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Research Paper

# Investigation of the interaction of biodegradable micro- and nanoparticulate drug delivery systems with platelets

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## Abstract

**Objectives** Biodegradable micro- and nanoparticles are being increasingly investigated for drug delivery and targeting of therapeutics. The size and surface properties of these particles are important factors influencing their interaction and uptake by various cells, tissues and organs. Optimising these properties, to enhance cellular uptake, may increase their potential for interaction with other physiological components such as platelets resulting in platelet activation and inappropriate thrombus formation. The aim of this study was to investigate the potential interaction of particulates with platelets.

**Methods** Biodegradable micro- and nanoparticles based on poly-lactide-co-glycolide (PLGA), poly-lactide-co-glycolide–macrogol (PLGA-macrogol) and chitosan were prepared using solvent evaporation, spray drying or solvent dispersion techniques.

**Key findings** Microparticles formulated had a median diameter (D50%) of 2–9 $\mu$m, while nanoparticles had an average diameter of 100–500 nm. The surface morphology ranged from smooth and spherical to irregular depending on polymer and preparation method used. Particles, reconstituted in the concentration range of 0.1–500 $\mu$g/ml, were tested for their ability to induce or inhibit platelet aggregation. No effects on either induction of platelet activity or inhibition of aggregation were detected.

**Conclusions** None of the particles examined were found to alter platelet activity. These results suggested that the biodegradable micro- and nanoparticles tested were safe for use as potential drug carriers of therapeutic agents.

**Keywords** aggregation; biodegradable; microparticles; nanoparticles; platelets

## Introduction

In recent years biodegradable polymeric micro- and nanoparticles have attracted considerable attention as drug delivery carrier systems for the controlled and targeted release of drugs, as carriers of DNA in gene therapy and in their ability to deliver proteins, peptides and genes through various routes of administration.[1,2] More recently, poly-lactide-co-glycolide (PLGA) microbubbles in the size range of 2.3 $\mu$m have been formulated for use as ultrasound contrast agents for myocardial perfusion echocardiography and are administered intravenously.[3] Biodegradable micro- and nanoparticles are also being studied for the delivery of cells and proteins in the treatment of defective tissues and for bone regeneration.[4] While currently there are only a small number of commercially available products that utilise this technology, with the recent advances in micro- and nanoparticle technology, their potential for application in medicine has increased and this confers enormous potential for human exposure and also for environmental release.[5] Due to their small sizes, these particles can enter the body by inhalation, and through the skin and intestinal tract.[6,7] Already, some evidence has been amassed to show the adverse effects of nanoparticles in the environment. Epidemiological studies have shown that urban pollution with airborne particulate matter, including nanoparticles, derived from combustion sources such as motor vehicle and industrial emissions, contributes to respiratory and cardiovascular morbidity and mortality.[8]

Micro- and nanoparticles investigated for drug delivery and targeting differ from other types of particulate matter in their size, chemical composition, structure, surface area and shape. These characteristics which make them useful in medicine may also contribute to the toxicity of these particles. Smaller particles and/or a non-smooth particle surface result in a

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larger surface area for the particles to interact with the cellular environment and may result in the enhancement of any intrinsic toxicity of these particles.[9] The biodegradable polymers, including those of PLGA, PLGA-macrogol (polyethylene glycol) and chitosan, are among the most frequently utilised polymers for the formulation of particulate drug delivery systems with application to both controlled release and targeted delivery and for administration by oral, pulmonary, nasal and parenteral routes.[2,10,11] PLGA is a polyester composed of one or more of three different hydroxy acid monomers, D-lactic, L-lactic and/or glycolic acids. The polymer can be processed into almost any shape and size (from microns to <200 nm) and has been used to encapsulate molecules of varying physicochemical properties and molecular weight. PLGA microspheres have a long safety record and are used in several different marketed products for the controlled release of peptides and proteins over periods of 1–12 months.[12,13] Such products are intended for administration by the subcutaneous route or by implantation at the target therapeutic organs and tissues. Recently, derivatised PLGA polymers such as polyethylene glycol derivatives (PLGA-macrogol polymers) have been investigated to reduce clearance of these micro- and nanoparticles by the reticuloendothelial cells, hence increasing their circulating half-life and enhancing the delivery of their therapeutic load. While the biocompatibility of PLGA is well known, there is a lack of information regarding the behaviour of the derivatised polymers and particulates in the systemic circulation.

Chitosan is a hydrophilic, biocompatible, and biodegradable polysaccharide consisting of glucosamine and *N*-acetylglucosamine derived by deacetylation of chitin. Chitosan has mucoadhesive properties as well as permeation enhancing properties and has been utilised as a pharmaceutical polymer for controlled and enhanced delivery of biological therapeutics and vaccines across the nasal and pulmonary mucosa.[14,15] Chitosan has haemostatic activity[16] and it is possible therefore that chitosan micro- and nanoparticles may have a pro-aggregatory activity on human platelets, limiting its use as a drug delivery carrier.

Mammalian platelets are small (2–4 $\mu$m), discoid, short lived fragments derived from megakaryocyte precursors.[17] Platelets play a crucial role not only in the formation of a normal haemostatic plug but also in the formation of a pathologic thrombus.[18] Platelets circulate in a resting state through blood vessels lined by endothelial cells. Following an insult to the endothelial cells platelets undergo a series of changes. Upon adhesion, platelets become activated, change shape, secrete granule contents and aggregate to prevent blood loss.[19] Micro- and nanoparticles entering the systemic circulation either from the environment or by administration for diagnosis or treatment may come into contact with platelets in circulation. It is possible that such contact or interaction may result in activation of the platelets leading to possible platelet aggregation and thrombus formation. Li *et al*.[20] reported that PLGA and chitosan nanoparticles did not modify platelet aggregation at concentrations below 10 $\mu$g/ml, irrespective of the surface charge of the nanoparticles. Other particle characteristics such as composition, surface morphology, size and surface area are factors contributing to cellular interaction and uptake and may also contribute to platelet interaction.

The objective of this study was to examine the potential of biodegradable micro- and nanoparticles of PLGA, PLGA-macrogol and chitosan, in the size range of <10 $\mu$m and <500 nm, to cause aggregation or inhibition of human platelets. The size ranges selected are commonly utilised for delivery and targeting of therapeutic molecules and vaccines by oral, nasal, pulmonary and parenteral routes or for diagnostic applications. These size ranges have been recognised as the size ranges which have the potential for cellular uptake and therefore may pose the most immediate safety concern.

## Materials and Methods

### Materials

PLGA (Resomer RG 504 H, molecular weight ~40–50 kDa) and PLGA-macrogol 5% (Resomer PEG RGP5055), of inherent viscosity 0.093 dl/g, were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Chitosan of medium molecular weight (240 kDa), polysorbate 80 (Tween 80), polyvinyl alcohol (PVA) of molecular weight ~30–70 kDa, and sodium citrate were obtained from Sigma-Aldrich (Ireland). Poloxamer 188 (Lutrol F68), was a gift from BASF (Burgburnheim, Germany). All other solvents and reagents used were purchased from Sigma-Aldrich.

### Preparation of micro- and nanoparticles

PLGA and PLGA-macrogol microspheres were prepared using a modification of the oil-in-water (o/w) microencapsulation method described previously.[21,22] The polymer was dissolved in ethylacetate and the solution was homogenised in an aqueous external phase containing 0.5% w/v Tween 80 using an Ultra Turrax high speed homogeniser (Janke-Kunkel, Staufen, Germany, type TP 25) operating at a speed of 17 500 rev/min. The emulsion was then stirred for 6–8 h to allow solvent evaporation and the microparticles were recovered by centrifugation (Hettich Zentrifugen, Tuttilingen, Germany; type Rotina 35 R) followed by lyophilisation using a Labconco FreeZone, model no. 7752030, freeze-drying apparatus. A batch of PLGA microparticles was spray dried after solvent evaporation to recover the microparticles formed. Chitosan microparticles with and without addition of a surfactant were prepared by spray drying a solution of chitosan in 1% v/v acetic acid using a Buchi-B290 mini spray drier at an aspirator setting of 80%, a feed flow rate of 3 ml/min and an inlet temperature of 140°C. PLGA microparticles were prepared by spray drying a solution of the polymer in ethylacetate using the method described in Clarke *et al*.[23]

PLGA, PLGA-macrogol and chitosan-coated PLGA nanoparticles were prepared using a modified solvent dispersion method.[24] Briefly, a solution of the PLGA or PLGA-macrogol in acetone was added dropwise to an aqueous solution containing 0.5% w/v Tween 80 with or without chitosan, under constant stirring. The resulting suspension was stirred for 6–8 h to allow solvent evaporation and the nanoparticles were recovered by centrifugation followed by lyophilisation.

### Characterisation of micro- and nanoparticles

The median diameter (D50%) of an aqueous suspension of the prepared microparticles was measured in triplicate using the

**Table 1** Characteristics of microparticles prepared by spray drying and solvent evaporation from emulsion

| Polymer used | Processing method | Surfactant | Median particle size D(50%) $\mu$m ± standard deviation |
|---|---|---|---|
| Chitosan | Spray drying | None | 2.74 ± 0.03 |
| Chitosan | Spray drying | Poloxamer (Lutrol F68) | 2.81 ± 0.04 |
| Chitosan | Spray drying | PVA | 2.71 ± 0.01 |
| Chitosan | Spray drying | Tween 80 | 4.24 ± 0.49 |
| PLGA | Spray drying | None | 8.98 ± 0.29 |
| PLGA (solvent evaporation-spray drying) | Solvent evaporation followed by spray drying | Tween 80 | 2.44 ± 0.08 |
| PLGA (solvent evaporation) | Solvent evaporation | Tween 80 | 6.21 ± 0.26 |
| PLGA-macrogol 10% (solvent evaporation) | Solvent evaporation | Tween 80 | 9.01 ± 0.26 |

PLGA, poly-lactide-co-glycolide; PVA, polyvinyl alcohol.

wet dispersion cell of the Mastersizer 2000 (Hydro 2000SM; Malvern Instruments, Malvern, Worcestershire, UK). The average size of an aqueous dispersion of the nanoparticles was measured using a high performance particle sizer (Malvern HPPS, Model HPP5001; Malvern Instruments). Measurement was carried out six times and the average value reported. The surface morphology of the micro- and nanoparticles was examined using a scanning electron microscopy (SEM) instrument (Hitachi Scanning Electron Microscope (model S 3500N), Tokyo, Japan). Samples were mounted on double-sided adhesive tape attached to an aluminium stub and were sputter coated with gold to approximately 30 nm (Polaron SC500, Gold Sputter Coater, Quotum Technologies, Newhaven, UK). The coated samples were viewed under a scanning electron microscope at 3 kV and at magnification of 5–20 k. The zeta potential of nanoparticles dispersed in deionised water ($n = 6$ measurements) was measured using a zetasizer instrument (Nanoseries, Nano-ZS; Malvern Instruments).

### Preparation of platelets

The preparation was based on methods published previously and briefly described here.[25] Whole blood was drawn from the antecubital vein of healthy volunteers who had abstained from taking nonsteroidal anti-inflammatory drugs in the previous 10 days. Ethical approval for collection of blood was obtained from the Royal College of Surgeons in Ireland Ethics Committee. To prevent coagulation 9 vol blood was added to 1 vol 3.8% sodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation of anticoagulated whole blood at room temperature at 150*g* for 10 min. Platelet poor plasma (PPP) was obtained by centrifuging the remaining whole blood at 650*g* for 10 min.

### Platelet aggregation and inhibition

Platelet aggregation was assessed by monitoring light transmission using a PAP-4 platelet aggregometer (Bio/Data Corp., Horsham, PA, USA) according to the method described by O'Brien *et al*.[25] Changes in light transmission were recorded against PRP (0% light transmission) and a blank of autologus PPP (100% light transmission). Platelets were tested for normal responses to adenosine diphosphate (ADP) agonist (20 $\mu$M) or arachidonic acid agonist (AA) (0.5 mg/ml). To investigate if the particles induced platelet aggregation, 50 $\mu$l each micro/nanoparticle suspension (in phosphate-buffered saline (PBS)) at concentrations of 0.01–500 $\mu$g/ml was mixed with 450 $\mu$l PRP. For inhibition studies, 50 $\mu$l each micro/nanoparticle suspension (in PBS) at concentrations of 0.01–500 $\mu$g/ml, was added to 400 $\mu$l PRP, incubated at 37°C for 1 min before adding 50 $\mu$l ADP. In control samples 50 $\mu$l PBS was added to the control sample (400 $\mu$l) for 1 min followed by 50 $\mu$l ADP. Changes in light transmission were recorded against autologous PPP (100% light transmission).

### Statistical analysis

The results are expressed as mean ± standard deviation of at least three separate samples/experiments and were analysed using SPSS, version 15. The differences between the samples were assessed by a Kruskal–Wallis test and Mann–Whitney test with a Šidàk correction to avoid inflation of type I error. A *P*-value of $< 0.05$ was considered significant.

## Results and Discussion

### Characteristics of micro- and nanoparticles prepared

Microparticles of PLGA, PLGA-macrogol and chitosan prepared using solvent evaporation from an o/w emulsion or by spray drying had a median particle size (D50%) in the range of 2.71–9.01 $\mu$m (Table 1). The median particle size of chitosan microparticles was smaller than for the PLGA and PLGA-macrogol microparticles, except for the microparticles recovered by spray drying after solvent evaporation. The addition of surfactant/surface stabiliser did not influence particle size or morphology of the chitosan particles prepared by spray drying except for the addition of Tween 80, which resulted in an increase in the median particle size.

Electron microscopy of the microparticles (Figure 1a–c) showed that PLGA and PLGA-macrogol microparticles prepared by solvent evaporation were spherical in shape, while spray-dried PLGA microparticles were collapsed. In contrast to PLGA microparticles, the surface of chitosan microparticles was irregular, offering a larger surface area than corresponding PLGA microparticles for potential interaction with platelets. The chitosan microparticles may therefore have a higher potential for causing platelet aggregation than the smooth surface PLGA microparticles. Chitosan microparticles prepared using Tween 80 as surfactant showed some

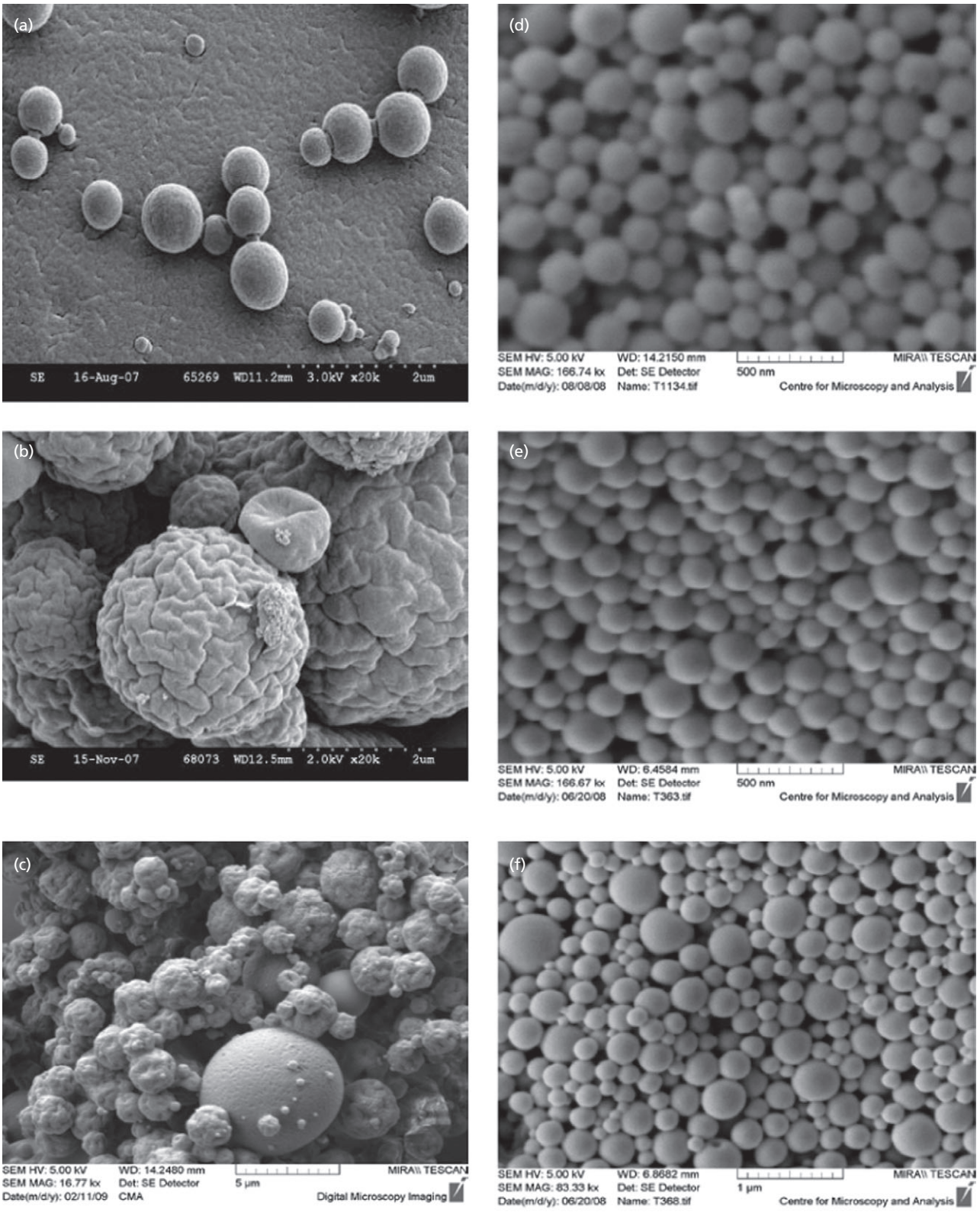


**Figure 1** Representative electron micrographs of microparticles and nanoparticles. (a) Poly-lactide-co-glycolide (PLGA)-macrogol microparticles prepared by solvent evaporation; (b) spray dried chitosan microparticles without surfactant; and (c) spray-dried chitosan microparticles formulated with Tween 80 surfactant. (d) PLGA-macrogol nanoparticles; (e) PLGA nanoparticles; and (f) chitosan-coated PLGA nanoparticles prepared by solvent dispersion.

**Table 2** Characteristics of nanoparticles prepared using poly-lactide-co-glycolide, poly-lactide-co-glycolide–macrogol and/or chitosan

| Polymer | Z average diameter (nm) | Polydispersity index | Zeta potential (mV) |
|---|---|---|---|
| PLGA | 209 | 0.138 | −31 |
| PLGA-macrogol | 138 | 0.247 | −38 |
| Chitosan (2.5%)-coated PLGA | 343 | 0.258 | +57 |
| Chitosan (15%)-coated PLGA | 443 | 0.257 | +55 |

PLGA, poly-lactide-co-glycolide.

agglomeration of the microparticles (Figure 1c), explaining the higher particle size observed for this batch.

Using the solvent dispersion method, nanoparticles with an average size below 500 nm were obtained. PLGA and PLGA-macrogol nanoparticles had an average diameter of 209 and 138 nm, respectively, while chitosan-coated PLGA nanoparticles were larger in size, with an average size of 343 and 443 nm for nanoparticles produced using 2.5 and 15% w/v chitosan, respectively (Table 2). The zeta potential of the nanoparticles ranged from −31 to +55, a negative zeta potential was observed for the PLGA and PLGA-macrogol nanoparticles as was expected due to the presence of carboxylic acid end groups for these polymers. Chitosan contains amino groups and is a positively charged polymer. A positive zeta potential was observed for the chitosan-coated PLGA nanoparticles, confirming the presence of the positively charged chitosan polymer on the surface of these nanoparticles. All nanoparticles formulated were discrete and spherical and had smooth surfaces irrespective of polymer composition, as shown in Figure 1d–f.

## Interaction of micro- and nanoparticles with human platelets

### *Effect of particles on aggregation of human platelets*

Incubation of platelets with chitosan, PLGA or PLGA-macrogol micro- or nanoparticles did not cause aggregation of platelets at any of the concentrations tested (Figures 2–4). The average percent aggregation of platelets caused by the micro- or nanoparticles was in the range of 5–15%, which was not significantly different to the average percent aggregation of platelets caused by the control vehicle at 7.8% ± 1.3 ($P > 0.05$). The average percent aggregation of platelets caused by the agonists AA or ADP was significantly different at 78 ± 5 and 72 ± 7%, respectively ($P < 0.01$). These data suggested that irrespective of the polymer composition used, particle size or surface morphology, none of the particles resulted in or showed the potential to induce platelet aggregation in the concentration range studied. Chitosan contains amino groups and is a positively charged polymer, while PLGA and PLGA-macrogol polymers have net negative charge due to the presence of carboxylic acid end groups resulting in the surface of the particles tested having a net negative or positive charge (Table 2). Nemmar *et al.*[26] showed that while unmodified polystyrene ultrafine nanoparticles had no effect on platelet aggregation, carboxylate-polystyrene particles weakly enhanced platelet aggregation, and amine-polystyrene particles induced platelet aggregation. The surface charge of the biodegradable nanoparticles or microparticles used in our study did not appear to influence platelet aggregation. Similarly, Li *et al.*[20] reported no effect of PLGA or chitosan nanoparticles in platelet aggregation.

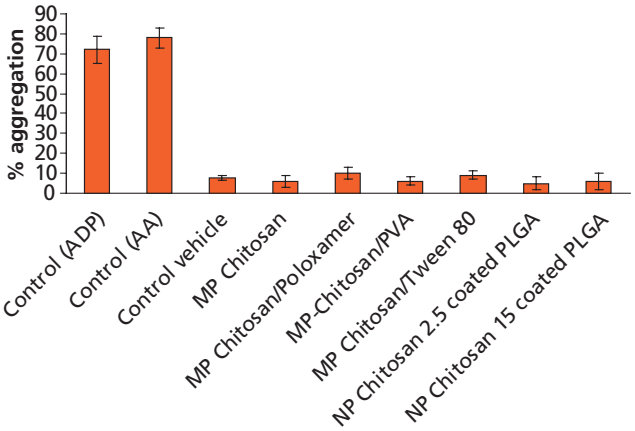


**Figure 2** Percent aggregation following addition of 500 μg/ml chitosan micro- and nanoparticles to platelet rich plasma. $n \geq 3$. AA, arachidonic acid; ADP, adenosine diphosphate; MP, microparticle; NP, nanoparticle; PLGA, polylactide-co-glycolide; PVA, polyvinyl alcohol.

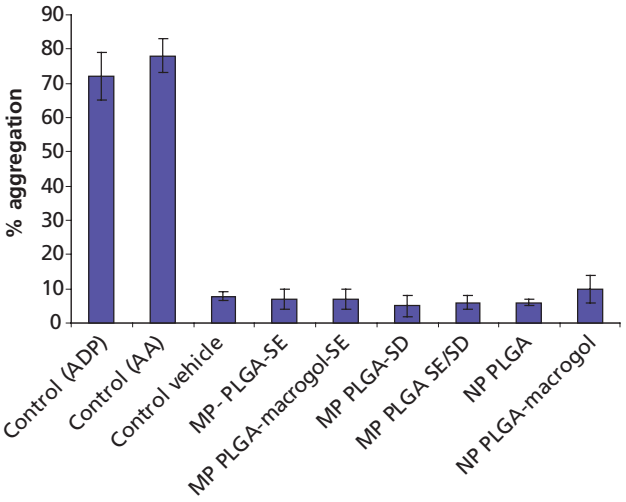


**Figure 3** Percent aggregation following addition of 500 μg/ml poly-lactide-co-glycolide or poly-lactide-co-glycolide-macrogol micro- and nanoparticles to platelet rich plasma. AA, arachidonic acid; ADP, adenosine diphosphate; MP, microparticle; NP, nanoparticle; PLGA, poly-lactide-co-glycolide; SD, spray drying; SE, solvent evaporation. $n \geq 3$.

The surface of the chitosan microparticles was not smooth (Figure 1b and c) and therefore offered a greater surface area

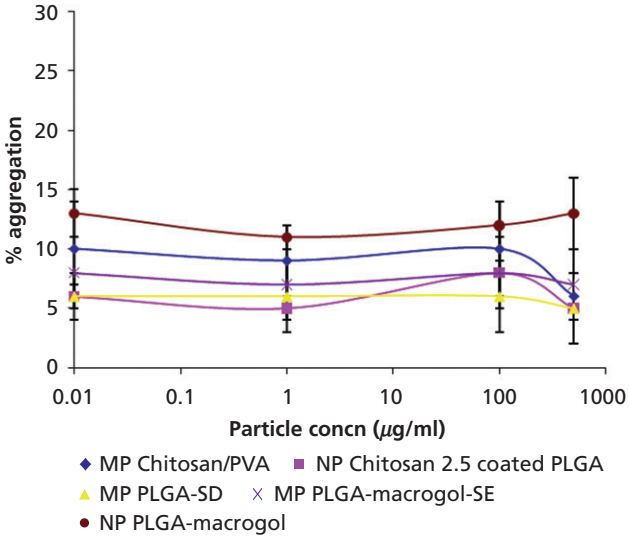


**Figure 4** Percent aggregation following addition of increasing concentrations of micro- or nanoparticles to platelet rich plasma. MP, microparticle; NP, nanoparticle; PLGA, poly-lactide-co-glycolide; SD, spray drying; SE, solvent evaporation. $n \geq 3$.

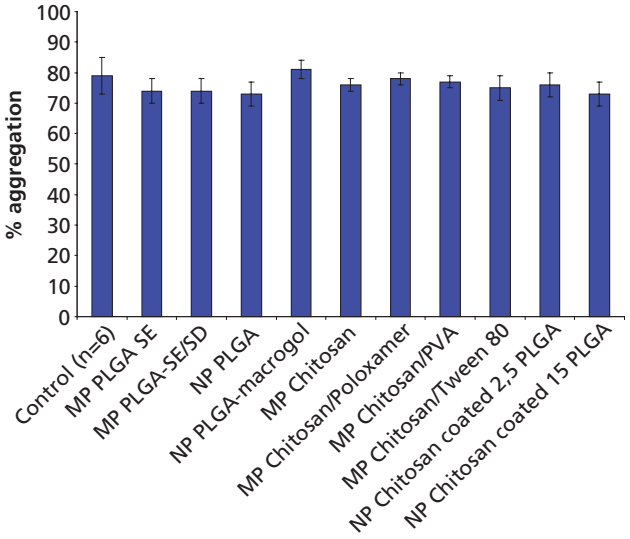


**Figure 5** Percent aggregation of platelets following addition of agonist adenosine diphosphate after incubation with 500 μg/ml biodegradable micro- and nanoparticles. MP, microparticle; NP, nanoparticle; PLGA, poly-lactide-co-glycolide; PVA, polyvinyl alcohol; SD, spray drying; SE, solvent evaporation.

for potential interaction, unlike the smooth surfaced PLGA or PLGA-macrogol particles of corresponding sizes. Chitosan is also a mucoadhesive polymer with potential to enhance the permeability of molecules across biological membranes and, as it is used in bandages as a haemostatic agent to stop bleeding, the potential of chitosan particles to interact with and possibly activate the platelets would therefore be higher than corresponding PLGA or PLGA-macrogol particles.[16,27] Interestingly, our study showed that chitosan when formulated as micro- or nanoparticles did not affect platelet function; this may have been due to the concentration of chitosan particles used or surface charge compared with chitosan bandages.

The presence or type of surfactant used during formulation of the particles was not found to influence interaction of the particles with the platelets. Surfactants such as polysorbate (Tween 80) and poloxamers (Lutrol F68) have been shown to increase drug transport across biological membranes by inhibiting P-glycoprotein drug efflux pumps or accelerating drug transbilayer movement or by membrane fluidisation.[28] This mechanism is being exploited as a formulation strategy in cancer therapy, targeting to the central nervous system and in drug delivery.[29] When included in the chitosan microparticles prepared by spray drying, Tween 80 resulted in particles being agglomerated possibly due to a decrease in the polymer glass transition temperature. While the inclusion of surfactants may influence particle morphology by fluidisation of the particle matrix, particularly in the case of Tween 80 which resulted in particle agglomeration (Figure 1c), our results showed that the presence of either Tween 80 or Lutrol F68 in the particles did not result in an increased interaction of the particles with the platelets ($P > 0.05$) compared with vehicle control or other particles tested. Further, no effect ($P > 0.05$) of particle concentration, and hence of the surfactant, on aggregation was observed (Figures 2 and 4). The low or negligible level of interaction with platelets and hence lack of platelet activation of the micro- and nanoparticles examined in this study supports the safety of their application in medical diagnosis and therapeutics.

Radomski *et al.*[30] showed that carbon nanoparticles induced platelet aggregation via activation of the fibrinogen receptor, GPIIb/IIIa. Platelet aggregation has also been shown to be induced by urban-type particulate matter.[8] In our study, the micro- or nanoparticles tested did not prevent platelet aggregation, meaning the particles did not bind to GPIIb/IIIa as if they had, they would have blocked fibrinogen binding, preventing platelet aggregation.

### *Effect of particles on inhibition of platelet aggregation*

The micro- and nanoparticles formulated were tested for inhibition of platelet aggregation. Incubation of platelets with chitosan, PLGA or PLGA-macrogol micro- or nanoparticles before the addition of ADP did not inhibit aggregation of platelets by ADP irrespective of the characteristics, composition or concentrations of particles (0.01–500 μg/ml) tested ($P > 0.05$, Figure 5). The average percent aggregation of platelets caused by the agonist ADP in absence of particles was 79 ± 6%. After incubation of the platelets with the micro- or nanoparticles, the percent aggregation observed after addition of ADP was in the range of 73 ± 4–81 ± 3%, respectively. Interestingly, Li *et al.*[20] reported that a high concentration of PLGA and chitosan particles of 100 μg/ml resulted in a weak inhibition of aggregation of 10–40%. Our results, however, demonstrated that the particles did not interact with platelets to induce aggregation, nor did they themselves prevent platelet aggregation, further adding to their safety profiles.

## Conclusions

This study has addressed an important consideration in the safety of systemic administration of biodegradable micro- and nanoparticles, in particular their ability to alter platelet activity, such as aggregatory activity leading to thrombus formation. The results demonstrated that biodegradable micro- and nanoparticles based on polymers of PLGA, PLGA-macrogol and chitosan had no affect on platelet aggregation and did not inhibit platelet aggregation at the particle concentrations tested. The polymers, formulation and processing methods used to prepare the particles studied spanned the wide range most frequently utilised in drug delivery and targeting and resulted in particles of various sizes, charge and morphology. This range of particle sizes and characteristics was recognised as the range of particles having the potential for cellular interaction and uptake and hence poses the most immediate safety concerns. However, unlike other airborne pollution and smoke particles, the biodegradable particles examined in this study were found to be safe, and hence were suitable for exploitation and use as drug delivery carriers for the controlled release and targeting application of therapeutic and diagnostic agents.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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