Optically controlled collisions of biological objects to evaluate potent polyvalent inhibitors of virus–cell adhesion

Mathai Mammen[1], Kristian Helmerson[2], Rani Kishore[2], Seok-Ki Choi[1], William D Phillips[2] and George M Whitesides[1]

Background: The biochemical and biomechanical determinants of adhesion between two biological objects following a collision are complex, and may vary from one system to another. We wished to develop an assay in which all the relevant factors, including the components of the solution, the relative orientation and the relative collision velocity, are under the user's control.

Results: A new assay is described in which two mesoscale particles are caused to collide using two independently controlled optical tweezers (optically controlled collision, OPTCOL). This assay enables precise examination of the probability of adhesion under biologically relevant conditions. The OPTCOL assay was used to evaluate the probability of adhesion of a single erythrocyte to a single virus-coated microsphere, in the absence and presence of a sialic acid-bearing inhibitor. Inhibition constants for the most effective inhibitors could not be measured using other types of assays. The best inhibitor prevented attachment 50 % of the time at a sialic acid concentration of 35 pmol l^{-1}; it is the most potent known inhibitor of attachment of influenza virus to erythrocytes.

Conclusions: OPTCOL is a versatile new bioassay for studying dynamic interactions in biochemistry. It offers an approach to investigating interactions between moving biological objects that is both quantitative and interpretable. The simplicity of the OPTCOL technique suggests broad applicability to the study of adhesion of mesoscale (1–100 μm) objects in the areas of cell biology, microbiology, medicinal chemistry, and biophysics.

Addresses: [1]Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA and [2]National Institute of Standards and Technology, Gaithersburg, MD 20899, USA.

Correspondence: Kristian Helmerson and George M Whitesides
e-mail: kristian@enh.nist.gov, gwhitesides@gmwgroup.harvard.edu

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Introduction

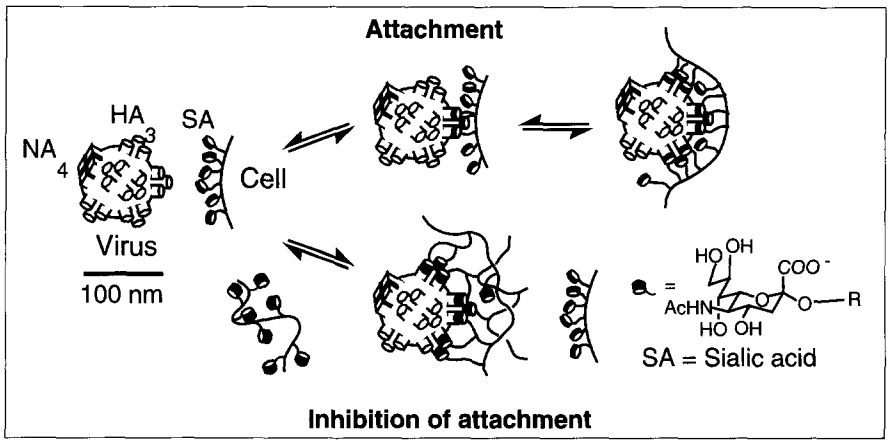
Optical tweezers use focused laser beams to trap and remotely manipulate dielectric particles, including cells and other biological objects [1–4]. Here we describe the use of dual optical tweezers to cause the collision of two mesoscale particles, and to measure the probability of their adhesion on collision. This measurement technique — optically controlled collision (OPTCOL) — is the basis for a new class of functional assays that provides information directly relevant to the dynamics of collision and adhesion.

To demonstrate the feasibility of OPTCOL, we have studied the inhibition of viral adhesion to a cell surface. The binding of influenza virus to its target cell (attachment) is the first step leading to invasion and infection. For the attachment of influenza to an erythrocyte (red blood cell) the interaction is specific for hemagglutinin (HA, a lectin found at 2–3 copies per 100 nm^2 on the surface of the virus) and sialic acid (SA, a 9-carbon sugar found at 150–200 copies per 100 nm^2 on the surface of the erythrocyte) [5]. The presence of SA-bearing molecules can inhibit viral attachment. Polyvalent, polymeric inhibitors that present multiple SA groups as side chains are particularly effective (Fig. 1) [5,6].

In this study, we caused a single microsphere covered with covalently bound influenza virus A (X-31) [7] to collide with a single chicken erythrocyte. Dual optical tweezers independently control the positions of the erythrocyte (a disk ~8 μm in diameter and 2 μm thick) and the microsphere (silica, 5 μm in diameter), their approach velocity, and their relative orientation. The probability of adhesion upon collision depends on both molecular and mechanical properties of the two colliding surfaces and of the solution (including the presence of any inhibitors of the interaction) [5]; using OPTCOL, we were able to measure this probability directly.

In the past, the inhibition of the viral adhesion to erythrocytes has been measured with techniques such as hemagglutination inhibition (HAI) assays [5]. During the HAI assay, erythrocytes fall under the influence of gravity to the base of the vessel, at a terminal velocity of ~5 μm s^{-1}. As the erythrocytes fall, they collide with viruses. If no inhibitor (or a concentration of inhibitor insufficient to block adhesion of viruses to erythrocytes) is present, viruses are 'captured' by the cell. When a sufficient quantity of viruses has been captured, the erythrocytes stick together, forming a macroscopic gel that serves as an indicator of

Figure 1



Schematic drawing of the attachment of influenza virus to a target cell, and inhibition of attachment by a polymeric, polyvalent inhibitor (see Fig. 3 for details). The hemagglutinin trimer (HA_3: 225 000 g mol^{-1} per trimer, with 200–300 trimers per virus) and the neuraminidase tetramer (NA_4: 240 000 g mol^{-1} per tetramer, ~20–60 tetramers per virus) protrude from the viral surface by ~110 Å and 60 Å, respectively. Both HA and NA have been demonstrated to be important in the binding of influenza virus to erythrocytes [10].

microscopic attachment. If sufficient inhibitor is present to block adhesion, the erythrocytes 'escape' these collisions and continue to fall to the bottom of the vessel.

For an assay such as hemagglutination to measure accurately the effectiveness of an inhibitor, the quantity of virus used in the assay must not deplete the concentration of this inhibitor. The concentration of the HA due to the virus ([HA]) must be small relative to the concentration of the SA ([SA]), otherwise the virus depletes the concentration of SA. In an extreme case, if [HA] >> [SA], the inhibitor can be highly effective (inhibiting attachment at extremely low concentrations), yet there may still be no inhibition of adhesion, since complete binding of the available SA to HA leaves most HA sites unblocked. Unfortunately there is a minimum usable concentration of virus (and therefore of HA) in the HAI assay; at [HA] < 1 nmol l^{-1}, the virus is unable to agglutinate the erythrocytes. Defining $K_i \equiv [SA]_{min}$ (the minimum concentration (referred to [SA]) of inhibitor that is effective at preventing attachment), the lowest measurable value of K_i in the HAI assay (K_i^{HAI}) is thus approximately equal to this minimum usable concentration of HA. Therefore all inhibitors with K_i^{HAI} < 1 nmol l^{-1} appear to be equally effective in the HAI assay.

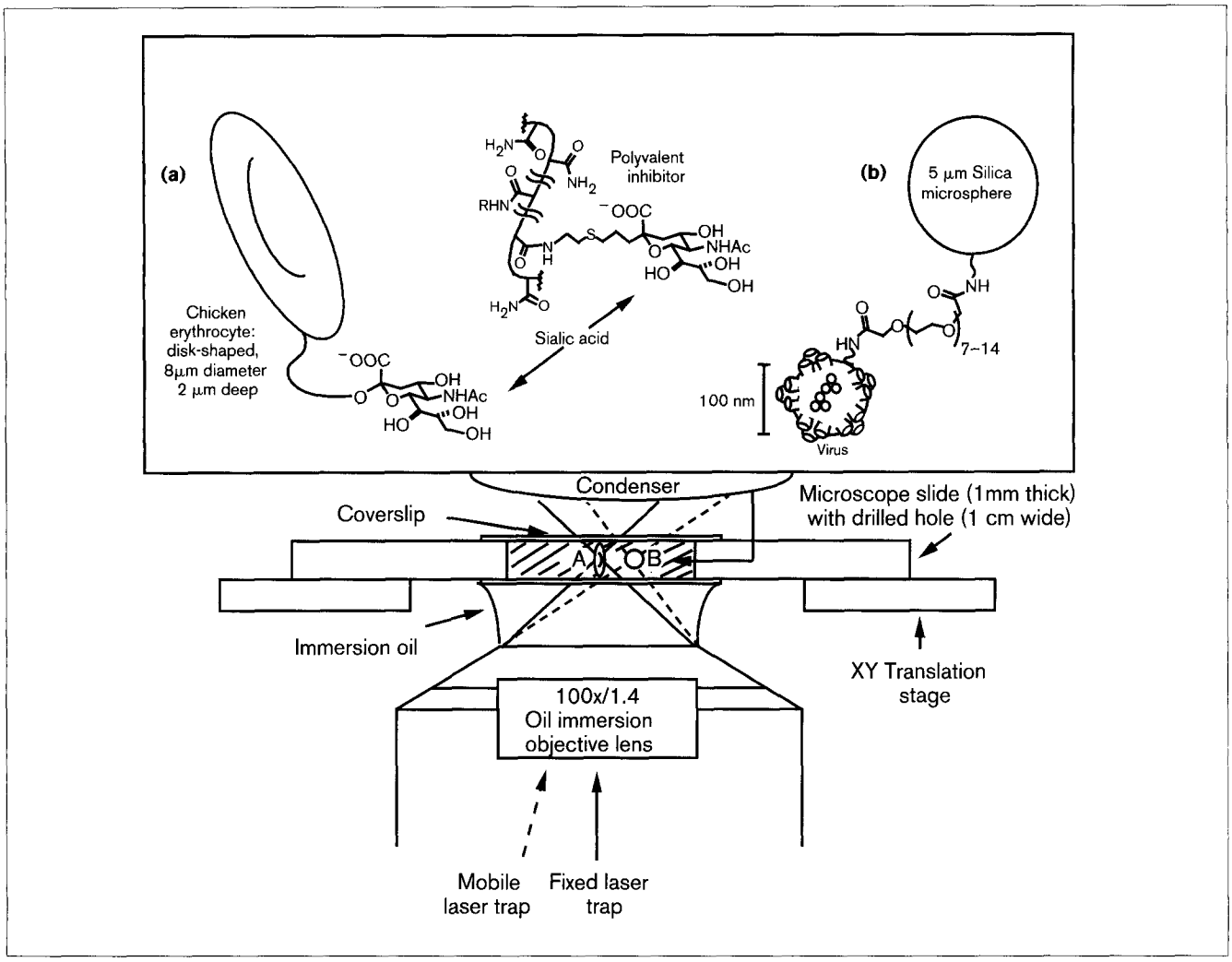
The key to the utility of OPTCOL in assaying highly effective inhibitors, where conventional assays fail, is that with OPTCOL the concentration of interactive particles can be made extremely low; in principle, one virus-coated microsphere and one erythrocyte in the experimental sample volume (100 µl in our case). This is much lower than the minimum concentration needed to observe hemagglutination.

Polyvalent, polymeric inhibitors (Fig. 2) can be >10^6 times more effective in inhibiting adhesion of virus to erythrocytes than analogous monovalent sialosides [5,6,8,9]. These polyvalent inhibitors function by a combination of high affinity binding to the surface of the virus, and steric stabilization of that surface [10]. The inhibition constants for these inhibitors have been measured by HAI assay. We examined three inhibitors that were so effective that HAI gave the lower bound of 1 nmol l^{-1} for all of them. Using OPTCOL, we were easily able to distinguish between them.

Results and discussion

We prepared silica microspheres (5 µm diameter) to whose surfaces influenza virus A (X-31) particles (approximately spherical in shape, 100 nm diameter) were covalently attached (see Materials and methods). Using dual, independent optical tweezers we measured the relative force required to separate a microsphere from an erythrocyte. This force is proportional to the optical power needed to keep the two particles trapped by their respective optical tweezers as they are separated. We inferred that the interaction between the virus-coated microsphere and erythrocyte was mediated by the HA–SA interaction from the following five experiments: (i) The virus-coated microsphere and the erythrocyte could not be separated even at maximum laser power, P > 500 mW (this value corresponds to a force greater than 200 pN); (ii) two polyethyleneglycol-modified microspheres (prepared as for virus coating but without the virus) could be separated at P < 12 mW; (iii) two virus-coated microspheres could be separated at P < 25 mW; (iv) the virus-coated microsphere could be separated from the erythrocyte (P < 25 mW) if the erythrocyte was pretreated with neuraminidase, an enzyme that specifically removes the sialic acid from the surface of the erythrocyte (treatment conditions were 30 min at 37 °C, pH 5); (v) if excess free virus was first added to the solution to bind to and cover the surface

Figure 2



A schematic diagram of the use of dual optical tweezers to control collisions (see text for details). Also shown are schematic drawings of **(a)** the erythrocyte (with SA covalently bound to various proteins and lipids embedded in the surface of the cell), **(b)** the microsphere (with virus tethered covalently to its surface) and (center) the polymeric, polyvalent inhibitor [5]. The erythrocyte is generally oriented in the trap so that its plane is along the direction of propagation of the laser beam; we believe that this orientation arises from asymmetry of the optical forces. The particles are held in a small vessel with a volume of ~100 μl.

of the erythrocyte, the virus-coated microsphere could be separated ($P < 25$ mW) from the erythrocyte.

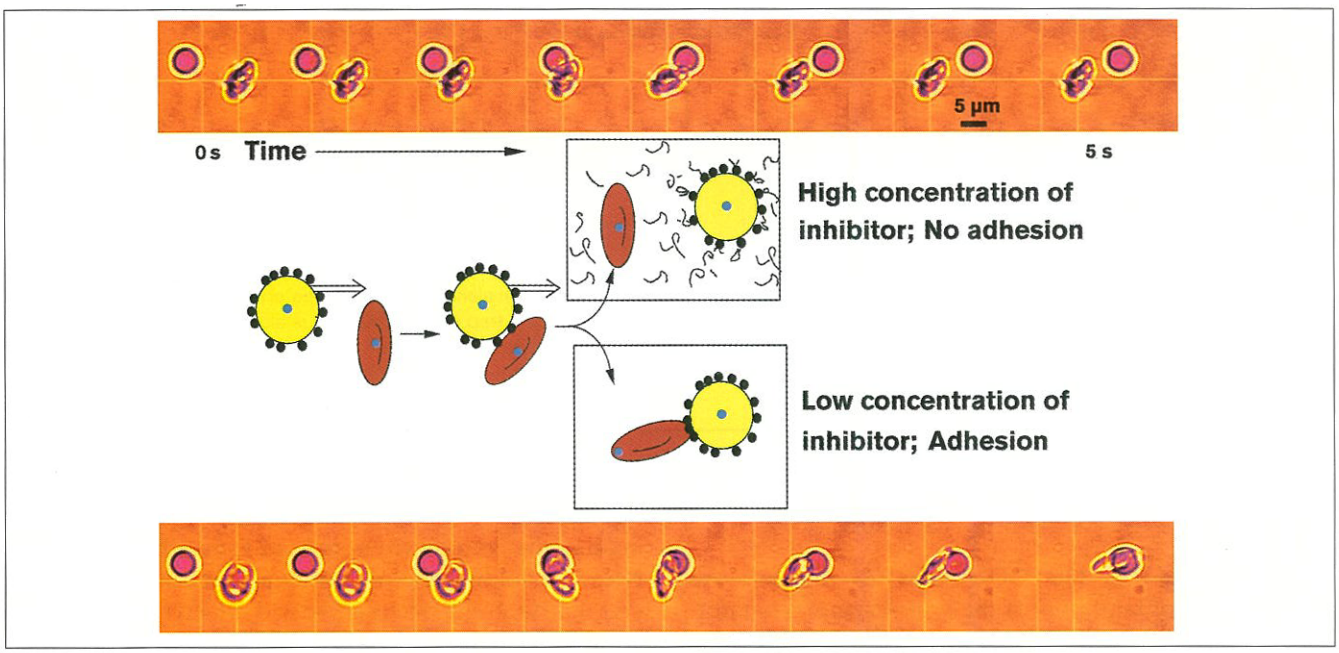
We next used the OPTCOL assay to evaluate the effectiveness of inhibitors of the virus-erythrocyte interaction through an isolated, controlled collision at a relative velocity of ~5 μm s^{-1}. The distance of closest approach of the centers of the colliding objects was chosen to be the radius of one microsphere. The microsphere and the erythrocyte were each held in optical tweezers with laser powers of 20 mW (as measured after passing through the objective lens), and their focuses located ~10 μm from the surface of the coverslip. The initial orientation of the plane of the erythrocyte is perpendicular to the collision plane; however, rotation of the erythrocyte often occurs during collision, which results in averaging over the possible orientations of the erythrocyte. The optical tweezers holding the erythrocyte were kept fixed while the optical tweezers holding the microsphere were moved past the erythrocyte. If the microsphere and the erythrocyte adhere after the collision, it is counted as a 'capture' event (see Fig. 3). The probability of capture is determined as a function of [SA] in solution. Figure 4a shows typical curves of probability of capture versus [SA] for three different inhibitors. We define K_i^{OPTCOL} to be that value of [SA] for which the capture probability is 50 %. Figure 4b compares values of K_i^{HAI} with K_i^{OPTCOL}. When K_i is >1 nmol l^{-1} (based on the concentration of SA groups), the value of K_i obtained using OPTCOL (K_i^{OPTCOL}) agrees very well with the K_i values obtained using the HAI assay (K_i^{HAI})

[5,11]. Inhibitors that had K_i values of < 1 nmol l^{-1} as measured by OPTCOL all gave the same values of K_i^{HAI}, even though the K_i^{OPTCOL} values were widely varying. Thus, the true K_i values could not be measured reliably using HAI assays, but were readily distinguished by the OPTCOL assay. One inhibitor in particular, a derivative of polyacrylamide [5] with 35 % of the side chains tethered to SA by a short flexible spacer (i.e., having a χ^{SA} value — moles of monomer containing SA divided by total moles of monomer — of 0.35) inhibited the interaction between influenza and erythrocyte at concentrations of sialic acid groups of 35 pmol l^{-1}. This polymer is therefore the most effective inhibitor of influenza viral attachment known: it is ~300 times better than equine α_2-macroglobulin, the most potent naturally occurring polyvalent inhibitor [12], and 10^8 times more effective than most monovalent derivatives of SA, which act exclusively through competitive binding and not through steric stabilization. For example, the value of both the inhibition constant of α-methyl O-glycoside of Neu5Ac (measured by HAI) and the dissociation of its complex with HA (as measured by fluorescence and NMR) is 2.5 mmol l^{-1} [13]. The inhibition constant for the monomer α-allyl C-glycoside of Neu5Ac was measured at 4 mmol l^{-1} using HAI.

These results demonstrate the usefulness of OPTCOL in a new type of assay that relies on the controlled collision of two mesoscale objects. This assay is particularly suited for biological systems that involve, or can be converted to, particulate form (cells, organelles, microspheres coated with molecules), and should be especially well suited for studies of polyvalency and of membrane-associated interactions. It remains to be investigated how the relative collision velocity, orientation (which we can fix using an asymmetric laser beam) and contact time influence the probability of adhesion.

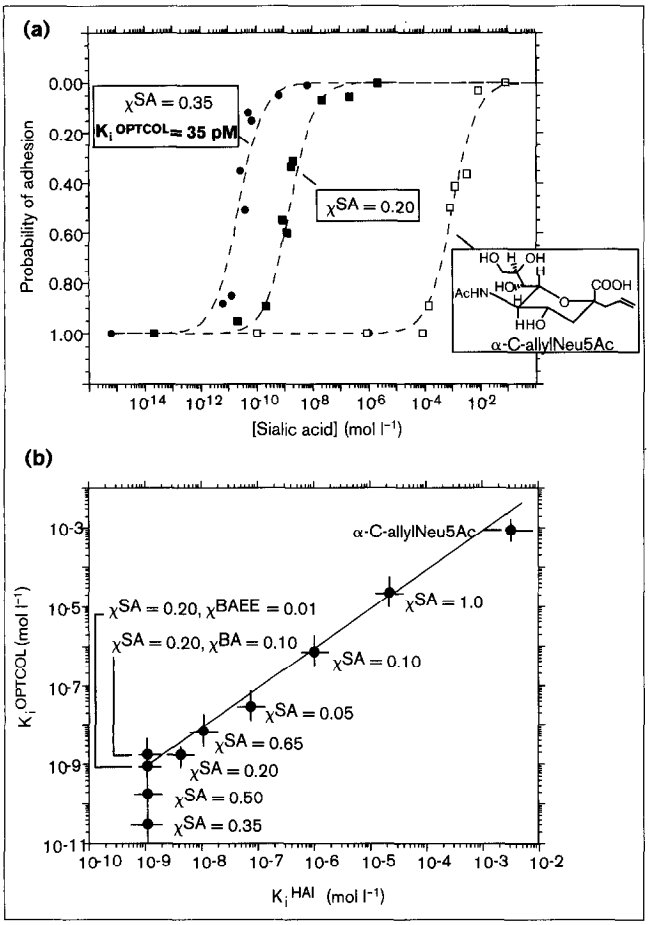
In addition to assaying the probability of adhesion, OPTCOL can be applied to biophysical investigations involving other parameters [14–19] such as deformation, compressibility and penetration depth. The OPTCOL method may complement the elegant methods based on deformation of membranes by Evans *et al.* [14] and other studies of cell adhesion using optical tweezers [20]. We have, for example, observed that adhesion of the virus-coated microspheres to the erythrocytes occurs preferentially at the edge of the disk-shaped cell. We have not established the origin of this phenomenon; it is, however, clearly relevant to the dynamics of biological adhesion,

Figure 3



Schematic representation of the OPTCOL assay. The virus-coated microsphere (in the mobile trap) is translated at a constant velocity and collides with the erythrocyte (in the stationary trap), above its center of mass; during this collision, the erythrocyte rotates. The erythrocyte remains in the stationary trap (the center of the righthand crosshair) as the microsphere collides with it. The microsphere continues to translate linearly at constant velocity until it reaches a point ~20 µm from the original position of the erythrocyte. If the concentration of inhibitor is sufficiently high, the cell loses its initial contact with the microsphere, and remains in the stationary trap as the microsphere translates past it. When no inhibitor, or too little inhibitor, is present, the cell adheres tightly to the passing microsphere, moves out of the stationary trap, and is 'captured.' A power of 20 mW was maintained in each of the two traps. Examining the probability of adhesion as a function of the concentration of the inhibitor's sialic acid groups yielded an inhibition constant. We performed multiple repetitions of collisions for multiple pairs of microsphere and erythrocyte. Micrographs of the virus-coated microsphere before and after collision with the erythrocyte are shown, with colors enhanced for clarity.

Figure 4



OPTCOL allows the accurate measurement of K_i values for highly potent polyvalent inhibitors. **(a)** Typical inhibition curves using OPTCOL showing probability of adhesion as a function of the concentration of inhibitor (expressed as the concentration of SA groups). The curves are fits of a simple binding isotherm (no cooperativity terms) with K_i as an adjustable parameter [5]. We conservatively estimate the uncertainty in our measurements by fitting the data with two limiting values of K_i that exclude most (90 %) of the data points. Probabilities were determined from at least 20 independent trials using 4–6 pairs of microsphere and erythrocyte. The values of K_i^{OPTCOL} are defined to be the value of [SA] for 50 % probability of adhesion. **(b)** The measured K_i^{OPTCOL} plotted against K_i^{HAI} show good agreement for $K_i^{OPTCOL} > 1$ nmol l^{-1}. BA = benzyl amine; BAEE = bis(aminoethyl)ether. The error bars corresponding to uncertainties in K_i^{HAI} correspond to plus or minus a factor of two; the values of K_i^{HAI} are obtained from [5]. For all inhibitors with $K_i^{OPTCOL} < 1$ nmol l^{-1}, K_i^{HAI} is ~1 nmol l^{-1}. The line shows the region in which $K_i^{OPTCOL} = K_i^{HAI}$.

and would be very difficult to investigate by most methods that are currently available. Microscopic techniques such as OPTCOL offer a new way to address the basis of phenomena of this kind.

Significance

The physical and chemical laws that determine the likelihood of stable interactions between moving mesoscale biological objects, such as cells, are complex.

We describe here a new method based on dual optical tweezers — optically controlled collisions, OPTCOL — for precise measurement of the probability of adhesion following collision under a wide range of conditions. Since the components of the solution, the relative orientation and the relative collision velocity are all under the user's control, OPTCOL can mimic closely the dynamics of a real encounter. OPTCOL can be used to examine collisions between both biological (e.g. cells, bacteria, viruses, ribosomes, liposomes, microspheres coated with biological objects) and non-biological (e.g. magnetic particles, colloids, organic clusters, crystals) objects.

Mean collision velocity is an example of a parameter that can vary substantially from one system to another, and is under precise control in the OPTCOL assay. An encounter between a neutrophil and an endothelial cell in fast flowing blood during recruitment to sites of inflammation is very different from a collision between sperm (moving very rapidly under its own power) and egg during fertilization. The encounter between a T cell and an antigen-presenting cell in the highly viscous matrix of a lymph node is also different from a T cell interacting with a foreign endothelial cell in rapidly moving mechanically pumped blood on the surface of a porcine-based human mitral valve transplant.

To our knowledge, OPTCOL is the only quantitative technique that can be used to investigate this class of biological interaction (that between moving particles). OPTCOL should open the way to a completely new type of mesoscopic-scale biological measurement.

Our study also illustrates another useful property of OPTCOL: it enables highly sensitive determinations of the effectiveness of inhibitors of the interaction between biological objects, as only a very low concentration of these objects (in principle, one of each in the entire sample vessel) is required for measurement. Using this assay, we were able to determine values of K_i for inhibitors of the influenza virus–erythrocyte interaction that could not be measured using other types of assays. The best inhibitor is significantly more potent than either the best natural or best synthetic inhibitor previously reported.

Materials and methods

Samples and sample vessels

The vessel was constructed by drilling a hole (~1 cm in diameter) in a glass slide (1 mm thick), and sealing the bottom of the vessel with a thin coverslip (0.085–0.13 mm thick) using silicone grease. Approximately 100 μl of sample was added, then the vessel was closed with a second coverslip. The sample solution was phosphate-buffered saline (pH 7.3) to which we added virus coated microspheres, erythrocytes, bovine serum albumin (BSA; 0.1 %) to minimize non-specific adsorption of microspheres and cells to the coverslip, and NaN_3 (0.03 %) to reduce

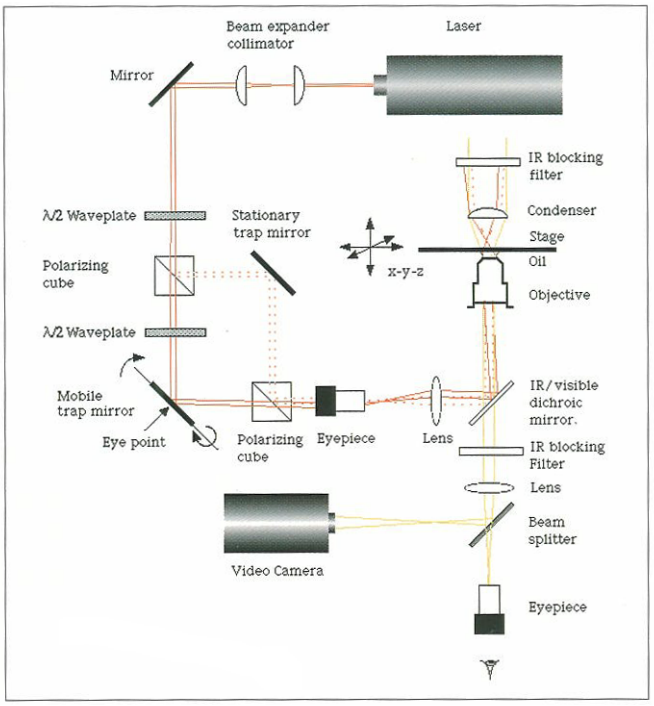
bacterial growth in the sample. The temperature of the sample did not rise more than 1 °C above the ambient room temperature, even after 1 h with the laser running at the combined power of 40 mW typically used in these experiments. The number of virus-coated microspheres used in a sample was typically such that the equivalent [HA] was ~10 fmol l^{-1}, thus limiting the sensitivity of our assay to inhibition constants greater than this value. This limit can be decreased further by increasing the volume of the sample.

Dual optical tweezer setup

The optical set-up used for the dual optical tweezers is shown in Figure 5. A beam from a linearly polarized, 2 Watt, 1.06 μm, Nd:YAG is expanded and collimated by a telescope and separated into two orthogonally polarized beams by the first polarizing beam-splitting cube; the relative amount of laser power in the two beams can be controlled by the first λ/2 waveplate. The stationary trap beam (dashed line) passes through the center of the eyepiece and lens and into the back of the high power (100x), high numerical aperture (1.4), oil immersion objective, which focuses the beam in the center of the field of view. The mobile trap beam (solid line) is passed through a second λ/2 waveplate, reflected off the mobile trap mirror and recombined with the stationary trap beam at a second polarizing cube. The second λ/2 waveplate and polarizing cube allow the intensity of the mobile trap beam to be adjusted independently of the fixed trap beam. The mobile trap mirror is located at the eye point of the eyepiece–lens–objective system, allowing the mobile trap beam focus to be smoothly and rapidly moved in the field of view, to any point within ~25 μm of the stationary trap [21]. The use of a dichroic mirror, which transmits visible light and reflects infrared, to couple the laser beams into the back of the objective lens allows the user to simultaneously manipulate and view the trapped objects, which are illuminated by the condenser.

The change in momentum of the light transmitted by the sphere exerts a restoring force on it; the sphere is thereby 'trapped' at the focal point of the objective lens [3,4]. The laser beam is split near its origin into two nearly parallel beams. The power of each beam is controlled independently. One focus (the fixed trap) is stationary at the center of the field of view. The motion of the second focus (the mobile trap) is determined by controlling precisely the angle of incidence of the laser beam into the back aperture of the objective lens [21].

Figure 5



A schematic diagram of the optical set-up for the OPTCOL technique. See Materials and methods for details regarding the optical components.

Preparation of virus-coated beads

Beginning with commercially available silica microspheres with aminopropyl groups on their surfaces (4 NH_2/100 $Å^2$), we first prepared an activated surface using the *bis* (*N*-hydroxysuccinimide) derivative of $HOOCCH_2(EG)_{7-14}CH_2COOH$ (Fig. 2). The activated microspheres were reacted with a concentrated solution of virus at pH 9, quenched with ethanolamine, and washed thoroughly with phosphate-buffered saline, pH 7.4. The quenching of the activated surface resulted in *N*-hydroxyethylcarboxamide-terminated polyethyleneglycol (PEG) groups, which help to reduce non-specific binding.

Preparation of polyvalent inhibitors

We prepared the inhibitors as in [5]. Briefly, a preactivated polymer (the poly(NHS)ester of poly(acrylic acid) was reacted sequentially with a limited number of equivalents of sialic acid tethered to a flexible linker, which is terminated with an amine (the ligand), then with a limited number of equivalents of a small organic amine, for example, benzyl amine (allowing modulation of molecular properties such as hydrophobicity), then with an excess of ammonium hydroxide (the quenching reagent, converting all unreacted esters into primary amides) to yield a polyfunctional, random co- or terpolymer. Virus coated microspheres are added to the inhibitors in buffer solution at the desired concentration and are allowed to incubate for 1 h. We then make a sample by adding erythrocytes to a small amount of the inhibitor and microspheres in solution.

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