JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Engineering the Protein Corona of a Synthetic Polymer Nanoparticle for Broad-Spectrum Sequestration and Neutralization of Venomous Biomacromolecules

Jeffrey O'Brien, Shih-Hui Lee, Shunsuke Onogi, and Kenneth J. Shea*

Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States

Supporting Information

ABSTRACT: Biochemical diversity of venom extracts often occurs within a small number of shared protein families. Developing a sequestrant capable of broad-spectrum neutralization across various protein isoforms within these protein families is a necessary step in creating broad-spectrum antivenom. Using directed synthetic evolution to optimize a nanoparticle (NP) formulation capable of sequestering and neutralizing venomous phospholipase A_2 (PLA_2), we demonstrate that broad-spectrum neutralization and sequestration of venomous biomacromolecules is possible via a single optimized NP formulation. Furthermore, this optimized NP showed selectivity for venomous PLA_2 over abundant serum proteins, was not cytotoxic, and showed substantially long dissociation rates from PLA_2. These findings suggest that it may show efficacy as an in vivo venom sequestrant and may serve as a generalized lipid-mediated toxin sequestrant.

Snake envenomation is recognized by the World Health Organization (WHO) as a neglected tropical disease.[1] Annually, 4.5 million people suffer from snakebites, 2.7 million suffer serious morbid injuries, and over 100 000 die as a result of snake envenomation.[2,3] The majority of the deaths occur in rural regions in South and Southeast Asia where individuals do not have immediate access to health care facilities capable of treating afflicted individuals.[4] In India alone, an estimated 35 000−50 000 people die annually from snake envenomation and 97% of these mortalities occur in rural regions.[5]

Current methods of treating snake envenomation rely on the acquisition and administration of polyclonal antibodies from surrogate animals.[6] Unfortunately, in many areas, this approach has not been effective due to variations in venom composition that exist between different species and even populations within the same species.[7,8] The interspecific and intraspecific variability of snake venom has led many experts in the field to abandon the idea of creating a broad-spectrum antidote for snake venom.[8] Moreover, the high cost of antivenom production has resulted in the discontinuation of African snake antivenom and coral snake antivenom from the world's leading suppliers.[9,10] This is a major concern in Africa, considering this region is home to many of the world's most venomous snake populations. Hence, there is a high global demand for an inexpensive and effective alternative to current antivenom therapeutics.

Although there is a great deal of variability across snake venoms, there is also a rather striking level of homology.[11] Venomous protein toxins can often be partitioned into a small number of protein families that are found abundantly in the venoms of numerous animals. For example, the protein family phospholipase A_2 (PLA_2) is found in many snake venoms, honey bee venoms, and scorpion venoms. Despite the fact that these families of protein toxins occur across multiple species, securing an effective antivenom is complicated by the diversity of protein isoforms found within each protein family. For example, over 300 different isoforms of PLA_2 have been sequenced.[12] PLA_2, like many protein families found in venom, contains a large number of highly conserved disulfide bonds.[11] This structural conservation and robust scaffold allows for a high degree of variation of exposed amino acid residues allosteric to the active site leading to diverse pharmacological profiles across the PLA_2 protein family. This diversity on the primary sequence level is likely the reason why immunoglobulin based antivenoms are highly specific to particular venom compositions and often show poor cross-reactivity.[8] Consequently, a challenge for any broad-spectrum antivenom is to develop an affinity reagent that recognizes features and similarities that are shared across venomous protein families but are unique with respect to abundant serum proteins to allow for selective sequestration.

Herein, we describe a novel approach to this problem. We have been developing strategies for the synthesis of abiotic protein/peptide affinity reagents. Nontoxic hydrogel copolymer nanoparticles (NPs) have been engineered with affinity and selectivity to peptide toxins, enzymes, signaling proteins and other large biomacromolecules (fibrinogen, IgG) through a directed synthetic evolution process.[13] These synthetic polymer NPs are analogous to biological protein affinity reagents (antibodies, aptamers, etc.). Examples include selective capture and programed release of lysozyme from complex protein mixtures (egg white),[14] a NP with specificity for the Fc domain of IgG,[15] in vitro inhibition of PSMα3, an amphiphilic defensive peptide secreted by methicillin-resistant *Staphylococcus aureus* that disrupts cellular membranes, in the presence of serum proteins,[16] and sequestration/neutralization of the peptide toxin melittin from the bloodstream of a living mouse.[17] More recently, a polymer NP was developed with engineered affinity for a vascular endothelial growth factor ($VEGF_{165}$). The NP

Received: October 21, 2016
Published: December 14, 2016

DOI: 10.1021/jacs.6b10950
J. Am. Chem. Soc. 2016, 138, 16604−16607

inhibits binding of the signaling protein to its receptor VEGFR-2, preventing receptor phosphorylation and downstream $VEGF_{165}$-dependent endothelial cell migration.[18]

These findings and the success of our in vivo synthetic toxin neutralization studies prompted the search for a synthetic NP composition capable of sequestering and neutralizing PLA_2 toxins from various venom extracts. Our NP selection process differs from that of the immune system. NP-protein affinity arises broadly from similarities that are characteristic of specific classes of toxins. PLA_2 interacts with lipid membranes and lipoprotein particles.[19] These features may be shared not only across isoforms but even different families of proteins. Hence, it is likely that during the process of formulating and optimizing a NP capable of neutralizing venomous PLA_2, the NP will also be able to sequester similar venomous proteins and peptides. Indeed, an ingenious use of biological membranes has been employed as a "toxin sponge" for toxins with membrane affinity.[20] However, because PLA_2, a membrane hydrolyzing enzyme, is a significant component of most snake venoms, this approach would not be successful. Our synthetic polymer NPs, which lack phospholipids, would not be vulnerable to PLA_2-catalyzed degradation.

Venomous PLA_2 enzymes are generally characterized by their low molecular weight (13−16 kDa), Ca^{2+}-dependency, and a conserved active site histidine/aspartic acid dyad.[21] Unlike mammalian PLA_2, which are relatively nontoxic and pharmacologically benign, venomous PLA_2 are capable of producing a variety of pharmacological effects, making them one of the most toxic components of snake, honey-bee, and scorpion venom.[22−24] In the presence of phosphatidylcholine, PLA_2 catalyzes the production of lysophosphatidylcholine, which is known to induce hemolysis.[25] This property allows for the activity of enzymatically active PLA_2 isoforms to be monitored indirectly through hemolytic assays.[26]

Using this assay, a first generation library (Table S1) of functionally diverse NPs were analyzed for their ability to inhibit PLA_2 induced erythrocyte lysis. It was discovered that NP 1_5, consisting of 20% acrylic acid, 40% *N*-phenylacrylamide, 38% *N*-isopropylacrylamide, and 2% *N*,*N*′-methylenebis(acrylamide) (Figure 1C), showed a decrease in erythrocyte lysis when tested against *Bungarus caeruleus* (Indian Krait) venom (Figure 1A). Furthermore, it appears that NP 1_5 does not prevent hemolysis by scavenging the lysophosphoatidylcholine (Figure 1B). Rather, it is likely inhibiting the production of lysophosphatidylcholine by directly interacting with PLA_2.

Using NP 1_5 as our lead formulation, a second generation library of NPs were synthesized with systematically varied feed ratios of the four monomers (Table S3). The optimization conditions used to evolve the lead formulation from the first generation were made more rigorous by using honey-bee venom instead of snake venom. Honey-bee venom contains two hemolytic principle components: PLA_2 and melittin. Melittin is a ∼3 kDa hemolytic pore forming peptide that comprises 50% of the dry mass of honey-bee venom.[27] The activity of melittin works synergistically with that of PLA_2 found in honey-bee venom by releasing phospholipids from lysed cellular membranes allowing for lipid shuttling to occur between melittin and PLA_2.[28] Thus, monitoring the inhibition of honey-bee venom induced hemolysis through an erythrocyte lysis assay allows for congruent analysis of anti-PLA_2 activity and antimelittin activity. Furthermore, this relatively simplified venom system can be used to test whether or not a single NP

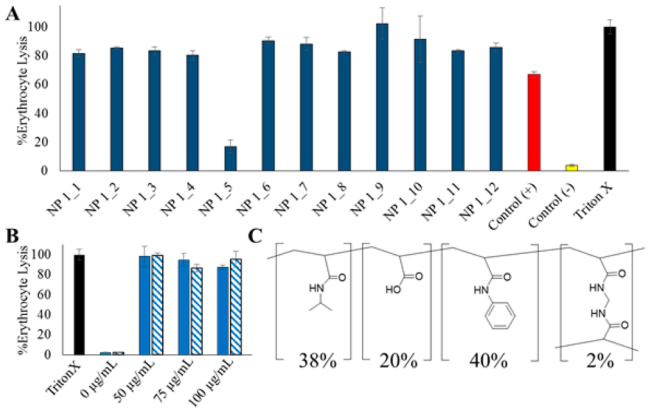

Figure 1. First round of NP optimization. (A) Erythrocyte lysis assay results for first generation library of NPs (500 μg/mL) incubated with Indian Krait venom (1 μg/mL) and phosphatidylcholine (100 μg/mL). Control (+, red) represents no NPs, Control (−,yellow) represents no venom or NPs, and all data was normalized to Triton X (black). (B) Erythrocyte lysis assay results for NP 1_5 (500 μg/mL) incubated with lysophosphatidylcholine (solid blue) versus lysophosphatidylcholine incubated without NPs (striped blue) at various concentrations of lysophosphatidylcholine. (C) Monomer feed ratio for NP 1_5.

composition can inhibit multiple venomous biomacromolecules simultaneously.

Using a concentration of 10 μg/mL of whole honey-bee venom, the second generation library of NPs were analyzed for their inhibitive properties (Figures S8−S18). Moreover, the experimental procedure was made more stringent by increasing the final incubation from 15 min to 1 h. Under these conditions, the inhibition of erythrocyte lysis when testing for anti-PLA_2 activity by NP 1_5 was overwhelmed. However, it was observed that the amount of cross-linker incorporated into the monomer feed ratio was directly correlated with the inhibition of erythrocyte lysis (Figure S13). The NP 2_12, synthesized with the greatest feed ratio of cross-linker (Figure 2C), was able to inhibit PLA_2 induced erythrocyte lysis at concentrations as low as 63 μg/mL (Figure 2B), and melittin induced erythrocyte lysis at concentrations lower than 0.3 mg/mL (Figure S16). Although the reasons for this trend in cross-linking are not obvious, it is clear that cross-linking significantly impacts the neutralization of venomous PLA_2. More importantly, these results suggest that dissimilar biomacromolecules can be neutralized by a single NP formulation, which is a necessary milestone in our pursuit of a broad-spectrum toxin sequestrant.

Although control experiments were performed on both generations of NPs to ensure that the NPs were not responsible for any observed erythrocyte lysis, the lead NP (NP 2_12) was also subjected to an MTT cell viability test using human immortalized myelogenous leukemia K562 cells. Control experiments were run to establish background absorption at 490 nm in PBS in the presence or absence of NP 2_12 when treated with the MTT dye. However, no cytotoxicity was observed even at NP concentrations as high as 500 μg/mL (Figure S20).

Following an envenomation, venom proteins are rapidly diluted in the bloodstream of the afflicted individual. Thus, selectivity for the targeted venom proteins over abundant serum proteins is required. This challenge must be addressed directly when developing a polymer sequestrant.

DOI: 10.1021/jacs.6b10950
J. Am. Chem. Soc. 2016, 138, 16604−16607

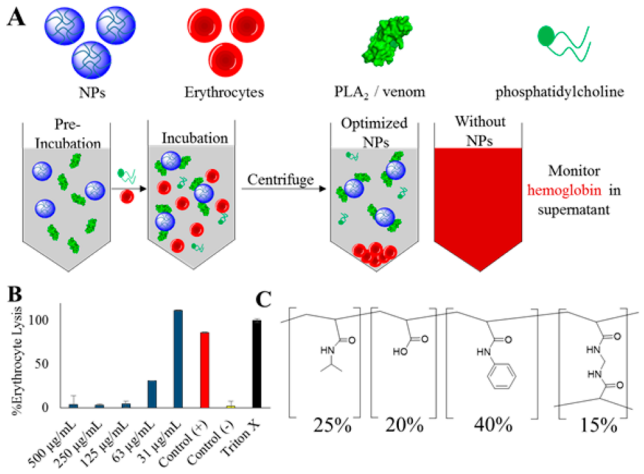

Figure 2. Second round of NP optimization. (A) PLA_2 activity assay used to analyze inhibition of enzymatic hydrolysis of phosphatidylcholine to lysophosphatidylcholine (hemolytic). (B) Erythrocyte lysis results using 10 μg/mL of whole honey-bee venom (~15% PLA_2) and various concentrations of NP 2_12 (blue). Control (+, red) represents no NPs, Control (−,yellow) represents no venom or NPs, and all data was normalized to Triton X (black). (C) Feed ratio of monomers used to synthesize NP 2_12.

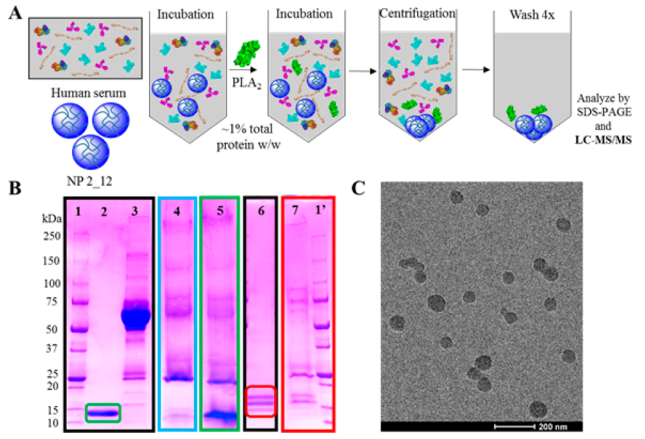

Figure 3. Selectivity experiments and TEM characterization of NP 2_12. (A) Strategy for analyzing selectivity for targeted venom proteins over human serum proteins. Serum was diluted to a final concentration of 25%, PLA_2 was diluted to a final concentration of 250 μg/mL, and NP 2_12 was diluted to a final concentration of 1 mg/mL. (B) PLA_2 selectivity experiments visualized by SDS-PAGE. (1/1′) molecular weight ladder. (2) Purified PLA_2 from *Naja mossambica* venom. (3) Serum control. (4) NP 2_12 incubated in serum only. (5) NP 2_12 incubated in serum and PLA_2 from *Naja mossambica* venom. (6) Purified PLA_2 from honey-bee venom. (7) NP 2_12 incubated in ovine plasma and PLA2 from honey-bee venom. (C) Unstained TEM image of NP 2_12. Scale bar = 200 nm. Full gel images available in SI (Figures S17−S19).

Exposing NPs to complex biological mixtures results in the rapid adsorption of biomacromolecules onto NPs.[29,30] This rapid adsorption event results in a new and more therapeutically relevant chemical identity termed the protein corona, which can be further divided into two domains: a hard corona and a soft corona.[31] The two protein corona domains can be differentiated by the differences in kinetic dissociation rates (k_{off}). The hard corona describes biomacromolecules that slowly dissociate from the NP and the soft corona describes a layer of proteins that rapidly dissociate from the NP or NP·hard corona complex. The composition of the hard and soft corona results from the cumulative contribution of a number of physicochemical parameters including size, shape, and the synthetic identity of the NP.[32,33]

Monitoring the entire protein corona composition has been met with considerable challenges due to the rapidly exchanging soft-corona. However, established methods have been developed for analyzing the composition of the thermodynamic hard-corona via SDS-PAGE and LC-MS/MS (Figure 3A).[32] For purposes of venom protein sequestration, it is necessary that venom proteins adsorb within the thermodynamic and slowly exchanging hard-corona.

Using concentrated human serum and purified fractions of PLA_2 from *Naja mossambica* venom (~1% w/w total protein), the hard protein corona of NP 2_12 was analyzed. In the absence of any PLA_2, it appears that the majority of the serum proteins have minimal affinity to NP 2_12 (Figure 3B4). However, upon introducing PLA_2 after a 5 min preincubation in serum, a significant amount of PLA_2 appears in the NP protein corona (Figure 3B5). This experiment demonstrates that serum proteins can be displaced by high-affinity venomous PLA_2 in the hard corona of the NP, which is a requirement for in vivo venom sequestration/neutralization. This experiment was conducted a second time using bee venom PLA_2, which also showed selective association to the NP over ovine plasma proteins (Figure 3B7). These findings demonstrate that a single synthetically optimized NP formulation (NP 2_12) can sequester a variety of PLA_2 isoforms and may function as a broad-spectrum venomous-PLA_2 sequestrant.

To understand the selectivity for PLA_2 over other serum proteins, the bands observable by SDS-PAGE were digested and analyzed by mass spectrometry (Table S5). Of the nine proteins found in the hard-corona, three were apolipoproteins (Apo-A1, Apo-E, and Apo-B100). In, particular Apo-A1 appears to be the major serum protein bound to NP 2_12. These proteins associate with lipoprotein particles and are important for trafficking lipids throughout the blood-stream.[34] This corona profile suggests that the NP is mimicking a natural substrate of PLA_2 (lipoprotein particles composed of glycerophospholipids) and explains why this NP composition is capable of sequestering PLA_2 with sufficient selectivity over abundant serum proteins. This also explains why NP 2_12 is capable of neutralizing the pore-forming toxin melittin from bee-venom and may act as a broad-spectrum sequestrant for lipid-mediated toxins.

Building on this discovery, we studied the dissociation kinetics for bee venom PLA_2·NP 2_12. From previous studies aimed at understanding hydrogel−biomacromolecule interactions, it was found that the interaction of NIPAm-based NPs with charged biomacromolecules is dominated by entropically driven processes which can be related to the expulsion of water from the NP hydrogel upon binding.[35] In the present system, a surface plasmon resonance (SPR) analysis revealed that following a rapid k_{on}, k_{off} was characterized by rapid desorption of a small (<20%) fraction of absorbed protein (perhaps from the soft corona) followed by a much slower desorption of the remaining (>80%) protein. Under the flow conditions of the SPR experiment, a significant fraction of the bound protein (~80%, largely PLA_2), remains tightly bound. The time needed to release the majority of bound PLA_2 from the NP is likely longer than the time needed to clear the PLA_2·NP 2_12

DOI: 10.1021/jacs.6b10950
J. Am. Chem. Soc. 2016, 138, 16604−16607

complex in vivo (Figure 4). This kinetic data lends credibility to the notion that broad-spectrum sequestration of lipid-mediated toxins may be accomplished via a single optimized NP formulation.

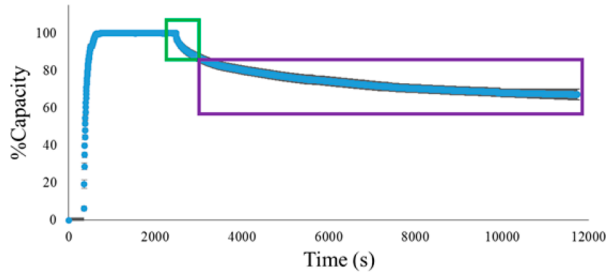

Figure 4. Kinetic analysis of the dissociation of bee venom PLA_2·NP 2_12 using NP 2_12 immobilized surface plasmon resonance (SPR) under flow conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b10950.

Experimental procedures and supporting data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*kjshea@uci.edu

ORCID

Kenneth J. Shea: 0000-0001-5926-0059

Author Contributions

The authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Mark Johnson for acquiring a TEM image of our NPs. This material is based upon work supported by the Defense Advanced Research Projects Agency (DARPA) under Contract No. W911NF-15-C-0068. Additional funding from the National Science Foundation NSF DMR-1308363 and the Institutional Chemical and Structural Biology Training Grant (National Institute of General Medical Sciences Grant T32GM108561) is also acknowledged.

■ REFERENCES

(1) Williams, D.; Gutierrez, J. M.; Harrison, R.; Warrell, D. A.; White, J.; Winkel, K. D.; Gopalakrishnakone, P. *Lancet* **2010**, *375*, 89.
(2) Gutierrez, J. M.; Williams, D.; Fan, H. W.; Warrell, D. A. *Toxicon* **2010**, *56*, 1223.
(3) Kasturiratne, A.; Wickremasinghe, A. R.; de Silva, N.; Gunawardena, N. K.; Pathmeswaran, A.; Premaratna, R.; Savioli, L.; Lalloo, D. G.; de Silva, H. J. *PLoS Med.* **2008**, *5*, e218.
(4) Alirol, E.; Sharma, S. K.; Bawaskar, H. S.; Kuch, U.; Chappuis, F. *PLoS Neglected Trop. Dis.* **2010**, *4*, e603.
(5) Mohapatra, B.; Warrell, D. A.; Suraweera, W.; Bhatia, P.; Dhingra, N.; Jotkar, R. M.; Rodriguez, P. S.; Mishra, K.; Whitaker, R.; Jha, P. *PLoS Neglected Trop. Dis.* **2011**, *5*, e1018.
(6) Angulo, Y.; Estrada, R.; Gutierrez, J. M. *Toxicon* **1997**, *35*, 81.
(7) Fry, B. G.; Wickramaratna, J. C.; Jones, A.; Alewood, P. F.; Hodgson, W. C. *Toxicol. Appl. Pharmacol.* **2001**, *175*, 140.
(8) Fry, B. G.; Winkel, K. D.; Wickramaratna, J. C.; Hodgson, W. C.; Wuster, W. *J. Toxicol., Toxin Rev.* **2003**, *22*, 23.
(9) Hoffman, R. S. *Clin. Toxicol.* **2014**, *52*, 157.
(10) Schiermeier, Q. *Nature* **2015**, *525*, 299.
(11) Reeks, T. A.; Fry, B. G.; Alewood, P. F. *Cell. Mol. Life Sci.* **2015**, *72*, 1939.
(12) Fry, B. G. *Genome Res.* **2005**, *15*, 403.
(13) O'Brien, J.; Shea, K. J. *Acc. Chem. Res.* **2016**, *49*, 1200.
(14) Yoshimatsu, K.; Lesel, B. K.; Yonamine, Y.; Beierle, J. M.; Hoshino, Y.; Shea, K. J. *Angew. Chem., Int. Ed.* **2012**, *51*, 2405.
(15) Lee, S. H.; Hoshino, Y.; Randall, A.; Zeng, Z. Y.; Baldi, P.; Doong, R. A.; Shea, K. J. *J. Am. Chem. Soc.* **2012**, *134*, 15765.
(16) Weisman, A.; Chen, Y. A.; Hoshino, Y.; Zhang, H. T.; Shea, K. *Biomacromolecules* **2014**, *15*, 3290.
(17) Hoshino, Y.; Koide, H.; Furuya, K.; Haberaecker, W. W.; Lee, S. H.; Kodama, T.; Kanazawa, H.; Oku, N.; Shea, K. J. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 33.
(18) Koide, H.; Hoshino, Y.; Lee, S.-H.; Okajima, A.; Ariizumi, S.; Narita, Y.; Yonamine, Y.; Weisman, A. C.; Nishimura, Y.; Oku1, N.; Miura, Y.; Shea, K. J. *Nat. Chem.*. **2017**, *9*, in press.
(19) Gazi, I.; Tsimihodimos, V.; Filippatos, T.; Lourida, E.; Elisaf, M.; Tselepis, A. *J. Hypertens.* **2006**, *24*, S395.
(20) Hu, C. M. J.; Fang, R. H.; Copp, J.; Luk, B. T.; Zhang, L. F. *Nat. Nanotechnol.* **2013**, *8*, 336.
(21) Scott, D. L.; White, S. P.; Otwinowski, Z.; Yuan, W.; Gelb, M. H.; Sigler, P. B. *Science* **1990**, *250*, 1541.
(22) Fry, B. G.; Roelants, K.; Champagne, D. E.; Scheib, H.; Tyndall, J. D. A.; King, G. F.; Nevalainen, T. J.; Norman, J. A.; Lewis, R. J.; Norton, R. S.; Renjifo, C.; de la Vega, R. C. R. *Annu. Rev. Genomics Hum. Genet.* **2009**, *10*, 483.
(23) Kini, R. M. *Toxicon* **2003**, *42*, 827.
(24) Kini, R. M.; Evans, H. J. *Toxicon* **1989**, *27*, 613.
(25) Bierbaum, T. J.; Bouma, S. R.; Huestis, W. H. *Biochim. Biophys. Acta, Biomembr.* **1979**, *555*, 102.
(26) Alabdulla, I. H.; Sidki, A. M.; Landon, J. *Toxicon* **1991**, *29*, 1043.
(27) Zhou, J. H.; Zhao, J.; Zhang, S. X.; Shen, J. Z.; Qi, Y. T.; Xue, X. F.; Li, Y.; Wu, L. M.; Zhang, J. Z.; Chen, F.; Chen, L. Z. *Anal. Biochem.* **2010**, *404*, 171.
(28) Cajal, Y.; Jain, M. K. *Biochemistry* **1997**, *36*, 3882.
(29) Monopoli, M. P.; Aberg, C.; Salvati, A.; Dawson, K. A. *Nat. Nanotechnol.* **2012**, *7*, 779.
(30) Walkey, C. D.; Chan, W. C. W. *Chem. Soc. Rev.* **2012**, *41*, 2780.
(31) Fleischer, C. C.; Payne, C. K. *Acc. Chem. Res.* **2014**, *47*, 2651.
(32) Lundqvist, M.; Stigler, J.; Elia, G.; Lynch, I.; Cedervall, T.; Dawson, K. A. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 14265.
(33) Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. *Nat. Mater.* **2009**, *8*, 543.
(34) Saito, H.; Lund-Katz, S.; Phillips, M. C. *Prog. Lipid Res.* **2004**, *43*, 350.
(35) Zeng, Z. Y.; Patel, J.; Lee, S. H.; McCallum, M.; Tyagi, A.; Yan, M. D.; Shea, K. J. *J. Am. Chem. Soc.* **2012**, *134*, 2681.

DOI: 10.1021/jacs.6b10950
J. Am. Chem. Soc. 2016, 138, 16604−16607