Measuring the Inhibition of Adhesion of Lectins to the Surface of Erythrocytes with Optically Controlled Collisions between Microspheres and Erythrocytes

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This paper describes the use of the OPTCOL (optically controlled collision) assay, an assay that uses optical tweezers to cause two biologically relevant particles to collide, to measure the potency of inhibitors that block the adhesion of wheat germ agglutinin (WGA) to the surface of erythrocytes. WGA was attached covalently to polystyrene microspheres (3 μm in diameter). Optical tweezers were used to cause an erythrocyte and a WGA-coated microsphere to collide in buffer at a controlled velocity. In the absence of inhibitor, or at low concentrations of soluble inhibitor, the microsphere adhered to the cell through polyvalent, biospecific interactions between WGA and *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (NeuAc) groups present on the surface of erythrocyte. At high concentrations of soluble inhibitors, adhesion was inhibited. The potency of inhibition was quantified by measuring the probability of adhesion as a function of the concentration of the inhibitor. The inhibition constants derived from measurements using OPTCOL agreed well with those obtained in hemagglutination inhibition assays, and they were also close to the dissociation constants measured by isothermal titration calorimetry. The experimental data suggest that the binding of WGA to erythrocyte is cooperative, but that the binding of WGA to soluble ligand is not. The ability to examine dynamic adhesion in a highly controlled fashion makes OPTCOL a useful bioassay with which to study inhibition of protein-cell adhesion under biologically relevant conditions.

Introduction

This paper describes a procedure based on optical tweezers[1−4] for measuring the potency of inhibitors that block the adhesion of proteins to the surface of biological cells. Optical tweezers use focused laser beams to hold and remotely manipulate dielectric particles, including cells and other biological objects.[5−8] We use optical tweezers to bring polystyrene microspheres to which lectins have been covalently linked into contact with the surface of erythrocytes in a well-controlled manner. When the lectin-linked microsphere and erythrocyte come into contact, they adhere. In the presence of a soluble ligand that binds to the lectin, the probability of adhesion is decreased; the probability of adhesion decreases as the concentration of the soluble ligand increases. The dependence of the probability of adhesion on the concentration of inhibitor correlates with the affinity of the inhibitor for the lectin. The results obtained using this procedure agree well with those obtained using hemagglutination assays[9] (another class of assays commonly used to measure the inhibition of adhesion of proteins to the surface of cells).

Cell-surface adhesion plays an important role in many biological processes, including blood clotting,[10] angiogenesis,[11] cancer metastasis,[12] inflammation,[13,14] and infectious disease.[15,16] Biospecific adhesion is mediated by the interaction between receptors on the surface of one cell and ligands on the surface of another; this interaction may be monovalent or polyvalent[17] depending on the system. For example, adhesion of circulating leukocytes to vascular endothelial cells during inflammation is initiated by the interaction of sialyl Lewis X on the surface of the leukocyte, and selectins on the surface of the endothelial cells.[18] Adhesion mediated by protein on the microorganisms and ligand on the host cell surfaces is an essential step in microbial colonization and development of infection.[19] Although the interaction between a single protein molecule and a carbohydrate is often weak (the dissociation constant is generally in the micromolar to millimolar range[20]), polyvalent interactions between multiple copies of proteins and multiple copies of ligands allows cells to attach strongly to other biological surfaces, even when exposed to shear forces from flowing liquid.[17,21]

As the biological importance of adhesion mediated by polyvalent protein−ligand interactions becomes clearer, the development of techniques to probe, assess, and quantify these interactions has become more important.[17] It is important to have a range of techniques available to explore the biophysics of adhesion and to evaluate potential inhibitors of adhesion. It is also important to understand the effects of polyvalency, since they can be both subtle and assay-dependent.[17] Enzyme-linked immunosorbent assays (ELISA)[22] and surface plasmon resonance (SPR)[23−25] are both used to assess binding affinities. Shear flow,[26,27] micropipet suction,[28] biomembrane force probe,[29,30] atomic force microscopy,[31−35] and microcantilever-based force measurements[36] have been developed to estimate the force required to disrupt binding. Hemagglutination inhibition (HAI)

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assays are commonly used in evaluating the effectiveness of inhibitors of bacterial and viral adhesion.[9]

The development of gradient force optical traps ("optical tweezers") has made it possible to manipulate dielectric particles in solution remotely using focused laser beams.[1−8] The ability to hold, orient and manipulate dielectric particles in a non-invasive and highly controllable fashion makes optical tweezers an attractive biophysical tool with which to study adhesion mediated by the interactions between receptors and ligands presented on the surface of cells, biological objects, microspheres, or organic thin films.[5−8,37,38] Mammen et al. first described optical tweezers as a component of an assay system for measuring adhesion of biological surfaces.[39] This assay method, OPTCOL (optically controlled collision),[39] measured the probability of adhesion of a single erythrocyte and a virus-coated microsphere, in the absence and presence of the inhibitor, when the two were caused to collide with a controlled velocity and geometry. Since the orientations of the colliding objects, their velocities, and the strength and duration of the collisions are all under the direct control of the experimenter, OPTCOL can be used to mimic the dynamics of a real encounter of two biological objects in flowing or quiescent biological fluids. OPTCOL is particularly useful in investigating adhesion between two moving objects; the ability to examine dynamic adhesion is a part of some other assays (e.g., hemagglutination), but these systems offer relatively little experimental control over the dynamics of collision.

Here we describe the use of OPTCOL to explore the ability of soluble sugars to inhibit the polyvalent adhesion of wheat germ agglutinin (WGA)[40] to the surface of erythrocytes. The surface of erythrocytes (∼12 μm in diameter) presents multiple oligosaccharides terminated with *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (NeuAc) (Figure 1A); both of these sugars are recognized by wheat germ agglutinin (WGA).[41−43] WGA is a lectin that has been characterized in atomic detail.[40,44] It consists of two identical 17 kDa subunits. Each subunit is made up of four structurally homologous, but spatially distinct, domains. In the dimer, the subunits associate to form four contact areas; the binding sites are located at the interfaces. We attached WGA covalently to polystyrene microspheres (3 μm in diameter) (Figure 1B), and used optical tweezers to bring an erythrocyte and a lectin-coated microsphere into contact. The data that follow indicate that in the absence of inhibitor, or at low concentration of soluble inhibitor, the microsphere adheres to the cell through polyvalent, biospecific interactions between WGA and NeuAc and GlcNAc. In the presence of high concentrations of soluble inhibitors, adhesion is blocked (Figure 1C). We evaluated the potency of the inhibitor by measuring the probability of adhesion as a function of the concentration of the inhibitor.

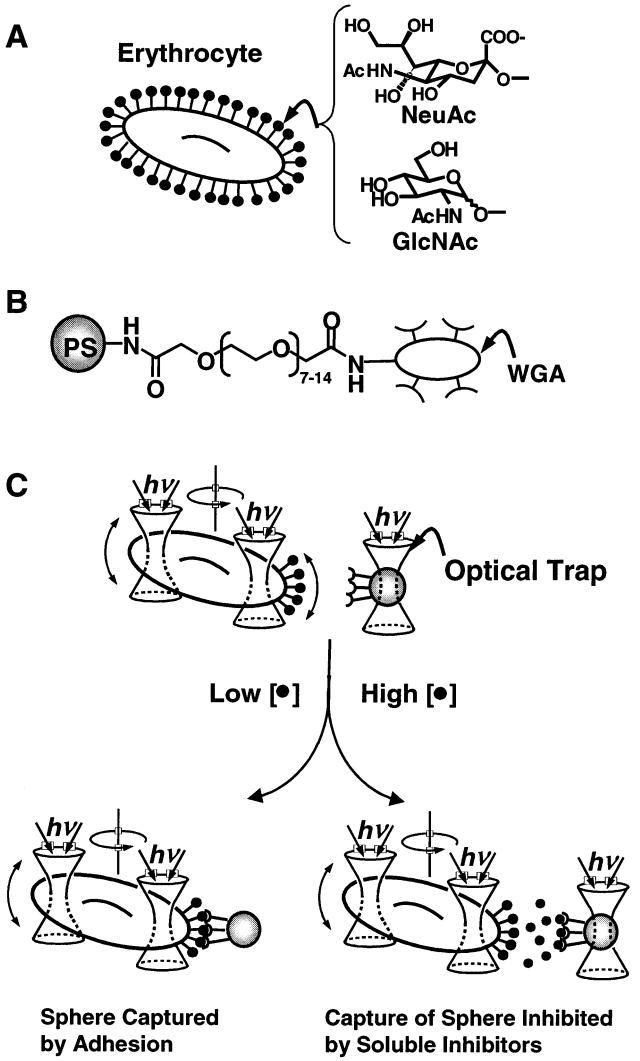


Figure 1. Components of OPTCOL. (A) The oligosaccharides on the surfaces of a chicken erythrocyte terminate in *N*-acetyl neuraminicacid (NeuAc) and *N*-acetyl glucosamine (GlcNAc). (B) A polystyrene microsphere (3 μm in diameter) was linked covalently to wheat germ agglutinin (WGA), a lectin that recognizes NeuAc and GlcNAc. (C) The OPTCOL assay involves using two optical traps to hold and orient an erythrocyte, and a third optical trap to hold a WGA-linked microsphere (for simplicity, the diagrams show only a few ligands on the surface of erythrocyte, and a few WGA molecules on the surface of the microsphere). The optical trap holding the microsphere was translated so that the microsphere and the cell collide. In the absence of a soluble inhibitor that binds to the WGA molecules on the microsphere, the microsphere adheres to the cell; we say the cell "captures" the microsphere. In the presence of concentrations of soluble inhibitor sufficiently high that the binding sites of the WGA molecules are fully occupied, the microsphere does not adhere to the cell; the capture event is blocked.

Experimental Section

Polystyrene microspheres were from Polysciences. Poly(ethylene glycol) 600 diacid ($HO_2CCH_2O(CH_2CH_2O)_n-CH_2-CO_2H$; $n = 7-14$; 0.05 g mL^{-1}) was from Fluka. Tridecafluoro-1,1,2,2-tetrahydrooctyl-1-methyldichlorosilane was from United Chemical Technologies. 1-[3-(Dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), phosphate buffered saline (PBS: 10 mM phosphate, 138 mM NaCl and 2.7 mM KCl), *N*-acetylglucosamine, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, *N*,*N*′-diacetylchitobiose ($GlcNAc_2$), tetra-*N*-acetylchitotetraose ($GlcNAc_4$), fetuin, bovine serum albumin (BSA), and wheat germ agglutinin were from Sigma-Aldrich.

Preparation of WGA-Linked Microspheres. Microspheres presenting primary amino groups (0.001 g mL^{-1}, polystyrene) were suspended in a phosphate-buffered solution (ca. pH 6) of poly(ethylene glycol) 600 diacid, EDC (0.4 M), and NHS (0.1 M). The suspension was agitated gently at room temperature for 12 h to couple the linker to the sphere. The beads were separated from the solution of reactants by centrifugation and washed with deionized water three times. The beads were resuspended in a solution of EDC (0.4 M) and NHS (0.1 M) and agitated gently for 15 min. The beads were isolated by centrifugation and resuspended in a solution of WGA (0.5 mg mL^{-1}) in phosphate buffer (0.1 M, pH 8.1); the suspension was

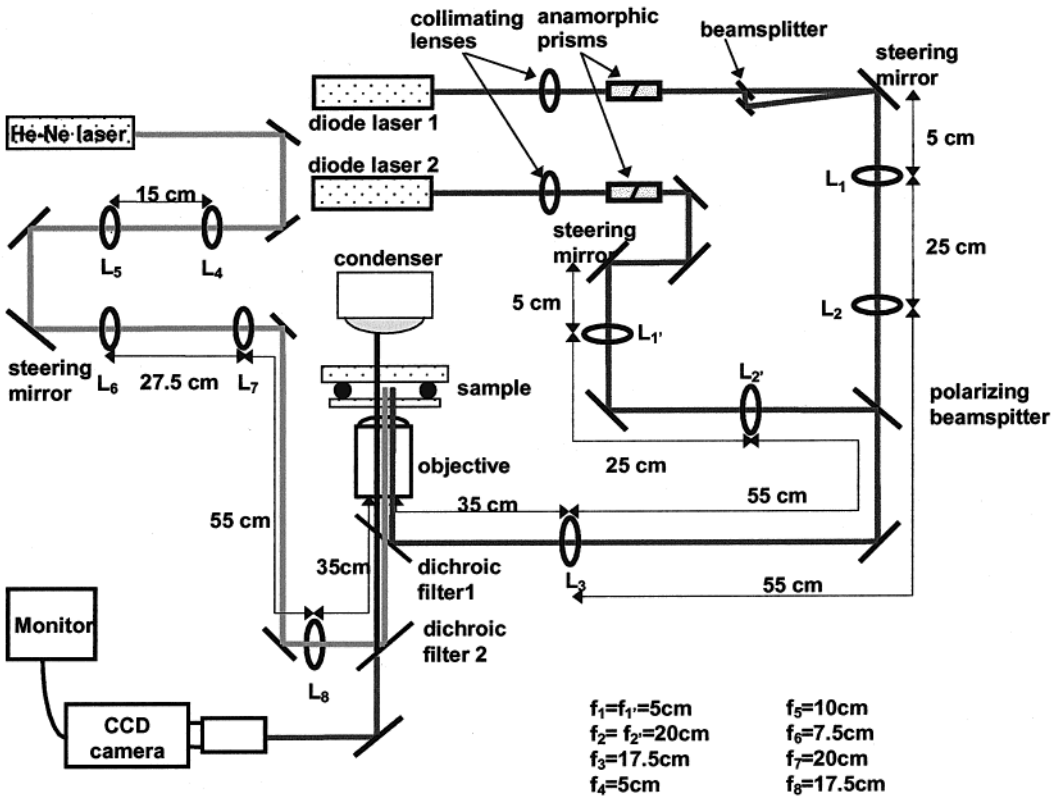


Figure 2. Schematic diagram of the three-beam optical tweezers used for OPTCOL experiments.

agitated gently for 8 h to couple WGA to active esters on the beads. The beads were isolated from the solution of WGA by centrifugation, washed with PBS three times, and stored in PBS containing 0.01% (w:w) NaN_3.

Assembly of Three-Beam Optical Tweezers Used for OPTCOL. We used three optical traps (optical tweezers) that could be manipulated independently (Figure 2). One trap was created using a collimated beam from a helium−neon (HeNe) laser (power = 22 mW). The beam was expanded in stages by two telescopes; a steering mirror was placed between these telescopes to allow us to adjust the position of the optical trap within the sample. A high power (100×, numerical aperture = 1.2) oil immersion microscope objective focused the beam into the sample. A lens placed before the objective converted the collimated telescope output into a beam with the appropriate size and curvature. A dichroic mirror that transmits most visible light but reflects the light produced by the HeNe laser (632 nm) was used to direct the beam into the objective while allowing us to image the sample with a CCD video camera. The remaining two traps were generated by two linearly polarized diode lasers (852 nm) whose powers could be adjusted from 0 to about 100 mW. Each beam was collimated and passed through an anamorphic prism so that they were both roughly circular. Telescopes expanded each beam as necessary. We placed mirrors before each telescope to be used to steer the beams. The polarization of each beam was adjusted so the beams could be passed through a polarizing beam splitter. The resulting output was directed through a lens to adjust the radius of curvature and the size of the beams, then directed through a dichroic mirror into the microscope objective and into the sample. This arrangement produced three optical traps within the sample. We used the near-infrared (852 nm) beams to trap the cells and the visible (632 nm) beam to trap the polystyrene microspheres.

Sample Vessel Construction. The glass cover slips used to support the sample suspension were treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-methyldichlorosilane under vacuum for 3 h and then soaked in a solution of BSA (0.05 g mL^{-1}) for at least 30 min. This procedure introduced a monolayer of BSA onto the surface; this monolayer blocks nonspecific adsorption to the glass. The sample vessel was constructed by attaching a Teflon O-ring to a glass cover slip. The bottom of the vessel was sealed with the BSA-coated cover slip. Phosphate-buffered saline was used as the sample solution, with the coated microspheres, erythrocytes, and ligand added to the solution.

Collision Procedure and Determining the Probability of Adhesion (P^{ADH}). P^{ADH} was determined through a series of controlled collisions between an erythrocyte and a coated microsphere from at least 20 independent trials using a new pair of microsphere and erythrocyte for each trial. Erythrocytes were held by two near-infrared optical traps, with the orientation of the plane of the erythrocyte perpendicular to the collision plane. By using a dual trap to position and orient the cells, we maintained their orientation through the collision event. Coated microspheres were manipulated using a single, steerable, trap. Once an erythrocyte and a microsphere were trapped and oriented, they could be caused to collide by holding the erythrocyte stationary and moving the microsphere. The relative velocity of the objects during the collision was roughly 3 μm/s, and the objects were positioned roughly 10 μm from the bottom surface of the sample vessel. A pair comprising an erythrocyte and a microsphere was caused to collide until they either (i) adhered or (ii) collided three times without adhesion. If adhesion occurred within the first three collisions, it was counted as a capture event; if adhesion did not occur within three collisions, it was a noncapture event. P^{ADH} was calculated as the ratio of capture events (m) to total number of capture and noncapture events (N) (eq 1).

$$P^{ADH} = \frac{m}{N} \tag{1}$$

Calculation of Error Bars on Values of P^{ADH}. The error bars assigned to each value of P^{ADH} were calculated were calculated by assuming a binomial distribution in the number of capture events (m) for a total number of capture and noncapture events (N). The error in P^{ADH} is given by eq 2.[45] When the value of in P^{ADH} approaches 0 or 1,

$$d(P^{ADH}) = \sqrt{\frac{P^{ADH}(1 - P^{ADH})}{N}} \quad (2)$$

$d(P^{ADH})$ approaches zero. For values of $P^{ADH} \leq 0.1$ or ≥ 0.9, we have arbitrarily assigned a value of 0.1 to estimate the error in these data.

Data Fitting. The experimental data were analyzed by nonlinear regression using KaleidaGraph 3.0 (Synergy Software).

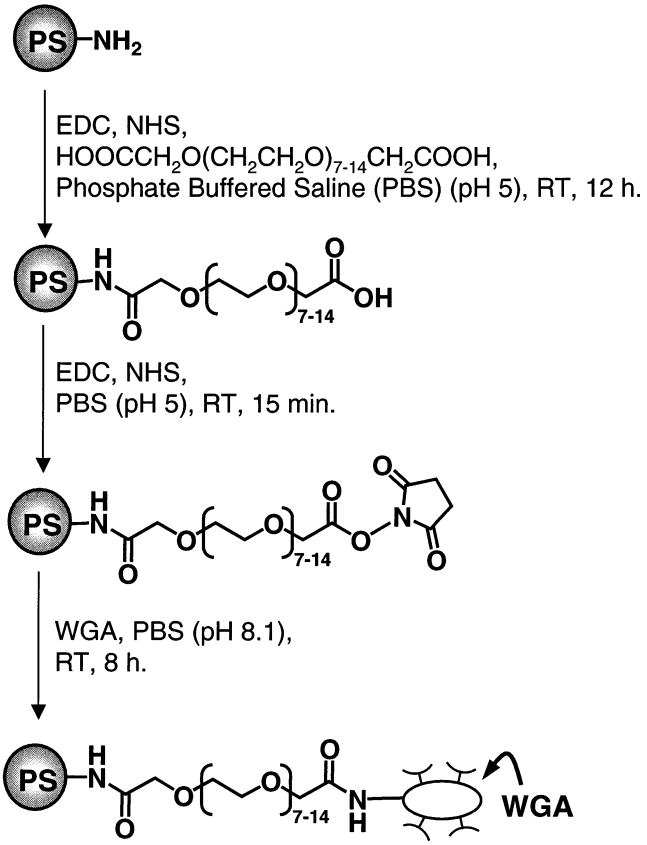
Results and Discussion

Linking Wheat-Germ Agglutinin to the Surface of Polystyrene Microspheres. Scheme 1 sketches the preparation of WGA-linked microspheres. Polystyrene microspheres presenting amino groups were treated with poly(ethylene glycol) 600 diacid, 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) to give carboxylic acid-presenting microspheres. We assume that no cross-linking occurs between two amino groups on the surface of the beads, since the dicarboxylic acid is in large excess, and only one carboxylic acid group is converted to the reactive *N*-hydroxylsuccinimidyl ester group in the reaction. The resulting carboxylic acid groups on the surface of microspheres were again converted to reactive NHS esters, which were allowed to react with the ϵ-amino groups of lysine residues on WGA to give WGA-linked microspheres.

Optical Tweezers Setup. We used three optical traps (optical tweezers) that could be manipulated independently (Figure 2). Erythrocytes were held by two near-infrared (852 nm) optical traps, with the orientation of the plane of the erythrocyte perpendicular to the collision plane. By using a dual trap to position and orient the cells, we maintained their orientation through the entire collision process. Microspheres were manipulated using a single, steerable visible (632 nm) optical trap. Once an erythrocyte/microsphere pair was trapped and oriented, they were caused to collide by holding the erythrocyte stationary while moving the microsphere toward the cell. The relative velocity of the objects during the collision was about 3 μm/s, and the objects were positioned roughly 10 μm from the bottom surface of the sample chamber.

Biospecific Adhesion of WGA-Linked Microspheres to the Surface of Erythrocytes. The WGA-linked microspheres adhered to the surface of the chicken erythrocytes when they were brought into contact using optical tweezers at a velocity of about 3 μm/s. They could not be detached using laser powers as high as ~70 mW. As control experiments, we tested microspheres coated with tri(ethylene glycol) groups and found that they did not adhere to the surface of erythrocytes; the individual WGA-linked microspheres also did not adhere to each other. These results indicate that the adhesion between the WGA-linked microsphere and the erythrocyte is biospecific. Although the interactions between the individual binding sites of WGA and carbohydrates are relatively weak (dissociation constants in the milimolar range[46]), the microspheres adhered strongly to the erythrocytes. We believe that the strength of this adhesion reflects polyvalency[17] in the interactions between multiple WGA molecules on the surface of the microsphere and multiple copies of sugar groups on the cell surface.

SCHEME 1. A Schematic Representation of the Preparation of WGA-Linked Microspheres



Inhibition of Adhesion of WGA to the Surface of Erythrocytes by Soluble Ligands. We studied the inhibition of adhesion of WGA-linked microspheres to the surfaces of erythrocytes in solutions containing soluble inhibitors: *N*-acetylglucosamine (GlcNAc) (**1**), *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2**), *N*,*N*′-diacetylchitobiose ($GlcNAc_2$) (**3**), tetra-*N*-acetylchitotetraose ($GlcNAc_4$) (**4**), and fetuin (**5**) (a glycosylated protein bearing oligosaccharides that terminate in NeuAc) (Table 1).

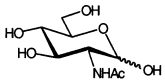
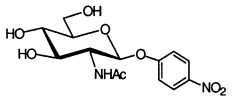
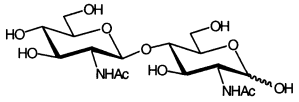
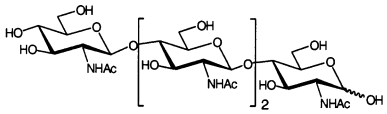
To evaluate the inhibitory potency of each ligand quantitatively, we measured the probability of adhesion (P^{ADH}) at different concentrations of the ligand. P^{ADH} was calculated as the ratio of capture events (m) to total number of capture and noncapture events (N) (eq 1) from at least 20 independent trials using a new microsphere and erythrocyte for each trial. We emphasize that P^{ADH} is independent of the power of optical traps: the laser power does not affect the probability of the capture event.

We measured P^{ADH} at different concentrations of the inhibitor. Figure 3 shows the P^{ADH} as a function of the concentration of the inhibitor. All the curves showed a characteristic sigmoidal shape. The data were fit to the Hill equation (eq 3)

$$P^{ADH} = \frac{1}{1 + ([I]/K_i^{OPTCOL})^n} \quad (3)$$

with K_i^{OPTCOL} and Hill coefficient, n, as adjustable parameters. K_i^{OPTCOL} is defined as the concentration of inhibitor required to achieve 50% probability of adhesion (or, equally, 50% inhibition), and the Hill coefficient, n, is the degree of

TABLE 1: Properties of Inhibitors Used in This Work

Entry	Inhibitor	K_d (M)[a]	K_i^{HAI} (M)[b]	K_i^{OPTCOL} (M)[c]	Cooperativity (n)[c]
(1)		2.5×10^{-3}	1.3×10^{-2}	$1.5 (\pm 0.06) \times 10^{-2}$	3.1 ± 0.4
(2)			1.0×10^{-4}	$5.5 (\pm 0.15) \times 10^{-4}$	3.3 ± 0.3
(3)		2.0×10^{-4}	1.5×10^{-4}	$3.7 (\pm 0.11) \times 10^{-4}$	3.3 ± 0.3
(4)		8.3×10^{-5}		$7.2 (\pm 0.37) \times 10^{-5}$	3.2 ± 0.5
(5)	Fetuin		2.1×10^{-5}	$2.3 (\pm 0.11) \times 10^{-5}$	3.3 ± 0.4

[a] Dissociation constant of soluble ligand with WGA measured by isothermal titration calorimetry (reference 46). [b] Concentration of inhibitor required for 50% inhibition of hemagglutination of human erythrocytes by WGA (reference 47). [c] This work.

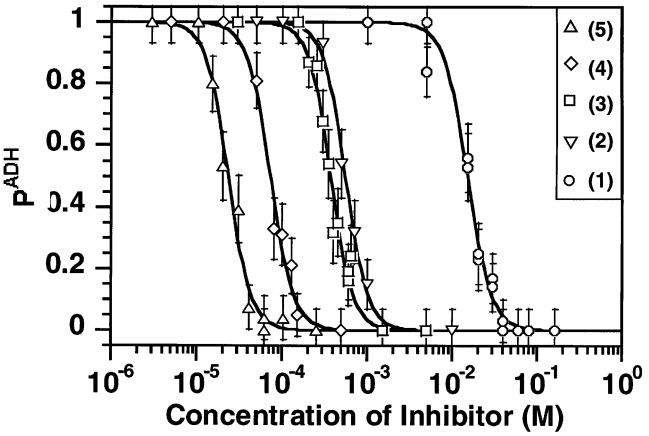


Figure 3. Plots of the probability of adhesion (P^{ADH}) of the microsphere to the erythrocyte as a function of the concentrations of different soluble derivatives of GlcNAc or NeuAc dissolved in the suspension of erythrocytes and microspheres. The different symbols represent experiments with different inhibitors; they are defined on the plot according to the entry number in Table 1 that corresponds to each compound. The curves are fits of the Hill equation with K_i^{OPTCOL} and Hill coefficient, n, as adjustable parameters (Equation 3).

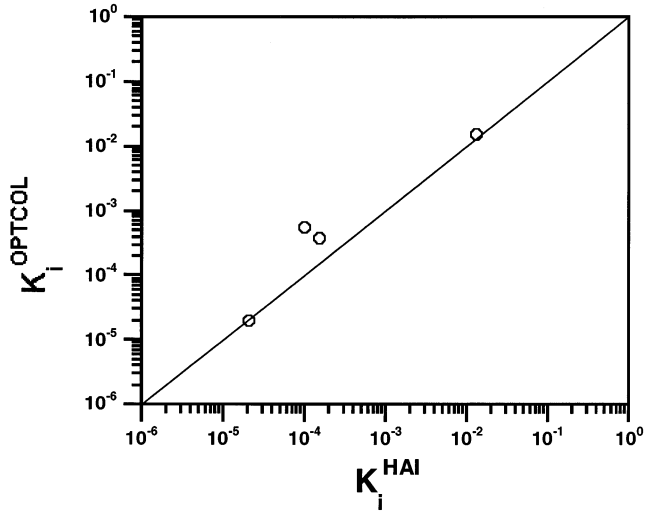


Figure 4. Plots of K_i^{HAI}, the concentration of soluble inhibitor that blocked 50% of hemagglutination by WGA, as a function of K_i^{OPTCOL}, the concentration of inhibitor that reduced P^{ADH} to 0.5. The symbols are defined on the plot with the entry number in Table 1 that corresponds to each inhibitor.

cooperativity for the adhesion of microsphere to the cell, or for the inhibition of adhesion; n has an upper bound value, N, the number of binding sites, for all-or-none binding in which the protein either has all or none of its binding sites occupied by the ligands. The values of K_i^{OPTCOL} and n for inhibitors **1**−**5** are listed in Table 1. The values of K_i^{OPTCOL} are in excellent agreement with values of K_i^{HAI} (the concentration of inhibitor required for 50% inhibition of hemagglutination of erythrocytes)[47] (Figure 4). The values of K_i^{OPTCOL} for inhibitors **3** and **4** were close to their dissociation constants; these values were determined using isothermal titration calorimetry.[46] For the fits plotted in Figure 3, the Hill coefficient, n, ranged from 3.1 to 3.3 ($\pm$ 0.4) (Table 1). The fact that the values were indistinguishable for different inhibitors suggests that the process that exhibited cooperativity was the adhesion of the microsphere to the cell, rather than the binding of the inhibitor to the WGA.

In general, the cooperative binding of lectins to cell surface receptors is believed to play a role in cross-linking and clustering of receptor with significant biological consequences.[17,44] Several studies of soluble ligands with WGA have, however, revealed that the binding data fit well a model that has four independent and equivalent binding sites in WGA (the crystal structure suggests there are eight possible binding sites[48,49]), and that there is no cooperativity in the binding of WGA to soluble ligands.[46,50,51] In contrast, positive cooperativity in the binding of WGA to erythrocytes was observed by Loverien and Anderson in their studies of WGA as an agent for controlling and protecting the morphology of human erythrocytes.[52] The cross-linked WGA structure revealed in the complex of WGA with a branched sialoglycopeptide also provided a structural basis for cooperative cell-lectin binding.[48] Our results also suggest that the binding of WGA to erythrocytes is cooperative, but that the binding of WGA to soluble ligands is not.

Conclusions

We have demonstrated that OPTCOL can be used to study protein−cell adhesion and to measure the potency of inhibitors that block the adhesion of proteins to the surface of biological cells. The inhibition constants derived from the measurements agree well with those obtained in hemagglutination inhibition assays, and with dissociation constants measured by isothermal titration calorimetry. Our experimental data from measuring the potency of inhibitors that block the adhesion of WGA-linked microspheres to erythrocytes suggest that the binding of WGA to erythrocytes is cooperative, but that the binding of WGA to soluble ligands is not. It is not obvious that a collision-based assay should necessarily correlate with an equilibrium (or quasi-equilibrium) assay, but the agreement of our results with those obtained from hemagglutination assays may be an indication that the collisions leading to sticking in OPTCOL are more closely related to the collisions that lead to the formation of a gel in hemagglutination. Further studies using OPTCOL may clarify the physical basis of hemagglutination.

OPTCOL assays have several useful characteristics for the study of adhesion of proteins to cells. OPTCOL is a quantitative technique that can be used to investigate biological interactions between two moving objects in a highly controllable fashion, since the relative orientation and velocities of the two colliding objects, as well as the strength and duration of the collisions, are all under the control of the user. As previously demonstrated by Mammen et al.,[39] OPTCOL makes it possible to measure dissociation constants of very tight-binding inhibitors, since very low concentration of biological objects (in principle, only one of each object), in a very low volume, is required for the measurement. OPTCOL is also a noninvasive assay; the viability of single cells, cell-to-cell variation, and cellular response can be monitored and studied during the measurement, if combined with other techniques such as fluorescent labeling.

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