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Synthesis of Potent Inhibitors of Anthrax Toxin Based on Poly-L-Glutamic Acid

Amit Joshi[†], Arundhati Saraph[†], Vincent Poon[‡], Jeremy Mogridge[‡], and Ravi S. Kane[†]

The Howard P. Isermann Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York, 12180, and Department of Laboratory Medicine and Pathobiology, University of Toronto, 1 King's College Circle, Toronto, Ontario M5S1A8, Canada

Abstract

We report the synthesis of biodegradable polyvalent inhibitors of anthrax toxin based on poly-L-glutamic acid (PLGA). These biocompatible polyvalent inhibitors are at least four orders of magnitude more potent than the corresponding monovalent peptides *in vitro* and are comparable in potency to polyacrylamide-based inhibitors of anthrax toxin assembly. We have elucidated the influence of peptide density on inhibitory potency and demonstrate that these inhibitory potencies are limited by kinetics, with even higher activities seen when the inhibitors are preincubated with the heptameric receptor-binding subunit of anthrax toxin prior to exposure to cells. These polyvalent inhibitors are also effective at neutralizing anthrax toxin *in vivo* and represent attractive leads for designing biocompatible anthrax therapeutics.

INTRODUCTION

Polyvalency refers to the simultaneous attachment of multiple ligands to multiple complementary receptors (1,2). Nature uses polyvalency in a variety of biological contexts, including interactions between bacterial toxins and cell surface receptors (1,3-6). Polyvalent interactions can collectively be much stronger than the corresponding monovalent interactions and several groups have successfully used this concept to enhance the potency of synthetic ligands (1,2,7-15). Linear polymers present an attractive scaffold for the polyvalent display of ligands because they are easily synthesized and their structure and the composition of ligands can be modulated (7,8,11,16-18). Poly-L-glutamic acid (PLGA) represents a particularly attractive scaffold for designing polymeric therapeutics because of its high water solubility, biodegradability, and low toxicity (19-21). Consequently, researchers have conjugated PLGA with drugs such as camptothecin, paclitaxel, doxorubicin or antibodies for drug delivery (21-26), with DNA for gene delivery (27-29), and with other ligands for synthesizing inhibitors of the influenza virus (20,30,31).

We report the design of potent polyvalent inhibitors of anthrax toxin based on PLGA. Anthrax toxin – responsible for the major symptoms and death in anthrax – is composed of three proteins: two enzymes, lethal factor (LF) and edema factor (EF), and a cell-binding protein, protective antigen (PA). PA is cleaved into a 63 kDa fragment that forms heptamers, [PA₆₃]₇, on the surface of target cells and translocates LF and/or EF into the cytosol wherein they exhibit their toxic effects (3). The biocompatible inhibitors reported in this work prevent the binding of LF to [PA₆₃]₇ and neutralize anthrax toxin both *in vitro* and *in vivo*.

To whom correspondence should be addressed. R.S.K.: tel, (518) 276 2536; fax, (518) 276 4030; e-mail, E-mail: kaner@rpi.edu. J.M.: tel, (416) 946 8095; fax, (416) 978 5959; e-mail, E-mail: jeremy.mogridge@utoronto.ca..

[†]Rensselaer Polytechnic Institute.

[‡]University of Toronto.

EXPERIMENTAL PROCEDURES

Materials and Methods

Poly-L-Glutamic acid sodium salt ($M_w = 7.5, 17.6, 41.4$ kDa), and 1-Hydroxybenzotriazole (HOBt), was purchased from Sigma (St. Louis, MO). 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) was purchased from Acros Chemicals (Somerville, NJ). Solvents were purchased from Acros Chemicals (Somerville, NJ) and were anhydrous unless stated otherwise. Peptide Ac-HTSTYWLDGAPK-Am was purchased from Genemed Synthesis, Inc. (South San Francisco, CA). Spectra/Por4 (MWCO = 12000–14000) dialysis membranes were purchased from Spectrum Laboratories (Rancho Dominguez, CA). ^1H and ^{13}C NMR spectra were recorded on a Varian 500-MHz spectrometer. Chemical shifts are reported in ppm relative to trimethylsilane (TMS) for the ^1H NMR spectra, and relative to DMSO- d_6 at 39.5 ppm for the ^{13}C spectra. Gel permeation chromatography (GPC) was carried out using a ViscoGEL column (GMPWXL, Mixed Bed, dimensions: 7.8 mm $\times$ 30 cm) using phosphate buffered saline as the eluent (pH 7.5, 20 mM NaH_2PO_4 , 130 mM NaCl, flow rate = 1 mL/min, $dn/dc = 0.190$ mL/g). Molecular weight was estimated using a light scattering instrument (Viscotek 270 Trisec Dual Detector; OmniSEC software, $\lambda = 670$ nm). Infrared measurements were made on an FT-IR spectrophotometer (Biorad FTS-3000 MX). CD spectra were recorded on a J-715 instrument (Jasco, Easton, MD) (Xe lamp, cell length 0.2 mm) using the Spectra Manager software.

Synthesis of activated PLGA

EDC (140 mg, 0.662 mmol) and a solution of HOBt (111 mg, 0.728 mmol) in *N,N*-dimethylformamide (DMF) (1 mL) were added to a solution of PLGA sodium salt (50 mg, 0.331 mmol) in water (1 mL) at room temperature (31). The reaction mixture was stirred at room temperature for 1 h. The resulting precipitate was washed with water and dried under vacuum. The residue was dissolved in DMF (2 mL) along with HOBt (111 mg), and a solution of EDC (280 mg) in water (1 mL) was added. The reaction mixture was stirred at room temperature for 1 h. The resultant precipitate was washed with water, followed by 2-propanol and dried under vacuum to give activated PLGA (60% yield, complete conversion of carboxylic acid groups to activated esters). ^1H NMR (DMSO- d_6): δ 1.6 – 2.0 (br, 2H), 2.0 – 2.4 (br, 2H), 3.8 – 4.3 (br, 1H), 7.2 – 8.4 (br, m, 4H). ^{13}C NMR (DMSO- d_6): δ 27.2, 30.2, 52.0, 109.9, 119.1, 124.4, 126.8, 127.9. IR (KBr): 3295, 3073, 1717, 1655, 1549, 1388, 1297, 1263, 1105, 777, 742 cm^{-1} . Elemental analysis was carried out after reaction of the activated PLGA with aqueous ammonium hydroxide. Calcd. (%) for $0.7(\text{C}_5\text{H}_7\text{NO}_3) \cdot 0.3(\text{C}_5\text{H}_8\text{N}_2\text{O}_2) \cdot 1\text{H}_2\text{O}$: C, 40.89; H, 6.34; N, 12.41; O, 40.31; Found: C, 39.60; H, 5.84; N, 12.68; O, 38.46.

Synthesis of polyvalent inhibitors of anthrax toxin

Activated PLGA was dissolved in DMF (5 mg/mL, 30 mM of repeat unit). Peptide, Ac-HTSTYWLDGAPK-Am, dissolved in DMF, was then added to the polymer solution followed by triethylamine (2 fold excess based on repeat unit). The mixture was allowed to react overnight at room temperature following which the unreacted active ester groups in the polymer backbone were quenched using ammonium hydroxide (5 fold excess based on repeat unit). The polymer was dialyzed extensively using MilliQ water for 2 days and lyophilized to yield a white powder. ^1H NMR (DMSO- d_6): δ 0.6 – 0.86 (br), 0.88 – 1.06 (br), 1.1 – 1.4 (br), 1.4 – 1.6 (br), 1.6 – 2.0 (br), 2.0 – 2.3 (br), 2.7 – 3.0 (br), 3.8 – 3.95 (br), 3.95 – 4.2 (br), 4.2 – 4.4 (br), 4.4 – 4.6 (br), 6.4 – 6.6 (br), 6.7 – 6.8 (br), 6.8 – 7.2 (br), 7.2 – 7.4 (br), 7.4 – 7.6 (br), 7.6 – 8.4 (br).

Estimation of molecular weight

Polymers obtained by hydrolysis of activated PLGA with 0.1 N NaOH were characterized using aqueous GPC with a ViscoGEL column (GMPWXL Mix Bed, dimensions: 7.8 mm × 30 cm) using phosphate buffered saline as the eluent. The molecular weight was estimated using a laser light scattering instrument (Viscotek 270 Trisec Dual Detector; OmniSEC 4.0 software, $\lambda = 670$ nm) inline with the column. A decrease in molecular weight was observed after activation, as reported earlier by Paterson and Leach (32); the polydispersity of the polymers was ca. 1.3. The average number of peptides per polymer chain was calculated using the number average molecular weight (M_n) and the peptide density (estimated by ^1H NMR spectroscopy), accounting for the error due to the polydispersity of the polymer and the error in estimating the peptide density by NMR spectroscopy.

Circular dichroism measurements

Circular dichroism (CD) measurements were carried out at room temperature (23 °C) on a JASCO J-715 spectropolarimeter. The spectra were scanned in a quartz optical cell with a path length of 0.2 mm. All spectra were recorded in 0.2 nm wavelength increments with a 4 sec response and a bandwidth of 1 nm and at a scanning speed of 100 nm/min. For observing the secondary structure of the polymers, the samples were dissolved in 20 mM sodium phosphate buffer (pH 7.5) and the spectra were scanned from 180 to 250 nm. Each spectrum is the average of 5 scans with a full scale sensitivity of 100 mdeg. All spectra were corrected for background.

Cytotoxicity assay

RAW264.7 cells were grown in RPMI medium supplemented with 5% fetal calf serum (Hyclone). The cells were plated on a 96-well plate and either left untreated or treated with PA (10^{-9} M), LF ($3 - 10^{-10}$ M) and various concentrations of the inhibitors and incubated for 4 h at 37 °C in 5% CO_2 . For the preincubation experiments, $[\text{PA}_{63}]_7$ (3×10^{-9} M) was incubated with various concentrations of inhibitors for 0, 1 or 2 h, before adding LF (10^{-9} M) and incubating the mixture with cells for 4 h at 37 °C in 5% CO_2 . 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)-2H-tetrazolium) and phenazine methosulfate (PMS) to each well and incubating for 1 h, according to manufacturer's instructions (Promega). The amount of soluble formazan product generated from the reduction of MTS by living cells was determined by measuring the absorbance at 490 nm. The IC_{50} of each inhibitor was determined using the software program PrismTM (GraphPad Software).

Rat Intoxication

Purified PA (40 μg) and LF (8 μg) diluted in PBS were mixed with either PBS, the monovalent peptide (300 nmol) in PBS, or the polyvalent inhibitor (300 nmol on a per-peptide basis) in PBS. Fisher 344 rats (Charles River Laboratories) were injected intravenously in the tail vein. The rats were injected with the different mixtures, and the appearance of symptoms of intoxication was monitored. When the symptoms were obvious, the rats were euthanized to avoid unnecessary distress.

RESULTS AND DISCUSSION

Synthesis and characterization of PLGA-based polyvalent inhibitors

To synthesize a PLGA-based polyvalent inhibitor, we activated the carboxylic acid groups of PLGA using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) and 1-Hydroxybenzotriazole (HOBt) (see Experimental Procedures). The activated polymer was allowed to react with the peptide Ac-HTSTYWWLDGAPK-Am, a peptide that binds $[\text{PA}_{63}]_7$ and inhibits the binding of LF (7). The remaining active ester groups were quenched

by a reaction with aqueous ammonium hydroxide (Scheme 1). We also synthesized unfunctionalized (control) polymers by reacting the activated polymers with aqueous ammonium hydroxide. Elemental analysis suggested that the polymer backbone of the control polymer was composed of a mixture of glutamine and glutamic acid units (3:7). This polymer had an aqueous solubility in excess of 30 mg/mL.

In vitro activity of polyvalent inhibitors

To test the ability of the PLGA-based polyvalent inhibitors to neutralize anthrax toxin *in vitro*, we incubated RAW264.7 cells with a mixture of PA and LF in the presence or absence of inhibitors. While the monovalent peptide had no activity at concentrations as high as 250 μ M (data not shown), the polyvalent inhibitor could inhibit toxin activity with a half-maximal inhibitory concentration (IC_{50}) of 20 nM on a per-peptide basis (Figure 1A). Polyvalency therefore enabled an enhancement in the potency of the peptide by over four orders of magnitude. Unfunctionalized polymers showed no appreciable activity (Figure 1A). Furthermore, the potency of the inhibitor based on the biocompatible polymer, PLGA, was comparable to that of a previously reported inhibitor based on a polyacrylamide backbone (IC_{50} = 50 nM) (Figure 1A) (33).

Influence of peptide density on the potency of the polyvalent inhibitors

To elucidate the influence of peptide density on inhibitory potency, we synthesized a series of polyvalent molecules based on PLGA backbones of three different molecular weights, with peptide densities varying between 0 and 18 %. For each backbone, we observed an initial sharp increase in potency (decrease in IC_{50}) with increasing peptide density, followed by a transition to a plateau, where the potency is independent of peptide density (Figure 1B). While the polydispersity of the backbone prevents a precise estimation of the number of peptide units per chain near the transition, the number was small (less than 10) for each backbone, and was also close to the number of peptide-binding sites on the heptameric target (seven).

Characterization of PLGA-based polyvalent inhibitors by circular dichroism spectroscopy

To further characterize these polyvalent molecules, we used circular dichroism (CD) spectroscopy. Characterization by CD spectroscopy revealed that the unfunctionalized (control) polymer obtained by the reaction of activated PLGA with aqueous ammonium hydroxide, exists in an unordered (random coil) structure as seen from the strong negative band near 200 nm (Figure 2A) (34). A similar spectrum was previously reported for PLGA (35, 36). The CD spectrum of the monovalent peptide shows two minima, one near 222 nm, which is characteristic of tryptophans (37), and the other near 200 nm (Figure 2B). In order to resolve the spectrum, we treated the monovalent peptide and the PLGA-based polyvalent inhibitor with N-bromosuccinimide (NBS), which is known to oxidize the indole of tryptophan to oxindole, resulting in the disappearance of the peak near 222 nm (38). The resulting spectra show that the monovalent peptide and the polyvalent inhibitor exist as unordered (random coil) structures (Figure 2A, B). The CD spectrum of the NBS-treated peptide is similar near 200 nm to that of the unmodified peptide, indicating that there is no significant change in the secondary structure of the peptide on modification.

Influence of kinetics on the potency of the polyvalent inhibitors

We hypothesized that the enhancement in potency obtained through polyvalency might be limited by kinetics since equilibrium between the polyvalent inhibitor and $[PA_{63}]_7$ might not be reached before the toxin is endocytosed. To determine if kinetics limit the potency of the polyvalent inhibitor, we preincubated the inhibitor with $[PA_{63}]_7$ for different amounts of time before adding LF and exposing cells to this mixture. If kinetics play a role in influencing the potency of the inhibitor, then one would expect the IC_{50} to decrease with increasing

preincubation time. We observed an order of magnitude decrease in the IC_{50} of the inhibitors following preincubation with $[PA_{63}]_7$ for 2 h (Figure 3), confirming that the enhancement in inhibitory potency is limited by kinetics.

***In vivo* activity of PLGA-based polyvalent inhibitors**

Biocompatible and biodegradable polymer scaffolds are particularly suitable for the synthesis of anthrax toxin inhibitors that can be used *in vivo*. We therefore tested the ability of PLGA-based inhibitors to neutralize anthrax toxin in Fischer rats. Although the inhibitor was co-injected with the toxin, neutralization could occur only *in vivo*, since the inhibitor binds to $[PA_{63}]_7$ and not to PA. As seen in Figure 4, 13 of 14 rats injected with toxin alone (40 μ g of PA and 8 μ g of LF) became moribund, while none of the rats that received the polyvalent inhibitor along with the toxin became moribund. The log-rank test indicates that the difference between these two groups is statistically significant ($P = 0.0002$). In contrast, the log-rank test does not indicate a significant difference between the group that received lethal toxin alone and the group that received the toxin along with the monovalent peptide ($P = 0.54$; see Figure 4).

CONCLUSION

In sum, we have designed inhibitors of anthrax toxin that are more than four orders of magnitude more potent than the corresponding monovalent inhibitors. This study shows that even a small number of pendant ligands, which is close to the number of binding sites on the target, may be sufficient to synthesize a potent polyvalent inhibitor. The enhancements in potency obtained from polyvalent systems may not be governed solely by thermodynamics, as is often assumed, but also by the kinetics of binding, suggesting that the intrinsic enhancements in affinity may be even greater than the already impressive numbers reported. These PLGA-based inhibitors are comparable in potency to the best polyvalent inhibitors of anthrax toxin assembly, and the biocompatibility of their scaffolds makes them attractive for *in vivo* use.

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Figure 1.

(A) Inhibition of cytotoxicity in cell culture by PLGA-based polyvalent inhibitor (□), polyacrylamide-based polyvalent inhibitor (▲) and control polymer (ammonium hydroxide quenched activated PLGA) (Δ). (B) Influence of peptide density on the potency of polyvalent inhibitors with different molecular weights $M_w = 4.5$ kDa (•), 12 kDa (■) and 29 kDa (□), average number of repeat units ca. 27, 70, 170 respectively.

Figure 2.

(A) CD spectrum of (i) ammonia hydrolyzed activated PLGA, (ii) NBS-treated PLGA-based polyvalent inhibitor. (B) CD spectrum of (i) monovalent peptide, (ii) NBS-treated monovalent peptide. $[\theta]$ is the mean residue molar ellipticity.

Figure 3.

Preincubation of [PA₆₃]₇ with a polyvalent inhibitor decreases the measured IC₅₀. [PA₆₃]₇ was incubated with various concentrations of two different molecular weight inhibitors for 0 h (■), 1 h (■) and 2 h (□). Following the preincubation period, the inhibitor-[PA₆₃]₇ mixtures were tested in cytotoxicity assays.

Figure 4.

PLGA-based inhibitor protects rats from lethal toxin challenge. Rats were injected with lethal toxin alone (▲, $n = 14$), lethal toxin plus Ac-HTSTYWWLDGAPK-Am peptide (○, $n = 12$), or lethal toxin plus PLGA-based inhibitor (■, $n = 7$). The log-rank test indicates a statistically significant difference between the group that received lethal toxin alone and the group that received the toxin plus the PLGA-based inhibitor ($P = 0.0002$), but no significant difference between the group that received lethal toxin alone and the group that received the toxin along with peptide ($P = 0.54$).

Scheme 1.

Synthesis of PLGA-based polyvalent inhibitor; (a) EDC, H₂O; (b) HOBt, DMF; (c) Peptide, DMF, TEA (d) NH₄OH, dialysis. The polyvalent inhibitor is a random copolymer composed of peptide conjugates (x repeat units), glutamine (y repeat units) and glutamic acid (z repeat units).