

CHAPTER 4- *In vitro* protein-binding and biodistribution of PLGA nanoparticles

Abbreviations

CLSM - confocal laser scanning microscope

INH - isoniazid

MPS - mononuclear phagocytic system

PEG - polyethylene glycol

PLGA - poly-(DL)-lactic-*co*-glycolic Acid

PVA - polyvinyl alcohol

PBS - phosphate buffered saline

RIF - rifampicin

4.1 Introduction

Knowledge of *in vitro* and *in vivo* characteristics of novel drug delivery systems such as polymer nanoparticles is imperative in pharmaceutical development. Developing a novel drug delivery system from laboratory to market takes many years. Both *in vitro* and *in vivo* assays are required to establish and validate novel drug delivery systems. However, although the necessity of *in vivo* assays cannot be excluded, a lot of time and money have to be invested to generate *in vivo* data. Therefore, establishing and validating *in vitro* methods to assay parameters of interest may considerably reduce the amount of *in vivo* assays required by generating *in vitro* data that would aid in the design of *in vivo* experiments. For instance, subsequent to oral administration of polymer nanoparticles, these particles have to traverse/permeate the gut wall to enter the systemic circulation and only particles, which are not bound to plasma proteins, will be distributed to target organs. This may thus suggest that an understanding of these parameters *in vitro*, i.e. protein-binding and permeability may provide insight into the *in vivo* biodistribution of these polymer nanoparticles. An example of how *in vitro* methods has been used to more accurately design *in vivo* assays in nanoparticle drug delivery was to predict the organ distribution of surface modified polymer nanoparticles by assessing their affinity for uptake by liver and spleen macrophages using *in vitro* cell culture assays (Müller *et al.* 1992:237). The study concluded that if a formulation coated with a specific surfactant did not show potential for reduced uptake *in vitro*, it could be excluded from *in vivo* studies. There has been increasing activity aimed towards developing *in vitro* methods to be correlated to *in vivo* assays (Emami, 2006:31).

4.1.1 Protein-binding on polymeric nanoparticles

Protein-binding generally refers to the binding of plasma proteins to drugs entering the systemic circulation. The mechanism of drug protein-binding is based primarily on the affinity of drugs to the plasma proteins as well as the amount of drug present. Thus, the drug concentrations in the blood determine the rate and extent of protein-binding. Drugs are most commonly bound by proteins such as albumin and α_1 -acid glycoproteins in a reversible manner, i.e. the drug-protein complex formed can dissociate and release free drug. Albumin is the most important plasma protein based on its concentration and binds mainly to acidic drugs. Neutral and basic drugs mostly bind to α_1 -acid glycoproteins that are present in much lower concentrations. In addition to these proteins, another group present in lower, more variable concentrations, the lipoproteins. The binding affinities of lipoproteins are similar to that of the glycoproteins with the exception of their affinity for drugs with a high degree of

lipophilicity (Mehvar, 2005:3). Plasma drug levels are measured as total drug concentration, which includes drugs, which are bound and unbound to plasma proteins. The intensity of the drug effect, i.e. pharmacodynamics is dependent on the concentration of free drug in the plasma water, since the concentration gradient is created here and results in the drug diffusing to its site of action (Sparreboom *et al.* 2001:197). The effect and/or relevance of the protein-binding of drugs on pharmacological activity have been the subject of much debate (Sparreboom *et al.* 2001:197). Potential pharmacologically significant protein-binding values should exceed 90% (Sparreboom *et al.* 2001:197). However, it is important to note that for a protein-drug complex to be of clinical importance, it would have to be of rather high stability and significantly reduce the amount of active, diffusible, unbound drug. Even at high protein-binding values (>90%) there are drugs that are still able to induce a sufficient pharmacological effect (Sparreboom *et al.* 2001). The pharmacological activity refers to both PK and PD parameters of the drug.

PK factors possibly affected by protein-binding are;

- 1) volume of distribution, which is also affected by binding to tissue proteins and the drugs' ability to cross membranes;
- 2) clearance, of which dependence on protein-binding is affected by the extraction ratio, i.e. low extraction drugs are dependent on protein-binding and high-extraction drugs are not;
- 3) bioavailability where protein-binding effects are based on first pass metabolism and thus route of administration; and
- 4) half-life which is determined by the volume of distribution and clearance and therefore protein-binding effects are similarly dependant on factors mentioned in (1) and (2). PD parameters affected by protein-binding are more difficult to determine since these parameters refer to drug action.

The PK parameters mentioned previously determine the amount of unbound drug which reaches the target site and exerts its therapeutic effect (PD) (Schmidt *et al.* 2010:1109). These are all important considerations when evaluating the effect of plasma protein-binding. Another class of proteins that play an important role in protein-binding are opsonins in a process called opsonisation during which these proteins cover foreign particles, making the foreign matter more visible to phagocytic cells which form part of the mononuclear phagocytic system (MPS). These opsonins are present throughout the blood and contact with

foreign particles is thought to occur by random Brownian motion (Owens & Peppas 2006:94). The binding of opsonins to foreign matter promotes the activation of the complement system as a result of opsonin recognition and facilitates phagocytic uptake by macrophages (Owens & Peppas 2006:94). This activation of the complement system would be relevant to polymer nanoparticles, which are foreign to the body (Chapter 3). When a foreign material, such as polymer nanoparticles enter the biological environment, they are immediately coated with a protein “corona” which is a dynamic layer of proteins and other biomolecules which adsorb to the nanoparticle surface (Cedervall *et al.* 2007:5856). This corona is expected to consist of approximately 3700 proteins. Of these, approximately 50 proteins have been associated with nanoparticles (Cedervall *et al.* 2007). Nanoparticle characteristics such as surface hydrophobicity, surface charge, size, morphology, shape and surface curvature determine the extent of adsorption of blood components (Lynch & Dawson 2008:42). Previously mentioned opsonins form part of the corona, which coats the nanoparticles. To minimise opsonisation, the surfaces of nanoparticles can be modified with biodegradable copolymers with hydrophilic segments such as polyethylene glycol (PEG), including poloxamines and polysorbate 80 which will eventually prolong the duration of systemic circulation of the nanoparticles (Mohanraj & Chen 2006:561). Of the above mentioned poloxamers, surface coating with PEG is the preferred method for imparting sterically stabilized properties to nanoparticles (Gref *et al.* 2000:301). This is done by a process called PEGylation, which is the covalently grafting, entrapping or adsorbing of PEG chains to the particle surface. This process has been previously described in detail in Chapter 2. These particles are often referred to as “stealth” nanoparticles because of their ability to avoid recognition by the MPS (Moghimi & Szebeni 2003:465). Plasma protein adsorption onto PEG-coated nanoparticles is largely dependent on the molecular weight (Mw) of PEG, i.e. the PEG chain length on the particle surface and PEG content in the particle formulation, i.e. the PEG chain density at the surface of the particles (Gref *et al.* 2000:302). For example, Gref *et al.* (2000:307) observed maximum reduction in protein adsorption for nanoparticle formulations coated with PEG with Mw 5 kDA. Furthermore, PEG content of 2- 5% weight per weight (w/w) demonstrated optimum reduction in protein adsorption. The surface modification of nanoparticles and the subsequent decrease in protein-binding/adsorption resulting in prolonged circulation time for these particles have also been reported to enhance their biodistribution.

4.1.2 Biodistribution of polymeric nanoparticles

Biodistribution is a term generally used to describe the “movement” of drugs or particulates to different organs in the body. It is the second step in drug disposition, i.e. absorption, distribution, metabolism and elimination (Wilkinson, 1975:12). The blood serves as the primary physiological medium of translocation and exchange for all tissues. However, each tissue only has access to a certain amount of blood and therefore ‘uptake’ and ‘washout’ of drugs and particles occur at different rates since each tissue has different blood flow (Wilkinson 1975:12). Once absorbed, these particles are exposed to blood proteins and other components described in section 4.1.1 and this interaction, although reversible, also contribute to the amount of particles distributed to different organs in a given time. Although prolonged circulation via surface modification is achieved, its effect on biodistribution is dependent on various factors. Firstly, particle size plays a major role in the rate of blood clearance, where particles larger than 200nm in size are cleared at a much faster rate than particles of approximately 70nm or less, regardless of whether or not the particle has been surface modified (Moghimi *et al.* 1993:235). Blood clearance refers to removal of particles from the blood by mechanisms of the MPS as well as clearance by eliminating organs such as the kidneys and liver. In addition to the blood clearance rate, particle size also has an effect on the biodistribution where PEGylated nanoparticles of 250nm and higher have been found to concentrate mainly in the liver and spleen and the PEGylated particles less than 150nm were primarily taken up by the bone marrow (Porter *et al.* 1992:65). Secondly, these size-dependent differences in biodistribution and clearance could be due to the presence of opsonins specific for splenic phagocytes and Kupffer cells, where the Kupffer cells may require larger particles for binding (Moghimi & Patel 1988:144). Thirdly, the PEG layer itself may contribute to the *in vivo* fate of surface-modified particles. Properties, such as thickness of the PEG layer, surface charge, surface density and functional groups may all contribute towards minimising opsonin recognition.

In a reported study, varying mixtures of PLGA with PLGA-PEG copolymers were evaluated. Each mixture contained different PEG concentrations. Each PLGA-PEG mixture was radiolabelled and evaluated following IV administration. Increasing concentrations of PEG on the surface of the particles resulted in a longer blood residence time and liver accumulation, suggesting MPS uptake was also observed. High accumulation in the blood demonstrated low accumulation in the liver and vice versa. This observation suggests that

even when blood circulation of particles is prolonged by PEGylation, eventual accumulation in the liver still occurs. The particles' surface charge was close to neutral, which would further account for prolonged blood circulation. The effect on the nanoparticle surface, i.e. PEG layer and size (increasing PEG reduced nanoparticle size) of the mixtures presented in this study was concluded to have influenced overall biodistribution by resulting in higher levels in the blood compared to the liver and other organs evaluated (Beletsi *et al.* 2005:236). In another study, PLGA nanoparticle blood clearance and MPS uptake were found to be dose dependent subsequent to IV administration, where this was not the case with PLGA-PEG nanoparticles. Many assays evaluate biodistribution of PLGA nanoparticles post IV administration. Understanding nanoparticle biodistribution following oral administration is important since factors such as gastrointestinal effects and intestinal permeability pose biodistribution effects that IV administration does not.

4.1.3 Objective of this study

The specific objective of this chapter is to evaluate the biodistribution of PLGA nanoparticles (described in chapter 3) to tissues with specific focus on the following:

- *in vitro* protein-binding of PLGA nanoparticles for formulations that are both surface modified with PEG and Pluronic F127 and 'naked' i.e. unmodified particles;
- *in vivo* biodistribution of the PLGA nanoparticles that are both surface modified with PEG and Pluronic F127 and unmodified particles.

4.2 Materials and methods

Materials used during the studies described in this chapter are summarised in Table 4.1.

Table 4.1 Summary of reagents used for *in vitro* and *in vivo* characterization

Product	Supplier
Polyvinyl alcohol	BASF, Germany
Polyethylene glycol	BASF, Germany
Pluronic-F127	BASF, Germany
Rifampicin	Linaria Chemicals, Thailand
Isoniazid	D.B. Fine Chemicals, South Africa
Rhodamine 6G	Sigma-Aldrich, Steinheim, Germany
Bradford Reagent	Sigma-Aldrich, Steinheim, Germany
Sodium dodecyl sulphate	BDH, Gauteng (SA)
Ammonium persulphate	Sigma-Aldrich, Steinheim, Germany
N,N,N',N'-tetramethylethylenediamine	Merck, Darmstadt, Germany
Bromophenol blue	Sigma-Aldrich, Steinheim, Germany
Glycerol	Sigma-Aldrich, Steinheim, Germany
Acrylamide/Bisacrylamide	Sigma-Aldrich, Steinheim, Germany
β -mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
PageRuler TM Prestained Protein Marker	Inqaba Biotechnologies (SA)

4.2.1 Nanoparticle preparation

Nanoparticles were prepared as described in Chapter 3 with slight variations. In the formulations encapsulating drugs, RIF was added in the oil phase with the polymer and INH was added in the aqueous phase in PBS.

For nanoparticle formulation coated with PEG, a 40 ml mixture was prepared consisting of 5 ml of 1% v/v PEG, 10 ml of 5% lactose, 15 ml of 1% v/v PVA and 10 ml of 0.3% chitosan during the second emulsion step. The first w/o emulsion was then dispersed in this mixture and emulsified. The second w/o/w emulsion was then spray dried. For formulations coated with Pluronic F127, a similar method was followed. PEG and Pluronic F127 were included to modify the nanoparticle surface to prolong blood circulation time as described in section 4.1.1. Rhodamine 6G- and coumarin-labelled PLGA nanoparticles were prepared using the same method, where these were added in the aqueous phase of the emulsion.

4.2.2 Particle size, zeta potential and surface morphology

Particle size and size distribution of nanoparticles were measured as described in Chapter 3.

4.2.3 Encapsulation efficiency and drug loading

The percentage encapsulation efficiency (EE) of the drugs was determined by UV-spectrophotometer readings of a representative sample of a nanoparticle batch. This EE determination is an indirect method that has been previously documented (Pandey *et al.* 2003:982; Pandey *et al.* 2005:270, Sharma *et al.* 2004:600a, Sharma *et al.* 2004:762b). Nanoparticles (10mg) were washed in 20ml of deionised water and the particles were collected by centrifugation. Free (unencapsulated) drug in the supernatant was analysed and quantified at absorbance wavelengths for RIF (330 nm) and INH (262 nm). The amount of drug analysed was calculated against the amount of drug in the original formulation. The percentage drug loading is calculated using the amount of drug in sample calculated from the encapsulation efficiency against the final yield of the batch. The following equations 4.1 and 4.2 were used to determine the encapsulation efficiency and drug loading:

$$\text{Encapsulation efficiency \%} = \frac{\text{Amount of drug in sample}}{\text{Amount of drug in formulation}} \times 100 \text{ Eqn. 4.1}$$

$$\text{Drug loading} = \frac{\text{Amount of drug in sample}}{\text{Yield (mg)}} \times 100 \text{ Eqn. 4.2}$$

4.2.4 Binding of plasma proteins to PLGA nanoparticles

To determine the binding of plasma proteins to PLGA nanoparticles encapsulating RIF and INH and drug free nanoparticles, a series of assays based on a published method were used (Stolnik *et al.* 2001:265). Both uncoated nanoparticles and nanoparticles coated with PEG and Pluronic F127 were evaluated.

The nanoparticle protein-binding was analysed using an adapted method as described previously for protein adsorption to polymer nanoparticles (Stolnik *et al.* 2001:265). 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 nanoparticles (10:90; 20:80; 40:60 (v/v)) to a total volume of 600 µl. The plasma/nanoparticle suspension were incubated for two hours at room temperature and then centrifuged at 14 000 rpm for 45 minutes to obtain a nanoparticle pellet. The pellet was

washed once with 600 μ l McIlvaine's buffer (91.7 ml 0.1 M Na_2HPO_4 + 8.3 ml 0.2M citric acid) at pH 7.5 to remove any additional unbound protein and centrifuged again at the same parameters. The supernatant was collected for protein analysis using the Bradford assay to determine the concentration of protein that did not bind to the nanoparticles. The pellets were re-suspended with 30 μ l of PBS pH 7.4 and 15 μ l reducing buffer (950 μ l sample buffer and 50 μ l β -mercaptoethanol (BME) to denature the proteins. The sample buffer used consisted of 3.55 ml distilled water, 1.25 ml 0.5M tris (hydroxy methyl) aminomethane (Tris-HCl) pH 6.8, 2.5 ml glycerol, 2.0 ml 10% SDS, 0.2 ml 0.5% bromophenol blue and made up to volume with 9.5 ml deionized water. This suspension was placed on a heating block for 10 minutes at 95°C to further denature the proteins thus removing the proteins from the nanoparticles, and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.5.1 Bradford assay

The Bradford reagent (brilliant blue G in phosphoric acid and methanol) was used for the quantification of bound and unbound plasma proteins. The supernatant collected (5 μ l) and denatured protein samples from re-suspended pellet (5 μ l) to be analysed was placed in a 96-well polystyrene plate. Bradford reagent (250 μ l) was added and the samples were incubated for 20 minutes. Bradford standards of 0.15, 0.25, 0.5, 1.0, 1.2, 1.4mg/ml were used according to manufacturer's instruction kit. The plate was then analysed on an absorbance plate reader at an absorbance of 595 nm. Both the supernatant and pellet solution was analysed using the Bradford assay. Absorbance readings were normalized with blank controls consisting of 5 μ l phosphate buffered saline with 250 μ l Bradford reagent.

4.2.5.2 SDS-PAGE analysis

The remainder of the pellet solution was analysed via SDS-PAGE gel for visual analysis. SDS-PAGE reagents used for protein-binding assays were sodium dodecyl sulphate (SDS), ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), bromophenol blue, Coomassie brilliant blue R250, glycerol, PageRuler™ Prestained Protein Marker(10-170 kDa) and 30% acrylamide/Bisacrylamide. The SDS-PAGE gels were prepared in 80x70x1.5 mm casting chambers. Resolving and stacking gels (6%) were prepared as indicated in Table 4.2. The percentage crosslink selected was based on obtaining adequate separation of the different size protein fragments. Separation of 30 μ l of samples was performed at a constant voltage of 200V on a Bio-Rad Power-Pac™. Protein fragments

were stained with Page Blue Stain™ for one hour and destained overnight with deionized water. The ChemiDoc XRS Plus gel dock system from Bio-Rad was used for visual documentation of the gels.

Table 4.2 Composition of gels for SDS-PAGE

Components	6% Resolving gel (ml)	6% Stacking gel (ml)
40% Bis/Acrylamide	1.5	1
Tris-HCl (pH 8.8)	2.5 (1.5 M)	2.52 (0.5M)
10% SDS	0.1	0.1
TEMED	0.005	0.01
10% APS	0.05	0.05
Deionized water	5.35	6.32

4.2.5.3 Equilibrium dialysis of free drugs

To determine the percentage protein-binding of the unencapsulated (free) drugs as positive controls, equilibrium dialysis was used. The method described in section 4.3.4 could not be applied for free drugs since the drug remained in solution after spiking into plasma and could not be precipitated. Free RIF and INH controls were prepared in the same ratios with human plasma as the nanoparticle formulations and placed in the sample chamber of an equilibrium dialysis device with buffer chamber containing PBS (pH 7.4). Diffusion against a concentration gradient was facilitated on an orbital shaker at 100 rpm for four hours at room temperature. The samples in both chambers were analysed using UV-spectrophotometry at 330 nm for RIF and 262 nm for INH. The samples collected from the sample and buffer chambers were analysed to determine the percentage of unbound drug. Percentage unbound drug was calculated as illustrated in Equation. 4.3.

$$\left(\frac{drug_{PBS}}{drug_{Plasma}} \times 100\right) = \%unbound$$

Eqn 4.3

$$\%bound = 100\% - \%unbound$$

4.2.6 *In vivo* evaluation of the biodistribution of fluorescently-labelled uncoated PLGA nanoparticles and PEGylated PLGA nanoparticles

Unchallenged Balb/C mice weighing between 20 to 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 Medical Research Council (MRC) Ethics Committee for Research on Animals (ECRA), Tygerberg, Cape Town, South Africa, see appendix A.

4.2.6.1 Tissue distribution assays of uncoated PLGA nanoparticles

To determine the biodistribution of the nanoparticles, particles labelled with rhodamine 6G and coumarin were orally administered to mice. The mice were grouped with three mice per group and the study was repeated three times. Mice were treated with 4mg of rhodamine-PLGA nanoparticles in 0.2 ml sterile saline by oral gavage. The fluorescent dye was used to determine if any differential biodistribution occurred based on the fluorescent dye. Furthermore, rhodamine was spray-dried without encapsulating into PLGA nanoparticles and orally administered to mice. In addition, 4 mg of FITC-fluorescently labelled polystyrene beads were orally administered as a control. The mice were sacrificed via cervical dislocation. The organs of interest harvested for analysis were the brain, heart, kidneys, liver, lungs and spleen. To determine circulating nanoparticles, plasma was collected at each time point (Days one, three and seven). Tissues were also collected from untreated mice to normalize the analysed homogenates for autofluorescence. The tissues were homogenized on ice in 2 ml PBS at pH 6.8. Due to the high concentration of the homogenate solution, a 10x dilution was prepared by aliquoting 20 µl of tissue homogenate into 180 µl of PBS. The resulting diluted homogenates were analysed for fluorescent particles on the FLx8000 Biotek plate reader at excitation and emission wavelengths of 488 nm and 525 nm, respectively. These analyses included relevant standard curves.

4.2.6.2 Confocal microscopy

Organs (described in section 4.2.6.1) were harvested from mice treated with rhodamine labelled PLGA nanoparticles and spray-dried rhodamine nanoparticles measured against blank tissue at Vetpath Onderstepoort, University of Pretoria. Analysts prepared the tissue section slides for confocal microscopy. After tissue fixation, tissue sections were selected from all the above mentioned organs and processed in an automated tissue processor. Wax blocks were produced and from these blocks, 5 µm sections were cut using a histological

microtome. Slides were mounted and tissues were analysed with the Zeiss LSM 510 Meta confocal microscope. The laser line used was the argon 514 nm green excitation for the detection of rhodamine dye. The confocal microscope has one Meta detector, a polychromatic detector. The confocal microscopy was used to determine if the fluorescence observed for the study conducted in section 4.2.6.1 was that bound to the nanoparticles or fluorescent particles which had degraded or been leached out. The sections were evaluated at 40x magnification.

4.2.6.3 Tissue distribution assays of poloxamer coated PLGA nanoparticles

To determine the biodistribution of PLGA nanoparticles surface-modified with different concentrations of PEG or Pluronics F127, these formulations were fluorescently labelled with rhodamine 6G and orally administered to mice at 4mg 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 0.2 ml saline. 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. Mice were euthanized at one, three or seven days post oral administration. The organ harvest, sample preparation and analysis were performed as in section 4.3.7.1. To confirm biodistribution data, confocal microscopy was performed. Three sets of tissue section slides were prepared, untreated tissues, tissues treated with spray-dried rhodamine only and tissues treated with rhodamine PLGA nanoparticles.

4.2.7 Statistical analysis

For statistical analysis, the F-test with two-sample for variances for the *in vivo* biodistribution assays was used to compare the difference in fluorescence detection between the organs evaluated. The Student's T-test for the *in vitro* protein-binding assays was used to statistically compare differences between the percentage protein-binding of the different formulations. The statistical parameters were calculated using Microsoft Excel 2010. The statistical data are given as mean $\pm$ SEM and the p-value for statistical significance.

4.3 Results

4.3.1 Nanoparticle preparation and characterization

Various formulations of PLGA nanoparticles based on different experiments (protein-binding and biodistribution assays) were prepared. Formulation 1: PLGA- drug free nanoparticles; Formulation 2: PLGA-drug free with 1% PEG (Mw 10 kDa) coating; Formulation 3: PLGA-

drug free with 1% Pluronic F127 (Mw 9 kDa) coating; Formulation 4: PLGA encapsulating RIF; Formulation 5: PLGA with 1% PEG coating encapsulating rifampicin; Formulation 6: PLGA with 1% Pluronic F127 encapsulating RIF; Formulation 7: PLGA encapsulating INH; Formulation 8: PLGA with 1% PEG coating encapsulating INH and Formulation 9: PLGA with 1% Pluronic F127 encapsulating INH. These formulations were prepared for the protein-binding assays described in 4.25. Formulations 10 and 11 were rhodamine labelled PLGA nanoparticles coated with 1% PEG and 1% Pluronic F127, respectively. These, along with uncoated rhodamine labelled PLGA nanoparticles were used to determine the biodistribution of these particles. Since PEG has a similar composition to PVA, which is also in the formulation, characterisation thereof would not be accurate. The particles were characterised as described in chapter 3 and the results are summarised in Table 4.3

Table 4.3: Summary of nanoparticle characterization showing the different formulations, size (nm), polydispersity index, encapsulation efficacy (%), drug loading (%) and zeta potential. Mean $\pm$ SEM is included.

No	Formulation	Size (nm)	Polydispersity index	Encapsulation efficiency (%)	Drug loading (%)	Zeta potential
1	PLGA-DF	265.5 $\pm$ 4.16	0.20 $\pm$ 0.14	N/A	N/A	+35.2
2	1% PEG-DF	342.0 $\pm$ 14.63	0.38 $\pm$ 0.20	N/A	N/A	+39.9
3	1% Pluronic-DF	310.5 $\pm$ 8.48	0.42 $\pm$ 0.02	N/A	N/A	+38.6
4	PLGA-RIF	382.3 $\pm$ 23.9	0.27 $\pm$ 0.05	69.2	7.64	+14.4
5	1% PEG-RIF	336.3 $\pm$ 2.12	0.44 $\pm$ 0.04	65.2	8.4	+19.1
6	1%Pluronic-RIF	260.1 $\pm$ 3.39	0.36 $\pm$ 0.02	67.34	8.53	+16
7	PLGA-INH	253.4 $\pm$ 14.2	0.12 $\pm$ 0.04	73.5	23.43	+15.8
8	1% PEG-INH	281.1 $\pm$ 6.92	0.35 $\pm$ 0.14	67.65	24.8	+8.52
9	1%Pluronic-INH	319.5 $\pm$ 4.80	0.35 $\pm$ 0.07	69	27.6	+13.7
10	PLGA-R (1% PEG)	292.8 $\pm$ 29.0	0.30 $\pm$ 0.12	N/A	N/A	N/A
11	PLGA-R (1% PLU)	229.5 $\pm$ 7.60	0.30 $\pm$ 0.01	N/A	N/A	N/A

PLGA-poly-lactic-glycolic acid; PEG-poly ethylene glycol; DF-drug-free; RIF- rifampicin; INH- isoniazid; R- rhodamine; PLU- Pluronic; N/A- not applicable

Nanoparticle size (nm) obtained was below 400 with a polydispersity index $\leq$ 0.4 for these formulations. The zeta potential for each of the formulations is indicated in Table 4.3. Particle size and polydispersity index for the uncoated PLGA nanoparticles were slightly higher as were observed for the nanoparticles used in chapter 3. However, a higher increase in particle size was observed when the nanoparticles were coated with the poloxamer

coatings as observed in Table 4.3. It was observed that the zeta potential was not significantly affected by presence or absence of poloxamer coating or drug encapsulation. PLGA has a low negative zeta potential, but the presence of chitosan, a positively charged ligand, in the formulation accounts for the positive zeta potential. Lactose was included in the formulation for surface modification as well as chitosan, which is a mucoadhesive polysaccharide. The encapsulation method applied, i.e. double emulsion spray-drying, resulted in %EE for RIF of $67 \pm 2\%$ and for INH, $70 \pm 3\%$.

4.3.2 Binding of plasma proteins to PLGA nanoparticles and free drugs

The positive controls for RIF and INH protein-binding resulted in values for plasma protein-binding at 20-40% for INH and 70-90% for RIF as depicted in Table 4.4. This corresponds to the findings of Woo *et al* (1996:176) of 70-80% protein-binding for RIF and 20% for INH. However, differences in protein-binding were observed for the different plasma to drug ratios as indicated in Table 4.4. Protein concentrations and percentage protein bound was calculated using the equation for the linear regression of the standard curve for the Bradford reagent (Figure 4.1). Standard curves for RIF and INH are shown in Figure 4.2. Percentage protein-binding was calculated using the concentrations of pellet solution (bound proteins) and supernatant (unbound proteins). Equation 4.3 was used to determine protein-binding to free drugs and standard curves are illustrated in Figure 4.3 A and B.

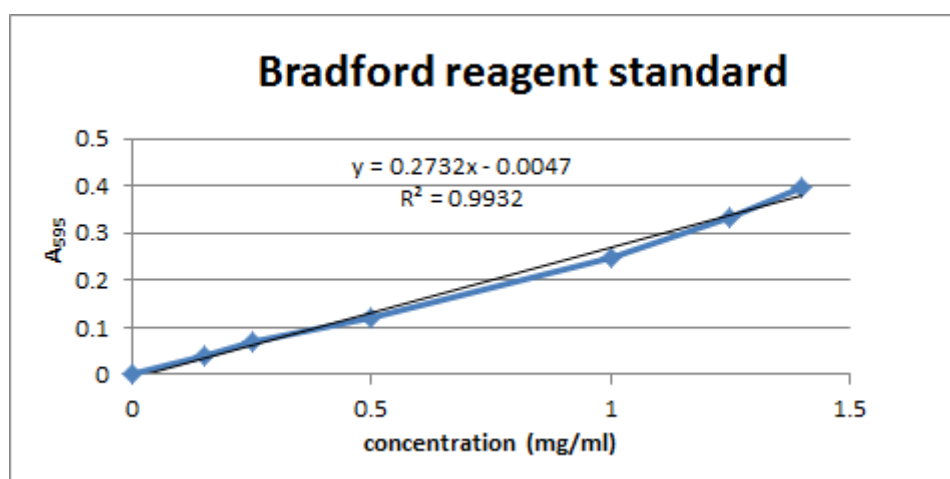
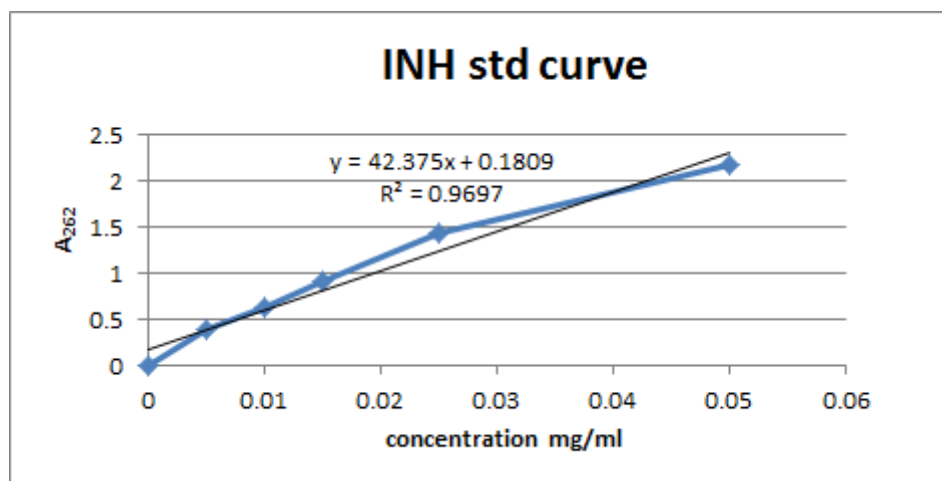
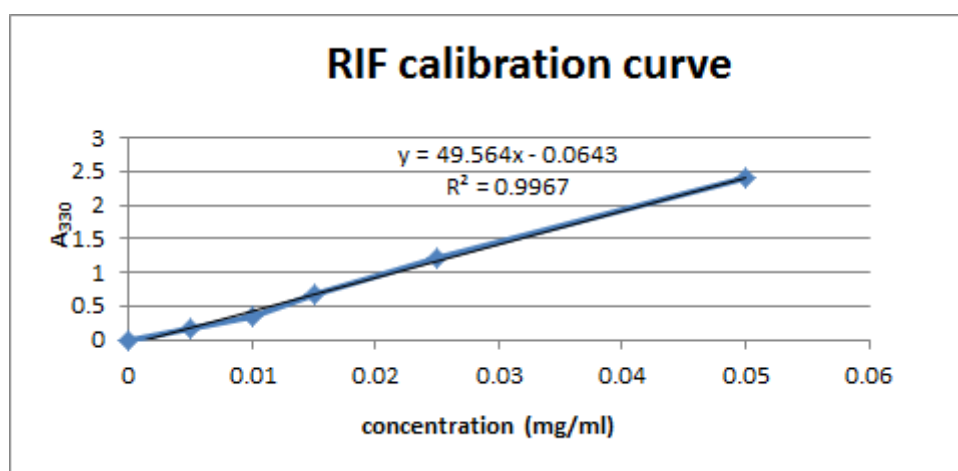


Figure 4.1 An example of the standard curve for Bradford reagent used in this study. Measured as absorbance (595nm) against concentration (mg/ml).



A



B

Figure 4.2 Example of the standard curves for (A) INH and (B) RIF analysed in the equilibrium dialysis analysis of drug protein-binding. Measured as absorbance (RIF 262nm, INH 330nm) against concentration (mg/ml).

Table 4.4 Protein-binding for various nanoparticle formulations with varying ratios of plasma: nanoparticle suspension. Values are depicted as mean with SEM shown in parentheses.

No.	Samples	10:90	20:80	40:60	p-value
1	PLGA-DF	25.02 (4.58)	22.03 (4.81)	20.91 (4.44)	> 0.01
2	1% PLURONIC	22.78 (6.49)	21.23 (6.62)	31.3 (9.76)	<0.01
3	1%PEG	31.41 (13.80)	20.57 (6.60)	14.32 (7.40)	<0.01
4	PLGA-RIF	23.95 (6.60)	18.83 (7.50)	15.40 (5.50)	> 0.01
5	1%PEG-RIF	10.16 (4.32)	16.87 (2.11)	12.92 (2.15)	> 0.01
6	1% PLURONIC RIF	17.31 (6.78)	17.58 (2.86)	16.57 (5.18)	> 0.01
7	PLGA-INH	19.80 (4.30)	13.15 (5.81)	15.07 (3.40)	> 0.01
8	1% PLURONIC INH	18.46 (3.88)	15.51 (6.63)	12.77 (9.76)	> 0.01
9	1% PEG-INH	18.94 (3.7)	14.40 (4.60)	15.80 (2.00)	> 0.01
10	Control RIF *	71.12 (0.78)	79.47 (1.60)	90.00 (1.38)	<0.01
11	Control INH *	43.37 (6.6)	29.96 (10.90)	23.00 (5.2)	< 0.01

* % protein bound was calculated as indicated in Eqn 4.3. Experiments were repeated three times (n=3.)

Table 4.5 is the statistical comparison of the different formulations analysed in this study. The comparisons 1 vs. 2, 1 vs. 3, etc. are based on the formulation numbers in the first column of Table 4.4.

At a 10% plasma volume, PLGA-DF formulations demonstrated an average protein-binding of $25.02 \pm 4.58\%$. A comparison between this formulation and a similar formulation coated with 1% Pluronic F127 illustrated no significant difference in plasma protein-binding. However, the formulation coated with 1% PEG resulted in a percentage protein-binding of $31.41 \pm 13.8\%$. This result was found to be significantly different when compared to the uncoated formulation ($p \leq 0.01$). Similarly, the percentage binding of the two coated formulations (1% PEG and Pluronic F127) also differed significantly ($p \leq 0.01$) as indicated in Table 4.5. 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.* 2000:306; Tan *et al.* 1993:827).

Table 4.5 Statistical analyses of the protein-binding properties for the various nanoparticle formulations. The significant differences are compared based on the formulation numbers allocated in the first row of Table 4.4.

Statistical parameter	1 vs. 2	1 vs. 3	2 vs. 3	1 vs. 10	3 vs. 10
	Based on the average of 3 repeats (10/90)				
p-value	0.43	0.02	0.02	0.00002	0.00001
Probability level 95%	not significant	significant	significant	significant	significant
	Based on the average of 3 repeats (20/80)				
p-value	0.700	0.500	0.800	0.000	0.000
Probability level 95%	not significant	not significant	not significant	significant	significant
	Based on the average of 3 repeats (40/60)				
p-value	0.000	0.013	0.007	0.000	0.000
Probability level 95%	significant	significant	significant	significant	significant
	4 vs. 5	4 vs. 6	5 vs. 6	4 vs. 10	5 vs. 10
	Based on the average of 3 repeats (10/90)				
p-value	0.01	0.17	0.01	0.000025	0
Probability level 95%	significant	not significant	significant	significant	significant
	Based on the average of 3 repeats (10/90)				
p-value	0.600	0.700	0.600	0.000	0.000
Probability level 95%	not significant	not significant	not significant	significant	significant
	Based on the average of 3 repeats (10/90)				
p-value	0.400	0.700	0.020	0.000	0.000
Probability level 95%	not significant	not significant	significant	significant	significant
	7 vs. 8	7vs 9	8vs 9	7 vs. 11	9 vs. 11
	Based on the average of 3 repeats (10/90)				
p-value	0.43	0.02	0.02	0.00002	0.00001
Probability level 95%	not significant	not significant	not significant	significant	significant
	Based on the average of 3 repeats (10/90)				
p-value	0.7	0.5	0.8	0	0
Probability level 95%	not significant	not significant	significant	significant	significant
	Based on the average of 3 repeats (10/90)				
p-value	0.000	0.013	0.007	0.000	0.000
Probability level 95%	not significant	not significant	not significant	not significant	significant

Furthermore, the effect of whole plasma content on the percentage protein-binding of these formulations was explored. A 10%, 20% and 40% v/v ratio of whole plasma to nanoparticle suspension were analysed. For the PLGA-DF formulations, no significant difference was observed between the ratios. Therefore, the affinity of these formulations for plasma proteins was not dependent on plasma content. However, a significant increase ($p \leq 0.01$) in protein-

binding was observed for formulations coated with 1% Pluronic F127 at 40% plasma content compared to the 10%. In contrast, the formulations coated with 1% PEG presented a significant decrease in protein-binding from $31.41 \pm 13.8\%$ at 10% to $14.32 \pm 7.4\%$ for 40% plasma content. The different protein-binding profiles observed for the two poloxamer formulations i.e. increased protein-binding for Pluronic F127 and decreased protein-binding for PEG, may suggest a difference in stability of the adsorbed layers for the two types of poloxamer formulations.

Formulations of coated and uncoated nanoparticles encapsulating RIF were compared. For PLGA-RIF, $23.95 \pm 6.6\%$ protein-binding was observed compared to $71.12 \pm 0.78\%$ for free RIF. This observation demonstrates a significant decrease in protein-binding. To further substantiate the findings, the same formulation was coated with 1% Pluronic F127 and 1% PEG. A 57% decrease in protein-binding was observed when PLGA-RIF nanoparticles were coated with 1% PEG ($10.16 \pm 4.32\%$) compared to unencapsulated (free) RIF. A similar significant reduction ($p \leq 0.01$) in protein-binding of 53% for 1% Pluronic F127 coated PLGA-RIF nanoparticles was also observed.

Based on these results, it can be suggested that nanoencapsulation could minimize exposure of the drugs to plasma proteins. Thus, more unbound drug would reach the site of action with potential for possible dosage adjustment. Furthermore, a slight decrease in percentage protein-binding was observed for PLGA-RIF formulation from 10%, 20% and 40% v/v plasma, but this was found to not be statistically significant. In addition, for PLGA-RIF formulations coated with 1% PEG and 1% Pluronic F127, no significant difference was observed at the different plasma concentrations either.

Table 4.4 includes data from an additional experiment in which the protein-binding of coated and uncoated nanoparticles encapsulating INH were evaluated. As previously discussed, INH has a very low protein-binding (20- 30%). However, when comparing encapsulated INH with unencapsulated (free) INH, a significant difference ($p \leq 0.01$) was observed. Free INH at 10% v/v plasma had a protein-binding of $43.4 \pm 6.6\%$ which was significantly decreased to $19.80 \pm 4.3\%$ following nanoencapsulation. Comparison of the uncoated and coated PLGA-INH formulation resulted in no significant difference in protein-binding. In addition, the effect of plasma content for these INH formulations was also evaluated. It was observed that

incubation of the nanoparticle suspensions with 20 and 40% v/v plasma content had no significant effect on the protein-binding compared to 10% v/v plasma content.

Figure 4.3 and 4.4 illustrates SDS-PAGE gel images for PLGA-drug free and PLGA encapsulating RIF, respectively. Since albumin is the major binding plasma protein for most neutral or acidic drugs (Woo *et al.* 1996:177), binding of albumin and binding of whole plasma is considered synonymous. The protein fragments with the strongest intensity appear in the marker range of 70 kDa in both images. This molecular weight range is reported to be plasma albumin (Carter *et al.* 1989:1196). In Figure 4.4, the fragment intensity at 70kDa is similar for both the coated and the uncoated formulations, with slightly stronger band intensity observed for PLGA with 1% PEG. In Figure 4.5, the uncoated PLGA-RIF formulations demonstrated the greatest intensity of the albumin fragment, with lower intensities for the formulations coated with 1% Pluronic and even lower intensities for the formulations coated with 1% PEG. The difference observed at different ratios can be interpreted as being a function of the different concentrations of the bound protein, in this case albumin. The observations discussed cannot be directly compared to Table 4.4, since these data reflect denatured proteins and not absolute protein concentrations as summarised in Table 4.4 since gel analysis is not quantitative. Other plasma proteins associated with these nanoparticles to a lesser extent were the apolipoproteins (Mw 28, 34 and 43 kDA), and possibly cholesteryl ester transfer protein (Mw 53 kDA) as reported in a previous study (Cedervall *et al.* 2007:5857)

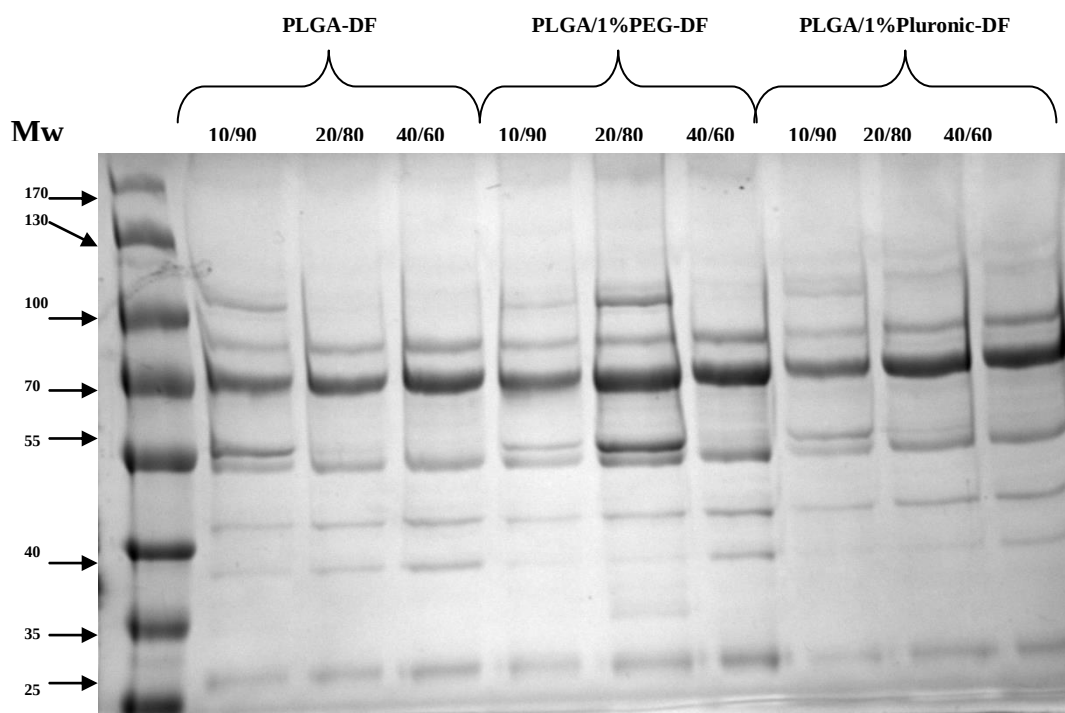


Figure 4.3 SDS-PAGE gel image of PLGA-drug free formulations coated with PEG/Pluronic F127. The image depicts the band intensities of the different proteins bound to the nanoparticles. Albumin with Mw 70 kDa demonstrated the strongest band intensity.

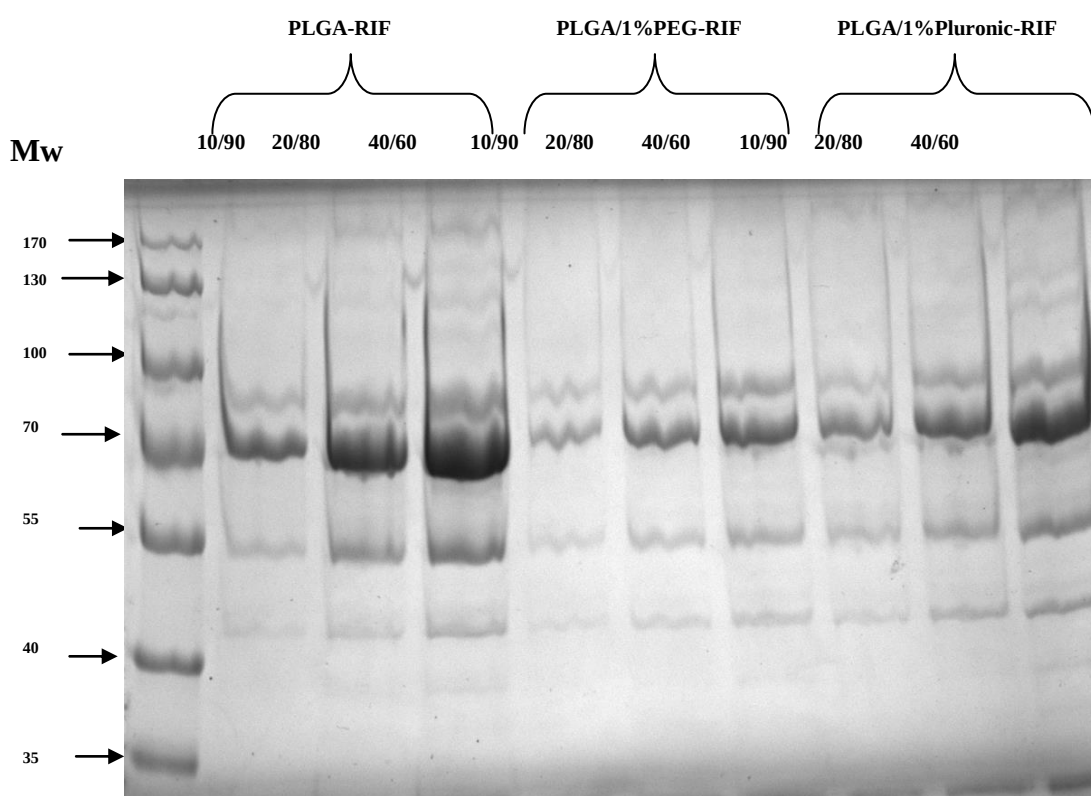


Figure 4.4 SDS-PAGE gel image of PLGA-drug free formulations coated with PEG/Pluronic F127. The image depicts the band intensities of the different proteins bound to the nanoparticles. Albumin with Mw 70 kDa demonstrated the strongest band intensity, but was weaker compared to the drug free analysis (Figure 4.3). The strongest band intensity was observed for the PLGA-RIF formulation at 40:60 suspensions.

4.3.3 Biodistribution of fluorescently labelled uncoated PLGA nanoparticles

The rhodamine and coumarin-labelled particles were initially not detected in the 5 μ m tissue sections via fluorescent microscopy, because of the intense autofluorescence of the tissues. Thus, a fluorometer was used to detect fluorescence in the tissue homogenates. The data was normalized with the negative control, which was tissue from mice treated with only saline. The background fluorescence from these tissues was subtracted from the experimental tissue fluorescence readings to exclude the effect of autofluorescence. The percentage particles detected was expressed as the ratio of the fluorescence unit of each tissue relative to the sum of fluorescence units of all tissues analysed and graphically illustrated in Figure 4.6 and listed in Table 4.6 for each tissue.

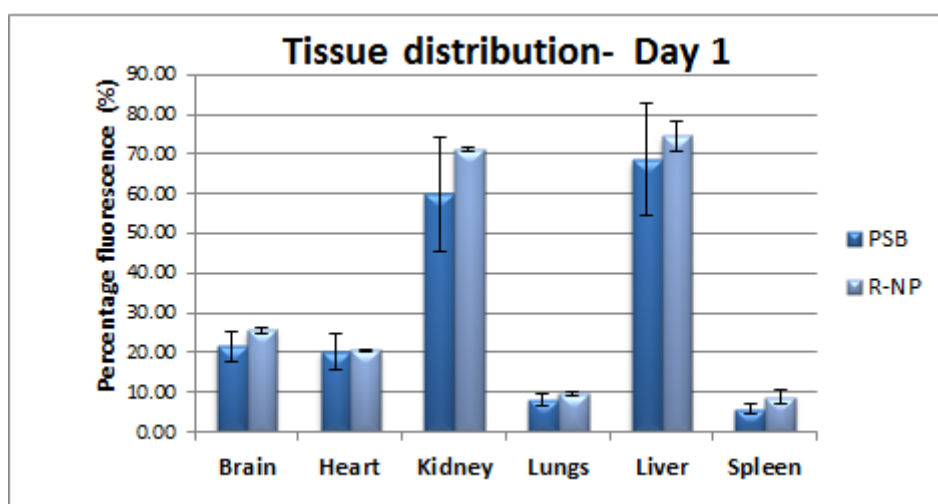
Table 4.6 Summary of percentage fluorescence intensities of particles detected in the organs as a function of mean fluorescence intensity detected evaluated over 7 days.

		Brain	Heart	Kidney	Lungs	Liver	Spleen
Day 1	PSB	21.49 (3.76)	20.28 (4.41)	59.91 (14.28)	8.07 (1.43)	68.65 (14.08)	5.70 (1.20)
	R-NP	25.52 (0.60)	20.51 (0.39)	71.09 (0.39)	9.66 (0.70)	74.62 (3.76)	8.83 (1.65)
	p-values	>0.01	>0.01	>0.01	>0.01	>0.01	>0.01
Day 3	PSB	21.98 (3.06)	14.44 (2.52)	53.90 (12.61)	8.21 (1.45)	58.25 (10.90)	6.35 (1.95)
	R-NP	16.17 (0.88)	15.39 (0.62)	49.22 (1.24)	9.28 (0.36)	78.44 (0.16)	8.05 (0.70)
	p-values	>0.01	>0.01	>0.01	>0.01	>0.01	>0.01
Day 7	PSB	15.52 (0.11)	9.62 (0.68)	19.44 (11.44)	3.28 (1.08)	62.29 (0.32)	3.07 (0.79)
	R-NP	13.23 (0.11)	16.81 (0.11)	54.89 (0.95)	15.61 (1.15)	48.48 (2.28)	5.73 (0.21)
	p-values	>0.01	>0.01	<0.01	<0.01	>0.01	>0.01

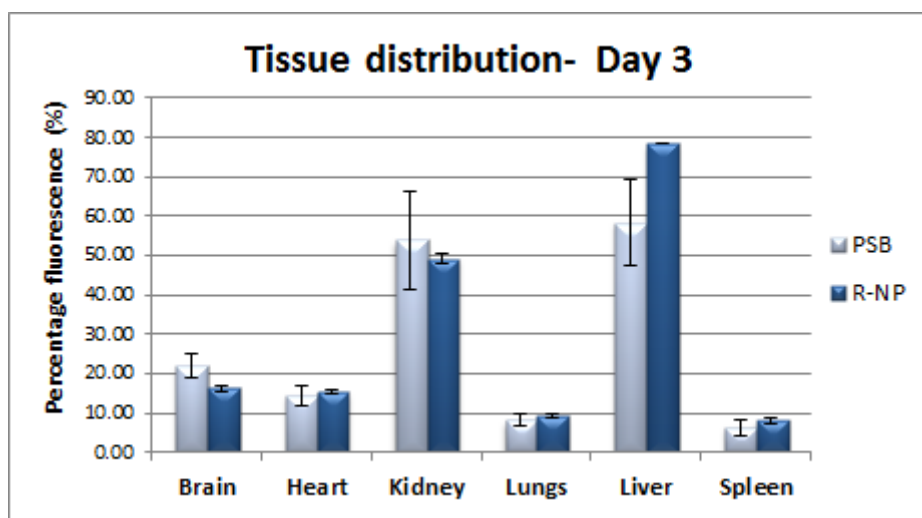
PSB- Polystyrene beads; R-NP- Rhodamine PLGA nanoparticles; Data expressed as a representation of percentage particles detected of the total particles, mean $\pm$ SEM in parentheses; n=6

From this data it is evident that most of the particles were detected in the liver at 74.62 $\pm$ 3.76% (Day 1), followed by the kidney (71.09 $\pm$ 0.39%, Day 1), brain (25.52 $\pm$ 0.60%, Day 1), and heart (20.51 $\pm$ 0.39%) as observed in Figures 4.5 (A). The lowest detection was observed in the lungs and spleen. The values observed were consistent on the third day of analysis (Figure 4.5 (B)) with a decrease observed at day seven, 48.48 $\pm$ 2.28 % and 54.89 $\pm$ 0.95% for the liver and kidney, respectively. Statistical comparison between the polystyrene bead control and the rhodamine labelled PLGA nanoparticles revealed no significant difference on the day one and three data analysis. On day seven, rhodamine labelled PLGA nanoparticles were observed to be significantly higher in the lungs and kidney compared to the polystyrene

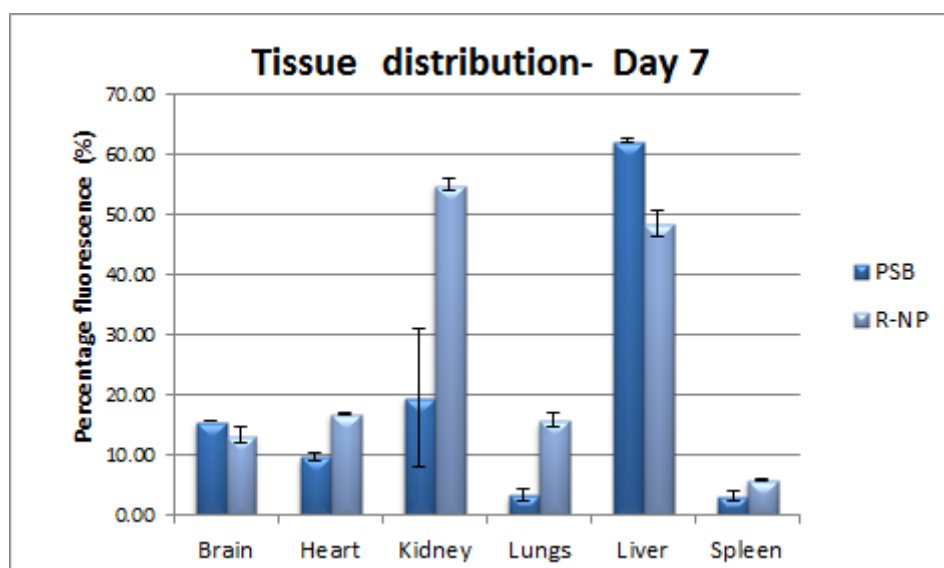
beads. This observation could be because of the polystyrene beads being more rapidly eliminated from the kidneys and possible accumulation of the PLGA nanoparticles in the lungs. Plasma samples collected from mice sacrificed on day three and seven were also analysed as indicated in Figure 4.5 (D), but only a very low percentage of the particles were detected.



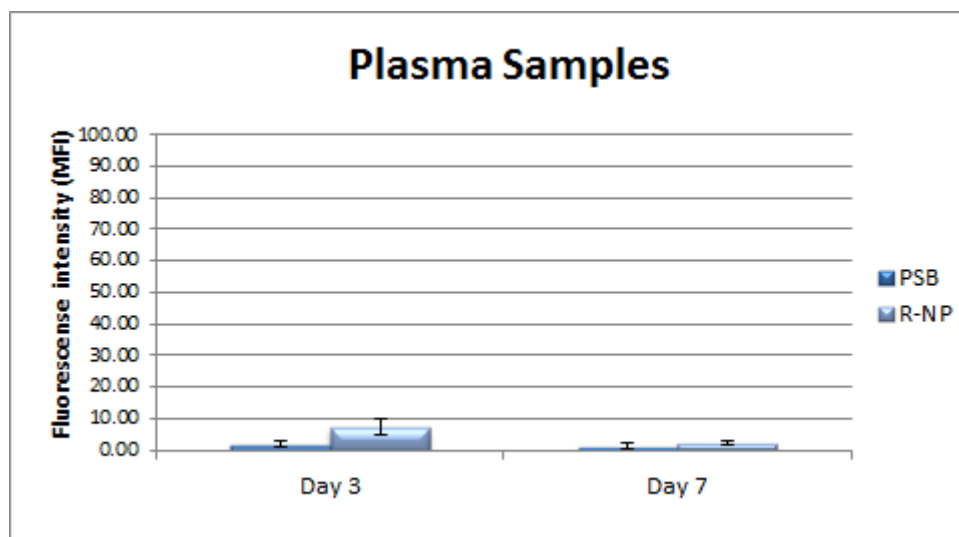
(A)



(B)



(C)



(D)

PSB - Polystyrene beads; R-NP- Rhodamine nanoparticles

Figure 4.5 (A), (B), (C) illustrates tissue distribution values for the organs of interest at day 1, 3 and 7, respectively as a function of the percentage fluorescence intensity detected per organ in polystyrene beads and rhodamine labelled nanoparticles. Figure 4.5 (D) illustrates fluorescence levels in plasma collected from the mice sacrificed on Day 3 and 7

To ensure that the fluorescence detected represents the detection of fluorescently-labelled nanoparticles and not leached or degraded fluorescence, an additional experiment was conducted administering nanoparticles consisting of spray-dried rhodamine-only without PLGA (Appendix C).

The highest percentage fluorescence of PLGA nanoparticles were observed for the liver and kidneys as was previously demonstrated (Table 4.6). The percentage fluorescence peaked on

day seven for the liver, lungs, spleen and heart, indicating possible accumulation. Fluorescence detection in the brain was consistent over the seven-day analyses. Excluding the day 1 observation for the spleen, the data demonstrates a significant difference in fluorescence detected for the rhodamine labelled PLGA nanoparticles compared to the rhodamine-only nanoparticles ($p < 0.01$). These results confirm that the fluorescence detected in the organs as previously established (Table 4.5) are PLGA nanoparticles and not leached or degrade fluorescence.

Confocal images of three sets of tissue sections were evaluated. The first was untreated tissues one day post oral administration. The second set was spray-dried rhodamine-only nanoparticles to distinguish between rhodamine-labelled nanoparticles and rhodamine released from the nanoparticles. The third set was confocal imaging of tissues following one day exposure to rhodamine-labelled PLGA particles. These are shown in Figure 4.6. This demonstrated that fluorescent particles can be detected within the various tissues. It has been reported that rhodamine displays long term release from nanoparticles of about 192 hours (Tosi, *et al.* 2007:5), and therefore, the detection of fluorescence in the different tissues can be considered that of the rhodamine associated with the PLGA nanoparticles. It must be emphasized that the confocal images do not correlate to the quantity of the particles in the tissues as indicated in Tables 4.6 and 4.7. These images only indicate the presence of the particles in the respective tissues and the particulate nature of the fluorescence. This observation suggests that the observed fluorescence is that of rhodamine in the nanoparticles and not leached rhodamine. The saline-treated tissues compared well to the spray-dried rhodamine only tissues, indicating that in tissues where rhodamine only was administered, the fluorescence was not visible by confocal imaging. For nanoparticle treated tissues, fluorescent “hotspots” are clearly visible in the lungs, kidneys and brain.

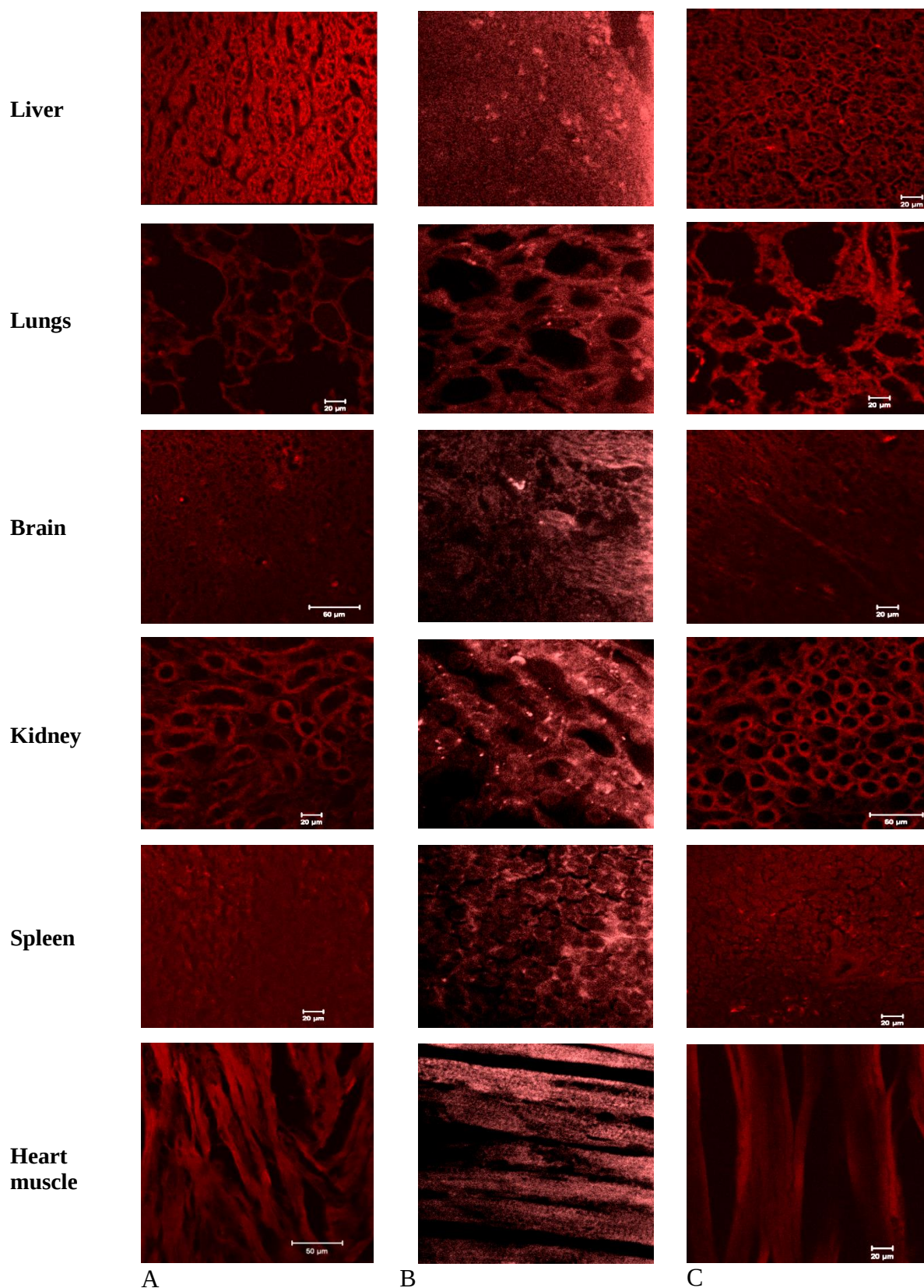


Figure 4.6 CLSM images of tissue collected from mice treated with saline (A), rhodamine-labelled PLGA nanoparticles (B) and spray-dried nanoparticles (C). Fluorescent nanoparticles are clearly visible in the liver, lungs, brain and kidney compared to blank tissue and rhodamine-only nanoparticles. Scale bar 20 μm .

The difference in fluorescence units observed for the different groups over 7 days together with the data presented in Figure 4.7, confirms that the fluorescence detected in the tissues were fluorescently labelled PLGA-nanoparticles and not leached or degraded fluorescence.

4.3.4 Biodistribution of fluorescently labelled poloxamer coated PLGA nanoparticles

The aim of this work was to evaluate PLGA nanoparticles coated with the poloxamers PEG and Pluronic F12, respectively. The percentage particles detected was expressed as the ratio of the fluorescence unit (FU) of each tissue relative to the sum of fluorescence units of all tissues analysed as shown in Table 4.8.

Table 4.8: Summary of percentage fluorescence detection of rhodamine labelled nanoparticles coated with poloxamers, PEG and Pluronic F127 at 1% concentration detected in the organs evaluated over seven days. Values are depicted as the mean $\pm$ SEM in parentheses.

		Brain	Heart	Kidney	Liver	Lung	Spleen	Plasma
Day 1	1% PEG	6.24 (1.50)	5.22 (1.26)	12.32 (3.56)	16.08 (4.19)	3.66 (1.47)	2.74 (1.50)	4.47 (2.97)
	1% Pluronic F127	4.45 (1.13)	4.13 (1.35)	15.70 (1.94)	18.27 (1.48)	3.43 (1.46)	2.46 (1.12)	1.57 (1.04)
Day 3	1% PEG	3.27 (1.06)	5.33 (1.09)	9.29 (2.88)	24.30 (1.14)	2.59 (0.34)	3.49 (0.93)	1.74 (0.46)
	1% Pluronic F127	2.51 (0.77)	4.61 (2.30)	6.72 (1.59)	31.34 (2.29)	3.02 (0.80)	1.64 (0.48)	0.24 (0.14)
Day 7	1% PEG	3.30 (0.89)	6.81 (2.15)	12.57 (3.76)	16.77 (2.82)	4.28 (2.13)	3.69 (1.85)	2.58 (1.22)
	1% Pluronic F127	7.97 (1.83)	10.71 (1.10)	15.07 (3.67)	4.91 (1.29)	6.51 (1.19)	3.94 (0.74)	0.89 (0.40)

Comparison of the two coated formulations demonstrated no significant difference for day one, three and seven of analysis. On day seven, significantly higher percentage fluorescence was observed in the liver for the PEG coated formulation ($16.77 \pm 2.82\%$) compared to the Pluronic F127 coated formulation ($4.91 \pm 1.29\%$, $p \leq 0.01$). The value observed for the Pluronic F127 coated formulation was also significantly lower than the value observed at day three ($31.34 \pm 2.29\%$), indicating that this formulation was eliminated more rapidly than the PEG coated formulation.

Table 4.9 is a summary of the p-values, i.e. statistical significance between the percentage fluorescence of rhodamine labelled PLGA nanoparticles detected in the organs analysed compared to the poloxamer coated nanoparticles. The percentage fluorescence observed for the poloxamer coated nanoparticles was significantly lower ($p \leq 0.01$), indicating that coating

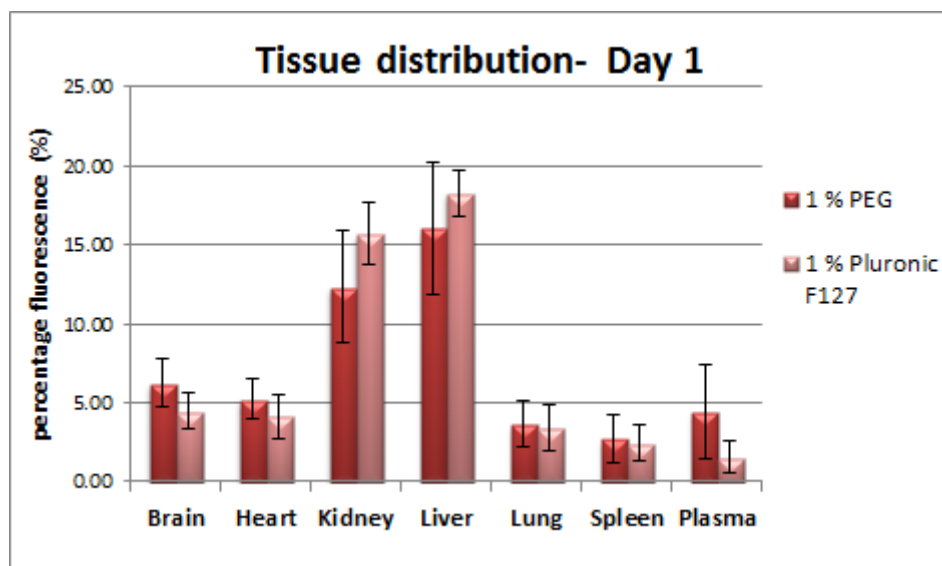
with a poloxamer could possibly result in an increased residence time of the particles as previously discussed (section 4.1.1).

Table 4.9 p-values comparing the uncoated PLGA nanoparticles in Table 4.5 and the two formulations coated with 1% PEG and Pluronic F127, respectively. P-values were calculated using the Student's T-test comparing data sets of the three formulations.

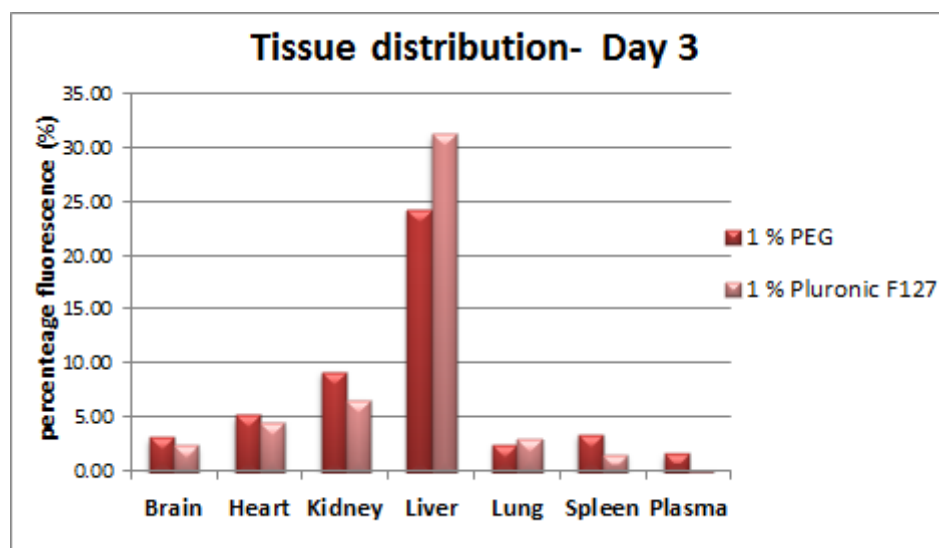
		Brain	Heart	Kidney	Liver	Lung	Spleen
Day 1	R-NP 1% PEG	0.000362	0.000179	0.000615	0.009565	0.001035	0.006105
	R-NP 1% Pluronic F127	0.000482	0.000135	0.000524	4.22E-05	0.001026	0.010212
Day 3	R-NP 1% PEG	5.99E-05	7.98311E-05	0.001824	2.59E-06	5.91E-05	0.001676
	R-NP 1% Pluronic F127	0.000139	0.000507	0.000523	1.84E-05	4.78E-05	0.000797
Day 7	R-NP 1% PEG	0.00486	0.014523	0.379888	0.000254	0.096033	0.79048
	R-NP 1% Pluronic F127	0.001351	0.000645	0.000493	0.00043	6.97E-05	0.160582

R-NP- rhodamine PLGA nanoparticles

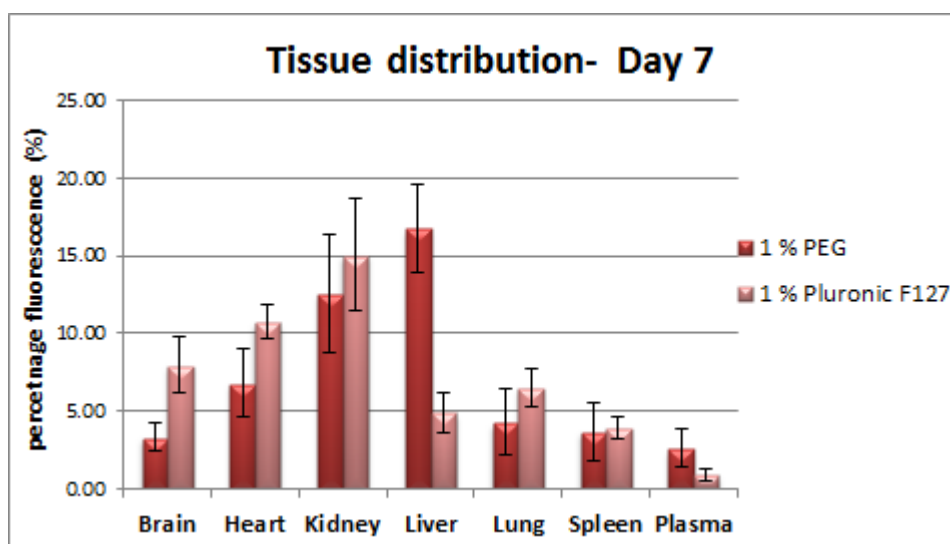
As illustrated in a section 4.3.3, PLGA particles with no poloxamer coating were detected in the liver, spleen, lungs, kidneys and the brain over a period of seven days. However, very low concentrations of particles were observed in the plasma over the same period when plasma collected on day three and seven was analysed. The biodistribution profiles observed for particles coated with 1% PEG or Pluronic F127 following orally administration, are depicted in Figure 4.7. The presence of PEG coated particles in the brain decreased over the seven days whereas the presence of particles in the heart, kidney, liver and lung remained relatively constant. A slight accumulation of particles was detected in the spleen, indicating uptake by the M cells of the Peyer's patches. However, Pluronic-F127 coated particles resulted in an accumulation in the brain over the seven days, similar profile to that of PEG coated particles was observed in the rest of the tissues. In both cases, plasma concentrations were significantly higher when compared to the previous experiment for uncoated PLGA particles. This increase in the residence time in plasma is in agreement with that of Stolnik *et al* (1994:1806). As discussed in section 4.3.3, the confocal imaging confirmed that the fluorescence detected in these tissues is of rhodamine in the nanoparticles and not leached rhodamine.



(A)



(B)



(C)

Figure 4.7 (A), (B) and (C) illustrates tissue distribution values for the organs of interest at day 1, 3 and 7, respectively as a function of the percentage fluorescence intensity detected per organ in rhodamine labelled PLGA nanoparticles coated with 1% PEG and 1% Pluronic F127.

4.4 Discussion

Nanoparticle characterization is an important consideration since chemical and physical properties of these particles determine its PK and biodistribution (Li & Huang 2008:496). For instance, opsonisation is a major factor in MPS uptake of polymer nanoparticles and therefore surface characteristics can greatly influence PK (Li & Huang 2008). Furthermore, considering the limited pore size of the epithelium wall, this is the primary delivery barrier for nanoparticles and therefore particles size plays an important role in nanoparticle biodistribution. The particle size of the nanoparticles used in this study were not below 100nm, but were still within a size range (229 ± 7.6 to 382 ± 23.9 nm) to promote longer circulation for the surface-modified particles. Although particles with neutral zeta potential have longer blood circulation times than charged particles, the positive zeta potential of the particles evaluated was still adequate to prolong circulation.

The development of surface-coated or “stealth” polymeric nanoparticles to reduce plasma protein adsorption in order to prolong the residence time of these particles in the blood has been well documented (Gref *et al.* 2000; Moghimi & Szebeni 2003; Tan *et al.* 1993; Stolnik *et al.* 2001). It has been reported that stealth nanoparticles have a half-life in humans as long as 45 hours following IV administration depending on the particle size and the characteristics of the coating polymer (Moghimi & Szebeni 2003:465). The most commonly used

poloxamers used in nanoparticle surface modification is PEG and Pluronic. Furthermore, it is well accepted that particle size and surface hydrophobicity also play an important role in plasma protein-binding, i.e. the smaller the particle, the lower the extent of protein adsorption (Li & Huang 2008). The drug concentrations in the blood determine the rate and extent of protein-binding. This may suggest that when the drug is encapsulated in a polymeric nanoparticle, the amount of drug available for binding is decreased by a controlled release mechanism, which results in a lower concentration of the drug at the time of analysis. In this study, the assay was conducted over a two-hour period, resulting in less drug concentration being released. This is compared to the bulk of drug that was available in this time with the unencapsulated drug. Therefore, the mechanism of protein-binding for the different formulations may be considered to be as a function of the affinity of the polymeric nanoparticles for plasma proteins as well as the concentration of drug available for binding which, at a specific time, is decreased by encapsulation.

The biodistribution of drugs into tissues is dependent on the fraction of unbound drug available which is controlled by a concentration gradient which is produced by the concentration of unbound drugs, since bound drugs does not readily leave the capillaries. The fraction of unbound drug not only affects the rate of distribution, but also the rate of drug elimination (Lindup & Orme 1981:213). RIF has been reported to have a low volume of distribution of approximately 1.44 L/kg (Nawaz, 1988:32) but is still pharmacologically effective despite the high percentage protein-binding. This therefore poses the question: why the need to evaluate the extent of protein-binding of nanoencapsulated RIF if it is pharmacologically effective? As discussed in Chapter 1, RIF has a high dosing frequency to achieve its pharmacological effect, i.e. once daily. Since, one of the aims of nanoencapsulation is to reduce dosing frequency by prolonging blood circulation of the drugs and facilitating controlled release, it is imperative to understand nanoparticulate interaction with blood proteins. This assumption is based on the fact that nanoparticles can be formulated to control drug release and with prolonged circulation and protection of the drug in the polymer core, drugs can reach target organs more readily with reduced protein interaction. Thus, dosing frequency can be reduced since it may be possible to maintain therapeutic drug levels for a longer period.

For the same reason, surface-modified nanoencapsulated INH was evaluated. The effect of surface modification became evident in the drug-free nanoparticles, where drug presence

could have no effect. This, however, was dependant on plasma concentration. The decrease in protein-binding between uncoated and 1% Pluronic coated nanoparticles were not significant, but at 40% plasma content nanoparticle protein-binding was decrease by 6% for 1% PEG coated particles. Therefore, protein-binding evaluation of nanoparticles encapsulating INH would not have a significant effect on PK and biodistribution of INH.

The significant decrease in protein-binding observed for nanoencapsulated RIF may support the postulated minimization of first pass metabolism (Italia *et al.* 2007:197), since the drug is not exposed to metabolic enzymes to the same extent as for conventional delivery systems due to protection of the drug in the polymer core. In principle, RIF is well absorbed, having a bioavailability of 90- 95% (Ahmad *et al.* 2006:409), however, it stimulates its own metabolism decreasing the plasma half-life by three hours (Chambers, 2001:806). Since half-life is determined by clearance and volume of distribution, this result may indicate that this effect on half-life may be improved. Therefore, potentially reducing the dosage and still exert an effective pharmacological effect.

This differences observed in plasma to drug ratio for the unencapsulated (free) drugs in Table 4.4 in this *in vitro* system may be explained by the Vroman effect. This effect states that the competitive binding of plasma proteins to binding sites is a function of the concentration of plasma proteins and the incubation period (Slack & Horbett 1995:112). The results demonstrate exactly this, the highest concentration of plasma proteins, i.e. for 40:60, plasma: drug solution, the highest percentage protein-binding was observed for RIF. However, the opposite effect was observed for INH, suggesting that protein-binding of INH may be because of plasma protein affinity for INH, since INH is a hydrophilic drug.

A possible explanation for the similar results in coated versus uncoated drug-free nanoparticles observed in this study is that the PEG and Pluronic-F127 surface coverage on the nanoparticle surface may not have provided sufficient protection against plasma protein-binding and the proteins still adsorb to the underlying PLGA core. Stolnik *et al* (2001:267) demonstrated that the mechanism of inhibition of protein adsorption to hydrophobic surfaces is that PEG blocks adsorption sites for proteins (Stolnik *et al.* 2001:275), thus resulting in a relatively low protein-binding. The PEG content for these experiments was selected at 1% since any further increase resulted in a substantial increase in nanoparticle size. A formulation coated with 2% PEG resulted in nanoparticles with a size of 607.8 nm versus

379.6 nm for 1% PEG, which is almost a two-fold increase in size. Pluronic F127 has similar hydrophilicity to PEG. However, Pluronic F127 is a poloxamer, which consists of a hydrophobic block of polypropylene glycol (PPG) that is flanked by two hydrophilic blocks of PEG, which makes it a tri-block copolymer. Thus, Pluronic will assume a brush-like morphology when adsorbed onto PLGA, where PPG would adsorb to PLGA via non-covalent binding and the hydrophilic PEG will extend away from the surface of the PLGA nanoparticle. Figure 4.8 illustrates this difference in terms of surface coverage of PEG. In the case of PEG, which only contains hydrophilic chains, surface coverage may be significantly reduced to that of Pluronic coated PLGA.

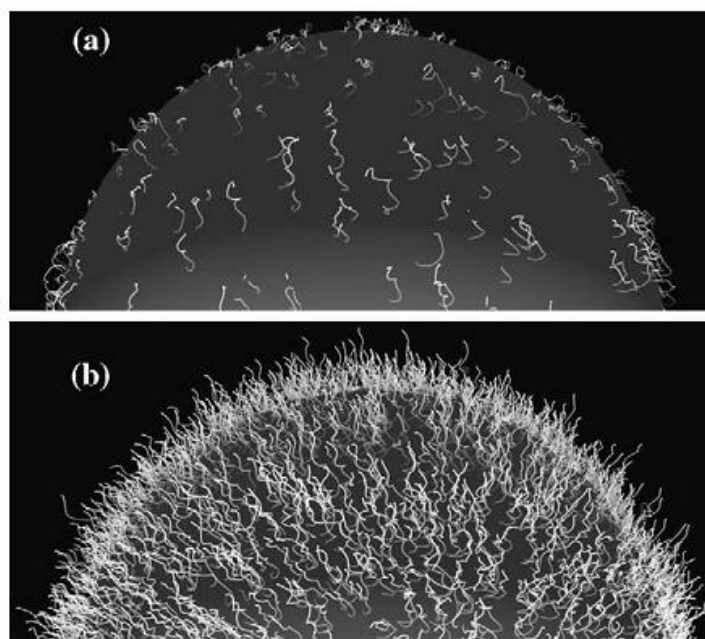


Fig. 4.8 Schematic diagrams presenting PEG configurations on the surface of a polymeric nanoparticle. (A) – depicts a low surface coverage of PEG chains which leads to the “mushroom” configuration observed. This is where most of the chains are located closer to the particles surface; (B) - depicts a high surface coverage. Here a lack of mobility of the PEG chains leading to the “brush” configuration where most of the chains are extended away from the surface are observed. Included with permission (Owens & Peppas 2006:98).

The observation of the differences in protein-binding for the PEG-coated nanoparticles encapsulating RIF compared to the drug-free nanoparticles discussed previously may be attributed to the increase in surface hydrophobicity with the inclusion of RIF which is not only present in the polymer matrix, but may also be on the surface of the nanoparticles. This may indicate that 1% coating with these poloxamers results in maximum reduction in protein-binding when a hydrophobic drug such as RIF is encapsulated. Thus, since the surface-modified polymeric nanoparticles demonstrated decreased protein-binding and may provide

an argument for dosage adjustment of highly bound drugs by nanoencapsulation. While the binding of these drugs may be reversible and therapeutic drug levels are eventually reached, higher drug concentrations have to be administered to ensure these drug levels are maintained. Therefore, if this binding can be delayed by nanoencapsulation, more unbound drug will be able to reach the target site and lower administered concentration can still reach therapeutic levels. The protein-binding data thus far further suggests prolonged circulation. This may result in improved biodistribution of these particles.

The biodistribution data demonstrate that following oral administration of fluorescently-labelled polystyrene beads and rhodamine-labelled PLGA nanoparticles to respective groups, nanoparticles were detected in all the tissues evaluated. The percentage order of the detected particles are liver>kidney>heart>brain (Table 4.6). Furthermore, Table 4.7 confirms that the fluorescence detected was from the nanoencapsulated fluorophores and not from leached fluorophore. This is demonstrated by comparing encapsulated fluorophores versus the spray-dried nanoparticulate fluorophores. The nanoparticulate fluorophores are observed in lower concentrations compared to encapsulated fluorophores over a seven-day period. This observation confirms that the nanoparticulate fluorophores are cleared within 24 hours post oral administration, whereas encapsulated fluorophores are present in higher concentrations up to seven days. For the nanoencapsulated fluorophores, the lowest concentrations were observed in the spleen and lungs. The increased percentage of nanoparticles observed in the liver has been reported to be because of rapid uptake of these uncoated nanoparticles by the hepatic macrophages or Kupffer cells (Owen & Peppas 2006:99). Peracchia *et al* (1999:126) demonstrated decreased nanoparticle accumulation in the liver with subsequent increased accumulation in the spleen as a result of PEGylation following 24 hour IV-administration. These reports together with the results presented here indicate the necessity for surface modification with hydrophilic molecules such as PEG/Pluronic to minimize opsonisation of the particles, therefore increasing blood circulation time as well as minimize the amount of nanoparticle accumulation in the liver. The experiments were therefore repeated with nanoparticles surface modified with 0.5 and 1% PEG and Pluronic-F127 coating to illustrate the effect of surface modification on biodistribution.

Comparison of particles coated with 0.5% poloxamer with particles coated with 1% poloxamer, illustrated that a higher concentration of particles were observed in the spleen, lungs and plasma for the latter formulation. The lower concentrations detected in plasma as

opposed to the higher proportion in the liver and kidney could be attributed to the surface heterogeneity in the population of PEG or Pluronics PLGA particles as previously described. For “naked” or uncoated particles, sequestration in the MPS organ occurs in a matter of minutes and particle accumulation will be observed in the liver and spleen. However, even for PEGylated particles, sequestration may not be as rapid due to longer blood circulation, but the eventual accumulation in these organs still occurs (Owen & Peppas 2006:99).

These results therefore suggest that more studies have to be conducted for oral formulation to give a better understanding of the biodistribution kinetics. Although the particles accumulated in the spleen and the brain, Semete *et al* (2010:695) reported the safety of these particles in these tissues at high doses of PLGA particles. The mice in the study appeared healthy throughout the seven-day study, which supports the findings observed in chapter 3 that despite presence, and in some cases accumulation, of nanoparticles the organs, no harmful immune response was elicited. This study suggests that the prolonged residence time in plasma may improve the plasma bioavailability of encapsulated drugs. The data further suggests a possible improvement of drug biodistribution by virtue of the fact that nanoparticles can localize drugs in tissues where conventional formulations present more limitations.

4.5 Conclusion

PEG and Pluronics have been extensively used in drug delivery to prolong the circulation time of particles in blood. The protein-binding data serve to provide further characterization for polymeric nanoparticles both uncoated and coated with poloxamers in oral formulations. Therefore providing an indication of the blood circulation residence time for these particles and its’ potential for use as a sustained release drug delivery system. The data also demonstrates that PEG-coated nanoparticles reduced protein-binding compared to uncoated particles in the presence and absence of encapsulated drug. Along with size and surface charge, protein-binding has been reported to be a key factor influencing biodistribution (Aggarwal *et al.* 2009:433). This is based on the changes brought about to the particle by the adsorbed proteins. Changes which affect the internalization process by macrophages and subsequently the overall biodistribution throughout the body (Aggarwal *et al.* 2009:432). However, the data suggests that poloxamer coating of polymeric nanoparticles results in a longer circulation time of these nanoparticles with a subsequent potential increase of nanoparticles accumulating in tissues. However, the biodistribution assays confirm that these

particles are able to cross the intestinal barrier as well as the blood brain barrier as was observed by confocal imaging as well as fluorescence detection. Future work should include more detailed protein-binding assays over a longer study period, because of the complexity of nanoparticle-protein interactions, which are not yet completely understood according to literature (Aggarwal *et al.* 2009; Lynch & Dawson 2002; Cedervall *et al.* 2007). Further analysis of the biodistribution of these nanoparticles would be needed to evaluate the drug biodistribution to determine whether therapeutic drug levels are released and present in tissues over the number of days evaluated. This will be one of the objectives evaluated in Chapter 5.

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