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In vitro characterisation of PLGA nanoparticles encapsulating rifampicin and isoniazid - Towards IVIVC

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INTRODUCTION

It has been postulated that antituberculosis drugs encapsulated in polymeric nanoparticles are able to control the release of these drugs *in vivo*. These biodegradable polymers facilitate sustained/controlled release by means of degradation of the polymer or by diffusion through the polymer matrix. For oral drug delivery, one of the most important parameters to be elucidated is the absorption of not only the drugs, but also of the nanoparticles. These nanoparticles are postulated to be absorbed in tact and be transported through the lymphatic system.

Once in the systemic circulation, the biodistribution of the particles is highly dependent on its response to the biological environment, mainly binding to plasma proteins. Nanoparticle characteristics such as surface hydrophobicity, size and polymer composition determine the extent of adsorption of blood components, mainly proteins such as albumin and glycoproteins[1]. For drugs with a high degree of protein binding, protein adsorption effects on volume of distribution are observed[2].

Another class of proteins that plays an important role in protein binding are opsonins. Binding of these proteins promotes the activation of the complement system and facilitates phagocytic uptake by macrophages[3]. 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[4].

The objective of the current study was to determine the effect that PLGA (coated/uncoated with PEG/Pluronic F127) nanoencapsulation of rifampicin (RIF) and isoniazid (INH) has on plasma protein binding of these drugs *in vitro*. Furthermore, the biodistribution of Rhodamine 6G labelled PEG-coated and Pluronic F127-coated nanoparticles was evaluated.

METHODS

Nanoparticle formulations

Poly-lactic-glycolic-acid (PLGA) nanoparticles were prepared by the double emulsion spray drying technique developed by the CSIR. Various formulations of PLGA nanoparticles were prepared and the results are summarised in **Table 1**. PEG (Mw 10 000) and Pluronic-F127 (Mw 9 000) were used to coat the formulations. RIF and INH were gifts from North-West University, Potchefstroom campus, South Africa. Also prepared for biodistribution assays were Rhodamine-6G labelled uncoated nanoparticles and Rhodamine-6G labelled nanoparticles coated with 1% PEG. These nanoparticles were prepared by including Rhodamine-6G in the aqueous phase of the first water-in-oil (w/o) emulsion. .

Protein binding assays

The nanoparticle protein binding was analysed using an adapted method as described previously for protein adsorption to polymer nanoparticles[5]. *In vitro* protein binding of polymeric nanoparticles was determined by preparing varying ratios of plasma to nanoparticles (10: 90; 20:80; 40:60 volume/volume (v/v)) to a total volume of 600 µl. The plasma/nanoparticle suspension was incubated for two hours at room temperature and then centrifuged at 14 000 rpm for 45 minutes to obtain a nanoparticle pellet, following a two-hour incubation in human plasma. The Bradford assay was used for the quantitative analysis of the pellet (bound protein) and the supernatant (unbound protein). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were used for qualitative analysis of the proteins in the pellet suspension. Protein binding of unencapsulated drug controls was analysed by equilibrium dialysis.

Biodistribution assays

To determine the biodistribution of PLGA nanoparticles of 1% PEG or 1% Pluronics-F127, these formulations were fluorescently labelled with Rhodamine 6G and orally administered to mice at 4 mg particles in 0.2 ml sterile saline by oral gavage. The biodistribution of the uncoated PLGA nanoparticles illustrated in **Figure 1** was reported previously[6]. The mice were grouped. Each group had three mice and the study was repeated three times. Group one was treated with drug-free PLGA-nanoparticles; group two with 1% PEG-PLGA nanoparticles and group three with 1% Pluronics F127-PLGA-nanoparticles. Oral administration was performed on the same day and the mice were euthanized at one, three or seven days post administration. The percentage detected was expressed as the ratio of the fluorescence unit of each tissue relative to the sum of fluorescence units of all tissues analysed.

Table 1: Summary of nanoparticle characterization

Formulation	Size (nm)	PdI	Encapsulation efficiency (%)	Drug loading (%)	Zeta potential
1. PLGA-DF	296.8	0.229	N/A	N/A	35.2
2. 1% PEG-DF	304.4	0.465	N/A	N/A	39.9
3. 1% Pluronic-DF	310.5	0.417	N/A	N/A	38.6
4. PLGA-RIF	399.2	0.325	69.2	7.64	14.4
5. 1% PEG-RIF	337.8	0.435	65.2	8.4	19.1
6. 1%Pluronic-RIF	260.1	0.355	67.34	8.53	16
7. PLGA-INH	253.4	0.12	73.5	23.43	15.8
8. 1% PEG-INH	281.1	0.35	67.65	24.8	8.52
9.1%Pluronic-INH	319.5	0.347	69	27.6	13.7
PLGA-Rhd(1% PEG)	313.3	0.303	N/A	N/A	N/A
PLGA-Rhd(1% PLU)	442.7	0.293	N/A	N/A	N/A

PLGA-poly-(lactic-co-glycolic) acid; PEG-poly ethylene glycol; DF-drug-free; RIF-rifampicin; INH- isoniazid; Rhd-Rhodamine; PLU- Pluronic

RESULTS

Protein binding

Table 2 illustrates the results observed for the different formulations. At a 10% plasma volume, PLGA-DF formulations demonstrated an average protein binding of 25.02% ± 4.58. A comparison between this formulation and a similar formulation coated with 1% Pluronic F127 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 31.41% ± 13.8. This result was found to be significantly different when compared to the uncoated formulation ($p < 0.01$).

Comparison of 10%, 20% and 40% v/v ratio of whole plasma to nanoparticle suspension demonstrated no significant difference for the uncoated drug free formulations. A significant increase was observed for Pluronic-F127 coated formulations whereas PEG coated drug free formulation demonstrated a significant decrease in protein binding with an increase in plasma volume. Therefore, the affinity of PEG coated nanoparticles for plasma proteins are dependent on plasma content. For nanoparticles (coated/uncoated) encapsulating RIF, a significant decrease in protein binding is observed when compared to unencapsulated (free) drug.

A 57% decrease in protein binding was observed when PLGA-RIF nanoparticles were coated with 1% PEG (10.16% ± 4.32 protein binding). For formulations encapsulating INH, no significant difference in protein binding was seen for the different formulations. However, nanoencapsulation facilitated decreased protein binding compared to free INH.

Table 2: Data summary of protein binding studies for various nanoparticle formulations with varying ratios of Plasma: Nanoparticle suspension

Nanoparticle formulations										
PLGA-DF	1% PLURONIC	1% PEG	PLGA-RIF	1%PEG-RIF	1% PLURONIC RIF	PLGA-INH	1% PLURONIC INH	1% PEG-INH	Control RIF *	Control INH *
Average percentage 10/90										
25.02 (4.58)	22.78 (6.49)	31.41 (13.80)	23.95 (8.60)	10.16 (4.32)	17.31 (6.78)	19.80 (4.30)	18.46 (3.88)	18.94 (3.7)	71.12 (0.78	43.37 (6.6)
Average percentage 20/80										
22.03 (4.81)	21.23 (6.62)	20.57 (6.60)	18.83 (7.50)	16.87 (2.11)	17.58 (2.86)	13.15 (5.81)	15.51 (6.34)	14.40 (4.60)	79.47 (1.60)	29.96 (10.90)
Average percentage 40/60										
20.91 (4.44)	31.30 (9.76)	14.32 (7.40)	15.40 (5.50)	12.92 (2.15)	16.57 (5.18)	15.07 (3.40)	12.77 (5.41)	15.80 (2.00)	90.00 (1.38)	23.00 (5.2)

* % protein bound was calculated as 100 minus % unbound. Standard deviations shown in parentheses. Experiments were repeated three time (n=3)

BIODISTRIBUTION

From this data illustrated in **Figure 1**, it is evident that most of the particles were detected in the liver at 40.04% ± 8.42%), followed by the kidney (25.97% ± 7.09%), heart (11.92% ± 3.16%), and brain (12.86% ± 2.82%) throughout all seven days in which the tissues were analysed. However, very low concentrations or no particles were observed in the plasma over the same period when plasma collected on day one, three and seven was analysed. The biodistribution for the uncoated nanoparticles warranted surface modification to minimise particle localisation in the liver.

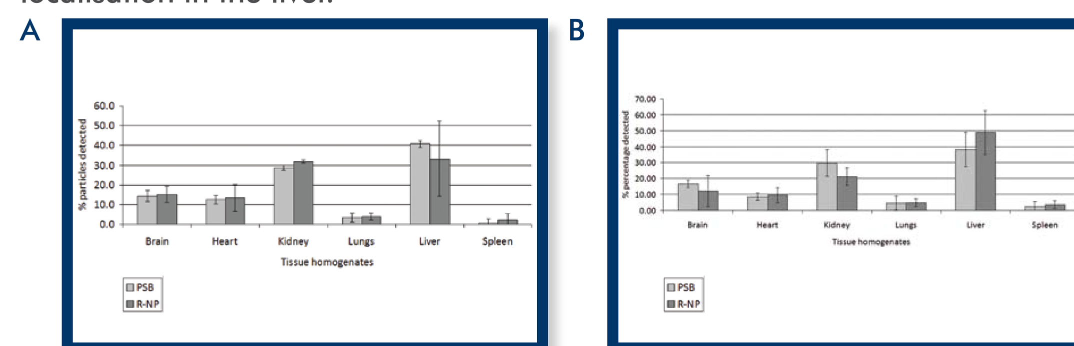


Figure 1:
A = biodistribution of coated PLGA nanoparticles after one day oral administration,
B = day three after oral administration,
C = day seven after oral administration. R-NP= rhodamine nanoparticles; PSB= polystyrene beads

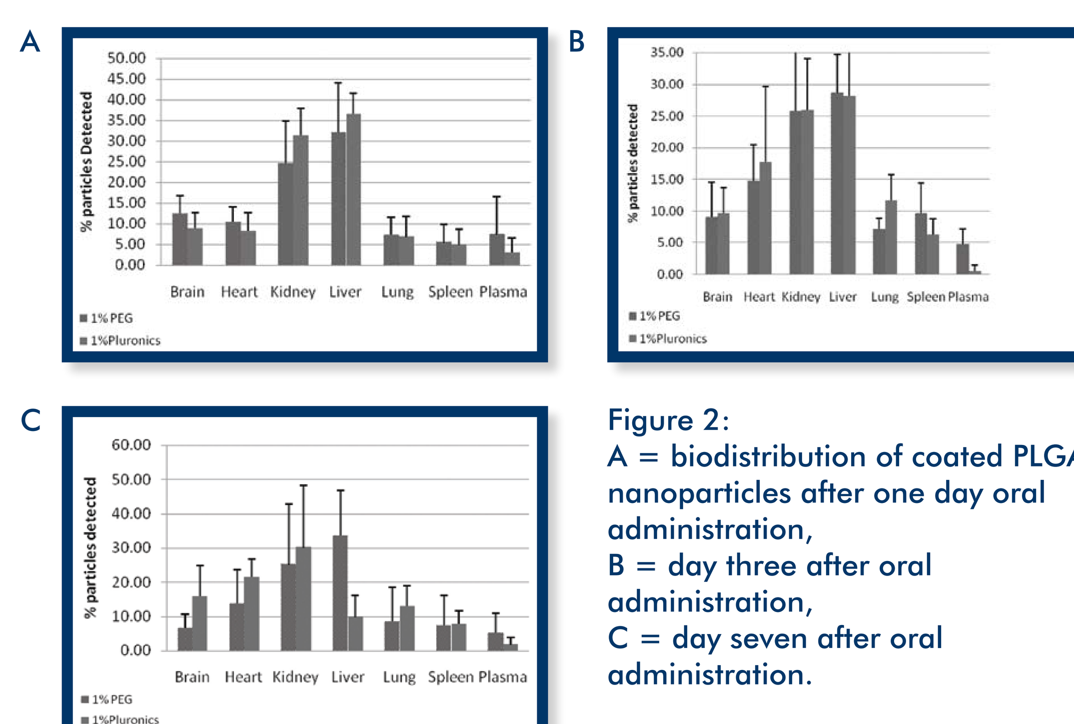
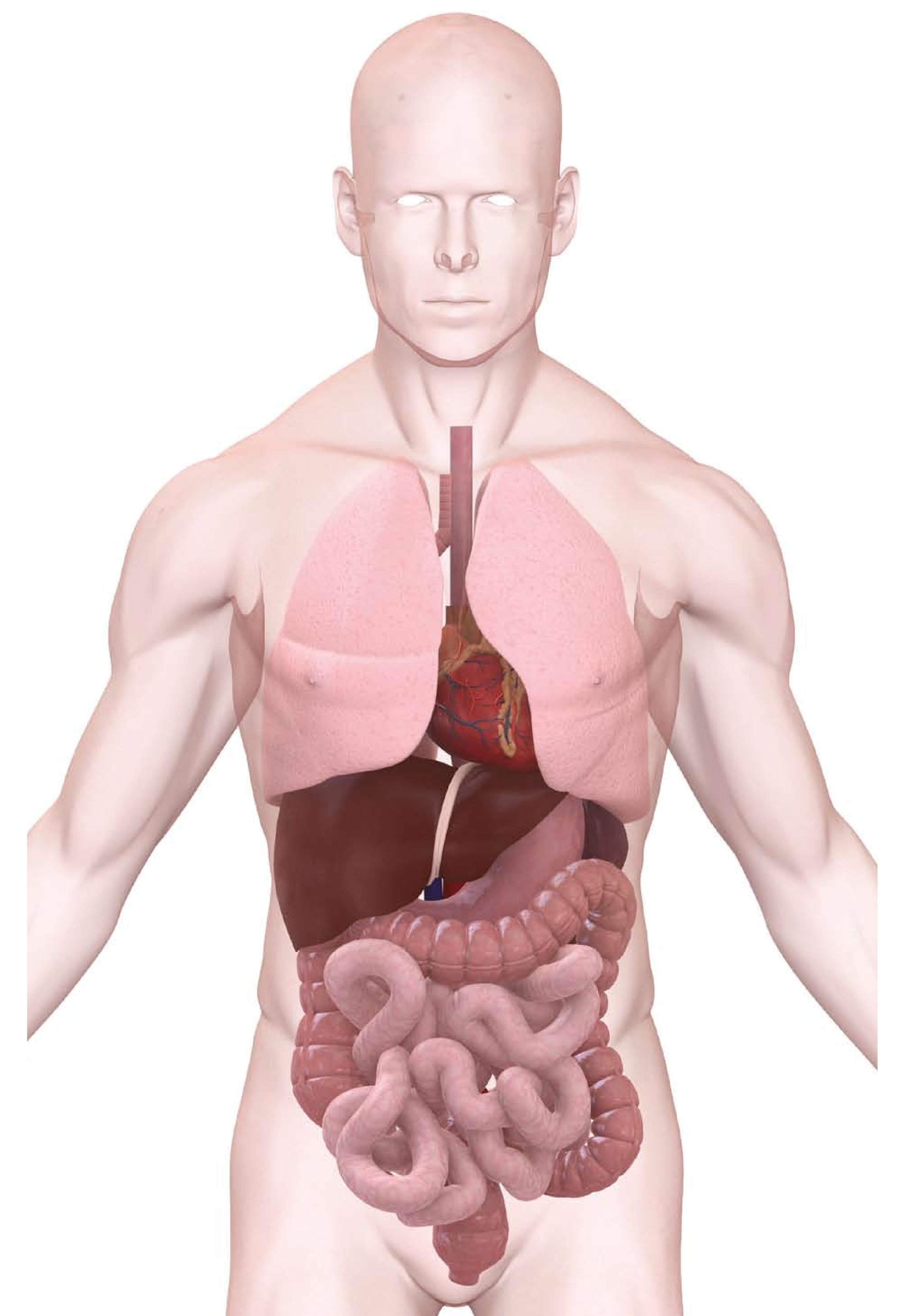


Figure 2:
A = biodistribution of coated PLGA nanoparticles after one day oral administration,
B = day three after oral administration,
C = day seven after oral administration.

Determining the effect of protein binding of nanoencapsulated drugs and its uptake in different organs.



Therefore, 1% PEGylated or pluronic F127 coated particles were orally administered and the biodistribution profile is indicated in Figure 2. The presence of PEG coated particles in the brain decreased over the seven days whereas the presence of particles in the heart, kidney and liver remained relatively constant. Increased accumulations in the lungs were observed. A slight accumulation of particles was detected in the spleen, indicating uptake by the M cells of the payers patches. However, Pluronic F127 coated particles resulted in an accumulation in the brain over the seven days. A similar profile to that of PEG coated particles was observed in the rest of the tissues. In both cases, plasma concentrations were significantly higher than those reported in Semete et al., 2010 for uncoated PLGA particles[6].

CONCLUSION

The decrease in protein binding of RIF observed in this study due to nanoencapsulation would result in higher drug concentrations being available to exert a therapeutic effect. Whether or not *in vivo* protein binding kinetics can be predicted using *in vitro* assays has been a subject of much debate. The *in vitro* data demonstrate decreased protein binding of polymeric nanoparticles coated with a poloxamer may facilitate minimum exposure to protein of highly protein bound drugs such as RIF as well as improve the biodistribution of nanoparticles. This study concludes that poloxamer coating of polymer nanoparticles presents a longer circulation time due to decreased protein binding with a subsequent increase of nanoparticles accumulation in tissues, primarily plasma and spleen.

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