International Journal of Pharmaceutics xx (2012) xxx–xxx

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International Journal of Pharmaceutics

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Graphical Abstract

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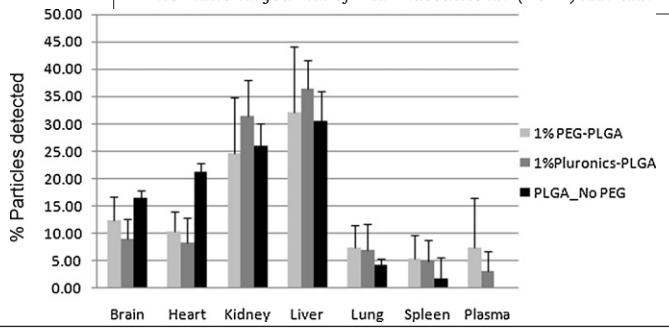

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Pharmaceutical Nanotechnology

Effects of protein binding on the biodistribution of PEGylated PLGA nanoparticles post oral administration

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ARTICLE INFO

Article history:
Received 8 September 2011
Received in revised form
21 December 2011
Accepted 22 December 2011
Available online xxx

Keywords:
Nanoparticles
PEGylation
Protein binding
Biodistribution

ABSTRACT

The surface of nanoparticles is often functionalised with polymeric surfactants, in order to increase systemic circulation time. This has been investigated mainly for intravenously administered nanoparticles. This study aims to elucidate the effect of surface coating with various concentrations of polymeric surfactants (PEG and Pluronics F127) on the *in vitro* protein binding as well as the tissue biodistribution, post oral administration, of PLGA nanoparticles. The *in vitro* protein binding varied depending on the polymeric surfactant used. However, *in vivo*, 1% PEG and 1% Pluronics F127 coated particles presented similar biodistribution profiles in various tissues over seven days. Furthermore, the percentage of PEG and Pluronics coated particles detected in plasma was higher than that of uncoated PLGA particles, indicating that systemic circulation time can also be increased with oral formulations. The difference in the *in vitro* protein binding as a result of the different poloxamers used *versus* similar *in vivo* profiles of these particles indicates that *in vitro* observations for nanoparticles cannot represent or be correlated to the *in vivo* behaviour of the nanoparticles. Our results therefore suggest that more studies have to be conducted for oral formulations to give a better understanding of the kinetics of the particles.

1. Introduction

The application of nanotechnology based drug delivery systems has been on the increase in the past two decades. It has been reported that by encapsulating drugs into nanoparticles, the bioavailability, tissue distribution and half-life can be improved and that toxicity of the drugs can be minimised (Bawarski et al., 2008; Li and Huang, 2008). Despite significant progress with nanoparticle-based drug delivery, shortcomings have been experienced, with rapid clearance of the particles from the blood in intravenously (iv) administered formulations (Moghimi and Szebeni, 2003; Owens and Peppas, 2006). This occurrence has been reported to be as a result of the adsorption of plasma proteins including opsonins on the surface of particles triggering recognition and uptake of the particles by the mononuclear phagocytic system (MPS) (Moghimi and Szebeni, 2003). This phenomenon has lead to the exploration of surface modification of the particles with non-ionic polymeric surfactants, to make these particles 'stealth'. Some of the extensively researched surfactants are poloxamers and poly-ethyleneglycol (PEG) (Moghimi and Szebeni, 2003; Stolnik et al., 1995). When particles are coated with these polymers, the recognition by plasma proteins is minimised, thus reducing the rate of MPS uptake. It is postulated that the presence of surfactants on the surface of the particles reduces the interparticulate attractive Van der Waals forces and increases the repulsive barrier between the particles (Owens and Peppas, 2006).

Much of the research conducted with stealth particles focuses on intravenously administered particles, however very little is known regarding the protein binding and thus tissue distribution of PEGylated particles when orally administered. Semete et al. (2010) evaluated the biodistribution of poly(DL-Lactic-*co*-Glycolic Acid) (PLGA) nanoparticles post oral administration into mice. Due to the preferential uptake of non-stealth particles by macrophages of the liver, *i.e.* the Kupffer cells, a greater proportion of particles were detected in the liver (Semete et al., 2010). Based on Semete et al. (2010) and other reports (Li and Huang, 2008; Owens and Peppas, 2006), it is well accepted that nanoparticles will generally be taken up by tissues with leaky endothelial walls such as the liver, spleen, bone marrow and tumours. It is postulated that when protein binding (primarily opsonisation) of the particles is minimised, this preferential uptake by macrophages and tissues will be reduced, however a balance needs to be obtained in that intracellular uptake of the particles is not compromised. In addition not much is known about the effect that minimised opsonisation will have on the biodistribution of orally administered stealth

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0378-5173/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2011.12.043

Table 1
Summary of nanoparticle characterisation.

Formulation	Ave size (nm)	Polydispersity index	Zeta potential (mV[a])
PLGA–Rhd[b]	296.8	0.229	+35.2
PLGA–Rhd (1% PEG)	313.3	0.303	+30.1
PLGA–Rhd (1% Pluronics F127)	442.7	0.293	+28.6
PLGA–Rhd (0.5% PEG)	340.2	0.145	+33.5
PLGA–Rhd (0.5% Pluronics F127)	442.7	0.293	+29.7

[a] mV: millivolts.
[b] Rhd: Rhodamine.

particles. Thus, in this study, we explore the effect of PEGylation on the biodistribution of PLGA particles, and furthermore ask to what extent the observed difference with the *in vitro* protein binding of nanoparticles can represent the *in vivo* observation.

2. Methods

2.1. Nanoparticle preparation

Nanoparticles were prepared with PLGA 50:50 (M_w: 45,000–75,000 Da) using a modified double emulsion solvent evaporation spray-drying technique. Briefly, aqueous phosphate buffered saline (PBS) pH 7.4 was emulsified for a short period with a solution of 100 mg PLGA dissolved in 8 ml of ethyl acetate (EA), by means of a high speed homogeniser (Silverson L4R) with a speed varying between 3000 and 5000 rpm. The resulting water-in-oil (w/o) emulsion was transferred into a specific volume of an aqueous solution of 1% w/v of the polyvinyl alcohol (PVA, M_w: 13,000–23,000 partially hydrolysed (87–89%)), 0.3% weight/volume (w/v) of chitosan and 5% (w/v) lactose to stabilise the emulsion. The mixture was further emulsified for 5 min by homogenisation at 8000 rpm. The double emulsion, *i.e.* water-in-oil-in-water (w/o/w) obtained was directly fed into a bench top Buchi mini-spray dryer (Model B-290) and spray dried at a temperature ranging between 95 and 110 °C, with an atomising pressure varying between 6 and 7 bar. PEG (M_w: 9000 Da) or Pluronics F127 (poly-ethylene oxide (PEO) and poly-propylene oxide (PPO) triblock, M_w: 10,000 Da. PEO is the hydrophilic polymer and PPO the hydrophobic polymer) were introduced in the formulations as excipients to increase the *in vivo* residence time of nanoparticles in the blood circulation (Torchilin and Trubetskoy, 1995). Rhodamine-6G labelled nanoparticles coated with either 0.5 or 1% volume/volume (v/v) PEG/Pluronics F127 were prepared for the biodistribution assays. These nanoparticles were prepared as described above by including Rhodamine-6G together with PLGA in the oil phase of the first w/o emulsion.

2.2. In vitro protein binding assays

The nanoparticle protein binding was analysed using an adapted method as described previously for protein adsorption to polymer nanoparticles (Stolnik et al., 2001). Pooled human plasma was donated by the Department of Pharmacology at the University of Pretoria and was stored at −20 °C until use. Briefly, samples were prepared in varying ratios of plasma to nanoparticle suspension (10:90; 20:80; 40:60 (v/v)) to a total volume of 600 μl. The plasma/nanoparticle suspension was incubated for 2 h at room temperature and then centrifuged at 14,000 rpm for 45 min to obtain a nanoparticle pellet. The pellet was washed once with 600 μl McIllvaine's buffer (91.7 ml 0.1 M Na_2HPO_4 + 8.3 ml 0.2 M citric acid) at pH 7.5 to remove any additional unbound protein and centrifuged again at the same parameters. The resulting supernatants from the washes and the original supernatant were combined for protein analysis using the Bradford assay to determine the concentration of protein that did not bind to the nanoparticles.

2.3. In vivo studies

2.3.1. Animals

Female Balb/C mice weighing between 20 and 25 g were selected and housed under standard environment conditions at ambient temperature of 25 °C. Animals were humanely cared for and supplied with food and water *ad libitum*. Ethics approval was obtained for this study from the Ethics Committee for Research on Animals (ECRA), Tygerberg, Cape Town, South Africa.

2.3.2. Tissue distribution assays of PLGA nanoparticles

In order to determine the biodistribution of surface functionalised PLGA nanoparticles with different concentrations of PEG or Pluronics F127, these formulations were fluorescently labelled with Rhodamine 6G and orally administered to mice at 4 ml particles in 0.2 ml sterile saline by oral gavage. The mice were grouped with three mice per group and the study was repeated three times. Group 1 was treated with PLGA-nanoparticles. Group 2 was treated with 0.5% PEG–PLGA nanoparticles; Group 3: 0.5% Pluronics F127–PLGA-nanoparticles, Group 4: 1% PEG–PLGA nanoparticles and Group 5: 1% Pluronics F127–PLGA–nanoparticles. Oral administration was performed on the same day and the mice were euthanised 1, 3 or 7 days post administration.

The mice were sacrificed by cervical dislocation. The brain, heart, kidney, liver, lung and spleen as well as plasma were collected and processed immediately for analysis. Briefly, the tissues were homogenised on ice in 2 ml PBS, and diluted 100 times. The resulting diluted homogenates were analysed for fluorescent particles on the FLx8000 Biotek plate reader at an excitation and emission wavelength of 488 nm and 525 nm, respectively.

3. Results

3.1. Nanoparticle formulation

Particles of sizes ranging between 250 and 440 nm with a polydispersity index less than 0.3 were prepared. It was observed that the zeta potential as indicated in Table 1 was not significantly affected by the presence or absence of poloxamer coating. Lactose was included in the formulation as drying aid agent together with

Table 2
Protein binding values of various nanoparticle formulations with varying ratios of plasma: nanoparticle suspension.

Plasma:nanoparticle ratio	Protein binding (%)		
	PLGA	1% Pluronics	1% PEG
10:90	25.02 (4.58)	22.78 (6.49)	31.41 (13.80)
20:80	22.03 (4.81)	21.23 (6.62)	20.57 (6.60)
40:60	20.91 (4.44)	31.30 (9.76)	14.32 (7.40)

% protein bound was calculated as 100 minus % unbound. Standard deviation is shown in parentheses.

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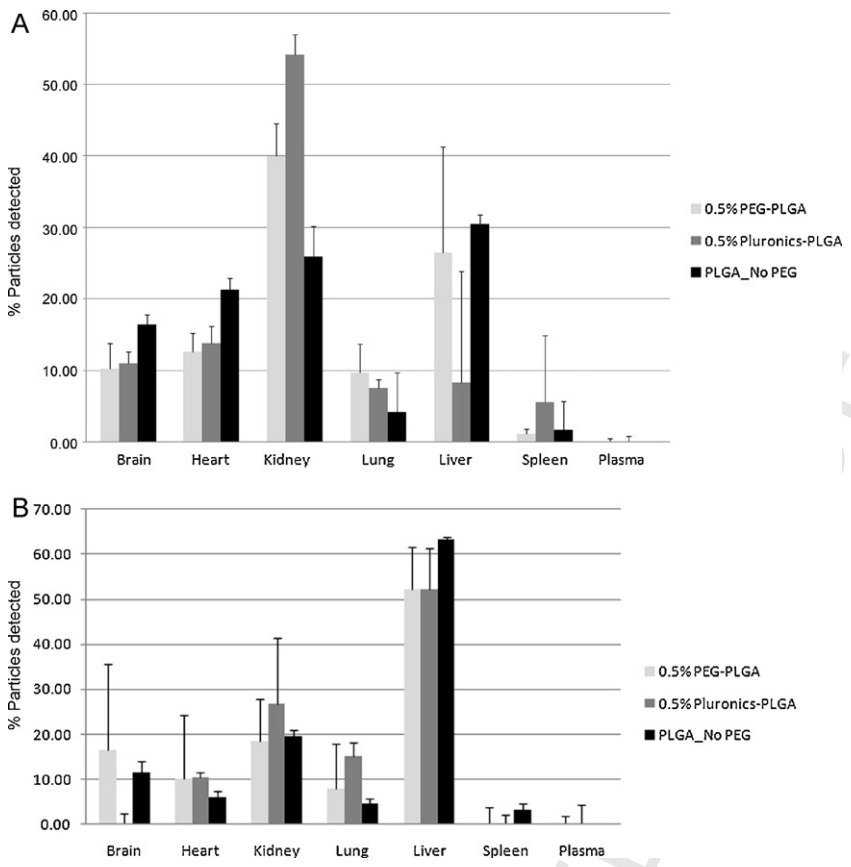

Fig. 1. Biodistribution of Rhodamine labelled PLGA nanoparticles coated with 0.5% PEG or Pluronics F127. (A) After 1 day oral administration, (B) after 3 days oral administration.

the mucoadhesive polysaccharide chitosan for surface charge modification. The inclusion of chitosan, a positively charged ligand, has been recommended in previous reports to enhance uptake through the gastrointestinal tract (Cui et al., 2006; Takeuchi et al., 2005). 0.5% and 1% w/w coated particles were prepared. When the concentration of the polymeric surfactants, *i.e.* PEG and Pluronics were increased beyond 1%, this led to an increase in the size of the particles (data not shown), possibly due to polymer chain entanglement.

3.2. In vitro protein binding of PLGA nanoparticles

Various concentrations of plasma: nanoparticle suspensions were included to evaluate the Vroman effect. This refers to a plasma protein concentration and exposure time dependent effect on the competitive adsorption of proteins for a finite number of surface sites on the particles (Moghimi and Szebeni, 2003). At a 10% plasma volume, PLGA formulations demonstrated an average protein binding of 25.02 ± 4.58%. A comparison between this formulation and a similar formulation coated with 1% Pluronics F127 as depicted in Table 2, illustrated no significant difference in plasma protein binding ($p > 0.01$, 95% confidence level (CI)). However, the formulation coated with 1% PEG resulted in a percentage protein binding of 31.4 ± 13.8% which was found to be significantly different when compared to the uncoated formulation ($p < 0.01$). Similarly, the percentage protein binding of the two coated formulations also differed significantly as indicated in Table 2. The increased protein binding for PEG formulations observed at 10% plasma volume was an unexpected result since surface modification with PEG has been well documented to reduce protein adsorption (Gref et al., 2000; Tan et al., 1993). At the 20% v/v plasma concentration, no significant difference was observed between the three formulations ($p > 0.01$). Interestingly, at 40% v/v plasma concentration, the 1% Pluronics F127–PLGA formulation resulted in a higher protein binding compared to both the uncoated PLGA and 1%PEG–PLGA formulations.

Comparisons of the same formulation at different plasma protein concentration revealed that for the uncoated PLGA formulations, no significant difference ($p > 0.01$) was observed between the plasma protein concentration, *i.e.* 10, 20 and 40%. Therefore this result suggests that the affinity of these formulations for plasma proteins was not dependent on plasma concentration. However, a significant increase ($p < 0.01$) in protein binding was observed for formulations coated with 1% Pluronics F127 at 40% v/v plasma concentration compared to the 10 and 20%. In contrast, the formulations coated with 1% PEG presented a significant decrease in protein binding from 31.41 ± 13.8% at 10% to 14.32 ± 7.4% for 40% v/v plasma concentration.

3.3. Biodistribution of PLGA nanoparticles

When the fluorescently labelled particles were orally administered to mice and the tissues analysed, the particles were initially not detected in the tissues (5 μm tissue sections) *via* fluorescent microscopy, as a result of the intense auto-fluorescence of the tissues. Thus, a fluorometer was used to detect fluorescence in the tissue homogenates. The data was normalised with the negative control, which was tissue from mice treated with saline only. The background fluorescence from these tissues was deducted from the control tissue fluorescent readings to exclude the effect of auto-fluorescence. The percentage particles detected was expressed as the concentration of the fluorescence unit (FU) of each tissue

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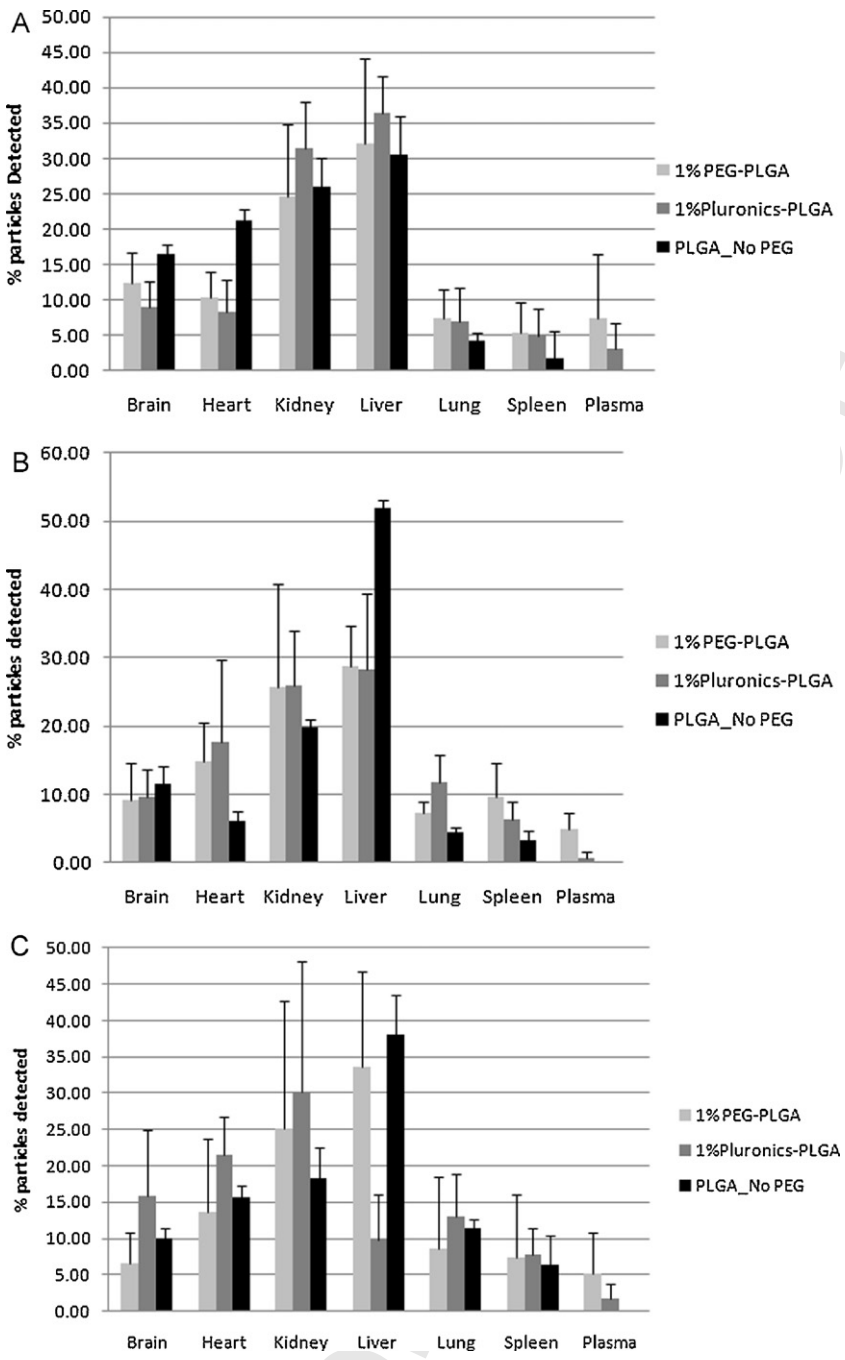

Fig. 2. Biodistribution of Rhodamine labelled PLGA nanoparticles coated with 1% PEG or Pluronics F127. (A) 1 day oral administration, (B) Day 3 after oral administration, (C) Day 7 after oral administration.

relative to the sum of fluorescence units of all tissues analysed and graphically illustrated.

As illustrated in our previous study (Semete et al., 2010) PLGA particles with no poloxamer coating were detected in the liver, spleen, lungs, kidneys, heart and the brain over a period of 7 days. However, very low concentrations or no particles were observed in the plasma over the same period. It was confirmed in Semete et al. (2010), *via* confocal imaging that the fluorescence detected in these tissues is of Rhodamine in the nanoparticles and not leached Rhodamine.

At 0.5% PEG and Pluronics F127 coating of the particles, no particles were detected in plasma over the 3 days as depicted in Fig. 1A and B. Furthermore, no significant difference between the three formulations ($p > 0.01$) was observed for the liver, heart, brain, spleen and lungs. Interestingly though, a significantly higher concentration of coated particles was detected in the kidneys compared to the uncoated formulation on day 1. This could indicate a possible renal clearance of the particles at this time point. These results indicate that at this specific surface coverage with 0.5% poloxamer coating, no significant difference in the biodistribution of PLGA nanoparticles is observed.

When 1% PEG or Pluronics F127 coated particles were orally administered, the biodistribution profile indicated in Fig. 2 was observed. The presence of 1% PEG–PLGA nanoparticles in the brain decreased over the 7 days, whereas in the heart, kidney, liver and lungs the % detected remained relatively constant. A slight

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accumulation of 1% PEG–PLGA nanoparticles was detected in the spleen, indicating uptake by the M cells of the Peyer's patches. Furthermore, these particles were detected in the plasma over the 7 days. An accumulation of 1% Pluronics F127–PLGA particles was observed in the brain over the 7 days as indicated in Fig. 2. A similar profile to that of 1% PEG–PLGA nanoparticles was observed in the rest of the tissues including the spleen and plasma. Plasma concentrations were significantly higher than those for uncoated PLGA particles. This increase in the residence time in plasma is in agreement to that of Stolnik et al. (1995).

4. Discussion

PEG and Pluronics have been extensively used in drug delivery to increase the circulation time of particles in blood. Much work has focused on intravenously administered particles, primarily liposomes, where stealth particles have been shown to circulate for prolonged periods of time with half-lives as long as 45 h (Moghimi and Szebeni, 2003). This study however focused on the effect of PEGylation and coating with Pluronics F127 on the *in vitro* protein binding as well as the biodistribution of PLGA particles post oral administration.

The *in vitro* protein binding of the different formulations indicated that when PLGA particles are made stealth, the protein binding varies depending on the polymeric surfactant used. In this case, 1% Pluronics F127–PLGA particles did not display the Vroman effect nor reduce the protein binding of the particles. However, for 1% PEG–PLGA particles, the plasma protein concentration had a significant effect on protein binding, with a lower protein binding at higher plasma protein concentration. This data is more physiologically relevant than the data at low plasma protein concentration because *in vivo*, the ratio of plasma protein will be high. The reduction of protein binding in 1% PEG coated nanoparticles could be attributed to the higher surface coverage which is obtained as a result of the conformation of the PEG chains in a 'brush-like' configuration as schematically illustrated in Fig. 3A. This conformation has been reported to result in a more efficient repulsion of protein (Owens and Peppas, 2006). On the other hand, the conformation of Pluronics on the surface of the particles as depicted in Fig. 3B would result in less surface coverage and thus a less efficient repulsion. The quantification of PEG and Pluronics in the formulation could not be carried out since PEG and Pluronics have similar composition to PVA which is also in the formulation, thus characterisation of the quantity of these poloxamers on the surface of the particles would not be accurate.

Particles were detected in all tissues over the 7 days and the plasma concentration of coated particles was higher than that of uncoated PLGA particles, indicating that the long residence time can also be achieved with oral formulations. Although accumulation of the particles was detected in the spleen and the brain, Semete et al. (2010) reported the safety of these particles in these tissues at high doses of PLGA particles. Furthermore, detection of the particles in the liver and the spleen irrespective of these particles being made stealth indicates that although opsonisation or protein binding is minimised, particle uptake/recognition by macrophages will still occur to some extent. In addition, the lower percentage of particles detected in plasma as opposed to the higher proportion in the liver, kidney and the lungs could be attributed to surface heterogeneity in the population of PEG or Pluronics coated PLGA particles. This surface heterogeneity and the hydrophobic nature of PLGA could further explain the presence of nanoparticles in the spleen (representing particles that are taken up by the M cells of the Peyer's patches *via* opsonisation) and the liver (representing particles that are taken up by the Kupffer cells of the liver).

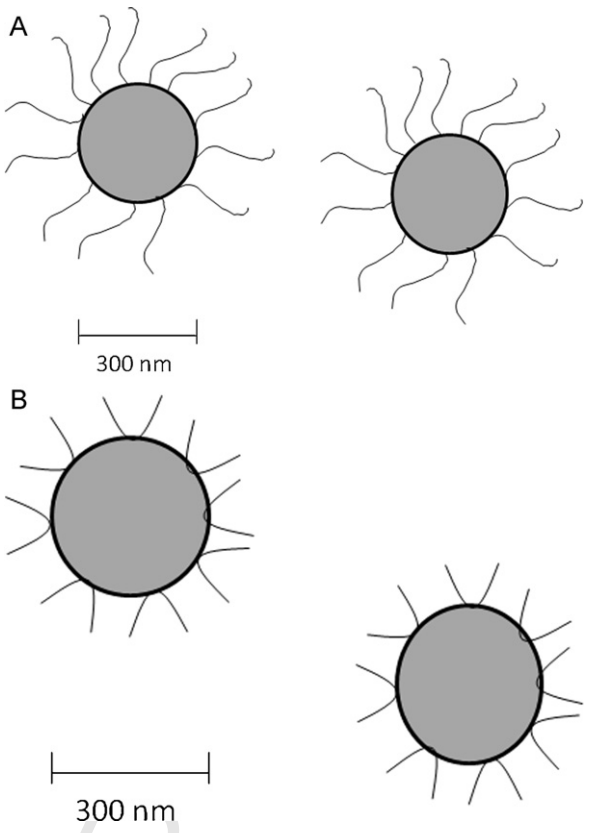

Fig. 3. Schematic illustration of the configuration of the poloxamers on PLGA nanoparticles. (A) Pluronics F127 and (B) PEG.

Although the 1% PEG–PLGA formulation resulted in reduced protein binding as per various reports (Owens and Peppas, 2006; Stolnik et al., 1995), when the same particles are administered orally, as much as there is a significant increase in the percentage detected in plasma, the distribution to various tissues is not significantly different to the non-stealth particles. Furthermore, these results indicate that for nanoparticle formulations *in vitro* observations cannot represent or be correlated to the *in vivo* behaviour of the nanoparticles. Our results therefore suggest that more studies have to be conducted for oral formulations to give a better understanding of the kinetics of the particles since they vary to that of intravenous formulations.

Conflict of interest

No conflict on interest exists.

Acknowledgements

We thank Mr Kobus Venter, at the Medical Research Council for assisting with the mice studies.

This study was funded by the South African Department of Science and Technology.

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