NANO LETTERS

2008
Vol. 8, No. 8
2180-2187

Method for Analysis of Nanoparticle Hemolytic Properties in Vitro

Marina A. Dobrovolskaia,* Jeffrey D. Clogston, Barry W. Neun, Jennifer B. Hall, Anil K. Patri, and Scott E. McNeil

Nanotechnology Characterization Laboratory, Advanced Technology Program, SAIC-Frederick Inc., NCI-Frederick, Frederick, Maryland 21702

Received February 25, 2008; Revised Manuscript Received May 2, 2008

ABSTRACT

Hemolysis (destruction of red blood cells) in vivo can lead to anemia, jaundice, and other pathological conditions; therefore the hemolytic potential of all intravenously administered pharmaceuticals must be evaluated. Nanotechnology-derived devices and drug carriers are emerging as alternatives to conventional small-molecule drugs, and in vitro evaluation of their biocompatibility with blood components is a necessary part of early preclinical development. The small size and unique physicochemical properties of nanoparticles may cause their interactions with erythrocytes to differ from those observed for conventional pharmaceuticals and may also cause interference with standardized in vitro tests. Separating true hemolytic responses from the false-positive or false-negative results caused by particle interference is important for correct interpretation of these tests. Here we describe validation of an in vitro assay for the analysis of nanoparticle hemolytic properties and discuss observed nanointerferences with the assay. We propose alternative methods to avoid misleading results from nanoparticles and discuss the potential relevance of nanoparticle in vitro hemolytic properties to in vivo systems.

. 1. Introduction. Nanoparticles are a relatively new class of biomedical products. Their potential use in medical devices or as drug carriers offers opportunities for novel therapy of complex disorders such as cancer and inflammatory and neurodegenerative diseases.[1,2] As with any device or pharmaceutical, nanoparticles intended for biomedical application must be subject to biocompatibility testing before regulatory approval for administration to patients. Although numerous efforts are underway to define the critical parameters which must be addressed during preclinical evaluation of nanomaterials engineered for use in medicine,[3–6] few, if any, harmonized protocols for testing nanoparticle biocompatibility are currently available.

Determination of hemolytic properties is one of the most common tests in studies of nanoparticle interaction with blood components.[7–25] Interpreting the results of these studies is complicated due to variability in experimental approaches and a lack of universally accepted criteria for determination of the test-result validity. Most in vitro studies of particle-induced hemolysis[7–14,17–20,22–25] evaluate the percent hemolysis by spectrophotometriclly detecting plasma-free hemoglobin derivatives after incubating the particles with blood and then separating undamaged cells by centrifugation. The incubation time, wavelength at which hemoglobin is quantified, and blood conditions (e.g., use of purified erythrocytes rather than whole blood, and inclusion of various anticoagulants) vary significantly from one study to another. In addition to these variables, differences in relative centrifugal force, blood storage time and conditions, and blood sources (human or rabbit) can further complicate meaningful comparison of the results from disparate studies.

Here, we integrate aspects of several methods for analysis of hemolytic properties into a 96-well plate assay and optimized this assay for testing nanoparticles. Our assay leverages an existing standard practice (ASTM F-756-00) for analysis of hemolytic properties of medical devices.[26] We scaled this standard practice to a 96-well plate format assay and conducted a 1 month validation aimed at determining its reproducibility, precision, and accuracy, as well as qualification of negative and positive nanoparticle-relevant controls. We refer to this initial phase of the validation as the prestudy validation. We subsequently used our assay to analyze various types of nanomaterials including polymers, gold nanoshells, nanoliposomes, nanoemulsions, fullerene derivatives, gold colloids, and dendrimers. We refer to this second phase as in-study validation. The second phase was conducted over a 2 year period and included identification and resolution of nanoparticle interference with the assay, in addition to evaluation of reproducibility, precision, accuracy, and control qualification. In this report we present assay performance from both phases (prestudy and in-study) of validation and discuss the observed interference with the assay which we ascribe to uniquely "nano" interactions. We

* Correspondence and requests for materials should be addressed to M.A.D. (marina@mail.nih.gov), Nanotechnology Characterization Laboratory, SAIC-Frederick Inc., NCI-Frederick, 1050 Boyles St., Bldg 469, Frederick MD, 21702; phone, (301)-846-6939; fax, (301)-846-6399; e-mail: ncl@mail.nih.gov.

10.1021/nl0805615 CCC: $40.75
Published on Web 07/08/2008

also provide practical solutions for overcoming these interferences and define critical parameters which must be monitored to avoid spurious (false-positive or false-negative) results.

2. Experimental Section. 2.1. Assay Development and Validation. The assay presented here is an adaptation of existing standard F-756-00,[26] which is based on colorimetric detection of red cyanmethemoglobin in solution. In this assay, analyte particles are incubated in blood, hemoglobin released by damaged cells is oxidized to methemoglobin by ferricyanide in the presence of bicarbonate, and then cyanide converts the methemoglobin to cyanmethemoglobin. The undamaged erythrocytes are removed by centrifugation, and the amount of cyanmethemoglobin in the supernatant is measured by spectrophotometry at its absorbance maximum wavelength, 540 nm. This measured absorbance is compared to a standard curve to determine the concentration of hemoglobin in the supernatant, and this hemoglobin concentration is compared to that in the supernatant of a blood sample not treated with nanoparticles to obtain the percentage of particle-induced hemolysis (referred to as percent hemolysis). The standard curve is created from a linear fit of several absorbance measurements made at 540 nm on a hemoglobin standard sample (treated with ferricyanide and bicarbonate) over a range of hemoglobin concentrations from 0.025 to 80 mg/mL (we refer to these samples as "calibration standards").

The precision of the measured hemoglobin concentrations (determined as percent coefficient variation, % CV) and accuracy (determined as percent difference from theoretical, PDFT), with the theoretical concentration corresponding to the value of the standard curve are calculated for each sample over all assay runs. $[\bar{h}]$ is the mean measured hemoglobin concentration for a particular sample over all runs, and % CV is the percentage of the mean of the standard deviation (% CV $= 100 \times$ SD/$[\bar{h}]$) and % DFT is the percent difference of the mean concentration from the theoretical concentration (PDFT $= 100 \times ([\bar{h}] - [h]_{\text{theory}})/[h]_{\text{theory}}$). In addition to the calibration standards, the assay includes measurement on hemoglobin standard samples (treated with ferricyanide and bicarbonate) with known concentrations, we refer to these samples as "quality controls".

The experimental procedure described in the ASTM standard was modified by: (1) scale to a 96-well plate format, (2) introduction of nanoparticle-relevant controls, and (3) modification of acceptance criteria to reflect ICHS6 requirements for bioanalytical method validation.[27] The results of this assay (expressed as percentage hemolysis with respect to negative control) are used to evaluate the acute in vitro hemolytic properties of nanoparticles. The detailed experimental procedure is available at the NCL Web site[28] and in the Supporting Information section.

2.2. Reagents. Cyanmethemoglobin (CMH) reagent and hemoglobin standard were purchased from StanBio. Ca^{2+}/Mg^{2+} free DPBS, poly(ethylene glycol) (average molecular weight 8000), and poly-L-lysine (PLL) hydrobromide (MW 150000−300000) were from Sigma-Aldrich. Polystyrene nanoparticles with sizes of 20, 50, and 80 nm were purchased from Duke Scientific Corp. Colloidal gold nanoparticles with sizes of 5, 10, 20, 30, 40, and 50 nm were from TedPella Inc. G5 and G6 PAMAM dendrimers with amine, carboxy, and hydroxyl surface were from Dendritic Nanotechnologies Inc. Nanoliposomes were kindly provided by Dr. Mark Kester (Pennsylvania State University). Nanoemulsions were kindly provided by Dr. Mansoor Amiji (Northeastern University). Triazine dendrimers and water-soluble fullerene derivatives (C3) were kindly provided by Dr. Eric Simanek (Texas A&M University) and C60 Inc., respectively. Abraxane, Propofol, and Doxil were purchased through the NIH pharmacy.

2.3. Research Donor Blood. Healthy volunteer blood specimens were drawn under NCI-Frederick Protocol OH99-C-N046. Blood was collected in BD vacutainer tubes containing lithium heparin (hemolsyis test) or sodium citrate (platelet aggregation test) as anticoagulant. Specimens from at least three donors were pooled.

3. Results and Discussion. 3.1. Assay Validation. The calibration standards and quality controls for both prestudy and in-study validation were prepared as described in the Experimental Section. Measurement of assay precision (determined as percent coefficient variation (% CV)) and accuracy (determined as percent difference from theoretical (PDFT), with the theoretical value corresponding to the value of the standard curve) were calculated from 7 and 12 standard curves for prestudy and in-study validation, respectively. The results of the first (prestudy) phase are presented in Table 1A. The results of the second (in-study) phase are summarized in Table 1B. In both phases we have observed high precision and accuracy, in that both the % CV and PDFT are less than 10%. The lowest calibration standard quantification was slightly less accurate (PDFT $= -14.3\%$) in the second phase (Table 1B). These values are within the limits suggested for analytical assays by the US FDA[29] and those used in industry in support of pharmaceutical development.[27,30]

To determine intra-assay (i.e., within one run) precision and accuracy, quality control hemoglobin standard samples were analyzed six times in one prestudy validation run. The results are presented in Table 1B. Both precision and accuracy in this test were high, as % CV and PDFT were below 10%.

Interassay precision and accuracy were determined by analysis of three quality control hemoglobin standard samples, each analyzed the prestudy and in-study validation, as described above. The quality control samples were prepared from a hemoglobin standard of a known concentration of 80.0 mg/mL. Theoretical (calculated) concentrations of the quality control samples were as follows: 0.0625 (low), 0.125 (mid), and 0.625 (high) mg/mL. The results for prestudy validation are presented in Table 1A. The results of in-study validation for quality controls are summarized in Table 1B. The data demonstrated high precision and accuracy (% CV and PDFT $<10\%$), with the exception of one low QC level in the in-study validation, for which variability between runs (% CV) was 12%. This low precision is still within the limits suggested by the FDA and was for the lowest concentration

Table 1. Assay Precision and Accuracy[a]

A. Prestudy Validation

	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	QC1	QC2	QC3	IA1	IA2	IA3
$[\bar{h}]$, mg/mL	0.800	0.399	0.201	0.100	0.050	0.025	0.635	0.124	0.059	0.606	0.122	0.057
$[\bar{h}]$ theory	0.800	0.400	0.200	0.100	0.050	0.025	0.625	0.125	0.063			
N	7	7	7	7	7	7	14	14	14	6	6	6
SD	0.0007868	0.00138013	0.00149603	0.001496026	0.002497618	0.001902379	0.009	0.003	0.002	0.002	0.003	0.002
% CV = 100 × SD/$[\bar{h}]$	**0.0983**	**0.346**	**0.743**	**1.49**	**5.02**	**7.74**	**1.462**	**2.505**	**3.589**	**0.404**	**2.281**	**3.760**
PDFT = 100 × $[\bar{h}]$ − $[\bar{h}]_{theory}/[\bar{h}]_{theory}$	**0.0536**	**−0.321**	**0.643**	**0.286**	**−0.571**	**−1.71**	**1.531**	**−0.686**	**−5.143**	**−3.040**	**−2.267**	**−9.067**

B. In-Study Validation

	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	QC1	QC2	QC3
$[\bar{h}]$ theory, mg/mL	0.8	0.4	0.2	0.1	0.05	0.025	0.625	0.125	0.0625
$[\bar{h}]$, mg/mL	0.801	0.398	0.199	0.106	0.050	0.021	0.624	0.124	0.059
N	12	12	12	12	12	12	24	24	24
SD	0.001	0.0026726	0.003512	0.0023094	0.000787	0.001773	0.013	0.007	0.007
% CV	**0.1248**	**0.671**	**1.763**	**2.18**	**1.57**	**8.28**	**2.021**	**5.677**	**12.007**
PDFT	**0.1562**	**−0.458**	**−0.417**	**5.750**	**0.167**	**−14.33**	**−0.200**	**−0.833**	**−6.133**

[a] Standard curves were created from linear fits to absorbance measurements made at 540 nm on six calibration hemoglobin standard samples (designated CAL1−CAL6) with theoretical hemoglobin concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 mg/mL. The measured hemoglobin concentrations are those predicted by the linear regression. $[\bar{h}]$ is the mean measured hemoglobin concentration for a particular sample over all seven runs. Quality controls (designated QC1−QC3) analyzed within each individual run and an additional set of quality controls analyzed in one run (designated IA1−IA3) were prepared by spiking various amounts of hemoglobin standard with known concentration into CMH reagent. Theoretical concentrations of quality controls were selected to fit high, middle, and low parts of the standard curve and were 0.625, 0.125, and 0.0625 mg/mL, respectively. % CV (precision) and PDFT (accuracy) and for all standard curves and quality controls are less than 15%, which is in agreement with current standards used by bioanalytical industry. Key: QC, quality control; Cal, calibrator; IA, interassay quality control; PDFT, percent difference from theoretical; SD, standard deviation.

calibration standard, which is most prone to variable absorbance readings.

This assay validation demonstrates that our 96-well format assay for quantitative determination of hemoglobin is reproducible and robust. Although there is no formal recommendation to the cutoff limit for precision and accuracy of the hemoglobin-specific assay, the obtained values are within requirements described for small molecules, i.e., within 15%.[27,29]

3.2. Selection and Qualification of Positive and Negative Controls. An important aspect of an accurate evaluation of a material's hemolytic properties is the use of relevant positive and negative controls. ASTM standard F-756-00 was developed for medical devices and uses controls which are impractical for the evaluation of nanoparticles. Since the small size and unique physicochemical properties of nanoparticles may cause a variety of nanoparticle-specific interferences, initially we aimed to include controls which were nanoparticles themselves. Poly(ethylene glycol) (PEG) solution was chosen as a negative control since this polymer is frequently attached to nanoparticles to increase compatibility with blood components.[1,31] Selection of the positive control was more challenging; originally we chose cationic polystyrene nanoparticles with nominal sizes of 20, 50, and 80 nm. Initially, high hemolytic activity was observed with each of these formulations (data not shown). However, when the surfactant/detergent Triton X-100 is removed from the polystyrene particle solution by dialysis prior to the assay, the particles adsorb plasma-free hemoglobin, aggregate, and are removed along with undamaged cells during centrifugation. Triton X-100 is known to be hemolytic,[10] and this was the reason for excluding polystyrene nanoparticles from positive control qualification. Another polymer which may produce a hemolytic response due to its high positive charge is poly-L-lysine (PLL). Initially, this polymer, when tested in our assay, produced percent hemolysis greater than 8%, which according to the ASTM standard F756 qualifies this material as a positive control. Interassay performance of the positive (PLL) and negative (PEG) control samples was evaluated during prestudy validation. We compared the percent hemolysis calculated for each control sample from six validation runs. The results are presented in Table 2A. To compare intra-assay performance, the positive and the negative control samples were analyzed six times in one validation run. The results are also presented in Table 2A.

Further studies of PLL in 96-well format assay revealed a high degree of interlot variability, depending on the lot of PLL and storage time; the percent hemolysis observed with this material varied from 5 to 70% (Figure 1). Although precision and accuracy of PLL in prestudy validation conducted using the same lot of polymer met the acceptance limits (i.e., % CV and PDFT <15%, Table 2A), the high degree of interlot variability (Figure 1) disqualified this material from further use as positive control in this assay.

The only material, which produced hemolysis consistently over the entire 2 year period was Triton-X 100 (Table 2B). This is consistent with previously published studies,[12] although other studies have used distilled water as a positive

Table 2. Qualification of Positive and Negative Controls[a]

A. Prestudy						B. In-Study				
	run no.	interassay NC	interassay PC	intra-assay NC	intra-assay PC		run no.	date	NC	PC
% hemolysis	1	BLOQ	35.7	BLOQ	33.9	% hemolysis	1	13 Dec 2005	4.7	98.500
		BLOQ	36.1	BLOQ	32.4				4.7	94.400
	2	BLOQ	38.4	BLOQ	32.1		2	17 Oct 2006	BLOQ	89.8
		BLOQ	37.9	BLOQ	31.5				BLOQ	90.7
	3	BLOQ	26.9	BLOQ	34.4		3	13 June 2006	BLOQ	94.7
		BLOQ	25.1	BLOQ	33.8				BLOQ	96.5
	4	BLOQ	26.8				4	15 Aug 2006	BLOQ	105.0
		BLOQ	27.1						BLOQ	104.0
	5	BLOQ	30.9				5	1 Aug 2006	BLOQ	100.0
		BLOQ	31.5						BLOQ	101.0
	6	BLOQ	29.3				6	20 Mar 2007	BLOQ	87.1
		BLOQ	29.6						BLOQ	88.1
							7	22 Mar 2007	BLOQ	81.3
N		12	12	6	6				BLOQ	85.6
mean		NA	31.3	NA	33.0		8	21 Mar 2007	BLOQ	99.8
SD		NA	4.66	NA	1.17				BLOQ	91.4
% CV		NA	14.91	NA	3.54		9	13 Mar 2007	3.700	112.0
									BLOQ	132.0
						N			18	18
						mean			4.4	97.3
						SD			0.58	11.59
						% CV			13.22	11.91

[a] Precision (% CV) of positive and negative controls was evaluated during prestudy (A) and in-study (B) validation. PBS was used as a negative control during both phases of validation. Poly-L-lysine was tested as positive control during prestudy validation (panel A), while TritonX-100 was tested during in-study validation (panel B). BLOQ is used to report results below lower limit of the assay quantification. Key: NC, negative control; PC, positive control; NA, not applicable; SD, standard deviation.

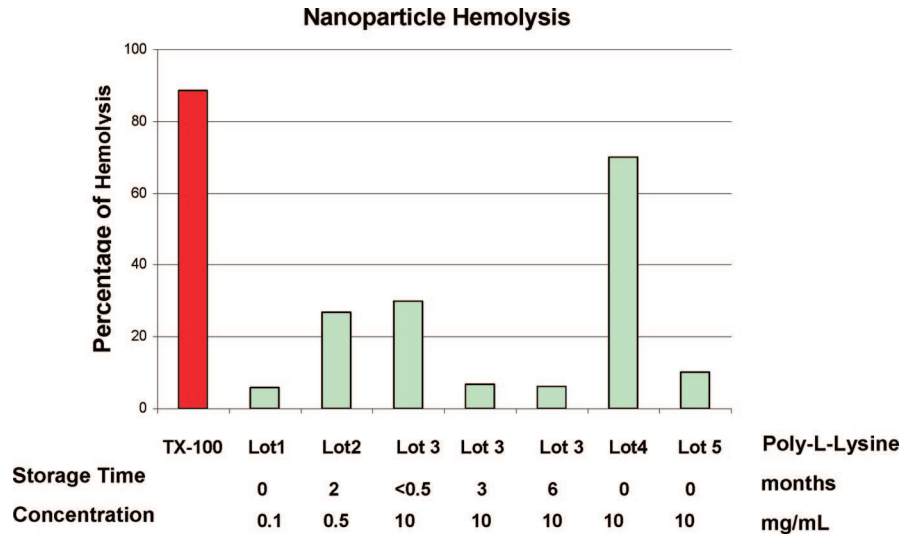

Figure 1. PLL disqualified as a positive control. Poly-L-lysine (PLL) is cationic polymer. Evaluation of PLL in 96-well format assay revealed a high degree of interlot variability. Although precision and accuracy of PLL in prestudy validation conducted using the same lot of polymer met the acceptance limits (i.e., % CV and PDFT <15%, Table 2A), the high degree of interlot variability disqualified this material from further use as positive control. Each bar represents the mean of at least two duplicate responses. Percent CV for each duplicate was less than 10.

control.[14] As with the calibration standards and quality controls, the variability in percent hemolysis (% CV) induced by Triton-X 100 and PEG in in-study validation has met the industrial requirement of being within 15%.[27,30]

Other parameters evaluated during assay validation included testing the effects of using freshly drawn blood versus stored blood, various blood incubation times with the test materials, and different types of mixing during incubation (rotation vs shaking every 30 min). The results of these tests indicated that freshly drawn blood can be stored up to 24 h at 2−8 °C with no appreciable effects on assay performance; prolonged storage (36 and 48 h) resulted in gradual increase in plasma-free hemoglobin (PFH), which disqualified blood samples with PHF levels above 1 mg/mL from the use in nanoparticle hemolysis test. The optimal time of sample incubation was 3 h, and there was no significant difference in test results when rotation was used instead of mixing every 30 min (data not shown).

3.3. Nanoparticle Interference with the Assay and Approaches to Overcome It. The protocol described herein relies on the use of human blood anticoagulated with lithium-heparin. Other anticoagulants are available commercially and

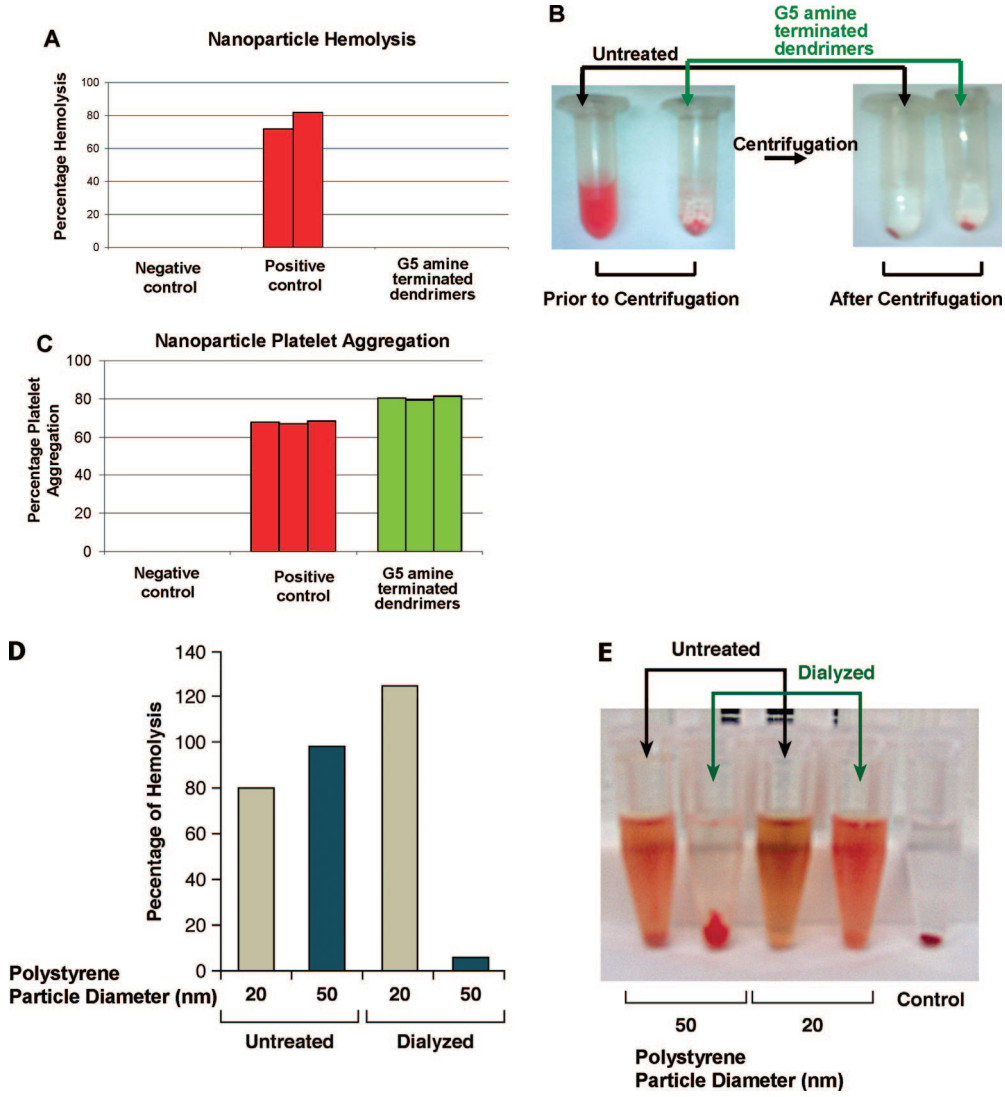

Figure 2. Nanoparticles interfere with the hemolysis assay. (A) G5 amine terminated dendrimers were analyzed in duplicate, and no plasma-free hemoglobin was detected in supernatants. Three tests were conducted for the dendrimers. Each bar represents the mean of the duplicate response with % CV less than 20. (B) The same samples were analyzed during various steps of incubation and rapid (within first 15 min) coagulation of blood was observed. Erythrocytes in the clot were protected from hemolysis, and blood clots were removed from the supernatants by centrifugation, giving a false-negative result. (C) The same samples were analyzed in a platelet aggregation test. Each bar is the mean of duplicate response with % CV less than 20. Three tests were conducted. (D) Analysis of 20 and 50 nm polysterene nanoparticles. Each bar represents the mean of three test results. Each test results was obtained from a duplicate response with % CV less than 20. (E) Visual inspection of the microcentrifuge tubes containing blood samples treated with 20 and 50 nm polysterene nanoparticles before and after dialysis.

may be used for this assay.[26] The same applies to the assay buffer. All nanoparticles in this study were suspended and diluted in calcium- and magnesium-free PBS. If a test nanomaterial is prone to agglomeration in PBS, other erythrocyte-friendly buffers, e.g., saline, may be used instead. We used this protocol for analysis of a variety of nanoparticles, nanoliposomes, PAMAM dendrimers, triazine dendrimers, nanoemulsions, water-soluble fullerene derivatives, and polystyrene nanoparticles, and repeatedly observed nanoparticle interference with the assay. Several examples of this interference are summarized below.

Generation 6 (G6) amine-terminated PAMAM dendrimers tested in this assay at concentration of 125 μg/mL resulted in ~5% hemolysis (data not shown). This was consistent with previous studies of various nanomaterials demonstrating that cationic particles are more "reactive" than anionic or neutral particles and can damage cells (reviewed in refs 1 and 7). When G5 amine terminated dendrimers were included in the analysis, no plasma-free hemoglobin was detected in supernatants (Figure 2A). When samples were analyzed during various steps of incubation, rapid (within first 15 min) coagulation of blood was observed (Figure 2B). Erythrocytes in the clot were protected from hemolysis, and blood clots were removed from the supernatants by centrifugation, giving a false-negative result. These findings were supported by a platelet aggregation study, in which G5 amine-terminated dendrimers caused approximately 80% platelet aggregation (Figure 2C).These data demonstrate that visual sample

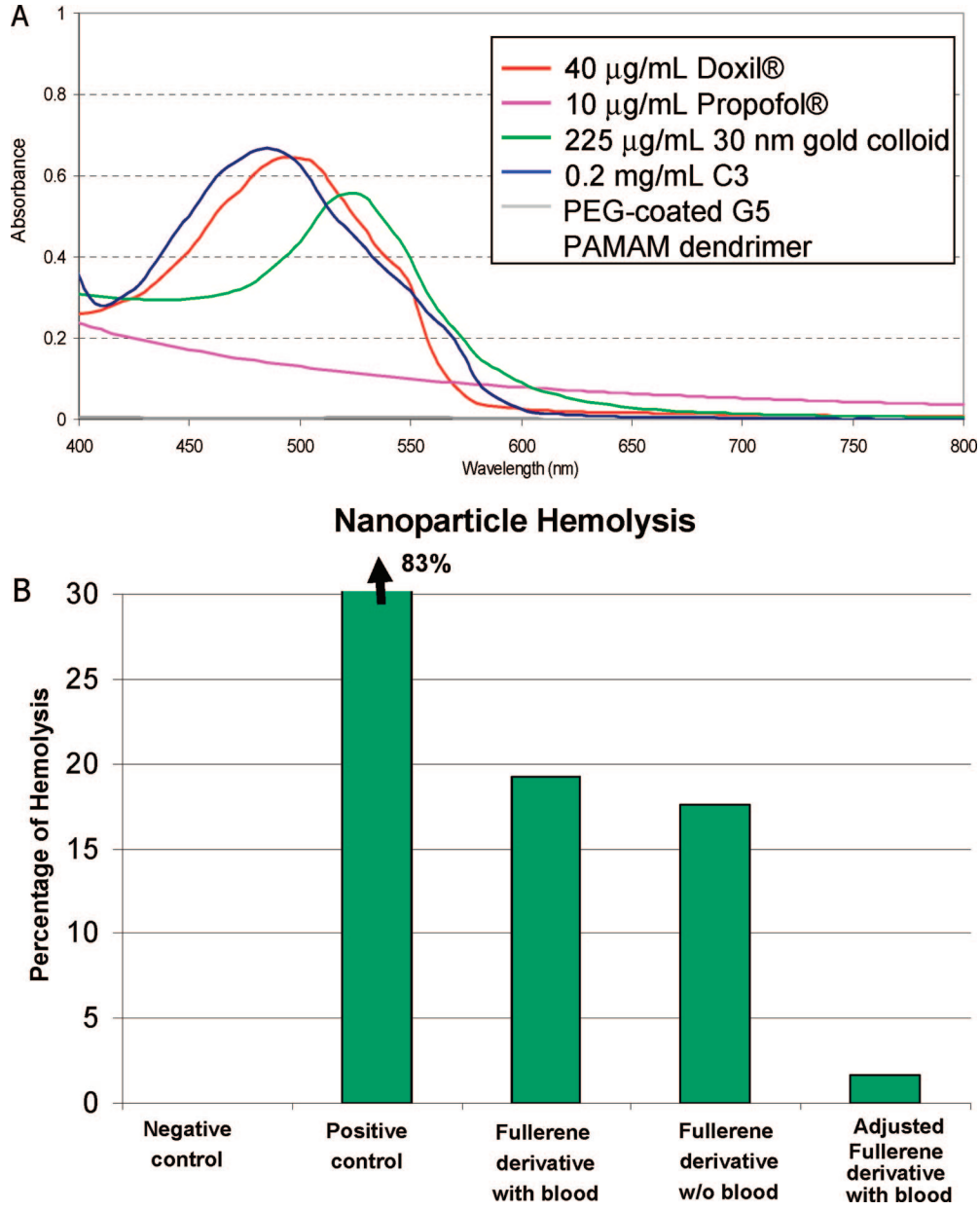

Figure 3. Nanoparticle optical properties as a source of interference with hemolysis assay. (A) UV−vis spectrum of several nanoparticle samples in the vicinity of the assay detection wavelength of 540 nm. Doxil, the 30 nm gold colloid, and fullerene derivative C3 at concentrations shown in the figure all have significant absorbance near 540 nm. UV−vis spectra were recorded using a Molecular Devices SpectraMax M5 (Sunnyvale, CA). Samples were prepared in Milli-Q water and measured in quartz microcuvettes (b = 10 mm, QS105.250, Hellma, Plainview, NY). Spectra were collected from 200 to 800 nm in 5 nm steps with Milli-Q water as the reference. (B) Analysis of fullerene derivative C3 in hemolysis assay. Results of one of three tests are shown. Each bar represents the mean of a duplicate response with % CV less than 15.

examination during all assay steps is important for the accurate interpretation of test results.

Another example of nanoparticle interference was observed when polystyrene nanoparticles were tested. Figure 2D shows the results of our assay for determination of the hemolytic properties of polystyrene nanoparticles. In this commonly used protocol, 20 and 50 nm polysterene nanoparticle size standards were incubated in whole blood, the blood was centrifuged to remove undamaged erythrocytes and nanoparticles, and the percent hemolysis was determined by colorimetric detection of hemoglobin in the supernatant. Under these conditions, untreated (i.e., commercially supplied) particles with 20 and 50 nm diameters were strongly hemolytic. In the case of the 50 nm particles, spectroscopic analysis indicated a reduction in hemolysis following dialysis. Visual inspection of the microcentrifuge tubes (Figure 2E), however, showed the dialyzed 50 nm particles adsorb hemoglobin (compared to control tube), and the adsorbed hemoglobin precipitates with the particles upon centrifugation—yielding a false negative result. This phenomenon was not observed with polystyrene nanoparticle less than 50 nm in size but was evident for larger, 80 nm, polystyrene nanoparticles.

The most common mechanism of interference is due to the nanoparticle absorbance at or close to the assay wavelength (540 nm). This can be seen from Figure 3A, which

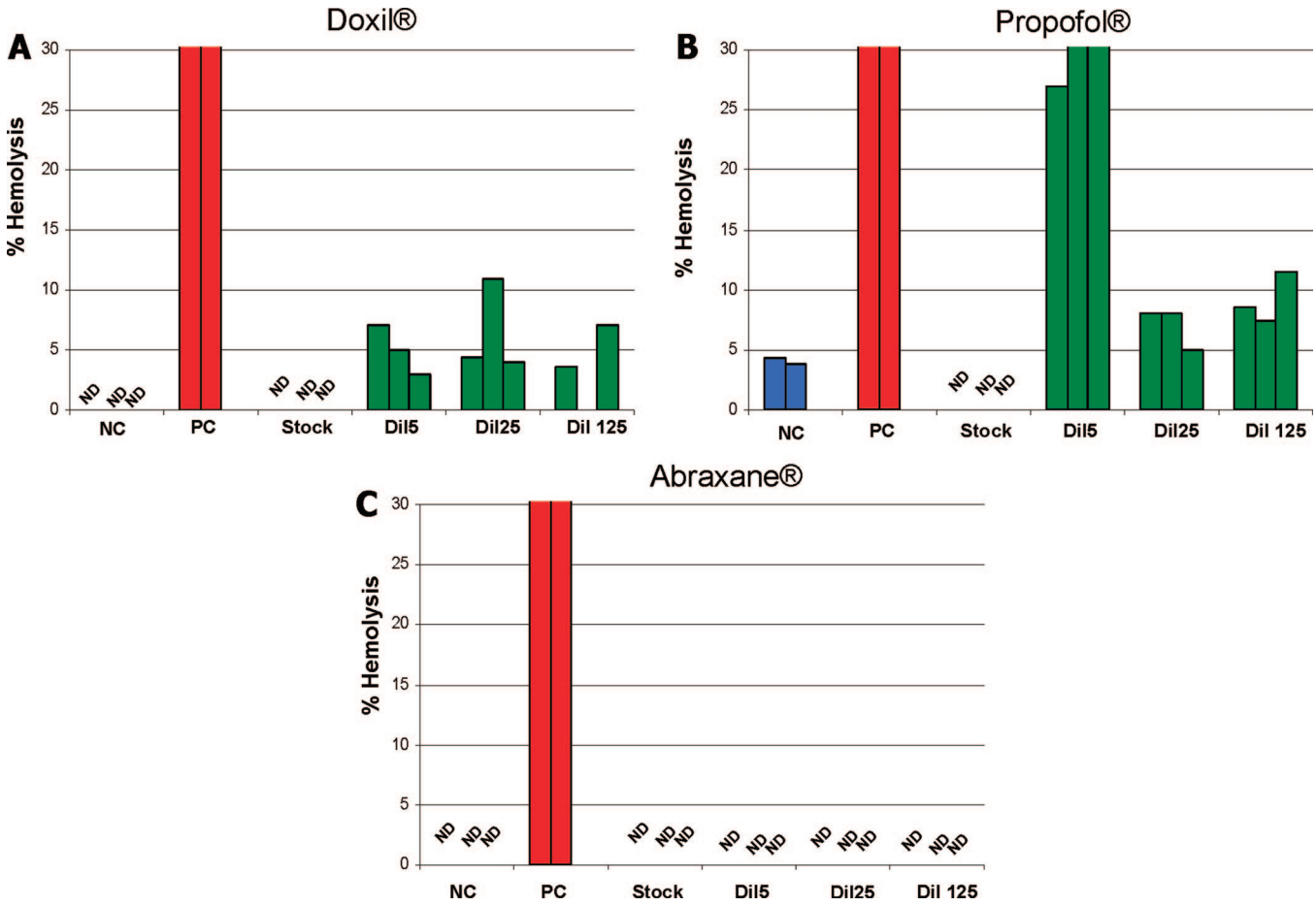

Figure 4. Hemolysis assay applied to nanoparticulate pharmaceuticals approved for clinical use. Hemolytic properties of commercially available nanoparticle formulations approved for clinical use, Doxil (A), Propofol (B), and Abraxane (C), were studied in vitro. Three tests were conducted for each nanoparticle sample. Each bar represents the mean of a duplicate response with % CV less than 15. Stock refers to nanoparticle formulations used directly from the commercially supplied vial. This stock was subjected to three serial 1 to 5 dilutions in PBS; these dilutions are labeled as Dil 5 (dilution of the stock 1 to 5), Dil 25 (dilution of the stock 1 to 25), and Dil 125 (dilution of the stock 1 to 125). NC is negative control (PBS). PC is positive control (Triton X-100). The scale was reduced to focus on nanoparticle percent hemolysis. Hemolysis observed for positive control samples was above 80%. ND refers to no detectable hemolysis.

shows the UV−vis spectra of several nanoparticle samples. The third column of Figure 3B shows that the fullerene derivative, C3, causes hemolysis of almost 20%. However, when the same nanoparticles were used in a control sample without blood (i.e., sample containing all assay components except the blood is substituted with PBS), the absorbance of this sample at 540 nm was also very high and when extrapolated against the hemoglobin standard curve corresponded to a percent hemolysis of 18% (Figure 3B fourth column). The same was true for citrated gold nanoparticles, some nanoemulsions, fullerene derivatives, and doxorubicin-loaded particles, all of which absorb near 540 nm (Figure 3A). For heavy particles (e.g., gold nanoparticles), an extra centrifugation step was used for removing the particles from the supernatant prior to evaluation on the plate reader. However, for the majority of nanoparticles, removal by centrifugation was not possible due to their small size and high solubility. For these particles, the results of the hemoglobin assay can be adjusted by subtraction of the absorbance of the no-blood control (i.e., sample containing all assay components except the blood is substituted by PBS). For example, for the C3 fullerene derivative, this adjusted result corresponds to a much lower percentage hemolysis (Figure 3B last column). If the degree of interference is very high (i.e., OD value of nanoparticle sample is above that of the highest calibrator in the hemoglobin standard curve), then dilution of the nanoparticle sample is required prior to the test in order to obtain accurate results after the adjustment procedure.

3.4. Relevance of in Vitro Assay to the in Vivo Testing. To evaluate the relevance of the described in vitro assay for analysis of nanoparticle hemolytic properties, we have tested three nanotechnology-derived formulations approved by the US FDA for use in clinical applications. This analysis included Doxil (a liposomal formulation of doxorubicin), Abraxane (albumin bound Paclitaxel nanoparticles), and Propofol (a nanoemulsion-based anesthetic agent). Doxil and Propofol interfered with the assay when used at high concentrations. The results shown in parts A and B of Figure 4 are adjusted to account for this interference. A low percentage hemolysis was observed with both formulations in vitro and it appeared to have no or weak relationship to the concentration of nanoparticle. This is in agreement with studies reporting low levels of erythrocyte damage by Doxil

in vivo, and Propofol in vitro.[32–35] In both cases it was dose dependent and minimized by using lower doses of formulations.[32–35] It is important to notice that when both Doxil and Propofol were analyzed at high concentration (labeled as "stock" in parts A and B of Figure 3), the OD value of nanoparticle only control was above that of the assay highest calibrator. Subtraction procedure applied to adjust test result for the interference as described in the section above may not be accurate due to the high degree of nanoparticle interference with the assay. According to several studies in vitro percent hemolysis is rated as "no concern' when it varies from 5 to 25%.[36–39] Our test results with Doxil and Propofol meet the criteria of some of these studies.[36] Abraxane did not induce any damage to red blood cells when tested in vitro in our assay (Figure 4C), and there is no report in the literature on the hemolytic activity of this drug in vivo.

Thus, the assay described in our study demonstrated comparable negative test results for nanotechnology derived formulations tested in vivo. Although more comprehensive study is required to prove in vivo relevance of this in vitro method, the described assay can be used to exclude potentially harmful formulations from early preclinical testing (if in vitro result is >50% hemolysis) and to suggest potential complications to monitor during in vivo studies of nanomaterials.

4. Conclusions. Many studies reported nanoparticle hemolytic propeties.[10–25] The majority of these studies utilized a spectrophotometric detection of plasma-free hemoglobin, and none of them reported specific controls to rule out nanoparticle interference. When it comes to preclinical safety testing of nanomaterials, ruling out false-positive or false-negative results is critical for accurate interpretation of data. Our study provides the first comprehensive insight to potential sources of this interference, demonstrates the usefulness of including "nanoparticle only" controls, and illustrates the importance of physicochemical characterization of nanoparticle formulations and visually monitoring test samples to avoid false-positive or false-negative results.

Acknowledgment. This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. We are grateful to Drs. Mansoor Amiji, Mark Kester, Eric Simanek and C60 Inc. for providing various nanoparticle formulations.

Supporting Information Available: Description of reagents and research donor blood used, assay procedures, experimental procedures, and calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) McNeil, S. E. *J. Leukocyte Biol.* **2005**, *78* (3), 585–594.
(2) Ferrari, M.; Downing, G. *BioDrugs* **2005**, *19* (4), 203–210.
(3) http://www.fda.gov/nanotechnology/.
(4) http://ncl.cancer.gov.
(5) http://www.astm.org(technical committee E56).
(6) http://www.iso.org(technical committee TC229).
(7) Brownlie, A.; Uchegbu, I. F.; Schatzlein, A. G. *Int. J. Pharm.* **2004**, *274* (1−2), 41–52.
(8) Cheng, F. Y.; Su, C. H.; Yang, Y. S.; Yeh, C. S.; Tsai, C. Y.; Wu, C. L.; Wu, M. T.; Shieh, D. B. *Biomaterials* **2005**, *26* (7), 729–738.
(9) Chouly, C.; Bordenave, L.; Bareille, R.; Guerin, V.; Baquey, A.; Pouliquen, D.; Baquey, C.; Jallet, P. *Clin. Mater.* **1994**, *15* (4), 293–301.
(10) Duguid, J. G.; Li, C.; Shi, M.; Logan, M. J.; Alila, H.; Rolland, A.; Tomlinson, E.; Sparrow, J. T.; Smith, L. C. *Biophys. J.* **1998**, *74* (6), 2802–2814.
(11) Dutta, T.; Agashe, H. B.; Garg, M.; Balasubramanium, P.; Kabra, M.; Jain, N. K. *J. Drug Targeting* **2007**, *15* (1), 89–98.
(12) Foger, F.; Noonpakdee, W.; Loretz, B.; Joojuntr, S.; Salvenmoser, W.; Thaler, M.; Bernkop-Schnurch, A. *Int. J. Pharm.* **2006**, *319* (1−2), 139–146.
(13) Goodman, C. M.; McCusker, C. D.; Yilmaz, T.; Rotello, V. M. *Bioconjugate Chem.* **2004**, *15* (4), 897–900.
(14) Guowei, D.; Adriane, K.; Chen, X.; Jie, C.; Yinfeng, L. *Int. J. Pharm.* **2007**, *328* (1), 78–85.
(15) Jin, Y.; Tong, L.; Ai, P.; Li, M.; Hou, X. *Int. J. Pharm.* **2006**, *309* (1−2), 199–207.
(16) Kainthan, R. K.; Gnanamani, M.; Ganguli, M.; Ghosh, T.; Brooks, D. E.; Maiti, S.; Kizhakkedathu, J. N. *Biomaterials* **2006**, *27* (31), 5377–5390.
(17) Kim, D.; El-Shall, H.; Dennis, D.; Morey, T. *Colloids Surf., B* **2005**, *40* (2), 83–91.
(18) Koziara, J. M.; Oh, J. J.; Akers, W. S.; Ferraris, S. P.; Mumper, R. J. *Pharm. Res.* **2005**, *22* (11), 1821–1828.
(19) Lim, S. J.; Lee, M. K.; Kim, C. K. *J. Controlled Release* **2004**, *100* (1), 53–61.
(20) Nimesh, S.; Goyal, A.; Pawar, V.; Jayaraman, S.; Kumar, P.; Chandra, R.; Singh, Y.; Gupta, K. C. *J. Controlled Release* **2006**, *110* (2), 457–468.
(21) Puzyr, A. P.; Neshumaev, D. A.; Tarskikh, S. V.; Makarskaia, G. V.; Dolmatov, V.; Bondar, V. S. *Biofizika* **2005**, *50* (1), 101–106.
(22) Schubert, M. A.; Muller-Goymann, C. C. *Eur. J. Pharm. Biopharm.* **2005**, *61* (1−2), 77–86.
(23) Verma, A. K.; Sachin, K.; Saxena, A.; Bohidar, H. B. *Curr. Pharm. Biotechnol.* **2005**, *6* (2), 121–130.
(24) Vittaz, M.; Bazile, D.; Spenlehauer, G.; Verrecchia, T.; Veillard, M.; Puisieux, F.; Labarre, D. *Biomaterials* **1996**, *17* (16), 1575–1581.
(25) Zobel, H. P.; Stieneker, F.; Atmaca-Abdel Aziz, S.; Gilbert, M.; Werner, D.; Noe, C. R.; Kreuter, J.; Zimmer, A. *Eur. J. Pharm. Biopharm.* **1999**, *48* (1), 1–12.
(26) *Standard practice for assessment of hemolytic properties of materials*; ASTM International: West Conshohocken, PA, 2000.
(27) DeSilva, B.; Smith, W.; Weiner, R.; Kelley, M.; Smolec, J.; Lee, B.; Khan, M.; Tacey, R.; Hill, H.; Celniker, A. *Pharm. Res.* **2003**, *20* (11), 1885–1900.
(28) http://ncl.cancer.gov/NCL_Method_ITA-1.pdf.
(29) www.fda.gov/cber/summaries/120600bio10.ppt.
(30) Smolec, J.; DeSilva, B.; Smith, W.; Weiner, R.; Kelly, M.; Lee, B.; Khan, M.; Tacey, R.; Hill, H.; Celniker, A.; Shah, V.; Bowsher, R.; Mire-Sluis, A.; Findlay, J. W.; Saltarelli, M.; Quarmby, V.; Lansky, D.; Dillard, R.; Ullmann, M.; Keller, S.; Karnes, H. T. *Pharm. Res.* **2005**, *22* (9), 1425–1431.
(31) Dobrovolskaia, M. A.; McNeil, S. E. *Nat. Nanotechnol.* **2007**, *2* (8), 469–478.
(32) Lewin, S. N.; Mutch, D. G.; Whitcomb, B. P.; Liapis, H.; Herzog, T. J. *Gynecol. Oncol.* **2005**, *97* (1), 228–233.
(33) Rivera, E.; Valero, V.; Arun, B.; Royce, M.; Adinin, R.; Hoelzer, K.; Walters, R.; Wade, J. L., 3rd; Pusztai, L.; Hortobagyi, G. N. *J. Clin. Oncol.* **2003**, *21* (17), 3249–3254.
(34) Chen, H.; Zhang, Z.; Almarsson, O.; Marier, J. F.; Berkovitz, D.; Gardner, C. R. *Pharm. Res.* **2005**, *22* (3), 356–361.
(35) Murphy, P. G.; Davies, M. J.; Columb, M. O.; Stratford, N. *Br. J. Anaesth.* **1996**, *76* (4), 536–543.
(36) Amin, K.; Dannenfelser, R. M. *J. Pharm. Sci.* **2006**, *95* (6), 1173–1176.
(37) Krzyzaniak, J. F.; Raymond, D. M.; Yalkowsky, S. H. *PDA J. Pharm. Sci. Technol.* **1996**, *50* (4), 223–226.
(38) Krzyzaniak, J. F.; Alvarez Nunez, F. A.; Raymond, D. M.; Yalkowsky, S. H. *J. Pharm. Sci.* **1997**, *86* (11), 1215–7.
(39) Krzyzaniak, J. F.; Yalkowsky, S. H. *PDA J. Pharm. Sci. Technol.* **1998**, *52* (2), 66–69.

NL0805615