Modification of the Copolymers Poloxamer 407 and Poloxamine 908 can Affect the Physical and Biological Properties of Surface Modified Nanospheres

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Purpose. To investigate the effects of the modification of the copolymers poloxamer 407 and poloxamine 908 on the physical and biological properties surface modified polystyrene nanospheres.

Methods. A method to modify poloxamer 407 and poloxamine 908, introducing a terminal amine group to each PEO chain has been developed. The aminated copolymers can be subsequently radiolabelled with Iodinated (I^{125}) Bolton-Hunter reagent. The aminated copolymers were used to surface modify polystyrene nanospheres. The physical and biological properties of the coated nanospheres were studied using particle size, zeta potential, *in vitro* non-parenchymal cell uptake and *in vivo* biodistribution experiments.

Results. The presence of protonated amine groups in the modified copolymers significantly affected the physical and biological properties of the resulting nanospheres, although the effects were copolymer specific. The protonated surface amine groups in both copolymers reduced the negative zeta potential of the nanospheres. Acetylation of the copolymer's free amine groups resulted in the production of nanospheres with comparable physical properties to control unmodified copolymer coated nanospheres. *In vivo*, the protonated amine groups in the copolymers increased the removal of the nanospheres by the liver and spleen, although these effects were more pronounced with the modified poloxamer 407 coated nanospheres. Acetylation of the amine groups improved the blood circulation time of the nanospheres providing modified poloxamine 908 coated nanospheres with comparable biological properties to control poloxamine 908 coated nanospheres. Similarly, modified poloxamer 407 coated nanospheres had only slightly reduced circulation times in comparison to control nanospheres.

Conclusions. The experiments have demonstrated the importance of copolymer structure on the biological properties of surface modified nanospheres. Modified copolymers, which possess comparable properties to their unmodified forms, could be used in nanosphere systems where antibody fragments can be attached to the copolymers, thereby producing nanospheres which target to specific body sites.

KEY WORDS: Poloxamer; poloxamine; nanospheres; drug targeting; copolymer modification.

INTRODUCTION

The surface modification of model polystyrene nanospheres, by the adsorption of poly(ethylene oxide) (PEO) containing block copolymers, has been demonstrated to reduce protein adsorption *in vitro* and extend the circulation time *in vivo* of the resulting nanospheres (1,2). Recently, we have shown that two such copolymers, poloxamer 407 (P-407) and poloxamine 908 (P-908) can also modify the surface of biodegradable PLGA nanospheres, producing corresponding biodegradable nanosphere systems which also displayed extended circulation *in vivo* (3). Such systems have potential for both targeted drug delivery and diagnostic imaging applications. However, the main limitation of surface modified nanospheres is the potential desorption of the adsorbed block copolymers from the nanosphere surface. Waltrous-Peltier *et al.* (4) suggested that desorption of Pluronic® F108 from polystyrene nanospheres can occur after 6 hours *in vivo* and Vandorpe *et al.* (5) also suggested from *in vivo* biodistribution studies that poloxamine 908 could be displaced from the surface of polyphosphazene nanospheres. To investigate this problem systematically, both *in vitro* and *in vivo*, first requires the production of radiolabelled block copolymers. In addition, it is essential that the radiolabelled copolymers retain the physical and biological properties of the original unmodified copolymers. We have recently reported the modification of poloxamer 407 and poloxamine 908 (6), introducing a terminal amino group into each PEO chain in the copolymers, to produce diamine poloxamer 407 (DA P-407) and tetramine poloxamine 908 (TA P-908). We then described the subsequent radiolabelling of the modified copolymers which allowed an investigation into the stability of adsorbed copolymer layers on the surface of polystyrene and PLGA nanospheres after incubation with serum *in vitro*.

The modified copolymers could have other applications apart from the ability to be radiolabelled and thereby allow an investigation of the stability of adsorbed copolymers. Recently, Zalipsky *et al.* (7) have developed liposomes containing amino-PEG-phosphatidylethanolamine which displayed comparably extended circulation times to the unmodified PEG-lipid containing liposomes. The amino group on the PEG-containing liposomes allowed the surface attachment of specific antibodies which could allow the liposomes to target specific body sites. The aminated forms of P-407 and P-908 could also be used to produce a comparable targeting nanosphere system, but only if the aminated copolymers retain the physical and biological properties of the unmodified copolymers.

In this paper, the effects of copolymer modification on the physical and biological properties of polystyrene nanospheres coated with the modified and radiolabelled copolymers was investigated. Firstly, the effect of the introduction of terminal amine groups into P-407 and P-908 was studied, where the amine groups were either uncapped (and hence protonated at physiological pH) or where the amino groups had been acetylated ('capped') to remove the positive charged groups in the copolymers. The particle size, zeta potential, *in vitro* non-parenchymal cell uptake and biodistribution of the nanospheres coated with the modified copolymers were studied and compared to nanospheres coated with unmodified forms of the copolymers. Secondly, the capped modified copolymers were used to investigate the possible desorption of block copolymers from nanospheres *in vivo*. The capped copolymers were used to coat nanospheres where either the copolymer or the nanosphere core was radiolabelled and from differences in the biodistribution of the two systems, desorption was assessed.

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METHODS

Copolymer Modification and Radiolabelling

Commercial grade P-407 and P-908 (gift from BASF, Parsippany, New Jersey, USA) were modified and radiolabelled as described in detail in Neal *et al.* (6) Briefly, the copolymers were aminated via a two stage reaction mechanism. A 20% w/v solution of copolymer in CH_2Cl_2 was reacted with a two fold molar excess of tosyl chloride and pyridine at room temperature for 24 hours. The tosyl ester product was obtained and subsequently reacted with 25% v/v NH_3 in H_2O for 6 hours at 120°C to produce the aminated copolymer (DA P-407 or TA P-908). The aminated copolymers (50 mg) were then radiolabelled with 6 MBq of I125 Bolton-Hunter reagent (185 MBq/ml) (Amersham International, Amersham, UK.) in 1 ml of tetrahydrofuran (THF) for 24 hours, with excess radiolabel removed by a combination of GPC and dialysis. The efficiency of the radiolabelling was approximately 20%. Unreacted amino groups were either left in the protonated form or acetylated ('capped') with acetic anhydride to remove the positive charge. A 5 molar excess of acetic anhydride was added to the reaction contents 24 hours after the addition of the I^{125} Bolton-Hunter reagent and the reaction was left to proceed for a further 24 hours before separation of the 'labelled capped' copolymers was carried out as before.

Nanosphere Coating

Polystyrene nanospheres (PS) (IDC, Washington, USA) 61 ± 9.1% nm in size were used for all the experiments and were coated with either modified or unmodified copolymers through a 12 hour incubation of equal volumes of a 1% w/v solution of copolymer with a 1% w/v suspension of nanospheres. Excess copolymer was removed from all systems by dialysis against double distilled water using Spectrapor CE (Spectrum Medical Industries, LA, USA) dialysis membrane (molecular weight cut off 100,000 Da). The nanosphere systems produced could be separated into two main groups:

Nanospheres Coated to Investigate the Effects of Copolymer Modification

The effect of copolymer modification, through the amination of P-407 and P-908, was investigated using three nanosphere systems. PS nanospheres were coated with either unmodified copolymers (P-407, P-908), uncapped modified copolymers (DA P-407, TA P-908) or capped modified copolymers (capped DA P-407, capped TA P-908). The unmodified copolymer coated nanospheres were prepared as the control systems, with two modified versions of the copolymers used to produce two other sets of coated nanospheres. The three sets of nanospheres were then used to investigate the effects of copolymer modification. For such experiments, the physical and biological properties of the nanospheres were studied.

Nanospheres Coated to Assess Copolymer Desorption In Vivo

As described above, the capped modified copolymers were also used to investigate the desorption of block copolymers from PS nanospheres *in vivo*. Two nanosphere systems were produced; where either the radiolabel was attached to the copolymer (unlabelled PS nanospheres coated with I^{125} labelled, capped copolymers) or the radiolabel was attached to the nanosphere (I^{125} labelled PS nanospheres coated with unlabelled capped copolymers). Differences in the biodistribution of the nanosphere systems would imply that some degree of copolymer desorption had occurred. Thus, the *in vivo* biodistribution of the nanospheres was followed.

Physical Characterisation of Nanospheres

The effect of copolymer modification on the physical properties of nanospheres, surface modified with P-407 and P-908 was assessed through a comparison of the particle size and zeta potential of the nanospheres. The particle size of the nanospheres was determined using a Malvern M4700 series PCS (Malvern Inst., UK) with the Z average and polydispersity determined from at least 3×6 measurements for each sample and using Contin analysis mode. The adsorbed copolymer layer thickness was calculated from the difference in particle size between the coated systems and naked PS nanospheres. The zeta potential of the nanospheres was determined in 1 mM HEPES buffer (Sigma, UK) pH 7.4 using a Malvern Zetasizer IV (Malvern Inst., UK). Each sample was measured three times with four measurements per run. The zeta potential is expressed as the mean ± s.d.

Biological Characterisation of Nanospheres

The biological properties of the modified copolymers were investigated *in vitro* and *in vivo*.

In Vitro *Uptake of Nanospheres by Non-parenchymal Liver Cells*

The uptake of nanospheres by non-parenchymal liver cells *in vitro* was determined for coated polystyrene nanospheres, which had been radiolabelled with $Iodine^{125}$ using the method of Huh *et al.* (8). Labelled nanospheres were coated with the modified or unmodified forms of P-407 and P-908 as described. The non-parenchymal cell uptake, both in the presence and absence of fresh rat serum was determined as described by Dunn *et al.* (9). Briefly, non-parenchymal cells were isolated from 200 ± 10 g female wistar rats using a collagenase liver perfusion method followed by low and high speed centrifugation before the use of a Nycodenz density gradient to separate the Kupffer and endothelial cells from cellular debris. The cells were then diluted to a concentration of approximately 2×10^6 cells/ml. 200 µg of each nanosphere system was then mixed, in triplicate, with 1 ml of cell suspension and 1 ml of HBSS buffer, gassed with 95% O_2: 5% CO_2 and then incubated for 1 hour at 37°C. For studies on uptake in the presence of serum, freshly prepared rat serum was added at a concentration of 5% v/v. After incubation, excess particles were removed from the cells by repeated washing, and the activity associated with the non-parenchymal cells was determined using a Gamma counter (LKB 182 Compugamma, LKB Wallac, Finland) and expressed as a percentage ± s.d. of the added nanosphere activity. Statistical differences between the non-parenchymal cell uptake of uncoated and coated nanospheres, and between the coated systems were evaluated using the student-t test ($P > 0.05$).

In Vivo *Biodistribution of Nanospheres*

The biodistribution of the nanosphere systems was determined in the rat model. The experiments were performed for three main reasons. Firstly, the effect of copolymer modification *in vivo* was investigated using the same systems used in the *in vitro* physical and cell uptake experiments. Secondly, as described, the production of the radiolabelled copolymers allowed experiments to be performed that would assess the extent of any desorption of I^{125} labelled P-407 or P-908 from PS nanospheres *in vivo*. Finally, the biodistribution of the free radiolabelled copolymers were determined.

The biodistribution experiments were performed using groups of three 150 ± 10 g female Wistar rats. Each group received an intravenous injection via the lateral tail vein of 1 mg of nanosphere system. 20 µl blood samples were taken from the contralateral tail vein after 5, 15, 30, 60, 120 and 180 minutes, at which point the animals were killed using intravenous injections of 0.4 ml pentobarbitone 60 mg/ml (Sagatal®). The liver, spleen, lungs, kidney and thyroid were then removed and along with blood samples, counted for radioactivity using a gamma counter. Carcass associated activity was determined using a well counter (Model SD1, Oakfield Instruments Ltd., Eynsham, U.K.). A blood volume of 7.5% body weight was assumed (10). The results were calculated as a percentage of the dose injected and are expressed as a mean ± s.d. Statistical differences between the nanosphere systems were evaluated using the student-t test.

RESULTS

Physical Characterisation of Nanospheres

The particle size, adsorbed layer thickness and zeta potential of PS nanospheres coated with the modified or unmodified forms of P-407 or P-908 are presented in Table I. The main effect of copolymer modification was to cause changes in the zeta potential of the coated nanospheres. Nanospheres coated with uncapped modified copolymers displayed a much less negative zeta potential than the unmodified systems; with the TA P-908 coated PS system even displaying a positive zeta potential. However, when the nanospheres were coated with the capped copolymers, the zeta potentials of the nanospheres was comparable to the nanospheres coated with the unmodified copolymers. The effect of copolymer modification on the adsorbed layer thicknesses produced by the copolymers was less clear with only small differences seen between the nanospheres. Thus, it would appear that amine substitution of the copolymers affected the zeta potential and to a small degree the adsorbed layer thickness of the resulting nanospheres. When the free amine groups were subsequently capped, the nanospheres displayed similar physical properties to the unmodified copolymer coated nanospheres.

Biological Characterisation of Nanospheres

In Vitro *Uptake of Nanospheres by Non-parenchymal Liver Cells*

The uptake of the nanospheres, coated with the modified and unmodified forms of P-407 and P-908, by the non-parenchymal liver cells, in the presence and absence of serum are shown in Table II. In the absence of serum, all the copolymer coated nanospheres showed a significantly reduced cell uptake. This is in agreement with previous studies (11). In the presence of serum, the uptake of all the nanospheres by the non-parenchymal cells were reduced, as seen previously by Muir *et al.* (12). The results obtained in the presence of serum also illustrate the effect of copolymer modification on particle uptake by non-parenchymal cells. For both the P-407 and P-908 systems, the highest cell uptake is seen for the nanospheres coated with the uncapped modified copolymers, suggesting that the free amine groups caused an increase in the phagocytosis of the nanospheres. Capping of the amine groups reduced the uptake of the nanospheres, however, the uptake of the capped modified systems was still significantly higher than for the control unmodified nanospheres.

In Vivo *Biodistribution Studies*

The *in vivo* biodistribution experiments were carried out to evaluate the effect of copolymer modification, to determine if any significant loss or desorption of radiolabelled copolymers from PS nanospheres occurred *in vivo* and to follow the biodistribution of the free radiolabelled copolymers.

Figures 1 and 2 and Table III show the circulation profiles and organ distributions three hours post dose of the nanospheres prepared to investigate the effects of copolymer modification. The data highlight that copolymer modification affected the biodistribution of coated nanospheres, but the effects were

Table I. Particle Size and Zeta Potential of PS Nanospheres Coated with Modified or Unmodified P-407 and P-908

Nanosphere system	Particle size z average (nm) mean ± SD	Adsorbed layer thickness (nm)	Zeta potential (mV) in HEPES 1 mM pH 7.4 mean ± SD
Uncoated PS 60 nm	73.8 ± 0.5	———	−55.8 ± 8.5
P-407 coated PS	89.0 ± 0.7	7.6	−18.3 ± 2.5
DA P-407 coated PS	91.0 ± 1.4	8.6	−4.7 ± 2.3
Capped DA P-407 coated PS	90.5 ± 0.6	8.4	−14.5 ± 1.2
P-908 coated PS	91.5 ± 0.9	8.9	−12.5 ± 2.1
TA P-908 coated PS	93.8 ± 0.8	10.0	+9.6 ± 0.9
Capped TA P-908 coated PS	94.1 ± 0.7	10.2	−10.6 ± 0.9

Table II. *In Vitro* Non-Parenchymal Cell Uptake of Modified and Unmodified P-407 and P-908 Coated PS Nanospheres

	% of Initial nanosphere activity associated with non-parenchymal cells, Mean ± SD	
Nanosphere system	Incubation without serum	Incubation with serum
Uncoated PS 60 nm	26.2 ± 1.2	0.7 ± 0.1
P-407 coated PS	4.8 ± 0.2*	0.5 ± 0.1*
DA P-407 coated PS	4.8 ± 0.3*	1.7 ± 0.1†
Capped DA P-407 coated PS	4.3 ± 0.2*	1.3 ± 0.1†
P-908 coated PS	3.5 ± 0.1*	0.4 ± 0.0*
TA P-908 coated PS	5.1 ± 0.2*†	1.3 ± 0.1†
Capped TA P-908 coated PS	3.6 ± 1.0*	1.0 ± 0.2†

* Significant reduction ($P > 0.05$) in cell uptake in comparison to uncoated nanospheres.
† Significant difference ($P > 0.05$) in cell uptake in comparison to unmodified copolymer coated nanospheres.

copolymer specific. With the P-407 coated nanospheres, copolymer modification had a dramatic effect on the biodistribution of the nanospheres, especially when the amine groups were uncapped and hence protonated *in vivo*. The uncapped DA-P-407 coated PS nanospheres showed a significantly faster clearance from the circulation than the control nanospheres which were in the form of nanospheres coated with unmodified P-407. This was reflected in the significantly higher liver and spleen uptake seen with the uncapped DA P-407 coated nanospheres. Acetylation of the charged amino groups increased the circulation times and reduced the liver uptake of the capped DA P-407 coated nanospheres in relation to nanospheres coated with uncapped DA P-407. However, the liver uptake and blood circulation levels three hours post dose were still significantly lower for the capped DA P-407 coated nanospheres as compared to the control (unmodified P-407 coated nanospheres), suggesting that the copolymer modification had altered the biological properties of the copolymers.

With the P-908 systems, the differences between the nanosphere systems were much smaller, implying that the copolymer modification of P-908 had a lower impact on the biological properties of the coated nanospheres when compared to the equivalent P-407 systems. The circulation profiles and liver uptake of the nanospheres were comparable. The only significant difference in the biodistribution of the nanospheres was in the spleen uptake values. The splenic uptake of uncapped TA P-908 coated nanospheres was twice that of unmodified P-908 coated nanospheres. Acetylation of the amine groups in the copolymer again reduced this uptake, although the splenic uptake of capped TA P-908 coated nanospheres was still significantly above that for P-908 coated nanospheres. The lack of any major differences in the biodistribution of the capped TA P-908 coated nanospheres and the control P-908 coated nanospheres suggests that copolymer modification, if charged groups are not present, does not generally affect the biological properties of P-908.

The capped copolymers, which have comparable (P-908) or only slightly changed (P-407) biological properties, with respect to the unmodified copolymers, were then used to investigate the possible desorption of copolymers from the surface of PS nanospheres *in vivo*. Table IV shows the organ distribution of the different nanosphere systems. The nanospheres were prepared where the radiolabel, used to trace the particles, was either associated with the nanosphere core or attached to the adsorbed copolymer. Therefore, differences in the *in vivo* behaviour of the two nanosphere systems would imply some

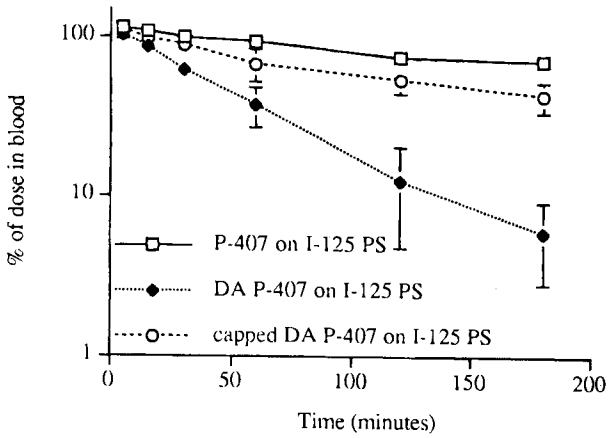

Fig. 1. Blood circulation profiles for modified and unmodified P-407 coated nanospheres.

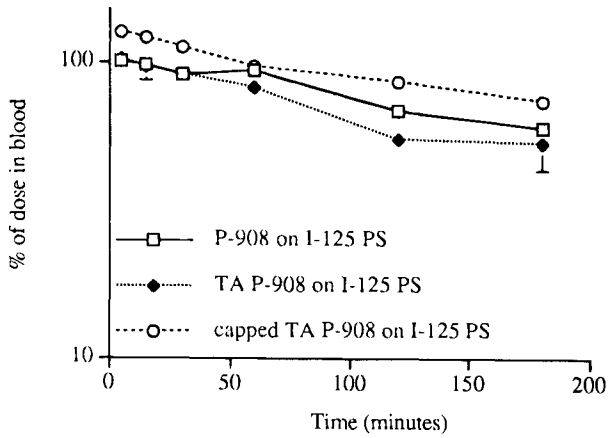

Fig. 2. Blood circulation profiles for modified and unmodified P-908 coated nanospheres.

Table III. Organ Distributions of PS Nanospheres Coated with Modified and Unmodified P-407 or P-908 3 Hours After Injection

	% of Dose injected, (mean ± S.D.)		
Organ	P-407 on PS	DA P-407 on PS	Capped DA P-407 on PS
Liver	17.8 ± 0.2	49.4 ± 5.8*	34.6 ± 5.9*
Spleen	4.4 ± 0.3	15.7 ± 1.5*	5.4 ± 1.1
Thyroid	0.8 ± 0.1	1.2 ± 0.1*	0.6 ± 0.2
Blood	70.1 ± 6.5	6.0 ± 3.2*	43.2 ± 9.1*
	P-908 on PS	TA P-908 on PS	Capped TA P-908 on PS
Liver	14.6 ± 2.4	15.0 ± 0.7	17.0 ± 0.3*
Spleen	6.4 ± 0.2	13.3 ± 1.2*	9.2 ± 0.3*
Thyroid	0.6 ± 0.2	1.9 ± 0.9	0.6 ± 0.2
Blood	60.2 ± 2.8	53.2 ± 9.6	73.6 ± 4.0*

*Significant difference ($P > 0.05$) in comparison to unmodified copolymer coated nanospheres.

degree of copolymer desorption. As before, the results were copolymer specific, although the blood circulation profiles for both sets of the copolymer coated nanospheres (data not shown) were very similar with only small, non-significant, differences identified. With the capped DA P-407 systems, differences were seen in the liver, spleen and blood levels between the I^{125} capped DA P-407 coated PS and the capped DA P-407 coated I^{125} PS, although only the difference in the liver uptake of the nanospheres was statistically significant. These results suggest that, either the presence of the radiolabel at the end of the copolymer molecules had affected the interaction of the coated nanospheres with the biological environment *in vivo*, or that some degree of desorption of the labelled DA P-407 had occurred. With the capped TA P-908 systems, there were no differences in the biodistribution of the two nanosphere systems, implying no desorption of capped TA P-908 within the limitations of the experiment.

A final set of biodistribution experiments was conducted to follow the biodistribution of the free radiolabelled copolymers. Figure 3 shows the organ distributions of both the uncapped and capped forms of radiolabelled DA P-407 and TA P-908. The circulation profiles of the copolymers (data not shown) revealed that all forms of the copolymers circulated for extended periods, with the liver being the only major organ showing uptake. However, the majority of the dose within the liver can be accounted for as "blood pool" (usually 25% of blood in the animal will be in the liver). Furthermore, the low levels of activity associated with the thyroid confirmed that the radiolabel on the copolymer was stable *in vivo*. These data, together with the *in vitro* stability data presented by Neal *et al.* (6), verify that the modified copolymers have been covalently labelled with I^{125} Bolton-Hunter reagent and the radiolabelled copolymers were stable both *in vitro* and *in vivo*.

DISCUSSION

The purpose of the present studies were twofold; firstly, to investigate the effect of copolymer modification and radiolabelling on the properties of polystyrene nanospheres that have been coated with the modified copolymers and, secondly, to investigate the stability of the adsorbed copolymer coating layers on polystyrene nanospheres *in vivo*. The main reason for using polystyrene nanospheres throughout these studies was the large body of evidence that is available on the physical and biological properties of polystyrene nanospheres surface modified with poloxamer and poloxamine copolymers. As the experiments described were planned to investigate the specific effects of copolymer modification on the properties of coated nanospheres, it was logical to use a highly studied model system as the reference point. In addition, polystyrene nanospheres

Table IV. Organ Distributions for the DA P-407 and TA P-908 *In Vivo* Desorption Experiments 3 Hours After Injection

	% of Dose injected, (mean ± S.D.)			
Organ	I^{125} capped DA P-407 on PS	Capped DA P-407 on I^{125}PS	I^{125}capped TA P-908 on PS	Capped TA P-908 on I^{125} PS
Liver	13.2 ± 6.5*	34.6 ± 5.9	18.0 ± 3.1	17.0 ± 0.3
Spleen	7.2 ± 2.3	5.4 ± 1.1	8.1 ± 1.8	9.2 ± 0.3
Thyroid	0.2 ± 0.0*	0.6 ± 0.2	0.2 ± 0.1*	0.6 ± 0.2
Blood	59.9 ± 16.7	43.2 ± 9.1	70.8 ± 12.3	73.6 ± 4.0

*Significant difference ($P > 0.05$) between nanosphere systems.

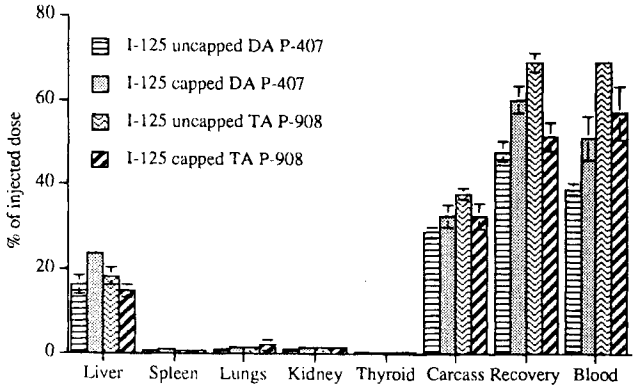

Fig. 3. Organ distributions for free radiolabelled copolymers three hours after injection.

are readily radiolabelled with I^{125} and the stability of the radiolabelling is good (13). This was important for the desorption experiments where instability in radiolabelling could have led to inaccurate conclusions.

The amination of the P-407 and P-908, not only allows their subsequent radiolabelling with I^{125} Bolton Hunter reagent, but also makes it possible to attach antibody fragments or specific proteins to the copolymers. Such polymers could be then used to produce nanospheres which target to specific areas of the body, as has been demonstrated in recent studies with liposomes (14). Reports into the modification of PEG containing block copolymers and how such modification can effect the biological properties of nanospheres surface modified by the copolymers are very limited. Tan *et al.* (15) described a method to radiolabel Pluronic® 108 in order to investigate the adsorption of the copolymer to polystyrene nanospheres. No account was made for possible changes in properties of the modified copolymers and the radiolabelled copolymer was not studied *in vivo*. Investigations have been made into the effect of the modification of PEG-lipid copolymers used in the production of sterically stabilised liposomes, although, all of the liposome studies have only evaluated the biological effects of copolymer modification. Zalipsky *et al.* (7) produced an amino functionalised PEG2000 conjugate of phosphatidylethanolamine (amino-PEG-DSPE), which was used to produce liposomes whose biodistribution was followed in comparison to liposomes produced with methoxy-PEG-DSPE. They found the two systems gave comparable results *in vivo*, with both systems displaying extended circulation. Blume *et al.* (16) produced liposomes with COOH-PEG_{3500}-DSPE which allowed the attachment of Glu-plasminogen to the terminal carboxyl groups. The carboxyl PEG containing liposomes displayed a slightly reduced circulation time in relation to the unmodified PEG-DSPE containing liposomes. The negatively charged carboxyl end group on the PEG was suggested as a possible explanation of the decreased circulation times. Allen *et al.* (17) used hydrazide modified PEG-DSPE containing liposomes to permit the similar attachment of antibodies to the liposomes. They found the PEG modification did not affect the *in vivo* distribution of the liposomes, which they stated was due to the fact that at physiological pH the hydrazide moiety was uncharged.

In the present work, we have not only investigated the effects of copolymer modification on the biological properties of the copolymer coated nanospheres, but we have also evaluated the changes in the nanosphere's physical properties. As with the liposome studies, we have also found that the presence of charged end groups was important to both the physical and the biological properties of the nanospheres coated with the modified copolymers. The observed changes in the zeta potential of the nanospheres coated with the uncapped modified copolymers are considered to be due to the free amine groups in the copolymers being protonated at pH 7.4. The presence of the protonated amine groups at the ends of the PEO chains would be expected to further reduce the negative zeta potential of the nanospheres. In addition, as TA P-908 contained 4 amine groups per molecule, in contrast to DA P-407, which had two amine groups per molecule, it was anticipated that the TA P-908 coated system would show the most reduced zeta potential. However, it is not just the amount of charge per molecule that is important; the number of copolymer molecules adsorbed per unit surface area is also an important factor. When the nanospheres were coated with the capped modified copolymers, the amine groups have been acetylated and hence the charge is removed. Therefore, the comparable physical properties of these nanospheres, in terms of zeta potential and particle size, in relation to the unmodified copolymer coated nanospheres, would have been predicted.

From the physical properties of the coated nanospheres, it was predicted that the nanospheres coated with the uncapped modified copolymers would show the most altered biological properties in relation to the unmodified copolymer coated nanospheres. This is because although no direct relationships between zeta potential and the *in vitro* or *in vivo* phagocytosis of nanospheres have been established, many research groups have shown the importance of surface charge on the *in vivo* handling of colloidal carriers (18–22). However, the magnitude of the changes in the biological properties of the nanospheres coated with the modified copolymers, especially with the P-407 coated nanospheres, was not totally expected. The uncapped DA P-407 coated nanospheres displayed a significantly reduced circulation in comparison to the unmodified nanospheres. When the amine groups were acetylated to leave uncharged end groups on the copolymers, the circulation of the nanospheres was improved, although the modification of P-407 resulted in a small alteration in the biological properties of the nanospheres. These observations are in contrast to Zalipsky *et al.* (7) who, as described, used protonated amino-PEG in liposomes which displayed unchanged extended circulation. Such differences may be due to the concentration of surface amine groups in the different systems. In addition, the effects of copolymer modification appear to be copolymer specific, since the effects of copolymer modification on the two related polymers used in this study were significantly different. As stated, the presence of protonated amine end groups in DA P-407 significantly reduced the circulation of the nanospheres. In contrast, protonated TA P-908 coated PS nanospheres, although displaying similar physico-chemical properties to the DA P-407 coated PS nanospheres, displayed comparable circulation times to the control nanospheres which were coated with the unmodified copolymers. The increased hydrophilicity of P-908 in relation to P-407 may explain why P-908 is able to form an adsorbed surface layer which is more able to compensate for any increased interactions with serum components caused

by the protonated amine groups. Conversely, the relatively less hydrophilic P-407 copolymer may produce an adsorbed layer which is only able to prevent opsonisation when the end groups are totally inert.

These experiments suggest that the theory of steric stabilisation can not totally explain the extended circulation of surface modified nanospheres, since small changes in the chemical structure of sterically stabilising copolymers can significantly alter the biological properties of the nanospheres. Through the removal of the protonated end groups in the copolymers we have produced a modified form of P-908 which had comparable physical and biological properties to the unmodified copolymer, in terms of the surface modification of polystyrene nanospheres, as well as a modified form of P-407, which can surface modify PS nanospheres such that the nanospheres displayed only slightly reduced circulation times *in vivo*.

The production of these modified radiolabelled copolymers has allowed the investigation of the possible desorption of the copolymers from the surface of the nanospheres *in vivo*. This desorption of copolymers from the surface of nanospheres could be a possible limitation of their extended use. Our studies have focussed on examining the differences in the biodistribution of nanospheres, where a radiolabel was either incorporated in the nanosphere core or on the surface of the adsorbed copolymer. Desorption, would then be identified by differences in the biodistribution of the two systems. However, it was not possible to identify any significant differences in the biodistributions of the nanospheres. Thus, it is difficult to conclude from these two sets of experiments whether any significant desorption of the radiolabelled forms of P-407 and P-908 actually occurred. However, *in vitro* experiments have been performed, investigating the stability of the adsorbed layers produced by the radiolabelled capped forms of P-407 and P-908 (both on polystyrene and PLGA nanospheres), after incubation of the nanospheres with serum. These experiments, described by Neal *et al.* (6), have clearly identified a serum linked desorption of both copolymers from polystyrene and PLGA nanospheres and hence this publication also contains a complete discussion of copolymer desorption. Briefly, the results indicated that the extent of the desorption was comparable for both of the copolymers. However, the desorption was found to be nanosphere dependent with the polystyrene nanospheres showing an approximate 20% loss of both I^{125} capped DA P-407 and I^{125} capped TA P-908 and the PLGA nanospheres showing a 71% loss of I^{125} capped DA P-407 and 78% loss of I^{125} capped TA P-908 after 24 hours incubation in serum. After 3 hours incubation, the time course used in the *in vivo* experiments presented in this paper, the *in vitro* desorption of the radiolabelled copolymers from polystyrene nanospheres was less than 10%. This relatively low level of desorption would be very difficult to identify *in vivo*, due to experimental error, and thus explains the uncertainty of the *in vivo* desorption experiments described above. To obtain conclusive evidence for the *in vivo* desorption of the radiolabelled copolymers, either an extended time course would be required or experiments could be conducted using dual labelled nanospheres, where both the copolymer and the nanosphere core would be radiolabelled with different radiolabels, which could then be followed directly for evidence of desorption.

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