Polyester–Poly(Ethylene Glycol) Nanoparticles Loaded with the Pure Antiestrogen RU 58668: Physicochemical and Opsonization Properties

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Purpose. The pure antiestrogen RU58668 (RU) was encapsulated within nanospheres (NS) and nanocapsules (NC) prepared from different polyester copolymers with poly(ethylene glycol) (PEG) chains. The influence of their physicochemical properties on drug release *in vitro* and their susceptibility to opsonization were evaluated.

Methods. RU-loaded PEG-bearing nanoparticles (NP) prepared by interfacial deposition of preformed polymer were characterized (size, zeta potential, percentage encapsulation and loading). *In vitro* release kinetics were studied in the presence of 10% fetal calf serum (FCS). Their opsonization in mouse serum was evaluated by silver staining of SDS-PAGE and Western blotting of desorbed proteins.

Results. The NS were smaller than NC and had a zeta potential close to zero and a higher percentage of loading. RU release from NS *in vitro* was reduced as compared with the dissolution profile of free RU in a serum-containing medium. Decreased opsonin adsorption at the surface of pegylated NS was observed.

Conclusion. Small nanoparticulate systems containing a high load of pure antiestrogen, showing reduced drug release, have been developed. Among the six nanosphere preparations containing RU, two show a size below 200 nm, and two others undergo reduced protein adsorption in the presence of serum, compatible with increased persistence in the blood.

KEY WORDS: nanospheres; nanocapsules; antiestrogen; opsonisation.

INTRODUCTION

In 2001, the American Cancer Society estimated that approximately 192,200 new cases of invasive breast cancers would be diagnosed among women in the United States. Moreover, according to the World Health Organization, more than 1.2 million people worldwide will be diagnosed with breast cancer this year. Tamoxifen (Tam) is considered as the antiestrogen of reference in the treatment of breast cancer. Nevertheless, the bioavailability of orally administered Tam citrate is very poor, and in addition, its agonist/antagonist activity leads to a number of undesirable effects. [see MacGregor and Jordan (1) for a review], and resistance often occurs after long-term treatment. RU 58668 (RU), a new pure antiestrogen that was able to overcome Tam-induced resistance and to induce up to 30% disappearance of MCF-7 breast cancer tumors implanted in nude mice (2–4), appears to be a powerful alternative treatment for this disease. However, because of the detrimental effects that could be produced by a pure antiestrogen on the skeleton (inhibition of bone resorption) as well as on the cardiovascular and central nervous systems (decrease of the incidence of coronary heart disease and improvement of cognitive functions, respectively), it is necessary to develop a system capable of delivering RU directly to the tumor.

Many anticancer drugs, and in particular doxorubicin, have already been associated with particulate drug delivery systems such as liposomes, micelles, or polymeric nanospheres in order to reduce their major side effects and to concentrate them in solid tumors [see Moghimi *et al.* (5) and references cited therein for a review]. When they are injected into the bloodstream, conventional drug delivery systems are rapidly recognized and removed from the circulation by the mononuclear phagocyte system (MPS) following opsonization by plasma proteins. The complement system, and in particular the adsorption of C3 and its cleavage to C3b, is essential for the recognition of foreign particles. Other proteins such as immunoglobulins (IgG) can contribute to the opsonization process (6).

An important strategy for avoiding opsonization, reducing the rate of uptake by the MPS, and concentrating anticancer drugs within tumor tissues is modification of the surface properties of the drug carriers. For example, the presence of hydrophilic chains such as poly(ethylene glycol) (PEG) at the surface of polymers or lipids drastically decreases the protein adsorption when compared with unmodified polymers or lipids, prolongs the circulation time of the encapsulated drug, and increases its probability of being taken up by tumor tissues after extravasation through discontinuous vascular endothelium (7).

In this study, we chose to take advantage of the protein-rejecting properties of PEG by associating RU with nanoparticles (NP) prepared from biodegradable diblock copolymers in which polyester blocks consisting of such as poly(lactide) (PLA), poly(ε-caprolactone) (PCL), and poly(lactide–co-glycolide) (PLGA), which differ by their degradation rates and their hydrophobicity, were covalently coupled to PEG. Two different types of NP were studied: nanospheres, which are matrix systems with the drug entrapped within the polymer matrix and/or adsorbed on the surface, and nanocapsules, vesicular systems in which the drug is confined to an oily cavity surrounded by a thin polymeric membrane.

After comparing the physicochemical properties of these two types of NP with different polyester cores, we determined the adsorption of specific opsonins onto their surface. The results obtained argue in favor of their prolonged persistence in the bloodstream, a prerequisite to tumor targeting. Because no long circulating drug delivery system containing

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ABBREVIATIONS: C3, C3b, complement proteins C3, C3b; FCS, fetal calf serum; IgG, immunoglobulin G; MPS, mononuclear phagocyte system; NC, nanocapsules; NP, nanoparticles; NS, nanospheres; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, PCL, poly(ϵ-caprolactone); PEG, poly(ethylene glycol); PLA, poly(D,L-lactide); PLGA, poly(D,L-lactide–co-glycolide); SDS, sodium dodecyl sulfate; RU, RU 58668 (11β-[4-[5-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]pentylo xy]phenyl]-estra-1,3,5(10)-triene-3,17β-diol; Tam, tamoxifen.

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pure antiestrogen compounds exists, and because the oral bioavailability of RU is low, the formulations developed in this work could be of potential therapeutic interest.

MATERIALS AND METHODS

Soy phosphatidylcholine Lipoïd S75® (~70% phosphatidylcholine) was purchased from Lipoid GmbH (Germany), and Synperonic F68 (poloxamer 188) from ICI (France). Miglyol 810N was kindly provided by Hüls (Germany). Poly(D,L-lactide) PLA_{50} of M_W 42 kDa was supplied by Phusis (France). Poly(D,L-lactide–co-glycolide) PLGA of M_W 75 kDa and poly(ε-caprolactone) PCL of M_W 40 kDa were purchased from Sigma Chemical Co. (USA) and Aldrich (France), respectively. PLA-PEG 45-5 (D,L-PLA_{50} M_W 45 kDa and PEG M_W 5 kDa), PLA-PEG 45-20 (D,L-PLA_{50} M_W 45 kDa, and PEG M_W 20 kDa), PCL-PEG 40-5 (PCL M_W 40 kDa and PEG M_W 5 kDa) and PLGA-PEG 45-5 (PLGA M_W 45 kDa constituted of 75% lactic acid units and 25% glycolic acid units and PEG M_W 5 kDa) were synthesized and characterized as previously described (8–10). They will be referred to below as PEG-PLA, PEG20-PLA, PEG-PCL, and PEG-PLGA, respectively.

The solvents were analytic grade, and all other chemicals were commercially available reagent grade. Water was purified by reverse osmosis (MilliQ, Millipore®). All micropipette tips and vials used were siliconized in order to avoid steroid adsorption.

Preparation of PEG-Bearing Nanoparticles

Nanospheres (NS) were prepared according to Fessi *et al.* (11), based on interfacial deposition of preformed polymer following solvent displacement. Briefly, 20 mg of polymer or copolymer were solubilized in 1 ml of acetone containing various concentrations (2×10^{-5} M to 10^{-3} M) of RU, then rapidly dispersed into 2 ml of demineralized water followed by acetone evaporation under nitrogen flow. For the PLGA nanospheres, 1% poloxamer 188 was added to the water phase in order to stabilize them.

In the case of nanocapsules (NC), only 5 mg of preformed polymer per milliliter of acetone containing various concentrations (2×10^{-5} M to 4×10^{-5} M) of RU were used, but a lipophilic surfactant (Lipoïd S75®, 7.5 mg/ml of acetone) and oil (Miglyol 810, 25 μl/ml of acetone) were added to the organic phase.

Control free RU without polymer and control particles without RU were prepared under the same conditions.

Physicochemical Characterization of Nanoparticles

The size of nanoparticles was measured by quasielastic laser light scattering using a Nanosizer N4 Plus (Coulter Electronics, USA) and the ζ potential measurements were made in a Zetasizer 4 (Malvern Instruments, UK) after dilution of NS or NC suspensions in 1 mM KCl by a constant factor of 1:40 or 1:250, respectively.

Drug incorporation efficiency was expressed both as percentage of loading (% w/w) and percentage encapsulation (%). The amount of RU associated with drug carriers was determined by an HPLC method [column, Hypersil Kromasil C1 (Thermoquest Corp., UK); mobile phase, acetonitrile/phosphate buffer 0.05 M, pH = 7 (53/47); flow rate, 1 ml/min, λ = 230 nm; sample volume, 70 μl; threshold of quantification, (10^{-7} M) as the difference between the total amount used to prepare the RU-loaded nanoparticles and the amount of free RU in the aqueous phase after separation by centrifugation (25,000 *g,* 12 min) for nanospheres or by ultrafiltration/centifugation using Ultrafree® Units (cutoff 0.1 μm; Millipore®, France) for nanocapsules.

Amount of RU Associated with NS as a Function of Preparation Method

Unloaded PLA and PLA-PEG nanospheres were incubated overnight under magnetic stirring with increasing amounts of RU at 20°C. Samples were then centrifuged (25,000 *g,* 12 min), the supernatants removed, and the pellets redispersed in a constant volume of water. The amounts of RU remaining in the supernatant and in the redispersed pellets were determined by HPLC as described above. The behavior of RU in these physical mixtures was compared with that of RU encapsulated within NS by the interfacial deposition method. The results were expressed as loading efficiency (μg RU/mg polymer).

Release Kinetics of RU 58668 from Nanoparticles

Release studies were carried out at 37°C under stirring in two different media: phosphate-buffered saline (PBS) pH = 7.4 and PBS containing 10% fetal calf serum (Eurobio, France). All nanosphere samples were prepared at the maximal drug loading (5×10^{-4} M RU in the preparation) and were diluted 1/10 in the release medium.

Following various incubation times (30 min, 1 h 30 min, 3 h, 5 h, and 24 h), samples were centrifuged (25,000 *g,* 12 min), the supernatants were removed, the pellets were washed with water, and finally the amount of RU remaining in the drug carriers was determined by HPLC. For each time point, duplicate samples were taken, and triplicate measurements were performed on each one. The results were expressed as the percentage of RU detected in the release medium with respect to the amount of RU encapsulated.

Adsorption of Opsonins onto Nanoparticles

Nanoparticle suspensions (500 μl) were incubated for 30 min with equal volumes of mouse serum (Sigma Inc.) then centrifuged (43,000 *g,* 45 min) and washed with demineralized water (1 ml) to remove proteins not firmly adsorbed onto the surface of NP. The washing step was repeated five times. The proteins were quantified by a sensitive method using Amidoschwartz dye with bovine serum albumin (BSA) as standard (12). After five washings, no protein was recovered in the supernatant, and proteins bound to NP were desorbed by one fifth (100 μl) of the incubation volume of PBS containing 1% SDS, then analyzed by SDS-PAGE (7.5 and 12.5% acrylamide) under reducing conditions. IgGs were identified with a rabbit antibody against mouse IgGs (Vector Laboratories, USA) and C3/C3b with a goat antiserum to mouse complement C3 (ICN, France), used at $1/1000^e$ and $1/200^e$, respectively. The detection of the antigen–antibody complexes was achieved by using a second biotinylated-conjugated antirabbit antibody for IgG detection, and a horse-radish peroxidase-labeled antigoat antibody for C3/C3b detection. The ECL system (Amersham Corp., USA) was employed for the de-

tection, and autoradiography was developed using BIOMAX films (Kodak).

In control experiments, after desorption of proteins adsorbed onto NP by SDS, nanospheres were degraded by an overnight incubation in NaOH 0.1 M, pH = 10. The resulting mixture was then analyzed by SDS-PAGE and Western blotting for IgG and C3/C3b.

RESULTS AND DISCUSSION

The aim of this study was to encapsulate RU within different types of nanoparticles and, furthermore, to determine their physicochemical properties and evaluate the ability of PEG chains at their surface to inhibit opsonin adsorption. The ideal carrier should be able to encapsulate a large amount of RU and to release it slowly. To achieve this, RU must have high affinity for the carrier. Moreover, the carriers should show reduced adsorption of serum proteins and remain in the circulation.

The high affinity and slow release from the carrier mainly depend on the nature of the carrier (type and composition of the core), whereas the size, surface properties, and ability to repel serum proteins are the key parameters responsible for the long-circulating behavior of NP after IV administration. Thus, size, zeta potential, charge, and release of RU were determined, as well as protein adsorption onto different types of NP (NS and NC) that differed by the nature of the polyester core and by the presence or absence of PEG.

Characterization of NP

Whatever the polymer used, the mean diameter of NC was between 200 and 300 nm (Table I). The presence of PEG chains tended to slightly reduce the mean size of NCs whatever the nature of the polyester core. However, no significant size difference could be observed between the various formulations of NC, whether loaded or not with RU or with or without PEG.

The NS (Table II) were always smaller than the NC (Table I) made of the same polymer, except for those prepared from PLGA coated with poloxamer 188, which had the same size. Similar observations have already been made for NS and NC prepared from PLA and/or PEG-PLA polymer by interfacial deposition (13). In contrast to NC, we observed that the nature of the polyester core influenced the mean size for NS, the largest NS being obtained with PLGA, the only ones for which poloxamer 188 was used as a stabilizing agent. Moreover, a drastic reduction of the mean diameter was shown with NS prepared with PEG–polyester copolymers, particularly with the PEG–PLGA polymer as compared with simple NS (80–100 nm vs. 250–260 nm). However, the resulting small size of all pegylated preparations whether nanospheres or nanocapsules, is compatible with extravasation through the tumor vascular endothelium following intravenous administration. Indeed, tumors have been shown to have a characteristic pore cutoff size, the majority ranging between 380 and 780 nm (14). Studies seeking to optimize the design of anticancer drug carriers have indicated that the optimal size for tumor accumulation of liposomes was 90–200 nm (15).

The zeta potential reflects the surface charge of NP, which in turn influences their fate following intravenous administration because highly positively or negatively charged particles are more rapidly cleared from the circulation than neutral ones. The zeta potential of NC was negative for all preparations as a result of the presence of lecithin, which was used as a lipophilic surfactant (Table I). The presence of PEG chains at the NC surface did not reduce the values of the zeta potential. Similar results obtained with NC made from the same polymers have been recently published, confirming that the nature of the polymer does not influence the zeta potential except at high PEG densities or if PEG chains are long enough to mask the lecithin present at the surface (13).

Table I. Composition, Zeta Potential, Size, Percentage of Encapsulation, and Percentage of Loading of Nanocapsule Formulations with or without RU (Final RU Concentration 10^{-5} M)

NC Designation	Zeta Potential (mV)	Size (nm)	Percentage Encapsulation	Loading (%) (w/w)
PLGA unloaded	−53.4 ± 0.5	258 ± 97		
PLGA + RU	−45.2 ± 1.9	252 ± 85	97.6%	0.5
PEG5–PLGA unloaded	−51.2 ± 3.7	246 ± 95		
PEG5–PLGA + RU	−56.5 ± 0.6	234 ± 59	>99%	0.5
PLA unloaded	−50.0 ± 1.1	245 ± 90		
PLA + RU	−50.2 ± 4	233 ± 75	>99%	0.5
PEG20-PLA unloaded	−44.4 ± 1.2	203 ± 67		
PEG20-PLA + RU	−42.2 ± 2.3	245 ± 87	98.9%	0.5
PEG5-PLA unloaded	−54.4 ± 1.2	203 ± 67		
PEG5-PLA + RU	−54.6 ± 0.5	210 ± 71	>99%	0.5
PCL unloaded	−62.3 ± 1.3	294 ± 95		
PCL + RU	−44.5 ± 1.4	287 ± 83	94.4%	0.5
PEG5-PCL unloaded	−49.8 ± 1.3	236 ± 81		
PEG5-PCL + RU	−51.3 ± 2.2	247 ± 83	>99%	0.5

Note: The results are expressed as the mean (n = 4) ± SD of measurements made on the same sample.

Table II. Composition, Zeta Potential, Size, Percentage Encapsulation, and Percentage Loading of Nanosphere Formulations with or without RU (Final RU Concentration 5×10^{-5} M)

NS designation	Size (nm)	Percentage Encapsulation	Loading (%)
PLGA unloaded[a]	262 ± 52		
PLGA + RU[a]	249 ± 64	94.10%	3.1
PEG5-PLGA unloaded	78 ± 26		
PEG5-PLGA + RU	99 ± 38	99.80%	3.3
PLA unloaded	204 ± 57		
PLA + RU	173 ± 33	99.80%	3.3
PEG5-PLA unloaded	133 ± 48		
PEG5-PLA + RU	129 ± 40	99.30%	3.3
PCL unloaded	194 ± 51		
PCL + RU	180 ± 45	>98%	3.3
PEG5-PCL unloaded	115 ± 42		
PEG5-PCL + RU	96 ± 38	98.97%	3.3

Note: The results are expressed as the mean ($n = 4$) ± SD of measurements made on the same sample.
[a] Final poloxamer 188 concentration 0.1% (w/w).

On the other hand, NS do not contain lecithin as a surfactant. NS prepared from polyester homopolymers had negative zeta potential values (–10 to –66 mV), which depended on the polymer (Fig. 1). This is in agreement with previous results recently reported with NS obtained from PLGA, PLA, and PCL polymers (16). However, the use of copolymers containing a PEG block yielded very different results (Fig. 1), with zeta potentials closer to zero, as suggested by previous reports (10,17). This is consistent with the concept that the presence of PEG governs the zeta potential of NS composed of copolymers, rendering it independent of the hydrophobic block. This is also in agreement with the observation that no variation of zeta potential of PLGA–PEG NS was observed with increasing molecular weights of PLGA (18). Moreover, Fig. 1 shows that encapsulating RU within

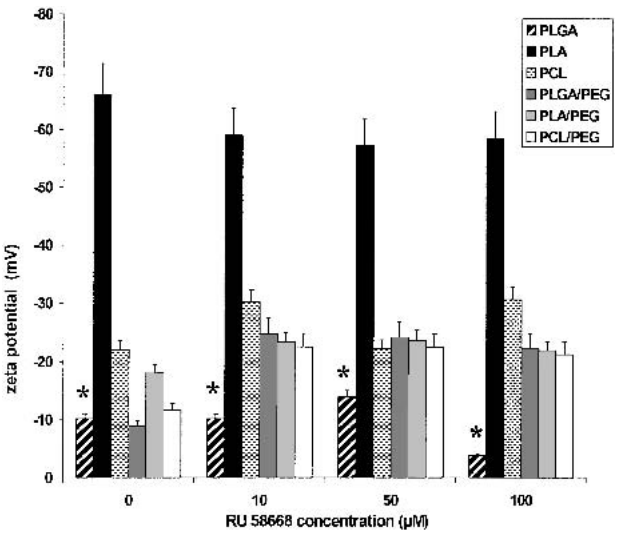

Fig. 1. Zeta potential of RU-loaded PLA NS, PCL NS, PLGA NS with or without grafted PEG chains of 5 kDa M_w containing different concentrations of RU (10^{-5} M, 5×10^{-5} M, 10^{-4} M). The results are expressed as the mean ($n = 4$) ± SD of measurements made on the same sample. Zeta potential of free RU solutions have been determined in same conditions (–33 mV). *Final poloxamer 188 concentration 0.1% (w/w).

PEG-coated NS leads to a constant value of –20 mV, corresponding to a small amount of RU remaining adsorbed at the surface of NS (the zeta potential of RU precipitated as particles alone without polymer was about –33 mV), whatever the initial RU concentration. Indeed, no variation of zeta potential was observed following a 10-fold time increase of RU loading. This may indicate that the colloidal stability of this system remains unaffected by increasing loading of drug, as shown with a water-soluble drug, procaine, incorporated into PLA–PEG NS (19).

In summary, the most important parameter governing the zeta potential of the NS prepared in this work appeared to be the presence of PEG chains at the surface rather than the nature and the molecular weight of the core and the percentage of loading.

The high percentage of encapsulation (Tables I, II), >94% in all cases for both NS and NC, could be explained by the physicochemical properties of RU (poor water solubility and high hydrophobicity). Comparable results were obtained for all NS preparations, whether with or without PEG, with final concentrations of RU varying between 10 and 500 μM for the same amount of polymer (data not shown). The values of RU encapsulation were not significantly different when a more hydrophilic polymer such as PLGA or a less hydrophilic one such as PCL was used. The maximal percentage of loading (RU/PLA) 3.3% (w/w) was obtained with NS at 32.5 μg RU/mg polymer (final concentration) (Table II) as compared with 0.5% (1.7 μg RU/mg polymer) for NC (Table I). The lower loading obtained with NC is related to the limited solubility of RU in oil.

The loadings achieved here are considerably higher than those obtained with tamoxifen (Tam), another antiestrogen, incorporated into nanospheres of poly(MePEGcyanoacrylate–co-hexadecylcyanoacrylate) (PEG–PHDCA) (20). Indeed, although the loading percentage of RU into PEG/PLA NS reached 3.3%, the maximal loading percentage of Tam incorporated into PEG–PHDCA was only 0.5%. This means that although it was possible to obtain a suspension of PEG–PLA NS containing 5×10^{-4} M RU, at the same polymer concentration PEG–PHDCA NS contained only $3.8.10^{-5}$M Tam, a poor loading probably incompatible with an antitumoral activity. In addition, most, if not all, of the associated Tam, were adsorbed at the surface of the NS (20). The high encapsulation rates of RU indicate a high affinity of this pure antiestrogen for the polyesters used to prepare NS.

We have tried to discriminate between RU adsorbed on the surface and that really incorporated into NP. Figure 2 shows that the adsorption of RU at the surface of unloaded NS is dose-dependent. The presence of PEG at the surface of PLA-based NS induced a four fold decrease in the amount of RU adsorbed onto the surface as compared to plain NS (Fig. 2), suggesting a repulsive activity of PEG for the adsorption of the hydrophobic RU. In contrast, when NS were loaded with RU during preparation, the presence of PEG had no effect on the amount of RU associated. Altogether, these data are consistent with an incorporation of RU within the polyester core of PEG-containing NS prepared by the interfacial deposition method, with a small proportion of the RU adsorbed at the surface.

In the following studies, we focused on NS rather than NC because they yielded higher drug loading which would be expected to be more effective in *in vivo* experiments.

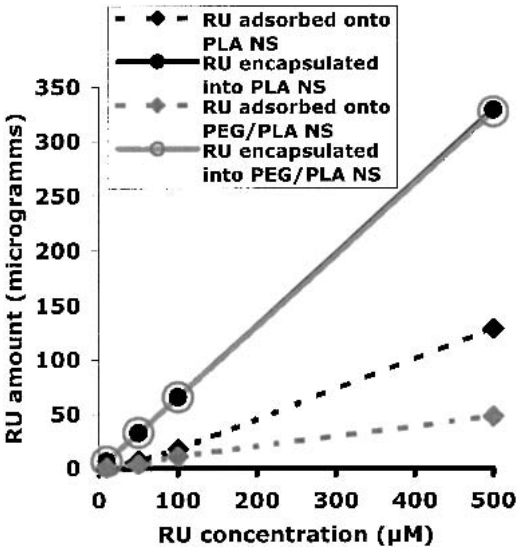

Fig. 2. Amount of RU associated with nanospheres as a function of the preparation method. Incubations of empty PLA (♦-♦) or PEG–PLA (♦—♦) nanospheres with increasing free RU concentrations (10, 50, 100, 500 µM) were performed at 20°C for 16 h. PLA (● - ●) or PEG–PLA (○ - ○) nanospheres loaded with equivalent amounts of RU were prepared by the interfacial deposition method. RU was further measured as described in Materials and Methods. The results are expressed as the mean ($n = 3$) ± SD (not visible on the figure because of their low values) of triplicate determinations in two independent experiments.

In Vitro Release Kinetics of RU 58668

In vitro RU release from nanoparticles of various compositions is shown in Fig. 3. Under sink conditions, RU concentrations in the release medium were below the limit of detection by HPLC. After 1/10 dilution of the NP in PBS 7.4, no free RU could be detected for any of the formulations tested (with or without PEG), whatever the polyester core used, suggesting a retention of the drug inside the formulations (data not shown). Similarly, a 24-h lag time for progesterone release from PLA–PEG–PLA nanoparticles after 1/20 dilution in PBS was observed; however, the use of a surfactant, polysorbate 80, drastically reduced this lag time and increased the release (21). Because steroids possess a relatively high affinity for serum proteins, and especially for serum albumin, and because the NP are destined for IV injection, release experiments in a protein-containing medium more closely resembling physiologic conditions appeared to be more appropriate. We therefore chose to follow *in vitro* release kinetics in 10% fetal calf serum in order to enhance the RU release by a probable partitioning between NS and serum proteins.

With regard to the possible release of RU from NS by an erosion mechanism, a previous paper (22) has presented strong evidence that this process does not occur with either PLA or PLA–PEG in the time course of our experiments but only after 15 days of incubation.

The dissolution kinetics of control free RU and RU release from the NP both displayed an initial rapid release. This may correspond to a fast transfer of adsorbed RU to hydrophobic serum lipoproteins. Thereafter, the kinetic profiles diverged, reflecting the different rates of release of RU from

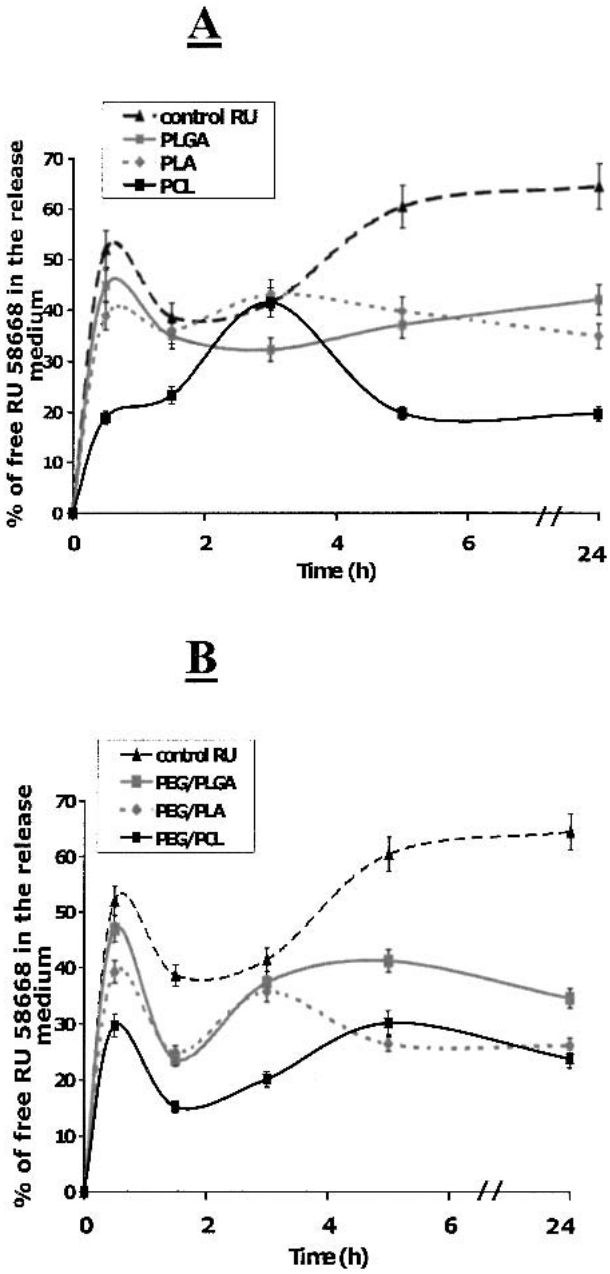

Fig. 3. *Panel A.* Kinetics of RU release from PLA NS, PLGA NS, and PCL NS, in PBS containing 10% fetal calf serum at 37°C, after separation by centrifugation (15,000 *g,* 12 min). The results are calculated as the percentage of RU detected in the release medium with respect to the amount of RU encapsulated (RU concentration: 32.5 µg/mg polymer). The results are expressed as the mean ($n = 3$) ± SD of triplicate determinations in two independent experiments. *Panel B.* Kinetics of RU release from PEG–PLA NS, PEG–PLGA NS and PEG–PCL NS in PBS containing 10% fetal calf serum at 37°C, after separation by centrifugation (15,000 *g,* 12 min). The results are calculated as the percentage of RU detected in the release medium with respect to the amount of RU encapsulated (RU concentration same as Fig. 3A). The results are expressed as the mean ($n = 3$) ± SD of triplicate determinations in two independent experiments.

different polymer formulations with variable hydrophilicity (PCL < PLA < PLGA). Surprisingly, at intermediate times, the amount of nonsedimentable RU in the release medium appeared to decrease. This could be explained both by the

association of RU with small lipoprotein particles and by the formation of insoluble RU aggregates (nonsink conditions), which were recovered by centrifugation with the NS in the pellets in which the measurements were made.

Comparison of the release kinetic profiles of RU released from NS with the dissolution profile of the free RU control shows that the entrapment of RU in the NS significantly retards its *in vitro* release (Fig. 3; compare control RU with other curves). In the presence of serum proteins, some authors (23,24) have also found complex kinetic patterns with an initial burst phase followed by a decrease of free drug in the medium, which could be caused by a complexation of the drug with serum proteins. Moreover, the release rate was enhanced in serum, which could be attributed to high affinity of the drug for serum proteins (23). In our case, the hydrophobicity of RU increases the time necessary for complete drug release, in accordance with previous data obtained with halofantrine incorporated into PEG-containing NC (24). In this latter case, the authors suggested that human serum acted as a pump that increased drug release from nanoparticle systems through the high affinity of the drug for plasma lipoproteins. Whether this was the case for RU incorporated into the formulations described here remains a matter for further investigations.

The composition of the NP affected the rate of RU release more than the nanoparticle size because PCL and PLA NS had similar size (Table II) but completely different release profiles. This is in contrast to previous results reporting that amounts of lidocaine released in PBS from PLA nanoparticles in the initial phase were related to the NS size (25); however, in this latter study only one polymer type was considered. The presence of PEG at the surface of NP affected the release kinetics only slightly (Fig. 3B), in agreement with previous data showing no significant variations in cyclosporin A release between pegylated and nonpegylated PLA NP (22). Figure 2 shows a large difference between the amount of RU adsorbed onto PLA NS and that adsorbed onto to PEG–PLA NS after overnight incubation. Because the presence of PEG at the surface of NP affected the release kinetic patterns of NS only slightly (compare PLA NS in Fig. 3A to PEG–PLA NS in Fig. 3B), the majority of the RU released from PEG–PLA NS would correspond to incorporated and not to adsorbed RU.

Effect of PEG Chains on Opsonin Adsorption onto NS

Nanoparticles with PEG chains covalently grafted on their surface have been described as long-circulating drug delivery systems with potential applications for intravenous drug administration. However, the rapid uptake of IV injected nanoparticulate drug carriers by cells of the mononuclear phagocyte system (MPS) is known to be the main limitation for drug targeting (26). Although reduction of opsonization is not the only parameter that render particles "Stealth®," it is a prerequisite for prolonged blood circulation time.

Despite the importance of the nature of protein interactions with injected colloidal drug carriers, only a limited number of studies dealing with the opsonisation process have been published. For example, a two-dimensional gel electrophoresis experiment has shown that the plasma protein adsorption on PEG-bearing PLA NS strongly depends on the PEG molecular weight as well as on the PEG content of the particles (27). In the same way, a decrease of protein adsorption onto hydrophilic poloxamine-modified polyvinyl pyrolidone nanoparticles, as compared with copolymer particles, conventional liposomes, and "Stealth®" liposomes has recently been reported (28). Likewise, a prolonged blood circulation time of lecithin-coated polystyrene microspheres was observed to be correlated to a smaller amount of serum protein adsorbed (29).

In the present study, opsonization was detected by SDS-PAGE followed by silver staining and Western blotting to identify specific opsonins (IgG and C3/C3b). Both unloaded NS and NS containing RU were used because drug adsorbed at the surface of the particles could influence interactions with proteins. Indeed, the silver-stained SDS-PAGE profiles shown in Fig. 4 reveal that when homopolymers without PEG were used to prepare NS, increased protein adsorption was seen on PLGA and PCL NS loaded with RU, compared with unloaded NS (see also Table III). On the other hand, whatever the polyester core used (PCL, PLA, or PLGA), the presence of PEG chains at the surface of NP led to a significant

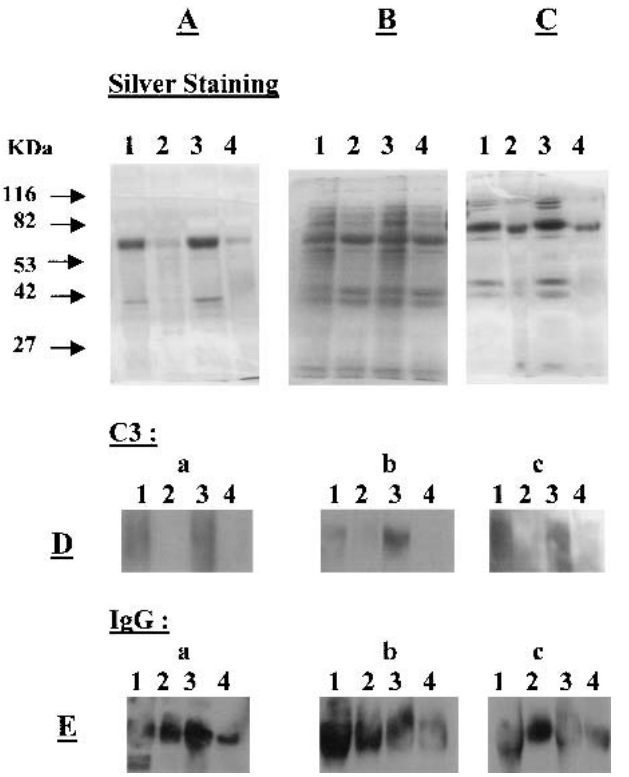

Fig. 4. Opsonin adsorption onto nanospheres. Nanospheres (100 μl) were incubated with 100 μl fresh mouse serum for 30 min. After extensive washing, adsorbed proteins were removed as described in Materials and Methods and analyzed on 12.5% SDS-PAGE (30 μg protein/lane) under reducing conditions, followed by silver staining (panels A, B, and C) or Western blot with goat antiserum to mouse complement C3 for C3b detection (panels Da,b,c) and antimouse rabbit antibody for IgG detection (panels Ea,b,c, showing only heavy chains). The positions of the molecular weight standards are indicated on the left. The protein pattern of the last wash is not shown because no protein could be detected (A and a, NS with a PLGA core; B and b, NS with a PLA core; C and c, NS with a PCL core; lane 1, unloaded NS without PEG; lane 2, unloaded NS with PEG; lane 3, loaded NS without PEG; lane 4, loaded NS with PEG).

Table III. Protein Binding as a Function of Nanoparticle Composition (RU Concentration 5×10^{-5} M)

	μg proteins / mg polymer
NS PLGA unloaded[a]	13.2
NS PLGA loaded[a]	15.1
NS PEG/PLGA unloaded	6.3
NS PEG/PLGA loaded	6.1
NS PLA unloaded	15.1
NS PLA loaded	14.9
NS PEG/PLA unloaded	11.5
NS PEG/PLA loaded	8.5
NS PCL unloaded	26.9
NS PCL loaded	40.0
NS PEG/PCL unloaded	15.0
NS PEG/PCL loaded	12.5

Note: Nanospheres (100 μl) were incubated with 100 μl fresh mouse serum for 30 min. After extensive washing, absorbed proteins were removed as described in Materials and Methods and quantified by the sensitive amidoschwartz dye method. The results are expressed as a ratio with NS weight.

[a] Final poloxamer 188 concentration 0.1% (w/w).

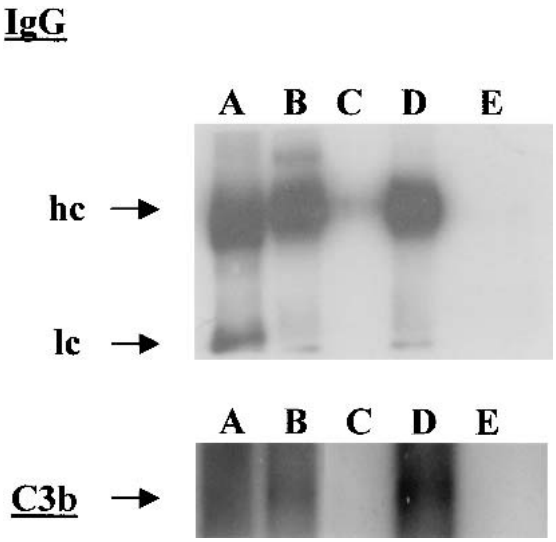

Fig. 5. Control experiments of opsonin adsorption onto nanospheres. RU-loaded PEG–PLA nanospheres (100 μl) were incubated with 100 μl fresh mouse serum for 30 min. After extensive washing, adsorbed proteins were removed as described in Materials and Methods and analyzed on 12.5% SDS-PAGE (30 μg protein/lane) under reducing conditions, followed by Western blot with antimouse rabbit antibody for IgG detection (upper panel) and goat antiserum to mouse complement C3 for C3b detection (lower panel). Similar profiles were obtained with unloaded PEG–PLA and/or PLA nanospheres (not shown) (lane A, control, serum incubated overnight in NaOH 0.1 M, pH 10; lane B, flow-through (first wash); lane C, last wash (100 μl, no protein measurable); lane D, adsorbed proteins onto PEG–PLA NS desorbed by SDS; lane E, RU-loaded PEG–PLA NS incubated with serum proteins, washed 5 times, then treated with SDS for protein desorption and, finally, incubated overnight in NaOH 0.1 M, pH 10 (100 μl loaded, no protein detected in the protein assay). hc, IgG heavy chains; lc, IgG light chains.

decrease of the total serum protein adsorbed (top panels in Fig. 4). This inhibition of protein adsorption by PEG was strongly marked for NS based on PLGA (Fig. 4A) and PCL (Fig. 4C) and was similar whether RU was incorporated or not, despite the fact that zeta potential measurement had indicated some RU at the surface of these NS. PLA NS showed a less marked effect of PEG. Indeed, the intensity of the silver-stained protein bands detected in the case of PLGA NS was only slightly reduced in the presence of PEG and RU (Fig. 4B). This is supported by the amount of adsorbed proteins measured after 30 min of incubation with serum (Table III), which was obviously influenced by the polymer used to prepare nanospheres without PEG. In Western blot experiments, the decrease of C3/C3b (Fig. 4D) observed in samples of proteins bound to various types of NS revealed that all the formulations bearing PEG repulse this opsonin, whether or not they contain RU. On the other hand, the repulsive effect of PEG was less visible for IgG heavy chains (Fig. 4E). The IgG light chains (not shown) showed a similar pattern.

These results assume that all adsorbed proteins were removed by the SDS washing procedure. We carried out control experiments to check this point. No IgG or C3 proteins could be detected in the last wash (Fig. 5, lanes C). Furthermore, we also performed electrophoresis on the degraded NS themselves to check for strongly adsorbed or covalently linked proteins. We confirmed that the degradation conditions did not affect the electrophoretic profile of the proteins (Fig. 5, lane A). No proteins were detected in the degraded NS, (Fig. 5, lanes E), whereas both IgG heavy and light chains and C3/C3b were clearly desorbed by SDS (Fig. 5, lanes D), indicating that the desorption procedure was adequate. Similar results were obtained with unloaded NS (not shown).

In the case of complement protein C3, covalent attachment might have been expected. However, depending on the nature of the surface, adsorption of C3 may or may not be followed by covalent linkage. Moreover, the polyclonal antibody used recognized both C3 and the cleavage product C3b. Thus, these results indicate that the complement C3 protein can be adsorbed onto NS but cannot give any information as to whether complement activation occurs. However, because adsorption is the first step of the activation process, the observation that the presence of PEG chains at the surface of NS reduces C3 adsorption is noteworthy.

Thus, with the use of Western blotting with specific antiopsonin antibodies, we found that the presence of PEG led to reduced adsorption of both IgG and C3, the latter being more efficiently repulsed than the former. This observation could be of importance because the adsorption of IgG or complement C3b fragment onto the surface of liposomes has been shown to promote their hepatic uptake via Fc receptor or complement receptor-mediated mechanisms [see Ogawara *et al.* (29) and refs cited].

CONCLUSION

The highly promising pure antiestrogen (RU), which is able to overcome tamoxifen-induced resistance and to induce the disappearance of MCF-7 breast cancer tumors, has been successfully incorporated into two types of PEG-bearing biodegradable polymeric carriers that could be expected to show prolonged circulation *in vivo.*

For the first time, the comparison between NC and NS has been made in terms of size, zeta potential, percentage loading, percentage encapsulation, and the influence of the polyester core. Compared with NC, NS showed physicochemical parameters (reduced size ~150 nm, better masking of the hydrophobic surface by PEG chains as shown by reduced zeta

potential, and higher percentage of loading: 3.3% w/w) that render them more likely to be effective carriers to tumors after intravenous injection.

Study of the opsonization process following incubation of NS in mouse serum indicates that the presence of PEG inhibits opsonization, but with an efficiency that depends on the polymer core composition. Although the presence of PEG led to a considerable reduction of protein adsorption on NS formed with a PLGA or PCL core, the effect of surface modification of PLA NS was more limited. This inhibition suggests that their capture by the MPS would be reduced. Finally, the three biodegradable polyesters studied (PLGA, PLA, and PCL) showed similar entrapment and reduced release of RU.

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