spleen (Fluoresbrite® nanoparticles).

studies realized on spleen cell-suspensions using poloxamine 908-coated nanoparticles (5).

### Influence of Intrasplenic Bloodstream on the Spleen Distribution of Nanoparticles

The intrasplenic bloodstream or the direct interactions between nanoparticles and phagocytozing cells could be responsible for such differences in nanoparticles repartition between mice and rats. In order to know which of these factors influenced the biodistribution, we have incubated uncoated or coated nanoparticles with thick spleen slices of mice and rats. These experiments allowed to study the spleen capture without involving the intrasplenic bloodstream.

A relation between *in vitro* and *in vivo* results was observed with uncoated nanoparticles for mice and rats. Indeed, in mice, the most part of these nanoparticles (Estapor® and Fluoresbrite®) was localized in the marginal zone of the spleen whereas in rats, these nanoparticles concentrated mainly in the red pulp.

Both in mice and rats, the absence of clear *in vitro* capture of coated nanoparticles in the cells, either in the marginal zone or in the red pulp, was in accordance with our histological results. These nanoparticles were probably not internalized in phagocyting cells and eliminated after rinsing.

These results suggested that the spleen distribution of plain nanoparticles did not depend on the intrasplenic bloodstream, but rather involved spleen structure and direct cell-nanoparticle interactions, for marginal zone macrophages in mice as well as for red pulp macrophages in rats. This is in accordance with results obtained by Moghimi *et al.* (7,11). On the contrary, as the coated nanoparticles were not found gathered into phagolysosomes of the capturing cells like the uncoated nanoparticles, it seemed that, *in vivo*, the coated nanoparticles were probably trapped and accumulated in the red pulp reticular meshwork by a sieving mechanism. The larger red pulp in rats than in mice could explain the higher retention of coated nanoparticles in rat spleen.

In conclusion, these results show different spleen uptake pathways for nanoparticles according to their surface characteristics and the rodent species. Thus, what may be observed for one type of nanoparticles in one animal species could not be extrapolated either to other types of nanoparticles or other animals. According to anatomical characteristics of human spleen (sinusoidal), rat may be suggested as being the most relevant model for testing spleen distribution of nanoparticles.

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