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Stable and Potent Polyvalent Anthrax Toxin Inhibitors: Raft-Inspired Domain Formation in Liposomes that Contain PEGylated Lipids

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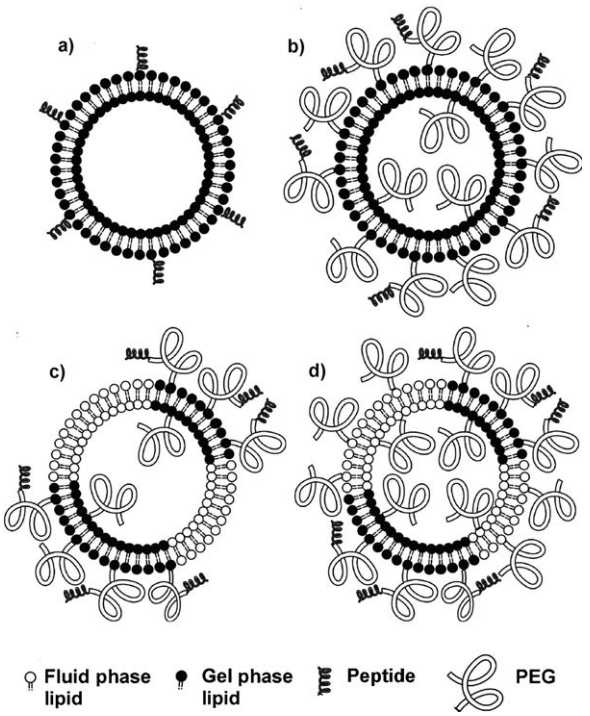
The design of polyvalent molecules,[1–5] which consist of multiple copies of a ligand attached to a suitable scaffold, represents a promising approach for designing potent inhibitors of pathogens and microbial toxins.[1,6–11] Liposomes are particularly attractive scaffolds for designing polyvalent inhibitors;[9,10,12–15] however, the poor colloidal stability of conventional liposomes and their short circulation times in vivo[16,17] are major obstacles that limit their therapeutic use. Herein, we describe the design of highly stable and active polyvalent anthrax toxin inhibitors based on liposomes that incorporate polyethylene glycol (PEG)-functionalized lipids (PEGylated liposomes). Furthermore, drawing from the concept of lipid rafts[15,18,19]—domains that are believed to exist in cellular membranes—we have designed heterogeneous domain-containing PEGylated liposomes that are considerably more active than their homogeneous counterparts (Scheme 1). These raft-mimetic PEGylated polyvalent liposomes are attractive not only for designing inhibitors for toxins and pathogens, but also for the design of efficient targeted drug-delivery systems.

While liposomes have been investigated extensively for applications in drug delivery, as described above, conventional liposomes are limited in effectiveness because of their low colloidal stability and their rapid uptake by macrophage cells of the immune system, predominantly in the liver and spleen.[20] The ability to enhance the physical stability and extend the circulation lifetime through modification with PEG, achieved by using lipids with PEG attached to their hydrophilic head groups, has proven to be useful in the context of drug delivery.[17,20–22] We first tested whether the use of PEGylated liposomes (Scheme 1b) would enable the design of stable and active polyvalent anthrax lethal toxin (LeTx) inhibitors.



Scheme 1. a) Conventional liposome functionalized with peptides, b) PEGylated liposome with a fraction of the PEG lipids functionalized with peptides, c) phase-separated liposome with PEG lipids present only in the gel phase, with a fraction of the PEG lipids attached to peptides, and d) phase-separated liposome with PEG lipids present in both phases, with peptides attached to only a fraction of PEG lipids in the gel phase. Figure not drawn to scale.

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To that end, we made liposomes (100 nm diameter) composed of a 19:1 mixture of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and a pyridyl dithiopropionate derivative of L-α-distearoyl phosphatidylethanolamine-*N*-[amino-(polyethylene glycol)2000] (DSPE-PEG2000-PDP). We functionalized these liposomes with an inhibitory peptide HTSTYWWLDGAPC[9,23] (4.7%) that binds to the heptameric cell-binding component of anthrax toxin, $[PA_{63}]_7$, thereby blocking the binding of the toxic enzyme lethal factor (LF). Inhibition of the binding of LF to $[PA_{63}]_7$ prevents the cytosolic delivery of LF, thereby inhibiting cell death. Peptide-functionalized PEGylated liposomes protected RAW264.7 cells from LeTx with a half-maximal inhibitory concentration (IC_{50}) of about 35 nM on a per-peptide basis; control PEGylated liposomes functionalized with thioglycerol showed no inhibitory activity (Figure 1a). Furthermore, as seen in Figure 1a, the activity of the polyvalent PEGylated liposomes (Scheme 1b) was comparable to that of conventional (non-PEGylated) DSPC-based liposomes (Scheme 1a) with the same peptide density (20 nM), which indicates that the use of PEGylated lipids does not compromise inhibitory activity. Polyvalent inhibitors that used PEGylated liposomes as a scaffold were over four orders of magnitude more potent than the corresponding monovalent peptide, which does not inhibit cytotoxicity, even at concentrations as high as 2 mM.

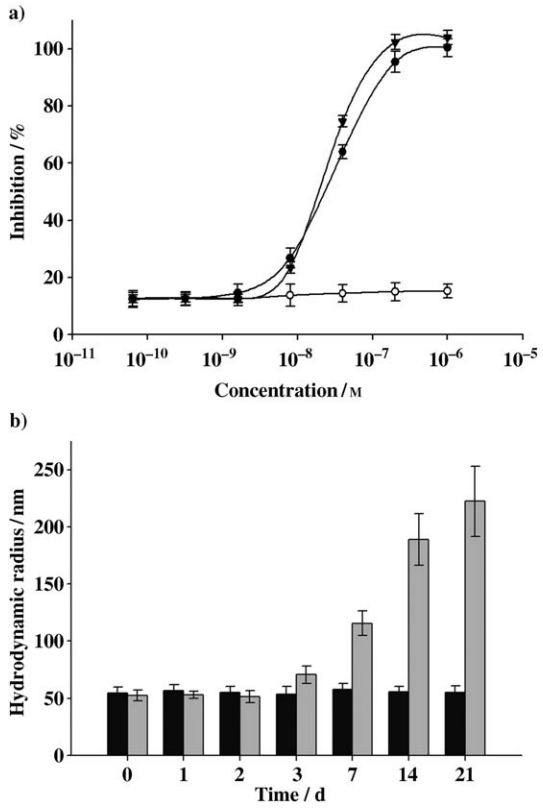

Figure 1. In vitro characterization of peptide-functionalized PEGylated liposomes. a) Percentage inhibition of cytotoxicity vs. concentration for peptide-functionalized PEGylated liposomes (●), thioglycerol-functionalized PEGylated liposomes (○), and DSPC-based non-PEGylated peptide-functionalized liposomes (▼). b) Hydrodynamic radii (R_H) determined by dynamic light scattering for PEGylated liposomes (black) and DSPC-based non-PEGylated liposomes (gray).

We monitored the physical stability of the peptide-functionalized liposomes as a function of storage time at 4°C by measuring their R_H value over a period of 21 d. For comparison, we also monitored the size of conventional liposomes over the same period. Conventional liposomes showed a consistent gradual increase in R_H over a 21 d period, whereas PEGylated liposomes showed no significant change in size (Figure 1b), which confirmed the greater colloidal stability of PEGylated liposomes as compared with the conventional liposomes.

Having demonstrated the ability to make stable and active polyvalent anthrax LeTx inhibitors based on PEGylated liposomes, we next tested the influence of the heterogeneity of the liposomal membrane on stability and inhibitory activity. We previously showed that lateral phase separation provides a general route to increase the efficiency of polyvalent recognition by conventional liposomes.[15] We hypothesized that laterally phase-separated PEGylated liposomes functionalized with an inhibitory peptide would be significantly more stable than the corresponding non-PEGylated or conventional liposomes, while still retaining their potency.

To that end, we made liposomes with three different compositions: 1) distearoylphosphatidylcholine (DSPC) and DSPE-PEG2000-PDP (molar ratio 19:1); 2) dioleoylphosphatidylcholine (DOPC), DSPC, and DSPE-PEG2000-PDP (molar ratio 75:23.8:1.2); and 3) DOPC, DOPE-PEG2000, DSPC, and DSPE-PEG2000-PDP (molar ratio 71.2:3.8:23.8:1.2). We reasoned that liposomes composed of gel-phase lipids DSPC and DSPE-PEG2000-PDP would be homogeneous (Scheme 1b); those composed of fluid-phase lipid DOPC and gel-phase lipids DSPC and DSPE-PEG2000-PDP would phase separate, with PEG lipids present primarily in domains enriched with gel-phase lipids (Scheme 1c); and liposomes of the third composition would phase separate and contain PEGylated lipids in both phases (Scheme 1d). Furthermore, we hypothesized that inhibitors based on phase-separated liposomes would be more potent than inhibitors based on homogeneous liposomes, and that phase-separated liposomes that contain PEGylated lipids in both phases (Scheme 1d) would be more stable than those that have PEGylated lipids in only one phase (Scheme 1c).

To visualize phase separation, we used confocal microscopy to examine giant unilamellar vesicles (GUVs) that incorporated 1% of the fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiIC), which partitions preferentially into gel-phase domains, or the fluorescent dye Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE), which partitions preferentially into fluid-phase domains. Consistent with our hypothesis, GUVs composed of DSPC, DSPE-PEG2000-PDP, and DiIC appeared to be uniformly fluorescent (Figure 2a), whereas

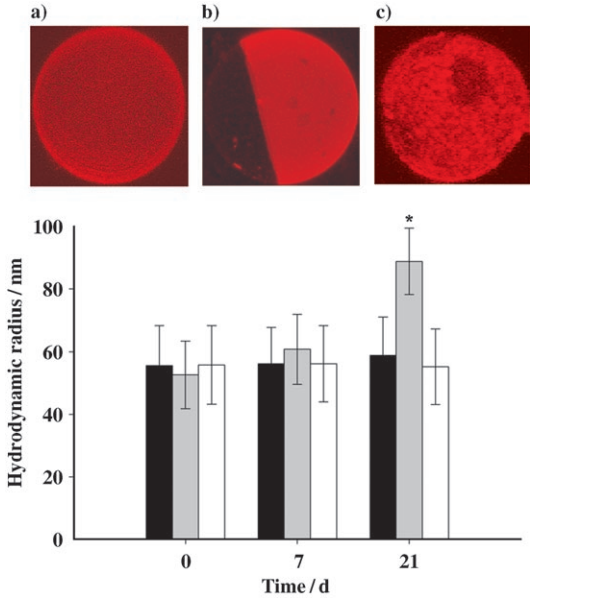

Figure 2. Characterization of homogeneous and heterogeneous PEGylated liposomes by confocal microscopy and dynamic light scattering. Top: Confocal micrographs of GUVs composed of a) DSPC/DSPE-PEG2000-PDP/DiIC, b) DOPC/DSPC/DSPE-PEG2000-PDP/TR-DHPE, and c) DOPC/DOPE-PEG2000/DSPC/DSPE-PEG2000-PDP/TR-DHPE. Bottom: R_H determined by dynamic light scattering for liposomes composed of DSPC/DSPE-PEG2000-PDP (black), DOPC/DSPC/DSPE-PEG2000-PDP (gray), and DOPC/DOPE-PEG2000/DSPC/DSPE-PEG2000-PDP (white). *: The difference in the R_H value is statistically significant compared with the other samples at 21 d. ($P < 0.02$; unpaired Student's T-Test).

GUVs composed of DOPC, DSPC, DSPE-PEG2000-PDP, and TR-DHPE (Figure 2b) and GUVs composed of DOPC, DOPE-PEG2000, DSPC, DSPE-PEG2000-PDP, and TR-DHPE (Figure 2c) showed the presence of dark phase-separated domains.

Next, to test the stability of liposomes that had the three different lipid compositions described above, we used dynamic light scattering to measure the hydrodynamic radii as a function of storage time (Figure 2, bottom). Again, consistent with our hypothesis, the dynamic-light-scattering data indicated that phase-separated liposomes that contained PEGylated lipids in both phases (Scheme 1d) were comparable in stability to homogeneous PEGylated liposomes (Scheme 1b) and significantly more stable than phase-separated liposomes that contained PEGylated lipids in only one phase (Scheme 1c).

Next, we tested the effect of domain formation on the potency of polyvalent anthrax LeTx inhibitors based on PEGylated liposomes. Homogeneous PEGylated liposomes (Scheme 1b) composed of DSPC and DSPE-PEG2000-PDP (molar ratio 95:5) and heterogeneous PEGylated liposomes (Scheme 1d) composed of DOPC, DOPE-PEG2000, DSPC, and DSPE-PEG2000-PDP (molar ratio 71.2:3.8:23.8:1.2) were allowed to react with $[PA_{63}]_7$-binding peptide HTSTYWWLDGAPC[9,23] and the remaining unreacted thiol-reactive groups on the liposomes were quenched with thioglycerol. We tested the ability of these polyvalent inhibitors to protect RAW264.7 cells from death caused by anthrax LeTx. The IC_{50} for inhibitors based on heterogeneous PEGylated liposomes was more than tenfold lower than that for homogeneous PEGylated inhibitors on a per-peptide basis, which is consistent with our hypothesis (Figure 3a). We used fluorescence resonant energy transfer (FRET) with fluorescein as the donor and rhodamine as the acceptor to confirm that the peptides cluster in lipid domains in the heterogeneous PEGylated liposomes. The homogeneous and heterogeneous PEGylated liposomes were treated with a mixture of fluorescein- and rhodamine-labeled $[PA_{63}]_7$-binding peptide (1:1 molar ratio; 0.85% total peptide density). The significant increase in donor quenching and acceptor emission for heterogeneous PEGylated liposomes relative to homogeneous PEGylated liposomes (Figure 3b) confirmed that the peptides cluster into domains in the heterogeneous liposomes.

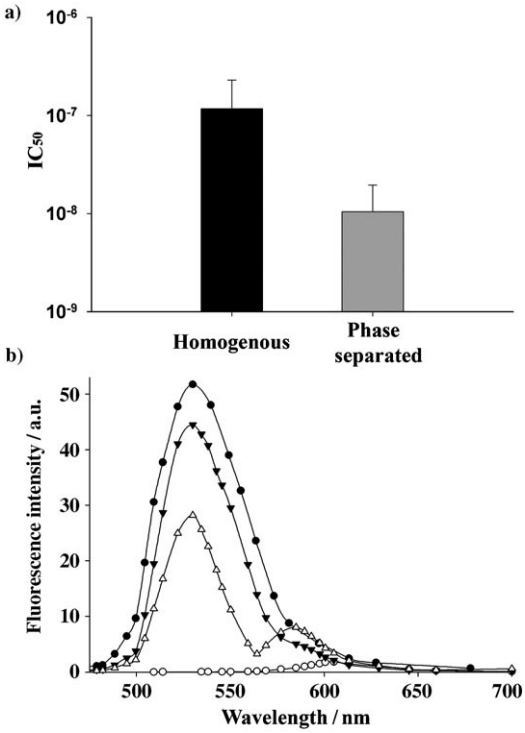

Figure 3. Characterization of phase-separated peptide-functionalized PEGylated liposomes. a) IC_{50} values for peptide-functionalized PEGylated liposomes (0.85% density) for homogenous PEGylated liposomes (black) and phase-separated PEGylated liposomes (gray). b) FRET data for phase-separated PEGylated liposomes functionalized with ●: fluorescein-labeled peptide only, ○: rhodamine-labeled peptide only, and △: a 1:1 mixture of fluorescein-labeled and rhodamine-labeled peptides, and ▼: homogenous PEGylated liposomes functionalized with a 1:1 mixture of fluorescein- and rhodamine-labeled peptides.

Collectively, our results demonstrate the ability to design highly active and stable polyvalent inhibitors based on laterally phase separated PEGylated liposomes. These raft-inspired stable liposomes are well suited for applications rang-

ing from the design of inhibitors for a variety of toxins and pathogens to the targeting of cells for imaging and drug delivery.

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Keywords: anthrax toxin · inhibitors · liposomes · PEGylation · phase separation

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