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Original research

Activity of microemulsion-based nanoparticles at the human bio-nano interface: Concentration-dependent effects on thrombosis and hemolysis in whole blood

Timothy E. Morey, Manoj Varshney, Jason A. Flint, Christoph N. Seubert, W. Brit Smith, David G. Bjoraker, Dinesh O. Shah and Donn M. Dennis*
*Departments of Anesthesiology, Chemical Engineering, and Pharmacology & Experimental Therapeutics, The Engineering Research Center for Particle Science & Technology, University of Florida, P.O. Box 100254, 1600 S.W. Archer Road, Gainesville, FL 32610-0254, USA; *Author for correspondence (Tel.: (352) 846-1355; Fax: (352) 392-4719; E-mail: DDennis@ufl.edu)*

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Abstract

Background: Although microemulsion-based nanoparticles (MEs) may be useful for drug delivery or scavenging, these benefits must be balanced against potential nanotoxicological effects in biological tissue (bio-nano interface). We investigated the actions of assembled MEs and their individual components at the bio-nano interface of thrombosis and hemolysis in human blood.

Methods: Oil-in-water MEs were synthesized using ethylbutyrate, sodium caprylate, and pluronic F-68 (ME4) or F-127 (ME6) in 0.9% $NaCl_{w/v}$. The effects of MEs or components on thrombosis were determined using thromboelastography, platelet contractile force, clot elastic modulus, and platelet counting. For hemolysis, ME or components were incubated with erythrocytes, centrifuged, and washed for measurement of free hemoglobin by spectroscopy.

Results and conclusions: The mean particle diameters (polydispersity index) for ME6 and ME4 were 23.6±2.5 nm (0.362) and 14.0 ± 1.0 nm (0.008), respectively. MEs (0, 0.03, 0.3, 3 mM) markedly reduced the thromboelastograph maximal amplitude in a concentration-dependent manner (49.0 ± 4.2, 39.0 ± 5.6, 15.0 ± 8.7, 3.8 ± 1.3 mm, respectively), an effect highly correlated ($r^2 = 0.94$) with similar changes caused by pluronic surfactants (48.7 ± 10.9, 30.7 ± 15.8, 20.0 ± 11.3, 2.0 ± 0.5) alone. Neither oil nor sodium caprylate alone affected the thromboelastograph. The clot contractile force was reduced by ME (27.3 ± 11.1–6.7 ± 3.4 kdynes/cm^2, $P = 0.02$, $n = 5$) whereas the platelet population not affected (175 ± 28–182 ± 23 10^6/ml, $P = 0.12$, $n = 6$). This data suggests that MEs reduced platelet activity due to associated pluronic surfactants, but caused minimal changes in protein function necessary for coagulation. Although pharmacological concentrations of sodium caprylate caused hemolysis ($EC_{50} = 213$ mM), MEs and pluronic surfactants did not disrupt erythrocytes. Knowledge of nanoparticle activity and potential associated nanotoxicity at this bio-nano interface enables rational ME design for *in vivo* applications.

Introduction

Although nanotechnology offers unprecedented opportunities for major medical advances, the potential nanotoxicological effects of these particles, and their components, at the interface of biological tissues and nanotechnology (i.e., bio-nano interface) must be carefully assessed prior to human use. For example, although microemulsion-based nanoparticles (MEs) have been proposed for both drug scavenging and pharmaceutical delivery via the intravenous route, minimal-to-no safety information is available

regarding the potential nanotoxicological actions of these MEs at two critical levels of the bio-nano interface for intravenous applications in humans: (1) effects on hemostasis (i.e., blood clotting functions), and (2) effects on the integrity of erythrocyte cellular membranes (i.e., hemolysis). The present authors previously described MEs that cause rapid detoxification of bupivacaine in rodent perfused heart by a mechanism involving sequestration of this local anesthetic from the free, active phase into or onto the nanoparticles (Morey et al., 2001). However, further development for use in intact animals using intravenous administration requires more knowledge of possible complications caused by these novel particles at the bio-nano interface. Similarly, although MEs have been safely used to deliver drugs via the topical and gastrointestinal routes to humans, intravenous injection has been studied as possible delivery vehicles for drugs in animal models only (Zabka & Benkova, 1995; Sakaeda & Hirano, 1998; von Corswant et al., 1998; Dietl, 2002). Although the aforementioned reports allude to the possibility of safe intravenous injection of MEs, additional examination of possible toxic effects of MEs are needed. These investigations are especially needed for drug scavenging by nanoparticles as this use is likely to require much greater concentrations of nanoparticles than are required for drug delivery. For these reasons, detailed studies of the activities of the nanoparticles at the bio-nano interface are merited.

In addition, while individual components of nanoparticles may be considered safe for human use, the assembly of these components into a new particulate entity, with particle sizes at the nanoscopic scale, may render nanoparticles with properties markedly different from those of the individual components. Previously, Lapré and colleagues noted such an event when they observed synergistically increased hemolysis caused by mixed micelle formation from fatty acids and bile salts compared to the individual components (Lapré et al., 1992). Although the constituents of MEs described herein are designated as being 'generally recognized as safe' (i.e., GRAS) by the U.S. Food and Drug Administration, it remains unknown if the assembly into nanoparticles will cause markedly increased nanotoxicological activity in blood. Therefore, direct investigations of the possible adverse actions of these nanoparticles on biological tissues are necessary.

Although many toxicological assays are available, we elected to study the actions of these nanoparticles on blood functions because this tissue is the first bio-nano interface encountered by intravenously injected nanoparticles. The potential effects of these nanoparticles on essential hematological functions (e.g., thrombosis, oxygen transport) will be important determinants of the overall safety profile for these compositions of materials and route of delivery. Therefore, we ascertained the effects of oils, nonionic surfactants, ionic surfactant, and these components' coassembly as MEs on: (1) thrombosis that depends on both platelets and plasma proteins by using thromboelastography (TEG), platelet contractile force, clot elastic modulus, and platelet counting, and (2) the structural integrity of erythrocytes by measuring hemolysis in human whole blood.

Methods

Microemulsions

Synthesis

Two different MEs were prepared by dissolving the appropriate amounts of oil, surfactant, and cosurfactant into normal saline bulk media to obtain thermodynamically stable, isotropic, clear, colorless solutions. These MEs were alphanumerically designated as ME4 and ME6. The composition of ME4 was (mM): ethyl butyrate (50), Pluronic F-68 (10), and sodium caprylate (6). ME6 had the following formulation (mM): ethyl butyrate (160), Pluronic F-127 (8), and sodium caprylate (8). For both MEs, the bulk media was $0.9\%_{w/v}$ NaCl (i.e., normal saline) that is isotonic to human whole blood. In the present study no distinction is made between a ME and a swollen micelle.

Particle size measurement

The effective particle size and polydispersity (i.e., distribution width) of the MEs were measured by the dynamic light scattering method using a submicron particle size analyzer (90Plus, Brookhaven Instruments Corporation, Holtsville, NY). This instrument measures particle sizes that range from about 2 to 3000 nm in any liquid. In these measurements, random intensity fluctuations arising from the Brownian motion of particles are analyzed using photon correlation spectroscopy (PCS), where measurements are made at a 90° scattering angle. Associated software performs statistical analysis of data and calculates mean diameter (e.g., effective diameter) and a measure of the distribution width (polydispersity) of the particles. The polydispersity index is a value calculated from

a simple fit of a parabola to the correlation data and ranges from 0.0 to 1.0. Values greater than 0.7 indicate that the sample has a very broad size distribution and is probably not suitable for the PCS technique. The mean particle diameters (polydispersity index) for ME6 and ME4 were 23.6 ± 2.5 nm (0.362) and 14.0 ± 1.0 nm (0.008), respectively.

Phlebotomy

After approval by the University of Florida Institutional Review Board and informed consent of subjects, blood was collected by percutaneous phlebotomy from human volunteers in the following manner. Following application of an elastic tourniquet to the upper arm, a 22-gauge hypodermic needle (Greiner Bio-One, Longwood, FL) was inserted into the median cubital or cephalic vein in the antecubital fossa. Subsequently, 50 ml of blood was aspirated using vacuum tubes (Vacu-Tainer, Becton Dickinson, Franklin Lakes, NJ). These tubes were previously treated by the manufacturer with either sodium heparin that is appropriate for hemolysis and hemogram assays or buffered sodium citrate (3.8%) to store blood for thromboelastographic analysis.

Thromboelastography

Sample preparation
Disposable cups and piston covers (2000/3000 series, Haemoscope Corp., Niles, IN) were inserted into two, identical coagulation analyzers (2000DT, Haemoscope Corp.). The TEG units were powered and allowed sufficient time to achieve an operational temperature ($37.0 \pm 0.5^{\circ}$C). Either three, serially diluted samples (30 μl) of an experimental solution (i.e., ME) or an equal volume of a normal saline control ($0.9\%_{w/v}$ NaCl, pH 7.44) was transferred to each of the four cups. Subsequently, citrated whole blood (240 μl) was added to the cups followed by 0.2 M $CaCl_2$ (30 μl). The pistons were then advanced and retracted from the mixture five times to ensure adequate mixing. The pistons were subsequently inserted fully into the cups. After stylus alignment to the zero point of clot amplitude, the charting mechanism was engaged with simultaneous notation of the starting point on the paper record. After 60 min, the experiment was terminated. Each assay was performed in triplicate using blood obtained from different subjects. The control (i.e., normal saline) lane was varied between and within coagulation analyzers for different specimens in order to obviate spurious control data that might arise from using a single control lane.

Thromboelastograph measurement
The following conventional parameters were measured from TEG analysis: r time, k time, α angle, and maximal amplitude (MA) as previously described (Bjoraker, 1991). In brief, the r time is the duration of time measured from the starting mark to the point where the full width amplitude of 2 mm is achieved (Figure 1). The k time is the duration of time measured from the point where the amplitude was 2 mm (i.e., r time) to the point where the amplitude achieved 20 mm. If the TEG never reached a full width of 20 mm, this k time was arbitrarily recorded as 60 min that was the terminal duration of the experiment. To determine the α angle, we first drew a longitudinal centerline on the TEG tracing. A second line was drawn from the centerline at a point 2 mm before the r time to tangentially touch the amplitude at the k time (Figure 1). The acute angle between these two lines is the α angle. If the MA never achieved 20 mm, the α angle was recorded as 0°. The MA was measured as the greatest full width that was perpendicular to the centerline of the TEG tracing irrespective of time.

Clot structure

Clot structure was also characterized by measuring the platelet contractile force and elastic modulus (Hemostasis System, Hemodyne, Inc., Richmond, VA) of polymerizing fibrin and cellular components (Carr, 1995). This system applies a calibrated compressive force periodically to an aliquot of whole blood undergoing thrombin-initiated clotting. Unlike the TEG, no similar clot adherence to surfaces is required. Compression elastic modulus, a measure of clot structural 'stiffness,' which is dependent on both platelets and fibrinogen, is calculated during each minute of the clotting process. Also, platelet contractile force, a force dependent on only platelet number and function is calculated. Data were measured 7678 s after addition of thrombin to human whole blood.

Hemogram

To determine the hemogram, blood (40 μl) was aspirated into an automated cell counter (Baker System 9000, Serono-Baker Diagnostics Inc.,

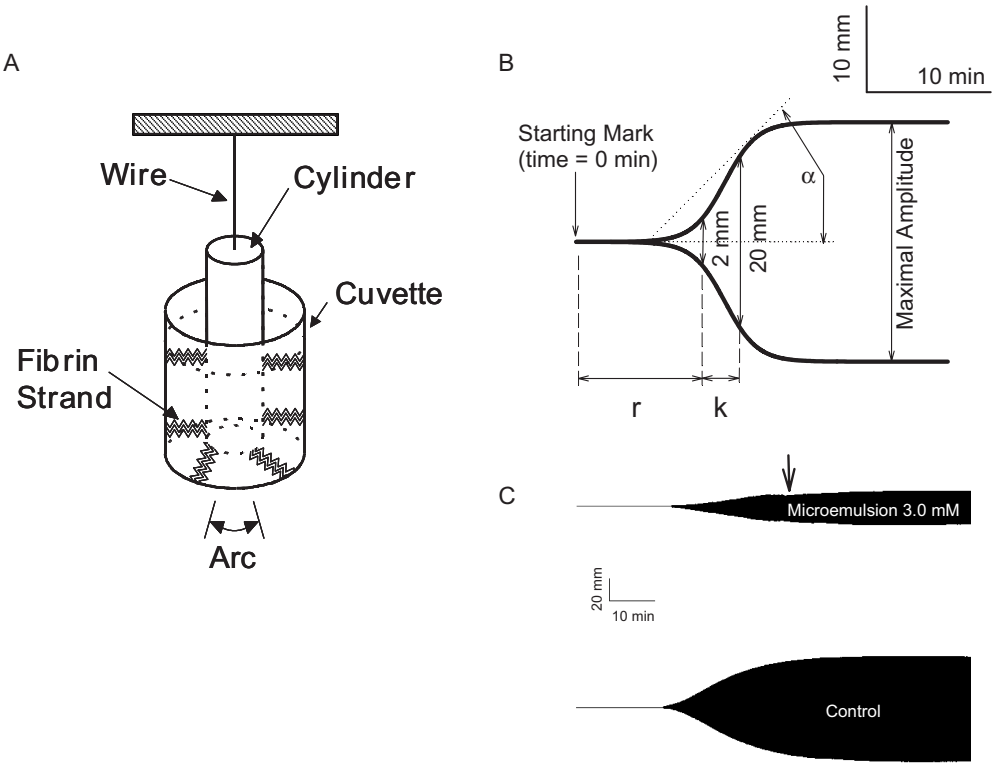

Figure 1. Schematic diagram of the operational principle of TEG device (Panel A), cartoon of idealized output plotted by the TEG device with endpoints noted (Panel B), and a sample tracing of a TEG determined from human whole blood in the presence and absence of microemulsion (Panel C). Panel A: a torsion wire suspends a metal piston that is immersed in a blood-filled cuvette. The disposable, plastic cuvette rotates back-and-forth in a 9.5° arc at a frequency of 0.1 Hz. As fibrin strands polymerize to connect the cuvette and piston surfaces, the back-and-forth movement of the piston commences. The magnitude of this piston rotation is amplified and recorded over time as illustrated in the next panels. Panel B: idealized data from a control specimen of blood. Noted are several parameters that characterize different portions of the thrombotic mechanism. The *r* time was defined to be the duration of time as measured from the starting mark (0 min) to the point where the full width amplitude achieved 2 mm. The *k* time was defined as the duration of time from the point where the amplitude was 2 mm (i.e., *r* time) to the point where the amplitude achieved 20 mm. The acute angle between the two dotted lines was defined to be the α angle. The MA was measured as the greatest full width that was perpendicular to the centerline of the TEG tracing irrespective of time. Panel C: sample TEGs of human whole blood in the presence and absence (Control) of a microemulsion. Note the overall diminished MA as well as the scallops (arrow) in the TEG tracing in the presence of microemulsion indicating clot instability. The control aliquot was normal saline (0.9% $NaCl_{w/v}$) whereas the microemulsion used in this example was ME4. See Methods for details measurement of TEG parameters and the synthesis and composition of this microemulsion. Each calibration bar applies only to its associated panel.

Allentown, PA) that relies on impedance technology to measure the hematocrit, leukocyte count, and platelet population. In all cases, the predilution setting was used.

Hemolysis

Cellular separation and incubation

Heparinized, whole blood (10 ml) was transferred into conical centrifuge tubes (15 ml, Fisher Scientific, Suwanee, GA) containing phosphate buffered saline (PBS, 4 ml). The PBS solution was formulated to the following composition (mM): 0.14 NaCl, 2.7 KCl, 10 Na_2HPO_4, and 1.8 KH_2PO_4. NaOH was added to the PBS solution to adjust the pH to 7.44 as measured by a calibrated, pH meter (320, Corning Inc., Corning, NY). The PBS and blood mixture was gently agitated by inversion and then spun at 2500 revolutions/min for 15 min in a fixed angle centrifuge (Marathon 8K, Fisher Scientific). Subsequently, the plasma supernatant was

decanted from the tube and replaced with an equal volume of PBS without disturbing the erythrocyte pellet retentate. Centrifugation and washing of erythrocytes was repeated twice more in this manner. After completion of the third washing, the erythrocyte pellet (3 ml) was removed, added to the PBS solution (11 ml), and gently mixed. This erythrocyte stock mixture was stored at room temperature (23°C) and subsequently used for incubation with control and experimental solutions.

Sample incubation

Aliquots (100 μl) of the erythrocyte stock mixture were added to experimental or control solutions (1000 μl) in polystyrene tubes. (Becton Dickinson, Franklin Lakes, NJ). Whereas the experimental solutions were MEs, the positive and negative control solutions were the deionized water (i.e., 100% hemolysis) and PBS (i.e., no hemolysis) solutions, respectively. Deionized water was obtained by ultrafiltration (D4200, Barnstead Thermolyne Inc., Dubuque, IA) of distilled water and had a minimal resistance of 18.2 MΩ-cm. Subsequently, the samples were mixed gently and incubated at room temperature for 15 min. Samples were then centrifuged at 2500 revolutions/min for 15 min to remove the erythrocytes and associated cellular debris. After aspiration of the supernatant (100 μl), a 40 : 1 ethanol : HCl solution (2000 μl) was added in order to ensure a clear solution. Each sample was then measured to determine the free hemoglobin concentration.

Free hemoglobin measurement

Supernatants were analyzed for the presence of free hemoglobin by UV-visible spectroscopy. The unshuttered output from a deuterium/tungsten light source (D_2Lite, World Precision Instruments, Inc., Sarasota, FL) was projected using a fiber-optic cable (FO-400-SMA-1M, World Precision Instruments, Inc.) through a quartz cuvette (3.5 ml, CUV2012-1, World Precision Instruments, Inc.) held by a sample holder (CUV, Ocean Optics, Inc., Dunedin, FL). Subsequently, the light was transmitted to a calibrated, single fiber-optic spectrometer (S-2000, World Precision Instruments, Inc.) with a bandwidth of 200–850 nm. The spectra were then recorded to the hard drive of a personal computer (Dimension XPS T700r, Dell Computer Corporation, Round Rock, TX) using software specifically designed for spectroscopic analysis (SpectraWare 2.8.1, World Precision Instruments, Inc.). Dark field data were performed manually and automatically subtracted from spectra. Reference data was also collected with a blank sample loaded into the cuvette in order to account for small fluctuations in the light source output. Absorbance was determined by measuring the optical density at 398 nm on the spectra. The absorbance of the solutions containing either PBS or deionized water was plotted against as 0% and 100% hemolysis, respectively, in order to generate a linear equation. This equation was used to transform hemoglobin absorbance in the experimental samples to percent of erythrocytes hemolyzed. All samples were repeated in duplicate.

Statistical analysis

All measurements are reported as mean ± standard deviation. Statistical analyses were performed using SSPS 11.5 (SPSS, Inc., Chicago, IL). Prior to parametric testing, the assumption of normality was validated using the Kolmogorov–Smirnov test with Lilliefors' correction. Paired, two-tailed t testing was used to analyze single comparisons between two groups (e.g., hemogram data). One-way or two-way analysis of variance followed by Tukey pairwise testing was used to analyze multiple comparisons among control and experimental values. Pharmacological curve-fitting software (Prism 2.01, GraphPad Software Inc., San Diego, CA) was used to perform the concentration-response data and generates pD_2 values where pD_2 equals $-\log_{10}(EC_{50})$ and EC_{50} is the concentration required to cause 50% of the maximal effect. Use of pD_2, vis-à-vis EC_{50}, values, is necessary to avoid heteroscedastic errors of linear regression when fitting data to untransformed equations of nonlinear regression analysis for concentration-response data. $P < 0.05$ was considered to be significant.

Results

Thrombosis

Thromboelastography

Microemulsions. The effects of synthesized MEs on TEG parameters were examined in human whole blood (Figure 2). Neither ME4 nor ME6 affected the r time ($P = 0.49$). However, the MEs significantly increased the k time in a concentration-dependent

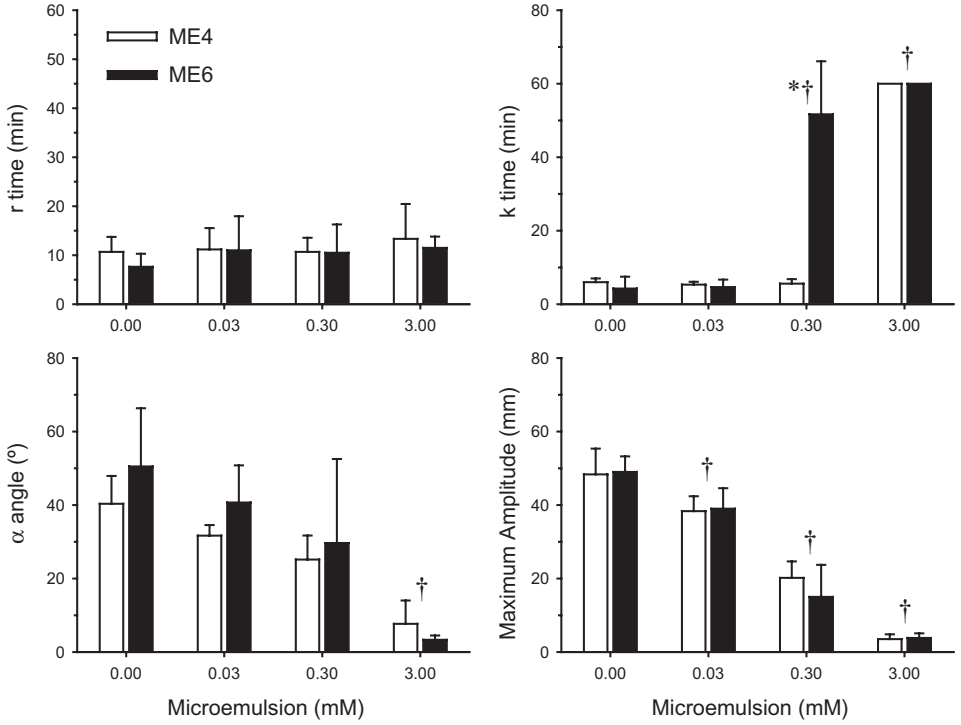

Figure 2. Concentration-dependent effects of two microemulsions designated ME4 and ME6 on the several indices of the TEG measured in human whole blood. Data expressed as mean ± standard deviation of 3–4 experiments as illustrated in Figure 3. The data point a 3.00 mM for the k time was always 0 min without variation for both ME4 and ME6. $P < 0.05$: $*$, ME4 compared to ME6 at 0.3 mM concentration only; †, ME4 and ME6 compared to all lesser concentrations. The legend applies to all panels. See Methods for details regarding the synthesis and composition of microemulsions.

manner with failure of the MA to achieve 20 mm necessary to measure the k time in all cases of 3.0 mM ME4 or ME6 (i.e., k values arbitrarily set to 60 min, see Methods). No statistical differences were observed between ME4 and ME6 on the k time except for the values noted at 0.30 mM ME ($P = 0.01$). The α angle was decreased in a concentration-dependent manner by the presence of MEs ($P < 0.001$), but did so irrespective of which ME (i.e., ME4 or ME6) was added to the cuvette. Similarly, the MA was significantly diminished in a concentration-dependent manner by increasing concentrations of MEs. However, no difference in reduction of MA was observed between a given concentration of ME4 or ME6 ($P = 0.68$). To further explore which components may potentially contribute to changes in TEG, the effects of oil as well as nonionic and ionic surfactant on thrombosis were studied.

Nonionic surfactant. Neither Pluronic F-127 nor F-68 significantly affected the r time ($P = 0.59$) as shown in Figure 3. Whereas F-127 and F-68 increased k time in a concentration-dependent manner ($P < 0.001$), no differences in effects were apparent between these nonionic surfactants ($P = 0.21$). Similarly, increasing concentrations of F-127 and F-68 markedly decreased the α angle ($P = 0.01$) although both did so with apparent equivalent magnitude ($P = 0.57$). In keeping with the k time and α angle observations, the MA was distinctly decreased by both F-127 and F-68 ($P < 0.01$), but in a similar manner when comparing F-127 to F-68 ($P = 0.24$).

We noted that the changes in MA observed with addition of nonionic surfactant (Figure 3) were similar to those seen after application of increasing concentrations of MEs (Figure 2). As written in Methods, F-127 and F-68 were the component nonionic surfactants of ME6 and ME4, respectively. Therefore, we replotted the MA data presented in Figures 2 and 4 for the effect of a ME and their constituent nonionic surfactants to examine the possibility of a relationship (Figure 4). There were no detectable differences between the

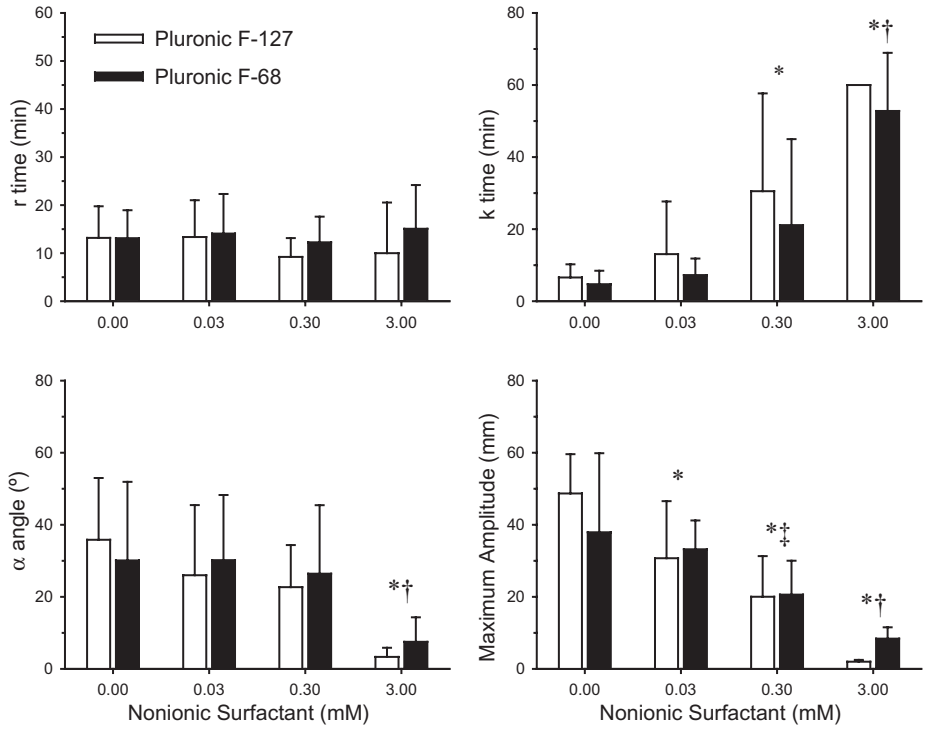

Figure 3. Effects of the nonionic surfactants F-127 and F-68 on the indices of the TEG measured in human whole blood. $P < 0.05$: ∗, compared to the absence of nonionic surfactant (i.e., 0.00 mM) for both groups; †, compared to 0.03 or 0.3 mM nonionic surfactant; ‡, compared to 0.03 mM only. Data expressed as mean ± standard deviation of 4–6 experiments. The legend applies to all panels.

effects of ME6 and F-127 (Figure 4A, $P = 0.61$) or ME4 and F-68 (Figure 4B, $P = 0.59$). Likewise, when the MA values for increasing concentrations of a ME and its nonionic surfactant were plotted against one another, high degrees of correlation were noted (Figure 4C and D).

Ionic surfactant. Sodium caprylate was selected as the ionic surfactant for both ME4 and ME6. When added alone to human whole blood ($n = 4$), sodium caprylate did not affect r time ($P = 0.97$), k time ($P = 0.78$), α angle ($P = 0.90$), or MA ($P = 0.89$) over a concentration range of 0–1 mM.

Oil. The addition of increasing concentrations of either ethylbutyrate or phenylbutyrate did not affect TEG parameters measured in human whole blood ($n = 4$) including the r time ($P = 0.74$), k time ($P = 0.12$), α angle ($P = 0.23$), and MA ($P = 0.70$). Phenylbutyrate was selected for study because this oil that has been injected intravenously into humans in large doses without apparent adverse effect (Gore et al., 2002).

Clot structure

Platelet and fibrin strand adherence to the surfaces of the piston and cuvette of the TEG may be artifactually reduced by the oily nature of ME constituents. Therefore, confirmation of the clot structure was characterized using the compression elastic modulus of the clot and the platelet contractile force. The elastic modulus measured in human whole blood ($n = 5$) after treatment with ME6 (3.0 mM) or an equal volume of normal saline was 6.7±3.4 and 27.3±11.1 kdynes/cm^2 ($P = 0.02$). The platelet contractile force in the presence and absence of ME6 was 3.65 ± 0.73 and 6.27 ± 3.00 kdynes ($P = 0.15$).

Platelet population

In order to determine if the ME affected clotting by reducing the number of platelets, hemograms were measured ($n = 6$). ME6 (3.0 mM) did not affect the platelet count compared to blood subjected to an equivalent volume of normal saline (i.e., control).

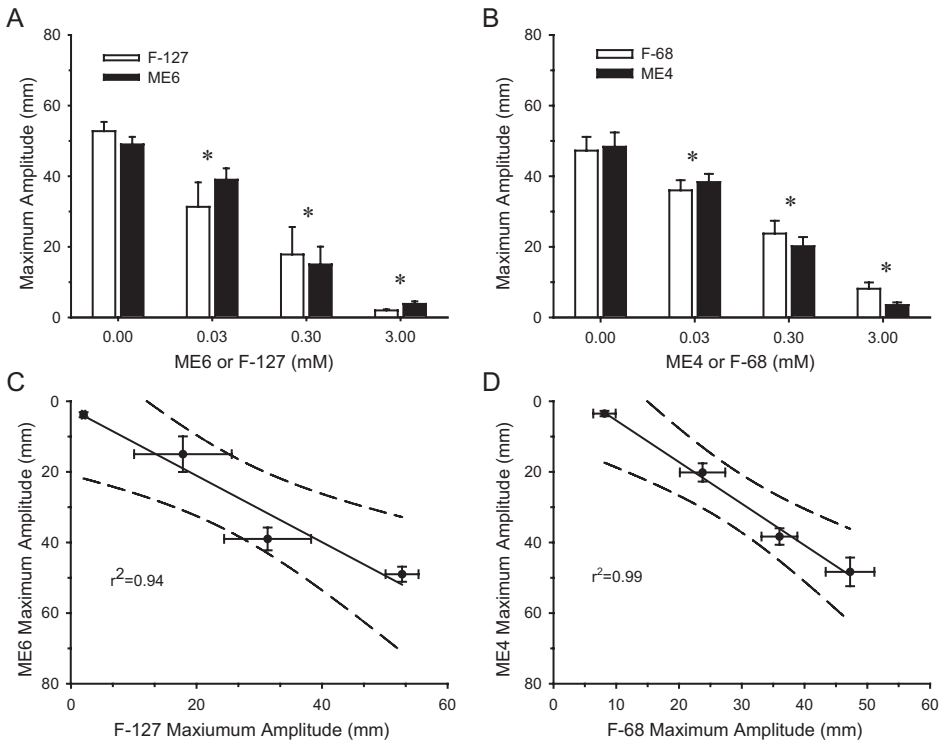

Figure 4. Replotted data illustrating the similar effects of increasing concentrations of microemulsions ME6 and ME4 (Figure 2) and nonionic surfactants F-127 and F-68 (Figure 4) on the MA measured in human whole blood by TEG. Panel A: effects of ME6 and its component nonionic surfactant F-127 on the MA. Panel B: effects of ME4 and its component nonionic surfactant F-68 on the MA. Panels C and D: linear plots of the effects of different concentrations of ME6 and F-127 (Panel C) or ME4 and F-68 (Panel D) on the MA with the correlation coefficients noted and dashed lines inscribing 95% confidence intervals. $P < 0.05$: $*$, ME6, ME4, F-127, or F-68 compared to all lower concentrations of ME6, ME4, F-127, or F-68, respectively. Data expressed as mean $\pm$ standard deviation of 3–6 experiments. The legends apply only to their respective panels.

That is, the platelet count determined after ME4 or control treatment was 182 ± 23 and 175 ± 28 10^6/ml, respectively ($P = 0.12$). Although platelet counting determines changes in the overall quantity of the platelet population, this method cannot assess changes in function of platelets caused by MEs.

Hemolysis

Microemulsions

Incubation of erythrocytes with ME6 did not cause significant hemolysis of these cells. That is, the relative absorbance of light at 398 nm was 3.143 ± 0.062 and 0.126 ± 0.003 in the presence of deionized water (i.e., positive control; 100% hemolysis) or PBS (i.e., negative control; 0% hemolysis), respectively. In the presence of ME6 ($n = 7$), the relative absorbance was 0.124 ± 0.003, a value not significantly different from the absorbance observed with addition of normal saline.

Ionic surfactant

Sodium caprylate caused significant hemolysis at concentrations equal to or greater than 200 mM (Figure 5). Using a sigmoidal concentration-response analysis to calculate the concentration of sodium caprylate necessary to cause half-maximal hemolysis, this ionic surfactant demonstrated a pD_2 (EC_{50}) value of 3.672 ± 0.002 ($\sim$213 mM).

Nonionic surfactant

Neither Pluronic F-68 nor F-127 caused significant hemolysis. That is, hemolysis values for erythrocytes exposed to these Pluronic surfactants (0.03, 0.3, 3 mM)

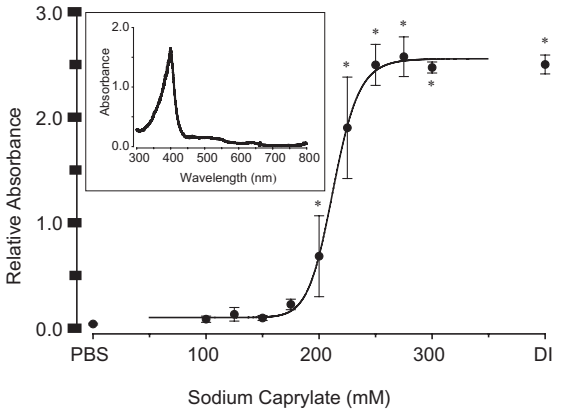

Figure 5. Typical UV-visible spectrum of free hemoglobin obtained from human whole blood and the effects caused by addition of sodium caprylate. Shown is the rise in relative absorbance at 398 nm (i.e., hemolysis) measured with increasing concentrations of the ionic surfactant sodium caprylate or during control incubation conditions with PBS or deionized water (DI). Inset: representative UV-visible spectrum recorded in the presence of sodium caprylate. Peak values of absorbance were measured at 398 nm. See Methods for details of sample preparation and hemoglobin measurement. $P < 0.05$: *, compared to PBS control. Data is expressed as mean ± standard deviation of 4–6 experiments in human whole blood.

did not significantly differ from hemolysis measured in the presence of isotonic PBS, a negative control.

Discussion

This study is the first to systematically detail the effects of MEs and their components on thrombosis and hemolysis in order to determine potentially limiting factors for *in vivo* use by these nanoparticles at this bio-nano interface. With respect to thrombosis, the data indicate that defects in coagulation noted at higher concentrations of MEs are primarily due to the presence of nonionic surfactants interfering with platelet function (qualitative defect), but not with the overall number of platelets (qualitative defect). In contrast, whereas neither ME6 nor nonionic surfactants hemolyzed blood, pharmacological concentrations of ionic surfactants caused erythrocyte membrane disruption. Knowledge of how the constituents and their coassembly into nanoparticles act at these critical elements of the bio-nano interface enable rational design and selection of nanoparticles (e.g., MEs) for novel, future nano-medical applications such as drug detoxification and delivery.

Thrombosis

The reductions in clotting parameters in human blood reported herein were primarily attributable to the actions of the nonionic surfactants as evidenced by the high degree of correlation between the effects of MEs and their respective Pluronic surfactants as well as the failure of other ME components (e.g., oil, sodium caprylate) to cause significant changes in the TEG profile. In addition, because the Pluronic surfactants markedly reduced the MA, a platelet-dependent phenomenon, while preserving the *r* time we propose that these nonionic surfactants selectively reduce platelet function with minimal effects on proteins essential to form clot. Although other parameters of protein function (i.e., *k* time, α angle) decreased, these changes were likely an artifact of the extreme decrements in MA at higher concentrations of MEs or Pluronic surfactants. That is, *k* time changes tended to reciprocate the reduction in MA whereas the α angle decreased proportionally with the MA. Probably, when MA fell precipitously to a value less than 20 mm, neither the *k* time nor the α angle that tangentially approaches the TEG at 20 mm amplitude could be reliably measured. For these reasons, we believe that the *r* time and MA more accurately reflect the effects of MEs and its constituents on coagulation as assessed by the TEG and imply that platelets were selectively affected.

These observations of diminished platelet activity in the presence of Pluronic surfactants are consistent with previous reports describing the anticoagulant properties of these nonionic surfactants. For example, Amiji and Park demonstrated that certain Pluronics inhibited platelet adhesion on dimethyldichlorosilane-treated glass and low-density polyethylene (Amiji & Park, 1992). In addition, the PEO component of Pluronics, when coated on the surfaces of certain biomaterials such as polyethylene terephthalate (i.e., Dacron) destined for contact with blood, markedly reduced platelet adhesion by a mechanism involving stearic repulsion (Park et al., 2000). Likewise, we found that platelet function was markedly decreased, but that the overall platelet population remained unchanged in the presence of MEs as determined by a cell counter. Besides the TEG results reported presently, the observations of clot elastic modulus and platelet contractile force also support the notion that the MEs primarily affect clotting by inhibiting platelet activity as these assays are triggered by thrombin and do

not depend on direct linkage to the surface of the TEG pistons (Amiji & Park, 1993). For these reasons, the concentration of nonionic surfactant in MEs is an important parameter affecting clotting and should be a consideration when designing MEs for *in vivo* use for various purposes such as drug delivery or removal. Reassuringly, however, this action at the bio-nano interface is seemingly direct and simple in that coassembly of Pluronics with other components into a ME does not cause synergistic decreases in the MA. In addition, ongoing investigations focused on the effect of ME-based nanoparticles into living rat demonstrate that changes in TEG are transient (data not shown). Finally, the clinical relevance of changes in TEG to anti-thrombotic properties cannot be fully delineated based on these *in vitro* studies. More thorough characterization of the effects MEs and Pluronic surfactants on coagulation requires *in vivo* testing in living animals.

Hemolysis

The possible destabilizing effects of ME-based nanoparticles on erythrocyte cytoplasmic membranes may reduce or preclude employment for *in vivo* administration. However, the data presented herein indicates that the ethylbutyrate-based ME6 caused minimal-to-no hemolysis in human erythrocytes. Several lines of mutually supportive evidence are concordant with this observation and suggest that these nanoparticles (and the constituents) do not destabilize erythrocyte cellular membranes and may be safe. First, at least two components of MEs, the nonionic surfactants and oil, used herein may be administered at relatively high concentrations to blood with minimal-to-no hemolysis. For example, Lowe and colleagues demonstrated that F-68 at concentrations up to $4\%_{w/v}$ did not cause hemolysis in rodent blood whereas 10% lysed only $0.5 \pm 0.3\%$ of red blood cells (Lowe et al., 1995). Likewise, studies conducted in human blood indicate that concentrations $\geq$20% emulsion that included F-68 did not affect the mechanical fragility of normal or sickled erythrocytes (Padilla et al., 1975). In addition, both F-68 and F-64 have been studied clinically without adverse event in order to reduce hemolysis for patients undergoing surgery requiring cardiopulmonary bypass or to reduce pain crisis severity in sickle cell patients, respectively (Danielson et al., 1970; Orringer et al., 2001). With respect to oil essential to construct a ME, phenylbutyrate has been administered intravenously complication and in large doses (375 mg/kg/day) for 6–12 weeks as a cell differentiator to patients suffering from myelodysplastic syndromes and leukemia (Gore et al., 2002). Unfortunately, the present methodology for hemolysis allows only aqueous experimental solutions and, thereby, precludes meaningful testing of oils. Second, some lipid emulsions significantly reduce the hemolytic activity caused by some profoundly lytic agents. That is, Jumaa and Muller used MEs to reduce the hemolysis caused by sodium oleate, a highly lytic agent (Jumaa & Muller, 2000). This protective effect was suggested to be due to incorporation of lytic agents within the core of the MEs wherein the sodium oleate would be unavailable to interact with erythrocyte membranes, a hypothesis that alludes to the possibility of *in vivo* drug detoxification. Third, MEs have been advocated and used as a drug delivery vehicle for extremely lipophilic drugs instead of more traditional emulsion-based vehicles such as Cremaphor that cause significant hypersensitivity reactions. For example, a ME (mean particle size 17.2 nm) that was synthesized primarily from egg phosphatidylcholine and Pluronic F-68 was safely used to solubilize and deliver paclitaxel, a chemotherapeutic agent, in guinea pig. Similarly, soy oil-based MEs (particle size range: 60–200 nm) were injected into rats without disturbing plasma electrolyte values (von Corswant et al., 1998). In these models, no adverse reactions such as hemolysis were reported.

Notwithstanding the present and previous observations regarding the relatively innocuous effects of MEs, oil, and nonionic surfactants on erythrocytes, cosurfactant fatty acids are known to cause hemolysis. However, both the length and presence of double carbon bonding in the carbon chain of the fatty acid are critical parameters affecting the hemolytic activity of these ionic surfactants. For example, Lapré and colleagues determined that a fatty acid of eight single bonded carbons (i.e., caprylate) had minimal-to-no hemolysis in human blood (Lapré et al., 1992). However, longer chains and double bonding markedly increased hemolysis although the relationship was complex and depended on incubation temperature (Csordas & Rybczynska, 1988; Lapré et al., 1992). In addition to these considerations, the overall concentration of these ionic surfactants at the bio-nano interface must be carefully considered with respect to the broader horizon of use. That is, we determined that a concentration as great as 150 mM sodium caprylate caused minimal-to-no hemolysis of human erythrocytes. In contrast, the concentration of sodium caprylate in

ME4 and ME6 was 45 and 8 mM, respectively. If one considers possible further dilution in the human intravascular space, ~5 l for a 70 kg adult, then the quantity of fatty acid cosurfactant rapidly diminishes to concentrations that are probably not hemolytic. In fact, sodium caprylate is used in the pharmaceutical armamentarium to stabilize proteins (e.g., albumin) for human use and has been employed without adverse event (Ebbesen & Brodersen, 1982). For these reasons, the data presented herein suggest that oil-in-water MEs are relatively innocuous with respect to erythrocyte lysis although the final concentration, chain length, and carbon bonding of fatty acid cosurfactant may be an important parameter to avoid erythrocyte hemolysis.

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Implications

These data suggest that whereas the oil in the MEs appeared relatively innocuous, both the nonionic and ionic surfactants have the capacity to cause defects in platelet function and hemolysis, respectively, if used in elevated concentrations. These properties of surfactants should be considered when designing nano-medical applications such as MEs-based nanoparticles for *in vivo* use (e.g., drug delivery or removal). However, whether the changes in thrombosis and hemolysis noted herein are directly applicable to the occurrence of adverse events in living animals deserve further consideration. Regardless, the results of this study are reassuring in the sense that a broad array of different types of molecules can be used alone or coassembled into apparently safe nanoparticles having great interfacial areas for a variety of nano-medical applications. Such knowledge is useful in order to enhance rational design of these nanoparticles for use in living rodent and other species.

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