New Macromolecular Carriers for Drugs. I. Preparation and Characterization of Poly(oxyethylene-*b*-isoprene-*b*-oxyethylene) Block Copolymer Aggregates

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SYNOPSIS

This work describes the formation of discrete micelles ($\simeq$ 0.1 μm) from ABA poly(oxyethylene-*b*-isoprene-*b*-oxyethylene) block copolymers in water. An efficient labeling of the micelles by polymerization of [^{14}C]-styrene within the hydrophobic core is also described. These micellar nanoparticles are being considered as promising materials for controlled release and/or site-specific drug delivery systems. In experimental animals the micelles remained in circulation with a half-life in excess of 50 h. Our results demonstrate the advantages of using block copolymers for the preparation of "perfect" biocompatible surfaces such as are required for well-tolerated, long-circulating particulate drug carriers.

INTRODUCTION

For any intervention in the body using a foreign material, the material's biocompatibility is one of the first criteria that must be satisfied. For the term *biocompatibility* to have a clear meaning, it must be defined with reference to the host response in a given application. For macromolecular and especially colloidal carriers for the parenteral, and more specifically intravenous administration, the required host response is ideally a negative response to the carrier itself and to the drug carrier conjugate.

It has now been demonstrated many times that to most colloids introduced into the vascular compartment of the body there is an overwhelming response from the mononuclear phagocyte system (MPS), resulting in a rapid removal of material from circulation.[1-7] Unless the required destination of the material is the liver and spleen, such host response is unacceptable.

Several decades ago it was already demonstrated that this uptake by the MPS of various particles depended on several related parameters such as size, dose, surface charge, and hydrophobicity.[8] The first step in this process of recognizing material as being foreign is likely to be the adsorption of circulating proteins to the particle surface, resulting in their conformational change.[9-12]

It would appear to be a reasonable assertion to make that for materials where interactions between circulating proteins and particles is prevented, there would be an increased chance of their "biocompatibility." Silberberg[13] explained very succinctly one approach to this: "Particles which have a saturated layer of macromolecules adsorbed to their surfaces, 'see' each other as do these macromolecules in solution. If the macromolecules give stable solution so do the coated particles." This being the case, the practical problem would appear to be a technical one—How does one prepare stable surfaces saturated with suitable macromolecules?

The conditions that lead to repulsion of protein from surfaces have recently been examined by Andrade et al.[14] for the case of polyoxyethylene (PEO) bound on to a model hydrophobic surface, with a

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protein of infinite size being considered as the adsorbing species. Their qualitative conclusions suggest that "high surface density and long chain length of PEO are necessary for protein resistance." Further, the surface density is predicted to have a greater effect than chain length on the steric repulsion and van der Waals attraction. The effect of other properties of materials such as surface charge, surface energy, interfacial free energy, and surface motion, together with the effect of specific compounds (e.g., albumin, glycoproteins, heparin) on blood compatibility have also been considered separately by Andrade et al.[15]

Practical attempts to modify the surfaces of many potential colloidal drug carriers have been made. Nonionic surfactants, Polyoxamers and Poloxamines, have thus been adsorbed onto hydrophobic particles to provide a sterically stabilized coating, causing particles to avoid clearance by the liver to some extent and to be diverted to other tissues.[16-18] Since Poloxamers are A-B-A block copolymers, where A is hydrophilic, i.e., poly(oxyethylene), and B is lipophilic, i.e., poly(oxypropylene), the hydrophobic part is adsorbed to the particle surface, with the hydrophilic portion forming an outer layer oriented toward the aqueous medium. However, the necessary assumption made by Silberberg about "irreversibly" adsorbed polymers is not fully satisfied here, and therefore this approach suffers from a major drawback in that Poloxamers can be relatively rapidly replaced at the particle surface by proteins.[19,20] Better stabilizing effect would be obtained when the hydrophilic polymer, poly(ethylene oxide), is attached covalently to the particulate carrier.

Very much according to the principle of "hydrated dynamic surfaces,"[21] lipid microspheres and other particles have been "stabilized by coating" with polysaccharide derivatives. A marginally increased biocompatibility, as judged by the length of survival of the particles in circulation, was reported.[22]

Geho et al.[23] patented a way of masking liposomes from the MPS by including in the composition of the liposomes lipids that have a trisialic acid as their head group. The role of sialic acid in controlling the nature and the timing of "recognition" in biological systems is well known.[24] Therefore there have been several attempts to utilize compounds that contain sialic acid for modifying surfaces of particles and liposomes.[25-27]

Most of the results indicate that a moderate increase in biocompatibility could be obtained by modifying the material's surface. It would also appear, however, that this so far limited success is due mainly to (a) changes in the surface due to desorption of the modifying species (e.g., Poloxamers) and (b) initial insufficient coverage of the surface with the modifying compound, giving an "imperfect" material (e.g., gangliosides in liposomes).

With the aim of examining a different way of making a "perfect surface," we have prepared prototype nanospheres utilizing the micellar behavior of A-B-A poly(oxyethylene-*b*-isoprene-oxyethylene) (POE–PI–POE) block copolymers in water. Further, in order to label the micelles for further *in vitro/in vivo* assays, a hydrophobic monomer ([^{14}C]-styrene) was polymerized within the hydrophobic core of the block copolymer micelles.

The present study describes the preparation, characterization, and some *in vivo* properties of the block copolymer micelles. A theoretical analysis of the density and conformation of polymer chains at the surface of such block copolymer micelles, and the micelle-unimer equilibria of our block copolymers, with respect to the use of such block copolymers in drug delivery, will be a subject of separate publications.

EXPERIMENTAL

Materials and Methods

The poly(ethylene oxide)-*b*-poly(isoprene)-*b*-poly(ethylene oxide) (POE–PI–POE) block copolymers used in this work were obtained from Prof. Riess and Dr. Abou Madi (Ecole Nationale Supérieure de Chimie, Mulhouse, France) under a collaborative agreement (cf. Table I for details).

The free radical initiators, benzoylperoxide and 2,2′-azobisisobutyronitrile, were obtained from

Table I Molecular Weight and Molecular Weight Distribution of Block Copolymers

Copolymer	M_w (M_w PI Block)[a]	M_n	M_w/M_n
9	24,800 (9,000)	17,400	1.43
11	13,900 (4,500)	8,500	1.64
19	6,500 (2,200)	1,900	3.42

[a] These values have been derived from the elemental analyses of the polymers.

Fluka Chemical Company. Styrene was purchased from BDH Chemicals and purified before use by distillation under reduced pressure at 46°C. [^{14}C]-styrene ($\simeq$ 4 mCi/mL) was obtained from NEN and used without further purification.

Characterization of Block Copolymers

The molecular weights of the polymers, and their molecular weight distribution, have been determined using size exclusion chromatography. Using Ultra-Styragel columns, tetrahydrofuran as a mobile phase, and poly(styrene) and poly(ethylene glycol) calibration standards, both the weight- and number-average molecular weights were derived using the universal calibration procedure[28] (Table I). The chemical composition of the polymers was confirmed by elemental and NMR analyses (data not shown).

Preparation of Micelles

Micelles were prepared by dissolving 0.1% (w/v) of a block copolymer in distilled water with stirring for 24 h at temperatures ranging from 20 to 70°C. (In some cases the polymers were first dissolved in an ancillary water-soluble solvent, e.g., tetrahydrofuran. The solvent was subsequently removed from the suspension by dialysis or by evaporation, or both.)

In order to reduce the polydispersity of the initial preparations, the dispersions were either filtered through 0.45- or 0.22-μm filters, or sonicated, or both (cf. Table II).

The core of the micelles were crosslinked by reacting the residual double bonds in the PI chains using UV radiations in the presence of a photoinitiator. Briefly, after bubbling nitrogen through 70 mL of a 0.1% (w/v) aqueous block copolymer micelle suspension, 1 mg of AIBN was added and mixed with stirring for 4 h. Crosslinked micelles (X micelles) were then obtained by exposure to UV radiations for 4 h.

Table II Size and Polydispersity of Micelles Prepared from Three Different Block Copolymers[a]

Copolymer	*z*-Average Diameter (nm)	Polydispersity
Copolymer 9	66.0 ± 2.9	0.403 ± 0.01
Copolymer 11	25.6 ± 0.4	0.458 ± 0.01
Copolymer 19	14.1 ± 0.4	0.721 ± 0.01

[a] Polymer concentration = 1.0 mg/cm^3; temperature of preparation = 35°C.

Table III Apparent Molecular Weight of Micelles and Their Unimer Aggregation Number

Copolymer Number	Molecular Weight of Micelles	Aggregation Number N[a]
9	5.85 × 106	236 ± 15
11	1.06 × 106	76 ± 3
19	2.80 × 105	43 ± 2

[a] Corresponding to the number of unimer molecules per micelle.

Radioactivelly labeled particles were prepared by copolymerizing [^{14}C]-styrene comonomer within the micellar core. Briefly, nitrogen was bubbled through 70 mL of a 0.1% (w/v) aqueous micelle suspension for 30 min. A 70-μL solution of styrene containing 1% (w/v) of a free radical initiator was added to the suspension and stirred for 24 h at room temperature. The micelle preparation was transferred into a thermostated photoreactor and polymerization with UV radiations was carried out for up to 4 h at 20°C, leading to sterically stabilized nanoparticles containing polystyrene within the core.

Characterization of Micelles

The mean diameter of the micelles and X micelles and their polydispersity index were assessed by photon correlation spectrometry (PCS) using a Malvern system (Malvern Instruments, UK) consisting of an E.M.I. 9863/100 KB photomultiplier and K7032 correlator with 64 delay channels. The optical source was a 2-W argon ion laser (Coherent, Innova 70) of which 488 nm wavelength was used. All the experiments were carried out at 25 ± 0.05°C.

The "*z*-average mean," defined as the mean weighted by the amount of scattered light (cf. the cumulants method developed by Koppel[29]), has been used as a measure of the hydrodynamic size of the micellar aggregates. Samples having the value of polydispersity (its value being derived from the width of the particle size distribution) between 0.0 and 0.05 can be considered "monodisperse." Although for values of polydispersity above 0.15 the polydispersity can be considered to have lost its significance as an accurate measure of the width of the size distribution, below a value of 0.5 useful comparisons between samples can still be made.

The translational diffusion coefficient of the block copolymer micelles has also been measured using

quasi-elastic light scattering (QELS) (or PCS). From this, their hydrodynamic radius have been derived and the corresponding micellar weights and the aggregation numbers (of unimers per micelle) have been calculated (Table III).

The morphology of the micelles and particles was examined by transmission electron microscopy (TEM). A small quantity (2 μL) of the micellar suspensions was pipetted onto a Formvar-coated 200-mesh gold electron microscope grid. After 1 min, the excess solution was drained off by touching the edge of the grid with a filter paper. The grid was then allowed to air-dry in a covered container before examination in a Philips CM10 transmission electron microscope. If no particle were visible, the grid was either exposed to osmium vapor in a closed Petri dish for 4 min or placed on a drop of osmium solution (1% osmium tetroxide in 0.1 *M* cacodylate buffer pH 7.3) for 1 min and finally rapidly washed using 3 drops of distilled water. The particle size distribution was obtained by analyzing electron micrographs of known magnification with a Joyce Loebl image analyzer.

In Vivo Examination of Block Copolymer Particles

[^{14}C]-labeled particles were administered intravenously to mice, and samples of blood were taken at various times over a period of 7 days (cf. Table VII). The amount of injected particles still remaining in circulation were determined from measuring the radioactivity of the blood samples.

RESULTS AND DISCUSSION

A number of copolymers we have examined can form stable colloidal particles in water (cf. our PCS and TEM data). A TEM examination (Fig. 1) showed the micelles to be spherical, discrete, and near monodisperse.

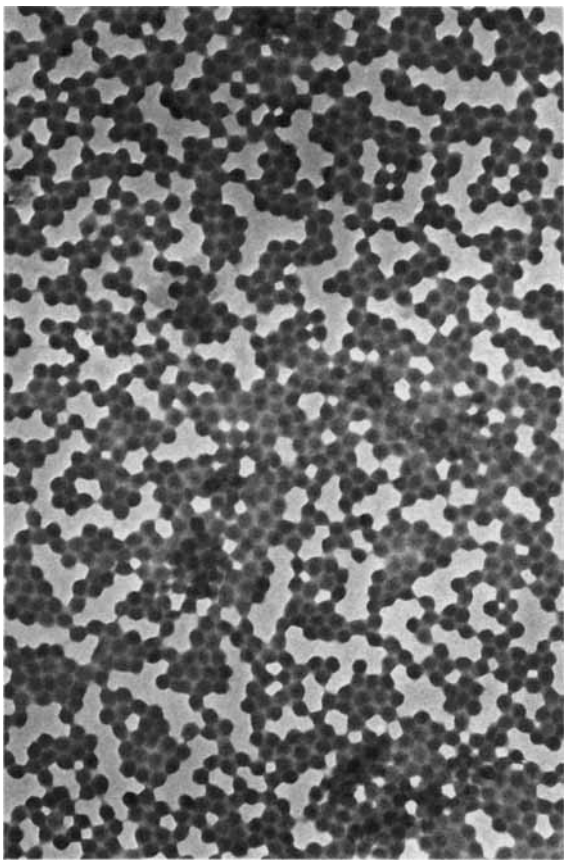

Figure 1 Transmission electron micrograph of copolymer 9 micelles.

As seen by PCS and TEM, crosslinking the chains of the micellar core did not dramatically reduce the size of the micelles (Fig. 2/Table V) and did not reduce their colloidal stability in water. TEM again showed the X micelles to be spherical, discrete, and

Table IV Influence of the Preparation Temperature on the Size and Size Distribution of Micelles Derived from Copolymer 9 (PCS Data)

Temperature (°C)	Micelle Size (nm)	Polydispersity
20	206	0.474
40	143	0.284
50	172	0.384
60	156	0.293
70	127	0.245
70	130	0.261
70	119	0.193

Table V Comparative Size and Size Distribution of Copolymer 9 Micelles and X Micelles in Water and Chloroform (PCS Data)

Sample	Mean Diameter	Polydispersity
Micelles	110.7	0.149
Micelles + AIBN	110.3	0.143
X micelles	95.3	0.142
Micelles in chloroform	Disolved	—
X micelles in chloroform	250	0.429

Table VI Effect of Ultrasonication Time on the Size and Polydispersity of Micelles and X Micelles Prepared from Copolymer 9 (PCS Data)

Ultrasonication Period (min)	Micelle Size (nm) + Polydispersity Index	X Micelle Size (nm) + Polydispersity Index
0	132 (0.229)	95 (0.142)
2	84 (0.055)	91 (0.118)
3	84 (0.069)	104 (0.177)
5	85 (0.104)	N.D.
10	87 (0.170)	N.D.
20	89 (0.172)	100 (0.138)

monodisperse. The occurrence of crosslinking was confirmed indirectly by comparing the solution properties of micelles and crosslinked particles. Thus, at the same concentrations, the micelles were completely soluble in chloroform and in tetrahydrofuran whereas the X micelles were insoluble in both solvents. The mean size and polydispersity index of both micelles and X micelles in chloroform were examined by PCS (Table V).

The formation of block copolymer micelles is in part governed by a unimer–micelle equilibrium.[30] It is reasonable to expect that when block copolymers of molecular size used in this work are involved, a true thermodynamic equilibrium may take a considerable time to be reached. With this in mind, the influence of sonication on both micelles and X micelles was examined (using Soniprep 150 for various periods of time (Table VI) (cf. also Table II). In the case of the micelles, a decrease in size and polydispersity was observed after short periods of ultrasonication (2–5 min). After longer ultrasonication times, the size remained unchanged while the polydispersity index slightly increased. The first reduction of both size and polydispersity index is probably

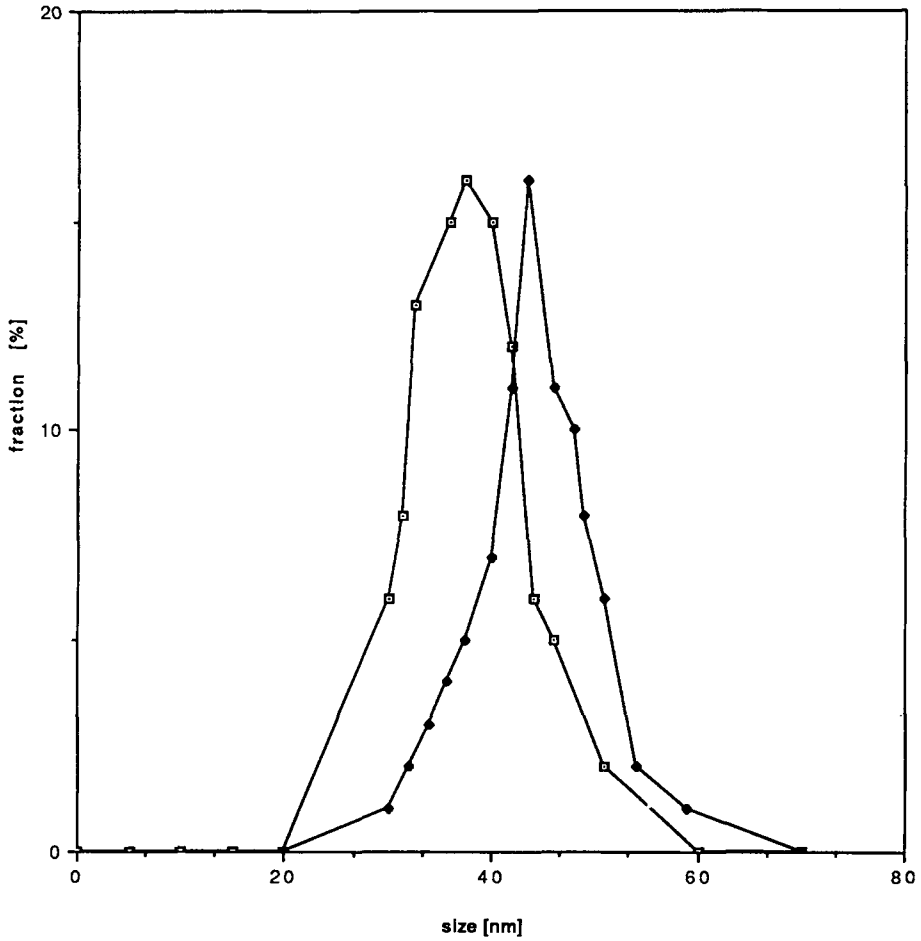

Figure 2 Copolymer 9 micelle (♦) and X micelle (⊡) (size distribution (analysis of the transmission electron micrographs).

due to the disintegration of a small number of large aggregates that might incidentally be present in the mixture. The increase of the index values after 10–20 min can be attributed to the formation of large micellar aggregates as observed by TEM (data not shown), as possibly caused by mechanical degradation of the individual block copolymer chains.

No such effect of ultrasonication on the X micelle size and size distribution were evidenced by PCS and TEM.

The effect of the temperature on the micelle size and size distribution was also examined (cf. Table IV). An increase of the preparation temperature leads to a decrease of both micelle size and polydispersity index.

In attempting to crosslink micelles in the presence of an auxiliary free monomer, there is always a possibility of homopolymer being formed both in the core of micelles and also in the aqueous solution. We examined two different photoinitiators for polymerizing and crosslinking styrene within the micellar core: benzoylperoxide and azo-bis-isobutyronitrile (AIBN). Both were effective, but interesting differences were observed.

In control experiments styrene polymerized in distilled water in the presence of benzoylperoxide when submitted to UV radiations, resulting in the formation of polystyrene particles with a mean diameter of 360 nm (polydispersity index = 0.35). In the same way styrene also polymerized in distilled water in the presence of AIBN, but the obtained polystyrene aggregates were larger ($\simeq$ 4 μm) with a very broad size distribution (0.79). This behavior is probably due to the differences in the solubility of the two initiators (e.g., benzoylperoxide being only poorly soluble in styrene ($<$ 1%) whereas AIBN being completely soluble at a concentration of 1% (w/v).)

Should any "aqueous solution polymerization" occur when micelles were being crosslinked, it would

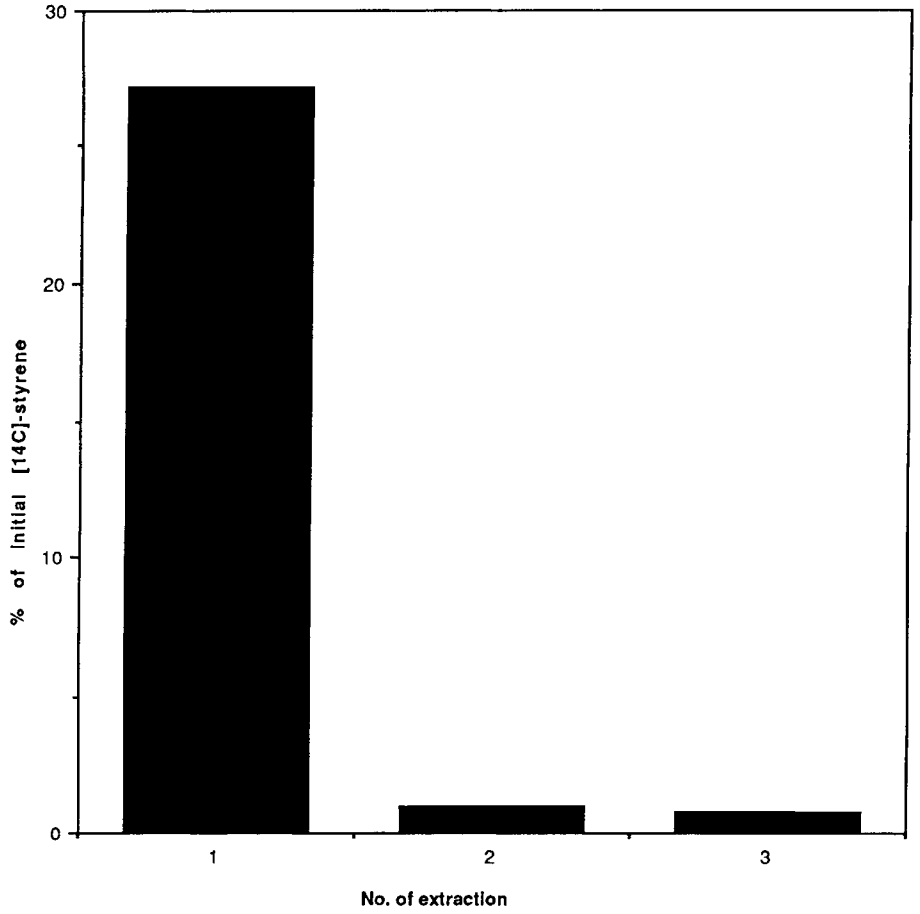

Figure 3 Extraction of the remaining [^{14}C]-styrene from labeled crosslinked copolymer 9 particles with petroleum ether 60/80.

be in the latter case easier to observe the formation of polystyrene particles.

Using our initiator of choice, AIBN, after 2 h polymerization there was still a high amount of styrene (67%) remaining in the mixture. Further, by TEM, "debris" and "bridges" between the particles could be observed in the polymerized mixture.

Changing the reaction conditions [styrene concentration, addition of NaCl (0.1 *M*) to the aqueous phase, and of 2 wt % toluene] did not alter this significantly. By increasing the polymerization time, the percentage of remaining styrene was reduced from 67% (2 h) to 47% (3 h) and even to 30% (4 h).

The system selected for this study (i.e., a high molecular weight block copolymer forming micelles in water; monomer soluble in the micellar core but also in the water phase; initiators also soluble, to different degrees, in both the aqueous and the nonaqueous environment) is clearly interesting and

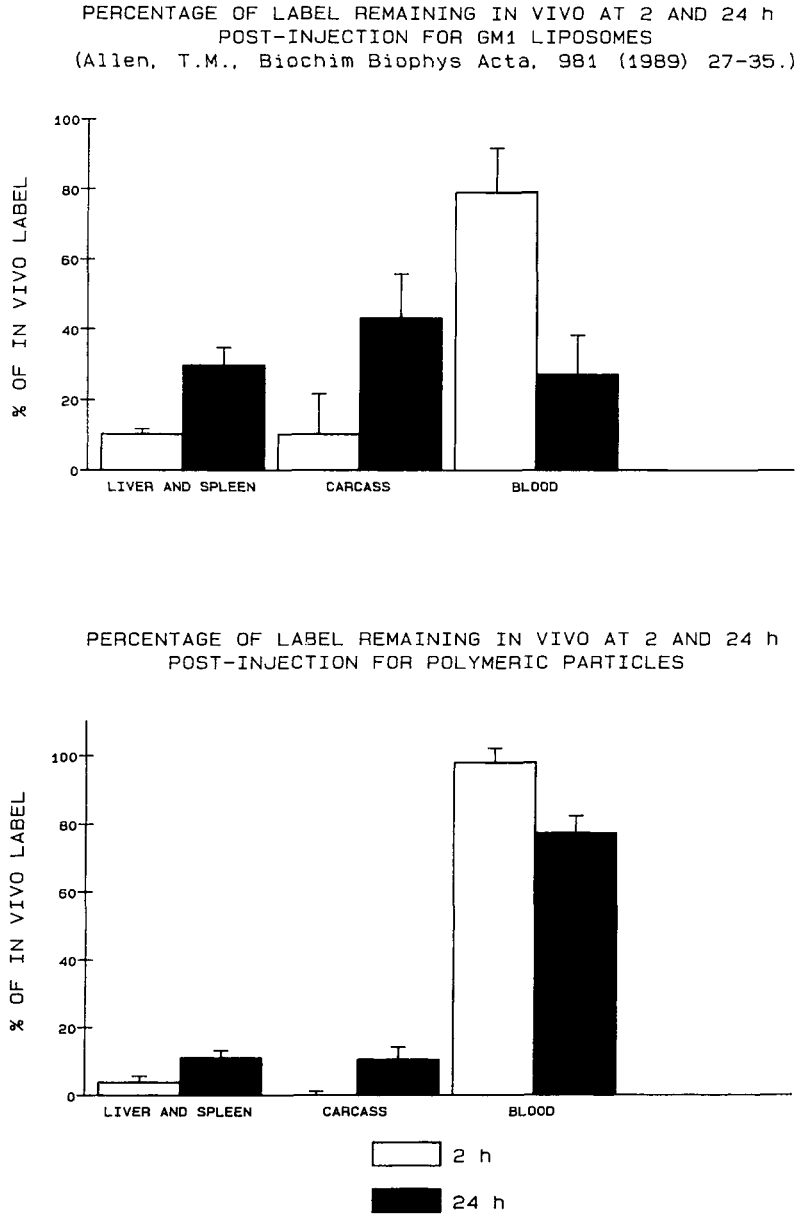

Figure 4 Comparison of the *in vivo* data obtained by Allen et al. with those obtained using our copolymer 9 particles.

Table VII Blood Levels (In % Total Recovered Dose) of Copolymers 9 and 11 Particles after Intravenous Injection in Mice

	Time (h)				
	2	24	72	96	168
Copolymer 9[a]	31.4	37.7	7.5	—	0.4
Copolymer 11[b]	93.9	88.5	—	7.7	0.9

[a] Average of three experiments.
[b] Average of two experiments.

merits further investigation into the processes that actually take place. However, this is beyond the scope of this contribution. We can say that the initiation probably takes place both within the aqueous phase and within the micellar core; that propagation also takes place in both environments. Transfer of radicals between the two environments is probably infrequent and is unlikely to influence significantly the overall outcome of the process.

Styrene was extracted with chloroform before and after 2, 3, and 4 h polymerization (micelle suspension–chloroform ratio = 1 : 9), and the organic extracts were analyzed by UV spectrophotometry. Using 0.1% (w/v) styrene in distilled water as a reference, it was demonstrated that the extraction efficiency was 100%.

For removing the debris and bridges between the particles, presumed to be mainly poly(styrene), five extractions with chloroform were required. The extracted nanoparticles were then analyzed by PCS, TEM, and IR.

Labeled nanoparticles were prepared as described earlier from copolymer 9 micelles and [^{14}C]-styrene. After irradiating the polymerization mixture for 4 h followed by passing nitrogen through the suspension for 2 h, about 80–85% of the initial radioactivity remained in the suspension. About 30% of free styrene was extractable from the micelles using petroleum ether 60/80 (cf. Fig. 3). After treating the particles as given earlier, a preparation having particle size 102 nm and the polydispersity index of 0.125 was obtained.

As already discussed, the fate of particulate drug delivery systems in the vascular compartment of the body is determined by the nature of the particle surface. The single factor curtailing most significantly the usefulness of exogeneous circulating putative drug delivery systems is their inability effectively to avoid their uptake by the "defense" systems of the body (typically residing in the liver, spleen, and bone marrow). Even for particles composed of "natural" materials, e.g., liposomes based on naturally occurring phospholipids, their survival in circulation is relatively short. The best results reported to date are those of Allen et al.[25] (cf. Fig. 4).

Our data, shown also in Table VII, support our view that carefully designed block copolymers offer an elegant way of preparing colloidal particles having "ideal" and "perfect" surfaces required by the stringent limits of biocompatibility.

Some practical applications of this general approach have already been mentioned in the literature.[31] The questions that need yet to be solved from the synthesis point of view are those of polymer degradation, controlled or site-selective drug release, and selective uptake of the particles by the cells of the body.

To summarize, based on the micellar properties of A-B-A block copolymers, a new type of macromolecular carrier with a reproducible and monodisperse size (< 0.1 μm) has been prepared. Micelles have been obtained from nonbiodegradable poly(ethylene oxide)/poly(isoprene) block copolymers and stabilized by crosslinking the chains of the micellar core. It has been demonstrated that crosslinking the PI chains did not dramatically decrease the micelle size, the stability of the X micelles being however significantly improved.

In order to use this new macromolecular carrier as a model to investigate *in vitro* and *in vivo* the steric stabilizing effect of the PEO chains, [^{14}C]-styrene has been successfully incorporated and polymerized within the micellar core, leading to monodisperse-labeled nanospheres.

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