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Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules

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Abstract

The aim of our work was to examine the relationship between modifications of the surface of nanocapsules (NC) by adsorption or covalent grafting of poly(ethylene oxide) (PEG), and changes in their phospholipid (PL) content on complement activation (C3 cleavage) and on uptake by macrophages. The physicochemical characterization of the NC included an investigation of their properties, such as surface charge, size, hydrophilicity, morphology and homogeneity. This is the first time that such properties have been correlated with biological interactions for NC, a novel carrier system with a structure more complex than nanospheres. C3 crossed immunoelectrophoresis revealed the reduced activation for NC with longer PEG chain and higher density, although all formulations induced C3 cleavage to a lesser or greater extent. NC bearing PEG covalently bound to the surface were weaker activators of complement than plain PLA [poly(D,L-lactide)] NC or nanospheres (NS). Furthermore, the fluorescent/confocal microscopy of J774A1 cells in contact with NC reveal a dramatically reduced interaction with PEG-bearing NC. However, the way in which PEG was attached (covalent or adsorbed) seemed to affect the mechanism of uptake. Taken together, these results suggest that the low level of protein binding to NC covered with a high density of 20 kDa PEG chains is likely to be due to the steric barriers surrounding these particles, which prevents protein adsorption and reduces their interaction with macrophages.

Keywords: Nanocapsules; Poly(D,L-lactide-*co*-ethylene oxide) copolymers; Complement activation; Cellular uptake; Physicochemical characterization; Surface properties

1. Introduction

When administrated intravenously, conventional colloidal drug carriers are rapidly cleared from the bloodstream by the mononuclear phagocyte system (MPS), mainly represented by the Kupffer cells of the liver and spleen macrophages [1]. This removal from the circulation generally occurs through specific recognition by cellular receptors specific for plasma proteins bound to the carriers rather than the carriers themselves. The patterns of protein absorption that determine colloid recognition by macrophages has been extensively studied and is a very useful tool to evaluate the ability of colloids that undergo delayed plasma protein adsorption [2–4] and thus reduced macrophage uptake. In particular, the complement system plays a major role in the immune system recognition of foreign particles [5]. The concept of surface modification of particulate carriers, developed in the last 10 years in order to control the opsonization process, the specific and non-specific interaction of particulate carriers with MPS and blood components, raises questions about the optimal surface properties of the carrier [1]. These properties have been modified by adsorption or by covalent attachment of hydrophilic polymers at the colloid surface. Surface charge, size, hydrophilicity and the conformation of the polymer chains are other factors that influence their interactions with biological media [6–9].

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Among the polymers able to drastically reduce interaction with blood proteins, thus also reducing complement activation, poly(ethylene oxide) (PEG) has been the most investigated. PEG is an uncharged, hydrophilic and non-immunogenic polymer that can be physically adsorbed onto or, preferably, covalently attached to the surface of hydrophobic colloids [8,10]. For example, the presence of the hydrophilic layer of PEG at the surface of the so-called Stealth®[1] liposomes and nanospheres, results in reduced clearance by the MPS and thus prolonged residence time in blood after intravenous administration [10–12].

In contrast to Stealth® liposomes [10] and nanospheres [8,9,11,12], surface-modified nanocapsules (NC) bearing adsorbed or covalent grafted poly(ethylene oxide) have not been thoroughly investigated as far as their physicochemical characteristics and their interactions with biological medium as a function of their components and surface properties are concerned. The advantages of NC obtained from polyesters such as poly(D,L-lactide) (PLA) or copolymers such as monomethoxypoly(D,L-lactide-*co*-ethylene oxide) (PLA-PEG) are their biodegradability, high lipophilic drug payload in the oily core, low polymer content compared with nanospheres and low inherent toxicity [13,14]. These systems represent an alternative colloidal carrier to nanospheres or liposomes when the solubility of the drug is higher in the oil phase of nanocapsules compared to the polymer (or lipid bilayer).

In previous work, a detailed investigation of the interactions between PEG surface-modified NC and macrophages was undertaken [13]. The hydrated PEG chains are flexible and decrease surface interactions with opsonins by steric repulsion. This effect is more dependent on the chain length and density of PEG on the particle surface. As a result, NC with a high PEG density showed a reduced interaction with J774A1 cells, with the best results being obtained with a longer PEG chain length (20 kDa) [13]. PLA-PEG diblock polymers are known to impair the complement activation when nanospheres are incubated in human serum [7]. On the other hand, for PLA-PEG nanocapsules, the direct correlation between the physicochemical properties, such as surface hydrophilicity and complement activation at the NC surface has not yet been studied.

In the present work we have investigated the influence of some parameters of NC composition, such as the nature of the hydrophobic polymer block, chain length and density of PEG and the phospholipid (PL) content, on the physicochemical and biological properties of NC. In particular, some properties (size, PEG density, surface charge, surface morphology and hydrophobicity) of PEG-modified and unmodified NC were correlated with their interactions with biological components such as human serum, complement proteins and macrophages. Complement activation was assessed by two-dimensional immunoelectrophoresis of complement component C3. Fluorescence microscopy of NC, labeled with a fluorescent oil dye, contributed to understanding the cellular pathways involved in NC interactions with phagocytic cells in vitro.

2. Materials and methods

Soy lecithin (Epikuron 170®, composed of approximately 70% of soy phosphatidylcholine) was purchased from Lucas Meyer (France) and Poloxamer-188 (Synperonic F68®) from ICI (France). Miglyol 810 N was kindly provided by Hülls (France). Poly(D,L-lactic acid) PLA^{50} with weight average molecular mass (Mw) of 42 kDa was supplied by Phusis (France), poly(ε-caprolactone) (PεCL) Mw 42.5 kDa and poly(D,L-lactide-*co*-glycolide) PLGA (75 : 25 wt. ratio lactic/glycolic acid) 75–120 kDa by Sigma-Aldrich (France). The diblock copolymers PLA-PEG 45–5 (PLA 45 kDa and PEG 5 kDa), PLA-PEG 45–20 (PLA 45 kDa and PEG 20 kDa), PLA-PEG 2–5 (PLA 2 kDa and PEG 5 kDa), PLGA-Peg 45–5 (PLGA 75 : 25 45 kDa and PEG 5 kDa), PεCL-PEG 45–5 (PεCL 45 kDa and PEG 5 kDa) were synthesized and characterized as described [4]. DiD oil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate) was supplied by Molecular Probes (The Netherlands). Density marker beads®, Percoll®, Gelbond® films and Agarose were obtained from Pharmacia (Uppsala, Sweden). The solvents used were analytical grade and all other chemicals were commercially available reagent grade. Water was purified by reverse osmosis (MillliQ, Millipore®).

2.1. Nanocapsule preparation and characterization

Nanocapsules were prepared by the method described by Fessi et al., based on interfacial polymer deposition following solvent displacement [15] and was previously described in detail [13].

"Naked" nanocapsules were prepared by dissolving 60 mg of polymer (PLA, PLGA or PεCL) in 8 ml of acetone which was added to an acetone solution (2 ml) containing 75 mg of lecithin and 250 μl of Miglyol 810 N. This organic solution was poured into 20 ml of external aqueous phase under magnetic stirring, and the solvent was evaporated to 10 ml under reduced pressure. A similar procedure was used to prepare PLA-POLOX NC except that 0.375% (w/v) of poloxamer 188, a hydrophilic surfactant, was included in the aqueous phase. For purposes of comparison, nanospheres (NS), were prepared by the same method using only polymer in the acetone phase and including poloxamer in the aqueous phase. They were dialyzed for 2 days against distilled water

[1] Stealth® is a trademark of Sequus Pharmaceutical Inc.

Table 1
Influence of PEG chain length and lecithin content on physicochemical characteristics of nanocapsules

NC formulation and polymer blend composition	PEG % w/w[a]	Lecithin[b] 0.75% w/v			Lecithin 0.30% w/v		
		Mean size ± SD[c] (nm)	Polydispersity index[d]	ζ potential (mV)	Mean size ± SD[c] (nm)	Polydispersity index[d]	ζ potential (mV)
Naked PLA	0	218 ± 52	0.130	− 51.8 ± 0.1	195 ± 42	0.061	− 47.7 ± 0.5
PLA-POLOX	—	211 ± 61	0.282	− 50.9 ± 0.2	246 ± 45	0.017	− 35.1 ± 0.1
PLA-PEG (45–5)	10	200 ± 51	0.056	− 51.0 ± 1.1	223 ± 45	0.050	− 40.6 ± 0.4
PLA-PEG (45–5)/ PLA-PEG (2–5) 2:1[e]	20	277 ± 95	0.217	− 56.2 ± 1.0	242 ± 42	0.036	− 41.9 ± 2.2
PLA-PEG (45–5)/ PLA-PEG(2–5) 1:2[e]	30	239 ± 88	0.295	− 50.0 ± 0.7	255 ± 44	0.021	− 35.6 ± 0.5
PLA-PEG (45–20)/PLA 1:2[e]	10	220 ± 71	0.174	− 45.9 ± 0.6	242 ± 55	0.079	− 36.3 ± 0.7
PLA-PEG (45–20)/PLA 2:1[e]	20	216 ± 65	0.146	− 45.9 ± 0.6	240 ± 26	0.022	− 22.2 ± 0.1
PLA-PEG (45–20)	30	193 ± 59	0.159	− 37.8 ± 1.1	226 ± 60	0.105	− 5.4 ± 0.2

[a] w/w of total polymer concentration (6 mg/ml).
[b] Lecithin is Epikuron 170® (with ~70% of soy phosphatidylcholine).
[c] Standard deviation of populations that were reported by the instrument ($n = 4$) on a typical preparation.
[d] Monodispersed samples (below 0.3).
[e] Blend ratio w/w.

(300,000 Da cut-off membrane) to obtain naked PLA NS, containing only a minimum of poloxamer at their surface. The poloxamer content was not measured after dialysis.

Surface-modified NC were prepared using diblock polymers (PLA-PEG 45–5, PLA-PEG 45–20, PLA-PEG 2–5) and PLA in various combinations as shown in Table 1 so as to obtain PEG contents of 10, 20 and 30% of PEG w/w with respect to total polymer in the acetone phase, with lecithin and Miglyol 810 N as above. No poloxamer was added to the aqueous phase in this case

In order to prepare fluorescent NC, a hydrophobic fluorescent marker, DiD oil, was incorporated into the oil phase (83.3 μg/ml of NC suspension) at high loading yields (> 99%) as described [13]. This probe has the advantage of possessing excitation and emission maximum at much higher wavelengths than those of cell components. The size of the nanocapsules was determined by quasi-elastic light scattering (QELS), with a Nanosizer (Coulter model N4 Plus, Coulter Electronics Inc., Hialeah, FL, USA) and ζ potential measurements were carried out (Zetasizer 4, Malvern Instr., UK) after dilution of nanocapsules by a constant factor of 1:250 in 1 mM NaCl (the conductivity was constant at 100 ± 5 μS/cm) except when otherwise stated.

2.2. Hydrophobic interaction chromatography

The hydrophobicity of the different surfaces of NC with or without PEG was investigated by hydrophobic interaction chromatography (HIC), used as previously described for the assessment of surface modification of polystyrene particles by ethoxylated surfactants [6]. The column was propyl-agarose (Sigma, France); its diameter was 1.0 cm, the height 16 cm, the void volume 5.5 ml and the flow rate 0.5 ml/min. The elution buffer was PBS and 0.1% Triton X-100 in PBS was used as the washing solution, after elution of the first 25 ml. The injected volume was 200 μl and the concentration was adjusted to yield elution peaks with a maximum absorption of 0.9 at 350 nm.

2.3. Density gradient studies

Separation of particles was achieved by development in situ of colloidal silica (Percoll® 54% v/v in NaCl 0.15 M, initial density 1.068 g/cm^3) gradients during centrifugation in a model SW41Ti rotor (Beckman) at 20°C and 20800 $g_{average}$ for 130 min. 11.8 ml of Percoll were added to 0.2 ml of initial colloidal suspension without prior concentration. In a separate tube, density marker beads®, of different pre-determined densities were added under the same conditions as samples and used for external calibration of the bands. Millimeter scaled paper strips were used to measure the distance from the top meniscus to the band limits. Particle densities were calculated from the curves plotting distance from the top versus the density of each band of marker beads. The encapsulated fluorescent dye (DiD) was a helpful tool to identify the separated bands.

2.4. Nanocapsule morphology by freeze-fracture scanning electron microscopy

A small drop of an aqueous particle suspension was deposited on a thin copper planchette, glycerol used as a cryoprotectant, and rapidly frozen by plunging into

liquid propane. Fracturing and shadowing, using Pt–C, were performed in a Balzers BAF 301 freeze unit. The replicas were observed in a Phillips 410 electron microscope.

2.5. Crossed immunoelectrophoresis of complement component C3

The specific activation of C3 complement component by different NC surfaces in human serum was assessed by comparative measurements of C3 cleavage, as previously described [16,17]. Human serum was obtained from healthy donors and stored at $-80^{\circ}C$ until use. Nanoparticles were incubated for 1 h at 37°C with human serum diluted 1/4 in veronal-buffered saline containing 0.15 mM Ca and 0.5 mM Mg ions (VBS^{2+}) with gentle agitation. To achieve a valid comparison of the different NC and NS batches, sample volumes with an equal surface area (500 cm^2) were incubated with 100 μl of human serum. The method of calculation of NC and NS surface area is described elsewhere [7,13]. All the samples (5 μl) were subjected to a first isoelectric focusing on 1% agarose gel. The second-dimension electrophoresis was carried out on Gelbond® films in agarose gel containing a polyclonal antibody to human C3, recognizing both C3 and C3b (complement C3 antiserum raised in goat, Sigma, France). The films were finally stained with Coomassie blue (Sigma). The ratio between the heights of the C3 and C3b peaks was calculated. Opsonized zymozan (Sigma), pretreated as described [18], was used as a positive control.

2.6. Fluorescence microscopy

The J774 A1 murine macrophage-like cell line was maintained as an adherent culture as previously described [13]. Cell viability was superior to 95% after 4 h incubation with particles as estimated by the MTT conversion test. One million J774A1 cells were previously seeded for 2 h onto sterile cover slips in 6-well plates (Costar) and incubated for 4 h at 37°C with DiD-labeled NC diluted 20 times in RPMI/10% (v/v) FCS medium without phenol red (4200 ng of DiD/ml). Incubations at 4°C were carried out in parallel. The cover slips were washed twice with ice-cold PBS and the living macrophages were placed in the closed chamber with fresh RPMI medium without FCS. The cells were immediately observed with a 63× objective in Nikon epifluorescence microscope (Optiphot-2, Japan) equipped with a Nipkow wheel coaxial-confocal attachment coupled to cooled camera (RTEA1317 K1CCD, Princeton Instruments). Fluorescence images were obtained with rhodamine filter set (BP546, FT590, LP600). Micrographs were transferred to a computer for processing with Adobe Photoshop® 4.0. Images were assembled and printed directly on photo quality glossy paper.

3. Results

3.1. Physicochemical characterization of nanocapsule surface

Table 1 shows the effect of PEG (content and chain length) and the soy lecithin concentration on NC size, polydispersity and ζ potential. The NC size was between 150 and 250 nm diameter depending on the formulation tested. Particle dimensions and polydispersities seemed to be more influenced by the amount of soy lecithin and by the blending of polymers, PLA-PEG 45–20 kDa and PLA, or PLA-PEG 45–5 kDa and PLA-PEG 2–5 kDa than by the characteristics of the polymers themselves. Lower polydispersities were obtained with lower amounts of lipids. The results shown in Table 1 are from one representative batch of each type of nanocapsule. In every preparation the same tendencies were observed: the use of blends of different polymers led to slightly larger NC than those prepared from only one polymer type, whatever the blend of polymer used.

Electrical potential distributions on the surface of PEG NC were examined by measuring ζ potential. When the ionic strength and concentration of NC in the suspension were kept constant it was possible to observe the effects of chain length, PEG content and soy lecithin content on the ζ potential. A strong influence of lecithin (PL) on ζ potential of NC was observed (Table 1). In fact, a high concentration of soy lecithin imparted a high negative ζ potential to the NC regardless of the polymer, the length and the content of PEG used. In this case, only PLA-PEG NC with PEG 20 kDa at 30% w/w content had an effect on surface charge compared with naked PLA NC (-38 to -51 mV). However, when lower amounts of soy lecithin (0.3% w/v) were used, increasing the PEG content led to ζ potentials closer to zero for surface-modified NC. This effect was much more pronounced for NC with PEG chains of 20 kDa Mw (-5 mV), showing an efficient shielding of the negative surface charges.

This strong influence of lecithin is confirmed in Fig. 1. The ζ potential profile of different PEG NC as a function of pH was similar to the profile of lecithin-based vesicles, and was related to the ionization of the phosphate (negative charge at higher pH) and choline groups (positive charge at lower pH) of phosphatidylcholine. At higher pH values, NC have more negative ζ potential values than lecithin vesicles, probably due to the ionization of free fatty acids from the oil and from lecithin mixture as previously discussed [19,20].

3.2. Hydrophobic interaction chromatography

Soy lecithin (PL) also seems to be responsible for the very hydrophilic character of the surface of the colloids formed, as shown in Fig. 2 by hydrophobic interaction

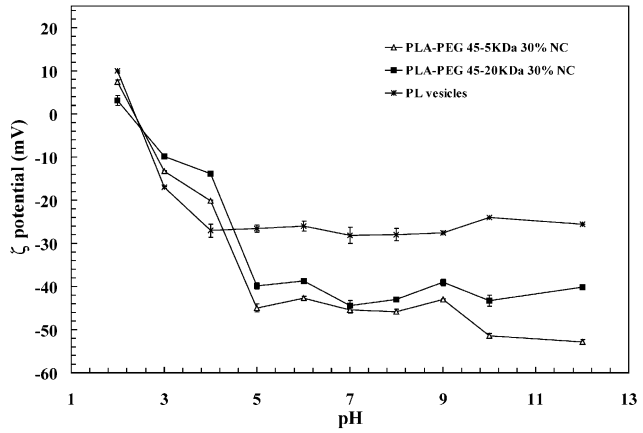

Fig. 1. The ζ potential of PEG surface modified NC as a function of the pH. All the formulations were obtained with lecithin at 0.75% w/v. The ζ potential measurement was performed after sample dilution 50 times in solutions of different pH adjusted with NaOH and HCl at constant ionic strength. Results are the mean of three measurements of two sample dilutions ($n = 6$) ± SD.

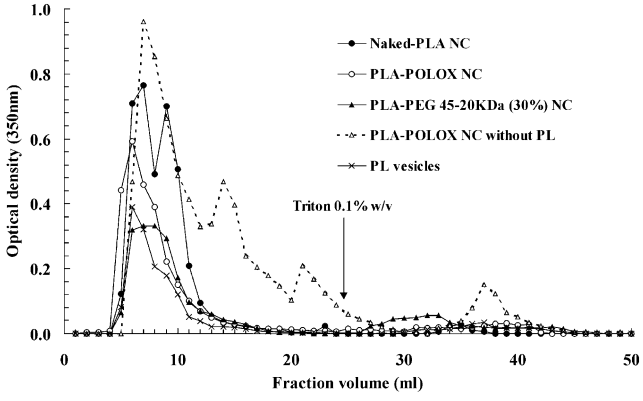

Fig. 2. HIC chromatogram of different NC formulations prepared by the solvent displacement method. The arrow represents the beginning of washing with Triton X-100. The phospholipid (PL) vesicles were prepared with 0.75% w/v of lecithin in water.

chromatography. The formulations containing lecithin (PL) and poloxamer showed little interaction with the column and were eluted rapidly. In contrast, the formulation without lecithin was the most retained on the column and exhibited a larger area peak. Three additional peaks were also observed in this case even after washing step indicating higher hydrophobic interaction with column. Naked-PLA NC, PLA-POLOX and PLA-PEG NC produced similar profiles with little interaction between particles and the gel matrix.

3.3. Nanocapsule homogeneity

Fig. 3 shows the density profiles obtained from different preparations after ultracentrifugation in Percoll® gradient. This technique allows the heterogeneity of the preparations to be assessed in terms of formation of different systems in the same preparation process. Increasing the amount of PEG reduces the polydispersity of the system and increases the homogeneity of the NC, with less formation of NS. Probably, the amphiphilic polymer containing PEG surrounds the oil droplets more effectively than the monoblocks of more hydrophobic PLA polymer. NC prepared from blends of polymer (e.g. PLA and PLA-PEG) were more heterogeneous than the NC obtained from only one polymer, as evidenced by thicker bands. PLA NS are present as contaminants only in formulations obtained from blends, but in very small amounts. The bands corresponding to the lower densities are probably due to the presence of small amounts of uncovered droplets as nanoemulsions (NE), that can be directly compared with the NE formulation. PL vesicles are very polydisperse. The presence of these vesicles seen in higher proportions in NE and PLA-POLOX NC than in the other NC preparations. These results indicate that, under our conditions, PLA-PEG 20 kDa NC at 30% PEG and PLA-PEG 5 kDa NC at 10% PEG yield the most homogeneous formulations of NC. These are not prepared from blends of polymers.

3.4. Nanocapsule morphology

Freeze-fracture electron microscopy revealed the presence of nanocapsules. Fig. 4 shows typical images. In both cases, PLA NC (not shown) and PLA-PEG NC, it was possible to obtain cross-fractured particles. This is the usual way of the propagation of the fracture of triglyceride microemulsions. However, we often noticed a plastic deformation on the border of the PLA-PEG NC which is probably related to the nature of the polymer.

3.5. Serum protein adsorption

Size and ζ potential measurements were used to detect the binding of serum proteins to naked NC and PLA-PEG NC, as previously described [21]. The choice of the incubation medium (serum or plasma) for investigating protein binding is important because the addition of anticoagulant may inhibit the activation of complement system by chelating Ca^{2+} and Mg^{2+} ions in plasma [2]. It was observed (Fig. 5A) that naked NC showed a reduction (−48.8 to −14.2) in their ζ potential value after incubation in 80% human serum, while the effect on PLA-PEG NC was less marked (−10.8 to −7.1). Moreover, the size of naked NC increased slightly, in contrast to that observed with PLA-PEG NC (Fig. 5B). These results suggest that less serum protein absorption occurs on PLA-PEG NC than on naked NC, although these measurements do not allow a quantitative estimate of protein binding.

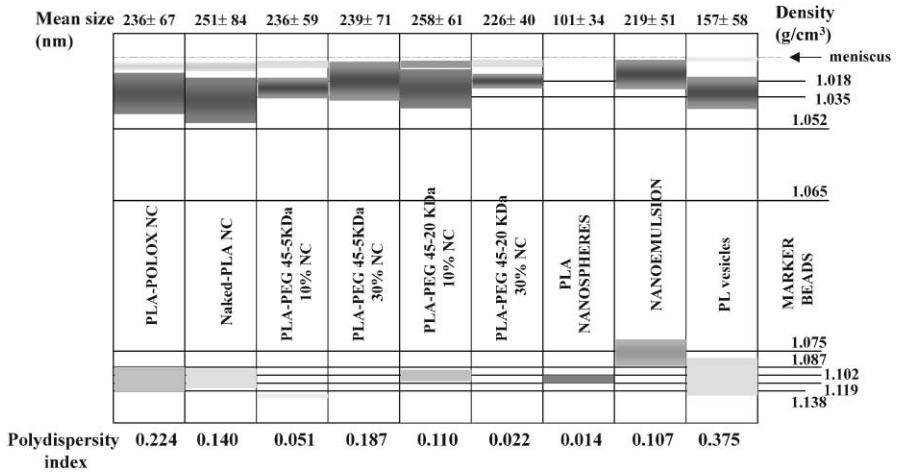

Fig. 3. Ultracentrifugation of different formulations loaded with DiD probe in a Percoll gradient. Gradient density was monitored using colored density marker beads in 0.15 M NaCl. The starting density was 1.068 g/cm^3. NC were obtained as described in Section 2. For comparison, nanoemulsion, nanospheres and phospholipids vesicles were obtained in the same way without polymer, without lecithin or oil, and with 0.75% w/v of lecithin only, respectively. The gray-scale intensity of the bands is proportional to their intensity in Percoll®.

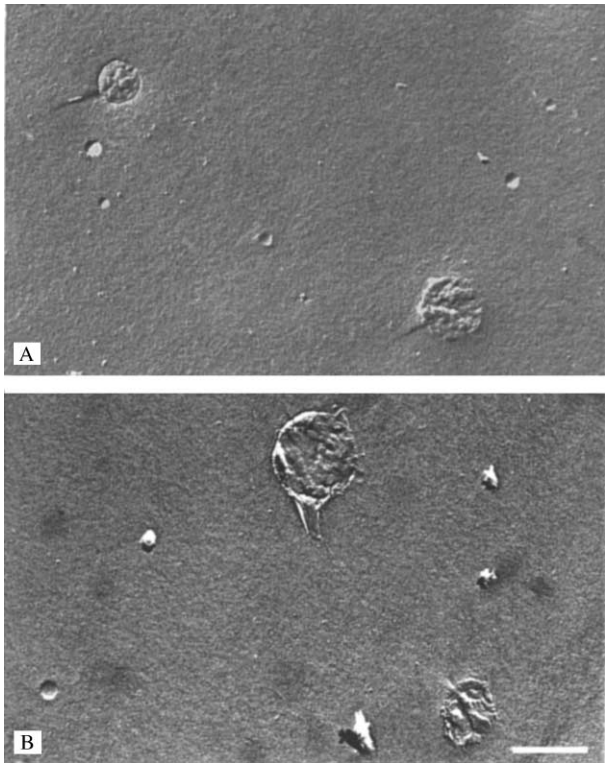

Fig. 4. Freeze-fracture electron microscopy. Visualization of naked PLA NC (A) and PLA-PEG NC (B). Bar = 200 nm, the two micrographs are at the same magnification. Large NC of about 200 nm of diameter are clearly identified. In (B) the border appears much more deformed than in (A). Both NC were prepared with a minimal concentration of lecithin (0.05% w/v).

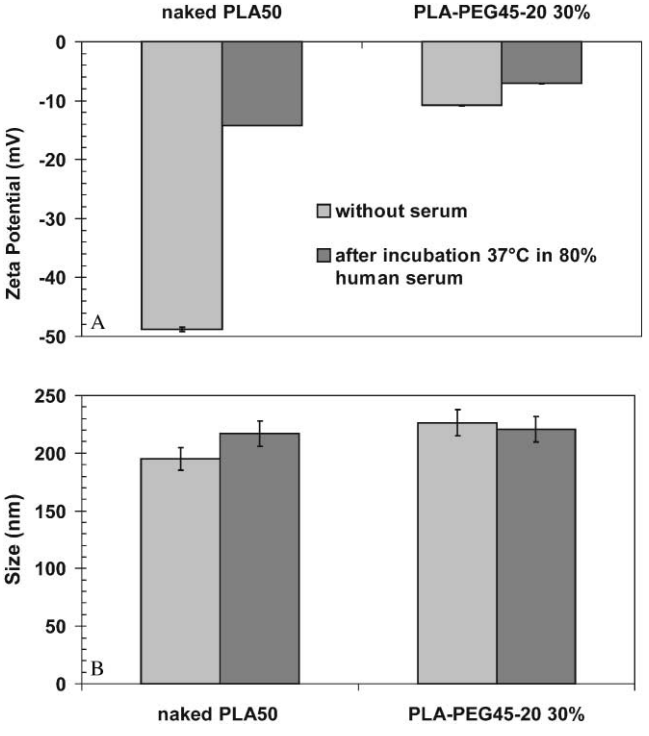

Fig. 5. Effect of human serum protein binding on ζ potential (A) and size (B) of NC. To determine the effect of serum proteins on ζ potential, 100 µl nanocapsules were incubated in 400 µl of human serum for 30 min at 37°C. The NC with or without serum were diluted 1:250 1 mM NaCl and the conductivity was adjusted to 160 ± 6 µS/cm for direct comparison.

3.6. Assessment of complement activation

Evidence of C3 cleavage was obtained by measuring C3 and C3b migration by crossed immunoelectrophoresis in the presence of a polyclonal antibody to human C3. This property was used as a way to characterize the avoidance of complement activation by surface-modified NC and allowed a semi-quantitative estimation of the

Table 2
Relationship between PEG content, distance, chain length, ζ potential and C3 activation of PEG and naked NC obtained from different biodegradable polymers

Nanocapsule formulation and polymer composition[a]	PEG % w/w[b]	ζ potential (mV)	*D* PEG-PEG (nm) calculated	Ratio height C3/C3b peaks
Naked PLA	0	− 47.7 ± 0.5	—	0.30
PLA-PEG 45–5 KDa	10	− 43.5 ± 0.5	4.17	0.42
PLA-PEG 45–20 KDa	30	− 3.9 ± 0.2	3.82	1.21
Naked PLGA (75:25)	0	− 43.8 ± 0.5	—	0.75
PLGA-PEG 45–5 KDa	10	− 41.7 ± 0.3	3.78	0.94
Naked PεCL	0	− 46.9 ± 0.4	—	0.70
PεCL-PEG 45–5 KDa	10	− 39.9 ± 0.3	4.02	0.70

[a] Lecithin at 0.3% w/v.
[b] w/w of total polymer concentration (6 mg/ml).

proportions of native and cleaved C3. Controls were carried out to estimate C3 cleavage in the absence of particles, in the absence of divalent ions in serum and in the presence of opsonized zymozan, a well-known complement activator. The relative sizes of the peaks of C3 and C3b fragments generated by incubation with naked PLA NC and PLA NS, PLA-POLOX NC and PLA-POLOX NC without lecithin were measured in order to compare with those generated by contact with PEG covalently bound to PLA NC.

Table 2 and Fig. 6 summarize the results obtained with the different NC formulations. To assess the effects of hydrophobic cores composed of different biodegradable polyesters, NC with increasing hydrophobicity related to the nature of side chain were prepared from PLGA, PLA and PεCL. Table 2 shows the effect of these different polymer blocks on complement activation in relation with their surface properties. NC diameter was between 180 and 270 nm and the polydispersity indexes of the populations were very similar (data not shown). The PEG distances and ζ potential were close, except for PLA-PEG 45–20 kDa NC, which show lower ζ potential values and the lowest complement activation. PLA, which has intermediate hydrophobicity, was the stronger activator in a NC system. The PεCL and PLGA NC activate C3 less than those with a PLA polymer. Moreover, the activation pattern for NC containing different polyesters was not greatly altered by PEG at similar chain length and distance except for the higher chain length (20 kDa), as shown in Table 2 for PLA-PEG 45–20 kDa 30% NC.

A clear difference in activation between NS and NC is shown in Figs. 6D and E. Naked-PLA nanospheres have a more activating surface than naked PLA NC under our experimental conditions. It is worth pointing out that the only difference between these formulations is the presence of lecithin and oil in the NC composition, which obviously changes the surface characteristics. Indeed, PLA-POLOX NC without lecithin showed a strong activation, indicating that lecithin plays a role in reducing C3 cleavage. At the same time, we observed a significant decrease of C3 activation on PLA-PEG 20 kDa NC compared with PLA-PEG 20 kDa NS, although the distance between PEG chains on the surface is twice as great in NC as in NS (Figs. 6G and H). PEG 5 kDa is not very effective at reducing complement activation at the NC surface even at small *D* values (Figs. 6I and J). The same observation holds for NC prepared from PLGA-PEG and PεCL-PEG copolymers with a PEG chain of 5 kDa (Table 2).

3.7. Uptake and localization in J774A1 cells

This cellular model has previously been used to quantify the association of NC with phagocytes and to suggest some possible uptake mechanisms [13]. In the present work, qualitative analysis of the fluorescence microphotographs allowed us to confirm the main mechanisms involved (Fig. 7). The nuclear area remained non-fluorescent in all cases. Association was markedly influenced by NC surface characteristics, with naked PLA NC, the probe was concentrated in large vesicular compartments (vacuoles), indicating an endocytic mechanism of NC uptake. Membrane fluorescence, mainly indicating binding of the particles, was significantly higher for naked NC. Furthermore, the diffuse fluorescence indicates that diffusion through the plasma membrane could also take place in this case (Fig. 7A2). Less-opsonized particles such as PLA-PEG NC (20 kDa 30%) seem to interact with cells by another non-specific mechanism, showing lower concentration of the fluorescent probe both outside and inside cells. Slight membrane-bound fluorescence (probably due to physical adsorption) and small spots of the probe inside cells can be observed in Fig. 7B2, for the PEG NC. PLA-POLOX NC show unexpected behavior, because fluorescence is concentrated inside cells in a punctuate pattern and very little probe is bound to the cell surface (Fig. 7C2). The confocal-like

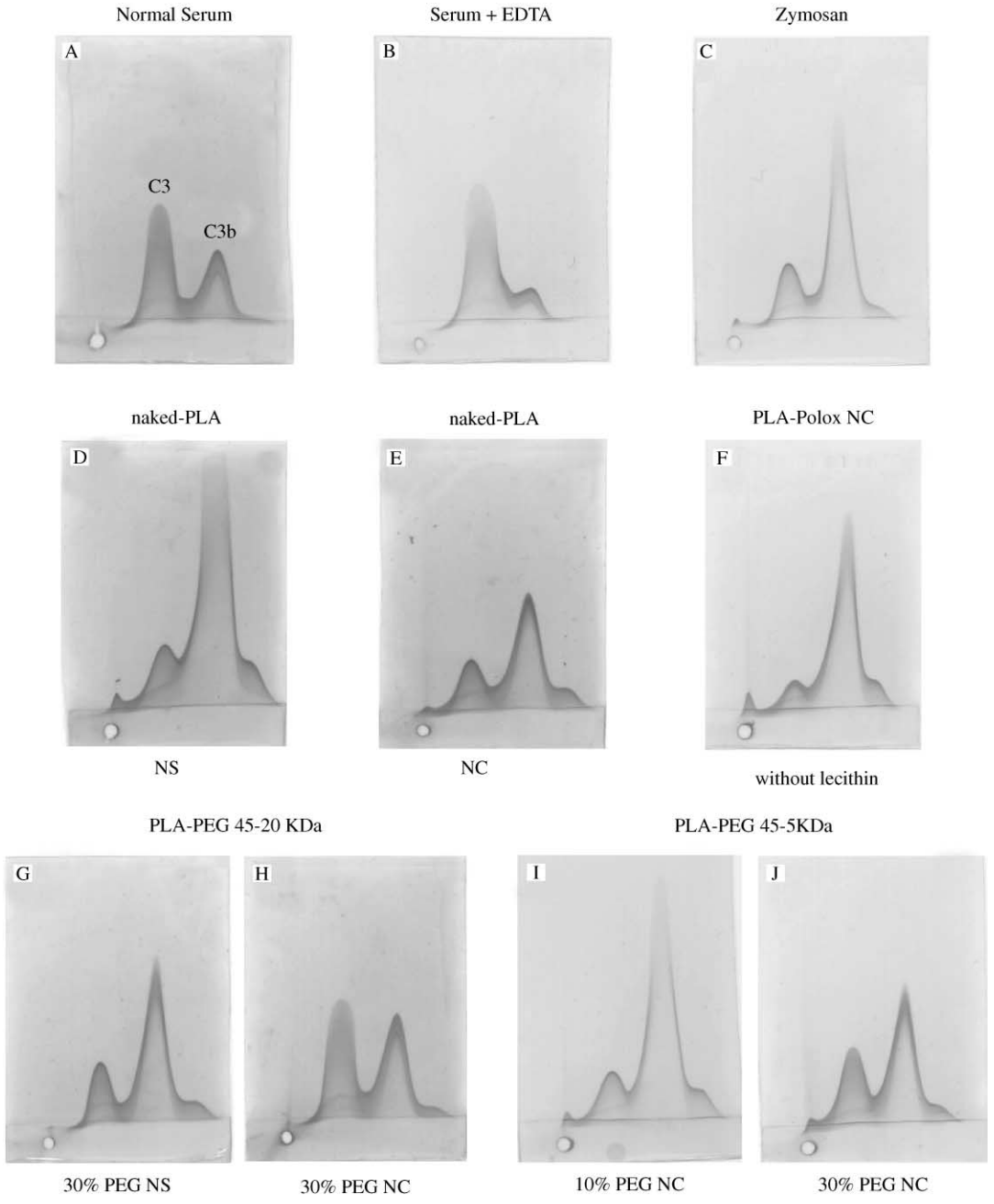

Fig. 6. Crossed immunoelectrophoresis of C3 antigens in human serum diluted 1/4 in VBS^{2+} after 60 min incubation with NP suspensions at constant surface area (500 cm^2). (A) Serum/VBS^{2+}; (B) serum/EDTA 10 mM 1 : 3 v/v; (C) serum/VBS^{2+}/zymosan; (D) naked-PLA NS 143 nm; (E) naked-PLA NC 206 nm; (F) PLA NC without lecithin 252 nm; (G) PLA-PEG 20 30% NS 128 nm, $D = 2.0$ nm; (H) PLA-PEG 20 30% NC, 241 nm, $D = 3.82$ nm; (I) PLA-PEG 5 10% NC 224 nm, $D = 3.48$ nm; (J) PLA-PEG 5 30% NC 255 nm, $D = 2.0$ nm.

fluorescent technique gives a semi-quantitative indication of the fluorescence intensity difference between the three formulations (Figs. 7A3, B3 and C3). A very low intensity was observed for PEG and PLA-POLOX NC. Reducing the incubation temperature from 37°C to 4°C led to a lower fluorescence association for all NC formulations, with dramatically reduced fluorescence inside cells consistent with a passive binding of NC to the J774A1 cell membrane (results not shown). These observations confirm our previous quantitative studies [13], summarized in Table 3.

4. Discussion

Zeta potential measurements have been found useful for characterizing the surface of Stealth® formulations. For liposomes and NS preparations, a "PEG" effect,

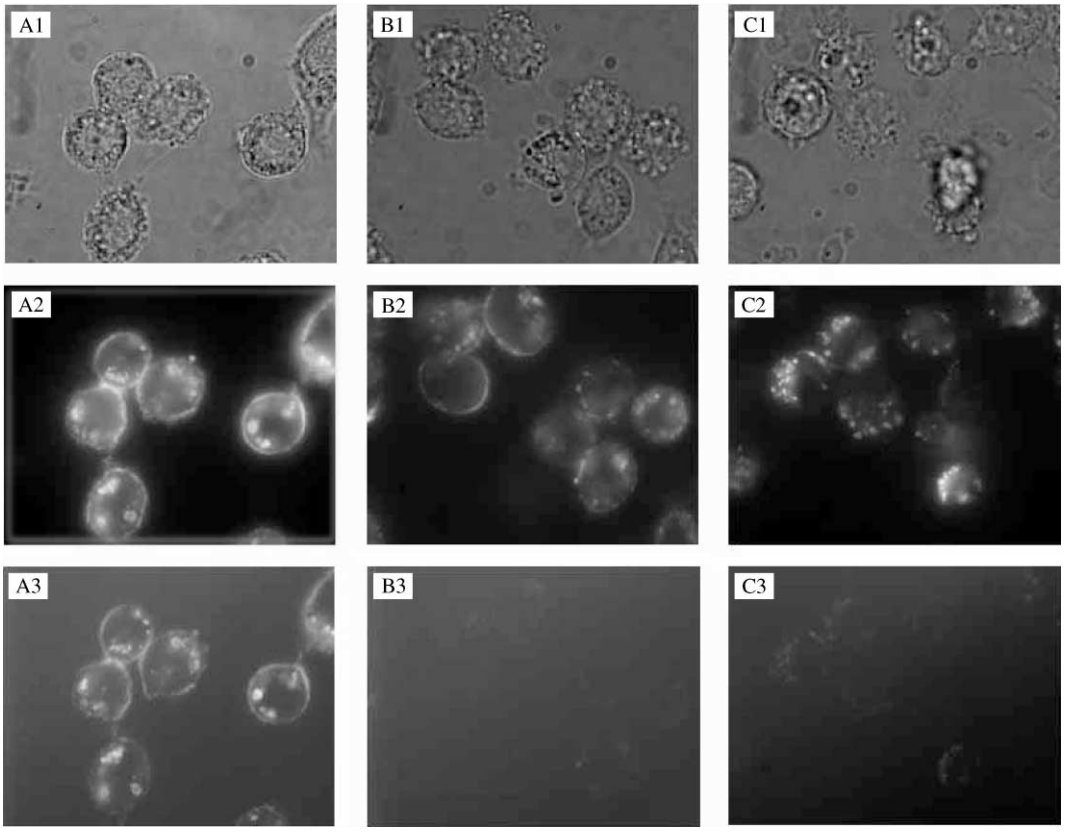

Fig. 7. Phase-contrast (1), fluorescent (2) and confocal-like (3) microphotographs of living J774A1 cells exposed to DiD-labeled NC. Cells were treated with naked PLA NC (A), PLA-PEG 45–20 KDa NC (30% of PEG) (B) and PLA-POLOX NC (C) at 300 μg of polymer/ml, at 37°C and 4°C for 4 h. Pictures were immediately taken after NC were removed from the medium using the same conditions of illumination exposure and were printed without modifications, to allow direct comparison. Three cover slips were observed for each formulation, representative areas are shown.

Table 3
Influence of *D* and chain length on the cellular uptake by J774A1

Nanocapsule formulation and polymeric blend composition[a]	PEG % w/w[b]	*D* PEG-PEG (nm) calculated	Cell-associated fluorescence (ng DiD/10^6 cell)[c]
Naked PLA	0	—	313
PLA-PEG 45–5	10	4.27	122
PLA-PEG 45–5/2–5 1 : 2	30	2.24	89
PLA-PEG 45–20/PLA 1 : 2	10	7.82	83
PLA-PEG 45–20	30	4.49	24

[a] Lecithin at 0.75% w/v.
[b] w/w of total polymer concentration (6 mg/ml).
[c] 300 μg/ml (4200 ng DiD/ml) of NC suspension was added to the culture medium for 4 h. Measured as described in Ref. [13].

reduction of the ζ potential as a result of the presence at the surface of the PEG layer that shifts the plane of shear to the outer boundary of the layer has been observed [8,22,23]. The surface charge of NC as reflected by their ζ potential is drastically reduced at higher PEG densities only at low soy lecithin content (0.3% w/v). The longer chain length (20 kDa) had a more marked effect in reducing the electro-kinetic mobility of these particles than had PEG 5 kDa. This ζ potential decrease could be due to an increase in the PEG density as the PEG content increases (10–30% w/w), ensuring an additional steric protection when a PEG chain length increases from 5 to 20 kDa. The 10–30% PEG content with respect to weight of total polymer seems at first sight to be a high proportion; however it represents only 1.7–5.3% of PEG w/w of dry NC suspension, as compared with 2–10% of PEG in long circulating nanospheres [4,8,24]. It is probable that in the case of NC, a more hydrophobic and complex core

(compared with NS) would demand a higher chain length and density of PEG (more bound water in the surrounding medium) to obtain the optimal colloidal steric stabilization.

The profile of ζ potential of PEG NC as a function of pH is very similar to that of lecithin (PL) vesicles (Fig. 1). Lecithin was used in NC preparations whatever the polymer, PLA or PLA-PEG. Indeed, in previous work it was shown that lecithin has a major role in determining surface properties because minor components of lecithin are able to impart a strong negative charge to the interface [20]. Thus, this result could explain in part the absence of the "PEG effect" on the NC surface charge at the higher lecithin content. However, after reducing the lecithin content the "PEG effect" can be observed more clearly.

The presence of ionizable groups, mainly phosphates and choline in the phospholipids used to stabilize NC (lecithin), confers hydrophilic properties to the interface of these systems, as observed in hydrophobic interaction chromatography experiments. The presence of polyethylene oxide chains at the surface, adsorbed or covalently grafted, is expected to rend these colloids even less hydrophobic (Fig. 2). It is interesting to note that some preparations are composed of different populations of particles of similar size but with different hydrophobicities, of varying densities, in accordance with the gradient density results shown in Fig. 3. These studies confirm the presence of PL vesicles in some NC preparations. Lecithin (PL) vesicles and PLA-PEG 45–20 30% NC are the least hydrophobic formulations. The latter is the only formulation for which it is possible to affirm that the hydrophilicity is mainly due to the PEG coating layer because PLA-PEG 45–20 30% NC is the most homogeneous formulation, as shown in Fig. 3, in which the presence of contaminant PL vesicles could not be detected. The preparation of PLA-PEG NC without lecithin yielded aggregated particles under our preparation conditions, regardless of their PEG content, which prevented their surface analysis. For all the other formulations, soy lecithin remains the principal hydrophilic component at the NC surface and masks the effect of different PEG chains. This could be due to the large volume of the hydrophilic head group of the lecithin phospholipids that could be placed at electrical double layer around the NC surface. It was previously shown that when 0.75% w/v of lecithin was used in the NC formulation a large contamination with phospholipids vesicles occurred but this was reduced by reducing the lecithin content to 0.15% in NC [20].

Particle size and ζ potential measurements can be measured easily and changes in these parameters can give information about protein absorption onto particles. Generally, the adsorption pattern can be different in serum when compared with plasma due to depletion of some proteins. However, the complement system remains functional in serum and was chosen for our experiments. The dramatic change in the ζ potential value of the naked NC after incubation indicates that a layer of protein was adsorbed at the NC surface, in contrast to the reduced effect on ζ potential and size for PLA-PEG NC (Fig. 5). The presence of a PEG layer at the NC surface reduces the charge-induced and hydrophobic binding of plasma, as discussed by other authors [25]. The analysis of the NC morphology by freeze-fracture electron microscopy shows indirectly but clearly that the nature of PLA-PEG and naked-PLA NC surfaces are different, influencing even the physical freeze-fracture properties (Fig. 4).

These differences in surface properties were confirmed by specific analysis of complement activation, which demonstrated the influence of the PEG density and chain length at NC surface on the interactions of these NC with biological systems. The direct correlation between PEG content and chain length in NC and reduced association with macrophages has been previously demonstrated using in vitro methods [13]. The influence of serum proteins was highlighted in that work without identifying which of them were the most important. However, complement proteins were expected to play a major role. The major opsonic fragments generated by complement activation are C3b and iC3b, both generated by proteolytic conversion of C3. C3 products can be generated by the classical or alternative pathways of complement activation, requiring Ca^{2+} and Mg^{2+} ions [5,26,27]. The activation of alternative pathway is regulated by the nature of surface interacting with the complement proteins. The cleavage of C3 by C3 convertase induced by functional groups at the surface of foreign particles results in C3b deposition onto them. Our results show that NC prepared from biodegradable polyesters, especially PLA, act as activators of complement. However, when they are coated with a PEG layer of high density and chain length this activation can be reduced. The hydrophobic (R) block of the amphipathic biodegradable polymers (PLA, PLGA or PεCL) used in NC preparation had only a small influence on C3 activation, as well as on the physicochemical properties of NC, although PLA NC seems to activate C3 more strongly, as shown in Table 2. When R-PEG 5 kDa copolymers were used only a slight reduction in C3 cleavage was observed. This was probably because the NC surfaces were not fully covered by the PEG chains at these distances ($D \sim 4.0$ nm) and the nature of the hydrophobic uncovered surface still determined the pattern of complement activation. However, with longer PEG chains (20 kDa) at the same density, the surface seem to be better covered by PEG chains, whose possible conformations would be a mixture of "brush" and "mushroom" arrangements. It can be hypothesized that these long PEG chains act through creating a PEG-hydrated cloud "shielding" over negatively charged groups located underneath it. A schematic and hypothetical representation of NC surface is

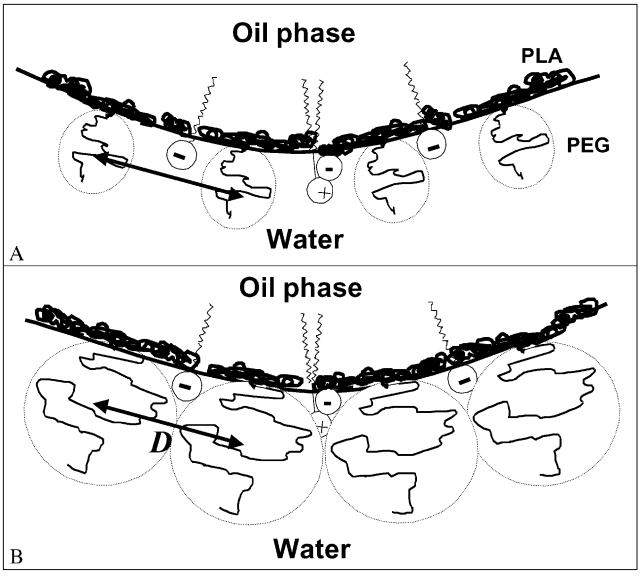

Fig. 8. A schematic and hypothetical representation of NC surface showing the PEG hydrated cloud shield over charged groups located underneath it and PLA blocks at the oil–water interface at the same surface distance (*D*). In (A) PEG 5 kDa and in (B) PEG 20 kDa chains. As the PLA blocks are insoluble in both phases they acquire the most energetically favorable "side-on" orientation at the oil–water interface [28].

proposed in Fig. 8, based on the PLA-PEG molecular chain arrangements at the oil–water interface as previously described [28]. It is widely expected that the preferential distribution of PEG layers at surfaces would change as the area per group decreases, from a "mushroom" in the dilute, unhindered state at low surface concentration, to an extended "brush" at high surface concentration, when interactions between adjacent head groups force each chain to extend further from the surface to which the PEG is attached [29], especially for higher PEG molecular masses.

It is noteworthy that for the same amount of polymer the NC surface is six to ten times greater than that of NS leading to lower concentration of PEG chains at the NC surface. The crossed immunoelectrophoresis results show that at equivalent surface area, reduced complement activation is obtained for NC compared to NS systems for both naked PLA and PLA-PEG particles. However, the values of *D* for nanospheres were lower than for NC (Figs. 6G and H). These differences between NC and nanospheres seems to be due to the presence of lecithin in NC formulations. Furthermore, the increasing complement activation with the PLA-POLOX formulation without lecithin provides additional evidence in this sense (Fig. 6F). Some preliminary results concerning complement consumption by PLA-POLOX NC were obtained using the CH50 method [7]. They indicated that a PLA-POLOX NC suspension/serum/VBS^{2+} at 2:1:1 ratio was able to induce 27% consumption of CH50 units at 5500 cm^2. This value is very low compared to PLA-F68 nanospheres consumption, estimated in a previous work as being greater than 100% for the same surface area [7]. This method did not allow a precise comparison between NC formulations because of the interference of NC turbidity when estimating hemolysis spectrophotometrically. However, this last result also indicates that lecithin probably play a role in reducing C3 activation when placed at the NC surface.

In our study, the PEG 5 kDa chains at higher density are more effective in reducing C3 cleavage than at lower density (Figs. 6I and J). Furthermore, PEG 20 kDa chains were also more effective in preventing C3 cleavage than PEG 5 kDa chains even at a lower PEG density. PEG 20 kDa chains might be more flexible than PEG 5 kDa chains at almost the same surface distance (*D*), thus reducing the accessibility to C3 fragments. Long hydrophilic chains could also exert steric effects of surface hindrance, thus shielding lecithin charge and further reducing C3 deposition, as well as the interaction with proteins in general as shown in Fig. 5.

To summarize, in this work two major effects were observed. The first is the effect of lecithin in NC surface and the second is the effect of PEG chains providing a steric stabilization. They seem to be additive in reducing complement activation of NC compared to NS. Furthermore, our results suggest the limit value ($D \sim 2.2$ nm) previously predicted for the NS system for protein rejection might to be higher for NC systems [4,7,8]. It could be speculated that the kind of polymeric carrier, NC or NS, would determine the response of complement system as a function of the other factors such as surface morphology and chemical topology of its structure, as already observed for nanospheres [3].

The fragments C3b and iC3b generated by complement activation provide specific recognition by type CR1 and CR3 receptors, respectively, on macrophages. Thus a reduced activation of complement system afforded by the PEG layer around the NC surface would be expected to reduce interactions with phagocytic cells and prevents direct contact with cell membrane. The results obtained in this study using fluorescence confirmed the conclusions of our recent paper [13], which is the first report using PLA-PEG diblock polymers to obtain surface-modified NC. NC containing PEG on their surface show reduced interaction with cells and different mechanisms of uptake. Table 3 illustrates the quantitative reduction of fluorescence association with macrophages as a function of PEG distance and chain length at the NC surface. The PLA-PEG 45–20 kDa NC with 30% of PEG yielded the lowest level of interaction (13 times less than naked-PLA NC) and were therefore used in the microscopic study.

In the present work, PLA-POLOX NC showed unexpected behavior, because the fluorescence was mainly concentrated inside cells in a punctuate pattern with very

little probe bound to the surface. It has been reported that poloxamer in micellar form enters the cell by fluid-phase endocytosis in bovine brain microvessel endothelial cells [30]. Since fluid-phase endocytosis in J774 macrophages usually involves vesicles of 30–300 nm diameter and does not require binding in the cell surface as a first step to internalization [31,32] it is possible that PLA-POLOX NC are taken up by this mechanism. Indeed, in Fig. 7C2, the vesicles inside cells are smaller than those in Fig. 7A2, indicating that different endocytic processes are taking place. This phenomenon merits further studies.

Taken together, the results suggest that the low level of protein binding in PLA-PEG 45–20 30% NC is likely to be due to the steric barrier surrounding these particles which prevents protein adsorption and reduces interaction with macrophages.

5. Conclusion

The in vivo behavior of surface-modified NC could be affected by many aspects of their formulation. The NC obtained by nanoprecipitation process are more or less heterogeneous depending on the polymers and the concentration of surfactants used. The PEG NC obtained with PLA-PEG 20 kDa copolymer are the most homogeneous according to density studies. It is necessary to consider the results of several different techniques (HIC, ζ potential, electronic microscopy) in order to have a clear picture of the nature of the NC surface. The techniques of crossed immunoelectrophoresis and fluorescent microscopy were able to demonstrate that the differences between PEG modified NC preparations are closely correlated with our previous results obtained in vitro with J774A1 cells [13]. All of our preparations activate complement C3 protein during 1 h of incubation. However, PLA-PEG NC seems to activate complement to a lesser extent than PLA-PEG nanospheres, although direct comparison is difficult because the NC surface is very different in nature from that of NS. More detailed studies are needed to understand the role of the phospholipids (lecithin) in the interactions with complement. PLA-PEG NC with higher PEG chain lengths (20 kDa) and densities showed a good correlation between some of their surface physicochemical aspects and biological interactions in vitro. These "Stealth" properties need to be confirmed by a study of the behavior of these NC in vivo; the results of such experiments will be the subject of a forthcoming paper.

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