# Development of Multivalent Macromolecular Ligands for Enhanced Detection of Biological Targets

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**Summary:** Macromolecular conjugates enable simultaneous binding of multiple ligands on one biological entity and these polyvalent interactions can be collectively stronger than the corresponding monovalent ligands. We have synthesized macromolecules and conjugated them with a lectin (Helix Pomatia lectin, HPA), and an antibody, both with shown affinities to certain bacteria. The binding ability was studied by flow cytometry and the results showed that the affinity of the biomolecules was greatly enhanced due to the polyvalent effect.

**Keywords:** bacteria detection, biomolecules, macromolecules, multivalent, polyvalent

## Introduction

Polyvalent interactions are a characteristic phenomenon in biology. Because these interactions can be collectively much stronger than the corresponding monovalent interactions, they would lower the needed concentration of biologial ligand molecules in sensor applications and thus increase the stability parameters of the sensors. Recently, one of the most extensively studied polyvalent interaction system has been inhibition of the adhesion of influenza virus A to erythrocytes.[1-7] The inhibition concentration of polymeric ligand could be $10^7$-$10^8$ times lower than that of monomeric ligands. Whitesides *et al* investigated the inhibition of agglunation of erythrocytes by influenza virus using polyacrylamides bearing pendant alfa-sialoside groups and showed that the cooperative binding was shown to be effective in the inhibition (1). Influenza virus binds tightly to cell with the recognition of sialic acid (SA) groups on the cell surface by a viral surface protein, hemagglutinin (HA). The binding is considered to be due the polyvalent interactions as solubilized HA binds only weakly to methyl alfa-sialoside. The extent of inhibition was shown to be a function of the conformation of the polymer and thus the function to the monomer size and concentration. For instance with high degree of substitution, the

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polymer exhibits extended conformation and is not effective in the inhibition. When the functional monomer concentration was below 0.05, no inhibition was observed. The concentration range 0.2-0.6 showed strong inhibition and with the concentration above 0.6, the polymer with extended conformation was not shown to be effective anymore. It was confirmed that the initial affinity of the ligand molecule is an essential starting requirement as polymer with non-specific interactions was not able to create inhibition. The quantitative affinity experiments did though show that in the polyvalent polymer, the affinity is weaker than in the individual molecules as the dissociation constant of the complex did not increase but actually showed less tight binding. The polyvalence was explained to be due to entropically enhanced binding expresses as enhanced probability, similar as in a chelate effect. Additional reasons for the inhibition were mentioned to be steric (colloidal) stabilization to prevent hemagglutination and possibly viscosity or the aggregation of the particles.

In the above studies, the biomolecules for conjugation were small molecules; sialic acids, mono- or disaccharides. Macromolecular conjugation with large molecules, such as antibodies or lectins would be much more difficult. Not only because the conjugating chemistry will become complicated due to the multifunctional groups in these large multifunctional molecules, but a blocking of the binding site can also be possible. Recently, Whitesides conjugated a dodecameric peptide (HTSTYWWLDGAP) into a polyacrylamide side chain and found that the inhibitiom ability was much higher than the monomeric peptide.[8]

We have investigated the surface composition of *B anthracis* and screened potential ligand molecules for the detection of these harmful bacteria. In this work we report that the conjugation of antibodies, lectins and heptapeptides with polymethacrylamide chains.

## Experimental

**Materials:** Lectin, Helix Pometia (HPA) was purchased from Sigma. The intact BD8 antibody was supplied by John Kearney in University of Alabama at Birmingham. Oregon Green 488 succinimide and 4'-(aminomethyl)fluoresein were purchased from Molecular Probe Co. N-(2-hydroxylpropyl) methacrylamide (HPMA) and N- (3-aminopropyl) methacrylamide (APMA)

were supplied by Polyscience, Inc.. Acryoyl-poly(ethylene glycol)-NHS (monomer 2) was purchased from Shearwater Polymers, Inc.

**Monomer synthesis:** NaOH (8g, 0.2mol) was slowly added with stirring to an aqueous 6-aminocaproic acid (13.1g, 0.1 mol, in 40 ml $H_2O$) in an ice bath. Methacroyl chloride (10.5g, 0.1mol) was dropped into the above solution. After additional stirring at ice bath, the reaction mixture was neutralized by diluted HCl to pH 2.0. $CHCl_3$ was used to extract the final product. Removal of the solvent gave a solid. The crude product (4g) was directly mixed with N-hydroxysuccinimide (NHS, 2.3g) in dry dioxane. DCC (4.1g) was added to catalyze the reaction. The reaction was monitored by TLC. The product was purified by column separation using hexane-ethyl acetate (1:1, v/v) as the eluent. Total yield: 65% mp.76-77°C. H-NMR ($CHCl_3$) δ (ppm) 5.68( $\underline{H}C=C$), 5.29($\underline{H}C=C$), 3.42(N-$\underline{H_2}$C-C), 2.64 (OC-$\underline{H_2}$C-C), 1.96 ($C\underline{H_3}$-C=), 1.4-1.8( -C$\underline{H2}$-C$\underline{H2}$-C$\underline{H2}$-).

The copolymerization of HPMA and APMA was initiated by 2-hydroxy-4'- (2-hydroxyethoxy)-2-methylpropiophenone (HHMP) with the irradiation of UV light at room temperature. The product was further reacted with predetermined amount of Oregon Green 488 succinimide, subsequently (about 2 hours) with excess of SMCC to give a copolymer with maleiimide functional groups and fluorescent chromospheres.

**The radical copolymerization was carried out followingly:** The two monomers, HPMA and the monomer (described above), and the initiator (AIBN) were mixed in dry acetone. After degassed, the polymerization solution was maintained at 60°C under $N_2$ for 24 hours. The copolymer was washed with cold acetone and further purified by dissolving in methanol and precipitated in acetone. This copolymer was stored in an inert environment at -20°C. Predetermined amount of 4'-aminomerthylfluorescein reacted with the polymer in dry DMF. Oregon Green 488 succinimide (5mg) in DMSO (0.5ml) was added to a HPA lectin (10mg) or BD8 antibody (3mg) in sodium bicarbonate buffer solution( pH 8.3). After 2 hours, the reaction mixture was dialysised against sodium bicarbonate buffer solution( pH 8.3).

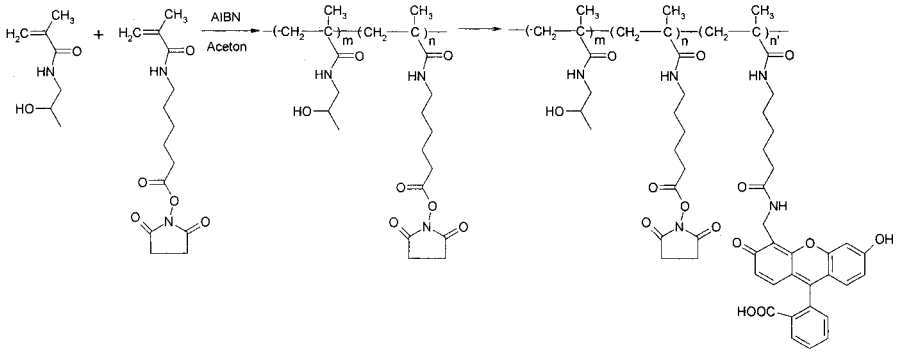
**HPA conjugation:** Oregon Green 488 labeled HPA was conjugated into polymer via the reaction of $NH_2$ groups in HPA and the NHS activated carboxyl groups. This amide formation was done by adding DMF solution of polymer into HPA in PBS buffer (pH 7.4) at 4°C. After overnight reaction, the conjugate was dialyzed against PBS buffer for 48 hrs. Antibody

conjugation: BD8 antibody conjugate was obtained similarly with HPA lectin conjugate, by the reaction of polymer with Oregon Green 488 labeled BD8 antibody.

Charaterization $^1$H NMR was recorded on a 400MZ Varian Unity spectrometer. GPC was performed on Waters using PBS buffer as eluent. Fluorescence spectra were obtained with a Perkin-Elmer spectrometer. HPA lectin and antibodies concentration in conjugates were determined by the protein assay. The peptide concentration in conjugates was measured by the method described in literature.[10]

## Results

The polymer synthetized had three different functionalities. First, certain monomer (HPMA) composition was to provide water solubility. The second monomer composition for to provide attachment of the fluorescent lable and the third was designed for the attachment of the ligand itself. An example of the polymer synthesized for the conjugation (experimental details described in the experimental section) is shown in Scheme 1.




Scheme 1.

Before the evaluation of the possible binding with the developed conjugates, a control experiment with unconjugated polymers was designed to confirm that the polymers do not create non-specific binding. In flow cytometry experiments, one follows the population of labeled species (y-axis) versus the changes in the fluoresence intensity. As shown in Figure 1, shifts in the x-axis can not be observed and thus the polymer does not bind the either bacteria.

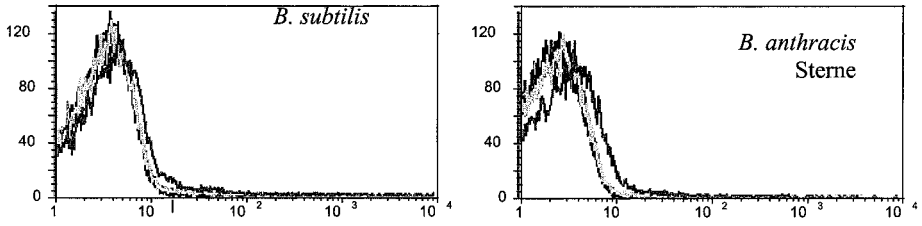



Fig. 1. Flow cytometry results on the non-specific binding of the polymers without the specific biological ligands.

HPA lectin is known to have affinity to selected sugar molecules. The binding experiments show that the labeled lectin interacts with *Bacillus thuriengiensis* at the highest concentration (Figure 2 A). The binding experiments with the lectin-polymer conjugate show a great enhancement as seen in Figure 2. The polymer conjugate (B) shows much higher binding compared to the labeled lectin alone (A). This not only confirms that sugars exist on the surface of the spores but also that polvalence increases the binding.

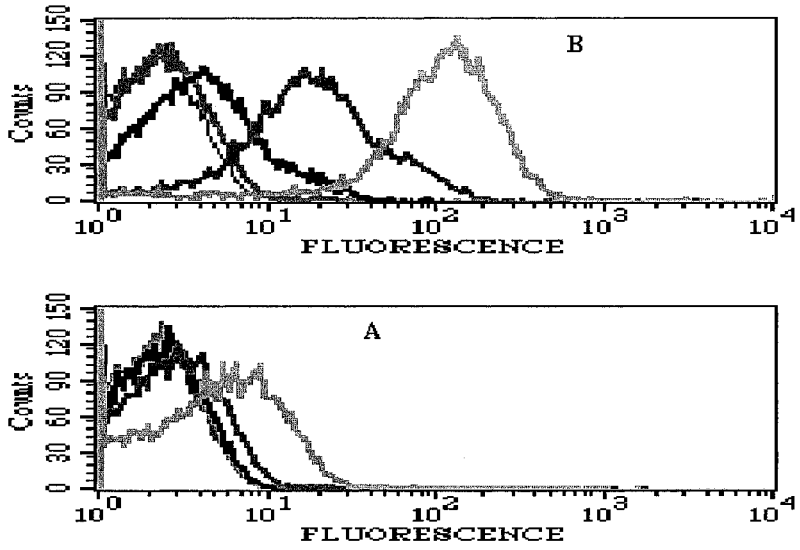



Fig. 2. Flow cytometry results on A. Lectin (HPA) and B. HPA-polymer conjugate with *B thuriengiensis.*

Lectin-sugar interactions are common in nature and lectins are large proteins so the use of these ligands are not optimal for the development of sensors. We were able to apply antibodies which had been developed in the presence of the exosporium of *B. anthracis.* We conjugated the antibody molecules as explained in the experimental section. Neither the antibody nor the antibody-polymer conjugate showed any binding to *B. subtilis* spores (the lowest picture in Figure 3). The antibody alone showed interaction with *B. anthracis* Sterne spores but only in the high concentrations. The antibody conjugates show the enhancement in a significant manner (the middle picture in Figure 3), not only the binding intensity has increased but the interactions can be observed at much lower antibody concentrations. The use of macromolecular conjugates is a potential method to build both multi- and polyvalent components for the detection of biological warfare agents.

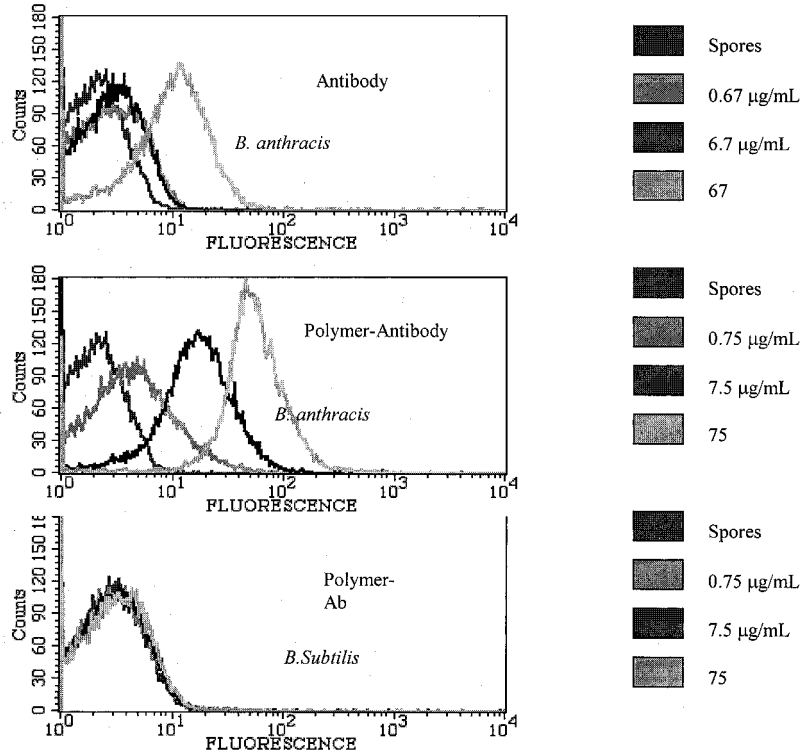



Fig. 3. Flow cytometry results for the anitbody and antibody-polymer conjugates.

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