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Phagocytic uptake of fluorescent stealth and non-stealth solid lipid nanoparticles

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Abstract

Fluorescent non-stealth and stealth solid lipid nanoparticles (SLN) were prepared using rhodamine B base as fluorescent marker. The steric stabilization of the nanoparticles was obtained using two lipid derivatives of monomethylpoly(ethylene)glycol 2000 (PEG 2000) as stealth agents: dipalmitoylphosphatidylethanolamine-PEG 2000 and stearic acid-PEG 2000. Stealth and non-stealth SLN were in the nanometer size range. Phagocytosis was evaluated by incubating SLN with murine macrophages and determining the extent of phagocytic uptake spectrofluorimetrically; stealth SLN inhibited phagocytosis to a greater extent than did non-stealth SLN. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Colloidal carriers; Macrophages; Phagocytosis uptake; Stealth solid lipid nanoparticles

1. Introduction

Many colloidal carriers, such as liposomes, nanocapsules and nanoparticles, have been developed as intravenous delivery systems for drugs. They are of interest because they may improve absorption of poorly water-soluble molecules, protect sensitive active molecules against in vivo degradation, modify the distribution of drugs in the body and increase the patient's comfort by avoiding repeated bolus injections.

The systemic use of these carriers is limited by the presence of the reticuloendothelial system (RES) which recognizes them as foreign products and quickly removes them from blood circulation. It is consequently necessary to avoid such recognition occurring. Colloidal carriers are generally recognized by the macrophages due to the physico-chemical characteristics, in particular size,

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surface charge and surface hydrophobicity (Illum et al., 1982; Müller, 1991); hydrophobic polymeric nanoparticles are efficiently cleared from circulation (about 90% in a few minutes), while more hydrophilic nanoparticles remain in circulation for longer times (Illum et al., 1987).

Various attempts have been made to achieve long blood circulation times by avoiding RES recognition, mainly by attaching or adsorbing appropriate polymers or molecules at the particle surface to minimize the interaction with opsonins. One of the most popular and successful methods is coating the nanoparticles with certain hydrophilic and flexible polymers (Moghimi et al., 1994; Gref et al., 1995; Moghimi, 1995).

Hydrophilic monomethyl poly(ethyleneglycol) (PEG) can be modified attaching a hydrophobic moiety, usually the residue of a diacylphosphatidyl amine or fatty acid (Klibanov et al., 1990; Zalipsky, 1995), to obtain an amphiphilic polymeric derivative capable of being incorporated into liposomes with the hydrophilic PEG chain on their surface.

Gref et al. (1994) developed monodispersed biodegradable nanospheres from amphiphilic copolymers composed of two biocompatible block-copolymers esterified with monomethyl PEG, in such a way that PEG attaches covalently to the nanosphere core. They obtained a dramatic increase of blood circulation times and reduced liver accumulation in mice.

Bazile et al. (1995) prepared stealth nanoparticles by using a blend of MePEG-PLA and PLA; these nanoparticles were more slowly captured by THP-1 monocytes than Pluronic F 68 coated PLA nanoparticles, in a PEG chain length dependent manner. Recently, stealth PLA-PEG nanoparticles have been developed for the transport of protein through the nasal mucosa (Tobio et al., 1998).

Solid lipid nanoparticles (SLN) have been proposed as alternative delivery systems of therapeutic agents and diagnostics by many authors (Schwartz et al., 1992; Westesen et al., 1993; Domb, 1995). We prepare SLN from warm oil-in-water (o/w) microemulsions (Cavalli et al., 1997); in previous research (Cavalli et al., 1998) we prepared sterically stabilized SLN, namely stealth SLN, using lipid molecules (dipalmitoylphosphatidylamine and stearic acid) covalently grafted with monomethyl PEG 2000 as stealth agent to form a hydrophilic steric barrier around the SLN. The presence of this hydrophilic PEG chain protected SLN from interaction with human serum albumin; we found a lower albumin absorption on stealth SLN than on non-stealth SLN.

The aim of the present study was to prepare fluorescent stealth and non-stealth SLN and to compare the uptake of stealth fluorescent SLN by murine macrophages with that of the non-stealth fluorescent SLN. Dipalmitoylphosphatidylethanolamine-PEG 2000 (DPPE-PEG) and stearic acid-PEG 2000 were used as stealth agents.

It has been shown that macrophages can be used as a model to study phagocytosis and therefore to point up the effect of modifying the nanoparticle surface on their uptake by cells. Four series of SLN were prepared from two warm oil-in-water (o/w) microemulsions; they differed in both stealth agent and biliar salt used as cosurfactant (glycocholate or taurocholate). Three different amounts of each of the two stealth agents

Table 1
Average diameter and polydispersity index of non-stealth SLN G and stealth SLN AG and BG

	Average diameter (nm)	Polydispersity index
SLN G (non-stealth)	90 (± 3)	0.14 (± 0.03)
SLN AG (0.20% DPPE-PEG 2000[a])	115 (± 4)	0.20 (± 0.03)
SLN AG (0.40% DPPE-PEG 2000[a])	135 (± 5)	0.22 (± 0.04)
SLN AG (0.70% DPPE-PEG 2000[a])	140 (± 6)	0.18 (± 0.05)
SLN BG (0.15% stearate-PEG 2000[a])	105 (± 3)	0.15 (± 0.03)
SLN BG (0.30% stearate-PEG 2000[a])	120 (± 4)	0.18 (± 0.04)
SLN BG (0.60% stearate-PEG 2000[a])	130 (± 5)	0.25 (± 0.04)

[a] The percentage of stealth agent refers to the warm microemulsion.

Table 2
Average diameter and polydispersity index of non-stealth SLN T and stealth SLN AT and BT

	Average diameter (nm)	Polydispersity index
SLN T (non-stealth)	45 (±3)	0.16 (±0.01)
SLN AT (0.20% DPPE-PEG 2000[a])	54 (±5)	0.18 (±0.02)
SLN AT (0.40% DPPE-PEG 2000[a])	66 (±3)	0.22 (±0.03)
SLN AT (0.70% DPPE-PEG 2000[a])	77 (±6)	0.23 (±0.03)
SLN BT (0.15% stearate-PEG 2000[a])	55 (±2)	0.16 (±0.04)
SLN BT (0.30% stearate-PEG 2000[a])	60 (±5)	0.20 (±0.03)
SLN BT (0.60% stearate-PEG 2000[a])	60 (±4)	0.22 (±0.03)

[a] The percentage of stealth agent refers to the warm microemulsion.

were added to each formulation to study their effect on particle size and on their phagocytic uptake and to establish the most suitable amount of stealth agent for this SLN formulation. Rhodamine B base was used as fluorescent marker to obtain fluorescent SLN for measurement in the phagocytosis assay.

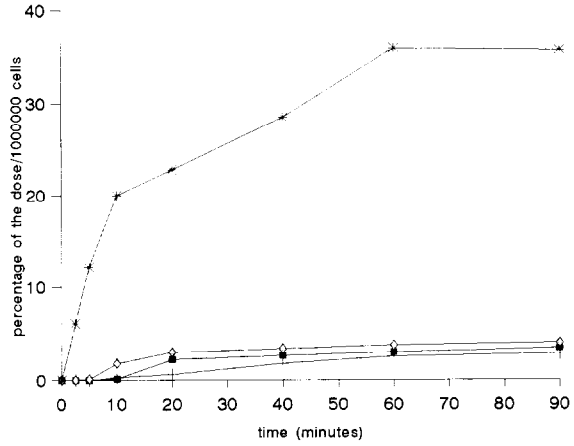

Fig. 2. Uptake of non-stealth SLN G and stealth SLN by macrophages. *, non-stealth SLN G; ◆, SLN BG with 0.15% of stearate-PEG 2000; +, SLN BG with 0.30% of stearate-PEG 2000; ■, SLN BG with 0.60% of stearate-PEG 2000. The percentages refer to the warm microemulsion.

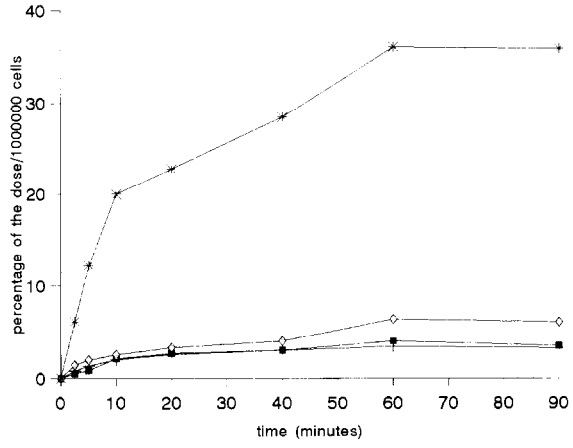

Fig. 1. Uptake of non-stealth SLN G and stealth SLN by macrophages. *, non-stealth SLN G; ◆, SLN AG with 0.20% of DPPE-PEG 2000; +, SLN AG with 0.40% of DPPE-PEG 2000; ■, SLN AG with 0.70% of DPPE-PEG 2000. The percentages refer to the warm microemulsion.

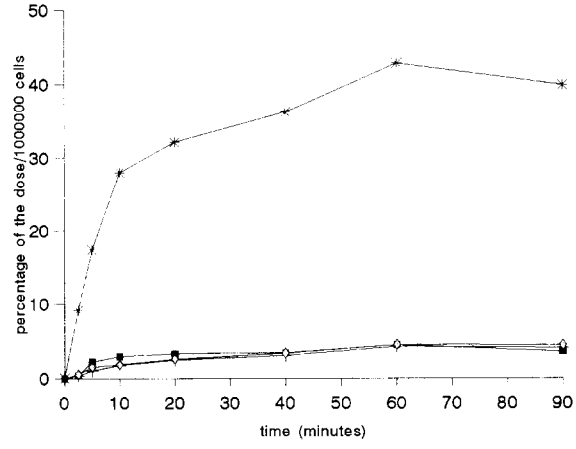

Fig. 3. Uptake of non-stealth SLN T and stealth SLN by macrophages. *, non-stealth SLN T; ◆, SLN AT with 0.20% of DPPE-PEG 2000; +, SLN AT with 0.40% of DPPE-PEG 2000; ■, SLN AT with 0.70% of DPPE-PEG 2000. The percentages refer to the warm microemulsion.

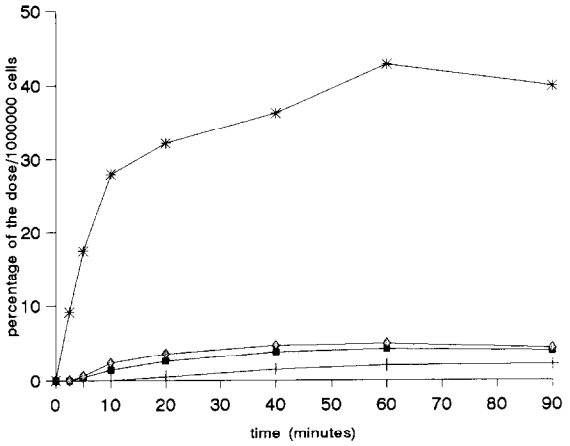

Fig. 4. Uptake of non-stealth SLN T and stealth SLN by macrophages. *, non-stealth SLN T; ◆, SLN BT with 0.15% of stearate-PEG 2000; +, SLN TG with 0.30% of stearate-PEG 2000; ■, SLN BT with 0.60% of stearate-PEG 2000. The percentages refer to the warm microemulsion.

2. Materials and methods

2.1. Materials

Rhodamine B base was from Aldrich Chimica (Milan, Italy). Monomethylpoly(ethylene)glycol (PEG) 2000 and stearic acid were from Fluka (Buchs, Switzerland); Epikuron 200 (soya phosphatidylcholine 95%) was a kind gift from Lucas Mayer (Hamburg, Germany); taurocholate sodium salt (TC) and glycocholate sodium salt (GC) were kind gifts from PCA (Basaluzzo, Italy); dipalmitoylphosphatidylethanolamine-PEG 2000 (DPPE-PEG) was from Avanti Polar Lipids (Alabaster, Al, USA). The other chemicals were of analitycal grade. Stearic acid esterified with monomethyl PEG 2000 was obtained as described elsewhere (Cavalli et al., 1998).

2.2. Culture of macrophages and phagocytosis assay

Murine macrophages cell line J774 A12 was employed to study the phagocytic uptake of SLN; the cells were grown in monolayers in RPMI 1664 medium (GIBCO, Italy) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

For phagocytosis quantification, the cells were incubated for 24 h at 37°C in a 24 well culture plate and then washed to remove non adherent cells; adherent cells were further incubated in DME F12 medium (GIBCO) with 10% fetal bovine serum. Cell numbers were adjusted so that each well contained about 5×10^5 cells. After 24 h incubation, 20 μl of aqueous dispersion of fluorescent SLN (containing 1.26 mg of SLN), either stealth or non-stealth, were added. Following incubation periods of 2.5, 5, 10, 40, 60, 90 min, the cells were washed and rinsed twice with the culture medium to remove the non-phagocytosed SLN. The extent of phagocytosis was assesed by measuring the concentration of the phagocytosed SLN in the resuspended cells as a function of intensity of fluorescence, measured with an LS5 spectrofluorimeter (Perkin Elmer) ($\lambda_{ex} = 540$ nm and $\lambda_{em} = 585$ nm) according to the phagocytosis assay proposed for liposomes (Perry and Martin, 1995). The results are expressed as percentage of the dose/1×10^6 cells. The experiment were performed in triplicate.

The viability of the cells was determined by the trypan blue dye exclusion method and found to be in the order of 95%.

2.3. Preparation of non-stealth fluorescent SLN (SLN T and SLN G)

Two types of non-stealth fluorescent SLN were prepared from two warm o/w microemulsions, G and T, differing only in the biliar salt used. The two microemulsions were prepared using stearic acid (0.70 mmol) as internal phase, Epikuron 200 (0.14 mmol) as surfactant, taurocholate sodium salt (SLN T) or glycocholate sodium salt (SLN G) as cosurfactant (0.69 mmol) and distilled water (111.10 mmol) as continuous phase and rhodamine B base (0.02 mmol) as fluorescent marker.

Epikuron 200 and warm water were added to melted stearic acid and rhodamine B at about 70°C. The cosurfactant was then added to the warm mixture, and a clear system was easily obtained under stirring.

G SLN and T SLN were obtained by dispersing the warm o/w microemulsions (about 70°C) in distilled cold water at a ratio of 1:10 (microemul-

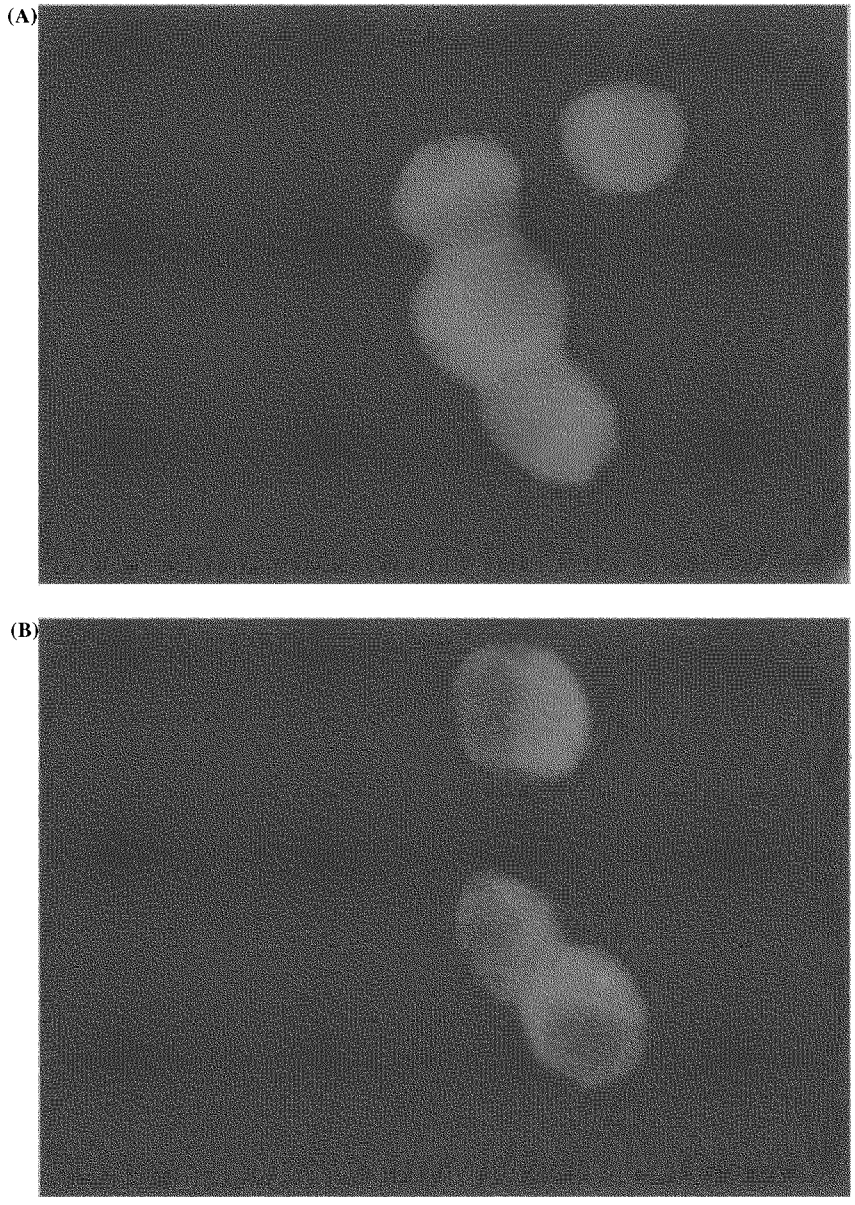

Fig. 5. Fluorescent staining of phagocytic uptake of non-stealth SLN T (A) and stealth SLN AT (B) with 0.2% of DPPE-PEG 2000 by J774-A1 cells.

sion:water, v/v) under mechanical stirring. The nanoparticle dispersions were washed twice with distilled water by diaultrafiltration with a TCF2 system (Amicon, Danvers, USA) using a Diaflo YM100 membrane (cut off 100000 Da) and then concentrated at a ratio of microemulsion:water ratio v/v. SLN dispersion were freeze-dried with a Modulyo freeze-dryer (Edwards, Crawley, UK).

2.4. Preparation of stealth fluorescent SLN (SLN AG, BG and AT, BT)

Four kinds of stealth fluorescent SLN were prepared. To obtain surface-modified SLN, i.e. stealth SLN, stearic acid-PEG 2000 or DPPE-PEG 2000 were added to previously melted stearic acid; then the two microemulsion formulations (G and T) were then prepared as reported above. Two series of SLN G (A and B) containing glycocholate as cosurfactant and three different amounts (1.8×10^{-3} mmol, 3.6×10^{-3} mmol or 7.0×10^{-3} mmol) of each stealth agent were prepared: series AG contained DPPE-PEG while series BG contained stearic acid-PEG as stealth agent. The percentage of DPPE-PEG on whole microemulsion was 0.20, 0.40 and 0.70% w/w, while the percentage of stearate-PEG was 0.15, 0.30 and 0.60% w/w.

Two other series of SLN T (A and B) containing taurocholate as cosurfactant were prepared: series AT contained DPPE-PEG while series BT contained stearic acid-PEG. The percentage of DPPE-PEG on whole microemulsion and stearate-PEG were the same as for SLN G.

Clear systems were easily obtained at about 70°C with each of the amounts of stealth agent.

Stealth SLN were obtained by dispersing the warm microemulsions (about 70°C) in distilled cold water at a ratio of 1:10 (microemulsion:water, v/v) under mechanical stirring. The nanoparticle dispersions were washed three times with distilled water by diaultrafiltration with a TCF2 system (Amicon, Danvers, USA) using a Diaflo YM100 membrane (cut off 100000 Da) and then concentrated at a 1:5 microemulsion:water ratio v/v. SLN dispersion were freeze-dried with a Modulyo freeze-dryer (Edwards, Crawley, UK).

2.5. Characterization of non-stealth and stealth fluorescent SLN

2.5.1. Photon correlation spectroscopy

The average diameter and polydispersity index of non-stealth and stealth SLN were determined by photon correlation spectroscopy (PCS) using an N4 instrument (Coulter) at a fixed angle of 90° and at a temperature of 25°C. Each value was the average of ten measurements. The polydispersity index is a measure of the distribution of nanoparticle population (Koppel, 1972).

2.5.2. Zeta potential measurement

Electrophoretic mobility and zeta potential were measured on dispersions diluted 1:10 v/v with bidistilled water, using a DELSA 440 instrument (Coulter).

2.5.3. Fluorescent microscopy

Fluorescent microscopy was used to observe the phagocytic uptake. Cells attached to coverslips were treated for 60 min with fluorescent non-stealth or stealth SLN, rinsed in phosphate buffered saline (PBS), fixed in *para* formaldehide and then in 95% ethanol. After rinsing in PBS, stained coverslips were mounted in Mowiol and observed under a Leitz DIALUX 20 microscope equipped with epifluorescence optics. Photographic recording was then obtained using a KODAK Ektachrome film.

2.5.4. Determination of rhodamine B incorporated into SLN

The amount of Rhodamine B incorporated into the SLN was determined spectrofluorimetrically using a LS5 Perkin Elmer Spectrofluorimeter ($\lambda_{ex} = 540$ nm and $\lambda_{em} = 585$ nm). Weighed amounts of non-stealth or stealth SLN were dissolved in methanol and analysed.

3. Results and discussion

Fluorescent non-stealth and stealth SLN were prepared using rhodamine B base as fluorescent marker; their spectroscopic characteristics were similar to those of free rhodamine B base solution.

Preliminary experiments were performed to verify the stability of fluorescent SLN in the cell culture medium used and the linearity between SLN (stealth and non-stealth) concentration and their fluorescence was established.

The amount of fluorescent SLN can be measured by fluorimetry at concentrations as low as 5

mcg of SLN/ml. The fluorescence of SLN can be followed in ex vivo uptake experiments and the fluorescence can be related to the concentration of SLN.

The average diameter and polydispersity index of fluorescent non-stealth (SLN G and SLN T) and stealth fluorescent SLN AG and BG and SLN AT and BT, respectively, are reported in Tables 1 and 2.

Fluorescent non-stealth SLN G, obtained from microemulsion G and containing glycocholate as cosurfactant, showed a larger average diameter than did fluorescent non-stealth SLN T, containing taurocholate. This difference in diameter had already been observed (Cavalli et al., 1998) and can be related to the different pK_a values of the two biliar salts used as cosurfactant: pK_a of glycocholate is 4.4 and pK_a of taurocholate was 1.4.

The simplest representation of the structural organization of a microemulsion is the droplet model, in which microemulsion droplets are surrounded by an interfacial film consisting of surfactant and cosurfactant molecules (Attwood, 1994). Three phases are thus present in a microemulsion: oil phase, water phase and interphase. A part of glycocholate at the pH of the microemulsion (pH 5.0) is in the undissociated form and some of it may be partitioned in the oil phase, and consequently increase the size of the SLN.

All stealth fluorescent SLN showed average diameters greater than those of the non-stealth version; as the percentage of the stealth agent added was increased, the average diameters also increased which suggests that the PEG chains are located on the surface of the SLN.

The increase is larger in the case of SLN G than in that of SLN T, and might be due to the different surface density of the layer of PEG chains. The specific surface of SLN G is smaller than that of SLN T, since the former are larger than the latter. Consequently, more stealth agent per unit area may be present on the SLN G. A higher density of stealth agent on the SLN surface may favour the interaction between SLN, increasing the average diameter value.

Indeed a small increase of the polydispersity indices of stealth SLN G was seen to confirm this hypothesis.

The zeta potential value were affected by the presence of the PEG chains; the zeta potential of the stealth fluorescent SLN (between -38.0 and -32.5 mV) are lower than those of the non stealth SLN (-47.5 mV). This decrease in the surface charge of stealth SLN indicates the presence of PEG chains on the surface.

A small quantity of rhodamine B was added to the warm o/w microemulsion in order to maintain the same physico-chemical surface properties as the non fluorescent stealth and non-stealth SLN previously employed in the experiments of albumin adsorption (Cavalli et al., 1998). The maintainance of the surface properties of the SLN, in particular of their hydrophobicity, is important because of parameters affecting the phagocytic uptake of nanoparticles. Müller et al. (1997) showed that the phagocytic uptake of polystyrene nanoparticles labelled with rhodamine B was very different according to the preparation method.

Due to the preparative method of the SLN (rapid crystallization of the oil droplet present in the microemulsion), rhodamine B is randomly dispersed in the lipid matrix of the SLN. Moreover fluorescent SLN were washed three times and consequently the rhodamine B present on the SLN surface was partially eliminated.

After washings, about 90% of rhodamine B was present in both non-stealth and stealth SLN.

The phagocytic uptake of fluorescent SLN by murine macrophages was determined over time for 90 min. The uptake kinetics of non-stealth SLN G and stealth fluorescent SLN AG and BG are reported in Figs. 1 and 2, while those of non-stealth SLN T and stealth SLN AT and BT are in Figs. 3 and 4. The figures showed that the uptake of SLN by macrophages increased from 2.5 to 60 min. The fastest uptake of SLN was found for non-stealth SLN G and T, while uptake of stealth SLN with monomethylPEG-stearate was the slowest. Indeed, the most marked inhibition of phagocytosis was observed in the presence of PEG stearate: in the first minutes the uptake was very slow, not detectable fluorimetrically.

In our experimental conditions, the increase in phagocytosis was non-linear and approached apparent saturation.

The different uptakes of stealth and non-stealth fluorescent SLN by macrophages was confirmed by morphologic observations after 60 min of incubation (Fig. 5) which confirmed intracellular accumulation. Stealth SLN seemed to be scarcely phagocytized by cells as is shown by the weak and discrete fluorescence throughout the cytoplasm. Non-stealth SLN, on the contrary, were apparently phagocytized more actively and accumulated in the cells as revealed by the bright and diffuse pattern of fluorescence observed.

Therefore, the experimental data showed substantial difference in phagocytosis uptake between stealth and non-stealth SLN. The phagocytosis of non-stealth fluorescent SLN was not marked, less than 36% of the initial dose incubated for SLN G and less than 42% for SLN T. This behaviour may depend on the small size of the non-stealth SLN and to the presence of lecithin on SLN surface. Illum et al. (1986) showed a lower uptake of polystyrene nanoparticles coated with lecithin.

The decreased uptake of stealth fluorescent SLN may depend on the presence of the PEG chains on the SLN surface, which form a hydrophilic cloud and the decreased zeta potential.

Fairly small amounts of stealth agent added to to the microemulsion (ranging from about 0.2 to 0.7% w/w) are able to decrease SLN uptake, probably because of the location of the hydrophilic PEG chains on the nanoparticle surface. The molecules of the two stealth agents consist of a hydrophilic and lipophilic moiety, the former being represented in both cases by a PEG 2000 chain. The droplet structure present in the o/w microemulsion should allow the stealth agent to locate at the microemulsion interphase with the hydrophilic PEG chains towards the aqueous phase and the lipophilic moiety in the oil phase. During quenching of the microemulsion in cold aqueous medium, stearic acid can incorporate the hydrophobic portion of the stealth agent, while the hydrophilic polymeric chain, protruding on the surface, remains exposed to the solution and can form a conformational hydrophilic cloud over the nanoparticles that protect the SLN, affecting their hydrophobicity and their charge. Higher amount of stealth agent ($\geq 1\%$ w/w) in the microemulsion did not enhance the protective effect.

In conclusion, the amount of phagocytic uptake of SLN is reduced using a stealth agent in the microemulsion formulation in order to modify the SLN surface. These results are in agreement with the albumin adsorption studies on stealth and non-stealth SLN.

Stealth SLN should have an increase of blood circulation time, if 'in vivo' administered. Works in progress (Zara et al., 1998) have confirmed that stealth SLN intravenously administered to rats showed a prolonged circulation time in comparison to non-stealth SLN.

Acknowledgements

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