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Multivalent Artificial Opsonin for the Recognition and Phagocytosis of Gram-Positive Bacteria by Human Phagocytes

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Abstract

Hospital-acquired infections (HAIs) remain a leading cause of death in the United States. Unfortunately, treatment of HAIs is complicated by the emergence of antibiotic-resistant bacterial strains. In an effort to enhance the body's natural immune response to infection, we have developed an artificial opsonin to promote the recognition, phagocytosis, and destruction of pathogenic bacteria by human phagocytes. The artificial opsonin is constructed from multivalent conjugates of poly(L-lysine)-*graft*-poly(ethylene glycol) with vancomycin and human IgG-Fc. Our approach utilizes vancomycin's inherent ability to bind to D-Ala-D-Ala terminated peptides present in the cell wall of Gram-positive bacteria. Here, we show that conjugation of vancomycin to PLL-g-PEG prevents its action as an antibiotic and allows vancomycin to function solely as a recognition molecule. Human IgG-Fc antibody fragment serves as a phagocyte recognition molecule and is recognized by the Fcγ cell surface receptors expressed on professional human phagocytes. Using flow cytometry, we found that a polysaccharide-encapsulated, methicillin-resistant strain of *S. epidermidis* is efficiently recognized by the artificial opsonin (nearly 100% of cells were opsonized) and that opsonin binding is specific since it can be inhibited by the soluble cell wall peptide analog acetyl-Lys-D-Ala-D-Ala. Opsonization of *S. epidermidis* resulted in an approximate 2-fold increase in phagocytosis by a human neutrophil cell line. Notably, *E. faecalis* VanB, a bacterial strain with inducible vancomycin resistance, was used to show that the artificial opsonin does not unintentionally induce antibiotic resistance mechanisms.

Keywords

bacteria; flow cytometry; immune response; immunostimulation; infection; neutrophil

1. Introduction

Although biomaterials have become indispensable in modern medicine, infection remains a major limitation to their use [1]. Infection of a biomaterial or indwelling medical device can lead to severe complications, including septicemia and sepsis, which are a leading cause of death in the U.S. [2,3]. The Centers for Disease Control and Prevention estimates that 1.7 million hospital-acquired infections occur annually [4,5] with approximately half attributed to indwelling medical devices [6]. Coagulase-negative staphylococci are the leading cause of biomaterials-related infections, with 80% of cases attributed to *Staphylococcus*

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epidermidis [7]. *S. epidermidis* has developed resistance to most classes of antibiotics [8–10], rendering traditional antibiotic therapy ineffective. Many bacteria, including *S. epidermidis*, have also developed traits to evade the host immune system [11]. These include the formation of a polysaccharide capsule [12] to conceal the bacteria from opsonins, such as antibodies and complement, and phagocytic immune cells, such as neutrophils and macrophages [13]. For this reason, novel antibody therapies, including passive and active immunization, also have little efficacy. The treatment of medical device infections must therefore rely on more innovative approaches.

One such approach is to enhance the body's natural immune response to infection. Several groups have developed bispecific recognition molecules (i.e., containing both pathogen and phagocyte recognition properties) to increase the phagocytosis of *Pseudomonas aeruginosa* [14], *Porphyromonas gingivalis* [15], *S. aureus* [16,17], *Escherichia coli* [18], and *Bacillus anthracis* [19]. The major disadvantage of the above bispecific molecules is their lack of broad pathogen recognition through the use of bacterial species- or strain-specific monoclonal antibodies or DNA aptamers. Additionally, these approaches would not have efficacy against encapsulated bacterial strains that are protected from antibody recognition and whose surface proteins are poorly immunogenic, such as *S. epidermidis*. Another limitation to the above designs is that most utilize a phagocyte recognition moiety (such as a monoclonal antibody fragment against a cell surface marker) which simply brings the pathogens within close proximity to the cells, but does not activate the phagocytes in order to elicit important antimicrobial functions such as the oxidative burst response.

Further, the above bispecific molecules employed monovalent ligands for targeting. Multivalency is a strategy commonly used in biological systems to enhance the affinity and specificity of interactions [20]. Krishnamurthy et al. [21] have taken advantage of this phenomenon by creating a bifunctional polyacrylamide presenting multiple copies of vancomycin and fluorescein. The vancomycin groups allowed for multivalent recognition of Gram-positive bacteria while the fluorescein groups served as model antigens. The bifunctional polyacrylamide was used to introduce the model antigens onto the surface of bacteria after which the bacterial cells could be opsonized by anti-fluorescein antibodies. This approach resulted in a 2-fold enhancement of *S. aureus* phagocytosis by a murine macrophage cell line. However, the use of fluorescein as a model antigen lacks clinical relevance and the two-step approach for opsonization would require pre-existing antibodies in patients, which may not be practical for those who are immunocompromised.

To overcome these limitations, we have developed a multivalent artificial opsonin to promote the recognition, phagocytosis, and destruction of Gram-positive bacteria by human phagocytes. The structure of our artificial opsonin consists of multiple copies of both bacterial and phagocyte recognition molecules attached to a soluble polymer support, poly(L-lysine)-*graft*-poly(ethylene glycol). Human IgG-Fc fragment serves as the phagocyte recognition molecule and is recognized by the Fc γ cell surface receptors (Fc γ R) expressed by professional human phagocytes (e.g., neutrophils, monocytes/macrophages, and dendritic cells) [22]. Fc γ R binding to IgG-Fc clustered on coated pathogens leads to cell activation to elicit the powerful antimicrobial functions of the phagocytes [23,24,25].

For bacterial recognition, we utilize the glycopeptide antibiotic vancomycin as a high affinity targeting molecule by exploiting the molecule's inherent ability to bind to D-Ala-D-Ala-terminated peptides naturally present in the cell wall of Gram-positive bacteria (as shown in Figure 1). In monomeric form, vancomycin has the ability to diffuse through the cell wall and bind to D-Ala-D-Ala-terminated peptidoglycan precursors at the plasma membrane before they are incorporated into the cell wall. This process inhibits cell wall synthesis and leads to death of the bacterium, as is the goal when vancomycin is employed

as an antibiotic. However, we have designed our artificial opsonin to have a MW larger than the exclusion limit of the cell wall (found by Scherrer et al. [26] to be ~120 kDa) in order to restrict access of the conjugated vancomycin to the non-vital D-Ala-D-Ala-terminated peptide targets present in the exterior portion of the cell wall. We hypothesized that this design would not only avoid direct killing of a targeted bacterium, but also prevent induction of vancomycin resistance.

Here, we investigate the ability of our artificial opsonin to recognize a broad range of Gram-positive bacteria, including multi-drug resistant strains which create a serious problem for the healthcare community. We utilize *E. faecalis* VanB, a bacterial strain with inducible vancomycin resistance, to determine whether or not the artificial opsonin would unintentionally induce antibiotic resistance mechanisms. Finally, we study the effects of opsonization on the phagocytosis of *S. epidermidis* by a human neutrophil cell line.

2. Materials and methods

2.1 Synthesis of poly(L-lysine)-graft-poly(ethylene glycol)

Poly-L-lysine hydrobromide (PLL; $M_n = 47,900$; $DP = 229$; $PDI = 1.1$; Sigma-Aldrich) was reacted with a 3-fold molar excess (based on PLL ϵ -amino groups) of succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol] ester (NHS-PEG₂-Mal; Pierce Biotechnology) for 30 min at room temperature to form PLL-*g*-PEG-Mal. Unreacted NHS-PEG₂-Mal was removed with a ZebaTM Spin Desalt Column (7 kDa MWCO; Pierce Biotechnology) and the concentration of maleimide groups in the grafted polymer determined by UV/Vis spectroscopy ($\epsilon_{300} = 649 \text{ M}^{-1}\text{cm}^{-1}$). The concentration of maleimide groups was also quantified by using a modification of the Thiol and Sulfide Quantitation Kit (Invitrogen) according to the manufacturer's instructions.

2.2 Vancomycin modification and conjugation

Vancomycin hydrochloride (Sigma-Aldrich) was reacted with a 10-fold molar excess of 2-iminothiolane (Traut's Reagent; Pierce Biotechnology) for 1 h at room temperature. Unreacted Traut's Reagent was removed by passing the reaction mixture through a column of Sephadex G-10 (700 Da MWCO; GE Healthcare) and the degree of vancomycin thiolation determined by an Ellman's assay (Pierce Biotechnology) according to the manufacturer's instructions. The purified vancomycin was immediately reacted with PLL-*g*-PEG-Mal at a final concentration of 5 mM (based on equimolar vancomycin and maleimide groups) to form PLL-*g*-PEG-Van. 25 mM tris(2-carboxyethyl)phosphine (TCEP; Pierce Biotechnology) was added to the reaction mixture to prevent formation of vancomycin dimers through disulfide bridging. Unreacted maleimide groups were blocked by the addition of 100 mM L-cysteine (Sigma-Aldrich). A control conjugate lacking vancomycin, denoted PLL-*g*-PEG-Cys, was prepared by conjugation of L-cysteine only.

Unreacted vancomycin was removed from the PLL-*g*-PEG-Van conjugate by size exclusion chromatography (SEC) using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) operated by an FPLC system. The degree of vancomycin conjugation was quantified by UV/Vis spectroscopy by subtracting the background PLL-*g*-PEG-Cys absorbance at 300 nm ($\epsilon_{300} = 0 \text{ M}^{-1}\text{cm}^{-1}$) from the PLL-*g*-PEG-Van absorbance ($\epsilon_{300} = 13,400 \text{ M}^{-1}\text{cm}^{-1}$) and subsequently calculating the number of vancomycin molecules conjugated per polymer from the molar absorption coefficient of vancomycin ($\epsilon_{300} = 430 \text{ M}^{-1}\text{cm}^{-1}$).

2.3 Human IgG-Fc modification and conjugation

Hydrazine and aldehyde functional groups were introduced at random sites into PLL-*g*-PEG-Van and human IgG-Fc (MW = 50 kDa; Bethyl Labs), respectively, after reaction of

their primary amines with NHS-activated modifying reagents (SoluLink). PLL-g-PEG-Van was functionalized by reaction with a 60-fold molar excess of sulfo-succinimidyl 6-hydrazinonicotinate acetone hydrazone sodium salt (sulfo-S-HyNic) while IgG-Fc was functionalized with an equimolar ratio of sulfo-succinimidyl 4-formylbenzoate sodium salt (sulfo-S-4FB). The molar substitution ratio of hydrazine (i.e., HyNic) and aldehyde (i.e., 4FB) functional groups into the PLL-g-PEG-Van and IgG-Fc, respectively, was determined by reaction of the modified products with 2-hydrazinopyridine dihydrochloride (2-HP; SoluLink) or 2-sulfobenzaldehyde (2-SBA; Sigma-Aldrich), respectively, and measuring the absorbance at 350 nm to quantify the formation of the chromophoric bis-aryl hydrazones ($\epsilon_{350, 4FB/2-HP} = 18,000 \text{ M}^{-1}\text{cm}^{-1}$; $\epsilon_{350, \text{HyNic}/2\text{-SBA}} = 20,000 \text{ M}^{-1}\text{cm}^{-1}$). The biological activity of the aldehyde-modified IgG-Fc was confirmed by binding to human complement C1q (EMD Chemicals) in an ELISA using methods similar to those described by Idusogie et al. [27]. The functionalized IgG-Fc and PLL-g-PEG-Van were mixed at a 5:1 molar ratio (IgG-Fc:PLL-g-PEG-Van), supplemented with 10 mM aniline (SoluLink), and allowed to react for 2 h at room temperature. Unreacted IgG-Fc was removed by SEC. The concentration of IgG-Fc in the purified conjugate was quantified by UV/Vis using the molar absorption coefficient of the bis-aryl hydrazone ($\epsilon_{354} = 29,000 \text{ M}^{-1}\text{cm}^{-1}$) and a particle agglutination assay (Easy-Titer® Human IgG (gamma chain) Assay Kit; Pierce Biotechnology).

2.4 Bacterial strains and culture

Table 1 lists the source of the bacterial strains used in the study and their corresponding vancomycin minimum inhibitory concentration (MIC). The vancomycin MIC was determined by the University of Washington Clinical Microbiology Laboratory using the Etest® (bioMérieux). All strains were grown in suspension in trypticase soy broth (TSB) at 37°C and 180 rpm for 16 h, transferred to fresh TSB and grown until mid- to late-log phase, unless otherwise specified. Vancomycin-resistant strains were grown in brain heart infusion broth (BHI) containing 2.7 µM vancomycin.

2.4 Mammalian cell culture

HL-60 human promyeloblast cell line (ATCC No. CCL-240) was grown in RPMI (Invitrogen) supplemented with 10% fetal bovine serum (Mediatech, Inc.) at 37°C and 5% CO₂. Cells were routinely passaged by dilution in fresh medium every 2–3 days. When used for experiments, cells were plated at the specified concentration in tissue culture polystyrene well-plates in medium supplemented with 100 nM phorbol-12-myristate-13-acetate (PMA; EMD Chemicals) to induce differentiation of precursor cells into neutrophil-like cells. Cells were incubated for 24 hours at 37°C with 5% CO₂ prior to performing an experiment.

2.5 Bacterial binding studies by flow cytometry

Bacteria were diluted to 10⁹ cells/mL in 2% w/v immunoglobulin-free bovine serum albumin (Sigma-Aldrich) containing 10–200 nM vancomycin-BODIPY (Invitrogen) or 2.8 µM (based on PLL) of Alexa Fluor 488-labeled PLL-g-PEG-Van, PLL-g-PEG-Cys, or PLL-g-PEG-Van-Fc. For inhibition studies, 10 mM acetyl-Lys-D-Ala-D-Ala (Sigma-Aldrich) was added to the solution prior to addition of the conjugate. Cells were incubated at 37°C for 30 min, rinsed with PBS, and the cell-associated fluorescence measured by flow cytometry. Bacteria were identified by forward and side angle light scatter and the fluorescence intensity of 10,000 events acquired with CellQuest software (BD). Data was analyzed using FlowJo software (Tree Star, Inc.) after gating cells that displayed fluorescence greater than 99% of the untreated control cells.

2.6 Bacterial viability assay

One μL /well of *S. epidermidis* RP62A culture was placed in a 24-well tissue culture plate containing 0.5 mL/well of either TSB alone or TSB containing 2.8 μM PLL, 70 μM vancomycin, or 2.8 μM (based on PLL) PLL-g-PEG-Van-Fc. The plate was incubated at 37°C and 180 rpm and the optical density at 600 nm (OD_{600}) measured with a microplate reader every hour until stationary phase in order to evaluate cell growth.

2.7 Phagocyte viability assay

HL-60 cells were plated at a concentration of 3.2×10^3 cells/well in a black 96-well tissue culture plate. The next day, medium was replaced with fresh medium containing 10% v/v alamarBlue® (Invitrogen) and PBS, 2.8 μM PLL, 70 μM vancomycin, or 2.8 μM (based on PLL) PLL-g-PEG-Van-Fc. Plates were incubated for 1 hour at 37°C with 5% CO_2 . Afterwards, the fluorescence intensity was measured with a microplate reader using excitation/emission wavelengths of 560/590 nm.

2.8 Antibiotic resistance study with *E. faecalis* VanB

According to the methods of Shlaes et al. [29], *E. faecalis* VanB was pre-grown overnight in 10 mL of either BHI alone, BHI containing a subinhibitory concentration of vancomycin ($\frac{1}{4}$ MIC $\approx$ 2.7 μM), or an equivalent concentration (based on conjugated vancomycin) of PLL-g-PEG-Van-Fc. The next morning, cultures were diluted 1:50 into the same medium, grown for 4 hours until mid-log phase and subsequently diluted 1:50 (to an OD_{600} of ~ 0.03) in BHI containing MIC vancomycin. The cultures pre-grown in BHI only were also diluted into control medium without vancomycin. Cell growth was monitored by measuring the OD_{600} every hour until stationary phase. A vancomycin-susceptible *E. faecalis* strain ($\frac{1}{4}$ MIC $\approx$ 0.17 μM) was used as a control to confirm that any differences in growth observed with the VanB strain could be attributed to its inducible resistant genotype.

2.9 Preparation of bacteria for phagocytosis assays

Live *S. epidermidis* RP62A cells were labeled with fluorescein-5-isothiocyanate (FITC; Invitrogen) and the viability after labeling confirmed to be $\geq 95\%$ by staining with 20 μM propidium iodide (Invitrogen). Bacteria were opsonized at 37°C for 1 hour by diluting FITC-labeled cells to a final concentration of 10^9 cells/mL in Hank's Buffered Salt Solution (HBSS) containing either 2.8 μM (based on PLL) of PLL-g-PEG-Van, 2.8 μM (based on PLL) PLL-g-PEG-Van-Fc, 20% v/v pooled normal human AB serum (Mediatech, Inc.), 20% v/v heat-inactivated human AB serum, or 2 mg/mL human IgG-Fc. Heat-inactivated serum was prepared by carefully heating serum to 56°C for 30 min in a water bath.

2.10 Phagocytosis assay

HL-60 neutrophil cells were plated at 2×10^5 cells/well in 24-well tissue culture plates and the cell density quantified by a CyQUANT® NF Cell Proliferation Assay (Invitrogen). Opsonized FITC-labeled bacteria were then added to the wells at a bacteria:phagocyte ratio of 10. Cytochalasin D (MP Biomedicals) was added to negative control wells at a final concentration of 10 μM to inhibit actin polymerization and prevent phagocytosis. The plate was centrifuged at $400 \times g$ for 2 min to sediment the bacteria and then incubated for 15 min at 37°C with 5% CO_2 to allow phagocytosis. Afterwards, the cells were rinsed with cold HBSS to arrest the phagocytosis process and removed from the plates by pipeting. Samples were kept on ice and the cell-associated fluorescence immediately measured by flow cytometry as described above. Intracellular bacteria were distinguished by quenching the extracellular fluorescence with 0.4% Trypan blue (Sigma-Aldrich).

2.11 Fluorescence deconvolution imaging

HL-60 neutrophil cells were plated on PLL-coated glass coverslips in 24-well plates and the phagocytosis assay performed as described above. Cells were fixed with a solution of 4% paraformaldehyde in PBS and stained with 500 nM propidium iodide for 5 min. Samples were rinsed with PBS and mounted onto glass slides using ProLong® Gold Antifade Reagent (Invitrogen). Fluorescence images were acquired with softWoRx™ Suite software (Applied Precision) using a z-section thickness of 0.15 μm . Image deconvolution was performed with softWoRx™ Suite and 3D image analysis was performed using Imaris (Bitplane).

3. Results and discussion

3.1 Fabrication and characterization of the artificial opsonin

We have developed an artificial opsonin constructed from multivalent conjugates of poly(L-lysine)-*graft*-poly(ethylene glycol) with vancomycin and human IgG-Fc. The structure of the opsonin is shown in Figure 1 with vancomycin's target, the terminal D-Ala-D-Ala dipeptide of mucopeptides found in the cell wall of Gram-positive bacteria. The opsonin was constructed in a stepwise manner. First, PLL was grafted with a heterobifunctional NHS-PEG²-maleimide linker to obtain the water-soluble product PLL-g-PEG-Mal. Approximately 70% of the PLL side chains were grafted, resulting in 160 maleimide groups per PLL, as determined by measuring the absorbance of the maleimide group at 300 nm by UV/Vis spectroscopy. The presence of PEG and maleimide groups in the grafted polymer was also confirmed by FTIR (see Figure 2) and ¹H-NMR (data not shown). A high grafting efficiency was chosen in order to minimize the toxicity of the cationic PLL side chains while maximizing the degree of vancomycin conjugation.

Next, vancomycin was derivatized with Traut's Reagent to introduce a single sulfhydryl group at its vancosamine residue with an average molar substitution ratio of 0.5–1.0 (moles sulfhydryl per mole vancomycin). This modification was found not to alter the antibacterial activity of vancomycin (data not shown), which is consistent with the results found by other groups that have modified vancomycin at the same location [30]. The thiolated vancomycin was conjugated to PLL-g-PEG-Mal to create PLL-g-PEG-Van. The remaining unreacted maleimide groups were blocked with an excess of L-cysteine, a sulfhydryl-containing amino acid, and PLL-g-PEG-Van purified by SEC to remove the unreacted vancomycin. A control conjugate lacking vancomycin, denoted PLL-g-PEG-Cys, was prepared by conjugation of L-cysteine only. Chromatograms of the PLL-g-PEG-Van and PLL-g-PEG-Cys conjugates obtained from SEC are shown in Figure 3A. Vancomycin conjugation results in a significant increase in molecular weight, which is signified by a shift to lower retention volume.

Using UV/Vis spectroscopy, it was determined that the PLL-g-PEG-Van conjugate contained approximately 25 vancomycin molecules. As shown in Figure 3B, the presence of vancomycin in the purified PLL-g-PEG-Van conjugate is demonstrated by a peak at 280 nm in the UV absorbance spectrum which corresponds to vancomycin's multiple aromatic moieties. As expected, the PLL-g-PEG-Cys control conjugate lacks this UV signature. FTIR and ¹H-NMR were employed as additional techniques to qualitatively confirm vancomycin conjugation (data not shown). Quantification of vancomycin conjugation by peak integration of the ¹H-NMR spectra was not possible due to the absence of unique peaks corresponding to PLL as well as extensive broadening and overlapping of the peaks corresponding to vancomycin. Similar peak broadening has been observed by other groups who have conjugated vancomycin to large polymers [31]. Based on the degree of vancomycin conjugation and PEG grafting efficiency found by UV/Vis spectroscopy, the theoretical

MWs of PLL-g-PEG-Van and PLL-g-PEG-Cys were calculated to be approximately 155 and 120 kDa, respectively.

Functionalization of human IgG-Fc was successfully performed to obtain a molar substitution ratio of 0.9–1.0 (moles aromatic aldehyde per mole IgG-Fc) in order to prevent attachment of a single IgG-Fc to multiple polymers. Functionalization of PLL-g-PEG-Van was optimized to obtain a molar substitution ratio of approximately 3.5 (moles aromatic hydrazine per mole polymer conjugate). IgG-Fc was conjugated to PLL-g-PEG-Van to obtain, on average, 1.5 IgG-Fc proteins per polymer as determined by UV/Vis spectroscopy based on the absorbance of the bis-aryl hydrazone conjugate bond formed. As shown in Figure 3C, homopolymers of PLL-g-PEG-Van and IgG-Fc were found to be measurable side products and their background absorbance at 354 nm was subtracted for accurate quantification of IgG-Fc conjugation. The homopolymers and unconjugated IgG-Fc were removed from the desired PLL-g-PEG-Van-Fc conjugate (i.e., “artificial opsonin”) by SEC and the presence of IgG-Fc in the purified conjugate verified by UV/Vis spectroscopy (Figure 3D). A particle agglutination assay was used to confirm that the conjugated IgG-Fc was biologically active (Figure 4) and to corroborate the quantification of IgG-Fc concentration by UV/Vis.

3.2 Specific recognition of Gram-positive bacteria

As vancomycin is typically used as an antibiotic and has rarely been employed as a targeting molecule, studies were performed to test the recognition properties of vancomycin to a range of Gram-positive bacteria, including antibiotic-resistant strains which currently pose a major health threat. As shown in Figure 5, a fluorescent conjugate of vancomycin was able to bind all Gram-positive strains tested, including a strain of *S. aureus* that is resistant to both methicillin and vancomycin (SA Mu50). The resistance mechanism utilized by the Mu50 strain has, thus far, only been observed in staphylococci [32] and involves a phenotypic change of the cell wall in which the bacterium remodels its peptidoglycan to form a very thick, highly uncrosslinked cell wall. This phenomenon results in a 3-fold increase in the number of non-vital D-Ala-D-Ala targets in the cell wall [33], which prevents access of the antibiotic to its vital targets at the plasma membrane [34,35]. For our purposes, this mechanism of resistance may be beneficial for the targeting of our artificial opsonins. Due to the similar binding results observed among the Gram-positive strains, *S. epidermidis* RP62A was chosen as a model strain to use for the remainder of the studies. *S. epidermidis* RP62A is a polysaccharide-encapsulated, methicillin-resistant strain which has been well-studied by our laboratory [36–39] as well as many others [40–42] and was originally isolated from a patient with intravascular catheter-associated sepsis [43,44].

The bacterial binding properties of the PLL-g-PEG-Cys, PLL-g-PEG-Van, and PLL-g-PEG-Van-Fc conjugates were investigated to verify that the conjugated vancomycin retained its specific recognition capabilities. Conjugates were labeled with Alexa Fluor 488 and incubated with *S. epidermidis* RP62A at a concentration of 2.8 μ M (based on PLL). The cell-associated fluorescence was then measured by flow cytometry. As shown in Figure 6, 99.6% of cells were bound by PLL-g-PEG-Van while no binding was observed with the PLL-g-PEG-Cys conjugate. The mean cell-associated fluorescence of bacteria treated with PLL-g-PEG-Van was more than 3-fold higher than those in the untreated and PLL-g-PEG-Cys treated controls. Notably, the binding of PLL-g-PEG-Van was completely inhibited by the addition of the cell wall peptide analog acetyl-Lys-D-Ala-D-Ala to the solution (Figure 6D). This result illustrates that the binding observed with the PLL-g-PEG-Van conjugate can be attributed to the recognition of D-Ala-D-Ala-terminated peptides in the bacterial cell wall by the conjugated vancomycin. The presence of the IgG-Fc protein in the complete PLL-g-PEG-Van-Fc opsonin was found not to hinder bacterial recognition by the conjugated vancomycin. 100% of *S. epidermidis* RP62A cells were positive for fluorescence after

incubation with PLL-g-PEG-Van-Fc, with a mean cell-associated fluorescence approximately 15-fold higher than those in the untreated control. The higher fluorescence observed with the PLL-g-PEG-Van-Fc conjugate compared to PLL-g-PEG-Van is simply due to a higher degree of Alexa Fluor labeling. Fluorescence microscopy confirmed that the opsonin bound to the exterior portion of the bacteria and did not cause cell aggregation (data not shown).

3.3 Cell viability

In order to determine if the artificial opsonin affected bacterial viability, *S. epidermidis* RP62A was grown in medium containing PLL-g-PEG-Van-Fc and compared to growth in medium alone or medium containing matching concentrations of PLL (2.8 μ M) or vancomycin (70 μ M). As shown in Figure 7A, *S. epidermidis* grew normally in the presence of PLL-g-PEG-Van-Fc (no significant difference compared to the untreated control) while no growth was observed with matched concentrations of PLL or vancomycin ($p < 0.05$). These results illustrate that neither the conjugated vancomycin nor the few remaining cationic side chain amines of the PLL-g-PEG backbone are toxic to bacteria at the concentrations tested. Notably, the amount of vancomycin present in the PLL-g-PEG-Van-Fc conjugate was equivalent to 70 μ M, which is fifty times higher than the MIC for *S. epidermidis* RP62A. This finding suggests that the conjugate has limited diffusion through the bacterial cell wall to the site of vancomycin's antibiotic action, as was intended in the design of our artificial opsonin.

Phagocyte viability in the presence of PLL-g-PEG-Van-Fc was measured by an alamarBlue® assay in which metabolically active cells reduce the cell-permeable nonfluorescent dye resazurin to the bright red-fluorescent molecule resorufin. As shown in Figure 7B, the viability of HL-60 neutrophil cells was not affected by PLL-g-PEG-Van-Fc or a matched concentration of vancomycin as demonstrated by equivalent fluorescence intensities as the untreated control. In contrast, PLL caused significant cytotoxicity, resulting in an 80% decrease in fluorescence intensity ($p < 0.05$). This finding again illustrates that the free cationic side chain amines of the PLL-g-PEG backbone were sufficiently blocked in the PEG grafting procedure.

3.4 Prevention of vancomycin resistance

After confirming that the artificial opsonin does not produce any evidence of cytotoxicity, we tested its ability to prevent the induction of antibiotic resistance in *E. faecalis* VanB. The property of inducible vancomycin resistance by *E. faecalis* VanB has been well-documented [29,45–49]; the strain contains a resistance gene cluster that is upregulated in the presence of subinhibitory concentrations (sub-MICs) of vancomycin, resulting in the expression of cell wall peptides terminated in D-Ala-D-Lac [32] and an approximate ~1,000-fold decrease in vancomycin binding affinity (from μ M to mM) [50]. Transcriptional activation of the *vanB* resistance genes is controlled by a two-component regulatory system, which includes a vancomycin sensor in the plasma membrane [51,52]. We hypothesized that by inhibiting the diffusion of the artificial opsonin through the cell wall, the sensor would not be activated and therefore the induction of antibiotic resistance would be prevented.

Upon exposure to sub-MICs of vancomycin, *E. faecalis* VanB remains viable but non-growing until resistance has been induced; the cells then begin to grow again, resulting in a slight lag in growth compared to non-induced or pre-induced cells [29]. These altered growth properties provide a convenient means to test for the induction of vancomycin resistance in the laboratory. In order to determine if the artificial opsonin would unintentionally induce antibiotic resistance, *E. faecalis* VanB was grown overnight during a “pre-growth” phase in BHI alone or BHI containing either sub-MIC vancomycin or a

matched concentration of PLL-g-PEG-Van-Fc. The next day, all samples were transferred to new medium containing sub-MIC vancomycin and the cell growth monitored during a “growth phase”. As shown in Figure 8B, pre-growth of *E. faecalis* VanB in sub-MICs of vancomycin improved its growth in medium containing vancomycin, while this was not true for pre-growth in medium alone or medium containing the PLL-g-PEG-Van-Fc conjugate. These data suggest that vancomycin resistance was not induced in *E. faecalis* VanB in the presence of the artificial opsonin during the pre-growth phase, but only when transferred to medium containing monomeric vancomycin during the growth phase. No difference in growth properties was observed with a vancomycin-susceptible control strain of *E. faecalis* for any of the conditions tested (Figure 8A), which demonstrates that the differences observed with the VanB strain can be attributed to its inducible *vanB* resistant genotype. Overall, this study suggests that our artificial opsonin can be used to treat infections without inducing vancomycin resistance in the targeted bacteria. The mechanism of vancomycin resistance employed by *E. faecalis* VanB has been found to be reversible in the absence of vancomycin [51]. For this reason, it may be possible to treat patients that are plagued by vancomycin-resistant infections if vancomycin treatment is ceased and artificial opsonins are administered.

3.5 Enhanced phagocytosis of *S. epidermidis*

We next investigated the ability of the artificial opsonin to enhance phagocytosis of FITC-labeled *S. epidermidis* RP62A by a human HL-60 neutrophil cell line. As shown in Figure 9A, bacterial opsonization with PLL-g-PEG-Van-Fc significantly enhances phagocytosis, with approximately 20% of the phagocyte population containing internalized bacteria, compared to opsonization with the PLL-g-PEG-Van control conjugate, of which no phagocytes contained bacteria. Bacterial opsonization with PLL-g-PEG-Van-Fc also resulted in a two-fold increase in the mean neutrophil cell-associated fluorescence, which is indicative of the relative number of phagocytosed bacteria, compared to non-opsonized bacteria (see Figure 9B). Similar results were observed when bacteria were opsonized with normal human AB serum (which contains both immunoglobulin and complement opsonic factors) or heat-inactivated human serum (which contains only immunoglobulin opsonic factors), although the extent of phagocytosis was slightly lower (13 and 15% of fluorescence-positive phagocytes, respectively). In contrast, bacterial opsonization with human IgG-Fc did not greatly enhance phagocytosis; only 2% of neutrophils contained internalized bacteria with a 25% increase in the mean cell-associated fluorescence intensity compared to non-opsonized bacteria. These results illustrate that the bacterial and phagocyte recognition capabilities extended by vancomycin and IgG-Fc in the artificial opsonin are both necessary components of the design in order to allow efficient phagocytosis of Gram-positive bacteria.

Appropriate controls were performed for each phagocytosis experiment to confirm complete fluorescence quenching of FITC-labeled *S. epidermidis* (data not shown) and verify bacterial internalization by the phagocytes. However, as a supplement to the flow cytometry data, we utilized deconvolution fluorescence microscopy in order to visualize and confirm phagocytosis by the HL-60 cells. As shown in Figure 9C, FITC-*S. epidermidis* is in fact internalized by the phagocytes. Most often, the bacteria are found associated with intracellular vacuoles.

3.6 Practical applications and limitations of artificial opsonins

The artificial opsonin we have described here holds significant clinical potential in that it could be used to treat infections caused by antibiotic-resistant and polysaccharide-encapsulated bacterial strains without promoting resistance mechanisms or killing beneficial resident microflora. Although the opsonin can target a broad range of clinically relevant

bacteria (i.e., Gram-positive bacteria), it cannot be used to target Gram-negative bacteria, yeast, or viruses. However, it may be possible to alter the design of the opsonin by attaching a combination of pathogen recognition molecules other than, or in addition to, vancomycin. The use of the artificial opsonin as a general therapeutic may also be limited by the high cost and heterogeneity of such an approach compared to cheaper and more well-defined alternatives such as antibiotics. For these reasons, artificial opsonins may be most useful for the treatment of infections caused by antibiotic-resistant bacterial strains. These strains, especially those that are resistant to multiple drugs, pose a major health threat since there are currently very few treatment options available. New therapeutics are desperately needed to treat these evasive strains as well as prevent the further spread of antibiotic resistance.

Studies are currently under way to investigate the potential of the artificial opsonin to promote the intracellular killing of Gram-positive bacteria and an oxidative burst response by primary human neutrophils. These two parameters would offer an additional indication of the efficacy of the artificial opsonin in a clinical setting. The further optimization of the targeting components, including the number of molecules attached and possible site-specific attachment of IgG-Fc, may also yield additional benefits to the design and performance of the artificial opsonin we have described here.

4. Conclusions

We have described the development of a multivalent artificial opsonin that can target a broad range of clinically relevant bacteria with high specificity. The artificial opsonin is not directly bactericidal, but rather can be used to enhance the body's natural immune response to infection by targeting the bacteria to human phagocytes for clearance. We have demonstrated that our approach has efficacy against antibiotic-resistant and polysaccharide-encapsulated strains that are notoriously difficult to treat with conventional methods. Importantly, the artificial opsonin did not induce antibiotic resistance in *E. faecalis* VanB, which illustrates that the opsonin holds promise as a therapeutic in its ability to prevent the induction and transfer of resistance genes.

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References

1. Gottenbos B, Busscher HJ, Van Der Mei HC, Nieuwenhuis P. Pathogenesis and prevention of biomaterial centered infections. *J Mater Sci-Mater M*. 2002; 13:717–722. [PubMed: 15348557]
2. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med*. 2003; 348(16):1546–1554. [PubMed: 12700374]
3. Wenzel RP, Edmond MB. The impact of hospital-acquired bloodstream infections. *Emerg Infect Dis*. 2001; 7(2):174–177. [PubMed: 11294700]
4. Pollack, A. Rising threat of infections unfazed by antibiotics. *New York Times*; 2010 February 27.
5. Kleven RM, Edwards JR, Richards CL Jr, Horan TC, Gaynes RP, Pollack DA, et al. Estimating health care-associated infections and deaths in U.S. hospitals, 2002. *Public Health Rep*. 2007; 122:160–166. [PubMed: 17357358]

6. Darouiche R. Treatment of infections associated with surgical implants. *New Engl J Med.* 2005; 350:1422–1429. [PubMed: 15070792]
7. Gotz F. *Staphylococcus* and biofilms. *Mol Microbiol.* 2002; 43(6):1367–1378. [PubMed: 11952892]
8. Otto M. *Staphylococcus epidermidis* – the ‘accidental’ pathogen. *Nat Rev Microbiol.* 2009; 7:555–567. [PubMed: 19609257]
9. Hellmark B, Unemo M, Nilsson-Augustinsson A, Söderquist B. Antibiotic susceptibility among *Staphylococcus epidermidis* isolated from prosthetic joint infections with special focus on rifampicin and variability of the *rpoB* gene. *Clin Microbiol Infect.* 2009; 15(3):238–244. [PubMed: 19196261]
10. Neu H. The crisis in antibiotic resistance. *Science.* 1992; 257:1064–1073. [PubMed: 1509257]
11. Foster T. Immune evasion by staphylococci. *Nat Rev Microbiol.* 2005; 3:948–958. [PubMed: 16322743]
12. Kocianova S, Vuong C, Yao Y, Voyich JM, Fischer ER, DeLeo FR, Otto M. Key role of poly- γ -DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. *J Clin Invest.* 2005; 115(3):688–694. [PubMed: 15696197]
13. Peterson PK, Wilkinson BJ, Kim Y, Schmeling D, Quie PG. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. *Infect Immun.* 1978; 19(3):943–949. [PubMed: 640738]
14. Lindorfer MA, Nardin A, Foley PL, Solga MD, Bankovich AJ, Martin EN, et al. Targeting of *Pseudomonas aeruginosa* in the bloodstream with bispecific monoclonal antibodies. *J Immunol.* 2001; 167:2240–2249. [PubMed: 11490011]
15. Kobayashi T, Takauchi A, van Spriel AB, Vilé HA, Hayakawa M, Shibata Y, et al. Targeting of *Porphyromonas gingivalis* with a bispecific antibody directed to Fc α RI (CD89) improves in vitro clearance by gingival crevicular neutrophils. *Vaccine.* 2004; 23:585–594. [PubMed: 15542178]
16. Gyimesi E, Bankovich AJ, Schuman TA, Goldberg JB, Lindorfer MA, Taylor RP. *Staphylococcus aureus* bound to complement receptor 1 on human erythrocytes by bispecific monoclonal antibodies is phagocytosed by acceptor macrophages. *Immunol Lett.* 2004; 95:185–192. [PubMed: 15388259]
17. Tomita GM, Wang Y, Paape MJ, Poultrel B, Rainard P. Influence of bispecific antibodies on the in vitro bactericidal activity of bovine neutrophils against *Staphylococcus aureus*. *J Dairy Sci.* 2000; 83(10):2269–2271. [PubMed: 11049067]
18. Kuhn SE, Nardin A, Klebba PE, Taylor RP. *Escherichia coli* bound to the primate erythrocyte complement receptor via bispecific monoclonal antibodies are transferred to and phagocytosed by human monocytes in an in vitro model. *J Immunol.* 1998; 160:5088–5097. [PubMed: 9590260]
19. Bruno JG, Carrillo MP, Crowell R. Preliminary development of DNA aptamer-Fc conjugate opsonins. *J Biomed Mater Res.* 2009; 90A:1152–1161.
20. Mammen M, Choi S-K, Whitesides GM. Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew Chem Int Ed.* 1998; 37:2754–2794.
21. Krishnamurthy VM, Quinton LJ, Estroff LA, Metallo SJ, Isaacs JM, Mizgerd JP, Whitesides GM. Promotion of opsonization by antibodies and phagocytosis of Gram-positive bacteria by a bifunctional polyacrylamide. *Biomaterials.* 2006; 27:3663–3674. [PubMed: 16527349]
22. Janeway, CA., Jr; Travers, P.; Walport, M.; Shlomchik, MJ., editors. The immune system in health and disease. 6. New York: Garland Science Publishing; 2005. Immunobiology.
23. Ivan E, Colovai AI. Human Fc receptors: critical targets in the treatment of autoimmune diseases and transplant rejections. *Human Immunol.* 2006; 67:479–491. [PubMed: 16829303]
24. Huynh, KK.; Grinstein, S. Receptor-initiated signal transduction during phagocytosis. In: Ernst, JD.; Stendahl, O., editors. Phagocytosis and bacterial pathogenicity. New York, NY: Cambridge University Press; 2006.
25. Fang FC. Antimicrobial reactive oxygen and nitrogen species: Concepts and controversies. *Nature Rev Microbiol.* 2004; 2:820–832. [PubMed: 15378046]
26. Scherrer R, Gerhardt P. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. *J Bacteriol.* 1971; 107(3):718–735. [PubMed: 4999413]

27. Idusogie EE, Presta LG, Gazzano-Santoro H, Totpal K, Wong PY, Ultsch M, et al. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol.* 2000; 164:4178–4184. [PubMed: 10754313]
28. Mack D, Siemssen N, Laufs R. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. *Infect Immun.* 1992; 60(5):2048–2057. [PubMed: 1314224]
29. Shlaes DM, Bouvet A, Devine C, Shlaes JH, Al-Obeid S, Williamson R. Inducible, transferable resistance to vancomycin in *Enterococcus faecalis* A256. *Antimicrob Agent Chemother.* 1989; 33(2):198–203.
30. Ge M, Chen Z, Onishi HR, Kohler J, Silver LL, Kerns R, et al. Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding D-Ala-D-Ala. *Science.* 1999; 284:507–511. [PubMed: 10205063]
31. 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(15):4534–4540. [PubMed: 12683824]
32. Fraimow, HS.; Courvalin, P. Resistance to glycopeptides in Gram-positive pathogens. In: Fischetti, VA.; Novick, RP.; Ferretti, JJ.; Portnoy, DA.; Rood, JI., editors. *Gram-positive pathogens.* Washington, DC: ASM Press; 2006. p. 782–800.
33. Hiramatsu K. Vancomycin resistance in staphylococci. *Drug Resist Updates.* 1998; 1:135–150.
34. Cui L, Iwamoto A, Lian J-Q, Neoh H-M, Maruyama T, Horikawa Y, et al. Novel mechanism of antibiotic resistance originating in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrob Agent Chemother.* 2006; 50(2):428–438.
35. Cui L, Murakami H, Kuwahara-Arai K, Hanaki H, Hiramatsu K. Contribution of a thickened cell wall and its glutamine nonamidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. *Antimicrob Agent Chemother.* 2000; 44(9):2276–2285.
36. Wagner VE, Koberstein JT, Bryers JD. Protein and bacterial fouling characteristics of peptide and antibody decorated surfaces of PEG-poly(acrylic acid) co-polymers. *Biomaterials.* 2004; 25(12):2247–2263. [PubMed: 14741590]
37. Cheng G, Zhang Z, Chen S, Bryers JD, Jiang S. Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces. *Biomaterials.* 2007; 28(29):4192–4199. [PubMed: 17604099]
38. Jarvis RA, Bryers JD. Effects of controlled fibronectin surface orientation on subsequent *Staphylococcus epidermidis* adhesion. *J Biomed Mater Res A.* 2005; 75(1):41–55. [PubMed: 16059880]
39. Sun D, Accavitti MA, Bryers JD. Inhibition of biofilm formation by monoclonal antibodies against *Staphylococcus epidermidis* RP62A accumulation-associated protein. *Clin Diagn Lab Immunol.* 2005; 12(1):93–100. [PubMed: 15642991]
40. Tojo M, Yamashita N, Goldmann DA, Pier GB. Isolation and characterization of a capsular polysaccharide adhesin from *Staphylococcus epidermidis*. *J Infect Dis.* 1988; 157:713–722. [PubMed: 3279138]
41. Wang IW, Anderson JM, Marchant RE. *Staphylococcus epidermidis* adhesion to hydrophobic biomedical polymer is mediated by platelets. *J Infect Dis.* 1993; 167(2):329–336. [PubMed: 8421167]
42. Cheung GY, Rigby K, Wang R, Queck SY, Braughton KR, Whitney AR, et al. *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. *PLoS Pathog.* 2010; 6(10):pii, e1001133. [PubMed: 20949069]
43. Christensen GD, Bisno AL, Parisi JT, McLaughlin B, Luther RW. Nosocomial septicemia due to multiply antibiotic-resistant *Staphylococcus epidermidis*. *Ann Intern Med.* 1982; 96:1–10. [PubMed: 7053681]
44. Christensen GD, Parisi JT, Bisno AL, Simpson WA, Beachey EH. Characterization of clinically significant strains of coagulase-negative staphylococci. *J Clin Microbiol.* 1983; 18:258–269. [PubMed: 6311870]

45. Baptista M, Depardieu F, Courvalin P, Arthur M. Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. *Antimicrob Agents Chemother*. 1996; 40(10):2291–2295. [PubMed: 8891132]
46. Arthur M, Depardieu F, Gerbaud G, Galimand M, Leclercq R, Courvalin P. The VanS sensor negatively controls VanR-mediated transcriptional activation of glycopeptide resistance genes of Tn1546 and related elements in the absence of induction. *J Bacteriol*. 1997; 179(1):97–106. [PubMed: 8981985]
47. Courvalin P. Vancomycin resistance in Gram-positive cocci. *Clin Infect Dis*. 2006; 42:S25–34. [PubMed: 16323116]
48. Dong S, Oberthür M, Losey HC, Anderson JW, Eggert US, Peczu MW, et al. The structural basis for induction of VanB resistance. *J Am Chem Soc*. 2002; 124:9064–9065. [PubMed: 12149006]
49. Swenson JM, Clark NC, Sahm DF, Ferraro MJ, Doern G, Hindler J, et al. Molecular characterization and multilaboratory evaluation of *Enterococcus faecalis* ATCC 51299 for quality control of screening tests for vancomycin and high-level aminoglycoside resistance in enterococci. *J Clin Microbiol*. 1995; 33(11):3019–3021. [PubMed: 8576364]
50. Walsh CT, Fisher SL, Park I-S, Prahalad M, Wu Z. Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. *Chem Biol*. 1996; 3:21–28. [PubMed: 8807824]
51. Arthur M. Vancomycin sensing. *Nat Chem Biol*. 2010; 6:313–315. [PubMed: 20404817]
52. Koteva K, Hong H-J, Wang XD, Nazi I, Hughes D, Naldrett MJ, et al. A vancomycin photoprobe identifies the histidine kinase VanSsc as a vancomycin receptor. *Nat Chem Biol*. 2010; 6:327–329. [PubMed: 20383152]

Figure 1.

Structure of the multivalent artificial opsonin. The polymer support, poly(L-lysine)-graft-poly(ethylene glycol), is shown in the upper right, where $m=69$, $n=160$. Sulfhydryl-reactive maleimide groups are present at the PEG termini to allow conjugation of ligand **R₁** while non-grafted PLL side chains allow for conjugation of ligand **R₂**. **(A)** Ligand **R₁** represents L-cysteine (84%) or thiolated vancomycin (16%). Vancomycin is shown complexed to its target, the D-Ala-D-Ala terminus of mucopeptides displayed in the cell wall of Gram-positive bacteria. The dashed lines represent intermolecular hydrogen bonds. **(B)** Ligand **R₂** represents unmodified PLL ϵ -amino groups (98%) or the bis-aryl hydrazone bond forming the human IgG-Fc conjugate (2%).

Figure 2. FTIR analysis of PEG grafting onto PLL. FTIR absorbance spectra of purified (A) PLL-g-PEG-Mal and (B) PLL control in KBr pellets. Distinct peaks at 1782 and 1704 cm⁻¹, corresponding to the maleimide group, and 1110 cm⁻¹, corresponding to the aliphatic ether bond of the PEG chain, in the PLL-g-PEG-Mal spectrum confirm grafting of the PLL with the NHS-PEG₂-Mal bifunctional linker.

Figure 3.

Analysis of vancomycin and IgG-Fc conjugation by SEC and UV/Vis spectroscopy. **(A)** SEC chromatograms representing PLL-g-PEG-Van (solid), PLL-g-PEG-Cys control (dashed), and PLL backbone polymer (dotted). Vancomycin conjugation results in a significant increase in molecular weight as signified by a shift to lower retention volume. **(B)** UV absorbance spectra of SEC-purified PLL-g-PEG-Van (solid) and PLL-g-PEG-Cys (dotted). A shift in the absorbance maximum to 280 nm for the PLL-g-PEG-Van conjugate signifies the presence of vancomycin. **(C)** SEC chromatograms representing PLL-g-PEG-Van-Fc (solid) and PLL-g-PEG-Van (dotted). IgG-Fc conjugation results in a shift to lower retention volume (signifying higher MW) and a slight increase in polydispersity compared to PLL-g-PEG-Van. Free IgG-Fc (peak at 83 mL), IgG-Fc homopolymers (peak at 72 mL), and PLL-g-PEG-Van homopolymers (peak at 45 mL) are easily removed from the PLL-g-PEG-Van-Fc conjugate (peak at 61 mL) by SEC. **(D)** UV absorbance spectrum of SEC-purified PLL-g-PEG-Van-Fc conjugate. The appearance of a peak at 354 nm, a signature of the 4FB-HyNic bis-aryl hydrazone bond, in the spectrum confirms IgG-Fc conjugation.

Figure 4.

Particle agglutination assay confirming IgG-Fc conjugation. The presence of IgG-Fc in the SEC-purified PLL-g-PEG-Van-Fc conjugate (open circles) is demonstrated by agglutination of anti-human IgG (Fc-specific) sensitized microspheres, which causes a decrease in absorbance at 405 nm with higher concentrations of IgG-Fc. In contrast, the PLL-g-PEG-Van conjugate (solid circles) lacking IgG-Fc does not agglutinate the microspheres.

Figure 5.

Broad recognition of Gram-positive bacteria by vancomycin. Cells were incubated with increasing concentrations of the fluorescent conjugate vancomycin-BODIPY and the cell-associated fluorescence measured by flow cytometry. A similar dose-dependent increase in the percentage of cells bound by vancomycin is observed by all Gram-positive strains tested. *P. aeruginosa* (PA 10145) is a Gram-negative strain which serves as a negative control to confirm the recognition specificity of vancomycin. Data are shown from a single representative experiment.

Figure 6.

Flow cytometry histograms representing specific binding of conjugates to *S. epidermidis* RP62A. Bacteria were incubated with (A) PBS or AlexaFluor 488-labeled (B) PLL-g-PEG-Cys, (C) PLL-g-PEG-Van, or (E) PLL-g-PEG-Van-Fc. The concentration of fluorescent conjugates was 2.8 μ M (based on PLL). Cell-associated fluorescence of ~10,000 events was measured after gating bacteria based on forward and side angle light scatter. The dotted line indicates the threshold for identification of fluorescence-positive cells compared to the untreated negative control (A). (D) Addition of 10 mM acetyl-Lys-D-Ala-D-Ala completely inhibited binding of PLL-g-PEG-Van to *S. epidermidis*, demonstrating specific recognition of the bacteria by the conjugated vancomycin.

Figure 7.

Effect of artificial opsonin on the viability of bacteria and phagocytes. **(A)** Viability of *S. epidermidis* RP62A in the presence of artificial opsonin. Bacterial growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) over time until stationary phase. No statistical significance was observed between cell growth in the presence of PLL-g-PEG-Van-Fc (solid squares) compared to the control grown in TSB medium (solid circles). In contrast, no growth occurred in TSB containing 70 μM vancomycin (open squares) or 2.8 μM PLL (open circles), concentrations which matched those of the conjugated vancomycin and PLL in the PLL-g-PEG-Van-Fc sample. Values are given as the mean ± std dev ($n \geq 3$). **(B)** Viability of HL-60 human neutrophil cell line in the presence of artificial opsonin. Phagocyte viability was measured by an alamarBlue assay which produces a bright red fluorescent signal when cells are metabolically active. Fluorescence data were normalized to the PBS (i.e., untreated) control for comparison purposes. Values are given as the mean ± std dev ($n \geq 6$).

Figure 8. Effect of artificial opsonin on the induction of vancomycin resistance. Growth curves of (A) vancomycin-susceptible control *E. faecalis* strain and (B) vancomycin-resistant *E. faecalis* VanB. For each strain, solid circles represent bacterial growth in BHI after pre-growth in BHI, while open circles, solid squares, and open squares represent growth in BHI containing 1/4 MIC vancomycin after pre-growth in 1/4 MIC vancomycin, BHI alone, and artificial opsonin, respectively. Data represents the average growth of at least three individual colonies for each sample with error bars indicating the std dev. Error bars are present in (A), but are not visible.

Figure 9.

Phagocytosis of *S. epidermidis* by HL-60 human neutrophil cell line. **(A)** Representative flow cytometry histograms showing the cell-associated fluorescence of the HL-60 population (~10,000 events) after gating cells based on forward and side angle light scatter. The dotted line indicates the threshold for identification of fluorescence-positive cells compared to the untreated negative control **(a)**. Fluorescence of extracellular FITC-labeled bacteria was quenched with Trypan blue prior to data acquisition. Bacterial opsonization with PLL-g-PEG-Van-Fc **(c)** results in a shift of HL-60 population to higher fluorescence intensity indicating a higher extent of phagocytosis compared to opsonization with PLL-g-PEG-Van **(b)**. A similar result is observed with bacteria treated with normal human serum **(d)** or heat-inactivated human serum **(e)**. In contrast, opsonization with human IgG-Fc **(f)** does not significantly enhance phagocytosis. **(B)** Mean fluorescence intensity of the HL-60 population (all events) measured by flow cytometry. Data is represented from a single experiment with error bars representing the std error of the mean. **(C)** Visualization of phagocytosis by deconvolution fluorescence microscopy. Deconvolved image of live FITC-labeled *S. epidermidis* (green) ingested by a neutrophil (red). Upper left image is in the horizontal x-y plane. Upper right (y-z plane slice) and lower (x-z plane slice) images confirm that the selected bacterium is contained within the phagocyte. Scale bar = 5 μ m.

Table 1

Bacterial strains used in the study and their corresponding vancomycin MIC.

Strain	Source ^a	Antibiotic Resistance	MIC (μM)
<i>Staphylococcus hominis</i> SCH1.1	catheter infection (Seattle Children's Hospital)	N/A	1.4
<i>S. epidermidis</i> SCH1.2, SCH1.3, SCH1.4, and SCH1.5	catheter infection (Seattle Children's Hospital)	N/A	1.4–2.8
<i>S. epidermidis</i> RP62A	catheter infection (ATCC 35984)	methicillin	1.4
<i>S. epidermidis</i> 1457	catheter infection [28]	N/A	2.8
<i>S. aureus</i>	ATCC 25923	methicillin	1.4
<i>S. aureus</i> Mu50	surgical wound infection (ATCC 700699)	methicillin, vancomycin	5.5
<i>Enterococcus faecalis</i>	ATCC 49332	N/A	2.8
<i>E. faecalis</i> VanB	peritoneal fluid (ATCC 51299)	methicillin, vancomycin, streptomycin/gentamicin	11–88
<i>Pseudomonas aeruginosa</i>	ATCC 10145	Gram-negative species (intrinsic resistance to vancomycin)	-

^a Abbreviations: ATCC, American Type Culture Collection. Catheter infections were associated with the use of intravascular catheters.