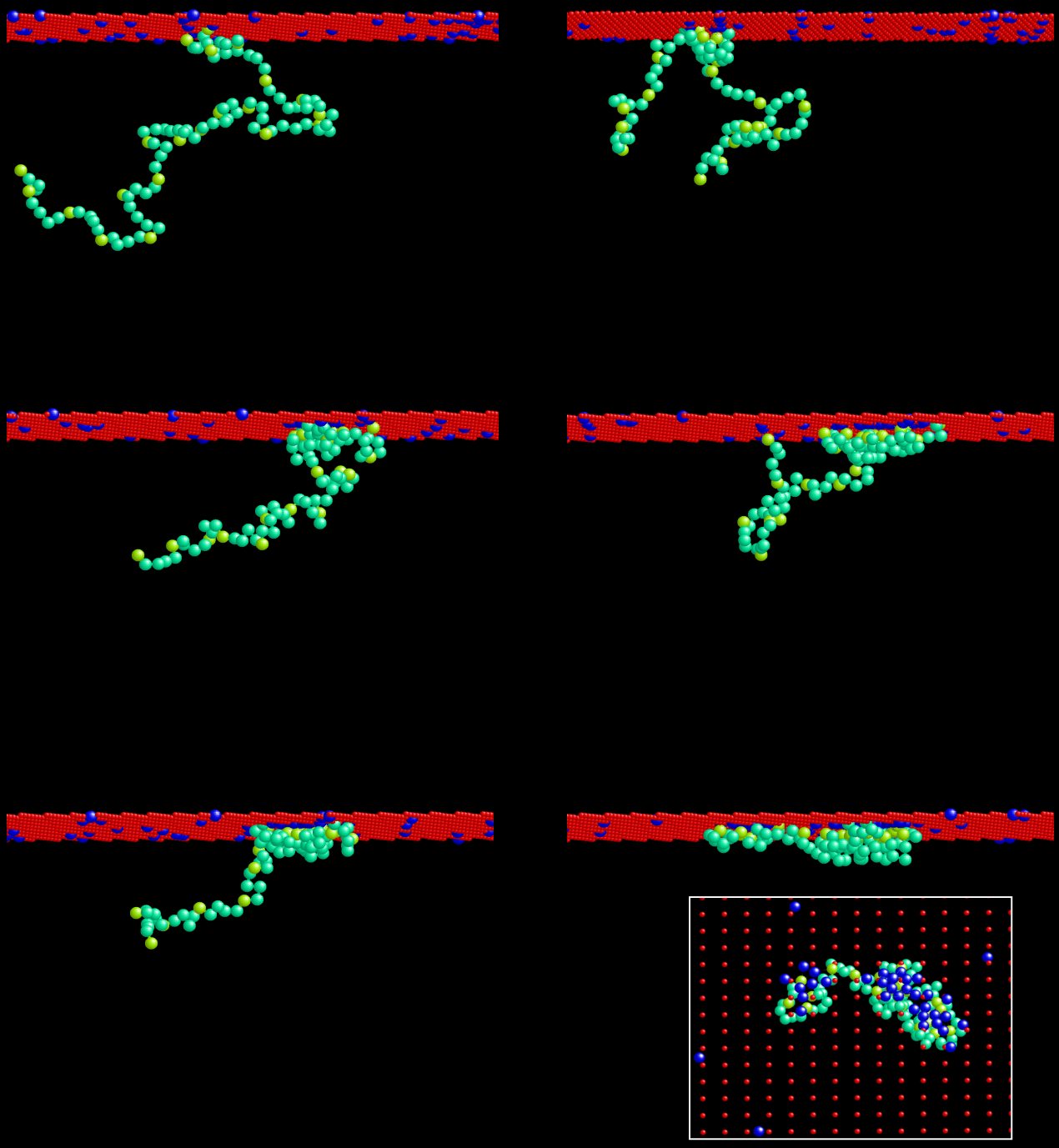
Polyvalent Inhibitors of *Pseudomonas aeruginosa* Adhesion



Polyvalent Inhibitors of *Pseudomonas aeruginosa* Adhesion

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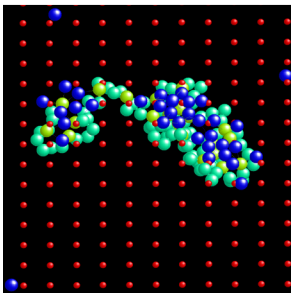

About the cover: A polymer binds to a surface in a computer simulation written by the author. The blue sites on the surface stick loosely to the green subunits of the polymer. The polymer can bind very tightly because of the combined attraction between multiple polymer sites and the surface. Notice that binding is not a discrete process: various parts of the polymer attach and detach before forming a tight bond. The time series goes from right to left, top to bottom. The inset shows a top view of the last frame. Images were rendered using RasMol, written by Roger Sayle.

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Acknowledgements

These experiments could never have gotten off the ground without the support of my mentor, Dr. Michael Liang, a postdoctoral fellow in the laboratory of Professor George Whitesides. His infectious enthusiasm has been inspiring, and he has guided me on the project in one way or another virtually every day. At the same time, he has given me a great deal of freedom to pursue my own ideas. Dr. Liang has shown me not only the laboratory techniques involved, but also the ideas and collaborations needed to put together a successful experiment.

Many other people have played a large role in this project. Prof. Whitesides provided invaluable advice on the most promising experiments to pursue. Prof. Gerald Pier and Dr. Tanweer Zaidi helped perform and interpret the biological assays. Prof. Eugene Shakhnovich provided guidance and encouragement on the theoretical studies.

Abstract

We synthesized polymers displaying multiple copies of a peptide from the cystic fibrosis transmembrane conductance regulator (residues 108-117). This peptide is the minimal sequence needed for binding of *Pseudomonas aeruginosa*. Using an *in vitro* bacterial internalization assay, our preliminary results indicate that such a polyvalent ligand can prevent *P. aeruginosa* from attaching to (and being internalized by) epithelial cells. The most effective polymer we synthesized was a polyacrylamide with CFTR peptide covalently attached to 0.5% of its acrylamide subunits. This polymer has an IC_{50} value of 700 pM, 40,000 times lower than monovalent CFTR peptide. Polyacrylamide at the same concentration does not inhibit internalization.

We also developed a simulation to qualitatively describe how a polyvalent ligand binds to a surface. This simulation demonstrates that the polymer binds to the surface cooperatively. Binding can be enhanced by a larger degree of polymerization, a larger fraction of polymer subunits that can attach to the surface, and greater diffusibility of binding sites on the surface.

Abbreviations

Ahx	aminohexanoic acid
asialo-GM_1	Gal-(β1-3)-GalNAc-(β1-4)-Gal(β1-4)-Glcβ1-ceramide
CFTR	cystic fibrosis transmembrane conductance regulator
Dde	(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl
DMF	dimethylformamide
FITC	fluorescein isothiocyanate
Fmoc	9-fluorenylmethyloxycarbonyl
HPLC	high performance liquid chromatography
LPS	lipopolysaccharide
NHS	N-hydroxysuccinimide
PBS	phosphate buffered saline
pA	polyacrylamide
pAA	polyacrylic acid
pLGA	poly-L-glutamic acid
pBMA	poly(butadiene-maleic anhydride)
pNAS	poly[N-(acryoyloxy)succinimide]

Peptides

CFTR	H_2N – S Y D P D N K E E R – COOH
CFTR-K	H_2N – S Y D P D N K E E R K – COOH
H_2N-Ahx-CFTR	H_2N – $(CH_2)_5$ – S Y D P D N K E E R – COOH
CFTR-K-FITC	H_2N – S Y D P D N K E E R K(FITC) – $CONH_2$
	(The ε-amino group of lysine is conjugated to fluorescein isothiocyanate)

Chapter 1: Experiment

INTRODUCTION

The interaction between pathogens (such as bacteria or viruses) and host cells is often polyvalent: multiple ligands on the pathogen simultaneously bind to multiple receptors on the host cell. This attachment can be a crucial first step towards infection, and it can be blocked by free monomeric host cell receptors. A polymer displaying multiple copies of the host cell receptor, however, could potentially be a more effective inhibitor because of its higher affinity for the pathogen and its steric blocking of additional pathogenic ligands.

In this study, we design and characterize polyvalent ligands to inhibit *Pseudomonas aeruginosa* adhesion to epithelial cells. Polyvalent ligands have been successfully used to block biological interactions, such as the binding of influenza virus to cells,[24] and we would like to test the generality of this strategy by extending it to a new system. Studying polyvalency in bacteria offers unique challenges because the types of adhesion receptors they display can be highly variable and no drug has yet been developed to successfully block bacterial attachment to cells. These experiments will help us understand the fundamental biochemistry and biophysics underlying polyvalent adhesion to microorganisms.

Polyvalency in biology

Polyvalent interactions play a major role in adhesion and molecular recognition in a diverse range of biological processes (comprehensive review of polyvalency in [25]). Table 1 and Figure 1 summarize some representative examples of these interactions.

Polyvalent receptors and ligands often can bind with much higher specificity and affinity than their monovalent counterparts. In essence, polyvalency provides a way to combine several weak interactions into a single stronger interaction. Many biological systems have taken advantage of this property in clever ways. For example, the Fc portions of individual antibodies cannot bind to low-affinity Fc receptors on macrophages. When several antibodies bind to a foreign particle, however, the array of antibodies presented on the surface can tightly bind to macrophage Fc receptors, activating the macrophage.

Polyvalency may also help improve the specificity of biological interactions by strengthening the desired ligand-receptor bond. Many of the examples listed in Table 1 require a fine degree of specificity. For example, several different species of abalone spew their sperm into the seawater during the same time of year, so it is critical for these sperm to only recognize eggs of the same species.[41] Many pathogens recognize specific receptors that allow them to infect a specific type of cell.

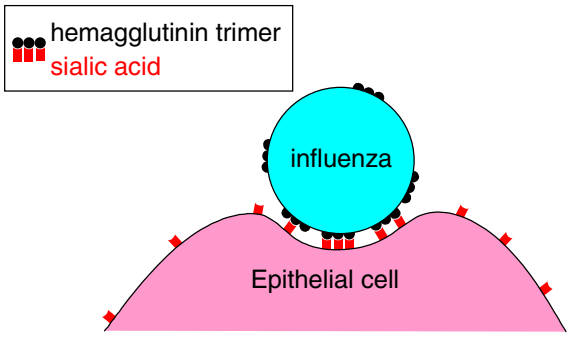


Figure 1. *Many pathogens attach to their hosts polyvalently. Figure not drawn to scale.*

Ligand	Receptor	Result of interaction	Ref.
Cell-cell interactions			
abalone sperm lysin	abalone egg vitelline envelope receptor for lysin	fusion of egg and sperm	[41]
Arg-Gly-Asp peptide from cell-surface adhesion molecules	cell-surface adhesion molecules	cell adhesion	[31]
sialyl-Lewis^x on leukocyte	E-selectin on vascular endothelium	weak attachment to endothelium	[14]
Molecule-cell interactions			
cholera toxin (pentamer)	GM_1 on cells	diarrhea	[37]
ricin (toxin from the bacteria *Ricinis communis*)	β-galactoside on cells	inhibition of protein synthesis	
antibody (two binding sites each, but IgA can form dimers, and IgM can form pentamers)	antigen	immune response	
Virus-cell interactions			
hemagglutinin trimers on influenza	sialic acid on cells	viral infection	
gp120 on HIV	CD4 on T cells and macrophages	viral infection	[43]
Bacteria-cell interactions			
PapG adhesin from P pili on uropathogenic *E. coli*	Gal-(α1-4)-Gal on urinary tract epithelium	bacterial colonization	[21]
S fimbriae on *E. coli*	sialic acid on cells	bacterial colonization	
FimH from type 1 fimbriae on *E. coli*	D-mannose on cells	bacterial colonization	[1]
LPS on *P. aeruginosa* or *S. typhii*	CFTR on cells	bacterial internalization	[29, 30]
Pili on *P. aeruginosa*	GalNAc-(β1-4)-Gal from asialo-GM_1 on cells	bacterial colonization	[38]
Protein-DNA interactions			
retinoid X receptor (can form tetramer or higher order complex)	adjacent DNA binding sites	increased gene transcription	[9]

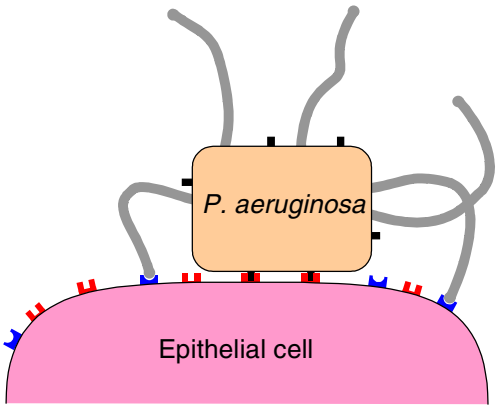
Table 1. *Selected examples of polyvalent interactions in biology. Highlighted interactions have been reproduced or antagonized in the laboratory with synthetic polyvalent ligands (see Table 2).*

Polyvalent ligands in *P. aeruginosa* infections

Pseudomonas aeruginosa is a ubiquitous extracellular gram-negative bacterium that causes recurrent opportunistic infections in patients with compromised respiratory function, in severe burn victims, and in cancer patients immunologically compromised by chemotherapy. This bacteria causes 10 – 20% of infections in most hospitals.[7] *P. aeruginosa* infections occur in 90% of cystic fibrosis patients and are the major cause of death for those patients.

P. aeruginosa has several receptors for binding to ligands on host cells. These receptors and their role in pathogenesis are discussed below and summarized in Figure 2. These interactions presumably occur polyvalently.

Lipopolysaccharide (LPS) on the surface of *P. aeruginosa* binds to the cystic fibrosis transmembrane conductance regulator (CFTR) on epithelial cells.[29] LPS is an



Interaction	In the lung …	In the cornea …
LPS (core oligosaccharide) CFTR (residues 108-117)	Promotes internalization and clearance	Promotes colonization
pili (residues 128-144 of pilin) asialo-GM_1 (GalNAc-(β1-4)-Gal)	Promotes colonization	(?)

Figure 2. *Molecular interactions important in* P. aeruginosa *binding to epithelial cells. Figure not drawn to scale.*

essential component of the outer membranes of all gram negative bacteria. Also known as endotoxin, it stimulates the inflammatory response and plays a large role in bacterial pathogenicity. CFTR was originally identified as a chloride channel, and cystic fibrosis patients have a mutation in this protein. The most common mutation, ΔF508, prevents the protein from being properly processed and targeted to the cell membrane.

Multiple lines of evidence support the biological importance of the interaction between LPS and CFTR. The interaction between *P. aeruginosa* and epithelial cells can be blocked by the LPS-core oligosaccharide, or by a peptide from the first predicted extracellular domain of the cystic fibrosis transmembrane conductance regulator (CFTR, residues 108-117).[28, 29] Normal epithelial cells internalize the bacteria after binding via LPS-CFTR. Cells homozygous for the temperature-sensitive ΔF508 mutation in CFTR are defective in this uptake at 37°C, but can be rescued by transferring to a permissive temperature (26°C), or by transfecting with wild-type CFTR.

Ingestion of bacteria by lung epithelial cells, followed by shedding of bacteria-laden cells, may be a clearance mechanism for *P. aeruginosa* infections in the normal lung. Cystic fibrosis patients lack this clearance mechanism, partly accounting for their increased susceptibility to *P. aeruginosa* infections. Bacterial counts in *P. aeruginosa* pulmonary infections in mice increased when bacterial internalization was inhibited with either LPS or the appropriate peptide from CFTR.[28, 29]

In the initial stages of a corneal infection, *P. aeruginosa* attaches to injured epithelial cells. Bacterial attachment in the cornea is also mediated by LPS; however, in contrast to pulmonary infections, which are cleared by *P. aeruginosa* attachment, corneal

infections are promoted by *P. aeruginosa* attachment, presumably because corneal cells are not as easily shed.[44]

The adherence of *P. aeruginosa* to epithelial surfaces can also be mediated by interactions between the pili on bacteria and the glycosphingolipids asialo-GM_1 or asialo-GM_2 on epithelial cells. Pili are thin flexible polymeric filaments made of pilin protein, and they average 2500 nm in length.[27] The C-terminal disulfide-bonded region of pilin (residues 128-144), which is only exposed at the tip of the pilus, binds to asialo-GM_1.[22] The disaccharide GalNAc-(β1-4)-Gal is the minimal carbohydrate sequence from from asialo-GM_1 and -GM_2 that binds to pilin. The dissociation constant for the disaccharide is around 2-3 μM, with some variability in binding pili from different strains of *P. aeruginosa*.[38]

In contrast to LPS-CFTR binding in the lung, attachment mediated by pili and asialo-GM_1 seems to promote bacterial colonization and infection. Cystic fibrosis epithelia express more asialo-GM_1, and *P. aeruginosa* binds with higher affinity to these cells. Adding asialo-GM_1 or an antibody to asialo-GM_1 inhibits this binding,[5, 17, 36] implicating asialo-GM_1 ligand as an important factor for the high frequency of *P. aeruginosa* infections among cystic fibrosis patients. There is mixed evidence on the role of GM_1 in corneal infections.[16, 45]

Designing polyvalent ligands

The special properties of polyvalent ligands, especially their high affinity, could form the basis for a whole new class of pharmaceuticals. This prospect has recently generated a great deal of interest, and several polyvalent ligands have already been designed to inhibit or promote specific biological interactions (Figure 3 and Table 2).

As shown in the table, the amount of enhancement expected from a polyvalent ligand varies greatly with the details of the system studied and the method of presenting the ligand polyvalently. Small changes in a polyvalent system can often drastically reduce the binding affinity, which may be a useful feature for designing highly specific ligands, but also makes the initial design of a polyvalent ligand more challenging.

Both entropic and enthalpic effects affect the affinity of a polyvalent ligand. The high affinity of polyvalent ligands is due to entropic stabilization: after one ligand has bound to its receptor, the other ligands are constrained to be near their receptors and can thus bind at a lower entropic cost. Ligands connected by a linker that is too long or too flexible will thus not bind as well because the ligands will move more independently. In addition, if the spacing between binding sites on a rigid polyvalent ligand does not precisely match the spacing between binding sites on a rigid polyvalent receptor, the strain induced upon binding of multiple sites will reduce the enthalpy of binding.

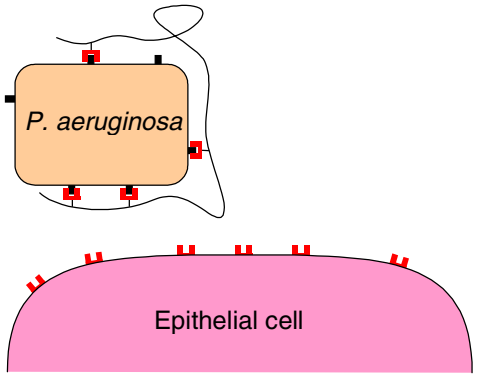


Figure 3. *Polymers displaying multiple copies of the normal host ligand for a pathogen could potentially block the pathogen-host interaction efficiently. Figure not drawn to scale.*

The example of trivalent vanco-

mycin and D-Ala-D-Ala illustrates some of the factors needed to design high affinity polyvalent ligands.[32] The antibiotic vancomycin binds to D-Ala-D-Ala, a component of bacterial cell walls, with $K_d = 10^{-6}$ M. By contrast, synthetic trimers of both molecules bind with $K_d = 4 \times 10^{-17}$ M. These binding constants indicate that binding energy of the trivalent ligands is almost three times the binding energy of the monovalent ligands. Near-optimal binding seen in this system is due in part to the relatively rigid linker that places the ligands in the correct geometry to bind.

Polyvalent ligands have also been designed to neutralize pathogens, most notably influenza (Table 2). The surface of influenza presents several hemagglutinin trimers that adhere to multiple sialic acid moieties on epithelial cells. *In vitro*, this interaction can be detected by hemagglutination, and the most effective known inhibitors of influenza-induced hemagglutination are polyacrylamides presenting sialic acid. These polymers can block hemagglutination at a concentration (K_i) of 600 pM, more than 6 orders of magnitude better than the monomer.[24]

Two major factors contribute to the enhanced inhibition of hemagglutination. Much of the inhibition is due to polyvalent enhancement of binding: a polymer that is 10^7 more effective than the monomer has an affinity for influenza 10^6 times higher.[25, 39] The polymer can also disable the virus by sterically blocking additional binding sites ("steric stabilization"). For example, the polymers prevent polyclonal antibodies from attaching to the virus.[39]

Target	Designed polyvalent ligand	K^{mono} (M)	K^{poly} (M)	$\frac{K^{mono}}{K^{poly}}$	Ref.
cell-surface adhesion molecules	self-assembled monolayer presenting Arg-Gly-Asp peptide	—	—	—	[35]
E-selectin	sialyl-Lewis^x liposomes	$1.5\ 10^{-3}$	$4\ 10^{-9}$	$4\ 10^{5}$	[40]
cholera toxin	dendritic polymer presenting GM_1 tetrasaccharide	(?)	(?)	(?)	[42]
ricin	pAA presenting galactosides	$4\ 10^{-5}$	10^{-7}	$3\ 10^{2}$	[10]
influenza	dendritic polymer presenting sialic acid	$4\ 10^{-3}$	10^{-7}	$4\ 10^{4}$	[34]
	pA presenting sialic acid	$2\ 10^{-3}$	$6\ 10^{-10}$	$3\ 10^{6}$	[24]
	pLGA presenting lysoganglioside GM_3	$3\ 10^{-9}$	$2\ 10^{-12}$	$2\ 10^{3}$	[18]
	liposome presenting sialic acid	$2\ 10^{-3}$	$2\ 10^{-8}$	10^{5}	[20]
P. aeruginosa	polyacrylamide presenting CFTR peptide	$3\ 10^{-5}$	$7\ 10^{-10}$	$4\ 10^{5}$	This paper
trivalent D-Ala-D-Ala	trivalent vancomycin	10^{-6}	$4\ 10^{-17}$	$3\ 10^{10}$	[32]

Table 2. *Synthetic polyvalent ligands that antagonize or promote the interactions shown in Table 1. The results of this paper are also included in the table for comparison.* K^{mono} *and* K^{poly} *are either binding constants (in the case of trivalent D-Ala-D-Ala) or inhibition constants. These values are not strictly comparable since they were measured in different ways, but they should provide a general indication of the effectiveness of the polyvalent ligand.* K^{mono} / K^{poly} *is the amount of polyvalent enhancement.*

METHODS

Reagents

Peptides were synthesized by SynPep Corp. (Dublin, CA), fluorescein cadaverine was obtained from Molecular Probes (Eugene, OR), Spectra/Por dialysis tubing (molecular weight cutoff 12,000 – 14,000) was obtained from Spectrum (Laguna Hills, CA), and fetal calf serum was obtained from HyClone (Logan, UT).

Peptide synthesis

CFTR peptide (H_2N – S Y D P D N K E E R – COOH) was synthesized to test monovalent inhibition of *P. aeruginosa* internalization; CFTR-K peptide (H_2N – S Y D P D N K E E R K – COOH) was synthesized to allow polymer conjugation by the C-

terminal lysine; H_2N-Ahx-CFTR peptide (H_2N – Ahx – S Y D P D N K E E R – COOH) was synthesized to allow N-terminal polymer conjugation via a six-carbon spacer. CFTR-K was synthesized with an Fmoc protecting group on the N-terminus, and both CFTR-K and H_2N-Ahx-CFTR were synthesized with a Dde protecting group on the ε-amino group of the internal lysine. These peptides were deprotected before use in any cellular assays.

Peptide deprotection

10 – 20 mg of protected peptide was dissolved in 5 mL of 10% hydrazine in DMF, mixed for 30 min, diluted in another 5 mL of 10% hydrazine and mixed for another 30 min to remove the Fmoc and Dde protecting groups. The reaction was then dried *in vacuo*, dissolved in 1 mL PBS, purified by HPLC, and lyophilized. Deprotection was confirmed by mass spectrometry.

Conjugation of peptides and fluorescent probes to polymers

pNAS was used as described previously[24] (Figure 4). Two different conjugation schemes were explored in this project: the C-terminal lysine of CFTR-K or the N-terminus of H_2N-Ahx-CFTR was coupled to a pA or pAA backbone. 30 mg pNAS, the appropriate mole fraction of protected peptide, and 2% mole fraction of fluorescein cadaverine (if applicable) were dissolved in DMF for a final volume of 1 mL. 30 µL diisopropyl ethylamine was added as a base catalyst, and the reaction was stirred at room temperature overnight. The unreacted NHS ester groups were quenched with 1 mL concentrated NH_4OH (to form a pA backbone) or 1 mL concentrated NaOH (to form a pAA backbone). The reaction was stirred at room temperature overnight, then hydrazine was

added to a final concentration of 5% to remove the protecting groups from the peptides. Using a 12,000 – 14,000 molecular weight cutoff dialysis bag, the completed reaction was dialyzed twice in H_2O for at least 4 hours each, dialyzed in 0.5M NH_4Cl for at least 4 hrs, then dialyzed three times in H_2O for at least 4 hours each. The dialyzed solution was then lyophilized and stored in solution with cell media containing 10% fetal calf serum.

When referring to these polymers, the attached groups and their mole fraction of substitution will be placed in parenthesis after the type of polymer backbone. For example, pA (0.5% CFTR-K) is a polyacrylamide with CFTR-K attached to 0.5% of acrylamide subunits.

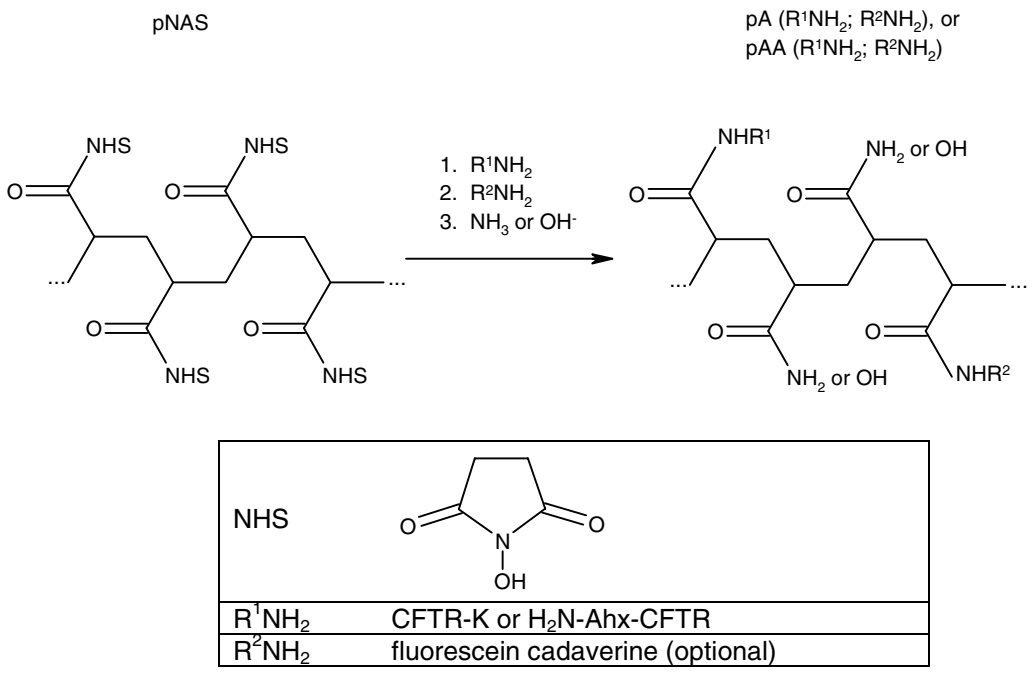


Figure 4*. Synthesizing a polyvalent ligand by attaching amines to pNAS. Several different amines can be simultaneously attached to the same polymer using this technique. Previous studies[39] showed that polyvalent ligands synthesized using pNAS are significantly more effective than ligands synthesized by copolymerization. One likely explanation is that differing rates of monomer addition create a non-uniform distribution of ligands on a polymer prepared by co-polymerization.*

In vitro infection inhibition assay

Bacteria that adhere to CFTR on epithelial cells are internalized, and we used an *in vitro* assay to test the efficacy of our polymers in preventing this internalization[29] (Figure 5). 200 µL of a 2x solution of the compound to be tested was mixed with 200 µL of the PAO1 strain of *P. aeruginosa* and incubated for 30 min at 37°C. 100 µL of this bacteria suspension was aliquoted into three wells containing confluent cells expressing CFTR (transfected murine C127 epithelial cells, grown in RPMI media; transformed human CFT1-LCFSN epithelial cells, grown in F-12 media; T84 cells, grown in RPMI media; or corneal epithelial cells, grown in F-12 media) that had been washed three times with the appropriate media. Each well contained approximately 10^5 cells and 10^6 bacteria. The bacteria were incubated with the cells for 3 hours, then the cells were washed three times with media, incubated for 1 hour with gentamycin to kill extracellular but not intracellular bacteria, washed three times with media, then lysed with 100 µL 0.05% Triton X-100 for 15 minutes. The cell lysates, which contain internalized bacteria, were plated and counted. Each polymer concentration was tested in triplicate.

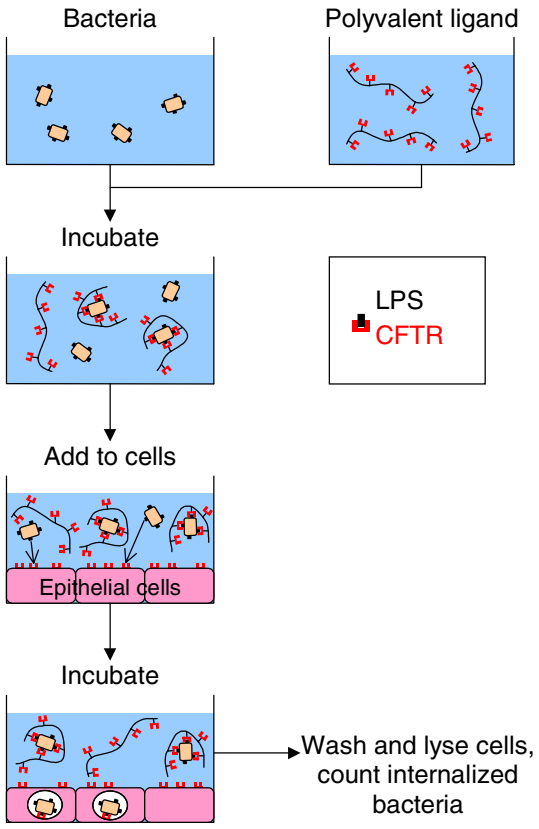


Figure 5. In vitro *bacterial infection inhibition assay. Figure not drawn to scale.*

All of the cellular incubations were

carried out at 37°C under a 5% CO_2 atmosphere.

Cytotoxicity assay

To test their cellular toxicity and their effect on bacterial growth, the polymers were separately incubated with bacteria and cells for 3 hours. Bacterial growth was measured by plating and counting colonies before and after the incubation, and cytotoxicity was measured with 2 minutes of Trypan blue staining of non-viable cells.

Measurement of fluorescent polymer binding to bacteria

500 µL of a 2x solution of the compound to be tested (CFTR-K-FITC, or a polymer with 2% fluorescein cadaverine covalently attached) was mixed with 500 µL of the PAO1 strain of *P. aeruginosa* (at 10^7 bacteria / mL for fluorescence microscopy and 10^9 bacteria / mL for fluorimeter measurements) and incubated for between 15 minutes and 4 hours at 37°C. Unbound fluorophore was removed by washing the bacteria twice in F-12 media containing 10% fetal calf serum.

For fluorescence microscopy, the final pellet was resuspended in 25 µL H_2O. 10 µL of this was heat fixed onto a microscope slide and examined under a Nikon fluorescence microscope using a 1000x oil immersion objective.

To quantitate the amount of fluorescence, the pellet was resuspended in 1 mL F-12 media containing 10% fetal calf serum, and the resulting sample analyzed on a fluorimeter.

RESULTS AND DISCUSSION

We synthesized and tested various polyvalent inhibitors of *P. aeruginosa* binding to epithelial cells. In an attempt to optimize the inhibition, we varied parameters such as the type of polymer, the amount of CFTR peptide linked to the polymer, and the method of linkage. All reagents and polymers were tested for cytotoxicity, and less than 2% cell death was found for three hour cellular incubations at the highest concentrations tested in the *in vitro* infection inhibition assay.

The effects of varying each of the polymer parameters is discussed below:

Polymer backbone

On average, over a wide range of concentrations tested, approximately 50% fewer bacteria were internalized when they were preincubated with polyacrylic acid versus polyacrylamide. One reasonable explanation for this is that the negative charges on polyacrylic acid interact favorably with positively charged components of the bacterial membrane, resulting in non-specific binding. Previous experiments with polyvalent inhibitors of influenza[24] also showed non-specific effects for polyacrylic acid.

Non-specific inhibition of bacterial internalization will complicate the analysis of polyvalent ligands made with polyacrylic acid, so we focused our experiments on polyacrylamide.

Peptide coupling method

The CFTR peptide exhibited comparable levels of inhibition when it was synthesized with a tri-alanine repeat on either the N- or C-terminus (data not shown). In addition, we linked the CFTR peptide to polyacrylamide via either an added C-terminal lysine

(CFTR-K) or an N-terminal six-carbon spacer (H_2N-Ahx-CFTR). Over the range of conditions tested (0.25% – 5% mole fraction substitution), both linkages produced similar results. These results indicate that blocking the CFTR peptide on either end does not interfere with bacterial recognition.

Mole fraction substitution

Figure 6 shows the activity of several different polyvalent ligands in the *in vitro* bacterial internalization inhibition assay. The IC_{50} values for these curves are shown in Figure 7. Polyacrylamide displaying CFTR-K at 0.25% to 1% of acrylamide sites ex-

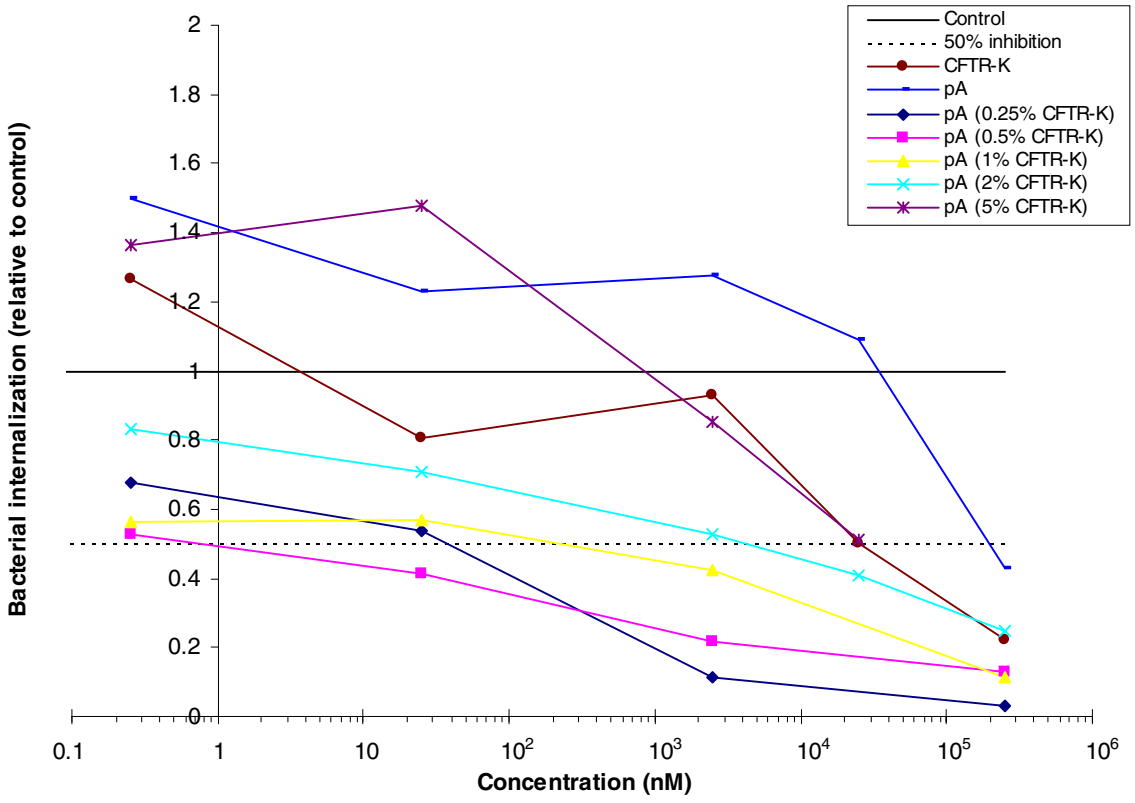


Figure 6. *Inhibition of bacterial internalization. The CFTR-K concentration is reported for pA displaying CFTR-K, and the acrylamide subunit concentration is reported for pA. Each data point is the average of at least three replicate measurements. The control had around 3×10^4 bacteria internalized per well, meaning that 3% of the added bacteria were internalized and each cell internalized an average of 0.3 bacteria.*

hibit 50% inhibition of bacterial internalization at concentrations where there is no detectable inhibition by pA and little (<20%) inhibition by CFTR-K. Our interpretation is that these polymers display the CFTR peptide in a manner that polyvalently enhances their affinity for bacteria, and the polymer then directly binds to or sterically blocks bacterial sites important for internalization.

The most effective polymer, pA (0.5% CFTR-K), yields 50% inhibition at a concentration of 600 pM, 40,000 lower than CFTR-K and 1,400 times lower than the pA backbone.

Using an estimate of 2×10^6 molecules of LPS per bacterium,[40] our experiments were conducted at an LPS concentration of 30 nM. The CFTR-K monomer requires a

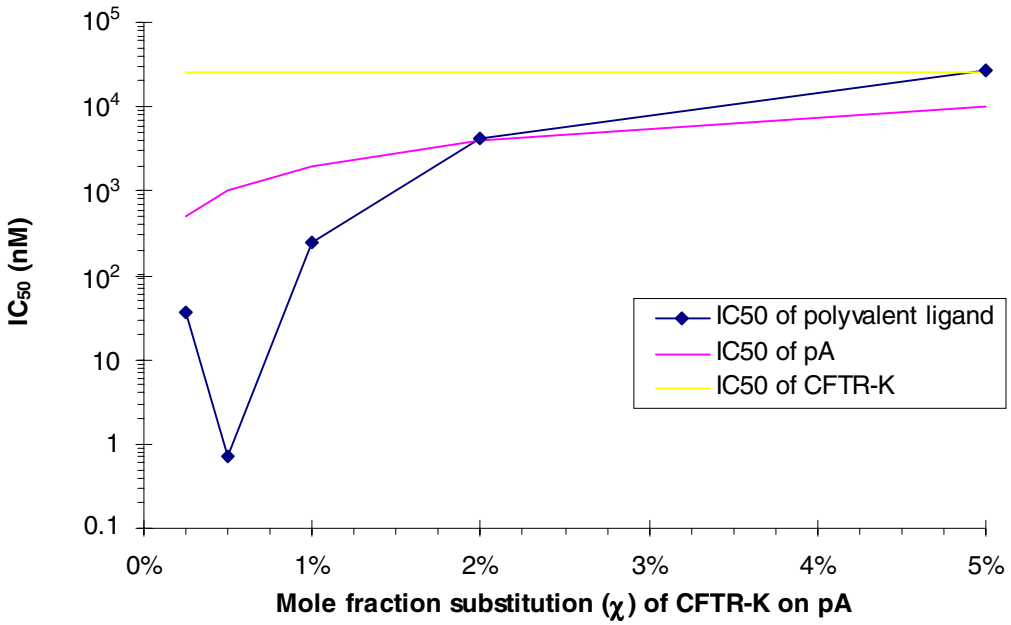


Figure 7. *pA (0.25% CFTR-K), pA (0.5% CFTR-K), and pA (1% CFTR-K) inhibit bacterial internalization at a lower concentration than the peptide or polymer backbone alone. IC_{50} values were interpolated from inhibition data shown in Figure 6, assuming a logarithmic dose-response curve between data points. The IC_{50} value for pA is 2×10^5 nM in terms of acrylamide concentration, or $(2 \times 10^5) \times \chi$ nM in terms of CFTR concentration on a polymer displaying a mole fraction substitution of χ CFTR. The latter value is plotted on the graph for direct comparison to IC_{50} values for pA displaying CFTR.*

1000-fold excess over this concentration to inhibit bacterial internalization by 50%, while the pA (0.5% CFTR-K) inhibits at 1/40th the LPS concentration.

At 5% CFTR-K occupancy on the polymer, inhibition is worse than would be expected from non-specific inhibition of the acrylamide backbone alone. At this high level of occupancy, the distance between peptide attachment sites on the polymer is comparable to the length of the peptide. Thus, it is likely that steric crowding decreases the effectiveness of this polymer. Even if the polymer does bind to the bacteria, it will stay close to the surface of the cell, decreasing the steric stabilization that results from loops of polymer protruding from the surface. Polyacrylamide displaying 20%, 40%, or 60% CFTR peptide does not inhibit bacteria any better than the polyacrylamide displaying 5% CFTR.

On the other hand, if the mole fraction of substituted sites is too low, the CFTR peptides will be tethered via a longer stretch of polymer, so they can move more independently of each other. As a result, the polyvalent effect will not be as pronounced.

Thus, the efficacy of polyvalent ligands decreases at both high and low mole fractions of substitution, suggesting that the polymer design must be finely tuned to produce good binding. In this system, 0.5% mole fraction of substitution produces optimal results, and the efficacy rapidly drops when the mole fraction is changed.

Polymer binding to bacteria

We attempted to directly measure binding of our polyvalent ligands to *P. aeruginosa* by attaching a 2% mole fraction of fluorescein to these ligands. The absorbance of the CFTR peptide is greatest at 300 nm, and this absorbance can be used to measure

[CFTR] > 100 μM. Fluorescence measurements, on the other hand, can detect fluoresceinated CFTR at concentrations as low as 1 nM.

We tested various CFTR concentrations up to 1.25 mM of CFTR-K-FITC, 12.5 μM pA (1% CFTR-K; 2% fluorescein), or 6.25 μM pA (0.5% CFTR-K; 2% fluorescein). These samples exhibit biological activity at the concentrations tested, but we detected very little binding to the bacteria (<1 nM), even after 4 hours of incubation. Because this was at the lower limit of the fluorimeter resolution, we were not able to characterize binding curves or kinetics any further. Furthermore, we have not reproducibly seen bacterial-associated fluorescence under a microscope.

These results are difficult to interpret, and additional studies are needed to quantify binding of our polyvalent ligands to bacteria.

CONCLUSIONS AND FUTURE WORK

We have shown that a polymer displaying multiple copies of a peptide from the CFTR can prevent *P. aeruginosa* internalization by epithelial cells. These polymers might be relevant for controlling *P. aeruginosa* corneal infections, although it is not clear whether they would have any effect after an infection has already been established. A murine model of *P. aeruginosa* corneal infections has been developed[44] and could be used to answer these questions. Along similar lines, the CFTR polymers might be useful in contact lens disinfectant solution, where it could potentially help dislodge *P. aeruginosa*.

CFTR is also important for the pathogenesis of other bacteria: *Salmonella typhi* attachment to CFTR allows it to pass through the intestinal wall and into the blood-

stream.[30] This uptake is inhibited by the same peptide that blocks *P. aeruginosa* uptake, suggesting that this type of polymer may have some clinical relevance in the control of typhoid.

Even without any clinical applications, the polymers designed in this experiment may be important as an experimental demonstration of polyvalency and as a test of the biological effects of blocking particular bacterial receptors.

Exploring further variations on these polymers, including linking antibiotics or other bacterial ligands to the polymers, should produce more efficient inhibitors.

Polyvalent inhibitors that also carry antibiotics or immunogens

Attaching other side chains to the polymer could target drugs to the bacteria, or label the bacteria for destruction by the immune system. The antibiotic protamine, which disrupts transport across the bacterial cell membrane,[2] could directly kill the bacteria, while a highly antigenic side chain could provoke an immune response to clear the infection. Adding these extra functional groups may be crucial for the prevention and control of a *P. aeruginosa* infection, especially in the lung where inhibition of CFTR-mediated binding would worsen the infection.

Polyvalent GalNAc-(β1-4)-Gal

A polymer displaying multiple copies of an asialo-GM_1 saccharide might help control *P. aeruginosa* lung infections. Asialo-GM_1 binds to the tips of the bacterial pili, which can extend far from the bacterial surface and thus may play a greater role in initial binding events than molecules such as LPS that are localized to the cell membrane.

Co-polymers displaying both CFTR peptide and GalNAc-(β1-4)-Gal would also be an interesting test case. For these copolymers, the ratio of CFTR peptide to GalNAc-(β1-4)-Gal might be varied to optimize inhibition.

Such a co-polymer would disable binding sites on both the pili and the bacterial surface. It might further cripple the bacteria by sterically blocking a whole host of possibly unknown bacterial ligands, especially if the polymer is engineered to have a few bulky side chains. Such a multi-pronged approach may be particularly applicable to neutralizing bacteria (as opposed to viruses or toxins), since bacteria can typically use several different types of receptor expressed at variable levels.[23]

Other polymers

Several factors are desirable in any polymeric ligand used to neutralize infections, including lack of cytotoxicity and intrinsic bacterial toxicity, biocompatibility, and ease of delivery to the infected tissue. To address these concerns, we have begun preliminary studies using other polymers, including poly-L-glutamic acid (pLGA) and poly(butadiene-maleic anhydride) (pBMA). pLGA is non-toxic and was successfully used to create a new picomolar inhibitor of influenza,[18] and pBMA is noted for its lack of toxicity when injected into rats.[11] In an attempt to reduce non-specific effects, we have also synthesized pNAS-based polymers quenched with $(EG)_3$ amine or dimethylamine.

Several other modifications to the polymer backbone would be worth testing. Increasing the degree of polymerization should enhance inhibition via both steric stabilization and entropically enhanced competitive binding. It might also be interesting to test the relative efficacy of a branched polymer of the same molecular weight. Adding just a

few large and flexible hydrophilic side chains might enhance steric stabilization without crowding the other side chains or making the polymer more rigid.[24]

Finally, the length of the spacer linking the ligand to the polymer might also be an important consideration. If it is too short, the ligand might not have enough rotational flexibility to bind to the bacteria, and if it is too long, there will be less entropic enhancement of binding.

Chapter 2: Theory

INTRODUCTION

Polyvalent ligands show a great deal of variation in the amount of binding enhancement over the corresponding monovalent ligand (Table 2, page 15). The theoretical studies in this section begin to examine how a polyvalent ligand binds to a surface and the factors that affect its binding affinity. The long term goal is to predict or explain the characteristics of the optimal polymer, allowing for more rational design of polyvalent inhibitors.

A wide range of mathematical models have been used to examine multivalent interactions, such as the binding of bivalent antibodies to a surface,[12, 26] multivalent antigen to a cell,[4] and multivalent antigen to one and two dimensional lattices.[13]

For our studies, we have decided to use computer simulations to determine how well various polymeric polyvalent ligands bind to a surface. These computer simula-

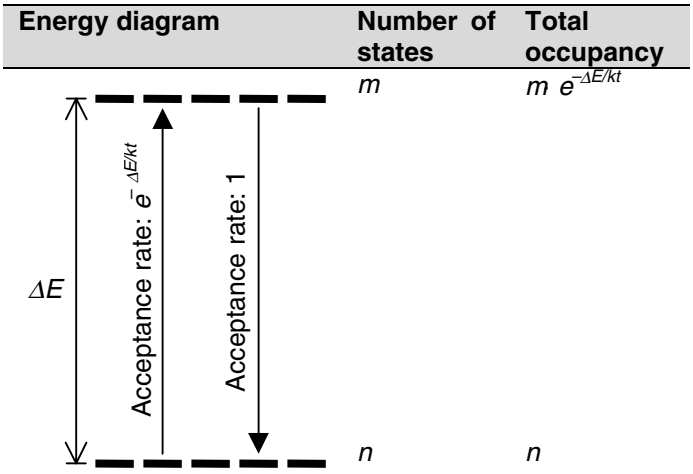


Figure 8. *Probabilities of accepting proposed moves in the Metropolis algorithm for Monte Carlo simulation. The equilibrium constant between two specific configurations with an energy difference of ΔE is $e^{-\Delta E/kt}$. The transition probabilities of the Metropolis algorithm recreate this equilibrium. Often, a system will have many different configurations at the same energy level, in which case the fraction of time the system spends at that energy is weighted by the number of configurations. The Metropolis algorithm properly accounts for this since it is more likely to propose moves to an energy level containing more configurations.*

tions incorporate details that would be more difficult to include in an analytical model, and they can provide a more detailed picture of polymer binding than would be possible in a purely mathematical treatment.

Our simulations use the Metropolis algorithm for Monte Carlo simulation,[6] which determines equilibrium constants of a system in thermal equilibrium with its surroundings. This algorithm involves randomly proposing changes in the system from a "move set," then accepting all moves that decrease the system's energy while accepting moves that increase energy with probability $e^{-\Delta E/kt}$. More entropic configurations are more likely to be proposed, while more energetically favorable configurations are more likely to be accepted. This arrangement results in the correct equilibrium between the system's various states, taking into account both energy and entropy (Figure 8).

A previous study using the Metropolis algorithm[8] demonstrated that a heteropolymer can specifically bind to a surface when the statistical distribution of binding sites on the polymer matches that on the surface.

SIMULATION DESCRIPTION

We examine binding of a heteropolymer composed of both inert subunits and a variable fraction (χ) of subunits that can bind to specific sites on a surface. These surface binding sites can be either fixed or diffusible (Figure 10). Binding between a polymer subunit and a surface site is a discrete recognition event that occurs at a specific distance. All particles have excluded volumes that almost never overlap because of the high energetic cost (Figure 9).

In all of our simulations, we use the same surface density of binding sites (0.086 / unit^2) and the same concentration of the polymer subunit that binds to the surface (6.4×10^{-5} / unit^3).

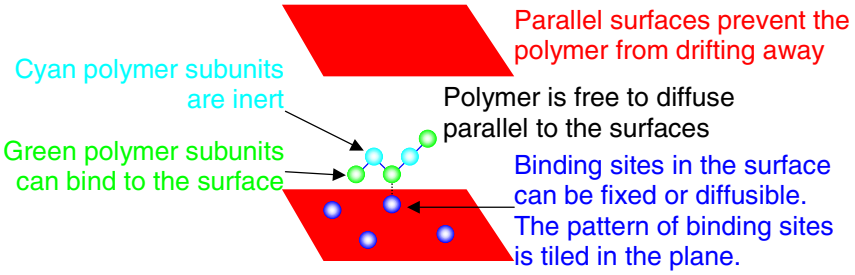


Figure 10. *The model system studied in this chapter.*

At each step of the Monte Carlo simulation, a randomly chosen particle is randomly moved in one of the ways shown in Figure 11. Monomers are diffused to a random location within a given radius of their current position. Polymers can be moved in two different ways. First, polymer subunits can be pivoted around the axis through the two adjacent subunits and the end subunits can be rotated about a random axis. Pivots are sufficient for the polymer to access all of its possible conformations. In reality, however,

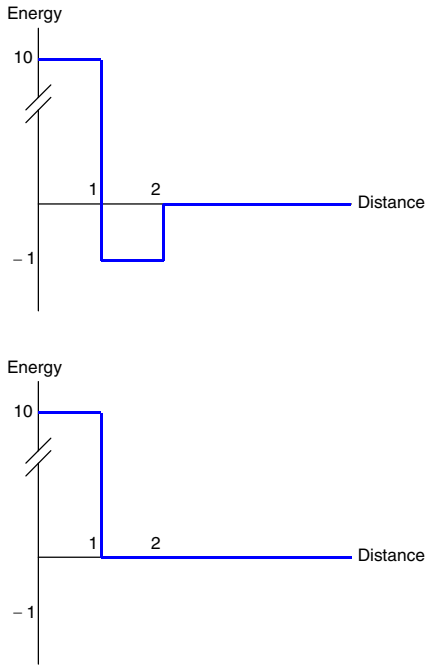


Figure 9. ***Top***: *The green polymer subunits binds to the blue surface sites with an energy equal to ambient thermal energy at a distance of twice the polymer bond length. At a distance equal to the polymer bond length, there is a significant hard-core repulsion energy.* ***Bottom***: *Other pairs of particles do not bind to each other, but they do repel each other when pushed close together.*

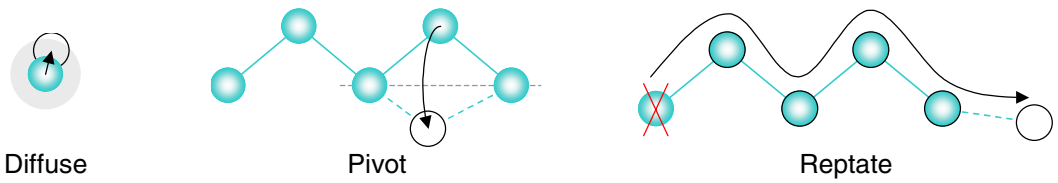


Figure 11. *The move set of the Monte Carlo simulation consists of diffusion, pivoting, and reptation.*

polymers can also exhibit a second motion called "reptation," whereby an end of the polymer moves in a random direction and the rest of the polymer follows along the path traced by the polymer, like a snake[3] (Figure 11). We have therefore made a small fraction (10%) of the proposed polymer moves reptations. This does not affect the equilibrium of the simulated system, but it may provide more realistic kinetics.

Following the Metropolis algorithm discussed above, a move is accepted if the total energy of the system decreases, and accepted with probability $e^{-\Delta E/kt}$ if the energy decreases.

In our simulations, we varied the degree of polymerization of our polyvalent polymers, the fraction of polymer sites that can bind to the surface (χ), and the diffusibility of surface sites. For each set of conditions, 10 independent restarts of 10^7 Monte Carlo steps each were run, and the simulation collected detailed kinetic and thermodynamic data.

The simulation code was written in C++ and run on a variety of Windows and Unix systems. The simulation code is optimized for maximum speed. For example, it records a table of all pairwise interaction energies so it only has to recalculate the energy of parts of the system that have changed. The simulation also records tables of which particles are near other particles, further cutting down the number of pairwise interactions that must be computed.

Executable (Windows 95 or higher) and source code are available upon request.

RESULTS AND DISCUSSION

Figure 12 shows the effects of various factors on the binding affinity of polyvalent ligands. Several important conclusions can be drawn from this graph. First, polyvalency does not always increase binding affinity. Some of the polymers with a low density of binding sites actually fare worse than the monomeric ligand. In these cases, the entropic stabilization that typically enhances the affinity of polyvalent ligands is too weak to overcome the decrease in affinity caused by steric interference with the bulky polymer backbone. Second, there is a relatively sharp transition to binding as the density of polymer binding sites increases. Third, increasing the degree of polymerization typically

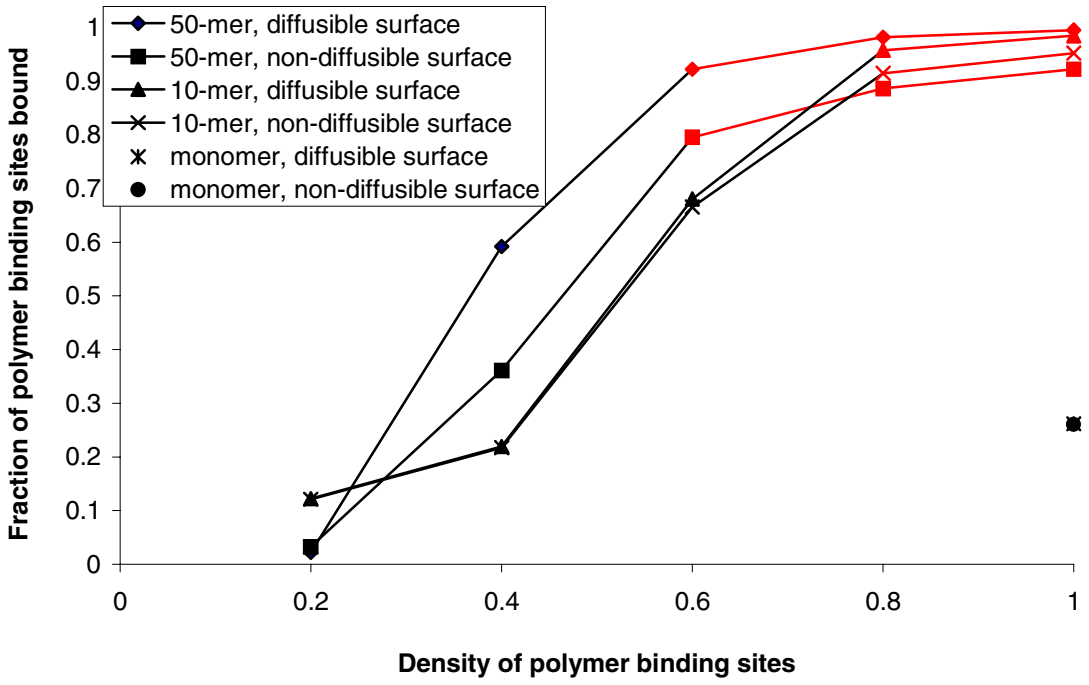


Figure 12. *Polymer binding as a function of the density of binding sites on the polymer, its degree of polymerization, and the diffusibility of binding sites on the surface. The red points indicate that polymer binding was irreversible (i.e. the polymer never completely dissociated) over the time scale of the simulation. Data points for the monomer binding to a diffusible versus non-diffusible surface overlap. The density of surface binding sites and the concentration of polymer binding sites was held constant in all of these simulations.*

increases the binding affinity, although there are deviations from this trend at both low and high densities of polymer binding sites. Fourth, polyvalent ligands can sometimes bind better to surfaces when the surface binding sites are free to diffuse. This is due to the extra time needed to "search" for a fixed configuration of non-diffusible surface binding sites that matches the fixed pattern of binding sites on the polymer. Also, diffusible surface binding sites can cluster at the site of polymer attachment and form additional favorable contacts (see cover picture).

Figure 13 and Figure 14 show snapshots from simulations of a 50-mer with two different densities of binding sites. In particular, they show that polymer binding is a co-operative process. The polymer is usually either completely unbound from the surface, or bound through multiple contacts. Intermediate states are unstable and short-lived.

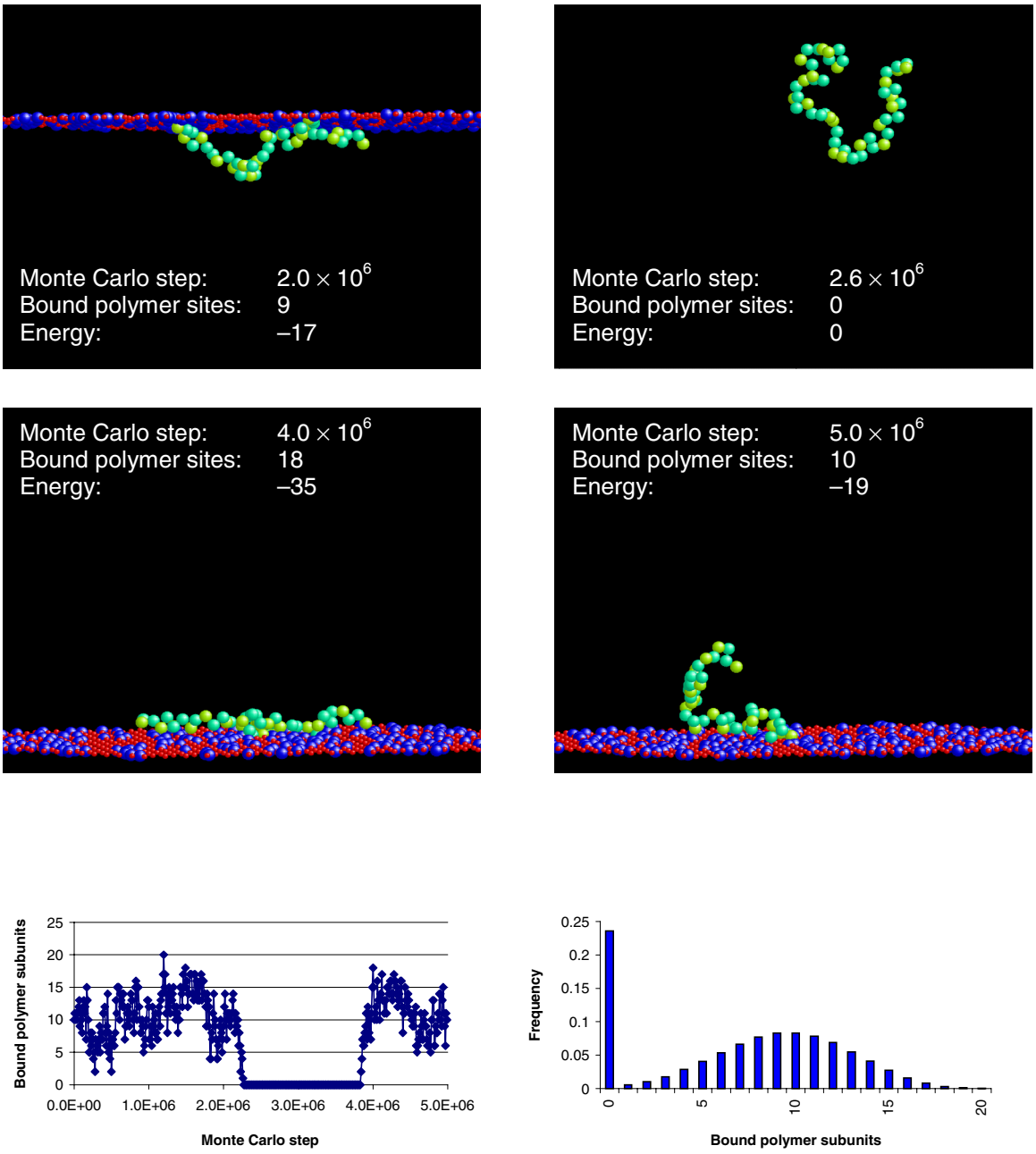


Figure 13. *A 50-mer with 20 binding sites binds and unbinds from a surface with non-diffusible binding sites. One in 10^4 Monte Carlo steps are plotted in the graph of bound polymer subunits as a function of Monte Carlo step. The histogram of bound polymer subunits includes data from all 10 independent restarts of the simulation.*

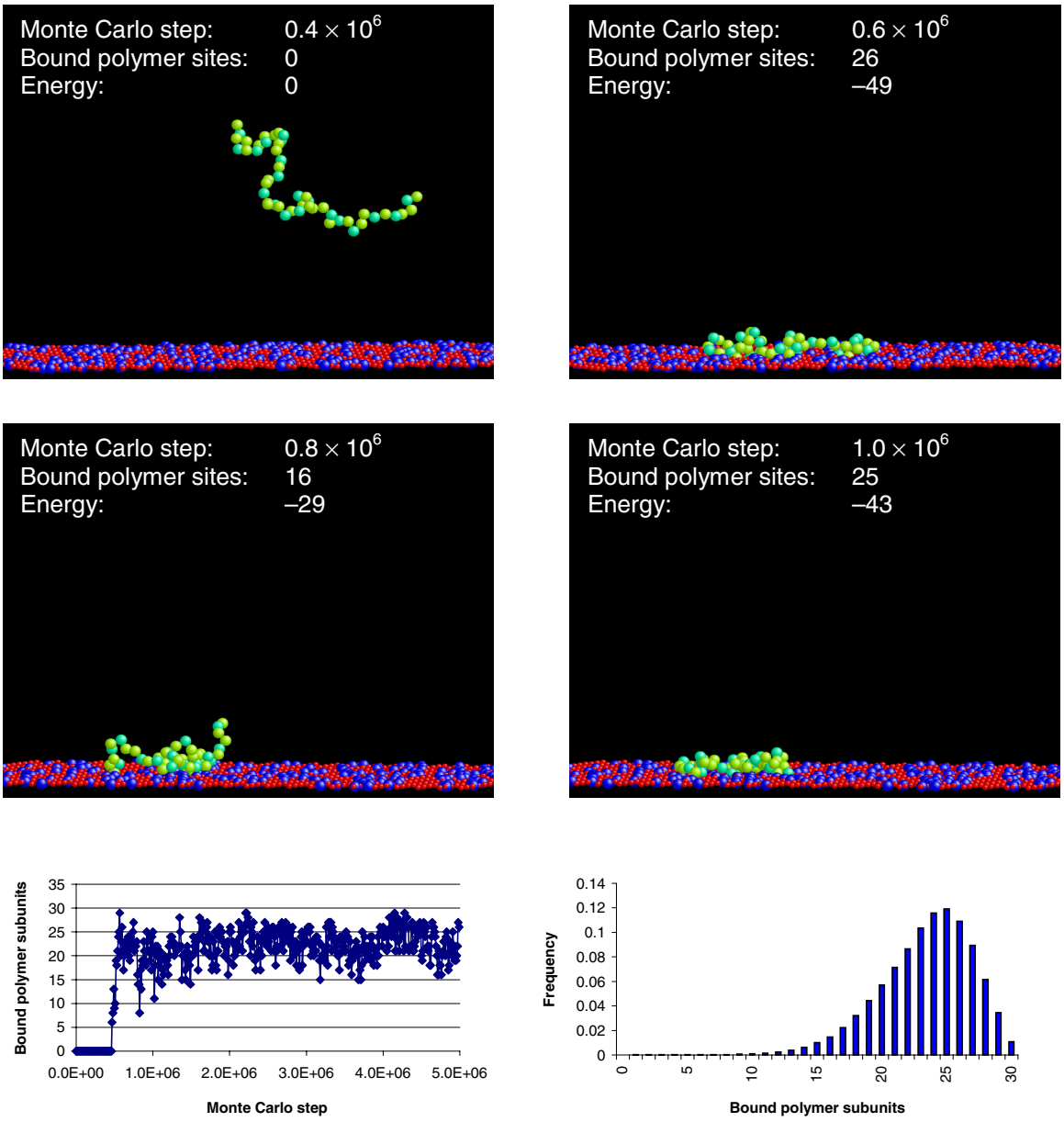


Figure 14. *A 50-mer with 30 binding sites binds tightly to a surface with non-diffusible binding sites. The histogram of bound polymer subunits was collected while the system was in equilibrium (i.e. it excludes the unbound polymer at the beginning of the simulation). See the caption for Figure 13 for additional information on the bottom two graphs.*

CONCLUSIONS AND FUTURE WORK

The simulations described in this chapter provide a controlled system for examining the fundamental concepts underlying polyvalency, and they allow us to examine polymer binding (albeit in an artificial system) in much greater detail than would be possible experimentally.

Future simulations could incorporate more realistic polymers, including charge, finite rigidity, and a more sophisticated interaction potential. In addition, a more rigorous treatment of the move set, or use of a molecular dynamics simulation, will be needed to obtain accurate kinetic data.

If we matched the parameters of these simulations to an experimental system, the binding data shown in Figure 12 could be a first step towards the rational design of polyvalent inhibitors. The amount of binding sets a lower limit on the optimal density of ligand that should be presented on the polyvalent inhibitor. Furthermore, as the density of ligand increases, the polymer becomes more collapsed onto the surface (compare Figure 13 and Figure 14). If steric stabilization is important, this consideration could set an upper limit on the optimal density of ligand. Steric interference between neighboring ligands would also set an upper limit on the optimal density of ligand.

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