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Characterization of PLGA Nanospheres Stabilized with Amphiphilic Polymers: Hydrophobically Modified Hydroxyethyl Starch vs Pluronics

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Abstract: Some Pluronics, particularly F127, are known to stabilize nanospheres and prolong their circulation time *in vivo*. However, these copolymers of poly(ethylene glycol) (PEG) and poly(propylene glycol) are not biodegradable, and despite the long history, there is no approved commercial product using F127 for parenteral administration until now. Meanwhile, hydroxyethyl starch (HES) is a biodegradable polymer that is currently investigated as a substitute for PEG. In order to produce a fully biodegradable amphiphilic polymer, we esterified different molar masses of HES with lauric acid to get different molar substitutions. These polymers, as well as Pluronic F68 and F127, were used to stabilize poly(lactic-*co*-glycolic acid) (PLGA) nanospheres prepared by nanoprecipitation. For physicochemical characterization, the particle size, zeta potential, and the thickness of the adsorbed polymer layer were measured. The ability of the polymer coating to prevent the adsorption of human serum albumin (HSA) and fibrinogen (FBG) was evaluated. Finally, the phagocytosis of the stabilized nanospheres by a monocyte macrophage cell line (J774.2) was assessed. Results show that the PLGA nanospheres had an average particle size of 110−140 nm. The thickness of the adsorbed polymer layer increases with the increase in molar mass, and is generally higher for HES laurates than the studied Pluronics. Pluronic F68, F127 as well as the HES laurates with low molar substitution prevented the adsorption of HSA. HES laurates with low molar substitution and F127, but not F68, prevented the adsorption of FBG. The phagocytosis experiments showed that the HES laurates, particularly the one with the highest molar mass, could reduce the uptake of the nanospheres better than F68 and comparable to F127. Finally, these results warrant *in vivo* experiments to evaluate how the HES laurates can affect the pharmacokinetics and fate of the nanospheres.

Keywords: Fatty acid esters of hydroxyethyl starch (HES); biodegradable polymers; nanoparticles; phagocytosis; Pluronics

Introduction

Polymeric nanoparticulate systems are promising drug delivery vehicles that can control the pharmacokinetics and biological fate of encapsulated drugs.[1] In this regard, surface properties proved to be crucial for the behavior of these nanoparticles, where untreated particles are rapidly removed from the circulation by the mononuclear phagocytic system (MPS).[2] This takes place because these particles can easily adsorb opsonins from the blood plasma, thus facilitating their

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identification and elimination from the circulation.[3] To prevent this rapid uptake, engineering the surface properties of nanoparticles is essential.[4] This can produce long-circulating or "stealth" particles and allow both passive and/or active targeting. The general conditions for the stealthiness of colloidal drug carriers were reviewed by Vonarbourg et al.[5] These include having a small size, with a neutral and hydrophilic surface, as well as a thick, well-anchored and flexible coating.

Accordingly, the use of hydrophilic polymer coatings to nanoparticulates is now a common practice to achieve a long circulation time. The most commonly used polymer for this purpose is poly(ethylene glycol) (PEG)[6] due to its high hydrophilicity and extreme flexibility. It is generally accepted that these PEG coats prevent opsonization by imparting an entropically driven steric hindrance for protein adsorption, which mainly depends on the degree of surface coverage and molar mass.[7] However, this may not be the only mechanism. Another possible explanation is that the PEG coats allow the selective adsorption of some serum proteins that are sometimes known as dysopsonins, i.e. plasma components which are believed to prevent opsonization.[8,9] This is supported by the observation that liposomes carrying gangliosides (a group of naturally occurring glycolipids) on their surface show an extended circulation time, despite the fact that their very thin hydrophilic coats cannot impart the necessary steric hindrance. This is explained by their ability to adsorb selectively some dysopsonins.[10] Additionally, the group of Müller has provided evidence that the selective adsorption of blood proteins can alter the fate of nanoparticles, by, for example, allowing their targeting to the brain, in what they called "differential protein adsorption".[11] To summarize, it may be the selective adsorption, and not the prevention of adsorption, that alters the pharmacokinetics and fate of nanoparticles.

In this study, Pluronics are used to modify the surface of poly(lactic-*co*-glycolic acid) PLGA nanospheres. Pluronics (also known as poloxamers) are a group of ABA triblock copolymers with hydrophilic PEG outer blocks and a hydrophobic poly(propylene oxide) (PPO) as a middle block. They have been extensively studied as surface coatings that can modify the pharmacokinetics and fate of nanoparticles.[12−16] For example, Pluronic F127 (poloxamer 407) was found to prolong the circulation of PLGA nanospheres, where 44% and 5.9% of the nanospheres were found in the blood after 3 and 24 h respectively, compared to 6.4 and 1.6% for the bare nanospheres.[14] However, Pluronics are not biodegradable, and despite the long history, no commercial product using F127 was approved for parenteral administration until now.

Polysaccharides have also been suggested as biomimetic polymer coatings for nanoparticles.[17] For example, a heparin coating on poly(methyl methacrylate) nanoparticles increased their half-life from a few minutes up to 5 h.[18] Similarly, coating superparamagnetic iron oxide nanoparticles (SPIONs) with dextran increased their half-life up to 4.5 h.[19] However,

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Table 1. Molar Mass and Fatty Acid Molar Substitution (Number of Fatty Acids per 100 Anhydroglucose Units (AGU)), as Determined by ^{1}H NMR, for the HES Laurate Samples Used in This Study from Ref 21

sample	HES $M_w \times 10^3$ g/mol[a]	$MS_{fatty\ acid}$ mol %
HES 70-L2	70	2.3
HES 200-L2	200	2
HES 450-L2	450	2
HES 70-L10.3	70	10.3
HES 200-L8.7	200	8.7
HES 450-L9.4	450	9.4

[a] As reported by the producer.

caution should be exercised upon clinical administration to avoid hypersensitivity reactions due to dextran.[17] Meanwhile, hydroxyethyl starch (HES) is a semisynthetic polysaccharide that is used as a plasma volume expander. HES is currently investigated at the industrial level as a biodegradable substitute for PEG, so that HESylation of proteins would substitute PEGylation,[20] however the use of HES for the surface modification of nanoparticulate systems was not studied before. We have reported earlier the synthesis, characterization and self-assembly of hydrophobically modified hydroxyethyl starch (HM-HES).[21] In this manuscript, the ability of HES laurate to stabilize PLGA nanospheres and the interaction of these stabilized nanospheres with phagocytic cells *in vitro* are reported, in comparison to Pluronics.

Experimental Section

Materials. Resomer RG 502 (PLGA, molar mass 12−13 kDa) was purchased from Boehringer Ingelheim, Germany, and Lutrol F68 (Pluronic F68, PEO_{79}-PPO_{28}-PEO_{79}, molar mass 8.594 kDa[22]) and Lutrol F127 (Pluronic F127, PEO_{101}-PPO_{56}-PEO_{101}, molar mass 12.154 kDa[23]) were purchased from BASF, Germany. Different HES laurate polymers were synthesized as described in ref 21 (see Table 1). Briefly, HES was dried and dissolved in dry DMSO. To the solution were added the fatty acid, dicyclohexyl carbodiimide (DCC) and dimethylaminopyridine (DMAP). The precipitated dicyclohexyl urea was removed by filtration. The fatty acid modified HES was precipitated by suitable solvent mixtures, washed and air-dried. The dry polymer was dialyzed against distilled water for 3 days and then lyophilized.

Triton-X 100, fibrinogen from bovine plasma (FBG) and the fluorescent probes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) were from Sigma-Aldrich, Germany. Human serum albumin (HSA) was from Pharma Dessau GmbH, Germany. All other chemicals and solvents were reagent grade and were used as received.

Nanosphere Preparation. A stock dispersion of the nanospheres was prepared by adding 3.5 mL of distilled acetone containing 25 mg of PLGA to 10 mL of bidistilled water with stirring at 1000 rpm. Acetone was removed by evaporation in a rotary evaporator, and the sample weight was adjusted to 10 g using bidistilled water. The polymer-stabilized nanospheres were prepared by dissolving the amphiphilic polymer in the nanospheres dispersion to a final concentration of 7.5 mg/mL.

For the cell uptake experiments (see below), a very hydrophobic fluorescent dye was entrapped in the nanospheres during preparation. One milligram of a fluorescent probe, DiI, was dissolved in 10 mL of acetone stock solution and used during the nanosphere preparation. Its final concentration in the nanospheres dispersion was 50 μg/mL. For these experiments, it was not possible to prepare a stock solution of PLGA nanospheres without using a stabilizer, since the nanospheres encapsulating the fluorescent probe precipitated during the evaporation of acetone. Therefore, the stabilizing polymers were dissolved in water before the addition of the organic PLGA solution. This led to the formation of stable nanospheres.

Physicochemical Characterization. Particle size was determined using dynamic light scattering (DLS). Samples were diluted 1:20 in bidistilled water, and the hydrodynamic diameter of the nanospheres was determined at 25 °C using the backscattering mode (HPPS, Malvern Instruments, U.K.).

The thickness of the adsorbed polymer layer (also known as fixed aqueous layer thickness, FALT[24]) was determined using DLS, as well as zeta potential (ζ) measurements as a function of electrolyte concentration.[25] In the case of DLS, the thickness of the adsorbed polymer layer (δ) was determined according to the following equation (eq 1):[15]

$$\delta = \frac{d_a - d_o}{2} \qquad (1)$$

where d_a is the hydrodynamic size after polymer adsorption and d_o is the hydrodynamic size of the bare nanospheres.

The zeta potential-based measurements of the adsorbed polymer layer are derived from the approximation of the Guy−Chapmann theory, which expresses the decrease of the

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electrostatic potential as a function of the distance from the surface as follows:[26]

$$\psi_x = \psi_o e^{-\kappa x} \quad (2)$$

where ψ_x is the potential at a distance x from the surface, ψ_o is the surface potential, and κ^{-1} is the Debye length, which is the length characterizing the decrease with distance of the potential in the double layer. The Debye length decreases as the concentration of electrolytes increases. This approximation applies only in case of using neutral polymers, and using monovalent electrolytes.[27] Since ζ is the potential measured at the slip plane (also called plane of shear), then a plot of ln ζ vs κ will give the thickness of the adsorbed polymer layer, where κ is equal to $3.33\sqrt{c}$ (c is the molality of electrolytes)[28] (Figure 2).

For zeta potential measurements, samples were diluted 1:10 with a NaCl solution (in bidistilled water) so that the final electrolyte concentration is 0, 1, 2, 4, 6, 8 and 10 mM. Samples were measured using a Zetamaster (Malvern Instruments, U.K.).

Protein Adsorption. In order to test protein adsorption, the nanospheres were prepared in bidistilled water or in a phosphate buffered saline (PBS) pH 7.4 (according to the EP). However in the latter case, bare nanospheres were not stable, probably because PBS reduces the zeta potential necessary for their stabilization. Therefore, the amphiphilic polymers were dissolved in PBS during nanospheres preparation to stabilize them. Please note that the HES laurates with high molar substitution were not soluble in PBS, and thus not used for this experiment.

Nanospheres stabilized with the different amphiphilic polymers were incubated with different concentrations of HSA (0, 10, 15 and 20 mg/mL dissolved in bidistilled water or in PBS pH 7.4) or FBG (0, 1, 2 and 3 mg/mL in PBS pH 7.4) for 3 h at room temperature, after which samples were diluted 1:20 and the hydrodynamic diameter was measured using DLS (HPPS, Malvern Instruments, U.K.).

Phagocytosis of the Nanospheres. The murine monocyte-macrophage cell line J774.2 was used to study the phagocytic uptake of the different PLGA nanospheres.[29,30] Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Biochrom GmbH, Berlin Germany) supplemented with 10% v/v fetal bovine serum (FBS, Biochrom) and 1% w/v antibiotic/antimycotic solution (Sigma, Deisenhofen, Germany) at 37 °C and 5% v/v CO_2. For phagocytosis experiments, the cells were scraped mechanically at 70 − 90% confluence from the bottom of tissue culture flask, and counted using a hematocytometer. The cells were suspended in DMEM with 10% v/v FBS at a concentration of 5×10^5 cells/mL.

100 μL of the cell suspension were added per well in a 96-well plate (Cellstar, Greiner bio-one GmbH, Germany), and incubated for 2 h. To each well, 100 μL of test suspension was added, consisting of 9 parts DMEM supplemented with FBS and 1 part nanosphere dispersion, and then incubated at 37 °C for 6 h. Thereafter, the wells were washed twice with DMEM and incubated for 1 h with 100 μL of 1% v/v Triton X-100 as a cell lysis solution. The resulting cell lysate was transferred to a black poly(propylene) 96-well plate to measure the fluorescence intensity using a fluorescent plate reader (Lumistar Optima, BMG, Jena, Germany) with excitation filter of 544 nm, and emission filter of 590 nm. The fraction of nanospheres phagocytosed was determined by dividing the measured fluorescence intensity to that of the original nanospheres preparation diluted 1:10 in 1% v/v Triton X-100 solution. Since phagocytosis does not take place at 4 °C,[31] the same experiment was carried out simultaneously at 4 °C to determine the extent of surface adsorption of the nanospheres to the cells.

Confocal Laser Scanning Microscopy (CLSM). Preparation of the DiO solution:[32] 3 mg of DiO was dissolved in 0.9 mL of ethanol plus 0.1 mL of DMSO. The solution was filtered through a 0.45 μm nylon filter; 0.7 mL of dye solution was diluted to 10 mL with DMEM to a final concentration of ca. 0.2 mg/mL.

For cell staining and fixation, glass cover slides 15 × 15 mm were placed into a 12-well plate (Cellstar, Greiner bio-one GmbH, Germany). To each well, 0.5 mL of the diluted DiO solution in the culture medium was added; 0.5 mL of the cell suspension in DMEM with 10% v/v FBS at a concentration of 5×10^5 cells/mL was added to each well. Cells were incubated in a CO_2 incubator at 37 °C for 2 h to allow cell adhesion and staining. Then, the medium was aspirated, and adherent cells were washed once with DMEM. One milliliter of DiI-encapsulating nanospheres, diluted 1:10 in DMEM medium with FBS, was added per well and incubated at 37 °C. After 6 h, the medium was aspirated, and the cells were washed with PBS. RotiHistofix was used for cell fixation, followed by washing with PBS. The cover

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Table 2. *Z*-Average Diameter, Polydispersity Index (PDI), Zeta Potential and the Thickness of the Adsorbed Polymer Layer Determined Using DLS and Zeta Potential Measurements for PLGA Nanospheres (NS) Stabilized with Different Amphiphilic Polymers

	Z-av ± SD	PDI	thickness of the adsorbed polymer layer from DLS [nm]	zeta potential [mV]	thickness of the adsorbed polymer layer from zeta potential [nm]
Bare NS	110.5 ± 1.1	0.06	0	−34.5	0
F68	116.9 ± 0.8	0.07	3.17	−23.1	5.4 ± 0.16
F127	122.8 ± 0.8	0.06	6.12	−23.5	8.9 ± 1.10
HES 70-L2	126.3 ± 0.9	0.07	7.87	−19.2	7.4 ± 1.40
HES 200-L2	132.9 ± 0.5	0.10	11.20	−16.4	11.9 ± 1.00
HES 450-L2	140.7 ± 1.2	0.07	15.07	−15.6	14.2 ± 2.40
HES 70-L10.3	116 ± 0.4	0.12	2.75	nd[a]	nd
HES 200-L8.4	118.8 ± 0.4	0.11	4.15	nd	nd
HES 450-L9.4	119 ± 0.9	0.12	4.25	nd	nd

[a] Not determined.

Table 3. Number of Monomeric Units for the Different Amphiphilic Polymers, Together with the Radius of Gyration in Theta Solvents and Good Solvents

	no. of monomers	R_g in theta solvent [nm]	diam in theta solvent [nm]	R_g in good solvent [nm]	diam in good solvent [nm]
one PEG chain of Pluronic F68	76	3.14	6.28	4.84	9.68
one PEG chain of Pluronic F127	100	3.6	7.2	5.7	11.4
HES 70-L2	379	10	20	18.15	
HES 200-L2	1084	16.96	33.92	34.1	68.2
HES 450-L2	2439	24.69	49.38	55.48	110.96

slides were mounted on objective glass slides using Mowiol 4-88 solution and then left to dry at 4 °C for at least 2 days before CLSM.

Images of cells and nanoparticles were obtained using a Leica DM-IRE2 confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) equipped with a computer-controlled, motorized scan stage. An argon laser for DiO excitation at 488 nm was used, while a 543 nm He−Ne laser was used for the excitation of DiI. Two band-pass filters were used between 493 and 538 nm and between 548−630 nm, for the DiO and the DiI emission signals, respectively. For each cell, 20 optical planes were scanned, each having a thickness of 0.3 μm.

Results and Discussion

Physicochemical Characterization. The process of nanoprecipitation is known to produce nanoparticles with a size range of 100−300 nm and with a narrow unimodal distribution.[33] Results in Table 2 show that the produced nanospheres follow the same pattern, with an average size of 110 nm for the bare nanospheres, and a PDI lower than 0.1. The thickness of the adsorbed polymer layer obtained from DLS measurements is close to those mentioned in the literature for Pluronic F68 (3−6 nm depending on particle size)[34] and F127 (7 nm).[14] In the case of HES laurate, it is possible to notice that the thickness increases with the increase of the average molar mass from 70 to 450 kDa, particularly in the group with low degree of substitution. It is also worth mentioning that the adsorbed layer is thinner in the case of high fatty acid substitution. This is probably because the macromolecule bends to form loops and tails that are anchored to the surface with the hydrophobic fatty acid. Due to the higher fatty acid content, the number of loops is higher and the thickness is thus lower (Figure 1).

As seen in Table 2, the increase of the molar mass (and thus the adsorbed layer thickness) is accompanied with a decrease in the zeta potential. This is due to the outward shift of the slip plane due to the increase in the thickness of the adsorbed polymer layer. The adsorbed layer thickness determined from zeta potential measurements is slightly higher for the Pluronics than that determined by DLS, but is nearly identical in the case of HES laurates. It is worth mentioning that the thickness of the adsorbed polymer layer is method-dependent. For example, Stolnik et al.[15] found a difference in the results of the thickness of the polymer layer when determined by field flow fractionation (FFF) and DLS.

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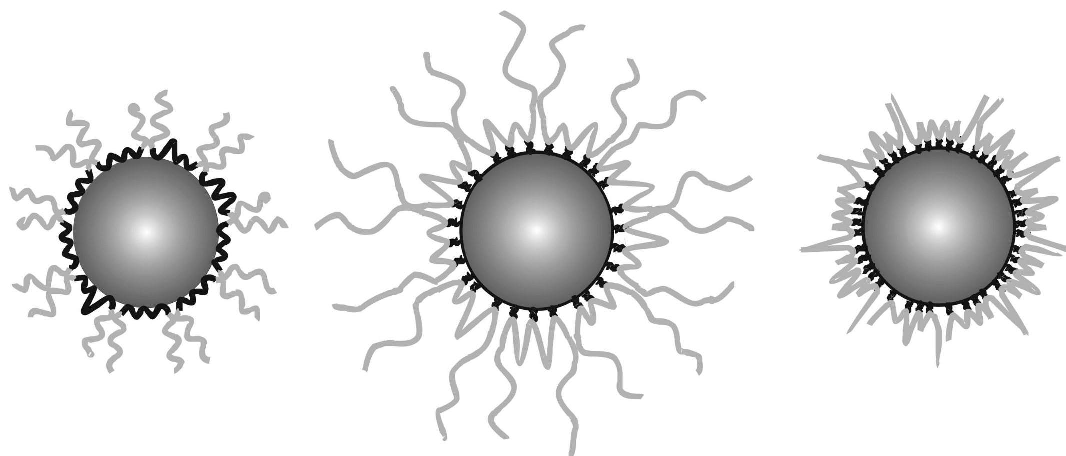

Figure 1. A simplified schematic representation showing the adsorption of Pluronics on the surface of PLGA nanospheres (left), where the PPO blocks (black) adsorb to the surface, while the PEG chains (light gray) extend to the surrounding aqueous phase. In the middle, the HM-HES with low substitution is anchored to the surface through the alkyl chains (black) and the HES backbone (light gray) forms loops and tails. To the right, the HM-HES with high substitution shows a more tight anchorage due to the higher number of fatty acid chains, and the thickness of the adsorbed layer is smaller.

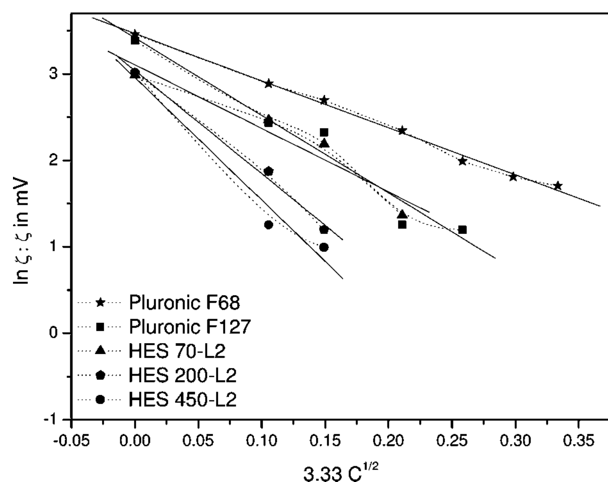

Figure 2. Plot of ln ζ vs $3.33\sqrt{c}$ ($=\kappa$). The slope gives the thickness of the adsorbed polymer layer.

Similarly, Rübe et al.[35] found the thickness of Pluronic F68 adsorbed on PLA nanocapsules to be 17 nm when using small angle neutron scattering. In this regard, the differences between the two methods used in this study can be regarded as minor.

Both Pluronic F68 and F127 have 2 PEG chains, each having an average of 76 and 100 monomeric units, respectively. (The average molar mass distribution which conforms to the USP for Pluronic F68 is 7680−9510 Da, and 9840−14600 Da for Pluronic F127. Batch-to batch variations and differences between different producers were reported.[36])

Note that the radius of gyration of a polymer in a theta solvent is equal to

$$R_g = aN^{1/2} \tag{3}$$

and in a good solvent

$$R_g = aN^{3/5} \tag{4}$$

where a is the segment length (which is 0.36 nm in case of PEG,[26] and 0.515 nm in case of the anhydroglucose unit (AGU)[37]), and N is the degree of polymerization. A comparison between the expected diameters from Table 3 and the thickness of the adsorbed polymer layers shows that the values for Pluronics are close to the theoretical values for a random coil, while they are much lower than theoretical for the HES laurates. This discrepancy could be for a number of reasons, including the fact that HES is a branched polymer with a rather large polydispersity, and more importantly, that HM-HES is expected to bend on the surface forming loops and tails in order to accommodate the hydrophobic fatty acid groups on the surface of the nanospheres.

Protein Adsorption. Figure 3 shows the results of incubation of the bare and the polymer-stabilized PLGA nanospheres with different concentrations of HSA in bidistilled water. The bare PLGA nanospheres show an increase in the hydrodynamic diameter of 33 nm with the 10 mg/mL HSA concentration, which then decreases to approximately 10 nm for the higher concentrations. Lee et al.[38] have reported similar results for HSA adsorption on polystyrene nanospheres. They attributed the initial high apparent increase in the hydrodynamic diameter at a protein/polymer ratio ≤5

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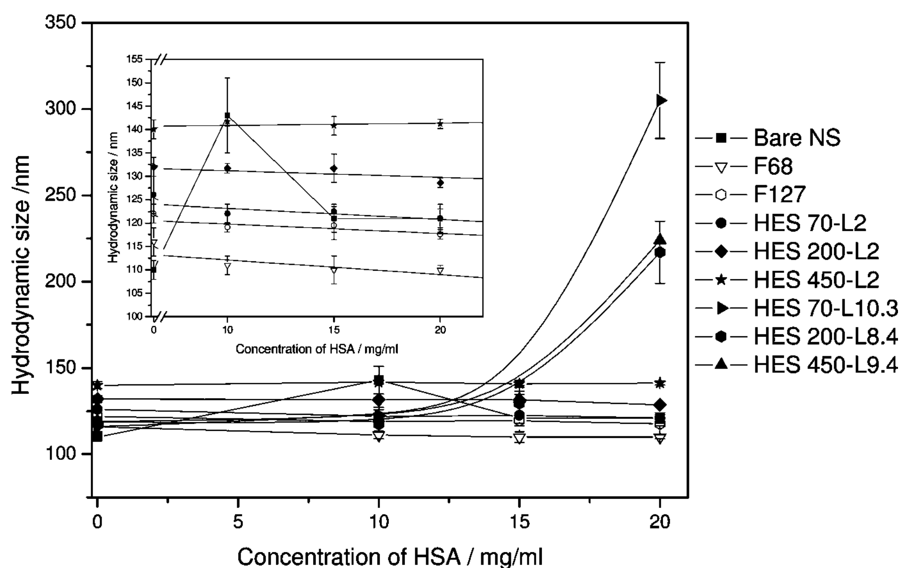

Figure 3. Hydrodynamic diameter of PLGA nanospheres stabilized using different amphiphilic polymers before and after incubation with 10, 15 and 20 mg/mL HSA in bidistilled water at room temperature for 3 h. For better visibility, the inset shows the same data excluding the results of HES laurates with high degree of modification.

due to bridging of nanospheres with the albumin molecules. Higher concentrations give a constant increase in thickness of approximately 8 nm due to a monolayer of adsorbed albumin.[38]

Tan et al.[13] reported that Pluronic F68 and F108 (the latter having two PEG chains each with approximately 129 monomeric units) were effective in preventing the adsorption of HSA on polystyrene nanospheres. Similarly, our results show that Pluronics prevent the adsorption of HSA on PLGA nanospheres as indicated by the constant hydrodynamic diameter of the nanospheres. On the other hand, not all the HES laurates behaved in the same manner. Those having a low degree of substitution were effective in preventing the protein adsorption, while the ones with a higher degree of substitution showed a slight increase in size with 15 mg/mL HSA probably due to protein adsorption, and a large increase in size with eventual precipitation with higher protein concentration due to bridging and destabilization of the nanospheres. Albumin is known to have four binding sites for fatty acids.[39] It is possible that some alkyl chains are extending to the aqueous environment in the case of the high degree of substitution, and thus act as adsorption sites and bridging points for HSA. Upon using PBS pH 7.4 instead of bidistilled water, no adsorption was observed in the case of Pluronics or low substitution HES laurates (Figure 4). It is worth mentioning that the increase in ionic strength due to using PBS reduces the effect of electrostatic repulsion. This indicates that indeed the prevention of adsorption was due to steric hindrance from the polymer coat. Please note that HES laurates with high degree of substitution were not used in this experiment because they were not soluble in PBS.

Fibrinogen is known to have a higher penetrating/anchoring ability due to its semiflexible α-chains, and thus a higher

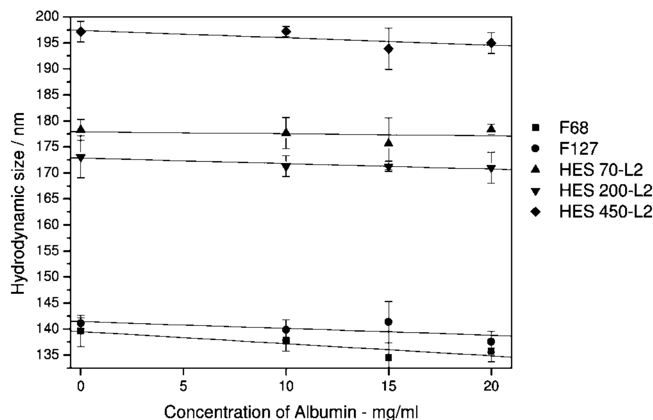

Figure 4. Hydrodynamic diameter of PLGA nanospheres stabilized using different amphiphilic polymers before and after incubation with 10, 15 and 20 mg/mL HSA in PBS pH 7.4 at room temperature for 3 h.

affinity to hydrophobic surfaces.[38] As seen in Figure 5, Pluronic F68 failed to prevent the adsorption of fibrinogen, while F127 as well as the low substitution HES laurates showed no adsorption.

Phagocytosis of the Nanospheres. Pluronic F68 and F127 are known to reduce the uptake of nanospheres by phagocytic cells *in vitro*.[14,16] However, the reduction of phagocytosis in the case of Pluronic F68 is concentration dependent, and decreases by dilution,[40] implying a reversible adsorption. Figure 6 shows the quantities of nanospheres associated with cells after 6 h of incubation with the particles at 4 and 37 °C. The fluorescence intensity (FI) was lower at 4 °C with no statistically significant differences between the various

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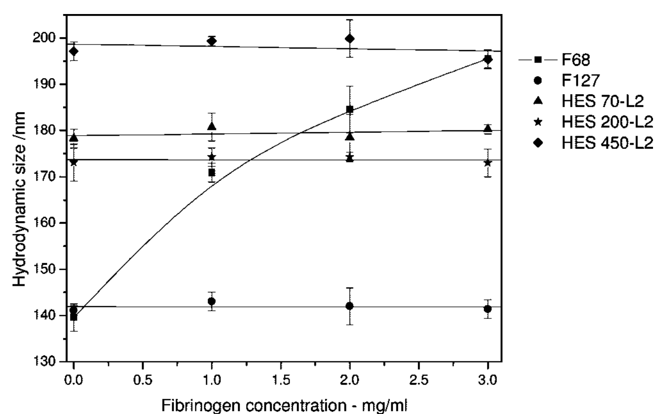

Figure 5. Hydrodynamic diameter of PLGA nanospheres stabilized with different amphiphilic polymers incubated with 0, 1, 2 and 3 mg/mL fibrinogen in PBS pH 7.4 at room temperature for 3 h.

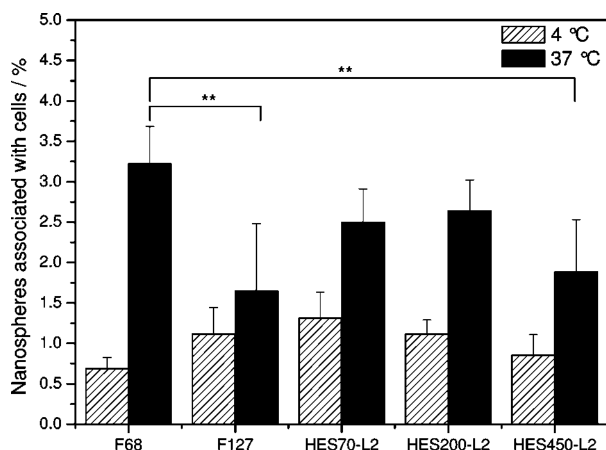

Figure 6. Adsorption and uptake of PLGA nanospheres stabilized with different amphiphilic polymers upon incubation with J774.2 phagocytic cell line for 6 h at 4 °C (dashed bars), and 37 °C (black bars). Statistical significance was carried out using 2-tailed unpaired *t* test, $P \leq 0.001$. (Average of 2 experiments, $n = 10$).

polymeric coats ($P \leq 0.001$). Since phagocytosis is inhibited at 4 °C,[31] one can assume that nanospheres are only adhering to the cell surface at this temperature. By contrast at 37 °C, significantly elevated FI can be observed for all nanospheres modifications indicating phagocytosis of nanospheres. Pluronic F68 expressed the highest FI indicating highest particle uptake, while Pluronic F127 and HES 450-L2 showed a statistically significant reduction (40−50% reduction in uptake) compared to Pluronic F68. Figure 7 shows the effect of the amphiphilic polymer concentration on the uptake of nanospheres at 37 °C. As expected for Pluronic F68, the reduction in uptake was concentration dependent, and increased with increasing concentration. By contrast, it was rather constant in the case of HES 450-L2 in the studied concentration range. This points to the relatively firm attachment of HES 450-L2 to the surface, which is probably due to the presence of multiple anchoring points, and the need for a statistically less likely cooperative motion of the polymer backbone for the desorption of the macromolecule.

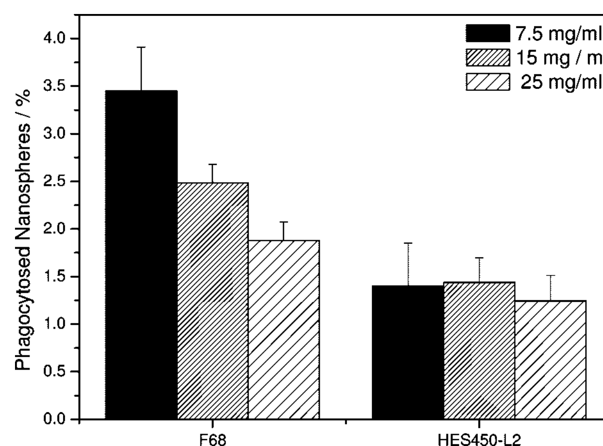

Figure 7. Effect of the amphiphilic polymer concentration on the phagocytic uptake of PLGA nanospheres stabilized with Pluronic F68 vs HES 450-L2 and incubated with J774.2 cell line at 37 °C for 6 h.

The confocal micrographs in Figure 8 represent optical sections in the middle of the cells (slice 11 out of 20). Cells were incubated with nanospheres, stabilized with different polymers, for 6 h at 37 °C. The micrograph in Figure 8a shows the appearance of cells labeled with DiO only, which stains mainly lipids. The cells have a strong green background, while the nuclear region appears black. A specific staining of internal vesicles, which may represent endosomes and other organelles, is visible. Cells incubated with Pluronic F68-stabilized nanospheres shown in Figure 8b, express a large number of red dots (see arrows), which represent nanospheres stained with DiI. These dots are mainly visible inside the cells. The orange color of most of them indicates a mixing with the green staining of intracellular vesicles. This is probably attributed to the trafficking of the nanospheres to cell organelles, such as the endosomes or lysosomes. By contrast, only a few nanospheres stabilized with F127 and HES 450-L2 (Figure 8c and d) can be seen associated with cells. Moreover, the nanospheres stabilized with F68 showed a higher tendency for surface adsorption than those stabilized with F127 or HES 450-L2 (see arrows in Figure 8b). Overall, confocal microscopy confirms qualitatively the findings of quantitative phagocytosis assay, that nanospheres stabilized with F68 undergo a considerable uptake while those covered with F127 or HES 450-L2 possess a stealth character since their uptake was considerably lower.

Conclusions

In this study, the ability of HES laurates—having different molar masses and different degrees of modifications—to stabilize PLGA nanospheres was evaluated, in comparison to Pluronic F68 and F127. Nanospheres with an average particle size of 110−140 nm were produced. The thickness of the adsorbed polymer layer increases with the increase in molar mass, and is generally higher for HES laurates than the studied Pluronics. The adsorption of HSA on the

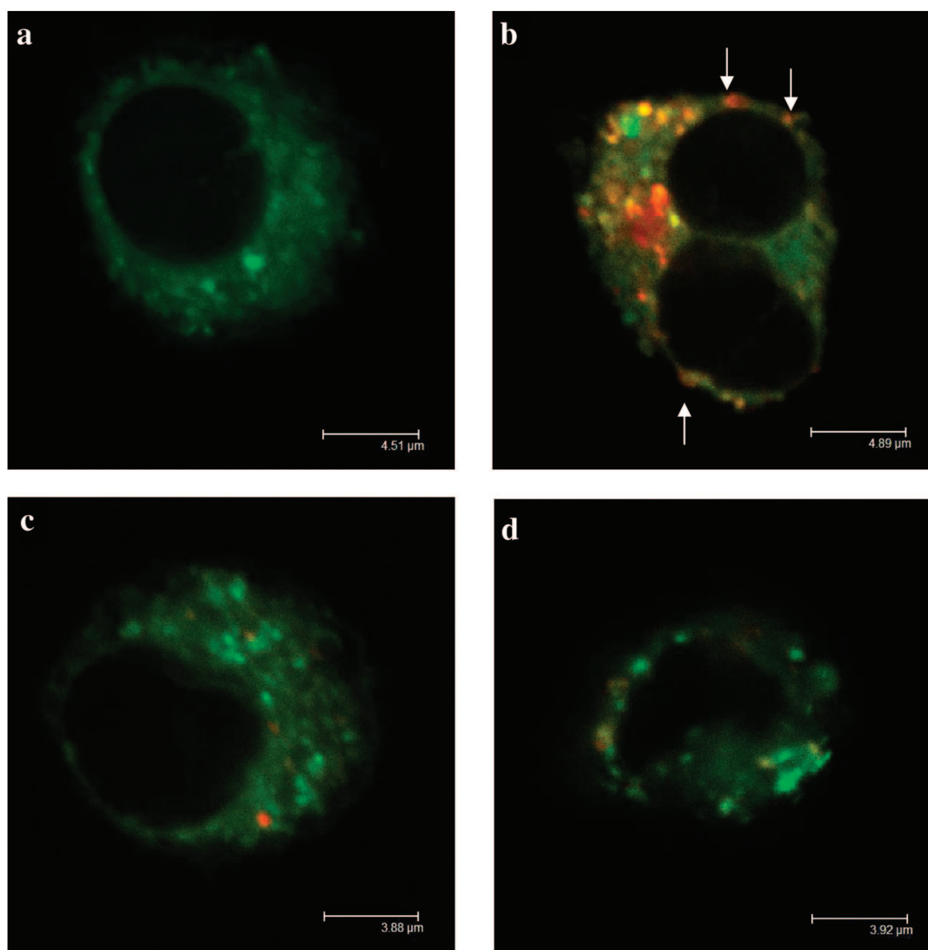

Figure 8. CLSM micrographs of a control cell (not incubated with nanospheres) stained with DiO (green) (a), cells incubated with PLGA nanospheres stabilized with Pluronic F68 (b), Pluronic F127 (c), and HES 450-L2 (d) at 37 °C for 6 h. Nanospheres are stained with DiI (red).

nanospheres was prevented by Pluronic F68, F127 as well as the HES laurates with low molar substitution, while the ones stabilized with medium substitution HES laurate aggregated in the presence of HSA. This is probably due to the presence of fatty acid binding sites on HSA, so that it acts as bridges between the nanospheres. Meanwhile, HES laurates with low molar substitution and F127, but not F68, prevented the adsorption of the more hydrophobic protein, fibrinogen. The phagocytosis experiments showed that the HES laurates, particularly HES450-L2, could reduce the uptake of the nanospheres better than F68 and comparable to F127. This points to the significant role of an efficient steric repulsion to prevent protein adsorption and phagocytosis. In general, HES laurates with low degree of substitution may be a biodegradable alternative to Pluronic F127 for stabilization and *in vivo* administration of PLGA nanospheres. However, the question remains open, how they would perform *in vivo*, which will be the focus of future investigations.

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