Toxin Inhibitors

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Raftlike Polyvalent Inhibitors of the Anthrax Toxin: Modulating Inhibitory Potency by Formation of Lipid Microdomains**

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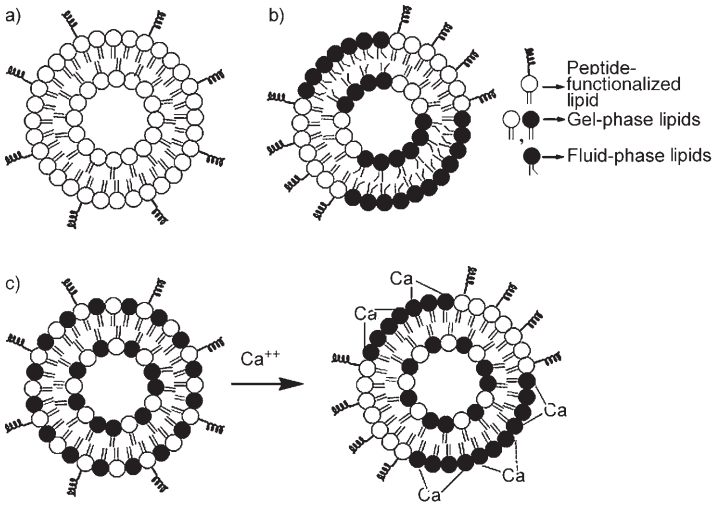
We describe the design of biomimetic anthrax toxin inhibitors that incorporate lipid microdomains. Cellular membranes are believed to contain microdomains (lipid rafts) that influence processes ranging from signal transduction to microbial pathogenesis.[1–4] We demonstrate that formation of raftlike membrane microdomains significantly enhances the potency of liposome-based polyvalent anthrax toxin inhibitors; phase separation in the liposomal membrane was used to cluster inhibitory peptides to increase the potency of these inhibitors (Scheme 1). We also synthesized "smart" inhibitors in which phase separation and potency can be modulated actively in response to an external stimulus (Scheme 1).



Scheme 1. a) Homogeneous gel-phase liposomes. b) Peptide clustering owing to phase separation in liposomes composed of a mixture of gel-phase and fluid-phase lipids. c) Active control of phase separation and peptide clustering in liposomes by adding calcium ions.

Liposomes represent simple models of cellular membranes and are also attractive scaffolds for the polyvalent display of ligands.[5–8] We showed previously that liposomes displaying multiple copies of an inhibitory peptide bound the heptameric cell-binding component of anthrax toxin, $[PA_{63}]_7$, and prevented it from binding the toxic enzyme lethal factor (LF). Blocking the binding of LF to $[PA_{63}]_7$ prevented the cytosolic delivery of LF, thereby inhibiting cell death. The density of peptide ligands on the surface of the liposome was optimal past a threshold peptide density that corresponded to the average distance between peptide-binding sites on $[PA_{63}]_7$.[8] We hypothesized, therefore, that peptides displayed at suboptimal density could be clustered into "raftlike" membrane microdomains to create regions of optimal ligand density. We demonstrate herein that the concentration of peptides into lipid microdomains facilitates toxin inhibition.

Coexisting liquid-ordered and liquid-disordered phases can be formed in membranes containing ternary mixtures of unsaturated lipids, saturated lipids, and cholesterol by increasing the amount of cholesterol in homogeneous model membranes.[4,9,10] We made liposomes from a mixture of dioleoylphosphatidylcholine (DOPC), diarachidoylphosphatidylcholine (DAPC), a thiol-reactive lipid (PDP-DPPE), and cholesterol. As the use of giant unilamellar vesicles (GUVs) enables phase separation to be visualized,[4,9] we first made GUVs composed of DOPC, the fluorescent dye Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE, which partitions preferentially into less-ordered liquid domains), DAPC, PDP-DPPE, and cholesterol at molar ratios of 79:1:10:5:5 and 64:1:10:5:20. Although GUVs containing 5% cholesterol showed a uniform distribution of fluorescence (Figure 1a,i), those containing 20% cholesterol showed the presence of dark phase-separated domains (Figure 1a,ii–iv). This is consistent with the coexistence of liquid-ordered and liquid-disordered domains.

Next, we tested the effect of domain formation on the potency of liposome-based anthrax toxin inhibitors. Liposomes composed of DOPC, DAPC, PDP-DPPE, and cholesterol (molar ratios of 80:10:5:5 and 65:10:5:20) were allowed to react with the peptide HTSTYWWLDGAPC, which binds to $[PA_{63}]_7$ and prevents the binding of LF;[8,11] the remaining unreacted thiol-reactive groups on the liposomes were quenched with thioglycerol. We tested the ability of

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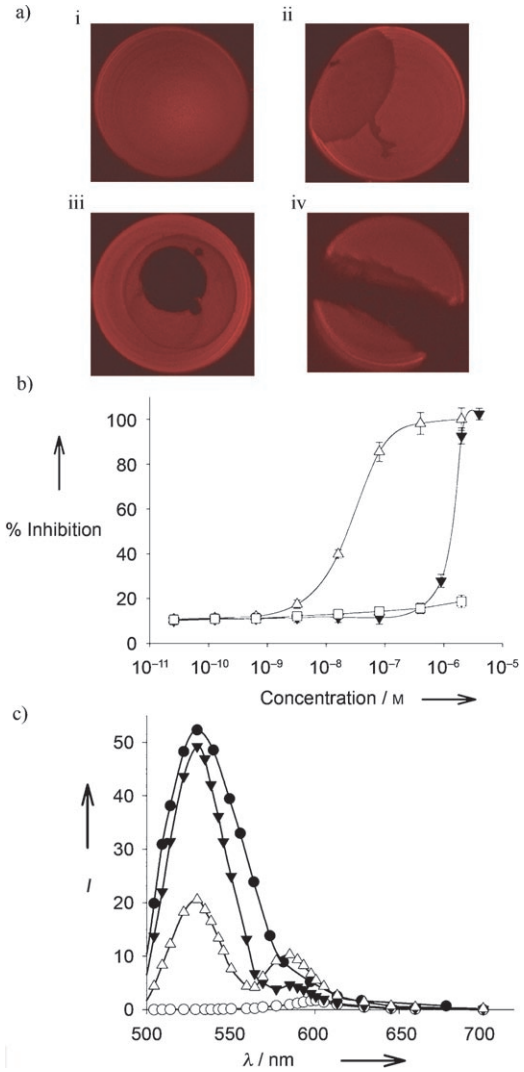

Figure 1. Characterization of microdomain-containing liposome-based inhibitors. a) Micrographs of GUVs containing i) 5% cholesterol and ii–iv) 20% cholesterol. b) Inhibition of cytotoxicity by peptide-functionalized liposomes containing 5% cholesterol (▼) and 20% cholesterol (△) as a function of concentration on a per-peptide basis and by control thioglycerol-functionalized liposomes (□). c) Characterization of peptide clustering in liposomes by FRET. Fluorescence intensity (*I*) as a function of wavelength (excitation at 450 nm) for liposomes functionalized with fluorescein-labeled peptide alone (●), rhodamine-labeled peptide alone (○), and for liposomes containing 5% cholesterol (▼) and 20% cholesterol (△) functionalized with a 1:1 mixture of fluorescein-labeled and rhodamine-labeled peptides.

these polyvalent liposome-based inhibitors (0.75% peptide density) to protect RAW264.7 cells from death caused by anthrax lethal toxin. Although the liposome-based inhibitors containing 5% cholesterol had a half-maximal inhibitory concentration (IC_{50}) of 1700 nM on a per-peptide basis, those containing 20% cholesterol had an IC_{50} of 16 nM on a per-peptide basis (Figure 1b). Liposomes presenting only thioglycerol showed no inhibitory activity (Figure 1b). These data suggest that formation of phase-separated domains results in over a 100-fold increase in the potency of these toxin inhibitors. Furthermore, the polyvalent inhibitors containing raftlike domains were at least five orders of magnitude more potent than the corresponding monovalent peptide, which does not inhibit cytotoxicity at concentrations as high as 2 mM.

To confirm that the peptides cluster in lipid microdomains, we characterized the liposome-based inhibitors by fluorescence resonant energy transfer (FRET) by using fluorescein as the donor and rhodamine as the acceptor. Liposomes composed of DOPC, DAPC, PDP-DPPE, and cholesterol (molar ratios of 80:10:5:5 and 65:10:5:20) were allowed to react with a mixture of fluorescein-labeled and rhodamine-labeled HTSTYWWLDGAPC peptide (1:1 molar ratio; 0.75% total peptide density). As seen in Figure 1c, a significant increase in donor quenching and sensitized acceptor emission was observed for liposomes containing 20% cholesterol relative to those containing 5% cholesterol. This is consistent with the segregation of the peptide into domains in the presence of 20% cholesterol. Analysis of the FRET spectra[12] indicated average interpeptide separations of approximately 88 and 45 Å for the liposomes containing 5% cholesterol and 20% cholesterol, respectively. Taken together, these results support the conclusion that the clustering of peptides in raftlike domains significantly increases the efficiency of inhibition of $[PA_{63}]_7$.

To test whether lateral phase separation provides a general route to increasing the potency of polyvalent anthrax toxin inhibitors, we made liposomes composed of distearoylphosphatidylcholine (DSPC) and PDP-DPPE (molar ratio of 3:1) and liposomes composed of DOPC and PDP-DPPE (molar ratio of 3:1). We reasoned that the liposomes composed of the gel-phase lipids DSPC and PDP-DPPE would be homogeneous, whereas those composed of the fluid-phase lipid DOPC and the gel-phase lipid PDP-DPPE would phase separate. Furthermore, we hypothesized that inhibitors based on the latter phase-separated liposomes would be more potent than inhibitors based on the former composition. GUVs composed of DSPC/PDP-DPPE appeared to be uniformly fluorescent (Figure 2a,i), whereas those composed of DOPC/PDP-DPPE showed the presence of dark phase-separated domains (Figure 2a,ii). The IC_{50} for inhibitors based on DOPC/PDP-DPPE liposomes was more than 50-fold lower than that for DSPC/PDP-DPPE-based inhibitors on a per-peptide basis (Figure 2b), which is consistent with our hypothesis. Characterization by FRET confirmed phase separation in liposomes composed of DOPC and PDP-DPPE (see Figure S1 in the Supporting Information).

Finally, we assessed whether the ability to induce phase separation in membranes could be used to actively modulate inhibitory potency. Membranes containing phosphatidylserine (PS) can be induced to form PS-enriched domains by the addition of Ca^{2+}.[1,13,14] We made liposomes composed of DSPC, DPPS, and PDP-DPPE (with a molar ratio of 3:6:1), functionalized them with the HTSTYWWLDGAPC peptide (1.8% peptide density), and tested their activity in a cytotoxicity assay. Preincubation of the liposomes with calcium chloride decreased the IC_{50} on a per-peptide basis by more than 30-fold (Figure 3a), indicating the ability to actively modulate inhibitor potency. Analysis of the liposomes by FRET revealed a significant increase in the

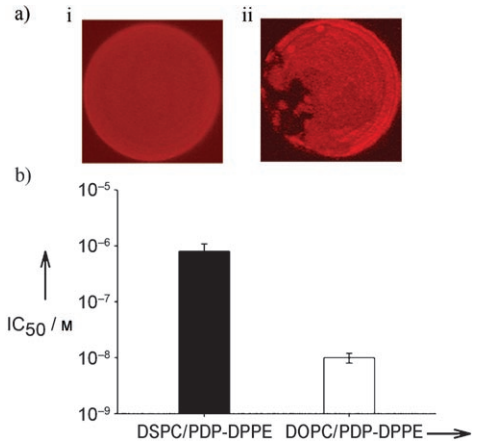

Figure 2. Influence of phase separation on potency of liposome-based inhibitors composed of a mixture of gel-phase and fluid-phase lipids. a) Micrographs of GUVs composed of i) Gel-phase lipids DSPC and PDP-DPPE with 1% fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiIC18(3)) and ii) a mixture of the fluid-phase lipid DOPC and the gel-phase lipid PDP-DPPE with 1% fluorescent dye TR-DHPE. b) IC_{50} for liposome-based inhibitors (1.8% peptide density) composed of gel-phase lipids (black bar) and a mixture of gel-phase and fluid-phase lipids (white bar).

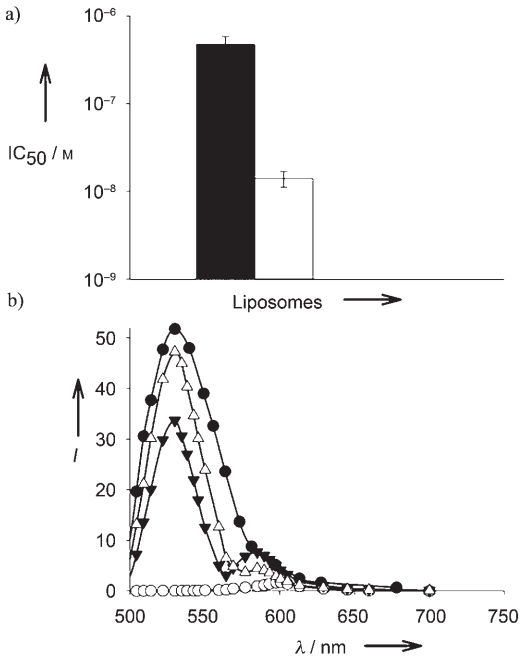

Figure 3. Active modulation of phase separation and potency of liposome-based inhibitors by the addition of calcium ions. a) IC_{50} on a per-peptide basis for peptide-functionalized liposomes (1.8% peptide density) containing DSPC and DPPS without Ca^{2+} (black bar) and with added Ca^{2+} (white bar). b) Characterization of peptide clustering by FRET. Fluorescence intensity (*I*) as a function of wavelength (excitation at 450 nm) for liposomes functionalized with fluorescein-labeled peptide alone (●), rhodamine-labeled peptide alone (○), and with a mixture of fluorescein-labeled, rhodamine-labeled, and unlabeled peptides (1:1:2) with Ca^{2+} added (▼) or without Ca^{2+} (△).

quenching of donor fluorescence for samples incubated with calcium chloride (Figure 3b), which is consistent with an increase in inhibitory potency owing to Ca^{2+}-triggered phase separation.

In summary, we demonstrate that segregation of biomolecules in membrane microdomains may be used to enhance the efficiency of polyvalent inhibition. Active control of phase separation in membranes by external stimuli such as ion concentration, temperature, light, and enzymatic activity provides an attractive method to tune the efficiency of polyvalent recognition with applications ranging from the design of inhibitors for toxins and pathogens to the design of targeted drug-delivery systems.

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