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PLGA nanoparticles of different surface properties: Preparation and evaluation of their body distribution

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Abstract

The opsonization or removal of nanoparticulate drug carriers from the body by the reticuloendothelial system (RES) is a major obstacle that hinders the efficiency of the nanoparticulate drug delivery systems. Therefore, several methods of camouflaging or masking nanoparticles (NPs) have been developed to increase their blood circulation half-life. In this study, rhodamine B isothiocyanate (RBITC) loaded NPs were fabricated by an emulsification/solvent diffusion method.

The surface of NPs was then modified using either poly ethylene glycol (PEG) or block copolymer of ethylene oxide and propylene oxide, Poloxamer 407 (POL). The surface treatment was carried out using two different methods: (a) co-incorporation of the surface modifying agents (SMAs) into NPs and (b) the external surface adsorptions method and both of these methods were done only by physical incorporation of the SMAs into the NPs, without the need of special chemical reagents. The biodistribution properties of the NPs were then measured.

The results confirmed that the surface treatment of the NPs using co-incorporation of the SMAs into NPs is more efficient in increasing the blood circulation half-life of the NPs when compared with the external surface adsorptions method.

Keywords: PLGA nanoparticles; Biodistribution; Surface treatment; Poly ethylene glycol; Poloxamer 407

1. Introduction

In order for a drug delivery device to achieve an increase in patient compliance and quality of life it must be present in the bloodstream long enough to reach or recognize its therapeutic site of action. However, the opsonization or removal of nanoparticulate drug carriers from the body by the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES), is a major obstacle to the realization of these goals (Gref et al., 1994).

Several methods of camouflaging or masking nanoparticles (NPs) have been developed, which allow them to temporarily bypass recognition by the MPS and increase their blood circulation half-life (Illum and Davis, 1984; Kaul and Amiji, 2002).

As a general rule, the opsonization of hydrophobic particles, as compared to hydrophilic particles, has been shown to occur more quickly due to the enhanced adsorption of blood serum proteins on these surfaces (Carstensen et al., 1992; Müller et al., 1992; Norman et al., 1992). Therefore, a widely used method to slow opsonization is the use of surface adsorbed or grafted shielding groups which can block the electrostatic and hydrophobic interactions that help opsonins bind to parti-

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cle surfaces. These groups tend to be long hydrophilic polymer chains and non-ionic surfactants. Some examples of polymer systems that have been tried in the literature as shielding groups include polysaccharides, polyacrylamides, poly(vinyl alcohol), poly(*N*-vinyl-2-pyrrolidone), PEG, and PEG-containing copolymers (Peracchia et al., 1999b).

Typically once a polymeric NP is opsonized and removed from the bloodstream; it is sequestered in one of the MPS organs. In the case of naked NPs, or NPs that have not been PEGylated and lack stealth properties, sequestration in the MPS organs is very rapid, typically a matter of minutes, and usually concentrates in the liver and spleen (Panagi et al., 2001). However, for PEGylated stealth NPs the speed of clearance and final biodistribution is dependent on many factors. The mode of PEG-attachment to NPs is carried out mainly by physical absorption or by covalent grafting (Stolnik et al., 1995; De Jaeghere et al., 2000).

Although a number of different polymers have been investigated for formulating biodegradable NPs, poly(D,L-lactide-*co*-glycolide) (PLGA) and poly lactic acid (PLA), biocompatible and biodegradable polymers, have been the most studied (Jain, 2000).

The goals of the present study were NP formulation, characterization, surface modification and in vivo biodistribution properties of the NPs in rats. In this way rhodamine B isothiocyanate (RBITC) loaded NPs were fabricated by an emulsification/solvent diffusion method, then surface treatment of the NPs was done in two different ways and their biodistribution properties were estimated as parentral drug delivery system. As in this method surface modification is done only by physical incorporation of the SMAs into the NPs, without the need of special chemical reagents, then the method is feasible and easy to use.

2. Materials and methods

2.1. Materials

RBITC and PVA (M_w 22,000, 88% hydrolized) were purchased from Sigma–Aldrich, USA. PLGA (50:50, Resomer® RG 504H, M_w 48,000 Da) was purchased from Bohringer Ingelheim, Germany. Poly(ethylene glycol) with M_w of 2000 and 35,000 Da (PEG 2000, PEG 35,000) were purchased from Fluka, Switzerland. Poloxamer 407 with M_w of 125,000 (POL) was provided by Synopharm GmbH, Germany. Dichloromethane (DCM), acetone and methanol (analytical grade) were purchased from Merck, Germany. Deionized water was used throughout the experiment. All other chemicals used were of reagent grade.

2.2. Methods

2.2.1. NPs preparation method

The RBITC loaded NPs were fabricated by a modified emulsification/solvent diffusion method (Esmaeili et al., 2007). Briefly, PLGA (200 mg) and RBITC as a fluorescent marker (0.05%, w/w, of polymer weight) were added into the mixture of DCM/acetone (10:60, v/v), which was suitably stirred to ensure that all material was dissolved. This solution of organic phase was slowly poured in 100 ml aqueous solution of PVA 1% (w/v) using a high-speed homogenizer (IKA, Ultra turrax, USA) at 24,000 rpm for 5 min. Stirring was continued for 3 h to allow the evaporation of the internal phase. The NPs were then isolated by using a centrifuge (Sigma 3K30, Germany) at 21,000 × *g* for 15 min and washed three times with deionized water. The produced suspension was freeze-dried for 48 h at −40 °C (Lyotrap Plus, LTE Scientific Ltd., UK) to obtain a fine powder of NPs, which was then kept in a desiccator.

2.2.2. Surface modification of the NP

Two different methods were investigated for NP surface modifications as described below:

2.2.2.1. Co-incorporation of SMAs into NPs (internal). In this protocol, PEG or POL as SMA was co-incorporated into the NP matrix during the NP formulation process. In this way PLGA (160 mg) and the SMA, PEG or POL (40 mg) were dissolved in the mixture of DCM/acetone (10:60, v/v). The RBITC was then dissolved in the above polymer solution and then emulsified into a PVA 1% (w/v) (100 ml) solution by homogenization to form an o/w emulsion. The emulsion was stirred for 3 h to evaporate the organic solvent. The NPs were then recovered by centrifugation and then lyophilized as described above.

2.2.2.2. Surface adsorptions (external). In this protocol PEG or POL as SMA, was dissolved in water to form a solution and was added to the produced suspension of the NPs before freeze drying, the amount of the SMA was calculated to produce the total 1% (w/v) of the SMA in the final solution (10 ml). The suspensions of NPs were then lyophilized for 48 h.

2.2.3. RBITC loading

A 20 mg sample of NPs powder was dissolved in 1 ml of acetonitrile followed by the addition of 2 ml of methanol to precipitate the polymer. The sample was then centrifuged for 10 min at 21,000 × *g* and the aliquot was taken from the supernatant and analyzed by a spectrofluorimeter (shimadzu, RF 5000, Japan) at λ_{ex} 540 nm and λ_{em} 580 nm. The RBITC loading was determined as the ratio between the weight of the RBITC in NPs to the weight of the NPs.

2.2.4. NPs characterization

The particle size, size distribution and zeta potential of the NPs were measured by laser light scattering (Malvern Zetasizer ZS, Malvern UK). The samples were examined to determine the volume mean diameter, size distribution, poly-dispersity and zeta potential.

Scanning electron microscopy (SEM, Philips XL 30 scanning microscope, Philips, the Netherlands) was employed to determine the shape and surface morphology of the produced NPs. Particles were coated with gold under vacuum before SEM.

2.2.5. Differential scanning calorimetry (DSC)

DSC scans of empty and RBITC loaded NPs were performed on a Mettler DSC 823 (Mettler Toledo, GmbH, Switzerland)

equipped with a Julabo thermocryostate model FT100Y (Julabo labortechnik GmbH, Germany). A Mettler Star software system, version 9.x was used for the data acquisition. Indium was used to calibrate the instrument. The samples were scanned at a speed of 5 °C/min in 30–250 °C temperature range.

2.2.6. *In vitro releases study*

Drug release from RBITC loaded NPs was studied using a modified dissolution method (Denkbas et al., 1995). The medium was a 0.05 M phosphate buffer solution. A known mass of NPs was suspended in tubes of buffer solution at pH 7.4. The pH experiments were repeated three times. The tubes were placed in a shaker bath (WB14, Memmert, Germany) at 37 °C and shaken horizontally at 90 cycles/min.

At selected time intervals, the tubes were centrifuged and an aliquot of 4.5 ml was taken from the supernatant. A volume of 0.5 ml of methanol was added and analyzed by spetrofluorimetry at λ_{ex} 540 nm and λ_{em} 580 nm. A calibration curve was prepared prior to the start of dissolution using a phosphate buffer–methanol (9:1) media. After the aliquots were removed, the entire supernatant was replenished in order to maintain sink conditions. Drug release data were normalized by converting drug concentration in solution to a percentage of the cumulative drug release.

2.2.7. *Body distribution of RBITC loaded NPs in rats*

Albino Wistar rats, body weight between 200 and 250 g (provided by Animal Care Center, Faculty of Pharmacy, Tehran University of Medical Sciences), were used for body distribution investigations. The Rats were fasted overnight but had free access to water. The RBITC solution or suspension of the RBITC loaded NPs was prepared in NaCl 0.9% and injected intravenously into the tail vein (200 μl of solution corresponding to 10 μg RBITC/kg). For each sample of the NPs, three animals were injected for each sample of RBITC loaded NPs. The animals were sacrificed by cervical dislocation 1 and 3 h post-administration. Organ samples, consisting lungs, liver and spleen were removed, washed with NaCl 0.9% and accurately weighed. The samples then homogenized and centrifuged at 21,000 × *g* for 10 min. Methanol was added to the supernatant (1:1) to precipitate the unwanted proteins and centrifuged (21,000 × *g* 10 min). The aliquots were assayed for RBITC by the spectrofluorimeter at λ_{ex} 540 nm and λ_{em} 580 nm to estimate the amount of RBITC in each organ. For calculations, standard curves of RBITC were prepared by addition of RBITC solutions in methanol to tissues following the same treatment steps (Yoncheva et al., 2005).

2.2.8. *Statistical analysis*

One-way analyses of variance (ANOVA) test was performed on the data to assess the impact of the formulation variables on the in vitro results ($n = 6$). The in vivo data were compared using the non-parametric Kruscal–Wallis test and Nemeny's multiple comparison test. *p*-Values of <0.05 were considered significant ($n = 3$). All calculations were performed using a statistical software program (SPSS® 11.5, Microsoft).

3. Results and discussion

3.1. *Physicochemical properties of the NPs*

PLGA NPs were prepared by modified emulsification/solvent diffusion method (Esmaeili et al., 2007). The basic characteristics of the NPs prepared in this study are presented in Table 1. As shown in Table 1, the drug loading was ranged from 0.4 to 0.8 μg/mg. The obtained NPs had a size range of 189–225 nm and were monodispersed (polydispersity <0.263).

Since the size of the colloidal carriers is a key for the biological fate of the NPs, and NPs of smaller than 100 nm usually bypass the MPS macrophages uptake, it is vital to obtain NPs of smaller particle size. Surface treatment did not affect the particle size ($p > 0.05$) when other preparation conditions were fixed.

Under SEM observation (Fig. 1), the NPs all had a fine spherical shape with a relatively monodispersed size distribution. The size of the particles viewed in the SEM pictures was in agreement with data obtained by laser light scattering.

Zeta potential of the NPs was negative (below −10 mV) due to the presence of terminal carboxylic groups of the PLGA (Müller, 1991). Zeta potential values for the surface modified samples was lower than the NT NPs ($p < 0.05$), it maybe because of a higher concentration of surfactant adsorbed on the particle surfaces with the formation of a denser surfactant film on the NP surface, thus, eliciting a reduced electrophoretic mobility, and a higher absolute potential value ensures a high-energy barrier that stabilizes the nanosuspension (Müller, 1991).

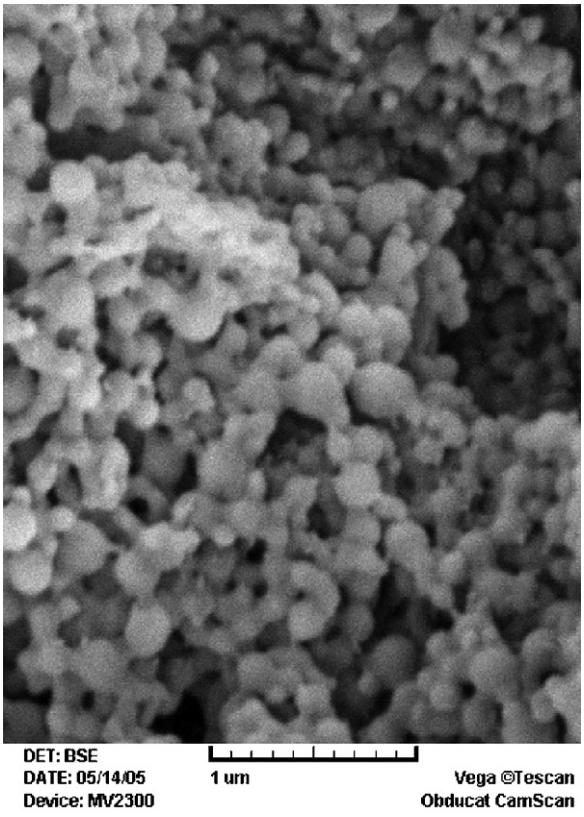

Fig. 1. SEM micrograph of nanoparticles showing the shape and the surface characteristics.

Table 1
Physicochemical characteristics of the nanoparticles

Sample	Size (nm)	PdI	Zeta potential (mV)	RBITC loading (μg/mg)
NT	218 ± 18	0.215	−11.9 ± 0.93	0.80 ± 0.02
PEG 2000-int NP	205 ± 21	0.197	−16.6 ± 0.80	0.57 ± 0.01
PEG 2000-ext NP	189 ± 14	0.142	−16.1 ± 0.12	0.40 ± 0.01
PEG 35,000-int NP	191 ± 16	0.117	−20.3 ± 0.65	0.61 ± 0.03
PEG 35,000-ext NP	225 ± 19	0.263	−19.6 ± 0.56	0.80 ± 0.02
POL-int NP	211 ± 20	0.175	−19.1 ± 0.71	0.55 ± 0.01
POL-ext NP	220 ± 19	0.214	−18.3 ± 0.58	0.60 ± 0.03

DSC studies were performed to investigate the physical state of the drug in the NPs, because this aspect could influence the in vitro and in vivo release of the drug from the systems. Different combinations of drug/polymer may coexist in the polymeric carriers, such as: (i) amorphous drug in either an amorphous or a crystalline polymer and (ii) crystalline drug in either an amorphous or a crystalline polymer. Moreover, a drug may be present either as a solid solution or solid dispersion in an amorphous or crystalline polymer (Musumeci et al., 2006). Fig. 2 shows the DSC thermograms of pure RBITC, Pure SMAs, PLGA RG504 and all NPs formulations.

RBITC showed no melting peak evidencing the absence of crystallinity. As shown in Fig. 2 all pure PEGs and POL have a sharp T_m (melting temperature) that corresponds to their crystal behavior. PLGA shows a T_g (glass to rubber transition temperature) and no T_m that means it is amorphous. Preparation of the NPs has increased the PLGA T_g to higher temperatures; it is because of the crystallization process during the preparation. In the NT NPs, it is obvious that preparation process caused to form two clear peaks in the temperature range of 145–165 °C, that may be attributed the residence of the PVA in aqueous solution during the process. It is clear that using the SMAs in the formulations could not change the crystal forms significantly. In PLGA/SMA physical mixture equal amounts of PLGA and SMA were physically mixed (Fig. 2e), when PEG 35,000 and PLGA were physically mixed (1:1, w/w), T_g of the polymer and T_m of the SMA were sharp and clear while in the prepared NPs (Fig. 2f and g) as the majority of the SMA on the surface of the NPs was removed after washing the NPs with deionized water, the T_m peak of the SMA was weaker.

In conclusion, when the SMAs were added internally or externally, the produced NPs did not differ from each other in crystal forms, on the other hand the SMAs did not enter into the PLGA texture and only was adsorbed on the surface of the NPs.

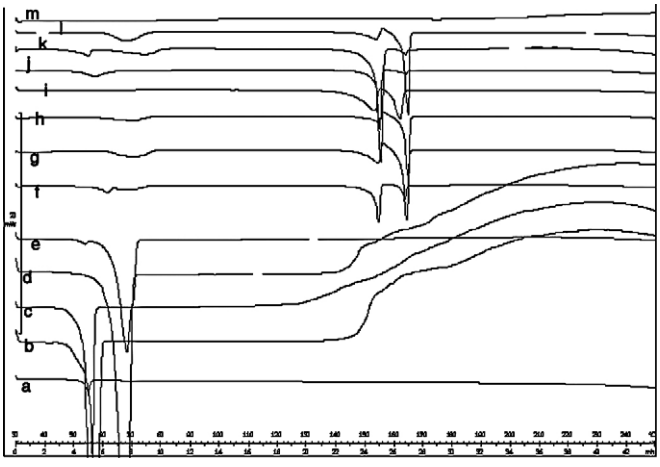

Fig. 2. DSC curves of PLGA Resomer 504H (a), POL 407 (b), PEG 2000 (c), PEG 35,000 (d), PLGA-PEG 35,000 physical mixture (1:1, w/w) (e), PEG 35,000-ext (f), PEG 35,000-int (g), PEG 2000-int (h), PEG 2000-ext (i), POL-int (j), POL-ext (k), NT (l), RBITC (m).

3.2. In vitro drug release study

The in vitro release behavior of the RBITC loaded NPs presented as the cumulative percentage release is shown in Fig. 3. The initial burst release was prominent for all formulations during the first hour of release, being greater than 14%, and for some formulations reach to 100% during the first day. The initial burst could be due to the diffusion release of RBITC distributed at or just beneath the surface of the NPs. Then release is mainly due to the diffusion of drug molecules through the polymeric matrix of the NPs afterwards, the matrix material would require time to erode in the aqueous environment, then the release mechanisms of surface release and polymer erosion might be the main causes of the release behavior (Holland and Tighe, 1992). Surface treatment caused a faster release and the presence of RBITC on the surface was larger in the presence of SMAs ($p < 0.05$). This is because of the fast hydration process at presenting the SMAs on the surface of the particles; however, there was no significant difference among the surface-treated NPs ($p > 0.05$). Hydration occurs very rapidly because of particles nanometric size. The

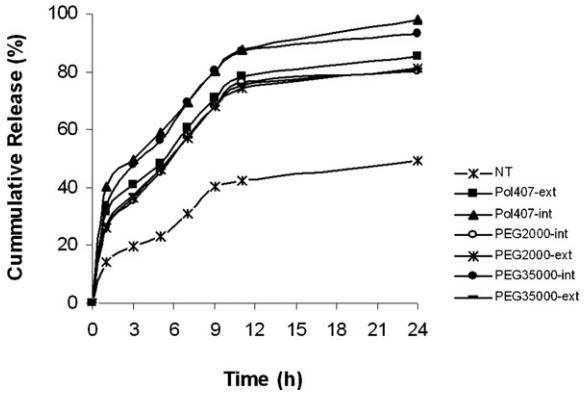

Fig. 3. In vitro release curves of RBITC loaded NPs in phosphate buffer.

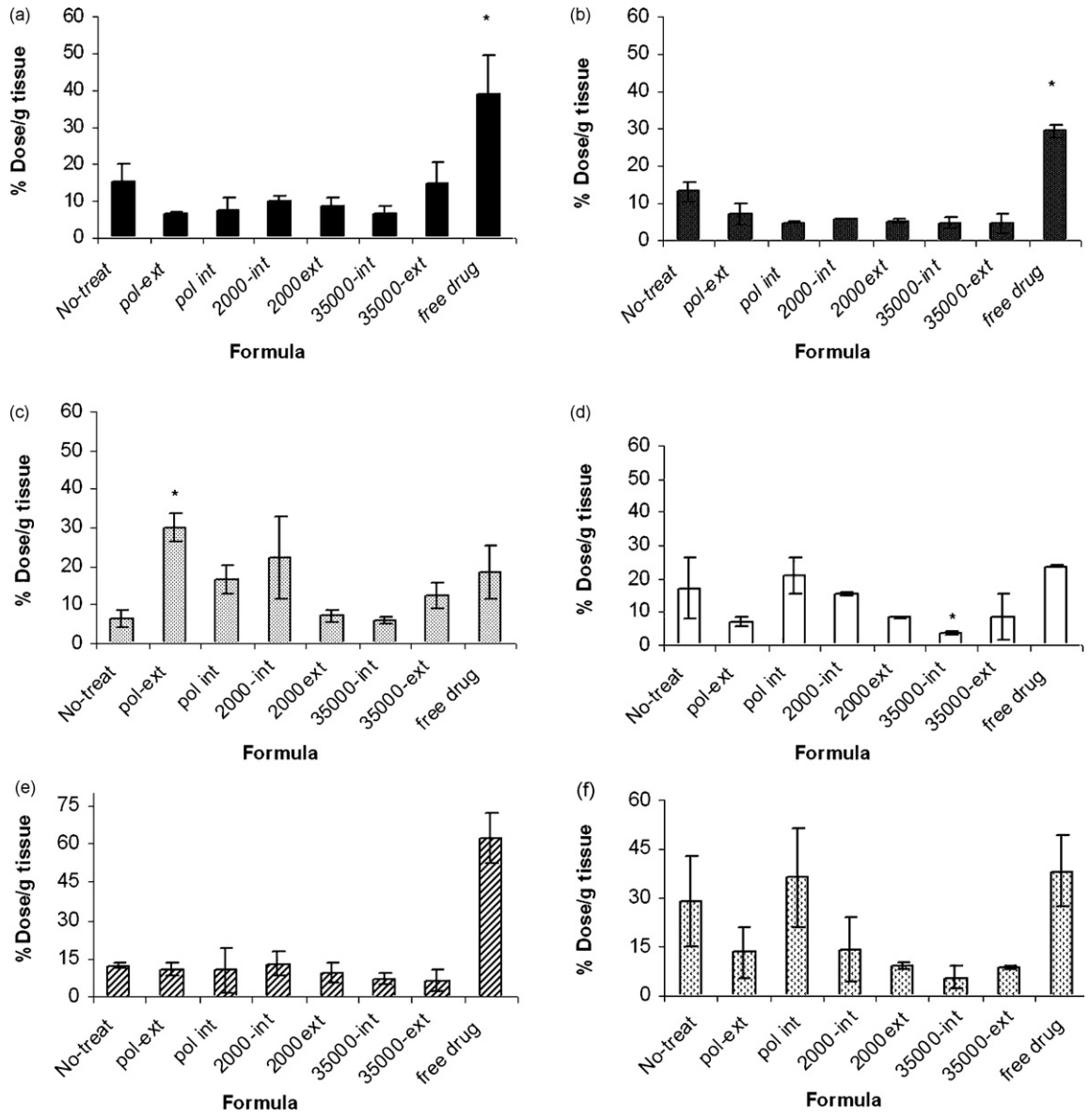

Fig. 4. The level of RBITC detected in the three organs of rat after IV administration. RBITC loaded NPs with different surface treatments. The data obtained from lung (a and b), liver (c and d) and spleen (e and f) in 1 h (a, c and e) and 3 h (b, d and f) after injection are calculated as a percent of the initial dose applied and presented as mean ± S.D. ($n = 3$), (*) shows the different group ($p < 0.05$).

hydrophilization of the matrix operated by the surfactants can account for a more rapid entry of water into the NPs, eventually accelerating the release of RBITC.

3.3. Body distribution of RBITC loaded NPs in rats

In vivo distribution studies were performed using prepared NPs loaded with a fluorescent marker (RBITC). Fig. 4 shows the RBITC percentage of the initial dose per gram of the tissues (lungs, liver and spleen) 1 and 3 h after IV administration of the NPs and RBITC free drug. The amount of the total amount of RBITC (both RBITC released from the NPs and RBITC still encapsulated within the NPs) was measured. Since the percentage of RBITC released from NT formula during the first 2 h was low, between 10 and 20% in PBS (Fig. 3), it could be assumed that the fluorescence measured after administration of the NT formula would be due to the RBITC associated to the NPs. As indicated in Fig. 4, the amount of RBITC in these tissues was different for different surface characteristics. Obviously, the percentage of the RBITC in free drug administration was more than NP formulations since time needed for release of the RBITC from the NPs. To ensure that the fluorescence determined in the organs was due to the RBITC associated NPs, in vitro release of RBITC was examined preliminary.

The non-parametric Kruscal–Wallis test was done to identify the between groups distribution differences. Results show that there was no significant difference ($p > 0.05$) in distribution of the formulations in spleen 1 and 3 h after IV administration (Fig. 4e and f). However, there were significant differences in lungs (1 and 3 h); [$\chi^2_{(7)} = 0.34$, $p < 0.05$] and [$\chi^2_{(7)} = 0.37$,

$p < 0.05$] and livers (1 and 3 h); [$\chi^2_{(7)} = 0.004$, $p < 0.05$] and [$\chi^2_{(7)} = 0.037$, $p < 0.05$] distribution among the formulations. Nemenyi's multiple comparison test (for identifying the within group) indicates that in lungs (Fig. 4a and b), distribution of all formulations was different from the free drug. On the other hand, the amount of the RBITC (% dose/g tissue) in liver after 1 h was the highest for POL-ext formula and the lowest amount in liver after 3 h was belonged to the PEG 35,000-int formula (Fig. 4c and d).

Furthermore, because the in vitro release profiles of RBITC from NPs were similar for all types of surface treated NPs, an eventual difference in their in vivo behavior would be consequence of their different biodistribution properties (Yoncheva et al., 2005). It is plausible to assume some formulations in body show different pattern of the release. Thus, the fact that RBITC amount in liver (1 h) was highest for POL-ext formula can be explained due to the faster release of the RBITC from POL-ext in vivo, because as explained above, the total amount of the RBITC was measured during the experiments. As shown in Table 2 for NT formula (naked NPs), the total RBITC in three organs after 1 h was about 33%, but this amount for the POL-ext was more than 47%, then this result is due to the faster release of this formula in vivo and then more amount of the RBITC in the liver.

In PEG 35,000-int formula treated animals, the lowest amount of the RBITC was detected in liver after 3 h. This is more likely because of the more hydrophilic surface properties of the NPs rather than lower release of the RBITC, since total RBITC dose percent after 1 h in three organs was the least for PEG 35,000-int (Table 2).

After 3 h, the percent RBITC in all three organs has slightly decreased for nearly all formulas. Although this difference is not statistically significant, the elimination process of the NPs can be involved.

Therefore, these results confirm that the surface treatment of the NPs by the first method (co-incorporation of SMAs into NPs (internal)) can be more effective to bypass the RES systems in the body than the second method (surface adsorptions (external)) and increase the blood circulation half-life of the NPs. This phenomena can be explained because of the more hydrophilic surface of NPs prepared in the first method. Furthermore, the higher molecular weights of the PEGs (e.g., PEG 35,000 in comparison with PEG 2000) have shown better results which are in agreement with findings of other studies (Fang et al., 2006; Beletsi et al., 2005). Moreover, the surface treatment of NPs by Poly ethylene glycol is more successful than Poloxamer 407 to increase elimination half-life.

Table 2
The amount of the RBITC percent initial dose measured after 1 and 3 h post-administration in total liver, lung and spleen of rats

Formula	RBITC % (1 h)	RBITC % (3 h)
NT	33.9 ± 2.2	59.3 ± 8.5
PEG 2000-int NP	45.1 ± 1.8	35.3 ± 10.6
PEG 2000-ext NP	25.1 ± 5.3	22.6 ± 18.9
PEG 35,000-int NP	19.7 ± 5.5	13.9 ± 5.3
PEG 35,000-ext NP	33.8 ± 2.6	21.8 ± 0.7
POL-int NP	34.1 ± 1.7	41.3 ± 1.9
POL-ext NP	47.9 ± 4.3	43.3 ± 3.3
RBITC (free drug)	119.8 ± 19.1	91.2 ± 4.3

The data presented as mean ± S.D. ($n = 3$).

4. Conclusions

A novel approach for comparison of the two method of surface treatment preparation of RBITC loaded NPs is proposed by applying homogenization-solvent diffusion method using a single o/w emulsification. Based on our results the co-incorporation of SMAs into NPs produced NPs with increased blood circulation half-lives compared with external surface adsorptions method.

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