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Variability of Complement Response toward Preclinical and Clinical Nanocarriers in the General Population

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Abstract

Opsonization (coating) of nanoparticles with complement C3 component is an important mechanism that triggers immune clearance and downstream anaphylactic and proinflammatory responses. The variability of complement C3 binding to nanoparticles in the general population has not been studied. We examined complement C3 binding to dextran superparamagnetic iron oxide nanoparticles (superparamagnetic iron oxide nanoworms, SPIO NWs, 58 and 110 nm) and clinically approved nanoparticles (carboxymethyl dextran iron oxide ferumoxytol (Feraheme, 28 nm), highly PEGylated liposomal doxorubicin (LipoDox, 88 nm), and minimally PEGylated liposomal irinotecan (Onivyde, 120 nm)) in sera from healthy human individuals. SPIO NWs had the highest variation in C3 binding ($n = 47$) between subjects, with a 15–30 fold range in levels of C3. LipoDox ($n = 12$) and Feraheme ($n = 18$) had the lowest levels of variation between subjects (an approximately 1.5-fold range), whereas Onivyde ($n = 18$) had intermediate between-subject variation (2-fold range). There was no statistical difference between males and females and no correlation with age. There was a significant correlation in complement response between small and large SPIO NWs, which are similar structurally and chemically, but the correlations between SPIO NWs and other types of nanoparticles, and between LipoDox and Onivyde, were not significant. The calculated average number of C3 molecules bound per nanoparticle correlated with the hydrodynamic diameter but was decreased in LipoDox, likely due to the PEG coating. The conclusions of this study are (1) all nanoparticles show variability of C3 opsonization in the general population; (2) an individual's response toward one nanoparticle cannot be reliably

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00496. Figures showing the association of C3 levels with age and gender and pair-wise comparisons of C3 levels across nanoparticles. (PDF)

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predicted based on another nanoparticle; and (3) the average number of C3 molecules per nanoparticle depends on size and surface coating. These results provide new strategies to improve nanomedicine safety.

Graphical Abstract

INTRODUCTION

The primary function of innate immunity is to immediately recognize and neutralize invading pathogens while being indiscriminate to an extremely diverse set of chemical groups and patterns.¹ Complement² is arguably the critical innate immunity arm that recognizes and neutralizes invading pathogens by (a) opsonizing the surface with cleavage products of the third complement component C3 (C3b, iC3b, C3dg, etc.), thereby promoting recognition by complement receptors C1qR, CR1, CR2, CR3, and CR4 on leukocytes and macrophages;^{3–5} (b) triggering proinflammatory response via liberation of C3a and C5a; and (c) forming the membrane attack complex C5b-C9 leading to lysis of bilayer-containing pathogens and cells.⁶

Complement activation by nanomedicines is considered one of the most serious immunological responses from the moment of infusion.^{7,8} Complement activation has been documented for preclinical and clinical nanoformulations,^{7,9} and a substantial amount of work has been done to understand mechanisms of activation by nanosurfaces.^{10–14} Activation of the complement is not desirable because it can lead to (a) the immune clearance of nanoparticles,^{15,16} (b) anaphylactic and proinflammatory response and immune cell activation,¹⁷ and (c) bystander damage to cells and tissues. Complement-induced pseudoallergy is a known phenomenon following infusion of liposomal doxorubicin, dextran–iron oxides, cremophor-cyclosporine A, etc.⁹ Complement is implicated in a variety of disease conditions;¹⁸ therefore, its enhanced activation could potentially result in pathophysiological misbalances and worsening of the disease rather than its alleviation.¹⁹

The variation of immune response between subjects in a population stems from a combination of individual traits that operate at the cellular and subcellular levels.²⁰ Previous studies demonstrated significant between-subject differences in complement reactivity that

present a predisposition to diseases (termed complotype).²¹ Additional evidence suggests that there are differences in complement response toward nanoparticles, particularly in the general population. Previous work demonstrated differences in the release of fluid-phase markers (C5a, Bb, C4d, and sC5-C9) after incubation of nanoparticles in sera in small cohorts of donors.^{22–24} At the same time, between-subject differences in C3 opsonization have never been studied. Here, we set out to answer several questions: (a) are there differences in complement C3 opsonization of nanomedicines in the general population? (b) Does the same person react the same way to all types of nanomedicine? (c) Finally, which factors determine the number of C3 molecules deposited per nanoparticle? Understanding these variables is critical to assess risks and to predict complement response to nanocarriers in general and diseased populations, as well as to develop strategies to block complement activation. Previously we found that dextran superparamagnetic iron oxide nano-worms (SPIO NWs), which are chemically and structurally similar to the clinically approved (and subsequently withdrawn) MRI contrast agents (Sinerem, Resovist, Feridex), are potent activators of complement in humans.^{25–28} Here, we synthesized SPIO NWs of two different sizes (58 and 110 nm) as well as obtained clinically approved nanoparticles: carboxymethyl dextran-coated iron oxide (28 nm) Feraheme, highly PEGylated liposomal doxorubicin (88 nm) LipoDox, and minimally PEGylated liposomal irinotecan (120 nm) Onivyde²⁹ to study variability of complement C3 opsonization in healthy human population. Our results show that all nanoparticle types become opsonized with C3, but the degree of opsonization is subject-dependent. Moreover, the individual response to one class of nanomedicine does not correlate with response to another class of nanomedicine. The additional determinants of nanoparticle opsonization are the hydrodynamic diameter and surface PEGylation. These results have important implications for the toxicity and immunocompatibility of nanocarriers.

RESULTS

The nanomedicines used in the study are described in Figure 1 and in Table 1. We prepared SPIO NWs of two different sizes by varying the ratio between dextran and iron salts during the precipitation reaction as described previously.^{25–28} A higher dextran-to-Fe ratio results in smaller NWs.²⁶ Transmission electron microscopy showed worm-like polycrystalline Fe₃O₄ core (Figure 1A,B). Larger SPIO NWs (hereafter L-SPIO NWs) had ~20 Fe₃O₄ cores per particle and a 110 nm hydrodynamic diameter (Table 1), whereas smaller SPIO NWs (hereafter called S-SPIO NWs) had ~10 Fe₃O₄ cores per particle and a 58 nm hydrodynamic diameter. The particles have a core–shell structure^{25–28} due to the 20 kDa dextran shell (not visible on transmission electron microscopy (TEM) images; Figure 1B).

Feraheme is the Food and Drug Administration (FDA)-approved iron supplement consisting of a reduced carboxymethyl dextran ultrasmall iron oxide nanoparticle (USPIO) (Figure 1A,B). Although Feraheme is prepared using the same Molday precipitation technique³⁰ and has the core–shell structure similar to SPIO NWs (Figure 1B), it has a much-smaller hydrodynamic diameter due to a single Fe₃O₄ crystalline core and has a negatively charged surface coating. LipoDox is a PEGylated liposomal doxorubicin (generic version of Doxil) approved for treatment of Kaposi sarcoma and several solid and hematological cancers.³¹ These liposomes are coated with a dense layer of polyethylene glycol and are loaded with

doxorubicin crystals (approximately 13 000 molecules per liposome). Onivyde is a recently FDA-approved liposomal irinotecan for the treatment of metastatic adenocarcinoma of the pancreas.

The composition and size of LipoDox and Onivyde liposomes are different (Table 1) because LipoDox has a highly dense layer of 1,2-distearoylphosphatidylethanolamine (DSPE)–PEG-2000 (over 5 mol %), whereas Onivyde has only 0.3 mol % DSPE–PEG-2000, likely to maintain colloidal stability. In addition, the internal content (doxorubicin versus irinotecan) can influence the liposome structure and eventually complement activation.¹⁰ Onivyde contains approximately 70 000 molecules of irinotecan hydrochloride per liposome and has a 120 nm hydrodynamic diameter.

SPIO NW Demonstration of a Significant Between-Subject Variation of C3 Opsonization in the General Population.

We collected sera from 47 healthy donors (20 males and 27 females, average age of 47 ± 16 ($\pm$ standard deviation) years). To quantify the bound C3, we used previously described immuno dot blot assay,^{11,27} in which nanoparticles were incubated with sera and washed by ultracentrifugation, and the amount of particle-bound C3 was quantified using purified iC3b as the standard (most bound C3 is the iC3b form¹¹). According to Figure 2A, all subjects had a significant opsonization of L-SPIO NWs with C3 (median: 9.0×10^{14} C3 molecules per milligram of Fe (range: 3.1×10^{14} to 5.5×10^{15}).

There was a significant level of between-subject variation between the donors (Figure 2A, covariance parameter estimate [SE]: 0.075 [0.018], p -value of <0.0001). Thus, 15% of the samples showed significantly higher levels of opsonization (nominal p -value of <0.05) than the modeled average, while 38% of samples showed significantly lower opsonization than the modeled average (nominal p -value of <0.05). There was no statistical association between the gender/age of the individuals and the levels of C3 opsonization (p -value of 0.69 for gender and p -value of 0.053 for age). C3 (C3b) deposition is the initial step in formation of C5 convertase (C3bBbC3b) that cleaves serum C5 into C5b and C5a. The levels of C3 opsonization (C3 molecules per milligram of Fe) correlated with C5a release (ng/mL serum) (correlation coefficient of 0.64; p -value of <0.0001 ; Figure 2B). Hence, C3 opsonization of nanoparticles determined with dot blot assay correlates with downstream complement activation (C5a release).

Smaller S-SPIO NWs behaved similarly to L-SPIO NWs (Figure 2C). S-SPIO NWs induced potent C3 opsonisation in all 47 individuals, with significant variation between individuals (covariance parameter estimate [SE]: 0.109 [0.024], p -value of <0.0001). The median number of C3 molecules bound per milligram of Fe was 1.0×10^{15} (range: 3.5×10^{14} to 1.2×10^{16}). Similarly to L-SPIO NWs, there was no statistical association between the gender and age of the individuals and the levels of C3 opsonization (p -value = 0.98 for gender and p -value = 0.44 for age; Supplemental Figure 1). There was a significant correlation between C3 opsonization and C5a levels (correlation coefficient = 0.70; p -value of <0.0001 ; Figure 2D).

Feraheme, LipoDox, and Onivyde Demonstrated a Significant Between-Subject Variation of C3 Opsonization.

We investigated whether the variability of complement activation observed for SPIO NWs pertains to Feraheme, which also belongs to dextran iron oxide nanoparticle type. Feraheme was incubated with 18 serum samples with high, intermediate, and low C3 opsonization levels based on reactivity toward L-SPIO NWs (Figure 2A). There was significant variation of complement activation between these 18 samples (covariance parameter estimate [SE]: 0.0029 [0.0013], p -value = 0.014; Figure 3A). The median number of C3 molecules bound per milligram of Fe was 1.4×10^{15} (range 1.2×10^{15} to 1.8×10^{15}).

Next, we tested C3 opsonization of nanoparticles structurally and physico-chemically unrelated to dextran iron oxides. Liposomes are perhaps the oldest and most commonly used drug delivery vehicles composed of significant proportion of FDA-approved clinical nanopharmaceuticals.³⁰ Both types of liposomes were incubated with serum (12 samples for LipoDox and 18 samples for Onivyde), washed by ultracentrifugation as described in the Materials and Methods section, and assayed by dot-blot. Both LipoDox (Figure 3B) and Onivyde (Figure 3C) bound C3 in all serum samples, and there was a significant between-subject variation for both (covariance parameter estimate [SE]: 0.0031 [0.0018], p -value = 0.037 for LipoDox; covariance parameter estimate [SE]: 0.0064 [0.0027], p -value = 0.009 for Onivyde), with as much as 2-fold difference between high and low activators. The median C3 molecules bound per mg doxorubicin was 2.6×10^{14} (range of 2.1×10^{14} to 3.0×10^{14}), and the median number of C3 molecules bound per milligram irinotecan was 5.0×10^{14} (range 3.9×10^{14} to 8.1×10^{14}). As for the case of SPIO NWs, there was no association between C3 opsonization and gender and age for LipoDox (p -value = 0.40 for gender and p -value = 0.92 for age; Supplemental Figure 1) or Onivyde (p -value = 0.41 for gender and p -value = 0.77 for age; Supplemental Figure 1).

Levels of C3 Opsonization Were Not Significantly Correlated between Nanoparticle Types.

In view of the observed between-subject variation in C3 levels for all nanoparticles, we questioned whether the same individual has the same reactivity toward different types of nanoparticles. There was a significant level of correlation between L-SPIO NWs and S-SPIO NWs (correlation coefficient = 0.40, p -value = 0.0056), which are similar chemically and structurally (shown in Supplemental Figure 2 and summarized as a color map in Figure 4). However, the correlations between SPIO NWs (either large or small) and Feraheme were not significant, suggesting that individual reactivity toward Feraheme could not be predicted based on the reactivity toward SPIO NWs. Importantly, correlation between LipoDox and Onivyde was also not statistically significant (correlation coefficient = 0.19, p -value = 0.681). There was also no correlation between SPIO NWs and the liposomes and between Feraheme and the liposomes (Figure 4).

C3 Opsonization Efficiency Associated with Size Differences between Nanoformulations.

To understand differences between nanoparticles in terms of their complement opsonization efficiency, we calculated the geometric means of C3 binding (C3 molecules per milligram of Fe or drug) in the donor population and converted to a number of C3 molecules per nanoparticle based on numbers provided in Table 1. The number of C3 per nanoparticle was

in the following order (Figure 5A): Onivyde > L-SPIO NWs > S-SPIO NWs > LipoDox > Feraheme. If LipoDox is excluded, there was a significant correlation between NP size and C3 number for SPIO NWs, Onivyde, and Feraheme (correlation coefficient = 0.95, p -value = 0.047).

At the same time, LipoDox was an outlier (red square in Figure 5A) and did not follow the trend. The low number of C3 per Feraheme nanoparticle could be explained by comparing the dimensions of Feraheme, C3b, and the alternative pathway convertase C3bBbProperdin (Figure 5B,C). It is likely that the bulky complement proteins cannot be accommodated on the surface of ultrasmall Feraheme. In comparison, larger-sized LipoDox can accommodate many more C3 molecules and convertase (Figure 5C), so the observed low level of C3 and LipoDox is likely due to the highly PEGylated coating that prevents binding of some of the protein corona proteins

DISCUSSION

Complement is one of the critical determinants of the between-subject variation of the innate immune response that can be dissected at the protein level (top down approach). Previous studies demonstrated donor-dependent differences in fluidphase activation markers (C5a, Bb, C4d, and sC5-C9) after incubation of perfluorocarbon nanoparticles, SPIO, and doxorubicin liposomes with serum.^{10,22,23} The difference between this work and that of others is that we determined between-subject variation of C3 binding, rather than fluid marker release and compared side-by-side different classes of preclinical and clinical nanoparticles. For the large and small SPIO NWs, the largest proportion of the total variance could be attributed to differences between subjects, whereas with Feraheme, Onivyde, and LipoDox, a lower proportion of the total variance could be attributed to differences between subjects, although there was a significant between-subject variance for each. The reason for the between-subject variation is not clear. While in all cases the differences were neither gender- nor age-dependent, we cannot rule out the role of genetics. Certain polymorphisms in C3, factor B, complement inhibitors factor H and I can lead to an increase or decrease in complement activation,^{21,33,34} and in such individuals, the complement system is expected to be overreactive toward wide range of pathogens and surfaces and predisposed for complement-related disorders.

Because C3 is the critical opsonin that enables recognition by a wide variety of immune cells, it would be interesting to test the hypothesis whether sera from high complement activators (green color in Figure 2–3) promotes a more-efficient uptake of nanoparticles by macrophages, neutrophils, and monocytes. Other well-documented consequences of uncontrolled complement activation include inflammation,⁶ immune cell activation,³⁵ increased vascular permeability,³⁶ and triggered tumor growth.^{37,38} At the same time, the role of high complement activation on the infusion related reactions is less clear. Thus, in our study LipoDox showed low variation of complement activation, while about 10% of patients show infusion-related reactions in the clinic. However, the incidence of infusion reactions with Feraheme, which in our study had higher population variation than Doxil, is less than 3% in the clinic. Despite that evidence in pig models points to the role of

complement in pseudoallergy,⁹ recent data suggest that initial interaction of nanoparticles with lung macrophages could also play a role in the infusion-related reactions.³⁹

We demonstrated a limited correlation of C3 levels among nanoparticle types in the same individual. Thus, while there was a significant correlation between small and large SPIO NWs, which are structurally and chemically similar, there were no significant correlations among unrelated nanoparticles types. The immediate conclusion is that the individual reactivity toward a certain nanoparticle type cannot predict the reactivity toward an unrelated nanoparticle type. Differences in complement activation pathways could be another reason. Thus, while complement activation of L-SPIO NWs and S-SPIO is almost predominantly via the alternative pathway,²⁶ liposomes activate complement via the alternative and the classical pathways.^{10,40} In addition, because C3 binding takes place on the surface of absorbed protein corona,¹¹ it is plausible to suggest that differences in corona composition formed on nanoparticles in sera of different individuals could be the reason. We recognize that with larger sample sizes for Doxil, Feraheme, and Onivyde, the correlations could become significant. Our ongoing studies are focused on correlation between protein corona composition and the efficiency of complement C3 binding in large donor cohorts.

Lastly, we showed that for Feraheme, SPIO NWs, and Onivyde, the number of C3 molecules per nanoparticle significantly correlated with the hydrodynamic diameter. Although it was previously suggested that complement activation pathway is determined by the surface curvature,²³ we believe this is the first demonstration of a relationship between size and number of C3 molecules per nanoparticle for different preclinical and clinical formulations. We suggest that ultrasmall nanoparticles below a certain diameter may not have sufficient surface for C3 convertase assembly (Figure 5C). Previous studies indicated much longer circulation time and lower immune uptake of smaller nanoparticles than larger nanoparticles,^{41,42} and one possible reason could be due to the lower complement opsonization. At the same time, highly PEGylated LipoDox appears to be an outlier as it had fewer C3 molecules relatively to its size. The significantly lower C3 opsonization is likely due to the ability of PEGylated layer to repel certain corona components, including complement. Therefore, we conclude that the level of C3 binding per particle is determined both by geometrical constraint (due to a relatively large size of C3 molecules and C3 convertases) and nanoparticle surface coating.

CONCLUSIONS

There is significant between-subject variability of complement C3 opsonization of nanoparticles. In the same individual, the efficiency of complement opsonization of one nanomedicine type cannot be predicted based on another nanoparticle type. This necessitates development of personalized approaches to predict complement activation by nanomedicines. PEGylated coatings and nanoparticle sizes smaller than 30 nm can significantly reduce but not mitigate complement opsonization.

MATERIALS AND METHODS

Materials.

All chemicals used for SPIO synthesis, including iron salts and 15–25 kDa dextran, were purchased from Sigma-Aldrich (Saint Louis, MO). Purified human complement component C3 and iC3b was purchased from Quidel Corporation (San Diego, CA), aliquoted and stored at -80°C . Goat anti-human complement C3 polyclonal antibody that recognizes most of the C3 cleavage products was purchased from MB Biomedicals (Solon, OH). A C5a ELISA kit was purchased from Sino Biological Inc. (Beijing, China) and used according to the manufacturer's instructions. IRDye 800CW-labeled secondary antibody (anti-goat) was from LI-COR Biosciences (Lincoln, NE). Feraheme, LipoDox, and Onivyde were obtained from the University of Colorado Hospital's pharmacy. Human sera (total 47 donors) were obtained within a 2 week period from consented healthy donors at the University of Colorado Blood Donor Center (under the Center's Institutional Review Board protocol for anonymous collection; only age and gender were made available to the investigators), according to the previously described protocol.⁴³ Briefly, blood was collected into Vacutainer Z (Beckton Dickinson), left to clot for 1 h at room temperature, and then centrifuged at 2500g for 15 min to separate serum from blood clots. Serum was aliquoted and stored at -80°C . Each aliquot was thawed and frozen no more than twice.

Synthesis and Characterization of Nanoparticles.

Large and small SPIO NWs (L-SPIO NWs and S-SPIO NWs, respectively) were synthesized from 15 to 25 kDa dextran, Fe(III) chloride, and Fe(II) chloride using a modified Molday precipitation method³⁰ as described previously.²⁶ The ratio between dextran and iron salts determined the final size of the nanoparticles.²⁶ Particles were resuspended in sterile phosphate-buffered saline (PBS), filtered through a $0.45\ \mu\text{m}$ filter, and stored at 4°C . Size and ζ potential were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). The intensity-weighted distribution was used to report the hydrodynamic diameter of nanoparticles and liposomes. For TEM analysis of S-SPIO NWs and L-SPIO NWs, the imaging of nonstained samples applied on carbon grid was performed using a FEI Tecnai Spirit BioTwin electron microscope at 100 keV. For TEM analysis of Feraheme, the imaging of nonstained particles was performed at Thermo Fisher Scientific (Americas Nanoport, Hillsboro, OR) using an FEI Apreo microscope. The imaging was performed in immersion mode with STEM 3+ detector and landing energy of 30 keV.

Quantification of Binding of C3 to Nanoparticles and Liposomes.

For binding assay of complement C3, a PBS solution containing 1 mg/mL (Fe) SPIO NWs or Feraheme or 1 mg/mL (drug) of LipoDox or Onivyde was incubated with freshly thawed serum at a 1:3 volume ratio for 30 min at 37°C . Each experiment was performed at least in triplicate. At the end of the incubation period, particles were washed 3 times with PBS at 70 000 rpm for 10 min at 4°C using a Beckman Optima TLX ultracentrifuge equipped with a TLA-100.3 rotor. The pellets were resuspended in PBS, and $2\ \mu\text{L}$ of sample was applied in triplicates onto a $0.45\ \mu\text{m}$ pore nitrocellulose membrane (Bio-Rad). Standard dilutions of purified iC3b (main C3 isoform found on nanoparticles) were applied on the same membrane in a $2\ \mu\text{L}$ volume in triplicate. The membranes were blocked using 5% (w/w)

nonfat dry milk in PBS-T (1× PBS with 0.1% Tween 20) for 1 h at room temperature and probed with the primary antibody for 1 h at room temperature, followed by washing the membranes 3 times with PBS-T and, finally, 1 h of incubation with the IRDye 800CW-labeled secondary antibody. The membrane was scanned using Odyssey infrared imager (LICOR Biosciences, Lincoln, NE) at 800 nm. The integrated intensities of dots in the scanned images were determined from 16-bit grayscale images by ImageJ software, and the number of C3 molecules per dot was calculated from a standard curve. The number of C3 molecules per milligram of iron or drug was determined after dividing the number of C3 molecules per dot by the applied amount of 0.002 milligrams of Fe per dot or 0.002 milligrams of drug per dot.

Calculation of Number of C3 Molecules Bound per Nanoparticle.

An average number of C3 molecules per nanoparticle was calculated by dividing the number of C3 molecules/mg (as determined above) by the number of iron oxide nanoparticle that contain 1 mg Fe or by the number of liposomes that contain 1 mg of drug. The numbers are provided in Table 1. Briefly, the concentration of L-SPIO NWs per milligram of Fe was determined with NanoSight (Malvern Instruments), and Fe concentration was determined with ferrozine iron assay as described.²⁸ S-SPIO NWs and Feraheme concentrations per milligram of Fe were determined theoretically, as described previously^{11,44} based on the average number of 10 magnetite crystals per particle and 1 magnetite crystal per particle, respectively. The concentration of Onivyde (number of liposomes per milligram of irinotecan hydrochloride) and LipoDox (number of liposomes per milligram of doxorubicin hydrochloride) was determined from calculations using the lipid headgroup cross-section area (0.5 nm² for PC and 0.27 nm² for cholesterol)⁴⁵ and liposome diameter (120 nm for Onivyde and 88 nm for LipoDox). These calculations agree with the previously published number of 70 000 irinotecan hydrochloride molecules per Onivyde³² and 10 000–15 000 doxorubicin hydrochloride molecules per Doxil molecule.⁴⁶

Statistical Analysis.

C3 molecules per milligram of Fe or drug were analyzed using a linear mixed model in SAS statistical software (Version 9.4; SAS Institute Inc., Cary NC) after values were transformed using log base 10. The model contained a fixed effect for batch and random effects for sample and the sample by batch interaction. Variance components were tested for significance using asymptotic standard errors and a Wald Z-test. To test for age and gender effects, each was added independently to the model as a fixed covariate. Batch-adjusted estimates for individual samples were calculated from the predicted random sample effects and the estimated model intercept. Correlation analyses were conducted and graphics were generated in R statistical software (version 3.4.0; www.rproject.org).

Modeling of Complement Convertase.

Crystal structure coordinates for the C3bBb complex⁴⁷ and the Properdin (P) monomer were downloaded from the Protein Data Bank (PDB ID: 2XWB and 1W0S, respectively). Protein structures were prepared, measured, and visualized using Biovia Discovery Studio 4.5 (Biovia, Inc., San Diego, CA). A complex of P trimer with three C3b and three Bb molecules was modeled based on published electron microscopy data.⁴⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Nanoparticles used in the study. (A) TEM images of nanoparticles were obtained in-house (L-SPIO NWs and S-SPIO NWs) by a third party (Feraheme) or were reported previously (Cryo-TEM images of Doxil,³¹ which is similar to LipoDox used in this study). The size bar is 50 nm for SPIO NWs and 100 nm for Feraheme and Doxil. There are no published images of Onivyde liposomes. (B) Schematic representation of nanoparticles based on the previous literature^{11,31,32} and TEM and DLS measurements (Table 1). SPIO NWs and Feraheme are coated with dextran chains (yellow). Liposomes are loaded with doxorubicin hydrochloride (red) or irinotecan hydrochloride (green mesh). LipoDox (Doxil) is a highly PEGylated liposome, whereas Onivyde is a minimally PEGylated liposome. The size bar is 100 nm.

Figure 2.

Between-subject variation of C3 levels and the association of C3 and C5a levels after exposure to S-SPIO and L-SPIO nanoworms. (A) Distribution of batch-adjusted C3 levels across subjects after exposure to L-SPIO NWs. A batch-adjusted estimate of C3 levels (molecules per milligram of Fe) for each sample is represented by a bar. Red bars indicate samples with C3 levels that were significantly lower than the model average ($p < 0.05$), and green bars indicate samples with C3 levels that were significantly higher than the model average. Sample labels indicate their gender (F or M) and their age at time of collection. Sample labels that end in “A” or “B” indicate that more than one sample had that combination of age and gender. (B) Association between batch-adjusted C3 levels and C5a concentration after exposure to L-SPIO NWs. Both C3 levels and C5a. Concentrations are plotted on the log base 10 scale. Each dot represents a separate subject and the color of the dot indicates the comparison to the model average as in panel A. The reported correlation coefficient and p -values are based on the Pearson correlation of the log base 10 values. (C)

Distribution of batch-adjusted C3 levels across subjects after exposure to S-SPIO NWs. See the description for panel A. (D) Association between batch-adjusted C3 levels and C5a concentration after exposure to S-SPIO NWs. See the description for panel B.

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Figure 3.

Between-subject variation of C3 levels after exposure to Feraheme, LipoDox, and Onivyde. A batch-adjusted estimate of C3 levels (molecules per milligram of drug) for each sample is represented by a bar separately for (A) Feraheme, (B) LipoDox, and (C) Onivyde. Red bars indicate samples with C3 levels that were significantly lower than the model average ($p < 0.05$), and green bars indicate samples with C3 levels that were significantly higher than the model average. Sample labels indicate their gender (F or M) and their age at time of collection. Sample labels that end in “A” or “B” indicate that more than one sample had that combination of age and gender. Fewer serum samples were tested with LipoDox than with Feraheme and Onivyde due to sample availability. (D) Sources of variation in C3 levels after exposure to different nanoparticles. The proportion of the total variance that can be attributed to variance between subjects (green), variance between replicate preparation of the same subject (blue), and the residual variance (red) that includes technical variation is indicated by the height of the bars and grouped by nanoparticle. SPIO NWs show the highest proportion of variance attributed to between-subject effects. LipoDox shows the lowest

proportion of variance attributed to between-subject effects and the highest proportion of variance due to preparation effects.

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Figure 4.

Association between C3 levels across nanoparticles. The correlation between C3 levels derived from different nanoparticles was calculated using log base 10 values and a Pearson correlation coefficient. The color of cells within the heat map represents the negative log base 10 p -values (higher value and smaller p -value). Comparisons to a p -value greater than 0.01 are white. The correlation coefficient is the first number reported in each cell followed by the p -value in parentheses. The p -values are not adjusted for multiple testing.

Figure 5.

Comparison of C3 opsonization efficiency per nanoparticle. (A) Number of C3 molecules per nanoparticle was calculated as “number of C3 molecules per milligram of Fe or drug” divided by “number of nanoparticles” as described in the Materials and Methods section and in Table 1. The number was plotted against average diameter for each nanoparticle (Table 1). Excluding LipoDox, there was a significant correlation between size and number of C3 molecules per particle. LipoDox does not fit the trend, likely due to a highly PEGylated coating. (B) Dimensions of the C3b molecule. (C) C3b and the alternative pathway convertase (C3b-green, Bb-red, and properdin-blue) in relation to the sizes of nanocarriers. The size of C3b and C3 convertase is comparable to the size of Feraheme, which could explain the low number of C3 per particle. LipoDox also showed low number of C3 per particle despite having much-larger diameters than Feraheme, most likely due to the

PEGylated coating. For simplicity, the protein corona, which is critical for complement binding and activation,¹¹ is not shown here.

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Table 1.

Description of Nanoparticles Used in the Study^a

nanoparticle name	size, intensity weighted ± width (nm)	bulk composition (mole ratio)	number of nanoparticles (per milligram of drug)	average content per nanoparticle
L(arge)-SPIO NWs	111.40 ± 48.10	20 kDa native dextran, Fe ₃ O ₄	3×10^{13}	~20 Fe ₃ O ₄ crystals
S(mall)-SPIO NWs	57.79 ± 22.21	20 kDa native dextran, Fe ₃ O ₄	6×10^{13}	~10 Fe ₃ O ₄ crystals
Feraheme	27.59 ± 10.60	10 kDa carboxymethyl reduced dextran, Fe ₃ O ₄	6×10^{14}	~1 Fe ₃ O ₄ crystal
LipoDox	87.63 ± 21.94	HSPC/Chol/DSPE-PEG-2000 (56.6:38.2:5.3)	8.1×10^{13}	~13 000 doxorubicin hydrochloride molecules
Onivyde	120.00 ± 33.88	DSPE/Chol/DSPE-PEG-2000 (59.8:39.9:0.3)	1.32×10^{13}	~70 000 irinotecan hydrochloride molecules

^aComposition of clinically approved particles was provided by the manufacturers. Concentration was determined by Nanosight or calculated as described in the Materials and Methods section.