Available online at www.sciencedirect.com
SCIENCE @ DIRECT®
ELSEVIER
Biomaterials 27 (2006) 3663–3674
Biomaterials
www.elsevier.com/locate/biomaterials

Promotion of opsonization by antibodies and phagocytosis of Gram-positive bacteria by a bifunctional polyacrylamide

Vijay M. Krishnamurthy[a], Lee J. Quinton[b], Lara A. Estroff[a,1], Steven J. Metallo[a,2], Jessica M. Isaacs[a], Joseph P. Mizgerd[b], George M. Whitesides[a,*]

[a]*Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA*
[b]*Physiology Program, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA*

Received 12 January 2006; accepted 8 February 2006
Available online 9 March 2006

Abstract

This paper describes the application of a bifunctional polyacrylamide (**pA–V–F**) presenting both vancomycin and fluorescein groups, to modify the surfaces of multiple species of Gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae,* and *Enterococcus faecalis*) to control molecular recognition of these surfaces. The vancomycin groups allowed the specific recognition of a structural component of the bacterial cell wall: peptides terminated in D–Ala–D–Ala. The fluorescein groups allowed the imaging of binding of polymer to the surfaces of bacteria by fluorescence, and are representative, low molecular weight haptens; their recognition by anti-fluorescein antibodies provides proof-of-principle that bifunctional polymers can be used to *introduce* haptens onto the surface of the bacteria. Flow cytometry revealed that polymer-labeled *S. aureus* and *S. pneumoniae* were opsonized by anti-fluorescein antibodies ~20-fold more than were untreated bacteria; nearly all (~92%) polymer-labeled *S. aureus*, and a large (76%) fraction of polymer-labeled *S. pneumoniae* were opsonized. The bound antibodies then promoted phagocytosis of the bacteria by cultured J774 macrophage-like cells. Flow cytometry revealed that macrophages ingested *S. aureus* decorated with the polymer-antibody complexes ~2-fold more efficiently than *S. aureus* in control groups, in spite of the high background (caused by efficient antibody-independent ingestion of *S. aureus* by macrophages). This paper, thus, demonstrates the ability of a bifunctional polymer to carry out three distinct functions based on polyvalent molecular recognition: (i) recognition of the surface of Gram-positive bacteria, (ii) modification of this surface to generate specific binding sites recognized by an antibody, and (iii) promotion of phagocytosis of the opsonized bacteria.
© 2006 Elsevier Ltd. All rights reserved.

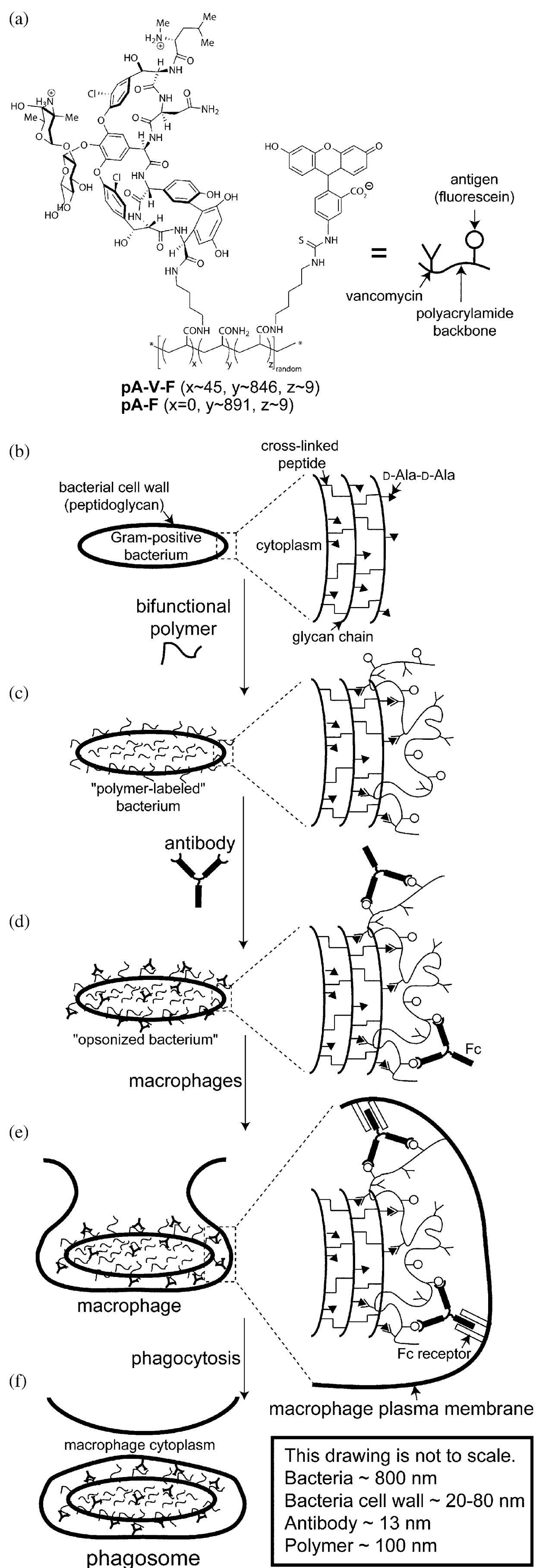
Keywords: Antibacterial agent; Bifunctional polyacrylamide; Specific immunostimulation; Antibody; Macrophage; Flow cytometry

1. Introduction

Gram-positive bacteria are important to public health, and many can cause bacterial infections, which remain an important cause of morbidity and mortality. Immunocompromised patients are particularly susceptible to such infections [1]. The effective design of vaccines against these infections has proven challenging because bacterial surfaces are often poorly antigenic [2–5]. Further, the rise of drug-resistant strains of bacteria has highlighted the need for antibacterial agents with new mechanisms of action [6]. The approach described in this paper begins to address this need by demonstrating the effective conversion of the surfaces of several types of Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*) into surfaces presenting controllable recognition elements by treatment with a bifunctional polymer, **pA–V–F** (a polyacrylamide presenting both vancomycin and fluorescein groups as side chains) (Fig. 1). The polymer-labeled bacteria effectively couple with components of the immune system (antibodies and macrophages) for destruction. We believe that this approach will provide the basis for new

*Corresponding author. Tel.: +1 617 495 9430; fax: +1 617 495 9857.
E-mail address: gwhitesides@gmwgroup.harvard.edu (G.M. Whitesides).
[1]Current address. Department of Materials Science and Engineering, Cornell University, 214 Bard Hall, Ithaca, NY 14853, USA.
[2]Current address. Department of Chemistry, Georgetown University, 37th and O Streets NW, Washington, DC 20057, USA.

0142-9612/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2006.02.006



methods of promoting interaction between components of the immune system and surfaces (e.g., bacteria, viruses, cancer cells) not normally recognized by the immune system.

Several investigators have employed strategies, related to the one we have used here, to attach monomeric, bifunctional molecules noncovalently to the surface of pathogens or to surface-immobilized purified proteins that occur on the surfaces of pathogens, and to recruit components of the immune system to the bound species [7–10]. As an example, this bifunctional, antibody-targeting approach has been applied to the Gram-negative bacterium *Escherichia coli*, exploiting the binding of mannose by FimH, the adhesion protein on the tips of type 1 pili on the bacterial surface, using small molecule conjugates [9,10] or polymers [11]. Bertozzi and Bednarski [9,10] synthesized a mannose-biotin conjugate to which they bound avidin and an anti-avidin antibody to generate a complex that displayed both mannose and antibody molecules. They then demonstrated binding of the mannose–avidin–IgG complex to the surface of *E. coli* and subsequent recognition of the IgG portion of the complex to activate complement and enable recognition and killing of the bacteria by macrophages [10]. In a conceptually related study, Li et al. [11] used a polymer that presented both mannoside units (to bind to the bacterial surface) and α-Gal units (with the potential to recruit innate anti-α-Gal antibodies to the polymer adsorbed on bacteria). The investigators, however, did not demonstrate such targeting of antibodies to a bacterial surface.

We have used a related but distinct approach that, unlike the previous approaches [7–11], is not directed towards a specific protein on the surface of a particular pathogen, but rather, targets ligands that are broadly distributed in many organisms. The ligands that we have targeted are D-Ala–D-Ala residues at the carboxy termini of some of the peptides

Fig. 1. The opsonization by antibodies and phagocytosis of a Gram-positive bacterium, directed by a bifunctional polymer (**pA–V–F**) that binds to the cell wall via vancomycin and displays fluorescein as an antigen. (a) The chemical structures of **pA–V–F** and a control polymer (**pA–F**) lacking vancomycin groups. The subscripts *x*, *y*, and *z* denote the average number of side-chains of vancomycin, unsubstituted amide, and fluorescein, respectively, per molecule of polymer. The ordering of these side-chains on both polymers is presumably random due to the method of synthesis of the polymers. (b) The cell wall of a Gram-positive bacterium is composed predominantly of peptidoglycan consisting of repeating disaccharide units with a peptide moiety (terminating in D-Ala–D-Ala; represented as filled triangles) acting as cross-linking agent. (c) The bifunctional polymer binds to the bacterial surface mediated by the vancomycin side chains (represented as "Y") interacting with D-Ala–D-Ala residues of the peptidoglycan. (d) The bacterial surface is subsequently recognized by anti-fluorescein IgG (from mouse) specific for the second group (fluorescein, represented as open circles) that the polymer presents. (e) Macrophages (cultured J774 cells from mouse) bind to the opsonized bacteria mediated by interactions with the Fc region of the bound IgG's, and (f) internalize the bacteria-polymer-antibody aggregate into a phagosome.

in the peptidoglycan cell wall of many bacteria (Fig. 1(b)) [12]. These residues provide recognition sites, dispersed throughout the cell wall, for the antibiotic vancomycin, which binds to them with good affinity (K_d~μM) [13,14]. Vancomycin serves as an antibiotic by inhibiting cross-linking of the D-Ala–D-Ala-terminated peptides during formation and remodeling of the bacterial cell wall by binding to these peptides at the membrane surface, and thus inhibiting the action of transpeptidases [15]. Vancomycin-resistant Gram-positive bacteria evade the cytotoxic properties of vancomycin by expressing three essential enzymes in response to the binding of vancomycin to VanS, a transmembrane sensor protein kinase [6,16]. The net effect of the action of these enzymes is to replace D-Ala–D-Ala in the bacterial cell wall with D-Ala–D-Lac; vancomycin binds to D-Ala–D-Lac with 10^3-fold lower affinity (K_d~mM) than to D-Ala-D-Ala, attenuating the potency of the antibiotic [16]. We included vancomycin groups in the bifunctional polymer, **pA–V–F**, to promote specific binding of the polymer to the surfaces of Gram-positive bacteria (Fig. 1(c)).

The approach that we present here exploits polyvalency, which can generate a high-avidity binder for a given surface from low-affinity recognition units even if not all of the recognition units are involved directly in binding to their cognate surface receptors [17–25]. We previously characterized the polyvalent binding of **pA–V–F** to a self-assembled monolayer (SAM) that presented D-Ala–D-Ala groups using surface plasmon resonance spectroscopy (SPR) [26]. These studies demonstrated that **pA–V–F** formed kinetically stable (k_{off}~$2 \times 10^{-6}\,s^{-1}$) complexes with high avidity at the surface presenting D-Ala–D-Ala groups. These studies also indicated that a thin (6.5 nm) layer of **pA–V–F** bound to the surface recruited anti-fluorescein antibodies to that surface almost as well as did a surface directly presenting fluorescein at a high surface density.

Our strategy in the current work was to use polyvalency and the molecular recognition of D-Ala–D-Ala by vancomycin (as opposed to the antibiotic properties of this molecule) to bind polymer with pendant vancomycin groups to the surfaces of Gram-positive bacteria; this strategy differs from that of a previous report on the use of monofunctional polymers of vancomycin as potential antibiotics [27]. The effective grafting of the polymer to the bacterial surface gives us the ability to modify the molecules displayed at the surface of the bacteria by incorporating additional groups (i.e., in addition to vancomycin) of arbitrary structure into the polymer.

As a model system, we elected to use fluorescein as a second group: this polymer (**pA–V–F**) allowed bacteria that had their surface modified by adsorption of polymer to be visualized by fluorescence, and also promoted subsequent binding of anti-fluorescein antibodies to the modified bacteria (Figs. 1(c) and (d)). These opsonized bacteria were then recognized and ingested by macrophages (Figs. 1(e) and (f)). To our knowledge, this paper is the first to report the coupling of antibodies and macrophages to the surfaces of pathogens using a bifunctional polymer.

2. Materials and methods

2.1. Materials

Unless otherwise specified, reagents were obtained from Sigma-Aldrich (St. Louis, MO) of the highest quality available and used as received. Fluorescein cadaverine, Syto-63, slow anti-fade reagent, rabbit *R*-phycoerythrin-conjugated anti-fluorescein IgG (polyclonal), and goat *R*-phycoerythrin-conjugated anti-mouse IgG (polyclonal) were obtained from Molecular Probes (Eugene, OR). Mouse anti-fluorescein IgG (clone 1.BB.75) was obtained from US Biologicals (Swampscott, MA). Mouse IgG2a isotype control (clone G155-178) was obtained from BD Biosciences Pharmingen (Chicago, IL). Dialysis cassettes (10,000 MWCO, 3–15 mL) were obtained from Pierce (Rockford, IL). Diffi-Quik stain set was obtained from Dade Behring (Marburg, Germany). *E. coli* (ATCC 53506), *S. epidermidis* (ATCC 35984), *E. faecalis* (ATCC 49332), *E. faecalis* VanB (ATCC 51299), and J774A.1 cells (ATCC TIB-67) were purchased from the American Type Culture Center (ATCC). *S. aureus* strain JL-243 was kindly provided by Dr. Jean Lee of Harvard Medical School. Confocal fluorescence microscopy was performed on a Leica confocal imaging system equipped with an Ar–Kr laser and using a 63× (N.A. = 1.4) or 100× (N.A. = 1.4) oil immersion objective. Flow cytometry was performed on a Coulter® Epics® Elite ESP flow cytometer.

2.2. Polymers

The bifunctional polyacrylamide, **pA–V–F**, and the control polyacrylamide containing only fluorescein groups, **pA–F,** were synthesized as previously reported [26]. Briefly, fluorescein cadaverine was added to a solution of poly(N-acryloyloxysuccinimide) (PNAS) [18] in DMF and allowed to react. A vancomycin derivative (vancomycin conjugated to 1,4-diaminobutane [28,29]) was then added, and finally the reaction was quenched with ammonium hydroxide (30%), which reacted with remaining NHS esters to yield amides [18]. **pA–F** was synthesized as above for **pA–V–F** with the omission of the vancomycin derivative. The polymers were dialyzed against water and stored frozen at −20 °C, or lyophilized and stored at −20 °C. The characteristics of the polymer backbone were determined by saponifying PNAS to polyacrylic acid and analyzing this product by GPC: $M_w = 96,500$; $M_n = 65,000$; PDI = 1.48; degree of polymerization ≈900. The side-chain % substitution of fluorescein and vancomycin on the polymer was determined by ^{1}H NMR and UV-Vis spectroscopies to be 1 and 5, respectively [26]. The polymers **pA–V–F** and **pA–F** were soluble in water and phosphate-buffered saline (PBS).

2.3. Culturing of bacteria

Cultures of *E. coli*, *S. epidermidis*, and *S. aureus* were grown as liquid cultures in 5 mL of trypticase soy broth (TSB). Cultures of *E. faecalis* were grown in 5 mL of brain-heart infusion broth. Cultures of *E. faecalis* VanB were grown in 5 mL of brain-heart infusion broth supplemented with PBS, vancomycin (5 μM), or **pA–V–F** (10 μM vancomycin, 2 μM fluorescein). All cultures were grown overnight at 37 °C with shaking at 200 rpm in Falcon 2059 tubes. All of the bacteria were harvested by centrifugation, and washed by resuspension in PBS and centrifugation.

For flow cytometry experiments, *S. pneumoniae* (ATCC 6303) or *S. aureus* (ATCC 25923) were grown on tryptic soy agar plates supplemented with 5% sheep blood for 16 h at 37 °C in an atmosphere containing 5% CO_2. Following the incubation, bacterial colonies were lifted from the plates and resuspended in PBS such that the optical densities (at 600 nm) for *S. pneumoniae* and *S. aureus* were 0.3 and 1.25, respectively. Actual numbers of viable bacteria were determined by plating known serial dilutions and determining the number of colony-forming units (CFU).

2.4. Fluorescence microscopy: binding of **pA–V–F** to bacterial surfaces

After washing, the bacteria were re-suspended in PBS containing Syto-63 and **pA–V–F**. The final OD_{600} was unity for the bacteria, and the final concentration of Syto-63 was 5 μM, and **pA–V–F** was 10 μM in vancomycin, 2 μM in fluorescein. The bacteria were incubated for 30 min with the dye and the polymer, and then 50 μL of the suspension was transferred to an ELF spin filter (0.2 μm pore size) and centrifuged to remove the free polymer and dye. The bacteria were washed by being re-suspended in 200 μL of PBS followed by removal of the solution by centrifugation in the spin filter. Samples were washed three times. After the final washing, the bacteria were resuspended in a 1:1 mixture of PBS and Slowfade antifade reagent or in PBS; no difference was found between the two preparations. Samples of *S. aureus* were prepared using this protocol except that only **pA–V–F** was included in the binding reaction; Syto-63 was not included. Control samples in which bacteria were incubated with Syto-63 and **pA–F**, or Syto-63 alone were also prepared and washed following the procedure given above. Samples were prepared for microscopy by placing 2–4 μL of bacterial sample on a glass slide and covering it with a no. 1 glass cover slip. The edges of the cover slip were sealed with clear nail polish.

Treatment with **pA–V–F** lead to no observed increase in the tendency of the bacteria to aggregate and no special treatment was required to disperse the cells before observation. To provide accurate images, the voltage of the PMT used for detection was adjusted to prevent any pixels from being saturated. Even in the presence of the anti-fade reagent, the sample (especially fluorescein) remained susceptible to photobleaching.

2.5. Fluorescence microscopy: opsonization by antibodies of **pA–V–F**-labeled bacteria

S. epidermidis was grown, harvested, and washed as previously described [26]. A portion of the bacteria was exposed to Syto-63 and **pA–V–F** and washed. This sample was then divided. One portion was exposed to 130 nM (20 μg mL^{-1}) *R*-phycoerythrin conjugated anti-fluorescein IgG (IgG(anti-fluor)$^{\text{Phyco}}$) for 2 h at room temperature in PBS SuperBlock buffer. A second portion of the sample was incubated for 2 h in the same buffer but without the antibody. Another portion of the washed *S. epidermidis* sample was exposed to Syto-63 (but not **pA–V–F**) for 30 min, washed three times using an ELF spin filter, then exposed to 130 nM IgG(anti-fluor)$^{\text{Phyco}}$ for 2 h in PBS SuperBlock buffer. All three samples were then washed by centrifugation in ELF spin filters followed by resuspension in PBS. Samples were washed five times, resuspended in a 1:1 mixture of PBS and Slowfade, and then analyzed by fluorescence microscopy.

2.6. Flow cytometry to quantitate opsonization of polymer-labeled bacteria

For opsonization experiments, 200 μL *S. pneumoniae* or *S. aureus* solutions (prepared as for studies in microscopy) were centrifuged (10 min; 16,000 × *g*) and resuspended in PBS containing no polymer, **pA–F** (2 μM in fluorescein), or **pA–V–F** (10 μM in vancomycin, 2 μM in fluorescein). Following a 30 min incubation at room temperature in the dark, bacteria were washed three times with PBS followed by centrifugation. After the third wash, bacteria were resuspended in PBS containing no antibody, 67 nM (10 μg mL^{-1}) isotype control antibody (IgG(control)), or 67 nM (10 μg mL^{-1}) anti-fluorescein antibody (IgG(anti-fluor)). Samples were then incubated for 30 min at room temperature, washed twice, and re-suspended in PBS containing no antibody (control/no polymer group only), or 67 nM (10 μg mL^{-1}) *R*-phycoerythrin-conjugated goat anti-mouse IgG (IgG(anti-mouse)$^{\text{Phyco}}$). After a final 30 min incubation, bacteria were washed twice, fixed in PBS with 4% paraformaldehyde, resuspended in 1 mL PBS, and examined by flow cytometry.

2.7. Preparation of *S. aureus* and J774A.1 cells (macrophages) for in vitro phagocytosis of opsonized *S. aureus*

S. aureus (286 μL; $OD_{600} = 1.25$) treated with **pA–V–F**/IgG(anti-fluor) or **pA–V–F**/IgG(control) were re-suspended in 1.0 mL modified Hank's Balanced Salt Solution (HBSS+) containing 1.0% Bovine Serum Albumin (BSA). J774A.1 cells were cultured at 37 °C (+5% CO_2) in RPMI medium supplemented with streptomycin (50 μg mL^{-1}), penicillin (50 IU mL^{-1}), L-glutamine (2 mM), and 10% fetal bovine serum. On the day of experiments, J774 cells were centrifuged (10 min; 300 × *g*), washed twice with PBS and re-suspended at a concentration of 2×10^6 cells mL^{-1} in HBSS+ containing 1% BSA. Bacteria (250 μL) and J774A.1 cells (250 μL) were combined resulting in a bacteria CFU:cell ratio of approximately 300:1, as verified by serial dilutions and plated colony counts. J774 cells were exposed to *S. aureus* for one or two hours in a 37 °C incubator containing 5% CO_2, washed twice with ice cold HBSS+, fixed for 30 min in PBS with 4% paraformaldehyde, and re-suspended in 0.5 mL PBS. Fixed J774A.1 cells were then examined by flow cytometry.

To visualize phagocytosis via microscopy, unfixed J774 cell monolayers were prepared by cytocentrifugation, stained using the Diff-Quik® stain set, and visualized by optical microscopy. Fluorescence microscopy was performed on unstained cells.

3. Results and discussion

3.1. Synthesis and characterization of the bifunctional polymer (**pA–V–F**)

The bifunctional polymer, **pA–V–F**, consists of a polyacrylamide backbone with pendant vancomycin and fluorescein groups (Fig. 1(a)). Vancomycin is present on the polymer at side-chain % = 5 and fluorescein is present at side-chain % = 1 (as determined by ^{1}H NMR and UV-Vis spectroscopies). In this context, we mean the "side-chain %" of a group to represent the percentage of side chains in the polymer containing that group. We also synthesized a control polymer, **pA–F**, consisting of a polyacrylamide backbone with only pendant fluorescein groups (Fig. 1(a)). We have described the synthesis and characterization of these polymers elsewhere [26].

In order to bind to the exterior surface of the microorganism, these polymers did not need to diffuse through the peptidoglycan layer (a three-dimensional structure with substantial porosity and an exclusion limit of ~100 kDa) [30] to the membrane surface (where the antibiotic activity of vancomycin is manifested); they could, therefore, be large. We confirmed that **pA–V–F** does not serve as an antibiotic at the concentrations used in our experiments through two control studies: (i) *S. aureus* treated with **pA–V–F** formed a number of colonies similar to that formed by bacteria that were not treated with polymer and (ii) *E. faecalis* grew normally in brain–heart infusion with (or without) **pA–V–F**. The average degree of polymerization for the polyacrylamide backbone was ~900; this value corresponds to an average of 45 vancomycin groups and nine fluorescein groups per molecule of **pA–V–F**, a molecular weight of 136 kDa after functionalization, and an extended, end-to-end length of the polymer of approximately 100 nm.

3.2. **pA–V–F** bound specifically to the Gram-positive bacteria *S. aureus*, *S. epidermidis*, and *E. faecalis*

Previously, we demonstrated that **pA–V–F** bound to the surface of a Gram-positive bacterium, *E. faecalis*, using fluorescence microscopy [26]. Here we examined the generality of **pA–V–F** labeling using two additional Gram-positive bacteria: *S. epidermidis* (Fig. 2) and *S. aureus* (data not shown). We treated a suspension of *S. epidermidis* with a solution of **pA–V–F** (10 μM in vancomycin, 2 μM in fluorescein) and the cell-permeable dye for nucleic acids Syto-63 (5 μM), in PBS. The bacteria were then washed with PBS and examined microscopically using an Ar–Kr laser for excitation of the fluorophores. The stained nucleic acid of the *S. epidermidis* was clearly visible when we selectively excited Syto-63 (Fig. 2). For a large fraction of bacteria, we observed fluorescence from fluorescein (on **pA–V–F**) that co-localized with the fluorescence from Syto-63 (Fig. 2); this result suggests a high labeling efficiency of the bacteria by **pA–V–F**.

We also investigated binding of the polymer to *S. aureus* (data not shown). Suspensions of this bacterium were incubated in a solution of **pA–V–F**, washed, and examined using fluorescence microscopy. Syto-63 was omitted in order to eliminate any possibility of interference from strongly fluorescent complexes of Syto-63 and nucleic acid. Similar levels of fluorescence from fluorescein were observed from the **pA–V–F**-labeled *S. aureus*, *S. epidermidis*, and *E. faecalis*. A control experiment with *S. aureus* and **pA–F**, a polymer that does not have vancomycin side chains, showed no detectable fluorescence from fluorescein on the bacteria (data not shown).

Gram-negative bacteria (e.g., *E. coli*) are not susceptible to the antibiotic activity of vancomycin because their cell wall is protected by an outer membrane that is impermeable to vancomycin [15]. A control experiment using *E. coli* incubated with Syto-63 and **pA–V–F**, revealed fluorescence from Syto-63 but not from fluorescein (Fig. 2). The results from these two control experiments support the inference that binding of **pA–V–F** to the surface of bacteria requires vancomycin-mediated molecular recognition.

We examined the binding of **pA–V–F** to vancomycin-resistant *E. faecalis* VanB to determine whether the polymer could be effective against these clinically important bacteria. While **pA–V–F** bound to vehicle (PBS)-treated VanB, it did not bind as effectively to VanB that had been grown overnight in 5 μM vancomycin (a concentration sufficient to induce expression of the vancomycin-resistance genes, which replace D-Ala–D-Ala at the surface of the bacteria with D-Ala–D-Lac) [6,16,31] (Fig. 2). This result suggests that **pA–V–F** is not able to form a kinetically stable complex with the bacterial surface displaying D-Ala–D-Lac (presumably due to the low affinity of vancomycin for this sequence, $K_d \sim$mM) [16], and confirms the specificity of the vancomycin/D-Ala–D-Ala interaction in the binding of **pA–V–F** to the surfaces of Gram-positive bacteria. In a second experiment, we grew

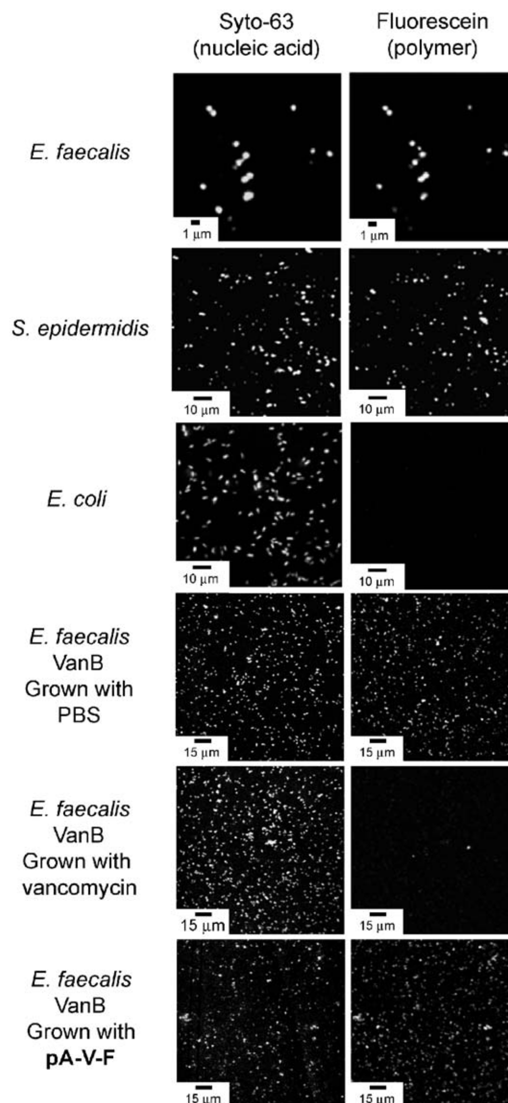


Fig. 2. Fluorescence micrographs demonstrating binding of **pA–V–F** to Gram-positive bacteria and not to Gram-negative bacteria. *E. faecalis*, *S. epidermidis*, and *E. coli* were grown overnight in media. *E. faecalis* VanB were grown overnight in media supplemented with vehicle (PBS), vancomycin (5 μM), or **pA–V–F** (10 μM vancomycin, 2 μM fluorescein). All of the bacteria were incubated with Syto-63 (a dye for nucleic acids used to visualize the bacteria) and **pA–V–F** (which displays fluorescein groups), and then rinsed with PBS to remove any material not associated with a cell. The treated bacteria were examined by confocal fluorescence microscopy using an Ar–Kr laser to excite the fluorophores. The Gram-positive bacteria, *S. epidermidis* and *E. faecalis* (vancomycin-sensitive and vancomycin-resistant VanB that had been grown with PBS or with **pA–V–F**) displayed fluorescence from fluorescein that coincided with fluorescence from Syto-63. *E. coli*, a Gram-negative bacterium, and *E. faecalis* VanB that had been grown with vancomycin only showed fluorescence from Syto-63 (i.e., fluorescein was not observed).

VanB in the presence of **pA–V–F** (at the concentration used in binding experiments) in order to determine whether the polymer would induce active expression of the vancomycin-resistance genes. Fig. 2 shows that **pA–V–F** bound to the surface of these treated bacteria (after rinsing with PBS and re-treating with **pA–V–F**); this result suggests that **pA–V–F** does not induce expression of these genes (i.e., the

bacteria were still displaying D-Ala–D-Ala on their surfaces). The observation that (monomeric) vancomycin triggers VanB to become resistant to binding both vancomycin and **pA–V–F**, while **pA–V–F** does not induce such a resistant phenotype, suggests that **pA–V–F** may have advantages over (monomeric) vancomycin for treating infections caused by VanB (and potentially, by other vancomycin-resistant bacteria) using the strategy that we describe below.

3.3. Opsonization of polymer-labeled bacteria by antibodies

After demonstrating binding of the bifunctional polymer, **pA–V–F**, to bacterial surfaces, we examined the ability of anti-fluorescein antibodies to bind to *S. epidermidis, S. pneumoniae*, and *S. aureus* that had been labeled with polymer. For the initial studies, we used fluorescence microscopy to examine qualitatively the binding of fluorescently labeled anti-fluorescein antibodies to the **pA–V–F**-labeled bacteria (Fig. 3). As a control experiment, we incubated *S. epidermidis* with Syto-63 and **pA–V–F** (as for polymer labeling studies). These bacteria displayed fluorescence from Syto-63 and fluorescein (on **pA–V–F**) but not from *R*-phycoerythrin (phyco) (Fig. 3); this result demonstrates that Syto-63 and fluorescein were not fluorescent at the wavelengths used to excite and detect phycoerythrin. *S. epidermidis* that were incubated with **pA–V–F**, Syto-63, and anti-fluorescein antibody labeled with *R*-phycoerythrin (IgG(anti-fluor)^{Phyco}) exhibited phycoerythrin (phyco) fluorescence (from the bound IgG(anti-fluor)^{Phyco}) that co-localized with fluorescence from fluorescein (and from Syto-63) (Fig. 3). Bacteria that were exposed to IgG(anti-fluor)^{Phyco} and Syto-63 but not **pA–V–F** did not display any phycoerythrin fluorescence (Fig. 3). These results demonstrate that **pA–V–F** must be bound to the bacteria to mediate binding by the antibody, and that every bacterium labeled with **pA–V–F** was recognized by *at least* one molecule of antibody.

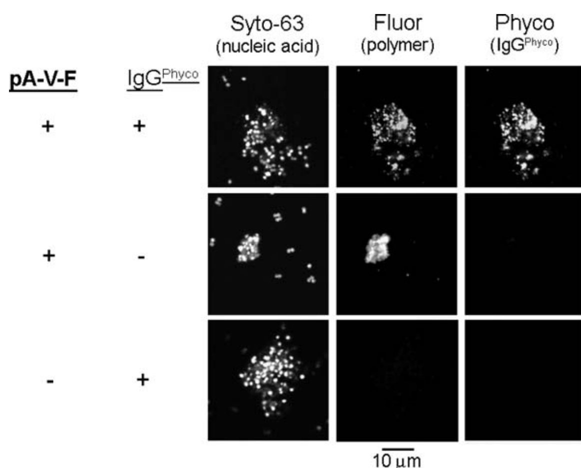


Fig. 3. Fluorescence micrographs demonstrating binding of **pA–V–F** to *S. epidermidis* and subsequent decoration with *R*-phycoerythrin-labeled anti-fluorescein IgG (IgG(anti-fluor)^{Phyco}), IgG^{Phyco}. *S. epidermidis* were incubated sequentially with Syto-63 (a dye for nucleic acids used to visualize the bacteria), **pA–V–F**, and IgG(anti-fluor)^{Phyco}, and then rinsed with PBS to remove any material not associated with a cell. IgG(anti-fluor)^{Phyco} or **pA–V–F** was omitted for control experiments. The treated bacteria were examined by fluorescence microscopy using an Ar–Kr laser to excite the fluorophores. *S. epidermidis* treated with Syto-63, **pA–V–F**, and IgG(anti-fluor)^{Phyco} displayed fluorescence from all three fluorophores that was coincident. Bacteria that were treated with Syto-63 and **pA–V–F** (i.e., no IgG(anti-fluor)^{Phyco}) displayed fluorescence from fluorescein that co-localized with that from Syto-63. The absence of fluorescence from phycoerythin demonstrated that fluorescein and Syto-63 were not visualized at the wavelengths used to stimulate and detect phycoerythrin. When *S. epidermidis* were only incubated with Syto-63 and IgG(anti-fluor)^{Phyco} (i.e., no **pA–V–F**), only fluorescence from Syto-63 was observed. No fluorescence due to phycoerythrin (or fluorescein) was observed in this case.

Flow cytometry measured the opsonization of **pA–V–F**-labeled bacteria (*S. aureus* and *S. pneumoniae*) by anti-fluorescein antibodies. This technique quantitatively and rapidly determines the fluorescence intensity of some thousands of bacteria. In addition, flow cytometry experiments do not require any dye for nucleic acids, because bacteria are identified by their ability to scatter light. We incubated the bacteria with polymer (**pA–V–F** or **pA–F**) and a primary antibody in a manner similar to that used in studies using microscopy. For flow cytometry, however, we used an unlabeled monoclonal IgG2a antibody (mouse anti-fluorescein antibody (IgG(anti-fluor))), so that we could use the same primary antibody in studies of both opsonization and phagocytosis (see Section 3.4). For control experiments, we used a mouse isotype-control IgG2a antibody directed towards an unrelated hapten (IgG(control)) to probe for non-specific interactions of the antibody (e.g., the Fc region) with the polymer-labeled bacteria. Finally, incubation with an anti-mouse secondary antibody labeled with *R*-phycoerythrin (goat IgG (anti-mouse)^{Phyco}) facilitated detection of primary antibody bound to the bacterial surface. We examined four groups of bacteria: untreated bacteria (no polymer or antibody) (**1**), **pA–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**2b**), **pA–V–F**/IgG(control)/IgG(anti-mouse)^{Phyco} (**3b**), and **pA–V–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**4b**) (Fig. 4).

We quantitated the changes in phycoerythrin fluorescence of the different groups of bacteria by determining the mean fluorescence intensity and the percentage of bacteria in each group that were more fluorescent than a threshold. The threshold was set to the highest fluorescence intensity observed for untreated bacteria (Fig. 5). Treatment of bacteria with **pA–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**2b**) controlled for any non-specific (i.e., independent of vancomycin) binding of the bifunctional polymer to the bacterial surface. This control group for both *S. aureus* and *S. pneumoniae* demonstrated a slight increase in phycoerythrin fluorescence intensity (mean fluorescence increased by ~2-fold, Table 1), and in the fraction of bacteria above the threshold (~16%, Table 1), relative to untreated bacteria (**1**). The possibility of non-specific binding of the antibodies to the bacterial surfaces was eliminated with a control experiment (see below). These increases are larger than the uncertainties in the measurements, and are

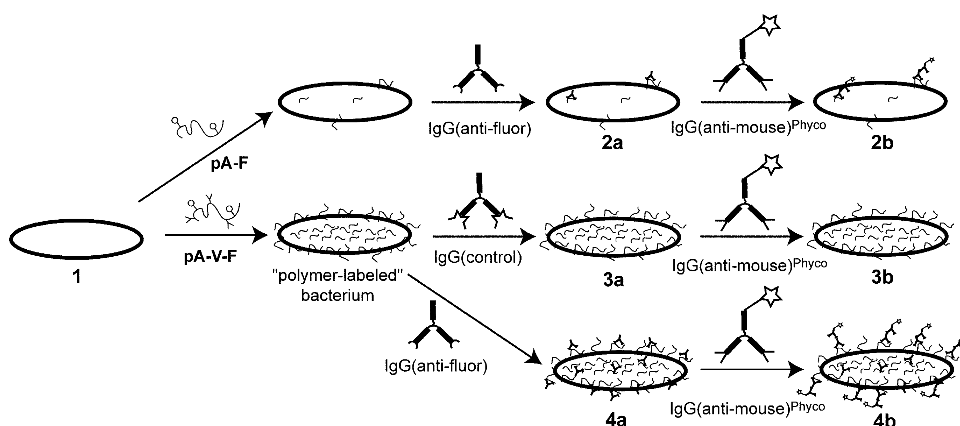


Fig. 4. Schematic representation of opsonization and phagocytosis experiments. The three groups of bacteria used in phagocytosis studies are untreated bacteria (no polymer or antibody) (**1**), **pA–V–F**/IgG(control) (**3a**), and **pA–V–F**/IgG(anti-fluor) (**4a**). The four groups of bacteria used in opsonization studies are untreated bacteria (no polymer or antibody) (**1**), **pA–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**2b**), **pA–V–F**/IgG(control)/IgG(anti-mouse)^{Phyco} (**3b**), and **pA–V–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**4b**). The amount of primary antibody (IgG(anti-fluor) and IgG(control)) bound to the bacterial surface is only qualitatively represented, and does not quantitatively reflect the relative amount of each antibody bound.

consistent with a low level of non-specific binding of the polymer to the surfaces of both bacteria by interactions that are independent of vancomycin.

Bacteria treated with **pA–V–F**/IgG(control)/IgG(anti-mouse)^{Phyco} (**3b**) displayed the same mean fluorescence intensity as untreated bacteria (within error) for both *S. aureus* and *S. pneumoniae*. The percentage of bacteria in this control group (IgG(control)) with fluorescence greater than the threshold was negligible (within error) for both bacteria. The absence of phycoerythrin fluorescence (over background) in these control experiments eliminated the possibility of non-specific binding to the bacterial surfaces of the antibodies: the Fc region of the IgG2a primary antibodies (IgG(control) and IgG(anti-fluor)) and the secondary antibody (IgG(anti-mouse)^{Phyco}).

Both *S. aureus* and *S. pneumoniae* treated with **pA–V–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**4b**) had mean phycoerythrin fluorescence intensities between 10- and 20-fold greater than control groups (**2b** and **3b**) based on statistical analysis. The percentage of bacteria that were above the fluorescence threshold was high in this group for both bacteria (*S. aureus*, $76\pm9\%$; *S. pneumoniae*, $92\pm6\%$). These values were much higher than those values observed in any of the control groups. For *S. pneumoniae,* the data from flow cytometry indicate that every bacterium is labeled by **pA–V–F** and IgG(anti-fluor) (the percentage of bacteria with fluorescence above the threshold was ~100%). For *S. aureus*, the data for the group incubated with **pA–V–F**/IgG(anti-fluor) show a high degree of labeling of bacteria by antibody (76% of bacteria have phyco fluorescence above threshold). We cannot, however, rule out the possibility that some fraction of the population of *S. aureus* did not bind to either IgG(fluor) or **pA–V–F**, given the overlap of the histograms for control and experimental groups (Fig. 5(a)). From both the flow cytometry and fluorescence microscopy results, we conclude that the polymer must have both vancomycin and fluorescein side-chains to opsonize *S. aureus* and *S. pneumoniae*: vancomycin, to adsorb the polymer to the bacterial surface via binding to D-Ala–D-Ala in the cell wall (cf. **2b** and **4b**, Fig. 5 and Table 1), and fluorescein, to localize anti-fluorescein antibodies to the bacterial surface (cf. **3b** and **4b**, Fig. 5 and Table 1) (Figs. 1(c) and (d)).

3.4. Association with macrophages and phagocytosis of opsonized S. aureus

Having demonstrated that antibodies recognize haptens (fluorescein) on the polymers bound to bacteria, we explored the ability of the bound IgG antibodies to interact with macrophages and to promote phagocytosis of the bacteria (Figs. 1(e) and (f)). Flow cytometry allowed us to follow the association of the fluorescent polymer-labeled bacteria with macrophages (Fig. 6). We incubated *S. aureus* that were pre-treated with **pA–V–F** and primary antibody (IgG(anti-fluor): **4a**, or IgG(control): **3a**) with cultured J774 cells (a mouse macrophage-like cell line, which we refer to below as macrophages). After incubation, we washed the macrophages and quantitatively examined macrophages associated with **pA–V–F**-labeled bacteria using flow cytometry (gating on the macrophages and detecting fluorescence from fluorescein) (Fig. 6). We also examined the macrophages qualitatively by optical microscopy (Fig. 7).

In the flow cytometry experiments, we could only observe bacteria that were labeled with **pA–V–F** because we detected fluorescence from fluorescein (on **pA–V–F**). We gated on the macrophages to exclude signals from free **pA–V–F** and **pA–V–F**-labeled bacteria that were not associated with the macrophages. The control experiments for phagocytosis, therefore, used fluorescent **pA–V–F**-labeled bacteria treated with IgG(control) (**3a**) to estimate the antibody-independent phagocytosis of *S. aureus* [32,33]. The control experiments for opsonization had

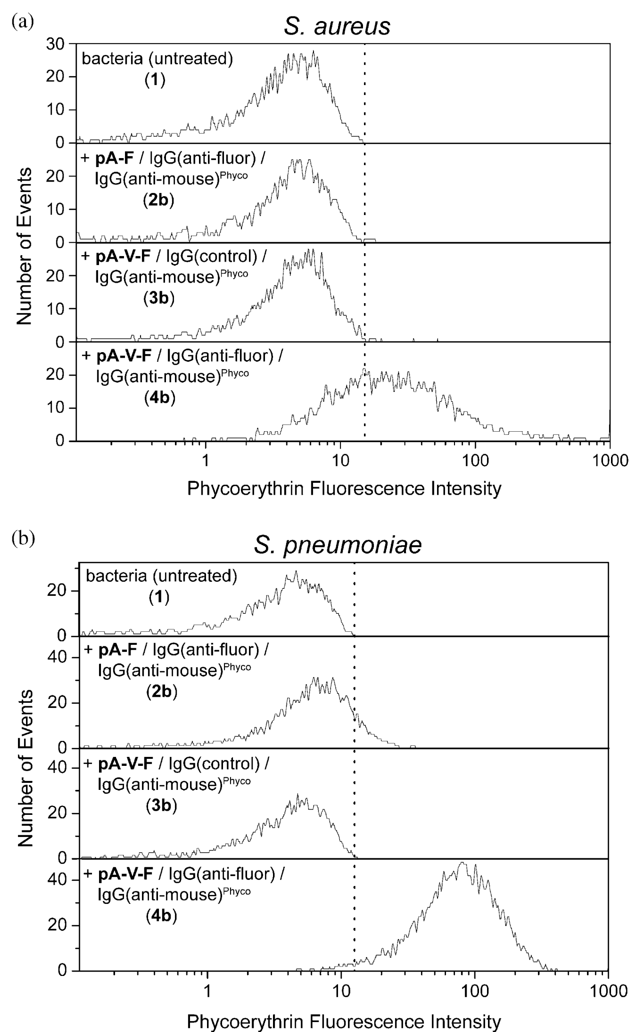


Fig. 5. Representative flow cytometry histograms demonstrating specific binding of anti-fluorescein IgG to **pA**–**V**–**F**-labeled bacteria. Bacteria (*S. aureus* (a) or *S. pneumoniae* (b)) were incubated sequentially with polymer (**pA**–**V**–**F** or **pA**–**F**), mouse anti-fluorescein IgG (IgG(anti-fluor)) or mouse isotype-control IgG (IgG(control)), and goat *R*-phycoerythrin-conjugated secondary IgG (IgG(anti-mouse)$^{\mathrm{Phyco}}$). Bacteria were also left untreated to serve as a control (untreated bacteria). Bacterial populations were identified and gated based on 90° light scatter, and ~10,000 events were analyzed for phycoerythrin fluorescence intensity. The dotted line indicates the maximum phycoerythrin fluorescence intensity observed for the untreated bacteria (threshold). There was a shift to higher phycoerythrin fluorescence of the entire population for both types of **pA**–**V**–**F** labeled bacteria when incubated with IgG(anti-fluor) (**4b**) than when left untreated (**1**). There was no such shift in fluorescence for the population of **pA**–**V**–**F** labeled bacteria that were incubated with IgG(control) (**3b**). There was a small shift towards higher phycoerythrin fluorescence for the *S. pneumoniae* when treated with **pA**–**F** and IgG(anti-fluor) (**4b**) than when left untreated (**1**) (a). Such an increase in this control group (**4b**) was not present for *S. aureus* (b) in this experiment.

demonstrated that IgG(control) does not bind to **pA**–**V**–**F**-labeled bacteria (Fig. 5, group (**3b**)). In the control phagocytosis experiment with IgG(control), approximately one-half (51±3%) of the macrophages had fluorescence from fluorescein above the threshold, which was set as the maximum fluorescence intensity observed for macrophages interacting with untreated *S. aureus* (i.e., background fluorescence). These results suggest a moderate level of association between **pA**–**V**–**F**-labeled *S. aureus* and macrophages independent of specific antibodies. These data are consistent with previous reports demonstrating that macrophages have antibody-independent mechanisms for phagocytosis of *S. aureus* [32,33].

There was a statistically significant increase in the percentage of macrophages above the fluorescence threshold (from 51±3% to 84±1%) when the **pA**–**V**–**F** labeled *S. aureus* were incubated with IgG(anti-fluor) (**4a**) as compared to being incubated with IgG(control) (**3a**) (Fig. 6(b)). This increased percentage of fluorescent macrophages represents an increase in the percentage of macrophages that are associated with **pA**–**V**–**F**-labeled bacteria in a specific antibody (IgG(anti-fluor))-dependent manner. The high background (antibody-independent) level of association between macrophages and **pA**–**V**–**F**-labeled *S. aureus* [32,33] (Fig. 6) limited the maximum effect that we could observe (theoretical maximum of 100% fluorescent macrophages). The results, nevertheless, demonstrate that **pA**–**V**–**F** is able to increase the percentage of macrophages that are associated with *S. aureus* in a manner that requires opsonization by anti-fluorescein antibodies.

The greater than two-fold increase in mean fluorescence for macrophages in the IgG(anti-fluor) group (**4a**) as compared to those in the IgG(control) group (**3a**) (Fig. 6(c)) indicates an increase in the average number of **pA**–**V**–**F**-labeled bacteria associated with each macrophage when there is IgG(anti-fluor) present on the surface of the bacteria. We believe that this increase in mean fluorescence underestimates the interaction between the **pA**–**V**–**F**-labeled bacteria and macrophages in the IgG(anti-fluor) group due to the quenching of fluorescence from fluorescein upon binding by IgG(anti-fluor). The addition of an excess of IgG(anti-fluor) to a solution of **pA–V–F** quenched the fluorescence from fluorescein by >95% (data not shown). We cannot comment on the quantitative amount that this quenching reduces the observed interaction between the **pA–V–F**-labeled bacteria and macrophages in the IgG(anti-fluor) group because we have no estimate of the number of free (not bound by antibody) fluorescein moieties per molecule of polymer associated with bacteria (see Refs. [17–20]). This quenching does not occur in the control samples incubated with IgG(control). The results with IgG(anti-fluor), even with the likely underestimation due to quenching of fluorescence by binding of antibody, demonstrate an increased association of **pA**–**V**–**F**-labeled bacteria with macrophages in a manner that is dependent upon an IgG with specificity towards the hapten (fluorescein) introduced by the polymer.

Macrophage studies included both one and two hour time points, in order to evaluate whether the enhanced phagocytosis of the IgG(anti-fluor) group relative to the IgG(control) group persisted. Similar to the earlier time point, IgG with specificity for the fluorescein hapten on **pA**–**V**–**F** (IgG(anti-fluor)) (**4a**) significantly increased

Table 1
Summary of flow cytometry data for opsonization of bacteria

	Mean fluorescence intensity[a]	Percentage of bacteria above threshold[a,b]
S. aureus		
Bacteria (untreated) (**1**)	4.5±0.3	
+**pA–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**2b**)	9±4	17±16
+**pA–V–F**/IgG(control)/IgG(anti-mouse)^{Phyco} (**3b**)	6±2	6±5
+**pA–V–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**4b**)	90±40[c]	76±9[c]
S. pneumoniae		
Bacteria (untreated) (**1**)	4.3±0.2	
+**pA–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**2b**)	8±3	15±11
+**pA–V–F**/IgG(control)/IgG(anti-mouse)^{Phyco} (**3b**)	4.5±0.2	0±0
+**pA–V–F**/IgG(anti-fluor)/IgG(anti-mouse)^{Phyco} (**4b**)	80±30[c]	92±6[c]

[a]Uncertainties represent standard errors of the mean from three independent experiments.
[b]Threshold was set based on the maximum fluorescence observed for untreated bacteria (and is shown as dotted line in Fig. 5). Percentages above threshold in group **1** are zero by definition.
[c]$p < 0.05$ vs. other polymer-treated groups. Comparisons were performed using a one-way analysis of variance (ANOVA) followed by a Duncan post-hoc test. When data did not pass Levine's test for normality and equal variance, they were logarithmically transformed (base 10) to meet ANOVA requirements.

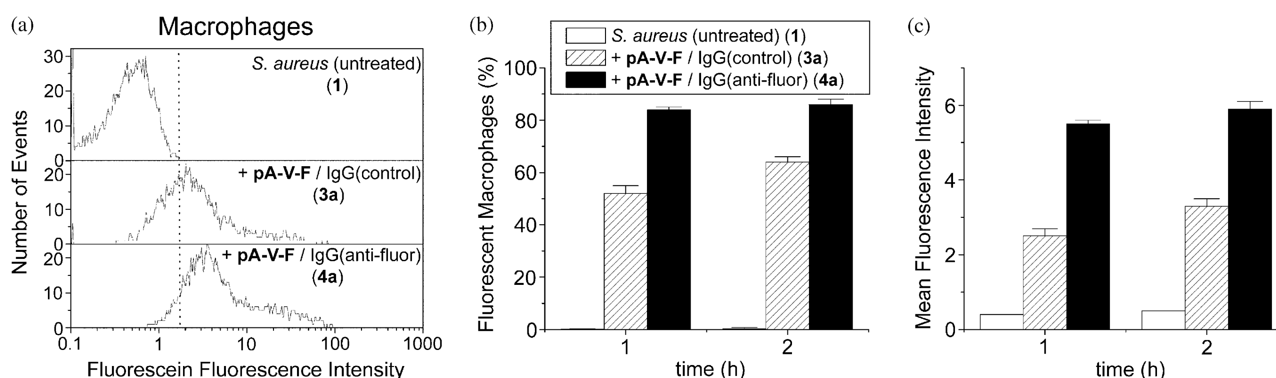


Fig. 6. Flow cytometry data demonstrating greater phagocytosis of **pA–V–F**-labeled *S. aureus* when treated with anti-fluorescein IgG (IgG(anti-fluor)) than when treated with isotype-control IgG (IgG(control)). Bacteria were treated with **pA–V–F** and then with either IgG(anti-fluor) (**4a**) or IgG(control) (**3a**). Cultured J774 cells (macrophages) were incubated with these potentially opsonized bacteria for 1 or 2 h, washed, and analyzed by flow cytometry. Macrophages were identified and gated based on forward and 90° light scatter, and fluorescence intensity from fluorescein was measured for 5000 events of the macrophage population. (**a**) Representative histograms (with 1 h incubation of bacteria with macrophages) showing the fluorescence intensity of 5000 events of the macrophage population. The dotted line indicates the fluorescence threshold that was set on the basis of the maximum fluorescence intensity from untreated control bacteria. The fluorescein fluorescence intensity shifts to higher values for the macrophage population when incubated with IgG(anti-fluor) than when incubated with IgG(control). Summarized data from four independent experiments are expressed as (b) the percentage of macrophages that are fluorescent (based on the threshold set in (a)), and (c) mean fluorescence intensity of all events. The error bars in (b) and (c) represent standard errors of the mean from the independent measurements. The legend in (b) applies to both (b) and (c). Comparisons were made among groups within each time point and the differences between all three groups were found to be statistically significant ($p < 0.05$) using one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test.

the percentage of macrophages associated with bacteria (Fig. 6(b)) and the number of bacteria per macrophage (Fig. 6(c)) relative to non-specific IgG (IgG(control)) (**3a**) after 2 h of incubation. The percentage of macrophages above the fluorescence threshold and the fluorescence per macrophage changed little between one and two hours for the IgG(anti-fluor) group; this result suggests that peak or near peak bacterial uptake in the hapten-specific antibody group was achieved by 1 h. The association of **pA–V–F**-labeled bacteria with macrophages achieved after two hours of incubation with IgG(control) failed to reach the levels achieved after only one hour of incubation with IgG(anti-fluor). Taken together, these findings suggest that the bifunctional polymer strategy (mediated by hapten-specific IgG) increases the net amount and the rate of bacterial association with macrophages for a significant time period relative to innate phagocytosis (independent of specific IgG) by the macrophages.

Flow cytometry does not distinguish between bacteria that are actually internalized and those that are merely associated with the periphery of the cell membrane of the macrophages. To address this issue, we examined

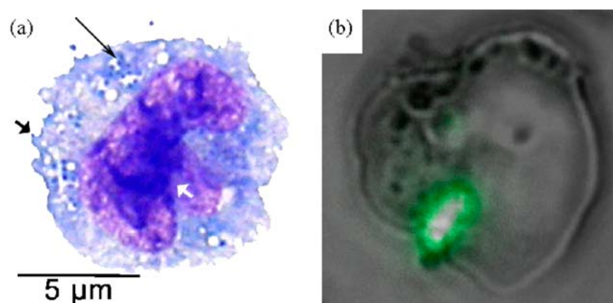


Fig. 7. *S. aureus* bacteria labeled with **pA–V–F** and anti-fluorescein IgG (IgG(anti-fluor)) are ingested by J774 macrophages. Macrophages were treated with **pA–V–F** and IgG(anti-fluor) as in Fig. 6. (a) Unfixed macrophages were prepared for visualization by optical microscopy by centrifugation and by staining with the Diff-Quik® stain set (Dade Behring). In this image of a representative macrophage, the *S. aureus* appear as small (0.8 μm diameter), darkly stained spheres (referred to as cocci). Cocci are evident both associated with the external cell membrane of the macrophage (thick black arrow) and internalized in intracellular phagosomes of the macrophage (thin black arrow). The large, dark object in the center of the macrophage (white arrow) is its nucleus. (b) Unstained macrophages were also imaged using fluorescence microscopy. This image is a fluorescence micrograph (fluorescence from fluorescein) merged with a phase contrast micrograph of the same field. The fluorescent signals (green or white) arising from the **pA–V–F**-labeled *S. aureus* appear to originate from within the macrophage. This intensity must arise from a large cluster of cocci; single cocci are not resolved using this technique. The scale bar applies to both images.

bacteria-associated macrophages (prepared as above) using optical microscopy (Fig. 7). Bacteria (diameter of 0.8 μm) are visible both inside of the macrophage in phagosomes (Fig. 7(a); thin black arrow) and associated with the external cell membrane (Fig. 7(a); thick black arrow). Moreover, overlaying a phase-contrast image with a fluorescence image (Fig. 7(b)) suggested an intracellular location of a fraction of the **pA–V–F**-labeled, fluorescent *S. aureus*. These images strongly suggest that the data from flow cytometry (increased mean fluorescence and increased percentage of events above threshold relative to control groups) for macrophages exposed to **pA–V–F**-labeled *S. aureus* and IgG(anti-fluor) is attributable to increases in both the amount of bacteria bound to and internalized by the macrophages; that is, some fraction of the macrophage-associated bacteria is phagocytosed.

3.5. *Strengths and limitations of the bifunctional polymer strategy*

There are several strengths to the bifunctional polymer approach. For the specific system studied here, vancomycin gives the polymer a broad, but specific target (all Gram-positive bacteria). The bifunctional polymer is modular in design: one component is the recognition element that binds the polymer to the surface, and the other component is the functional element. This modularity makes this strategy a general approach to the design of a wide range of polymers with different specificities (by varying the recognition element) and functions (by varying the secondary or functional element). These bifunctional polymers function sequentially: (i) the polymer binds to the surface of the target mediated by one type of side-chain on the polymer, (ii) antibodies bind to the other side-chain functionality on the polymer, and (iii) the opsonized bacteria are recognized and ingested by macrophages. This step-wise mode of action is in contrast to previous approaches using *monomeric* bifunctional molecules (e.g., chimeric or small-molecule conjugated proteins [7,8,34,35] or pre-assembled complexes [9,10]) and has potential benefits in future applications of these polymers (see below).

In the specific system studied here, **pA–V–F** cannot target Gram-negative bacteria, viruses, or other cell types and is thus not general for all pathogens. The use of fluorescein as our functional element (i.e., hapten) required using anti-fluorescein, an antibody directed towards an unnatural hapten, in the opsonization step. These specific limitations could, theoretically, be overcome by varying the recognition and functional elements of the polymer, as discussed above. Bifunctional polymers (like all polymers) are subject to certain limitations in therapeutic applications: they are not orally bioavailable and their innate polydispersity has hampered their FDA approval [36]. We believe, however, that polymers will be particularly effective in places where their large size (and thus, low oral bioavailability) is an advantage rather than a disadvantage. Examples could include administration to appropriate compartments, such as the digestive tract, respiratory system, eye, superficial soft tissue infections, and vagina, where retaining the polyvalent ligand in that organ or structure is useful, and where release into the systemic circulation may be undesirable.

As a general approach, bifunctional polymers might be useful therapeutically to target antibodies to pathogens or cancer cells to accelerate their destruction. An appropriate recognition element for the target of interest can be incorporated into the polymer and, given the polyvalent nature of the interaction, weak monovalent interactions would be acceptable in this role. The functional element on the polymer can be either a synthetic (as demonstrated here) or a natural hapten (e.g., part of a vaccine). A synthetic hapten would require the subsequent administration of antibodies directed towards that hapten, while a natural hapten would allow antibodies of the host to target the polymer-labeled target. Bifunctional polymers may also be useful in analytical applications. Binding to cells or viruses by a molecule that has both a tunable recognition component and a tunable labeling component could be useful in approaches designed to quantitate specific populations of cells [37] (e.g., by flow-cytometry).

4. Conclusions

We used a bifunctional polyacrylamide, **pA–V–F** (Fig. 1(a)), to form complexes with the surfaces of several representative Gram-positive bacteria (*S. aureus,*

S. epidermidis, S. pneumoniae, and *E. faecalis*) and to "decorate" these bacteria with a synthetic molecule (fluorescein); this molecule served as a hapten that was recognized by antibodies (IgG(anti-fluor)) in a second step. These antibodies, which were bound to the bacterial surfaces, interacted with macrophages (presumably via interactions with the Fc region of the antibody) and promoted phagocytosis of the opsonized, polymer-labeled bacteria (Fig. 1).

This polyacrylamide might be useful therapeutically in the clearance from appropriate biological compartments of bacterial infections caused by Gram-positive bacteria. We are currently investigating these applications using in vivo models of bacterial infections in the respiratory system. Further, the binding of **pA–V–F** to *E. faecalis* VanB after overnight incubation with **pA–V–F** (but not after incubation with monomeric vancomycin itself), and preliminary results that demonstrated that **pA–V–F** was able to mediate opsonization of some fraction of induced VanB by anti-fluorescein antibodies, suggest that the bifunctional polymer approach using vancomycin as the recognition moiety could be successful in targeting even these vancomycin-resistant bacteria, which pose a serious public health problem, for phagocytosis in vitro and in vivo.

Most importantly, the studies reported here offer a proof-of-principle demonstration that bifunctional polymers can target microbes for antibody-mediated immunity. By appropriate selection of the recognition moiety, this polyvalent approach should allow the selective destruction of pathogens (or other cells) of interest by coating their surfaces with hapten, to which antibody-mediated host defenses can be targeted.

Acknowledgments

This research was supported by the National Institute of Health (GM30367, HL68153, and ES00002). V.M.K. acknowledges a pre-doctoral fellowship from the NDSEG. L.J.Q., L.A.E., and S.J.M. acknowledge postdoctoral fellowships from the NIH (HL07118, EB003361, and AI010013, respectively). J.M.I. acknowledges support from the NSF R.E.U. program. We thank Amy Imrich (Harvard, School of Public Health) for expert technical assistance with flow cytometry. We thank Professor Daniel Kahne (Harvard, Department of Chemistry and Chemical Biology) for use of his tissue culture facility for handling *E. faecalis* VanB.

References

[1] Graham NM. The epidemiology of acute respiratory infections in children and adults: a global perspective. Epidemiol Rev 1990;12: 149–78.

[2] Picard C, Puel A, Bustamante J, Ku CL, Casanova JL. Primary immunodeficiencies associated with pneumococcal disease. Curr Opin Allergy Clin Immunol 2003;3:451–9.

[3] Verbrugh HA, Peterson PK, Nguyen BY, Sisson SP, Kim Y. Opsonization of encapsulated *Staphylococcus aureus*: the role of specific antibody and complement. J Immunol 1982;129:1681–7.

[4] Casal J, Tarrago D. Immunity to *Streptococcus pneumoniae*: Factors affecting production and efficacy. Curr Opin Infect Dis 2003;16: 219–24.

[5] Bogaert D, Hermans PWH, Adrian PV, Rumke HC, de Groot R. Pneumococcal vaccines: an update on current strategies. Vaccine 2004;22:2209–20.

[6] Hubbard BK, Walsh CT. Vancomycin assembly: Nature's way. Angew Chem Int Ed Eng 2003;42:730–65.

[7] Byrn RA, Mordenti J, Lucas C, Smith D, Marsters SA, Johnson JS, et al. Biological properties of a CD4 immunoadhesin. Nature 1990;344:667–70.

[8] Shokat KM, Schultz PG. Redirecting the immune response: ligand-mediated immunogenicity. J Am Chem Soc 1991;113:1861–2.

[9] Bertozzi CR, Bednarski MD. Antibody targeting to bacterial-cells using receptor-specific ligands. J Am Chem Soc 1992;114:2242–5.

[10] Bertozzi CR, Bednarski MD. A receptor-mediated immune-response using synthetic glycoconjugates. J Am Chem Soc 1992;114:5543–6.

[11] Li J, Zacharek S, Chen X, Wang J, Zhang W, Janczuk A, et al. Bacteria targeted by human natural antibodies using α-Gal conjugated receptor-specific glycopolymers. Bioorg Med Chem 1999;7: 1549–58.

[12] Rogers HJ, Perkins HR, Ward JB. Microbial cell walls and membranes. New York: Chapman and Hall; 1980.

[13] Nieto M, Perkins HR. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. Biochem J 1971;123:789–803.

[14] Nieto M, Perkins HR. Physicochemical properties of vancomycin and iodovancomycin and their complexes with diacetyl-L-lysyl-D-alanyl-D-alanine. Biochem J 1971;123:773–87.

[15] Barna JC, Williams DH. The structure and mode of action of glycopeptide antibiotics of the vancomycin group. Annu Rev Microbiol 1984;38:339–57.

[16] Walsh CT. Vancomycin resistance: decoding the molecular logic. Science 1993;261:308–9.

[17] Mammen M, Helmerson K, Kishore R, Choi SK, Phillips WD, Whitesides GM. Optically controlled collisions of biological objects to evaluate potent polyvalent inhibitors of virus-cell adhesion. Chem Biol 1996;3:757–63.

[18] Mammen M, Dahmann G, Whitesides GM. Effective inhibitors of hemagglutination by influenza-virus synthesized from polymers having active ester groups—insight into mechanism of inhibition. J Med Chem 1995;38:4179–90.

[19] Lees WJ, Spaltenstein A, Kingerywood JE, Whitesides GM. Polyacrylamides bearing pendant α-sialoside groups strongly inhibit agglutination of erythrocytes by influenza a virus: multivalency and steric stabilization of particulate biological systems. J Med Chem 1994;37:3419–33.

[20] Sigal GB, Mammen M, Dahmann G, Whitesides GM. Polyacrylamides bearing pendant α-sialoside groups strongly inhibit agglutination of erythrocytes by influenza virus: the strong inhibition reflects enhanced binding through cooperative polyvalent interactions. J Am Chem Soc 1996;118:3789–800.

[21] Mourez M, Kane RS, Mogridge J, Metallo S, Deschatelets P, Sellman BR, et al. Designing a polyvalent inhibitor of anthrax toxin. Nat Biotechnol 2001;19:958–61.

[22] Sanders WJ, Gordon EJ, Dwir O, Beck PJ, Alon R, Kiessling LL. Inhibition of L-selectin-mediated leukocyte rolling by synthetic glycoprotein mimics. J Biol Chem 1999;274:5271–8.

[23] Yang ZQ, Puffer EB, Pontrello JK, Kiessling LL. Synthesis of a multivalent display of a CD22-binding trisaccharide. Carbohydr Res 2002;337:1605–13.

[24] Gestwicki JE, Strong LE, Kiessling LL. Tuning chemotactic responses with synthetic multivalent ligands. Chem Biol 2000;7: 583–91.

[25] Gestwicki JE, Strong LE, Borchardt SL, Cairo CW, Schnoes AM, Kiessling LL. Designed potent multivalent chemoattractants for *Escherichia coli*. Bioorg Med Chem 2001;9:2387–93.
[26] Metallo SJ, Kane RS, Holmlin RE, Whitesides GM. Using bifunctional polymers presenting vancomycin and fluorescein groups to direct anti-fluorescein antibodies to self-assembled monolayers presenting D-alanine-D-alanine groups. J Am Chem Soc 2003;125: 4534–40.
[27] Arimoto H, Nishimura K, Kinumi T, Hayakawa I, Uemura D. Multi-valent polymer of vancomycin: enhanced antibacterial activity against VRE. Chem Commun 1999;15:1361–2.
[28] Sundram UN, Griffin JH. General and efficient method for the solution- and solid-phase synthesis of vancomycin carboxamide derivatives. J Org Chem 1995;60:1102–3.
[29] Rao J, Lahiri J, Weis RM, Whitesides GM. Design, synthesis, and characterization of a high-affinity trivalent system derived from vancomycin and L-Lys-D-Ala–D-Ala. J Am Chem Soc 2000;122: 2698–710.
[30] Schrerrer R, Gerhardt P. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. J Bacteriol 1971;107: 718–35.
[31] Baptista M, Depardieu F, Courvalin P, Arthur M. Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. Antimicrob Agents Chemother 1996;40:2291–5.
[32] Thomas CA, Li YM, Kodama T, Suzuki H, Silverstein SC, El Khoury J. Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. J Exp Med 2000;191:147–55.
[33] Stuart LM, Deng JS, Silver JM, Takahashi K, Tseng AA, Hennessy EJ, et al. Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol 2005;170:477–85.
[34] Capon DJ, Chamow SM, Mordenti J, Marsters SA, Gregory T, Mitsuya H, et al. Designing CD4 immunoadhesins for AIDS therapy. Nature 1989;337:525–31.
[35] Traunecker A, Schneider J, Kiefer H, Karjalainen K. Highly efficient neutralization of HIV with recombinant CD4-immuglobulin molecules. Nature 1989;339:68–70.
[36] Duncan R. The dawning era of polymer therapeutics. Nat Rev Drug Discovery 2003;2:347–60.
[37] Disney MD, Zheng J, Swager TM, Seeberger PH. Detection of bacteria with carbohydrate-functionalized fluorescent polymers. J Am Chem Soc 2004;126:13343–6.