Targeting of Gram-Positive Pathogens to Phagocytes by Multivalent Artificial Opsonins

Kristy N. Katzenmeyer, James D. Bryers
University of Washington, Department of Bioengineering, Seattle, WA 98195 USA

Statement of Purpose: Although biomaterials have become indispensible in modern medicine, infection remains a major limitation to their use (Darouiche RO. N Engl J Med. 2004; 350:1422-9). In an effort to enhance the body's natural immune response, we have developed multivalent artificial opsonins by conjugation of vancomycin and human IgG-Fc to poly-L-lysine (PLL) via water-soluble carbodiimide and aldehyde chemistry, respectively. Conjugation of vancomycin to PLL prevents its action as an antibiotic and allows it to function solely as a bacterial recognition molecule (Krishnamurthy VM. Biomaterials. 2006; 27:3663-74), thus minimizing the chance that bacteria would develop resistance mechanisms. We hypothesize that our artificial opsonins will promote the recognition, phagocytosis, and destruction of pathogenic bacteria by human phagocytes.

Methods: A solution of 20 mg/mL (13.5 mM) vancomycin (MW 1485, Sigma-Aldrich, St. Louis, MO), 0.22 mg/mL (4.6 μM) PLL (MW 47900, PDI 1.1, Sigma-Aldrich) and 19 mg/mL (0.1 M) 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (EDC; Pierce, Rockford, IL) in MES buffer, pH 6.0 was incubated for 2 hours at 25°C to create Van-PLL. Van-PLL was purified by size exclusion chromatography (SEC) and conjugation efficiency determined by UV spectrophotometry (UV/Vis). Van-PLL and human IgG-Fc (Bethyl Laboratories, Montgomery, TX) were functionalized with sulfo-S-4FB and sulfo-S-HyNic (SoluLink, San Diego, CA) in PBS, pH 7.4 to introduce aromatic aldehydes and hydrazides, respectively, on free primary amines. The molar substitution ratio of functional groups was quantified by measuring the absorbance at 350 nm after reaction with 0.5 mM 2-hydrazinopyridine (SoluLink) or 2-sulfobenzaldehyde (Sigma-Aldrich), respectively, in MES buffer, pH 5.0 at 37°C. Functionalized Van-PLL and IgG-Fc were conjugated in PBS, pH 6.0 containing 10 mM aniline using the desired molar excess of IgG-Fc. For bacterial binding studies, laboratory *Staphylococcus* strains or clinical isolates acquired from Seattle Children's hospital from patients with infected intravascular catheters were cultured in suspension in tryptic soy broth at 37°C and 180 rpm until log phase. Bacteria were diluted to 10^9 cells/mL in SuperBlock (Pierce) containing various concentrations of vancomycin-BODIPY (Invitrogen, Carlsbad, CA) or human IgG-Fc and incubated at 37°C with gentle rotation to allow binding. To detect IgG-Fc binding, a FITC-labeled secondary antibody was used. For inhibition studies, a concentration of 50 nM of vancomycin-BODIPY was used and increasing concentrations of acetyl-Lys-D-Ala-D-Ala (Sigma) were added to the solution. Cells were rinsed and the cell-associated fluorescence quantified by flow cytometry.

Results: Vancomycin was conjugated by its carboxyl group to primary amines on PLL to create Van-PLL, which was determined by UV/Vis to contain 12 vancomycin molecules per polymer. The number of IgG-Fc proteins conjugated to Van-PLL was able to be varied simply by changing the number of functional groups on each molecule followed by purification by SEC. The biological activity of aldehyde-functionalized IgG-Fc was confirmed by a human complement C1q enzyme-linked immunosorbent assay. As shown in Figure 1, functionalized Van-PLL and IgG-Fc spontaneously react to form a stable bis-aryl hydrazone bond that exhibits a unique absorbance peak at 355 nm. The addition of 10 mM aniline to the reaction mixture was found to decrease reaction time from more than 8 hours to 45 minutes with reaction yields greater than 95%.

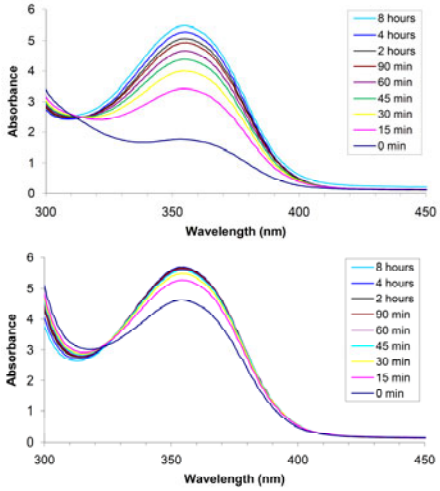

Figure 1. Real-time spectrophotometric monitoring of hydrazide-PLL and aldehyde-Fc conjugation reaction. The reaction is catalyzed by the addition of aniline. (Top) 0 mM and (Bottom) 10 mM aniline.

Preliminary studies performed to investigate the bacterial binding properties of vancomycin and IgG-Fc found that little nonspecific binding occurs with IgG-Fc and that nearly 100% of cells can be targeted at nM concentrations of vancomycin. Importantly, vancomycin was able to bind to a vancomycin-resistant strain of *S. aureus* with equivalent efficacy, further demonstrating the significance of our approach. The addition of 5 mM acetyl-Lys-D-Ala-D-Ala completely inhibited vancomycin binding, confirming the molecule's specificity for D-Ala-D-Ala terminated peptides naturally present in the cell wall of Gram-positive bacteria.

Conclusions: We have developed methods to fabricate and characterize multivalent artificial opsonins with the flexibility to alter the number of IgG-Fc proteins per opsonin. Preliminary evidence indicates that our opsonins may be effective against virulent drug-resistant and encapsulated strains as well as prevent the spread of antibiotic resistance. Future studies will use flow cytometry and fluorescence microscopy to evaluate the efficacy of the artificial opsonins in promoting bacterial phagocytosis.