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Accelerated Blood Clearance of Antibodies by Nanosized Click Antidotes

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Abstract

Long blood half-life is one of the advantages of antibodies over small molecule drugs. At the same time, prolonged half-life is a problem for imaging applications or in the case of antibody-induced toxicities. There is a substantial need for antidotes that can quickly clear antibodies from systemic circulation and peripheral tissues. Engineered nanoparticles exhibit intrinsic affinity for clearance organs (mainly liver and spleen). *trans*-Cyclooctene (TCO) and methyltetrazine (MTZ) are versatile copper-free click chemistry components that are extensively being used for *in vivo* bioorthogonal couplings. To test the ability of nanoparticles to eliminate antibodies, we prepared a set of click-modified, clinically relevant antidotes based on several classes of drug carriers: phospholipid-PEG micelles, bovine serum albumin (BSA), and cross-linked dextran iron oxide (CLIO) nanoparticles. Mice were injected with IRDye 800CW-labeled, click-modified IgG followed by a click-modified antidote or PBS (control), and the levels of the IgG were monitored up to 72 h postinjection. Long-circulating lipid micelles produced a spike in IgG levels at 1 h, decreased IgG levels at 24 h, and did not decrease the area under the curve (AUC) and IgG accumulation in main organs. Long-circulating BSA decreased IgG levels at 1 and 24 h, decreased the AUC, but did not significantly decrease organ accumulation. Long-circulating CLIO

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ASSOCIATED CONTENT

Supporting Information

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Elimination profile of IgG; reactivity of CLIO-TCO and CLIO-NW-TCO with IgG in mouse blood; reaction between CLIO-TCO and IRDye 800CW-IgG-MTZ *in vitro*; elimination half-life of IgG-MTZ with or without CLIO-TCO detected with secondary antibody; liver accumulation of IgG-MTZ with or without CLIO-TCO; hematological counts of mice injected with IgG-MTZ or IgG-MTZ followed by CLIO-TCO; H&E staining of organs of mice injected with IgG-MTZ or IgG-MTZ and CLIO-NW-TCO; and elimination half-life of CLIO-TCO and CLIO-NW-TCO with or without conjugation to IgG-MTZ (PDF)

Notes

The authors declare no competing financial interest.

nanoworms increased IgG levels at 1 h, decreased IgG levels at 24 h, did not decrease the AUC, and did not decrease the organ accumulation. On the other hand, short-circulating CLIO nanoparticles decreased IgG levels at 1 and 24 h, significantly decreasing the AUC and accumulation in the main organs. Multiple doses of CLIO and BSA were not able to completely eliminate the antibody from blood, despite the click reactivity of the residual IgG, likely due to exchange of IgG between blood and tissue compartments. Pharmacokinetic modeling suggests that short antidote half-life and fast click reaction rate should result in higher IgG depletion efficiency. Short-circulating click-modified nanocarriers are the most effective antidotes for elimination of antibodies from blood. This study sets a stage for future development of antidotes based on nanomedicine.

Graphical Abstract

Keywords

nanoparticle; CLIO; SPIO; albumin; antibody; click chemistry; antidote

The renaissance of monoclonal antibodies has revolutionized medicine and the pharmaceutical industry. There are hundreds of clinically approved antibody drugs on the market and several hundreds at different stages of clinical testing or approval.¹ The majority of antibodies are designed for therapy, but some antibodies are also being tested for imaging, for example, near-infrared dye-labeled anti-epidermal growth factor (cetuximab) and antivascular endothelial growth factors (bevacizumab) for perioperative imaging.^{2,3} Unlike small molecule drugs and other biologics, antibodies possess intrinsically long half-lives that can be further enhanced through engineering. Among the factors affecting antibody longevity are affinity for FcRn (neonatal Fc receptor) that enables recycling after the internalization, overall charge, presence of sugar moieties, and molecular weight.⁴⁻⁶

The need for accelerated clearance of monoclonal antibodies was recognized many years ago when radionuclide-labeled antibodies were first tested in imaging applications, primarily in cancer.⁷ The wide body distribution and long circulation can lead to unnecessary exposure to radiation, prompting the development of a pretargeting approach, wherein non-radioactive

antibody tagged with streptavidin was injected first, followed by injection of a biotinylated radioactive molecule^{7–9} This pretargeting concept resulted in a much better signal-to-background ratio and image quality. At the same time, the long-circulating properties of targeting antibodies were still a problem as one had to wait weeks until the antibody was sufficiently cleared for the imaging procedure to take place. Therefore, clearing strategies were developed, using neutralizing antibodies, galactosylated biotin albumin,¹¹ biotin–albumin, avidin,^{12,13} complementary oligonucleotides,¹⁴ or extracorporeal affinity tags,¹⁵ in order to quickly eliminate the antibodies from the systemic circulation. Although some of these approaches, mostly biotin–streptavidin pair, have been tested in nuclear imaging and therapy in patients,¹⁶ there is still a substantial risks of immunogenicity,^{7,11} as well as suboptimal clearing efficiency. More recently, several interesting clearing approaches to block FcRn recycling by *in vivo* PEGylation of the Fc portion¹⁷ or by anti-FcRn antibody¹⁸ have been reported. However, these approaches still retain IgG in tissues, require introduction of non-natural amino acids into the antibody sequence, or interfere with metabolism of natural immunoglobulins.

With the expanded range of antibody applications in clinical use, we believe that there is a substantial unmet need in antibody antidotes. For example, immune checkpoint inhibitors (anti-CTLA-4 and anti-PD-1) cause serious dermatologic and neurological toxicities, whereas anti-EGFR antibody causes severe skin toxicity,^{19–21} and there are no effective strategies to eliminate these drugs from the body once the adverse effects appear. In addition, antibodies for infrared perioperative imaging are directly labeled and may take days until cleared from circulation.² Bioorthogonal click chemistry,²² in particular, copper-free Diels–Alder additions involving strained *trans*-cyclooctene (TCO) and methyltetrazine (MTZ), has been proven to be versatile due to very fast second-order reaction rates resulting in the formation of a stable covalent bond.²³ The efficient pretargeting^{24,25} and *in vivo* cell surface modification²⁶ using the TCO-MTZ pair have been previously demonstrated. We set out to explore a set of clinically relevant nanosized drug carriers with different circulation properties available in our laboratory as click chemistry antidotes. We reasoned that click-modified nanoparticles should interact with click-modified antibodies *in vivo* and trigger elimination from the bloodstream, due to the general propensity of nano-medicines to be cleared by the liver and spleen.²⁷ Our results suggest that the clearance efficiency is dictated by the elimination half-life of the antidote as well as the tissue/blood distribution of the antibody. This study lays the groundwork for development of cost-effective, clinically viable, antibody-clearing nanoantidotes.

RESULTS

Click-Modified Nanocarriers.

In order to develop antidotes that capture and eliminate antibodies *in vivo*, we prepared click-modified carriers of different chemistries and sizes, based on the expertise in our laboratory (Figure 1A). Phospholipid micelles are some of the most attractive delivery systems, and PEGylated phospholipids are FDA-approved components of liposomal drugs (*e.g.*, Doxil, Onivyde). We prepared the MTZ derivative of DSPE-PEG₃₄₀₀ (hereafter DSPE-PEG-MTZ) as described in Materials and Methods. The lipid formed heterogeneous

assemblies sized between 17 and 257 nm and a negative ζ -potential of -6 mV due to the presence of the phosphate group. Theoretical binding capacity (based on weight fraction of MTZ groups) was $4241.5 \mu\text{g}$ of IgG per μg of lipid (Table 1). The actual binding capacity is likely to be lower because the surface area of the lipid assemblies should limit the number of bound IgG molecules. To prepare a macromolecule-based antidote, we conjugated NHS-PEG₄-TCO to bovine serum albumin (BSA). BSA-TCO was 8 nm in diameter with a ζ -potential of -20 mV, and a theoretical binding capacity of 2 IgG/BSA or $2.3 \mu\text{g}$ IgG/ μg BSA. Superparamagnetic crystalline iron oxide (SPIO) nano-particles are an important magnetic resonance imaging contrast agent also used as a component of multifunctional theranostic nanomedicines for imaging and treatment.^{28–30} To prepare nanoparticle-sized antidotes, we synthesized aminated crosslinked 20 kDa dextran CLIO nanoworms (CLIO-NW) and aminated cross-linked 10 kDa dextran CLIO nanoparticles (CLIO, Figure 1A). According to the transmission electron microscopy (TEM) images (Figure 1B), CLIO-NWs contained mostly worm-like cores composed of several Fe₃O₄ crystals,³¹ whereas CLIO had smaller size cores and there were many single-crystalline nanoparticles. Each CLIO-NW particle contained ~ 30000 amino groups, and each CLIO particle contained ~ 6000 amino groups. These amines were further modified with NHS-PEG₄-TCO in order to form CLIO-NW-TCO and CLIO-TCO. The hydrodynamic diameter of CLIO-NW-TCO and CLIO-TCO was 70 and 55 nm, and ζ -potential was -2.7 and $+8$ mV, respectively. Theoretical binding capacity (based on the surface area) was 16095 and 95160 IgG/particle or 2.31.9 and $1.92.3 \mu\text{g}$ IgG/ μg Fe, respectively (Table 1).

To measure the blood half-life of the antidotes, DSPE-PEG-MTZ micelles were labeled with DiR, whereas BSA-TCO, CLIO-NW-TCO, and CLIO-TCO were labeled with IRDye 800CW. The elimination half-life following i.v. injection into female BALB/c mice was in the following order (slow phase): DSPE-PEG-MTZ (14 h) > CLIO-NW-TCO (6.7 h) > BSA-TCO (4 h) >> CLIO-TCO (6 min) (Figure 1C–F). The shorter half-life of CLIO-TCO *versus* that of CLIO-NW-TCO was probably due to excess positive charge on the former because positively charged particles are usually cleared faster than negatively charged ones.³² All the antidotes accumulated predominantly in the liver and spleen, with minor accumulation in the kidney (Figure 1C–F). In addition, DSPE-PEG-MTZ and BSA-TCO showed significant accumulation in the lungs (Figure 1C,D).

***In Vivo* Efficiency of the Antidotes.**

In order to test the depletion efficiency of the antidotes, female BALB/c mice were injected with $25 \mu\text{g}$ of IRDye 800CW-labeled IgG followed by PBS (control) or the antidote, and the levels of IgG in blood (both antidote-bound and free) were measured at different times post-IgG injection (Figure 2A). IgG alone has a long biexponential blood half-life of 32 h (slow phase, Supplemental Figure 1); however, in order to account for experimental variability and batch effect of the modified antibodies, each depletion experiment always included an “IgG only” group along with a “IgG+antidote” group. To test the ability of DSPE-PEG-MTZ to decrease total blood levels of IgG (both antidote-bound and free), mice were injected with IRDye 800CW-IgG-TCO followed 1.5 h later with the lipid (1 mg, 1660-fold molar excess of MTZ groups). Surprisingly, there was a 72% increase in the blood levels of IgG at 2 h (p value 0.0007, two-sided t test, $n = 3$) and a 68% decrease at 24 h (p value 0.00018, two-sided

t test, $n = 3$) post-IgG injection, compared to the control IgG group (Figure 2B). Overall, however, the injection of DSPE-PEG-MTZ did not significantly decrease the AUC_{24h} (17.7%; p value 0.056, two-tailed *t* test, $n = 3$). To test the depletion efficiency of BSA-TCO, we injected mice with 25 μ g of IgG-MTZ followed by BSA-TCO at 2 and 3 h post-IgG injection (500 μ g each; ~400-fold excess of TCO; 92-fold excess of surface-binding capacity). BSA-TCO decreased the blood level of IgG by 40% at 3 h (Figure 2C; p value <0.0001, two-sided *t* test, $n = 3$) and by 53% at 24 h, and the AUC_{24h} was significantly decreased (41%; p value <0.0001, two-tailed *t* test, $n = 3$) compared to the control group.

In view of the large excess of the injected lipid and BSA over IgG, it is unlikely that insufficient binding capacity is responsible for the observed difference in the depletion efficiency of these antidotes. To further investigate which factors contribute to the depletion efficiency, we compared CLIO-TCO and CLIO-NW-TCO that have similar sizes but different blood half-lives (Figure 2E,F). Both nanoparticles showed similar IgG-MTZ binding capacity in BSA and mouse blood (Supplemental Figure 2). We injected 25 μ g of IRDye 800CW-IgG-MTZ followed 1.5 h later with long-circulating CLIO-NW-TCO (290 μ g Fe; ~5200-fold molar excess of TCO, 26.5-fold excess of surface-binding capacity). The injection resulted in a 68% increase in the blood level of IgG at 2 h post-IgG injection (Figure 2D; p value 0.01, two-sided *t* test, $n = 3$) and nonsignificant increase at 24 h, compared to the control group (Figure 2D; p value 0.12, two-sided *t* test, $n = 3$). The AUC_{24h} was significantly increased (85.6%; p value <0.0001, two-tailed *t* test, $n = 3$). On the other hand, injection of short-circulating CLIO-TCO 1 h after IgG (290 μ g; ~1400-fold molar excess of TCO over IgG, 22-fold excess of surface-binding capacity) resulted in 56% decrease in IgG 3 h post-IgG injection (p value 0.0022, two-sided *t* test, $n = 3$), 54% decrease at 24 h, and significant decrease in AUC_{24h} (62%; p value <0.0001, two-tailed *t* test, $n = 3$) compared to the control IgG group (Figure 2E). Conjugation of IgG-MTZ to CLIO-TCO did not result in any decrease in the fluorescence of the antibody (Supplemental Figure 3A). We also confirmed that CLIO-TCO specifically binds to IgG *via* click chemistry (Supplemental Figure 3B), which confirms that the observed decrease in IgG blood fluorescence was due to depletion. Furthermore, to exclude the possibility that near-infrared dye IRDye 800 detached from the antibody in the circulation, we confirmed the similar efficiency of depletion of IgG-MTZ by CLIO-TCO using secondary anti-human antibody (Supplemental Figure 4).

To study the depletion in the peripheral organs, we scanned the main organs 48–72 h after the antidote injection with a Li-COR Odyssey NIR scanner. The mean fluorescence reflects both the deposited IgG and the circulating IgG (either free or antidote-bound). According to Figure 3A,C, DSPE-PEG-MTZ and CLIO-NW-TCO did not cause any decrease in the fluorescence in the organs. BSA-TCO (Figure 3B) actually increased the liver fluorescence (p -value 0.02, two-way ANOVA with multiple comparisons, $n = 3$) but did not decrease the level of fluorescence in other organs. CLIO-TCO (Figure 3D) that had the best depletion efficiency in blood also decreased fluorescence by 50% in the liver and the lungs (liver p value <0.0001; lung p value <0.001; two-way ANOVA with multiple comparisons, $n = 3$). The decreased fluorescence in the organs probably reflected the decrease in the blood pool levels of IgG (the antibody was still in circulation when the animals were euthanized). Liver is the main clearance organ for CLIO-TCO (Supplemental Figure 1). In order to understand

how the antidote injection affects distribution of IgG in the liver, we injected mice with IRDye 800-IgG-MTZ alone or followed by Cy3-CLIO-TCO, euthanized them 3 h post-antidote injection, and imaged the histological sections with a fluorescence microscope. As shown in Figure 4A, antidote-injected mice showed colocalization of the nanoparticles and the antibody in the Kupffer cells, whereas control IgG-injected mice showed diffuse and widespread distribution consistent with the antibody being in the blood pool and also in hepatocytes and endothelial cells (Figure 4B). A similar result was obtained by indirect immunofluorescence using a secondary anti-human antibody to detect human IgG in the liver (Supplemental Figure 5). These data suggest that the antidote mainly directs the antibody to the Kupffer cells in the liver.

Two injections of BSA-TCO and a single injection of CLIO-TCO showed efficient depletion, but there was still a substantial fraction of non-eliminated IgG in blood (Figure 2C,E). Injection of mice with 25 μg of IRDye 800CW-IgG-MTZ followed with three injections of BSA-TCO at 1.5, 3, and 23 h post-IgG (500 μg each; ~ 1200 -fold excess TCO, ~ 140 -fold excess of surface-binding capacity) showed a 38% decrease in AUC_{24} (p value < 0.0001 , two-tailed t test, $n = 3$), which was not different from that achieved after two injections of BSA-TCO (Figure 5A,C). Three injections of CLIO-TCO at 2, 3, and 4 h post-IgG (150 μg each; ~ 2300 -fold excess TCO, 34-fold excess of surface-binding capacity) showed 62.7% decrease in $\text{AUC}_{24\text{h}}$ (p value < 0.0001 , two-tailed t test, $n = 3$), which was not different from that achieved after a single injection of 290 μg of CLIO-TCO (Figure 5B,C). These data suggest that multiple injections of the antidote are not able to clear the remaining circulating IgG. To exclude the possibility that the remaining fraction of IgG does not have the reactive MTZ group, we drew blood samples from CLIO-TCO-injected mice and control mice at 4.5 and 24 h (Figure 2E) and added excess CLIO-TCO. The particles were pelleted with ultracentrifugation, and the supernatant NIR fluorescence was measured with Li-COR Odyssey. According to Figure 6, IgG was completely pulled down by the nanoparticles at both time points, ruling out the possibility that the remaining circulating IgG was not reactive.

Lastly, we questioned whether CLIO-TCO nanoparticles that were the most efficient depletion agent in our study induce toxicity. Nanoparticle toxicity often causes challenges in clinical development of nanomaterials.³³ Analysis of blood cell profile of mice injected with IgG-MTZ followed by CLIO-TCO (4 h post-IgG injection, Supplemental Figure 6) and hematoxylin–eosin staining of the main organs (Supplemental Figure 7) did not reveal any gross differences with IgG-MTZ injected mice and no signs of inflammatory response.

Modeling of Parameters That Determine Depletion Efficiency.

CLIO and CLIO-NWs have similar size, IgG binding efficiency, and biodistribution but different half-lives and strikingly different depletion efficiency. In order to understand the factors that determine the depletion efficiency, we developed a compartmental pharmacokinetic (PK) model that takes into account blood elimination rates of the antidote and the antidote–IgG complex, click reaction rates, and the antibody exchange rate between blood and tissue compartment (Figure 7A). Using our previously described PK modeling program Boomer,³⁴ we calculated the volume of distribution V and tissue/blood exchange

rates k_{12} and k_{21} of IgG only (Table 2 and Supplemental Figure 1). These parameters were adjusted for each experiment by the software in order to obtain the best fit to the antidote data. The elimination rate of CLIO-TCO and CLIO-NW-TCO conjugated to IgG-MTZ was the same as that of free antidotes (Supplemental Figure 8). The model produced an accurate fit for IgG blood profile (Figure 7B,C) after injection of CLIO-TCO (one injection) (Table 2 for the fitted parameters) and a reasonable fit for CLIO-TCO (three injections). The model predicts that multiple injections of CLIO-TCO do not improve depletion of blood IgG at early time points (Figure 7C) but predicts a slightly better depletion at 24 h than observed experimentally. The mechanism of incomplete IgG depletion despite click reactivity of the remaining antibody (Figure 6) is likely due to the exchange of the IgG between blood and tissue compartments, which may not be fully accounted for by the PK model. The model produced an excellent fit for long-circulating CLIO-NW-TCO and predicted the observed spike in IgG at 2 h and decrease at 24 h (Figure 7D). Interestingly, the simulation also explains the result obtained with DSPE-PEG-MTZ, which had a long circulation time, and showed the spike in IgG levels and depletion at late time points. Simulations of click reaction rate constant showed strong effect on the depletion efficiency (Figure 7E). Click rates *in vitro* reach up to $3.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ but the rate *in vivo* could be lower and depend on the interaction between antidote, blood cells, and blood proteins. Finally, we simulated the IgG depletion efficiency by a hypothetical antidote that has a long blood half-life but exhibits accelerated clearance upon click reaction with IgG (Figure 7F). The modeling predicts that such an antidote should exhibit the best efficiency due to long residence in blood and quick removal of the IgG that diffuses from the tissue back into blood compartment.

DISCUSSION

There is an unmet preclinical/clinical need for agents that can eliminate therapeutic and diagnostic antibodies from the body. Previously described approaches were mostly based on streptavidin-labeled antibodies and albumin-labeled biotin. Whereas the affinity and specificity of streptavidin–biotin binding is extremely high ($K_d \sim 10^{-15} \text{ mol/L}$), there is substantial immunogenicity of streptavidin that prevents repeated injections.³⁶ Click chemistry presents an attractive alternative due to efficient *in vivo* reaction rates,³⁷ small size of the reagents, and potentially lower immunogenicity. We explored nanosized clinically relevant antidotes with different physicochemical properties and pharmacokinetics. The straightforward comparison of CLIO-TCO and CLIO-NW-TCO suggests that nanocarriers with short circulation times are more efficient, and the PK modeling confirms that hypothesis. Nevertheless, even short-circulating CLIO-TCO did not eliminate completely the antibody from blood and peripheral organs. Liver, muscle, gut, kidney, and skin are the organs with the highest FcRn activity.³⁸ The fact that clearing agents were not able to completely deplete the antibody could be due to distribution of the latter into the tissue compartment and continuous diffusion back into the blood compartment, but we did not perform additional experiments to prove this hypothesis. On the other hand, the spike in IgG concentration soon after injection of CLIO-NW-TCO and DSPE-PEG-MTZ suggests that the long-circulating antidotes keep the antibody in blood and from elimination. This mode of sequestration may be an interesting alternative to the immediate elimination and could be

used to restrict the antibody to the blood compartment. In addition, we included albumin, which was used in previous approaches as the clearing agent, either in biotinylated or galactosylated/biotinylated form.^{8,11} The performance of albumin varied from ineffective to 90% effective in other publications,^{39,40} necessitating comparison in this work using the same chemistry. The ultrasmall highly aminated CLIO-TCO showed efficiency better than that of BSA-TCO in our study, most likely due to fast elimination rate of the former. At the same time, long-circulating albumin was more efficient than CLIO-NW-TCO and DSPE-PEG-MTZ, suggesting that half-life plays only a partial role in the antidote efficiency. It is plausible that *in vivo* reaction rate for BSA-TCO is much faster than that for CLIO-NW-TCO and DSPE-PEG-MTZ. In addition, albumin distributes into tissues *via* the same FcRn pathway as IgG,⁴¹ which could lead to a more efficient capture.

The PK modeling suggests that a long-circulating nano-antidote that clears immediately upon binding of IgG should demonstrate the most efficient depletion. At the same time, due to a complicated relationship between redistribution in tissues, circulation half-life of the antidote, reaction efficiency, and binding capacity, the model cannot fully predict the *in vivo* performance. Moreover, an antidote has to be tailored to a specific antibody due to differences in IgG pharmacokinetic profiles and clearances.^{38,42} Finally, we must point out the limitations on the translational use of nanoparticles as antidotes. Nanomedicines potentially activate complement,^{43,44} and antibodies attached to nanoparticles can further enhance the complement activation.⁴⁵ The biodistribution of nano-carriers is of concern because other organs besides liver and spleen can accumulate nanoparticles. For instance, mesoporous silica nanoparticles with affinity for kidneys have been reported,⁴⁶ and our data showed accumulation of lipid nanoparticles and BSA in the lungs (Supplemental Figure 1). Although we demonstrated no effect on blood cell counts and gross organ histology after injection of CLIO-TCO, there is a need in further assessment of safety of nanoparticle-based antidote approaches. We did not assess clinically approved nanoparticles such as liposomes and ultrasmall iron oxide Feraheme. These carriers usually show somewhat prolonged circulation times^{47,48} and are expected to be less effective in this particular application.

CONCLUSIONS

We demonstrated efficient and fast elimination of antibodies by the click nanoantidote with intrinsically short half-life. Antidote half-life and reaction kinetics are the critical parameters. The removal of IgG from blood was not complete, likely due to exchange of IgG between tissue and blood compartment. This study provides important guidelines for further development of click antidotes based on nanomedicine. It remains to be demonstrated if the level of depletion in blood and organs achieved using click nanoantidotes is sufficient to enable shorter imaging times or to reduce toxicity.

MATERIALS AND METHODS

Materials.

Iron salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). DSPE-PEG3400-amine was from Laysan Bio. Bovine serum albumin was from Sigma (St. Louis, MO, USA).

Methyltetrazine (MTZ)-PEG₄-NHS ester, *trans*-cyclooctene (TCO)-PEG₄-NHS ester, and MTZ-NHS ester were from Click Chemistry Tools (Scottsdale, AZ, USA). IRDye 800CW-NHS ester was from Li-COR Biosciences (Lincoln, NB, USA). Purified human IgG was from Jackson ImmunoResearch (West Grove, PA, USA). Zeba desalting columns (7 and 40 kDa) were from Thermo Fisher. Cy7-NHS ester and Cy3-NHS ester were from Lumiprobe Inc. (Hunt Valley, MD, USA). Goat anti-human IRDye 680 antibody was from Li-COR. Lipophilic carbocyanine dye DiOC18(7) ("DiR") was from Thermo Fisher.

Synthesis of DSPE-PEG-MTZ.

DSPE-PEG-MTZ was synthesized according to scheme below by reacting methyltetrazine NHS ester (4.33 mg, 13.2 μ mol) with DSPE-PEG₃₄₀₀-NH₂ (M_w = 3400, 30 mg, 8.8 μ mol) and DIEA (3.42 mg, 26.47 μ mol) in 1 mL of anhydrous DMSO at room temperature for 6 h. The crude product was purified by C18 reverse-phase HPLC, and the product eluted with 95% methanol/water to obtain DSPE-PEG-MTZ as a red solid (20.2 mg, 63% yield). ¹H NMR (400 MHz, CDCl₃): δ 0.90 (m, 10H); 1.27 (m, 56H); 1.46–1.57 (m, 9H); 2.23–2.56 (m, 18H); 3.12 (s, 3H); 3.39–3.52 (m, 3H); 3.66 (m, 269H); 3.82 (s, 3H); 4.08 (s, 2H); 4.09–4.26 (m, 4H); 4.35 (m, 1H); 5.20 (m, 1H); 6.43 (m, 1H); 7.28 (d, J =8.40 Hz, 2H, Ar-H); 7.82 (s, 1H) 8.57 (d, J = 8.36 Hz, 2H, Ar-H).

Synthesis of BSA-TCO and IRDye 800-BSA-TCO.

Bovine serum albumin (10 mg/mL in 100 μ L PBS) was combined with a 10-fold excess of TCO-PEG₄-NHS ester (2 μ L of DMSO). The reaction was allowed to incubate at 4 °C for 24 h. Alternatively, BSA in PBS (100 μ L, 5 mg/mL) was combined with a 10-fold excess of IRDye 800CW-NHS ester (in 2 μ L of DMSO) for 15 min before adding a 20-fold excess of TCO-PEG₄-NHS ester (in 2 μ L of DMSO). All the reactions were purified using a 7000 molecular weight cutoff Zeba spin column.

Synthesis of CLIO and CLIO-NWs.

CLIO-NWs were synthesized from native 20 kDa dextran (Sigma), whereas CLIO were synthesized from reduced T-10 (10 kDa) dextran (Pharmacosmos). Dextran, Fe(III) chloride, and Fe(II) chloride were mixed in DDW and titrated with ammonia using a modified Molday precipitation method.^{52,53} The ratio between dextran and iron salts determined the final size of the nanoparticles.⁵³ Excess dextran was removed with ultrafiltration, and the particles were cross-linked with epichlorohydrin as described.³¹ Excess epichlorohydrin was

removed with ultrafiltration, and particles were aminated by addition of ammonium hydroxide (CLIO-NWs) or diaminoethane (CLIO) overnight at 4 °C. Excess amines was removed by ultrafiltration; particles were resuspended in sterile water, filtered through a 0.45 μm filter, and stored at 4 °C. Size and ζ -potential were determined using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). The intensity-weighted distribution was used to report hydrodynamic diameter. For TEM analysis, the imaging of unstained samples applied on the carbon grid was performed using a FEI Tecnai Spirit BioTwin electron microscope at 100 keV. The amino group content was determined by reaction with Cy7-NHS. Particles were reacted in PBS with excess dye and then precipitated with 99% ethanol. The amount of the reacted dye was determined by the difference in the supernatant fluorescence between the reaction tube and control tube (no particles). The number of amines per nanoparticle was determined to be ~ 100000 per CLIO and CLIO-NW.

Synthesis of CLIO-TCO and IRDye 800CW-CLIO-TCO.

Aminated CLIO in H_2O (100 μL , 4 μM) was combined with a 100-fold excess of TCO-PEG₄-NHS ester (in 2 μL of DMSO). The reaction was allowed to incubate at 4 °C for 24 h and was purified with a 40 kDa molecular weight cutoff Zeba spin column. Alternatively, aminated CLIO in H_2O (100 μL , 4 μM) was combined with a 30-fold excess of IRDye 800CW-NHS ester (in 2 μL of DMSO) for 15 min before adding a 100-fold excess of TCO-PEG₄-NHS ester (in 2 μL of DMSO). The reaction was allowed to incubate at 4 °C for 24 h and was purified using a 40 kDa cutoff Zeba spin column.

Synthesis of CLIO-NW-TCO and IRDye 800CW-CLIO-NW-TCO.

Aminated CLIO-NW in H_2O (100 μL , 500 nM) was combined with a 100-fold excess of TCO-PEG₄-NHS ester (in 2 μL of DMSO). The reaction was allowed to incubate at 4 °C for 24 h. Alternatively, aminated CLIO-NW in H_2O (100 μL , 500 nM) was combined with a 30-fold excess of IRDye 800CW NHS ester (in 2 μL of DMSO) for 15 min before adding a 100-fold excess of TCO-PEG₄-NHS ester (in 2 μL of DMSO). The reaction was allowed to incubate at 4 °C for 24 h and was purified using a 40 kDa cutoff Zeba spin column.

Synthesis of IgG-MTZ, IgG-TCO, IRDye 800-IgG-MTZ, and IRDye 800-TCO.

Human polyclonal IgG in PBS (100 μL , 10 mg/mL) was combined with a 20-fold excess of MTZ-PEG₄-NHS ester or TCO-PEG₄-NHS (in 2 μL of DMSO). The reaction was allowed to incubate at 4 °C for 24 h and was purified using a 7 kDa cutoff Zeba spin column. Alternatively, IgG in PBS (100 μL , 10 mg/mL) was combined with a 10-fold excess of IRDye 800CW-NHS ester (in 2 μL of DMSO) for 15 min before adding a 20-fold excess of methyltetrazine-PEG₄-NHS ester (in 2 μL of DMSO). The reaction was allowed to incubate at 4 °C for 24 h and was purified using a 7 kDa cutoff Zeba spin column. Conjugation efficiency was about 1–2 IRDye 800/IgG as determined by UV absorbance and the dye extinction coefficient of $160000 \text{ M}^{-1} \text{ cm}^{-1}$.

In Vitro Reactivity of Nanoparticles.

To compare the efficiency of reaction of CLIO-NW-TCO and CLIO-TCO with IgG-MTZ in mouse blood, IRDye 800-IgG-MTZ was added to fresh heparinized mouse blood or 1%

BSA/PBS at 15 $\mu\text{g}/\text{mL}$. CLIO-NW and CLIO-NW TCO were added next at 90 $\mu\text{g}/\text{mL}$. The reaction was mixed at 37 $^{\circ}\text{C}$ for 10 min and then centrifuged at 450000g in a TLA-100.3 rotor (Beckman Optima ultracentrifuge) in order to pellet blood cells and nanoparticles. Supernatants were collected into a 384-well black/transparent plate and scanned for NIR fluorescence with Li-COR Odyssey at 800 nm. The fluorescence of supernatant without added particles (control) was set as 100%.

For measurement of quenching of IRDye 800CW fluorescence, CLIO-TCO were mixed at different weight ratios with IRDye 800CW-IgG-MTZ or IRDye 800CW-IgG (control) and incubated for 15 min, and the fluorescence was scanned at 800 nm with Li-COR Odyssey. IgG fluorescence without added nanoparticles was set as 100%.

To test the specificity of reaction of CLIO-TCO, 1.5 μg of IRDye 800CW-IgG-MTZ was added to 100 μL of FBS. Aminated CLIO or CLIO-TCO were added in triplicate to FBS (15 μg Fe/sample), incubated for 1.5 h at room temperature, and pelleted at 450000g for 15 min by ultracentrifugation as described above. Two microliters of supernatant was blotted for each of the samples on a nitrocellulose membrane and scanned at 800 nm.

In order to test the reactivity of the residual circulating antibody that was not depleted by CLIO-TCO, mice (injected with IgG only (control) or IgG followed by CLIO-TCO) were bled at 4.5 and 24 h post-IgG injection. After the blood samples (~ 50 μL) were lysed, the lysates were split into two groups. One group had 200 μg of CLIO-TCO added to it, and the other group had PBS. After 3 h of incubation, both groups were spun down at 450000g, and the lysates were blotted on a nitrocellulose membrane and scanned by Li-COR at 800 nm.

Mouse Experiments.

The University of Colorado Institutional Animal Care and Use Committee (IACUC) approved all animal experiments. Mice were treated according to regulations provided by the Office of Laboratory Animal Resources at the UC Denver. Female BALB/c mice 6–8 weeks old were used for the experiments and bred in house. In order to determine circulation half-life of NIR-labeled antibodies and antidotes, 2 μL blood was collected at different time points through the retroorbital vein or mandibular vein and were applied in duplicates on a 0.22 μm nitrocellulose membrane and scanned at 800 nm using Li-COR Odyssey. The spot integrated density of a 16-bit TIFF image was measured with ImageJ and plotted as a function of time. The fluorescence of dots at initial time point was used as 100%. For organ distribution, mice were euthanized at 48–72 h postinjection, and the organs were placed in wells on a 12-well plate. Organs were scanned with Li-COR Odyssey at 800 nm. Mean fluorescence was determined from 16-bit images using ImageJ software by subtracting the background, drawing a region of interest around the organs, and using a measure function to determine mean gray value.

In order to compare elimination half-lives of the antidotes alone and conjugated to IgG, IRDye 800CW-CLIO-NW-TCO, and IRDye 800CW-CLIO-TCO were treated with excess IgG-MTZ in 10% FBS in PBS overnight (4 $^{\circ}\text{C}$). The antidotes with or without IgG (30 μg Fe) were injected in three mice per group.

Pharmacokinetic Modeling.

IgG measurements *versus* time data without the antidote were modeled with a one- or two-compartment pharmacokinetic model using the pharmacokinetic modeling software Boomer.³⁴ Each data point was weighted equally. The two-compartment model was found to provide a better fit to the IgG data after consideration of the Akaike information criterion values, weighted residual plots, and observed *versus* calculated concentration *versus* time plots. Data collected before and after administration of one or more doses of the antidote were added to the model and fitted simultaneously with a parallel model (Figure 7A, top and bottom diagrams show the model without and with antidote, respectively), which included a second-order interaction between IgG and the antidote and elimination of the antidote–IgG complex. In the presence of the antidote, the IgG signal included both the free and bound IgG. In each case, a fit to both the free IgG and antidote-bound IgG resulted in random weighted residual plots and good correspondence between observed and calculated data *versus* time plots.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Antidotes used in the study. (A) Types of nanocarriers used to prepare antidotes. The nanocarriers were modified with click groups (DSPE-PEG₃₄₀₀ with MTZ, BSA with TCO, CLIO-NW and CLIO with TCO) as described in Materials and Methods. (B) TEM images of iron oxide cores of CLIO-NWs and CLIO. Cross-linked dextran shell is not visible. Scale bar = 50 nm. (C–F) Elimination profile of the antidotes and organ distribution (NIR images). Labels are as follows: k, kidney; s, spleen; lv, liver; i, intestine; lu, lung; h, heart. DSPE-PEG-MTZ, BSA-TCO, and CLIO-NW-TCO are long-circulating particles, whereas CLIO-TCO are short-circulating particles. Each time point shows mean and SD; $n = 3$ mice per group.

Figure 2.

Depletion efficiency of IgG *in vivo* by antidotes. (A) IgG was labeled with IRDye 800CW and either TCO or MTZ and injected into female BALB/c mice (25 μ g/mouse), followed by antidote or PBS (control). Blood IgG levels were measured at different time points, up to 72 h postinjection. (B) DSPE-PEG-MTZ. Inset shows early time points for DSPE-PEG-MTZ; (C) BSA-TCO; (D) CLIO-NW-TCO; (E) CLIO-TCO. Black line is “IgG only”, and green line is “IgG+antidote”. Time of IgG injection is “0” on the X-axis. The antidotes were injected at different times after IgG injection, as marked by red arrows. First blood draw was at 1 h post-IgG injection (100%). The IgG only (control) group was included in every experiment. Each time point shows mean and SD. Two-tailed *t* test, *n* = 3 mice per group.

Figure 3.

Organ distribution in control and antidote-injected groups. (A) DSPE-PEG-MTZ; (B) BSA-TCO; (C) CLIO-NW-TCO; (D) CLIO-TCO. Graphs show mean gray value of fluorescence scans, and images show colored images in the 800 nm channel. Organs were scanned in 24-well plates; each organ's image corresponds to the bar graph below; representative images are shown. The signal represents both circulating IgG and organ-deposited IgG. Brightness and contrast were adjusted to the same extent in control and treated groups of the same experiment. Due to scanner settings, antibody batch and time when the experiment was terminated, the gray values show differences between experiments. Two-way ANOVA test with multiple comparisons, $n = 3$ mice per group. Means and SD of experimental replicates are shown.

Figure 4. Histological images of liver of mice injected with IgG-MTZ or with IgG-MTZ followed by CLIO-TCO. In the “IgG+CLIO-TCO” group, the antibody and the nanoparticles mostly colocalize in Kupffer cells (yellow) with some endothelial staining, whereas in the “IgG only” group, there is a diffuse fluorescence of IgG, with some of the antibody accumulating in hepatocytes and sinusoidal endothelium. The experiment was done in two mice per group; representative images are shown.

Figure 5.

Multiple injections of antidotes do not improve the efficiency of depletion. IgG-MTZ was injected in mice and then followed with three injections of CLIO-TCO (A) or BSA-TCO (B). Red arrows show injection of antidote; black lines are the IgG only group, and green lines are the antidote group. First blood draw was at 1 h postinjection of IgG (100%). None of the antidotes was able to deplete all IgG after multiple injections. Each time point shows mean and SD; $n = 3$ mice per group. (C) AUC_{24h} summary for all the experiments shows no difference in depletion efficiency between 1 and 3 injections of CLIO-TCO and no difference between 2 and 3 injections of BSA-TCO. Note lack of depletion by DSPE-PEG-MTZ and increased AUC_{24h} by CLIO-NW-TCO, suggesting that nanoparticles keep the antibody from elimination (two-sided t test, $n = 3$ mice per group).

Figure 6.

Remaining IgG in blood is click-reactive. Mice were injected with IRDye 800CW-IgG-MTZ (control) or with IRDye 800CW-IgG-MTZ followed by CLIO-TCO as described in Figure 2E, and blood samples were collected at 4.5 and 24 h postinjection. CLIO-TCO was added to blood samples ex vivo and pulled down with ultracentrifugation as described in Materials and Methods. Bar graphs and corresponding dots (three mice) show supernatant NIR fluorescence. CLIO-TCO was able to completely pull down the remaining antibody, suggesting the click reactivity. Note the difference in blood IgG levels between control and CLIO-TCO injected mice at both time points. Means and SD of three mice are shown.

Figure 7.

Pharmacokinetic modeling of depletion efficiency. (A) Schematic of the PK model. Top model was used to fit IgG (control) data, and bottom model was used to fit IgG+antidote data. Red circles denote blood compartment, and black circles denote the tissue compartment. IgG data are blood data for control and antidote injected mice, respectively; k_{12} and k_{21} are exchange rate constants with the tissue compartment; k_R is the click reaction rate constant (second order); k_{el} is the IgG elimination rate constant; k_A is the antidote elimination rate, and k_{A-IgG} is the antidote–IgG elimination rate constant. The fitted and

model-adjusted values are in Table 2. Details of the modeling are in Materials and Methods. (B) Model-fitted (solid line) and actual (solid black symbols) blood IgG levels for CLIO-TCO (one injection, Figure 2E). (C) Model-fitted (solid line) and actual (solid black symbols) blood IgG levels for CLIO-TCO (three injections, Figure 5A). (D) Model-fitted (solid line) and actual (solid black symbols) blood IgG levels for CLIO-NW-TCO. Note that the model accurately predicts the spike in IgG concentration at early time points. (E) Simulation of the effect of click reaction rate constant on IgG elimination profile. Higher click reaction rate constant leads to better elimination efficiency. For comparison, the fitted data for CLIO-TCO are shown (blue line). (F) Simulation of an ideal hypothetical antidote (pink line) that has a slow elimination rate constant but 100 times faster elimination rate constant once bound to IgG. For comparison, the fitted data for CLIO-TCO are shown (blue line).

Table 1.

Measured and Calculated Parameters of the Antidotes^a

name	type	diameter (nm)	PDI	ζ -potential (mV)	particles/ mg	theoretical IgG/NP (mole ratio)	Capacity (μ g IgG/ μ g NP)
DSPE-PEG-MTZ	lipid assembly	17 (43%), 257 (57%)	N/A	-5.94	N/A	N/A	~42
BSA-TCO	macromolecule	8	N/A	-20.3	9×10^{12}	~2	~2.3
CLIO-NW-TCO	cross-linked 20 kDa dextran iron oxide	70	0.3	-2.7	$\sim 6 \times 10^{10}$	~160	~2.3
CLIO-TCO	cross-linked 10 kDa dextran iron oxide	55	0.27	7.9	$\sim 8 \times 10^{10}$	~95	~1.9

^a BSA-TCO size and ζ -potential are similar to those of native BSA values reported in the literature.^{49,50} Particle concentration of BSA was determined based on M_w of 67 kDa; CLIO and CLIO-NW concentration was determined as described before.⁵¹ Particle concentration of lipid assemblies could not be estimated due to size heterogeneity. Theoretical surface-based capacity (bound IgG/particle) was determined from particle surface area and assuming that each IgG molecule occupies a cross section of 100 nm^2 . The binding capacity was calculated from surface-based capacity, or in case of DSPE-PEG-MTZ based on percent weight of MTZ groups (M_w 3613 Da).

Table 2.

Parameters of the Model^a

parameter	experiment			
	IgG/CLIO-TCO (Figures 2E, 5B)	IgG/CLIO-NW-TCO (Figure 2D)	IgG only (Supplemental Figure 1)	
k_{e1} (1,2)	0.0264	0.0345	0.031	
k_{12}	0.426	0.16	0.106	
k_{21}	0.655	0.188	0.181	
V_{IgG}	0.168	0.213	7.068 mL	
$V_{IgG-antidote}$	0.62	0.233	N/A	
$k_A : k_{A-IgG}$	1.33/1.174	0.0632/ 0.087	N/A	
k_R	0.0213	0.0168	N/A	

^aThe parameter labels correspond to the scheme in Figure 7A. Parameters such as apparent volume of distribution V in the antidote experiments are unitless due to fitting to the percent IgG over time profile. All k rate constants are h^{-1} . The program (Boomer) was allowed to adjust the parameters to obtain the best fit. Note that the model-adjusted parameters are close to, but not the same as, the parameters obtained from fitting actual data (**bold**). Elimination rate constants of antidotes and IgG-antidote complexes are assumed to be similar and monoexponential (for the sake of simplicity of the model). IgG disposition was found to be biexponential due to distribution into the tissue compartment.