Articles

Synthesis of Polyvalent Inhibitors of Controlled Molecular Weight: Structure−Activity Relationship for Inhibitors of Anthrax Toxin

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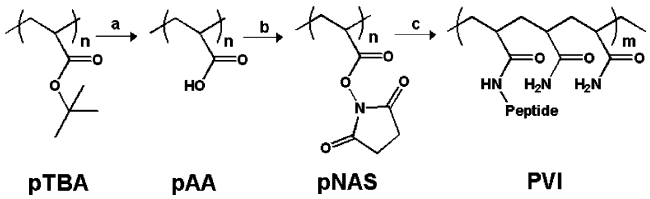
We describe a novel method to synthesize activated polymers of controlled molecular weight and apply this method to investigate the relationship between the structure and activity of polyvalent inhibitors of anthrax toxin. In particular, we observe an initial sharp increase in potency with increasing ligand density, followed by a plateau where potency is independent of ligand density. Our simple strategy for designing polyvalent inhibitors of controlled molecular weight and ligand density will be broadly applicable for designing inhibitors for a variety of pathogens and toxins, and for elucidating structure−activity relationships in these systems. Our results also demonstrate a role for kinetics in influencing inhibitory potency in polyvalent systems. Finally, our work presents a synthetic route to polyvalent inhibitors that are more structurally defined and effective in vivo. This control over inhibitor composition will be generally useful for the optimization of inhibitor potency and pharmacokinetics, and for the eventual application of these molecules in vivo.

Introduction

Polyvalency, which refers to the simultaneous interaction between multiple ligands on one entity and multiple receptors on another, is a phenomenon that is ubiquitous in nature.[1−4] Polyvalent interactions can have affinities that are orders of magnitude greater than the corresponding monovalent interactions, and several groups have successfully used this concept to enhance the potency of synthetic ligands.[1,2,5−10]

Linear polymers represent simple and commonly used scaffolds for the polyvalent display of ligands,[1,2] and the reaction of ligands with activated polymers is a particularly attractive approach to synthesize polyvalent molecules.[5,6,10−15] An advantage of this approach is that the properties of the molecule can be easily modulated by varying the amount or nature of the ligand and by using two different ligands to create bifunctional molecules.[16,17] Activated polymers of controlled molecular weight would be particularly useful for determining the influence of molecular weight and ligand density on inhibitor potency. Attempts to synthesize controlled molecular weight activated polymers by using controlled polymerization techniques have, however, primarily been restricted to relatively low molecular weights.[18,19]

Scheme 1. Scheme for Synthesis of Polyvalent Inhibitor of Controlled Molecular Weight[a]



[a] (a) TFA/CH_2Cl_2. (b) *N,N*′-Carbonyldiimidazole, *N*-hydroxysuccinimide, pyridine, 110 °C. (c) i. Peptide, DMF. ii. NH_4OH.

We describe a method to synthesize the activated polymer poly(*N*-acryloyloxysuccinimide) (pNAS, Scheme 1) of controlled molecular weight. We focused on pNAS, because it has been used to design inhibitors for a variety of pathogens and toxins,[5,10,14,20,21] including one that provided efficacy in vivo.[10] We apply this method to investigate the relationship between the structure and the potency of polyvalent inhibitors of anthrax toxin. This method enables the design of potent controlled molecular weight inhibitors that neutralize anthrax toxin both in vitro and in vivo.

Experimental Section

Materials and Methods. Poly(*tert*-butyl acrylate) (pTBA, M_p = 28.4, 69, 100, 150 kDa) was purchased from Polymer Standards Service (Silverspring, MD). The polydispersity of the polymers ranged from 1.03 to 1.2. Solvents were purchased from Acros Chemicals (Somerville, NJ) and were anhydrous unless stated otherwise. Peptide Ac−HTSTYWWLDGAPK−Am was purchased from Genemed Synthesis,

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Inc. (South San Francisco, CA). Spectra/Por4 (MWCO = 12 000−14 000) dialysis membranes were purchased from Spectrum Laboratories (Rancho Dominguez, CA).

Characterization. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 500-MHz spectrometer. Chemical shifts are reported in parts per million relative to trimethylsilane (TMS) for the ^{1}H NMR spectra, and relative to DMSO-d_6 at 39.5 ppm for the ^{13}C NMR spectra. Gel permeation chromatography (GPC) was carried out on poly(acrylic acid) samples obtained by hydrolysis of pTBA and poly(*N*-acryloyloxysuccinimide) (pNAS) on a ViscoGEL column (GMPWXL, Mixed Bed, dimensions: 7.8 mm × 30 cm) using phosphate buffer as the eluent (pH 7.0, 100 mM NaCl, 25 mM NaH_2PO_4, 25 mM Na_2HPO_4, flow rate = 1 mL/min, d*n*/d*c* = 0.165 mL/g). Molecular weight was estimated using a light scattering instrument (Viscotek 270 Trisec Dual Detector; OmniSEC software, λ = 670 nm). Infrared measurements were made on an FT-IR spectrophotometer (Biorad FTS-3000 MX).

Synthesis of Poly(*N*-acryloyloxysuccinimide) of Controlled Molecular Weight. Poly(*tert*-butyl acrylate) was hydrolyzed using trifluoroacetic acid in dichloromethane (20% v/v) to yield poly(acrylic acid). The precipitated polymer was washed with dichloromethane and freeze-dried from 1,4-dioxane containing small amounts of methanol. Poly(acrylic acid) was dissolved in pyridine and heated to 110 °C. Activation of the carboxylic acid groups was carried out by reaction with *N*,*N*′-carbonyldiimidazole for 30 min at 110 °C.[24] *N*-Hydroxysuccinimide was then added, and the mixture was allowed to react overnight at 60 °C. The activated polymer was precipitated in acetone and dried under vacuum to give pNAS (60% yield). The conversion of the carboxylic acid groups along the backbone to active esters was quantitative as confirmed by ^{1}H NMR spectroscopy (data not shown). ^{1}H NMR (DMSO-d_6): δ 1.8−2.3 (br, 2H), 2.7−2.9 (br, 4H), 3−3.2 (br, 1H). ^{13}C NMR (DMSO-d_6): δ 25.3, 32.9, 37.8, 16.8, 172.7. IR (KBr): 3010, 2949, 3005, 1783, 1737, 1206, 1068 cm^{-1}.

Synthesis of Polyvalent Inhibitors of Anthrax Toxin. The controlled molecular weight activated polymer, pNAS, was dissolved in DMF (5 mg/mL, 30 mM of active ester group). A solution of the peptide Ac−HTSTYWWLDGAPK−Am in DMF was then added to the polymer solution followed by triethylamine (2 equiv per active ester group). The mixture was allowed to react overnight at room temperature. The unreacted active ester groups along the polymer backbone were quenched using ammonium hydroxide (fivefold excess per repeat unit). The polymer was dialyzed extensively using MilliQ water for 48 h and lyophilized to yield a white, fluffy powder. ^{1}H NMR (D_2O): δ 0.55−0.65 (br), 0.8−0.85 (br), 0.95−1.0 (br), 1−1.7 (br), 1.75−1.85 (br), 2−2.2 (br), 2.5−2.95 (br), 3.55−3.85 (br), 4.0−4.5 (br), 4.7−4.8 (br), 4.9−4.95 (br), 6.5−6.8 (br), 7−7.4 (br).

Cytotoxicity Assay. PA (10^{-9} M), LF (3×10^{-10} M), and various concentrations of the inhibitors were added to RAW264.7 cells plated in a 96-well plate and incubated for 4 h at 37 °C. For the preincubation experiments, PA_{63} (3×10^{-9} M) was incubated with various concentrations of inhibitors for indicated times before adding LF and incubating the mixture with cells for 4 h. Cell viability was assessed by adding 20 μL of a mixture of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) and phenazine methosulfate (PMS) to each well and incubating for 60 min according to manufacturer's instructions (Promega). The absorbance at 490 nm was measured to determine the amount of soluble formazan product generated from the reduction of MTS by living cells. The IC_{50} was determined using Prism software (GraphPad Software).

Rat Intoxication. Fisher 344 rats (Charles River Laboratories) were injected intravenously in the tail vein with a mixture of PA (40 μg) and LF (8 μg) or with a mixture of PA (40 μg), LF (8 μg), and polyvalent inhibitor (300 nmol on a per-peptide basis). Three rats were used per group (per experiment), and the appearance of symptoms of intoxication was monitored. When the symptoms were obvious, the rats were euthanized to avoid unnecessary distress. The experiments were repeated on two separate days.

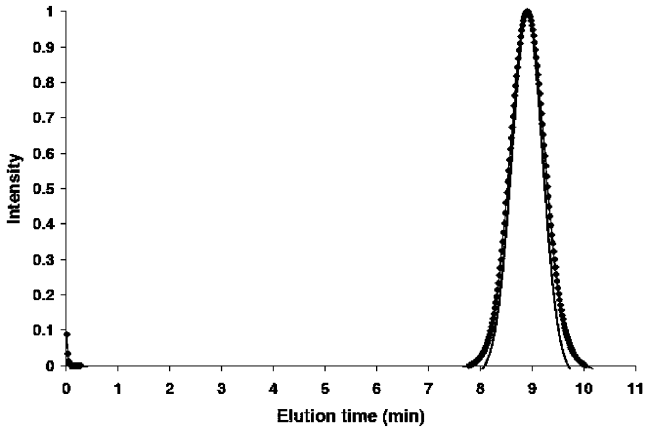

Figure 1. Gel permeation chromatograms of poly(acrylic acid) obtained by the hydrolysis of poly(*tert*-butyl acrylate) (−) and poly(*N*-acryloyloxysuccinimide) (◆).

Results and Discussion

Synthesis of Active Ester of Poly(acrylic acid) of Controlled Molecular Weight. Since controlled molecular weight poly(acrylic acid) is not readily available commercially, we developed a synthetic route (Scheme 1) that starts with poly(*tert*-butyl acrylate) (pTBA). Controlled molecular weight pTBA can be synthesized by anionic polymerization[22,23] and is commercially available in a range of molecular weights (27−650 kDa) with narrow polydispersities. Reaction of pTBA with trifluoroacetic acid yields poly(acrylic acid) (pAA, Scheme 1), which on further reaction with *N*,*N*′-carbonyldiimidazole and *N*-hydroxysuccinimide yields pNAS.[24]

Validation of the Backbone Integrity of the Activated Polymer. Poly(acrylic acid) obtained from the hydrolysis of controlled molecular weight pNAS was characterized by gel permeation chromatography (see Experimental Section). Chromatograms for poly(acrylic acid) obtained by the hydrolysis of pNAS were compared to those for poly(acrylic acid) obtained by the hydrolysis of the starting material—pTBA. As seen in Figure 1, these chromatograms are virtually identical, confirming that there was no degradation of the polymer backbone during the conversion of pTBA to pNAS. The polydispersity of the poly(acrylic acid) obtained by the hydrolysis of pNAS was narrow and similar to that for pTBA.

Synthesis of Polyvalent Inhibitor of Anthrax Toxin. We synthesized a series of controlled molecular weight pNAS polymers (average number of repeat units, *N*, was varied from ca. 200−1350). To demonstrate the utility of these activated polymers, we used them to synthesize controlled molecular weight polyvalent inhibitors of anthrax lethal toxin (LeTx). LeTx is a bipartite protein toxin that is responsible for symptoms and death in anthrax.[25] It consists of an enzymatic moiety (LF) and a cell-binding moiety, protective antigen (PA). PA is proteolytically cleaved at the mammalian cell surface, resulting in the formation of heptamers ($[PA_{63}]_7$) that bind LF. Mourez et al.[10] previously identified a peptide HTSTYWWLDGAP, which binds to $[PA_{63}]_7$ and inhibits toxin assembly—the binding of LF to $[PA_{63}]_7$. The inhibitory potency of this peptide was enhanced by several orders of magnitude by attaching multiple copies to a polyacrylamide backbone. However, there is limited understanding of the relationship between the composition of these linear polyvalent inhibitors and their potencies. We therefore synthesized polyvalent LeTx inhibitors of controlled molecular weight (PVI, Scheme 1) by allowing the pNAS polymers to react first with varying amounts of the peptide Ac−

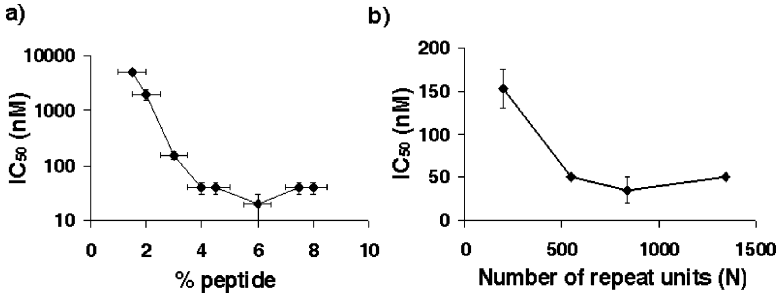

Figure 2. (a) Influence of peptide density on the potency of a polyvalent anthrax toxin inhibitor. Number of monomer repeat units in the backbone, *N* ca. 200. (b) Influence of number of monomer repeat units on the inhibitory potency (peptide density ca. 3%). IC_{50} values are reported on a per-peptide basis.

HTSTYWWLDGAPK−Am and then with ammonia. These polyvalent inhibitors were then tested for their efficacy in preventing cell death due to anthrax toxin; the inhibitory potency was quantified in terms of the half-maximal inhibitory concentration (IC_{50}), which we report on a per-peptide basis.

Influence of Peptide Density on the Potency of the Polyvalent Inhibitor. We first synthesized a series of polyvalent inhibitors in which the number of repeat units in the backbone was held constant (ca. 200) and tested the effect of peptide density on potency (Figure 2a). We observed an initial sharp increase in potency with increasing ligand density, followed by a plateau where potency is independent of ligand density. The peptide density at the transition corresponds to an average number of peptides per polymer chain of 6 ± 2; significantly, this number is small and is also close to the number of peptide binding sites on the heptameric target (7). The use of backbones with a narrow molecular weight distribution was critical for determining the relationship between peptide density and inhibitor potency. For instance, at a peptide density of 2%, the value of IC_{50} changes fivefold—from 2 μM (Figure 2a) to 400 nM—when only 10% of the peptides in the mixture are attached to a higher molecular weight backbone (*N* ca. 550 instead of *N* ca. 200; data not shown).

Influence of the Molecular Weight of the Backbone on the Potency of the Polyvalent Inhibitor. Next, we tested the influence of the molecular weight of the polymer backbone on the inhibitory potency; for these experiments, the peptide density was held constant at 3% (Figure 2b). As seen in Figure 2b, the potency of the inhibitors is weakly dependent on the number of repeat units in the polymer backbone in the range tested, with values of IC_{50} close to those seen in the plateau region in Figure 2a.

Influence of Kinetics on the Potency of the Polyvalent Inhibitor. Next, we compared these experimental results to theoretical predictions. Gargano et al.[29] used the concept of effective concentration to derive an expression for the enhancement in potency of polyvalent inhibitors as a function of the number of bound ligand−receptor pairs (*n*). For our system ($n = 7$), this model predicts a very large enhancement in potency of 5.6×10^{12}, which greatly exceeds experimental observations.[10] We wished to test whether kinetics, i.e., the relative binding rates of the polyvalent inhibitor and LF to $[PA_{63}]_7$, might limit the observed enhancement in potency because the model of Gargano et al.[29] assumes equilibrium binding. If the enhancement in potency of the polyvalent inhibitor is limited by kinetics, then preincubating the inhibitor with the target protein ($[PA_{63}]_7$) in the absence of LF should lead to an increase in potency (decrease in IC_{50}). As seen in Figure 3, the potency of the inhibitors increased with increasing preincubation time, indicating that the potency of the polyvalent inhibitor is not governed solely by thermodynamics as is often assumed, and that kinetics plays a role. We note that a long time may be required to reach equilibrium for a polyvalent interaction—a phenomenon that is well-appreciated in the polymer adsorption literature.[26−28]

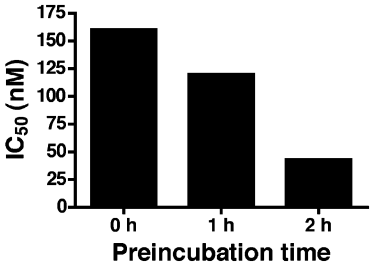

Figure 3. Preincubation of $[PA_{63}]_7$ with a polyvalent inhibitor decreases the measured IC_{50}. $[PA_{63}]_7$ was incubated with various concentrations of inhibitor for indicated times. LF was then added, and the mixture was incubated with cells for 4 h. Viability was assessed using the MTS assay. Results shown are representative of three independent experiments.

Table 1. Inhibition of LeTx Action in a Rat intoxication Model by a Controlled Molecular Weight Polyvalent Inhibitor (*N* ca. 550)

inhibitor	amount of peptide (nmol)	moribund/total
none	0	6/6
polyvalent inhibitor	300	0/6

It would be interesting to test whether a similar phenomenon is observed in other polyvalent systems, where the observed enhancements in potency, while significant, might still be limited by kinetics. We also note that the model of Gargano et al.[29] does not incorporate certain entropic terms, such as the loss of conformational entropy[30] of the polymer on binding. While the model works remarkably well in a number of systems, it significantly overestimates the degree of enhancement for some polyvalent inhibitors.[29]

In Vivo Activity of Polyvalent Inhibitors of Controlled Molecular Weight. Finally, we tested the ability of these "defined" controlled molecular weight polyvalent inhibitors to neutralize LeTx (40 μg PA plus 8 μg LF) in vivo, in Fisher rats. As seen in Table 1, none of the six rats that received the inhibitor along with the toxin became moribund. In contrast, all six rats that received toxin alone became moribund.

Conclusion

In sum, this work is significant for several reasons. First, we have developed a simple strategy to design polyvalent inhibitors of controlled molecular weight and ligand density; this strategy will be broadly applicable for designing inhibitors for a number of pathogens and toxins[1] and for elucidating structure−activity relationships. Second, our results illustrate a role for kinetics in influencing potency in polyvalent systems. While polyvalency

has provided impressive enhancements in potency in several systems,[1] our results suggest that the intrinsic enhancement in affinity may be even greater than that initially estimated. Finally, we have demonstrated a synthetic route to polyvalent inhibitors that are more structurally defined, and effective in vivo; this control over inhibitor composition will be generally useful for the optimization of inhibitor potency and pharmacokinetics and for the eventual application of these molecules in vivo.

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