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Polymeric Microtubes on Cell Surfaces*

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Orientation-Specific Attachment of Polymeric Microtubes on Cell Surfaces

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Understanding and controlling the interaction of living cells with synthetic micro or nanoparticles is becoming increasingly important for a number of biomedical applications, driven largely by the desire to limit cytotoxicity and produce more efficient drug delivery systems. To enhance drug delivery efficiency, for example, material characteristics such as particle shape and surface chemistry have been manipulated to tune the half-life, biodistribution and cellular uptake of synthetic particles.^[1, 2] From such studies it has been established that the local shape or orientation of a particle when it contacts a cell surface drastically alters the internalization rate.^[3, 4] Therefore of particular interest are strategies to control the orientation of a particle on a cell surface.^[5] Chemically non-uniform, Janus or 'patchy' particles have been utilized to control local orientation in colloid systems by presenting heterogeneously defined regions of attractive or repulsive chemistry.^[6] These simple building blocks can also self-assemble into a variety of higher order structures depending on the placement and interaction strength of the heterogeneous regions.^[7] In this work, we demonstrate a simple method to create functionally heterogeneous hollow microtubes whose interactions with living cells exploit both the effects of particle shape and

chemistry. The net result is an ability to control the orientation of a tube shaped microparticle on cell surfaces, thus opening up new design space for the fabrication of novel cell-biomaterial hybrids.

In addition to considering the characteristics and potential of synthetic particles as vehicles for drug delivery, the functionalization of a cell surface with synthetic materials to create cell-biomaterial hybrids is an evolving new method to control cell phenotype, fate and function.^[4, 8, 9] A logical extension of this approach is to attach synthetic particles directly to the surface of living cells to enhance their native functionalities and provide additional capabilities not found in nature.^[4, 9, 10] Toward this goal, prior work in our group has investigated the attachment of payload carrying cellular backpacks onto immune system cells for cell-mediated drug delivery, imaging and magnetic manipulation.^[4, 11] These disc-shaped cellular backpacks, which are microns wide and a few hundred nanometers thick, are able to resist internalization by macrophages due to their unique shape.^[4] The ability to attach functional, cargo carrying backpacks onto living immune cells without inducing phagocytosis provides new opportunities for extracellular targeted drug delivery. In this work, we explore the use of hollow microtubes with a controllable, non-uniform presentation of functional groups as a new type of biomaterial with the means to control particle orientation on cell surfaces.

Controlling the orientation of a microparticle on a cell surface requires the use of an anisotropic particle with distinct surface regions that promote and resist cellular attachment. Surfaces that are highly hydrated are commonly used to resist cell attachment,^[12] while surfaces presenting particular ligands promote cell attachment.^[2] Polyelectrolyte multilayers (PEMs) can achieve such surface characteristics simply by choice of polyelectrolytes and assembly conditions.^[12, 13] In this work, poly(acrylic acid)/poly(allylamine hydrochloride) (PAA/PAH) multilayers assembled at pH 3 were used to create microtube surfaces that resist cell interactions due to their highly hydrated nature.^[12] To create regions of the microtubes

that strongly interact with the lymphocyte B-cells used in this study, multilayers containing the biopolymers hyaluronic acid (HA) and chitosan (CHI) were used. Previous studies have shown that the B-cell CD44 receptor binds strongly and specifically to HA present in a film.^[13] HA containing multilayers have also been used to interact with other cell types including macrophages and T-cells.^[4, 11] Rhodamine (Rhod) labeled PAH and fluorescein (FITC) labeled CHI were used to identify the two different multilayer systems (depicted as red and green colors respectively in all figures).

To fabricate anisotropic, chemically non-uniform microtubes, we used a simple template approach involving the use of a sacrificial track-etched membrane.^[14-16] The PEM assemblies were designated as follows: (poly1/poly2)_z, where poly1 and poly2 represent the polymers used to assemble the multilayer and z is the total number of bilayers deposited (1 bilayer = poly1+poly2). The fabrication sequence for microtubes is shown in **Figure 1**. To fabricate chemically uniform tubes, the entire membrane, including the pores, was coated conformally with a PEM (Figure 1 step i).^[17] For completely cell-resistant tubes, multilayers of (PAA/PAH-Rhod) were assembled.^[12] The coated membrane was then plasma treated to remove the polymer selectively from the top and bottom surfaces of the membrane; previous work has demonstrated that the polymers within the pores are protected from the plasma etching process (Figure 1 step ii).^[15] Carbodiimide chemistry was then used to cross-link the PAH amine groups to the PAA carboxylic acid groups to form stable amide bonds. The membrane was then selectively dissolved and single component cell-resistant tubes filtered out (Figure 1 step iii).

To fabricate orientation-specific tubes, a cell-adhesive multilayer containing HA and CHI was deposited onto a membrane previously coated with (PAA/PAH-Rhod) (Figure 1 step iv). Another plasma treatment step removed the polymer film from the exposed surfaces, leaving the polymers only in the pores (Figure 1 step v). The resultant heterostructured tubes in the membrane pores have cell-adhesive multilayers on the inside of the tube and therefore

will be largely shielded from interacting with cells. However, as will be shown, when a cell approaches the end of the tube, the polymer from the interior of the tube is sufficiently exposed to participate in cell adhesion. The tubes were then cross-linked before the polycarbonate membrane was dissolved and the tubes filtered out (Figure 1 step vi). The fabrication method yields millions of stable tubes per membrane and aqueous suspensions containing around 10^6 tubes/mL. Scanning electron micrographs of typical microtubes are seen in Figure S1.

To investigate how the structure of the microtubes affects the orientation on cell surfaces, three microtube groups were fabricated and imaged with confocal microscopy (**Figure 2**). The first group (Figure 2a) consists of red labeled cell-resistant (PAA/PAH-Rhod) microtubes that were fabricated by ending at step (iii) in Figure 1. The second group (Figure 2b) consists of heterostructured microtubes with red labeled cell-resistant (PAA/PAH-Rhod) multilayers on the outside and green labeled cell-adhesive (HA/FITC-CHI) multilayers on the interior which were fabricated by continuing to step (vi) in Figure 1. The third group (Figure 2c) is produced by reversing the order of film deposition such that green labeled cell-adhesive (HA/FITC-CHI) multilayers are on the outside and red labeled cell-resistant (PAA/PAH-Rhod) multilayers are on the interior of the tube. Figure 2b shows the green FITC-CHI presence at both ends, and in most cases localized at the ends of the tubes. The clumping at the ends of the tubes is believed to be caused by the (HA/FITC-CHI) multilayer clogging or plugging the tube during fabrication. In Figure 2c the overlay images do not distinctly show the green FITC-CHI around the tube exterior surface since the layer containing FITC labeled chitosan is thin, but as seen in the inset, upon splitting into independent FITC and rhodamine channels, the full coating of chitosan is displayed. Additional images of the three tube types are shown in Figure S2.

To study the cell-microtube interaction, tubes suspended in water were added to a solution of B-cells. B-cells were chosen since they are non-adherent lymphocytes that are

integral to the adaptive immune response and thus are therapeutic targets in immune system diseases.^[18] After 1-2 hours confocal imaging was used to analyze attachment orientation.

The quantitative results shown in **Figure 3a** were obtained by analyzing over 100 tubes each in at least 3 different experiments and assigning each interaction to one of three possible categories; i) unattached, ii) end-on attachment or iii) side-on attachment. The first group of (PAA/PAH-Rhod) tubes minimally associated with cells due to the highly hydrated, non-adhesive nature of the (PAA/PAH-Rhod) multilayer and therefore >80% of the tubes were unattached to any cell. The second group of tubes with (HA/FITC-CHI) multilayers on the interior attached preferentially end-on, consistent with the cell interacting with HA on either end of the microtubes. The third group of tubes with (HA/FITC-CHI) multilayers on the outside preferentially attached side-on, consistent with the presentation of cell-adhesive regions on the outside of the tube. Confocal microscopy images of cell-tube interactions are shown in Figure 3b with larger images and a movie in the supporting information (Figure S3 and Movie S1). Approximately 50% of the tubes in groups 2 and 3 did not attach after 1-2 hours of agitation and incubation. This time was chosen as the analysis point since longer intervals of agitation and incubation led to increased attachment and the formation of large aggregates, limiting the ability to determine the attachment orientation. Conditions were not optimized to achieve the highest levels of tube attachment; future work focuses on immobilizing cells on a surface to minimize the aggregation potential. Nevertheless, these data clearly show a preference for microtube attachment based on the location of the cell binding (HA/FITC-CHI) multilayers. Figure 3c compares the ratio of tubes end-on to side-on and further supports the notion that the presentation of HA on the ends of the tubes favors end-on cell surface orientation. Some of the side-on orientation observed with the (HA/FITC-CHI) multilayers on the inside of microtubes may simply be due to the cell binding the HA from both ends of the tube at the same time. We are confident that HA is not diffusing through the (PAA/PAH-Rhod) outside multilayer, since previous work has documented the

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effective blocking properties of PAH-containing layers.^[19] Regarding the issue of cytotoxicity, Figure 3d shows that the microtubes did not decrease cellular viability.

In conclusion, we have developed a novel and reproducible fabrication method that allows for the presentation of a selected polymer preferentially on the ends of microtubes. Our method provides a general platform that could be expanded to many applications in biomaterials and responsive materials. In particular, we have shown in the present work that by limiting the cell-adhesive polymer to the end of the microtube, we can control the orientation of an anisotropic microtube on cell surfaces. These results point to the possibility of using microtubes as part of a bottom-up cell scaffold in the future. Controlling the interaction points on the microparticle may allow for structures such as linear or branched ‘polymerized’ chains of cells. In addition, regions of the microtube can be designed to respond to stimuli such as pH, salt or temperature, opening the possibility of stimuli sensitive polymer tubes for drug delivery or controlled assembly.^[14, 20] In general, we believe that controlling the orientation of microparticles on the cell surface could open new design space for applications in tissue engineering and drug delivery.

Experimental

Materials: PAA (Aldrich, M=450kDa), PAH (Aldrich, M=56kDa), HA (from Streptococcus equip, Fluka, M~1580 kDa), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Sigma), N-hydroxylsuccinimide (NHS, Sigma), NHS-Rhodamine (Pierce), Nuclepore track-etch polycarbonate membrane 3.0 μ m (Whatman), Fluorescein isothiocyanate (FITC, Sigma), Millex-LCR 0.45 μ m hydrophilic PTFE 25mm membranes (Millipore) and low molecular weight CHI (deacetylation 0.9, M=50-190kDa, Sigma) were used as received. FITC-chitosan was made from in a 1% aqueous acetic acid–methanol (1:2 v/v) solution of pH 4.5 at room temperature as described [21]. Rhodamine-PAH was synthesized by reaction

between PAH and NHS-rhodamine in 50mM MES buffer at pH 8 for 2hrs, after the solution was dialyzed into water. Detailed tube fabrication steps are given in supporting information.

Cell Study: CH27 B-cells were grown in RPMI media with 10% fetal calf serum and 1% penicillin/streptomycin. Before incubation with microtubes the cells were washed with Hanks Buffered Salt Solution and resuspended at 10^6 cells/mL in complete media. The tubes and cells were iteratively incubated on a shaker plate for 15 minutes at 100rpm and then incubated for 15 minutes for 1-2 hours before analysis. Confocal images were taken on a Zeiss LSM 510 using a 488nm Argon laser for FITC excitation and a 543nm HeNe laser for rhodamine excitation. The images were visually analyzed to determine the orientation of the microtube. A minimum of 100 interactions were analyzed per sample. Cell viability was determined by trypan blue staining.

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Supporting Information is available online from Wiley InterScience.

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Figure 1. Fabrication Scheme of Orientation Controlled Microtubes

Figure 2. Confocal microscopy images of heterostructured tubes dried on a slide along with cartoons of the structure. Red represents the cell-resistant rhodamine conjugated (PAA/PAH) multilayers and green represents the cell-adhesive FITC conjugated (HA/CHI) multilayers. a) (PAA/PAH-Rhod)₄₀ b) (PAA/PAH-Rhod)₄₀ outside and (HA/FITC-CHI)₄₀ inside c) (HA/FITC-CHI)₄ outside and (PAA/PAH-Rhod)₄₀ inside. Full image scale bars are 10µm and inset scale bars are 5µm.

Figure 3. Controlled Orientation of Microtubes on the Surface of B-cells: a) Samples were analyzed using confocal microscopy and microtubes with cell-adhesive (HA/CHI) on the inside caused more end-on attachment. b) Confocal images of cell tube interactions i) (PAA/PAH-Rhod)₄₀ ii) (PAA/PAH-Rhod)₄₀ outside and (HA/FITC-CHI)₄₀ inside iii) (HA/FITC-CHI)₄ outside and (PAA/PAH-Rhod)₄₀ inside. Scale bar 10 µm. c) Ratio of ‘End-on’ to ‘Side-on’ showing effect of tube structure d) Cellular viability of the B-cells after 24 hr incubation with tubes. Viability was not altered by the addition of (HA/CHI) outside tubes added at a 1:1 cell to tube ratio.

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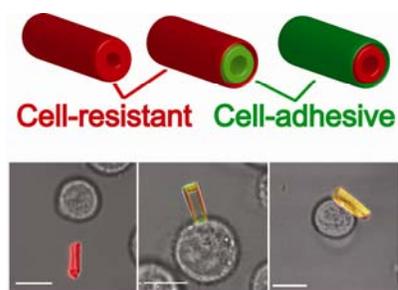
Tubular particles presenting heterogeneous regions of chemistry on the tube-ends versus the side are fabricated and are shown to control the particle orientation on the surface of live lymphocytes. Controlling the orientation of anisotropic microparticles on cell surfaces is of interest for biomedical applications and drug delivery in particular, since it can be used to promote or resist particle internalization.

Keywords: (polyelectrolyte multilayer, anisotropic, polymer, drug delivery, Janus)

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Title: Orientation-Specific Attachment of Polymeric Microtubes on Cell Surfaces

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Supporting Information

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Orientation-Specific Attachment of Polymeric Microtubes on Cell Surfaces

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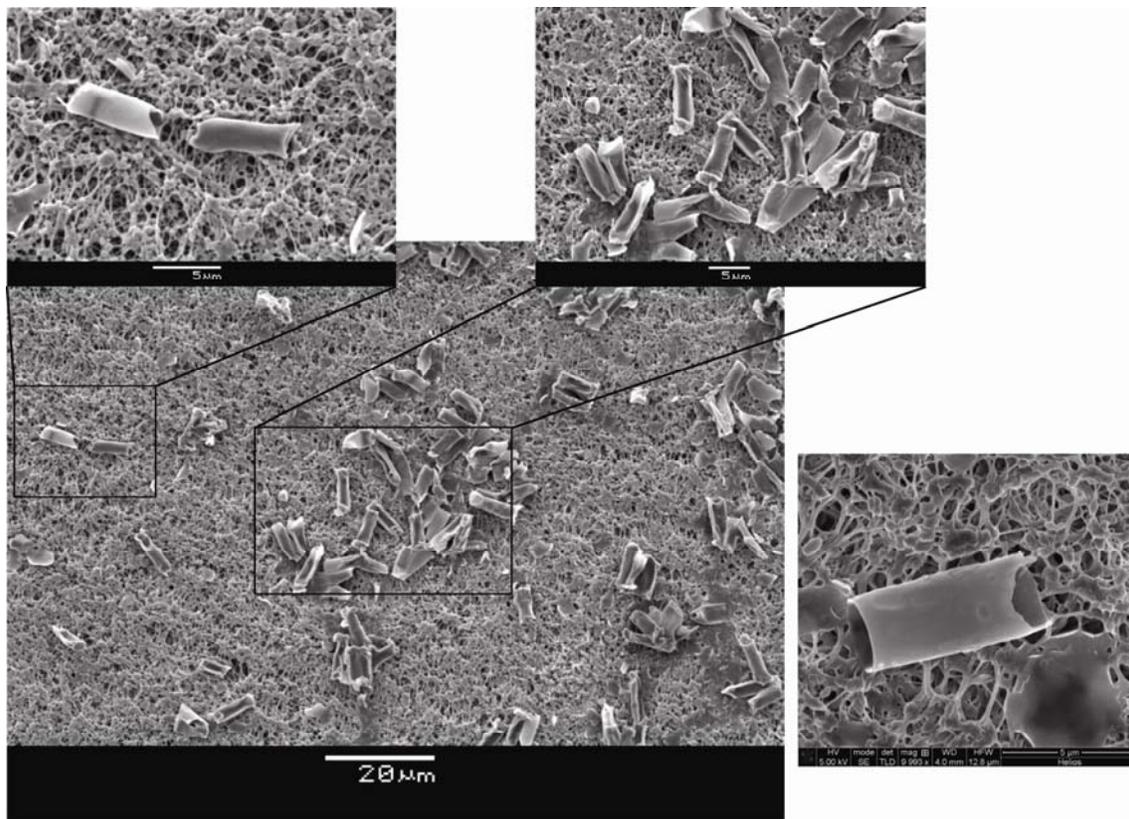


Figure S1. Scanning electron micrographs of cell tubes with (PAA/PAH-Rhod)₄₀ outside and (HA/FITC-CHI)₄₀ inside on the surface of a syringe filter.

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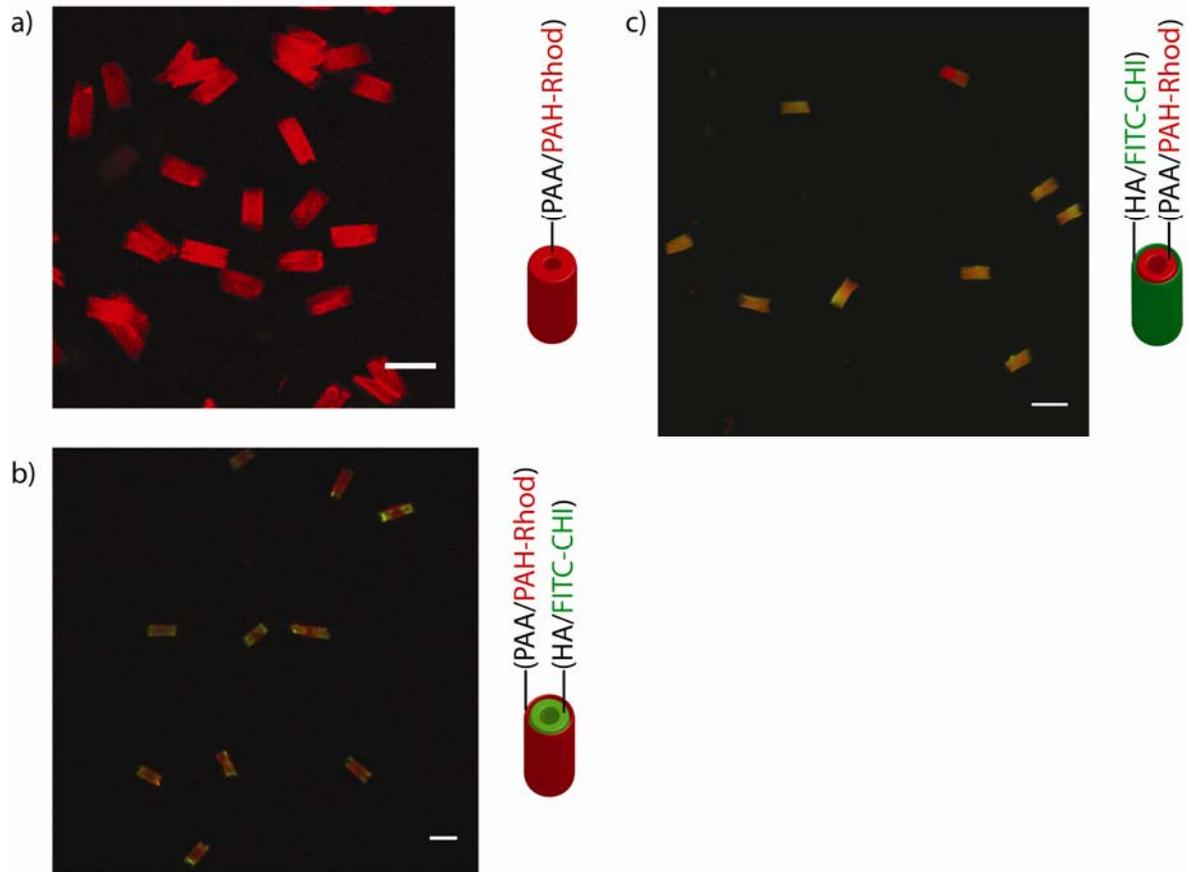


Figure S2. Confocal microscopy images of heterostructured tubes dried on a slide along with cartoons of the structure. Red represents the cell-resistant rhodamine conjugated (PAA/PAH) multilayers and green represents the cell-adhesive FITC conjugated (HA/CHI) multilayers. a) $(\text{PAA/PAH-Rhod})_{40}$ b) $(\text{PAA/PAH-Rhod})_{40}$ outside and $(\text{HA/FITC-CHI})_{40}$ inside c) $(\text{HA/FITC-CHI})_4$ outside and $(\text{PAA/PAH-Rhod})_{40}$ inside. Image scale bars are $10\mu\text{m}$.

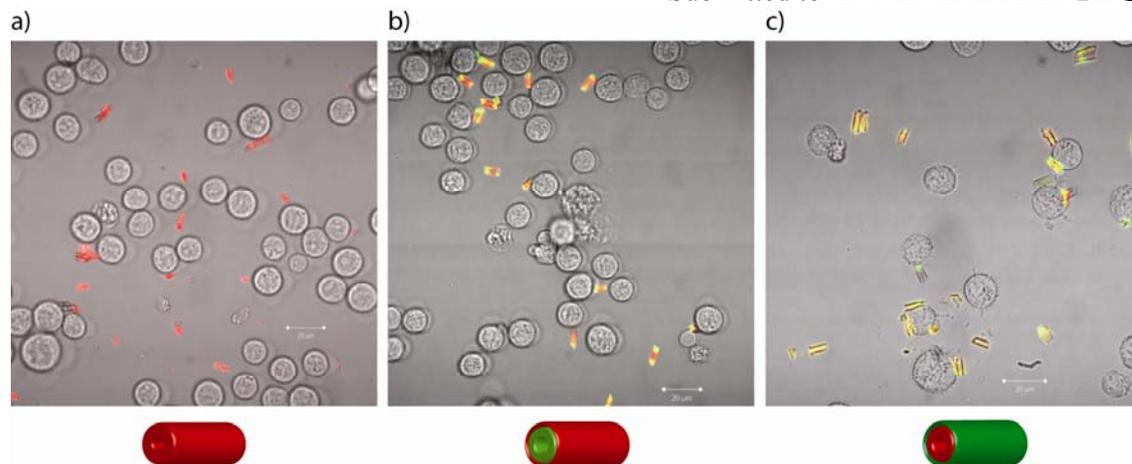
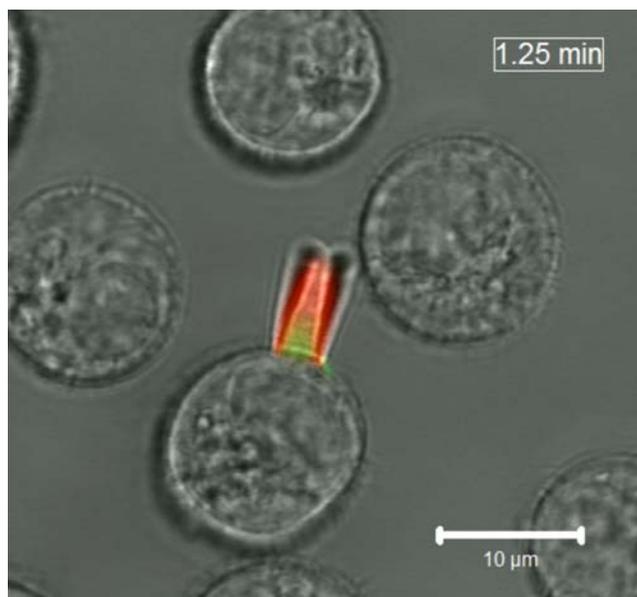


Figure S3. Representative confocal images of cell-tube orientation study after 1-2 hours a) (PAA/PAH-Rhod)₄₀ b) (PAA/PAH-Rhod)₄₀ outside and (HA/FITC-CHI)₄₀ inside c) (HA/FITC-CHI)₄ outside and (PAA/PAH-Rhod)₄₀ inside.



Movie S1. Movie of a B-cell interacting with a (PAA/PAH-Rhod)₄₀ outside and (HA/FITC-CHI)₄₀ inside microtubule

Supplemental Experimental Information:

Fabrication of Microtubes: Polymers solutions were made from Milli-Q 18.2MΩ water. Solutions of PAA and PAH-Rhod were 0.01M and solutions of HA and FITC-CHI were 0.1%

and 0.05% (w/v) respectively, FITC-CHI had 0.1M of acetic acid added to aid dissolution. HA and CHI solutions had 100mM NaCl added to the solution. All solution pHs were adjusted to pH 3.0 with 1M HCl. Track-etch polycarbonate membranes were immobilized on teflon holders and sequentially dipped in the polymer solutions using a nanoStrata dipping unit. Polymer depositions were done for 10 minutes, followed by three pH3 Milli-Q water rinses: one for 2 minutes and two for 1 minute, while the substrate was rotating at 100rpm. Both sides of the membrane were exposed to plasma etching simultaneously. The chamber was flushed with O₂ twice before igniting the plasma at 150 mTorr. (PAA/PAH-Rhod)₄₀ and (HA/FITC-CHI)₄₀ layers required 20 min and 60 min of etching respectively. Following plasma etching, the membrane was gently rubbed with a moist q-tip to fully remove the damaged film. The tubes were cross-linked by EDC/NHS at a concentration of 60mM EDC and 24mM NHS in 50mM MES buffer at pH 5 for 15 minutes. The membrane was dissolved in dichloromethane and the tubes filtered with a Millex-LCR filter before rinsing with 10mL of dichloromethane. The filter surface was wet with water and gently rubbed with a cell scraper for 5 minutes to transfer the microtubes. The concentration of tubes were measured using a standard hemocytometer on a fluorescent microscope. If aggregation occurs, light sonication can disperse the tubes.

Track-etch
poly(carbonate) membrane

