Pharmaceutical Research, Vol. 22 No. 11, November 2005 (© 2005)
DOI: 10.1007/s11095-005-7547-7

Research Paper

Blood Compatibility of Cetyl Alcohol/Polysorbate-Based Nanoparticles

J. M. Koziara,[1] J. J. Oh,[2] W. S. Akers,[2] S. P. Ferraris,[3] and R. J. Mumper[1,4]

Received June 27, 2005; accepted July 18, 2005

Purpose. Pegylated and nonpegylated cetyl alcohol/polysorbate nanoparticles (E78 NPs) are being tested as drug carriers for specific tumor and brain targeting. Because these nanoparticle formulations are designed for systemic administration, it is important to test the compatibility of these lipid-based NPs with blood and blood cells.
Methods. The hemocompatibility of E78 NPs was evaluated with a particular focus on hemolytic activity, platelet function, and blood coagulation. Human red blood cell lysis was determined by measuring hemoglobin release. Activation and aggregation of human platelets were determined using flow cytometry and aggregometry, respectively. Finally, the whole blood clotting time was measured using human blood.
Results. E78 NPs did not cause *in vitro* red blood cell lysis at concentrations up to 1 mg/mL. In addition, under conditions tested, E78 and polyethylene glycol (PEG)-coated E78 NPs (PEG-E78 NPs) did not activate platelets. In fact, both NP formulations very rapidly inhibited agonist-induced platelet activation and aggregation in a dose-dependent manner. Additionally, E78 NPs significantly prolonged *in vitro* whole blood clotting time at a concentration of 500 μg/mL or greater.
Conclusions. It was concluded that PEG-coated and nonpegylated E78 NPs have potential blood compatibility at clinically relevant doses. Based on the calculated nanoparticle-to-platelet ratio, the concentration at which E78 NPs could potentially affect platelet function *in vivo* was approximately 1 mg/mL.

KEY WORDS: blood clotting; hemocompatibility; platelet activation; platelet aggregation; platelets; red blood cell lysis.

INTRODUCTION

Nanoparticles have received a great deal of attention as drug delivery carriers for systemic administration. They have been shown to deliver drugs in a controlled manner and are being developed as targeted drug delivery systems for therapeutic and imaging agents (1,2). Considering the fact that the majority of nanoparticle formulations are intended for systemic administration, many researchers are concerned with general biocompatibility of nanoparticles (3–5). However, there are very few reports on nanoparticle interaction with blood and blood cells, although researchers have attempted to study the interaction of liposomes with blood cells, particularly platelets (6–11). Nevertheless, there are no standard methodologies for the *in vitro* assessment of the blood compatibility of particulate carriers such as liposomes or nanoparticles.

The thrombogenic potential of liposomes was studied by determining the influence of surface charge on a variety of *in vitro* parameters including whole blood clotting time (WBCT), recalcification time, thrombin time, prothrombin time, and platelet aggregation in platelet-rich plasma (PRP) (10). Additionally, platelet aggregation and whole blood clotting time were determined *in vivo* after liposome infusion into guinea pigs (10). In general, Zbinden *et al.* showed that only negatively charged liposomes induced platelet activation as evidenced by a reduction in clotting time and the formation of reversible liposome/platelet aggregates both *in vitro* and *in vivo*. Based on these observations, it was concluded that negatively charged liposomes have the ability to activate contact factors such as Hageman factor and possibly factor XI, which further leads to activation of the blood clotting system (10). The association of various types of liposomes with blood cells was also studied by Constantinescu *et al.* (6). Liposomes were mixed with whole blood, and association with platelets, red blood cells, and leukocytes was determined using flow cytometry. All liposomal formulations tested, irrespective of their composition, associated with cells very rapidly in a dose-dependent manner, both *in vivo* and *in vitro*. Interestingly, liposomes appeared to associate with the various blood cell types to the same extent.

[1] Department of Pharmaceutical Sciences, Center of Pharmaceutical Science and Technology, College of Pharmacy, University of Kentucky, 725 Rose Street, Lexington, Kentucky 40536-0082, USA.
[2] Department of Pharmacy Practice and Science, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0082, USA.
[3] Department of Surgery, College of Medicine, University of Kentucky, Lexington, Kentucky 40536-0293, USA.
[4] To whom correspondence should be addressesd. (e-mail: rjmump2@email.uky.edu)

Additionally, the interaction was independent of the presence of plasma proteins suggesting that liposomes interacted directly with cells (e.g., no opsonization was necessary to trigger interaction). Constantinescu *et al.* (6) hypothesized that phospholipids from the liposomes fuse with cell membranes. However, no studies were performed to investigate the influence of liposomes on the function on each blood cell component and hemostasis. Miyamoto *et al.* investigated the interactions of latex nanoparticles of different sizes and surface charge with platelets (12) and the mechanisms of blood coagulation induced by the nanoparticles (13). In the first study, the effects of particle size, surface charge, and hydrophobicity on platelet aggregation in PRP (single cell count and TEM) and platelet activation [monitored by the release of adenosine triphosphate (ATP)] were investigated. It was observed that all types of particles tested induced platelet activation and aggregation (12). Particle size was an important factor affecting nanoparticle–platelet interaction as larger nanoparticles induced platelet aggregation more readily. Additionally, nanoparticles having more hydrophobic surfaces resulted in greater platelet activation and aggregation (12). Similarly, negatively charged nanoparticles induced platelet aggregation to a greater extent than cationic or neutral nanoparticles (12). Interestingly, it was also noted that an increase in the nanoparticle-to-platelet ratio over a certain threshold value resulted in an inhibition of aggregate formation. However, there were no changes in ATP release with an increase in nanoparticle concentration, e.g., no inhibition of platelet activation. Additionally, it was calculated that the number of nanoparticles required to inhibit platelet aggregation was approximately equal to the number of nanoparticles needed to cover the available surface area of the platelets. It was concluded that at a certain nanoparticle-to-platelet ratio, nanoparticles inhibited aggregate formation by suppressing platelet-to-platelet contact (12). The ability of the nanoparticles to inhibit platelet aggregation was dependent on particle size as well as surface hydrophobicity and charge.

Our laboratory recently reported on the feasibility of using novel cetyl alcohol-based nanoparticles (E78 NPs) for specific tumor and brain targeting (14–17). Initial blood compatibility of E78 NPs overcoated with PEG_{3300}-folate was evaluated (17). The thrombogenic potential of nanoparticles was determined based on changes in light absorbance in mouse platelet-rich plasma (PRP) (17). Nanoparticles did not cause neutrophil activation or platelet aggregation. The purpose of the present studies was to 1) continue the evaluation of the hemocompatibility of these cetyl alcohol-based nanoparticles with particular focus on hemolytic activity, platelet function, and blood coagulation and 2) work toward the development of standard methodologies for the *in vitro* assessment of the hemocompatibility of particulate carriers.

MATERIALS AND METHODS

Materials

Emulsifying wax (E. wax) was purchased from Spectrum Chemicals (New Brunswick, NJ, USA). Polyoxyl 20-stearyl ether (Brij 78) was obtained from Uniqema (Wilmington, DE, USA). DSPE-PEG_{2000} was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Human α-thrombin (THR), gly-pro-arg-pro peptide, epinephrine, collagen, and sodium chloride were purchased from Sigma Chemicals (St. Louis, MO, USA). [1*S*-[1α,2α(*Z*),3β(1*E*,3*S*),4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo-[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Fluorescently labeled antibodies [IgM–fluorescein isothiocyanate (FITC), PAC-1–FITC, and CD42-PE] were obtained from BD Biosciences (San Jose, CA, USA). All materials were used as obtained. For all experiments, deionized water was filtered through 0.22-μm filters (Nalgene International, Rochester, NY, USA).

Preparation of Nanoparticles from Microemulsion Precursors

Microemulsion precursors were prepared as reported by Oyewumi and Mumper (18) with slight modifications. Briefly, 2 mg of E. wax as the oil phase and 4.5 mg Brij 78 as the surfactant were weighed out into glass vials, and the vials were heated to 50–55°C. To the melted mixture of oil and surfactant, deionized and 0.2-μm filtered water was added to obtain a final volume of 1 ml and final Brij 78 surfactant concentration of 4 mM. Microemulsions formed spontaneously at this elevated temperature. Upon simple cooling in the mixing vial, warm oil-in-water microemulsion precursors solidified into nanoparticles (E78 NPs). To form polyethylene glycol (PEG)-coated E78 NPs (PEG-E78 NPs), 60 μL of a chloroform solution of DSPE-PEG_{2000} (1 mg/mL) was added to the vial containing the melted mixture of oil phase and surfactant. The chloroform was evaporated, and nanoparticles were engineered as described above. Nanoparticles were coated with 3% w/w DSPE-PEG_{2000}. For the RBC lysis, whole blood clotting time, and aggregometry experiments, nanoparticles were formulated in 150 mM sodium chloride. All NP formulations were prepared at a final E. wax concentration of 2 mg/mL.

Nanoparticle Characterization

Particle size was measured at 25°C using a Coulter N4 Plus Sub-Micron Particle Sizer (Coulter Corporation, Miami, FL, USA) at 90° light scattering for 90 s. Prior to size determination, nanoparticle suspensions were diluted with filtered water to ensure light scattering intensity within the required range of the instrument (5×10^4–1×10^6 counts/s). Surface charge of nanoparticles was measured using Zetasizer 2000 (Malvern Instruments, Southborough, MA, USA).

Red Blood Cell Lysis

Fresh citrated blood was obtained from healthy human volunteers. Blood was mixed at 1:1 (v/v) ratio with water (positive control) and nanoparticle suspension in 150 mM NaCl at final concentrations ranging from 0 (negative control) to 1 mg/mL. Samples were incubated at 37°C for 30 min on a Labquake Orbital Shaker. After the incubation, intact red blood cells were separated from plasma by centrifugation at 600 × *g* for 5 min. Supernatant was

collected and incubated for 30 min at room temperature to allow for hemoglobin oxidation. Oxyhemoglobin absorbance was measured spectrophotometrically at 540 nm. To evaluate the influence of time on red blood cell (RBC) lysis in the presence of nanoparticles, blood was incubated with 1 mg/mL of E78 NPs for up to 6 h. At predetermined time points, oxyhemoglobin level was measured as described above. The percentage of RBC lysis was calculated based on the assumption that 100% red blood cell lysis resulted from a 1:1 (v/v) mixing of blood with water. Additionally, RBC lysis was evaluated in the presence of an increasing concentration of Brij 78. Equal volumes of blood and Brij 78 solution (0–4 mM) in saline were incubated for 30, 60, and 120 min at 37°C on a Labquake orbital shaker. The percentage of RBC lysis was determined as above.

Platelet Activation–Flow Cytometry Studies

Fresh citrated blood was obtained from healthy human volunteers. To minimize *ex vivo* platelet activation, the first collection tube was discarded. Whole blood was diluted with platelet buffer 1:8 (v/v) and incubated with platelet activators (final concentration) α-THR (1 U/mL), I-BOP (1 μM), or adenosine diphosphate (ADP; 10 μM) in the presence or absence of nanoparticle formulations at concentrations ranging from 1 to 150 μg/mL for 10 min at 37°C. Resting platelets (no agonists) were used as a negative control. All samples were incubated with the antibodies IgM-FITC (control), PAC-1–FITC, and CD42b-PE at room temperature for 20 min and protected from light. CD42b recognizes the membrane glycoprotein Ib (GP Ib), found on the surface of resting and activated platelets, and was used to identify platelets for flow cytometry analysis. PAC-1 antibody recognizes the glycoprotein IIb/IIIa (GP IIb/IIIa) on the surface of activated platelets. Samples were fixed with 2% paraformaldehyde and further diluted with the platelet buffer. Fixed samples were stored up to 24 h in the refrigerator until analyzed by flow cytometry. Samples were evaluated in the Flow Cytometry Core Research Facility at the University of Kentucky using FACSCalibur by Becton Dickinson (San Jose, CA, USA) and analyzed using Cell Quest software, version 3.4 (BD Biosciences).

Aggregometry Studies

Platelet aggregation in the presence and absence of nanoparticles was monitored using Chrono-Log Whole Blood Impedance Aggregometer (Chrono-Log Corporation, Havertown, PA, USA). Nanoparticle suspensions in saline were mixed with citrated blood at a 1:1 (v/v) ratio. Mixtures were equilibrated to 37°C under constant stirring, and the baseline current was set by immersing electrodes in the citrated blood/nanoparticle mixture. Aggregation was assessed as maximal amplitude at 6 min after the addition of agonists (20 μM of ADP, 20 μM I-BOP, or 0.1 U/mL of α-THR). Nanoparticle concentrations ranged from 5 to 200 μg/mL. A saline solution was used as a positive control instead of nanoparticles. Additionally, nanoparticles (200 μg/mL) in saline with no blood were used as a negative control. All blood was used no later than 3 h from the time of collection. Data were analyzed using AGGRO/LINK® software (Chrono-Log Corporation).

In Vitro Whole Blood Clotting Time

Freshly collected citrated human blood was mixed 1:1 (v/v) with saline (control) or nanoparticles (final concentration 25–1000 μg/mL) in borosilicate glass vials. Samples were incubated for 5 min at room temperature after which 25 μL of 50 mM calcium chloride ($CaCl_2$) solution was added to the vials. The time from $CaCl_2$ addition to the first visible signs of clot formation was determined as the whole blood clotting time. This assay is commonly used in clinical hospitals to determine WBCT.

RESULTS

Preparation and Characterization of Nanoparticles

E78 NPs and PEG-E78 NPs had similar sizes and zeta potentials. E78 NPs had a particle size and zeta potential of 67.0 ± 17.5 nm and −40.0 ± 2.2 mV, respectively. PEG-E78 NPs had a particle size and zeta potential of 67.0 ± 11.7 nm and −40.2 ± 2.0 mV, respectively. These data are in agreement with previous findings (14).

RBC Lysis

Incubation of blood with an equal volume of water (as a positive control) resulted in instantaneous red blood cell lysis. Addition of increasing concentration of Brij 78 to whole blood led to a significant RBC lysis (Fig. 1A). The RBC lysis in the presence of surfactant was concentration- and time-dependent (Fig. 1A). For example, the presence of Brij 78 at final concentration of 2 mM resulted in 54, 62, and 82% RBC lysis after 30-, 60-, and 120-min incubation, respectively. In contrast, nanoparticle concentrations ranging from 0 to 500 μg/mL did not cause hemoglobin release after 30-min incubation with blood (data not shown). The higher concentration of 1000 μg/mL E78 NPs led to detectable release of hemoglobin (Fig. 1B). The percentage of RBC lysed, following 30-min incubation, was determined to be 0.89 ± 1.1% based on osmotic cell lysis with water. As demonstrated in Fig. 1B, nanoparticles at the highest final diluted concentration of 1 mg/mL incubated with blood over time resulted in time-dependent increase in RBC lysis. After 6 h, the calculated percentage of RBC lysed was 4.7 ± 1.5%. There was no spontaneous cell lysis over time when blood was diluted with physiological saline (as a control).

Flow Cytometry Studies

To evaluate the potential thrombogenic activity of nanoparticles, E78 NPs and PEG-E78 NPs were incubated with blood, and platelet activation was determined based on the efficiency of PAC-1 binding to the activated platelets. As shown in Fig. 2, a strong increase in fluorescence signal was observed after incubating blood with α-THR, a potent platelet agonist. However, both types of nanoparticles at final diluted concentrations of 50, 100, and 150 μg/mL did not cause changes in PAC-1 binding when compared to rested platelets (control). Of particular interest was that there was an inhibition of agonist-induced platelet activation when platelet agonists were added to the mixture of blood and E78

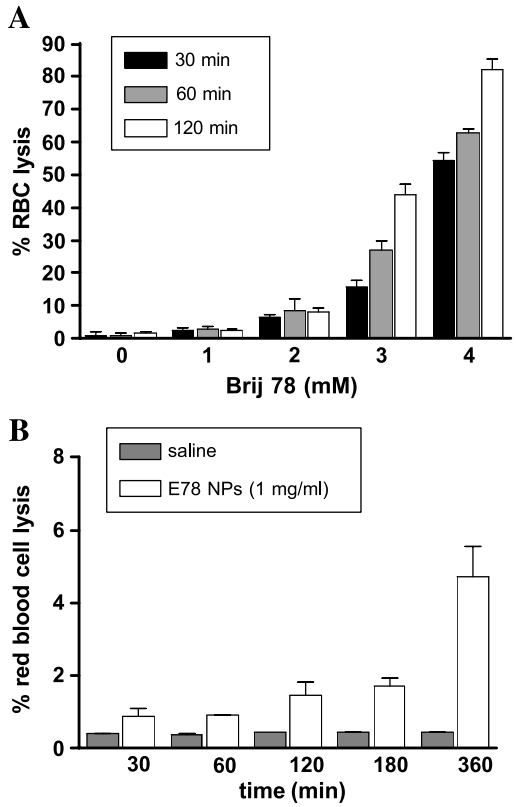

Fig. 1. *In vitro* red blood cell (RBC) lysis. (A) Equal volume of Brij 78 (0–4 mM) was added to the whole blood and incubated at 37°C; RBC lysis was determined spectrophotometrically (λ = 540 nm) based on oxyhemoglobin level in plasma. (B) Time course of RBC lysis in the presence of E78 NPs (final NP concentration 1 mg/mL) and saline (control). Data represent mean ± SD, n = 3.

NPs (100 μg/mL). Moreover, this inhibition was agonist-independent as shown in Fig. 3. To further investigate inhibition of agonist-induced platelet activation by the nanoparticles, a dose response study was performed. The final diluted concentration of E78 NPs ranged from 1 to 100 μg/mL. Furthermore, to evaluate whether nanoparticle surface properties influenced nanoparticle–platelet interaction, the

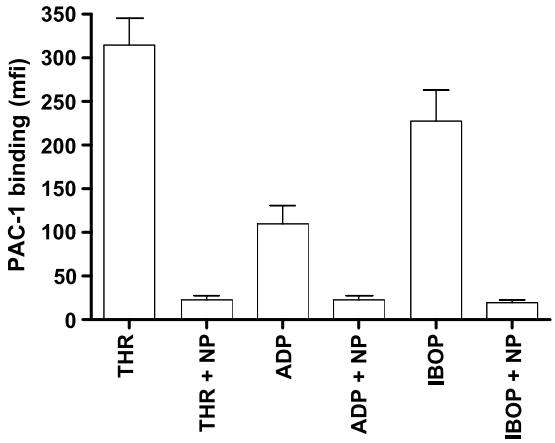

Fig. 3. Mean fluorescence intensity of PAC-1 binding to agonist-induced activated platelets in the presence and absence of E78 NPs (100 μg/mL). Data suggest that inhibition of agonist-induced platelet activation by E78 NPs is agonist-independent. Data represent mean ± SEM, n = 3.

experiment was repeated for PEG-E78 NPs. As evidenced in Fig. 4, there was a significant decrease in α-THR-induced PAC-1 binding when the nanoparticle concentration was increased. There were no statistical differences between E78 NPs or PEG-E78 NPs.

Whole Blood Impedance Aggregometry

The influence of nanoparticles on the aggregation processes in whole blood was determined based on changes in electrical resistance between two probes immersed in the sample. It was shown that there was no increase in resistance due to the presence of negatively charged nanoparticles (200 μg/mL) and 0.1 U/mL of α-THR in normal saline (Fig. 5A). After the addition of α-THR to a whole blood sample with no nanoparticles, there was a change in the electrical resistance resulting from platelet aggregation and adhesion to the probes (Fig. 5B). In the presence of 5 μg/mL of E78 NPs, the current amplitude dropped from 14 Ω (positive control) to 6 Ω indicating partial inhibition of α-THR-induced platelet aggregation. There were no apparent

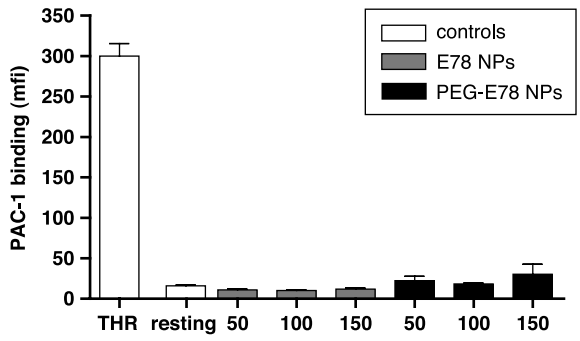

Fig. 2. Mean fluorescence intensity of PAC-1 binding to resting platelets (negative control) and platelets in the presence of thrombin (positive control) and nanoparticle formulations. Lack of significant fluorescence in presence of E78 NPs and PEG-E78 NPs indicates that nanoparticles do not cause platelet activation. Data represent mean ± SEM, n = 3.

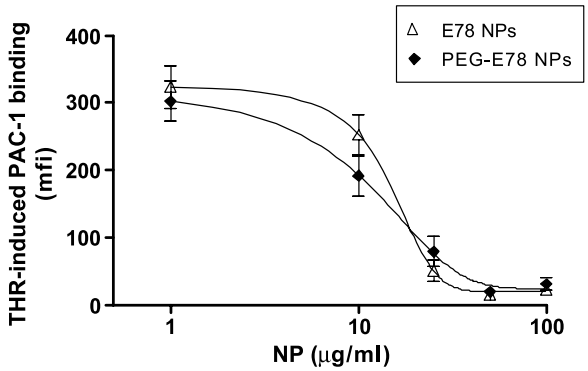

Fig. 4. Mean fluorescence intensity of PAC-1 binding after thrombin-induced platelet activation in the presence of increasing concentrations of E78 NPs and PEG-coated nanoparticles. There is a dose-dependent inhibition of agonist-induced platelet activation by nanoparticles. Data represent mean ± SEM, n = 3.

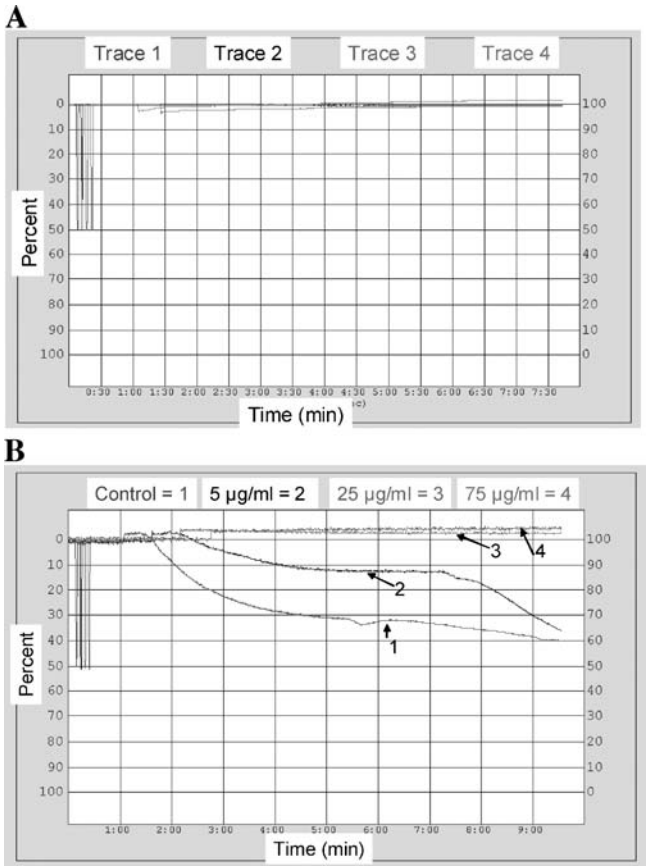

Fig. 5. Impedance aggregometry. (A) Negative control: E78 NPs (200 µg/mL) incubated with saline and challenged with 0.1 U/mL α-THR. Data suggest that in the presence of nanoparticles, but the absence of platelets, there are no significant changes in electrical resistance. (B) Blood was incubated with saline (control, no. 1), E78 NPs (5 µg/mL, no. 2; 25 µg/mL, no. 3; 75 µg/ml, no. 4), and challenged with 0.1 U/mL THR. Data suggest that nanoparticles inhibit platelet aggregation in a dose-dependent manner.

changes in resistance at nanoparticle concentrations of 25 and 75 µg/mL, indicating a lack of platelet aggregation. Dose-dependent inhibition of platelet aggregation was also confirmed in the presence of agonists such ADP and I-BOP (data not shown).

In Vitro Whole Blood Clotting Time

As shown in Table I, the time required to start clot formation in whole blood–saline mixture (1:1 v/v) upon the addition of calcium ions was determined to be 157 ± 19 s. When blood was preincubated with E78 NPs at varying concentrations, a dose-dependent prolongation of whole blood clotting time was noticed. There were no differences in clotting time with nanoparticles at a concentration up to 250 µg/mL when compared to the saline control. At 500 µg/mL of E78 NPs, the first signs of clot formation were noticed after 280 s corresponding to a 1.8-fold increase in WBCT. In addition, a further doubling of the E78 NPs to 1000 µg/mL resulted in a ~3-fold increase in WBCT when compared to control.

DISCUSSION

With increasing use of drug carriers such as polymeric nanoparticles and solid lipid nanoparticles, it is of critical importance to establish methodologies to systematically investigate the hemocompatibility of these carriers with whole blood and various blood components such as RBCs, platelets, and white blood cells.

The cetyl alcohol/polysorbate-based nanoparticles used in these present studies were made with Brij 78, a known surface active agent by itself. Because it is well known that surface-active agents can compromise membrane integrity (19), the effect of E78 NPs on red blood cells was investigated. It was hypothesized that as a result of nanoparticle formation from oil-in-water microemulsion droplets, the hydrophobic portion of the surfactant was immobilized within nanoparticle matrix, and hydrophilic head was localized on the surface of the particles. This unique arrangement of Brij 78 implies that Brij 78 no longer retained its surface-active properties and therefore should not have caused red blood cell lysis. As demonstrated in Fig. 1A, the addition of an increasing amount of Brij 78 (final concentration 0–2 mM) led to a significant time-dependent hemoglobin release. In contrast, nanoparticles made with Brij 78 did not cause hemoglobin release at relevant concentrations, i.e., concentrations that would be practical in therapeutic applications after systemic injection into the blood. Even at the highest concentration of 1 mg/mL, which equals to 1:1 (v/v) dilution of blood with the nanoparticle formulation, only a small percentage of red blood cell lysis was observed. A final nanoparticle concentration of 1 mg/mL corresponds to a final Brij 78 concentration of 2 mM. However, as demonstrated in Fig. 1A and B, there are significant differences between the extent of RBC lysis in presence of 1 mg/mL of nanoparticles and 2 mM free Brij 78. These findings confirmed the hypothesis that Brij 78 on the surface of E78 NPs had different physical properties from free surfactant. Nonetheless, there is the possibility of some free Brij 78 in the water phase at equilibrium. This free surfactant molecules could possibly be responsible for the observed RBC lysis in the presence of the highest nanoparticle concentration as shown in Fig. 1B. Similar studies have been reported in the literature with superparamagnetic nanoparticles. These studies revealed

Table I. The Effect of E78 NP Concentration on the *In Vitro* Whole Blood Clotting Time

E78 NPs (µg/mL)	Clotting time (s)
0	157 ± 19
50	154 ± 19
100	145 ± 5
150	144 ± 5
250	174 ± 16
500	281 ± 52*
1000	463 ± 46*

Whole citrated blood was mixed at 1:1 (v/v) ratio with saline (control) or E78 NPs at various concentrations in borosilicate glass and incubated for 5 min at room temperature. An aliquot of 50 mM calcium chloride was added, and the time was measured until the first signs of visual clot formation.

that incubation of 70 nm magnetide/dextran particles with blood resulted in 60% hemolysis; however, this lytic activity was considered clinically irrelevant due to extremely high nanoparticle concentration used for the *in vitro* studies. Indeed, when clinically relevant doses of the magnetide/dextran particles were applied, no hemolysis occurred (20).

It is known that blood contact with negatively charged surfaces such as glass or kaolin often results in the contact phase activation of the plasma coagulation system (21,22). Thus, it is possible that negatively charged carrier systems may lead to thrombosis upon systemic administration. As demonstrated by Miyamoto *et al.* (12), latex nanoparticles having the most negative zeta potential aggregated platelets more readily than neutral or cationic particles. Additionally, nanoparticles were rapidly phagocytosed by platelets that subsequently resulted in platelet aggregation (12,23). The potential thrombogenic activity of E78 NPs was investigated by *in vitro* aggregometry, which is considered the gold standard for the assessment of platelet function. Most commonly used aggregometry procedures are based on turbidometric changes in light transmittance using platelet-rich plasma samples. However, it is known that platelet activity is modulated by red and white blood cells (13). Therefore, it is more reliable to test platelet function in physiological milieu such as whole blood. For this reason, flow cytometry studies as well as whole blood impedance aggregometry were employed in the present studies. Whole blood impedance aggregometry when compared to turbidometric methods offers advantages such as increased sensitivity and the ability to test platelet function in whole blood (24). This allows for maintaining physiological environment for platelets and eliminates sample (PRP) preparation step, which is time-consuming and may cause platelet damage. As demonstrated in Fig. 2, incubation of nanoparticles with whole blood did not result in an increase in PAC-1 binding indicating that E78 NPs and PEG-E78 NPs did not activate platelets and were not thrombogenic. However, it was found that nanoparticles do interact with platelets leading to inhibition of agonist-induced platelet activation and aggregation. As reported by Miyamoto *et al.* (12), latex nanoparticles can activate platelets, but at the same time, at high concentrations, nanoparticles disrupt the formation of platelet aggregates by inhibiting cell to cell contact. Likewise, in these present flow cytometry studies, E78 NPs inhibited platelet activation and prevented platelet aggregation. Therefore, it is possible that nanoparticles coated the surface of platelets and limited the access of antibody to the cell surface. Alternatively, Brij 78 present on the surface of nanoparticles and/or excess of free surfactant may interact with the surface of the platelets thereby inhibiting antibody recognition. Indeed, the ability of certain polyethylene glycol–polypropylene glycol copolymers (poloxamers) to inhibit platelet aggregation has previously been demonstrated (25). Poloxamer 184 at concentration of 2 mM significantly inhibited ADP-induced platelet activation as determined by flow cytometry analysis. Further, this inhibition was independent of agonist tested (ADP and TRAP). It was also demonstrated that the same surfactant markedly reduced the ability of the platelets to bind fibrinogen and antibody PAC-1 (25). It was therefore suggested that the mechanism underlying Poloxamer 184 effect on platelets may be due to its binding to GP IIb/IIIA on the surface of activated platelets. Similarly, in the case of E78 NPs, a decrease in PAC-1 binding was observed in the presence of nanoparticles (Figs. 2–4). However, no changes in CD-42 antibody binding to the platelets were noted in the presence and absence of nanoparticles (data not shown), indicating that there was no physical barrier that would non-specifically inhibit access of the antibody to the platelet surface. Additionally, inhibition of agonist-induced platelet activation by E78 NPs was evident using P-selectin as an activation marker (data not shown) suggesting that there were no specific interactions between nanoparticles and/or Brij 78 and GP IIb/IIIa. Juliano *et al.* reported that positively charged liposomes inhibited ADP-induced platelet aggregation in platelet-rich plasma (PRP) in dose-dependent manner. However, when liposomes were incubated with washed platelets rather than PRP, negatively charged liposomes prevented thrombin-induced aggregation (7). The authors concluded that the interaction of cationic liposomes with platelets was indirect because it occurred in PRP but not with isolated and washed platelets. This indirect interaction could be potentially triggered by plasma protein coating on the surface of the liposomes. In contrast, anionic liposomes interacted with platelets directly. It is possible that negatively charged liposomes first interacted with thrombin, a positively charged protein. This interaction would result in a lowering of the free thrombin concentration, which could subsequently lead to an apparent inhibition of platelet aggregation. Thrombin was shown to coat the surface of negatively charged E78 NPs (data not shown). Thus, to avoid artifacts due to nanoparticle/thrombin interactions, a series of other agonists such as ADP, I-BOP (Fig. 3), epinephrine, TRAP, and collagen (data not shown) were also tested. Importantly, it was determined that E78 NPs inhibited platelet activation irrespective of these additional agonists tested. These findings suggest that nanoparticles interact directly with platelets and not first with the agonist and/or detector antibody. Using impedance aggregometry, it was confirmed that E78 NPs inhibit agonist-induced platelet aggregation in dose-dependent manner, and that this process is agonist-independent.

The results of the present studies suggest that the process of nanoparticle–platelet interaction is very rapid and occurs in the timeframe of seconds. It was observed that when agonist was added to the blood sample first and then followed by nanoparticles, there were no differences in platelet activation as compared to the positive control. However, when the order of addition was reversed, a significant inhibition of platelet activation was observed. The rapid interaction of nanoparticles with platelets agrees with other reports (6). Constantinescu *et al.* (6) observed that association of liposomes with blood cells was in most cases complete within 1 min.

Polyethylene glycol and polyethylene oxide (PEO) molecules are capable of resisting plasma protein adsorption (26–28) and platelet adhesion (29–32). Therefore, pegylation of surfaces is commonly used for the purpose of increasing hemocompatibility of biomaterials. The DSPE-PEG molecules are hypothesized to be oriented in a similar manner as Brij 78 during microemulsion formation; for example, the lipid–DSPE is embedded in nanoparticle matrix, and the PEG molecules are located on the surface of nanoparticles. In case of E78 NPs, there were no differences in platelet interaction between E78 NPs and PEG-E78 NPs (Fig. 4),

indicating that the character of the interaction is most likely not related to the surface of the nanoparticle. In fact, similar findings were reported by Constantinescu *et al.* (6) where kinetics of liposomes/platelet association were the same for uncoated liposomes and PEG-coated liposomes.

E78 NPs and PEG-E78 NPs have been tested for numerous applications *in vivo* by intravenous injection of doses up to 40 mg/kg into mice, which would result in nanoparticle blood concentrations of approximately 400 μg/mL. No gross effects of this highest dose with respect to prolonged clotting time and/or bleeding problems have been observed (unpublished observations). In an attempt to understand the potential discrepancies between the flow cytometry results and *in vivo* observations, *in vitro* clotting time experiments were performed. At tested conditions, nanoparticles up to 250 μg/mL did not have any significant effect on whole blood clotting time. However, at E78 NPs concentration of 500 μg/mL in a 1:1 (v/v) blood/saline mixture, prolongation of clotting time was found to be significant. To further correlate these results, the ratio of nanoparticles to platelet was calculated based on the assumption that there was the same number of platelets in each original blood sample. At a concentration of 500 μg/mL of blood/saline mixture (1:1), the nanoparticle-to-cell ratio was approximately 10,000 to 1. In flow cytometry studies, this ratio would be approximately equivalent to a nanoparticle concentration of 100 μg/mL. Using these calculations, a relatively strong correlation between both experiments was obtained. Some discrepancy may be explained based on the employed assumptions and/or the inherent error in the visual estimation of the endpoint during clotting time experiments. Nevertheless, it was concluded that the concentration of E78 NPs in whole blood (e.g., *in vivo* conditions), which potentially could affect platelet aggregation, was approximately 1000 μg/mL, which is at least 2-fold greater than the highest NP concentration in blood routinely tested in animal studies. The concentration of 1000 μg/mL was calculated with the assumption that the *in vivo* injection volume will not result in significant blood dilution.

The actual mechanism underlying inhibition of platelet activation and aggregation by E78 NPs still remains to be elucidated. Numerous reports suggest, however, that platelets have the ability to phagocytose drug carriers such as liposomes both *in vitro* and *in vivo* (7,8,33). In addition, *in vitro* assays such as aggregometry, serotonin release, and platelet membrane integrity demonstrated that liposome uptake by platelets did not alter cell functions (8). Moreover, the uptake of liposomes did not impair platelet circulation and function *in vivo* (33). On the other hand, various studies have indicated that liposomes can affect platelet function both *in vitro* (7,10) and *in vivo* (10). Therefore, it could be hypothesized that E78 NPs may be rapidly taken up by platelets. Once in the cell, nanoparticles may alter cellular pathways controlling platelet activation and subsequent aggregation. In addition, if nanoparticles are found to alter these pathways, what are the kinetics of these processes and are they reversible or irreversible? Experiments testing this hypothesis are being currently designed. In addition, initial experiments have been performed that demonstrate that these nanoparticles are extensively metabolized *in vitro* by alcohol dehydrogenases over 24 h suggesting that the effects of E78 NPs, if any, may be short-lived.

Based on literature review, it was concluded that nanoparticle or liposome interaction with blood cells is highly dependent on particle size and surface properties, particularly surface charge. Present studies with E78 NPs demonstrated that when evaluating the influence of drug carriers on platelet function, it is critical to assess the potential thrombogenic properties and also the ability of the carrier to inhibit platelet activity. Overall, E78 NPs do not cause red blood cell lysis and are not thrombogenic. Nanoparticles at high nanoparticle-to-platelet ratios inhibited agonist-induced platelet activation and aggregation. Additionally, above certain concentrations, E78 NPs were observed to prolong *in vitro* clotting time. However, at clinically relevant doses, cetyl alcohol/polysorbate nanoparticles have potential blood compatibility.

REFERENCES

1. J. Kreuter. Nanoparticles and microparticles for drug and vaccine delivery. *J. Anat.* **189**(Pt 3):503–505 (1996).
2. P. R. Lockman, R. J. Mumper, M. A. Khan, and D. D. Allen. Nanoparticle technology for drug delivery across the blood–brain barrier. *Drug Dev. Ind. Pharm.* **28**:1–13 (2002).
3. A. K. Gupta and M. Gupta. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* **26**:3995–4021 (2005).
4. A. K. Gupta and A. S. Curtis. Surface modified superparamagnetic nanoparticles for drug delivery: interaction studies with human fibroblasts in culture. *J. Mater. Sci. Mater. Med.* **15**: 493–496 (2004).
5. A. Brownlie, I. F. Uchegbu, and A. G. Schatzlein. PEI-based vesicle-polymer hybrid gene delivery system with improved biocompatibility. *Int. J. Pharm.* **274**:41–52 (2004).
6. I. Constantinescu, E. Levin, and M. Gyongyossy-Issa. Liposomes and blood cells: a flow cytometric study. *Artif. Cells Blood Substit. Immobil. Biotechnol.* **31**:395–424 (2003).
7. R. L. Juliano, M. J. Hsu, D. Peterson, S. L. Regen, and A. Singh. Interactions of conventional or photopolymerized liposomes with platelets *in vitro*. *Exp. Cell Res.* **146**:422–427 (1983).
8. R. Male, W. E. Vannier, and J. D. Baldeschwieler. Phagocytosis of liposomes by human platelets. *Proc. Natl. Acad. Sci. USA* **89**: 9191–9195 (1992).
9. L. W. Reinish, M. B. Bally, H. C. Loughrey, and P. R. Cullis. Interactions of liposomes and platelets. *Thromb. Haemost.* **60**:518–523 (1988).
10. G. Zbinden, H. Wunderli-Allenspach, and L. Grimm. Assessment of thrombogenic potential of liposomes. *Toxicology* **54**: 273–280 (1989).
11. L. M. Pinto, R. Pereira, E. de Paula, G. Nucci de, M. H. Santana, and J. L. Donato. Influence of liposomal local anesthetics on platelet aggregation *in vitro*. *J. Liposome Res.* **14**:51–59 (2004).
12. M. Miyamoto, S. Sasakawa, T. Ozawa, H. Kawaguchi, and Y. Ohtsuka. Platelet aggregation induced by latex particles. I. Effects of size, surface potential and hydrophobicity of particles. *Biomaterials* **10**:251–257 (1989).
13. M. Miyamoto, S. Sasakawa, T. Ozawa, H. Kawaguchi, and Y. Ohtsuka. Mechanisms of blood coagulation induced by latex particles and the roles of blood cells. *Biomaterials* **11**:385–388 (1990).
14. J. M. Koziara, P. R. Lockman, D. D. Allen, and R. J. Mumper. *In situ* blood–brain barrier transport of nanoparticles. *Pharm. Res.* **20**:1772–1778 (2003).
15. J. M. Koziara, P. R. Lockman, D. D. Allen, and R. J. Mumper. Paclitaxel nanoparticles for the potential treatment of brain tumors. *J. Control. Release* **99**:259–269 (2004).
16. P. R. Lockman, J. M. Koziara, R. J. Mumper, and D. D. Allen. Nanoparticle surface charges alter blood–brain barrier integrity and permeability. *J. Drug Target.* **12**:635–641 (2004).
17. M. O. Oyewumi, R. A. Yokel, M. Jay, T. Coakley, and R. J. Mumper. Comparison of cell uptake, biodistribution and tumor

retention of folate-coated and PEG-coated gadolinium nanoparticles in tumor-bearing mice. *J. Control. Release* **95**:613–626 (2004).
18. M. O. Oyewumi and R. J. Mumper. Gadolinium-loaded nanoparticles engineered from microemulsion templates. *Drug Dev. Ind. Pharm.* **28**:317–328 (2002).
19. S. Shalel, S. Streichman, and A. Marmur. The mechanism of hemolysis by surfactants: effect of solution composition. *J. Colloid Interface Sci.* **252**:66–76 (2002).
20. C. Chouly, L. Bordenave, R. Bareille, V. Guerin, A. Baquey, D. Pouliquen, C. Baquey, and P. Jallet. *In vitro* study of the hemocompatibility of superparamagnetic contrast agent for magnetic resonance imaging. *Clin. Mater.* **15**:293–301 (1994).
21. C. M. Jackson and Y. Nemerson. Blood coagulation. *Annu. Rev. Biochem.* **49**:765–811 (1980).
22. T. Matsuda, H. Takano, Y. Taenaka, K. Hayashi, and T. Nakamura. Role of surface charge on blood coagulation. *Polym. Preprints Jpn.* **30**:1668–1671 (1981).
23. A. L. Colpey. Platelets and physiological defense mechanism. In G. Gaetano and S. Garattini (eds.), *Platelets. A Multidisciplinary Approach*, Raven Press, New York, 1977, pp. 161–197.
24. Y. Ozaki. Measurements of platelet aggregation and attempts for standardization. *Sysmex J. Int.* **8**:15–22 (1998).
25. F. Ahmed, P. Alexandridis, H. Shankaran, and S. Neelamegham. The ability of poloxamers to inhibit platelet aggregation depends on their physicochemical properties. *Thromb. Haemost.* **86**: 1532–1539 (2001).
26. R. Gref, M. Luck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, and R. H. Muller. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids Surf., B Biointerfaces* **18**:301–313 (2000).
27. H. Otsuka, Y. Nagasaki, and K. Kataoka. PEGylated nanoparticles for biological and pharmaceutical applications. *Adv. Drug Deliv. Rev.* **55**:403–419 (2003).
28. D. Bazile, C. Prud'homme, M. T. Bassoullet, M. Marlard, G. Spenlehauer, and M. Veillard. Stealth Me.PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system. *J. Pharm. Sci.* **84**:493–498 (1995).
29. H. Fujii, S. Fujii, H. Togashi, M. Yoshioka, K. Nakai, H. Satoh, I. Sakuma, O. Kenmotsu, and A. Kitabatake. Attenuation of hypothermia-induced platelet activation and platelet adhesion to artificial surfaces *in vitro* by modification of hemoglobin to carry S-nitric oxide and polyethylene glycol. *Thromb. Res.* **100**: 519–528 (2000).
30. M. Amiji and K. Park. Surface modification of polymeric biomaterials with poly(ethylene oxide), albumin, and heparin for reduced thrombogenicity. *J. Biomater. Sci., Polym. Ed.* **4**:217–234 (1993).
31. C. Pan, J. Wang, H. Sun, P. Yang, Y. Leng, J. Chen, G. Wan, and N. Huang. Research of plasma adsorption and action of platelet adhesion of Dacron modified by plasma surface modification. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi* **21**:536–540 (2004).
32. J. E. Burchenal, C. R. Deible, T. E. Deglau, A. J. Russell, E. J. Beckman, and W. R. Wagner. Polyethylene glycol diisocyanate decreases platelet deposition after balloon injury of rabbit femoral arteries. *J. Thromb. Thrombolysis* **13**:27–33 (2002).
33. R. Male, D. G. Moon, J. S. Garvey, W. E. Vannier, and J. D. Baldeschwieler. Organ distributions of liposome-loaded rat platelets. *Biochem. Biophys. Res. Commun.* **195**:276–281 (1993).