The Effect of Polymer Chain Length and Surface Density on the Adhesiveness of Functionalized Polymersomes

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Giant cell-like polymer vesicles, polymersomes, made from the diblock copolymer poly(ethylene oxide)−polybutadiene (PEO−PBD), have bilayer structures similar to the cell membrane but have superior and tunable properties for storage and stability. We have modified the terminal hydroxyl of the hydrophilic block with biotin−lysine (biocytin), a biologically derived group that imparts specific adhesiveness to a polymer colloid coated with avidin. The functionalized polymer will form vesicles, either on its own or when mixed with unmodified block copolymers that also form vesicles. The incorporation and mixing of the functionalized polymer into vesicle bilayers is measured using a fluorescent version of biocytin with confocal microscopy. The fluorescence signal associated with the vesicle is in proportion with the concentration of functional polymer added during vesicle construction. The adhesiveness of polymer vesicles containing functionalized biotinylated polymer to avidin coated microspheres is measured with micropipet aspiration. Two types of polymer vesicles were constructed: one where the functionalized polymer (molecular weight (MW), 10 400 Da) was longer than the surrounding unfunctionalized polymer (MW, 3600 Da) and one where the functionalized polymer (MW, 10 400 Da) was the same length as the unfunctionalized polymer. In all cases, the avidin−biotin bonds form kinetically trapped crossbridges that impart little tension as they form but require significantly more tension to break. The relative length of the functionalized polymer on the surface of the vesicle is an important determinant for the adhesion of a polymer vesicle but not for the adsorption of soluble avidin. Greater adhesion strengths are seen where the functionalized polymer is longer than the surrounding polymer. The concentration of functionalized polymer at which adhesion is maximal depends on the relative lengths of the polymers. When the functionalized polymer is the same length as the surface brush of the polymersome membrane, the critical tension is maximal at 10 mol % functionalized polymer concentration. However, when the biocytin groups are attached to a polymer which is larger than the surface brush, the critical tension is maximal at 55 mol % functionalized polymer. These results indicate that polymer mixing and length can control the interfacial adhesion of polymer brushes and must be understood to tune polymersome adhesiveness.

Introduction

Gene therapy and drug delivery are important areas of research, and the development of sophisticated delivery devices for treatment or diagnostic purposes remains a challenging area of research. In recent years, cell-like vesicles[1] made from purely synthetic block copolymers, polymersomes,[2] have gained increased attention because of their unique properties. Synthetic block copolymers can self-assemble into bilayer structures, similar to the plasma membrane of cells, but form vesicles that have superior material properties for storage and stability.[3] Several of the material properties, such as the area elastic and bending moduli, of the polymersome and phospholipid bilayer membranes have been studied using micropipet aspiration.[2,4−9] The bilayer membrane can deform and stretch under tension when the membrane is fluidlike. Polymersomes are tougher than phospholipid vesicles,[2] as the area strain achievable by a polymersome membrane is an order of magnitude higher than that of a liposome made of diacyl phospholipid and depends on the molecular weight (MW) of the polymers that comprise the membrane. The similarity in structure between the cell-like polymer vesicles and cells provides the opportunity to use polymersomes as models to investigate membrane mediated processes, such as adhesion.

Micropipet aspiration is a useful technique for studying the adhesion[10−16] of bilayer membranes. Red blood cells,

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(1) Hammer, D. A.; Discher, D. E. *Annu. Rev. Mater. Sci.* **2001**, *31*, 387−404.

(2) Discher, B. M.; Won, Y. Y.; Ege, D. S.; Lee, J. C.; Bates, F. S.; Discher, D. E.; Hammer, D. A. *Science* **1999**, *284*, 1143−1146.

(3) Lee, J. C.; Bermudez, H.; Discher, B. M.; Sheehan, M. A.; Won, Y. Y.; Bates, F. S.; Discher, D. E. *Biotechnol. Bioeng.* **2001**, *73*, 135−145

(4) Evans, E.; Yeung, A. *Chem. Phys. Lipids* **1994**, *73*, 39−56.

(5) Evans, E.; Needham, D. *J. Phys. Chem.* **1987**, *91*, 4219−4228.

(6) Bo, L.; Waugh, R. E. *Biophys. J.* **1989**, *55*, 509−517.

(7) Bozic, B.; Svetina, S.; Zeks, B.; Waugh, R. E. *Biophys. J.* **1992**, *61*, 963−973.

(8) Waugh, R. E.; Song, J.; Svetina, S.; Zeks, B. *Biophys. J.* **1992**, *61*, 974−982.

(9) Heinrich, V.; Waugh, R. E. *Ann. Biomed. Eng.* **1996**, *24*, 595−605.

(10) Evans, E. A. *Biophys. J.* **1985**, *48*, 175−183.

(11) Evans, E. A. *Biophys. J.* **1985**, *48*, 184−192.

(12) Evans, E.; Berk, D.; Leung, A. *Biophys. J.* **1991**, *59*, 838−848.

(13) Evans, E.; Berk, D.; Leung, A.; Mohandas, N. *Biophys. J.* **1991**, *59*, 849−860.

(14) Berk, D.; Evans, E. *Biophys. J.* **1991**, *59*, 861−872.

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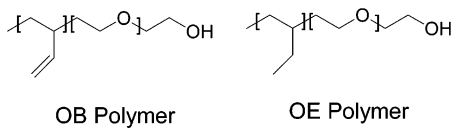

Figure 1. Chemical structures of polymers. The structure on the left-hand side is a representative structure of a poly(ethylene oxide)−polybutadiene polymer, and the structure on the right-hand side is a representative structure of a poly(ethylene oxide)−polyethylethylene polymer.

Table 1. Vesicle Formation Block Copolymers

designation	block copolymer	AMW[a] (Da)	F_{EO}[b]
OB-2	$PEO_{26}-PBD_{46}$	3600	0.28
OB-18	$PEO_{80}-PBD_{125}$	10400	0.29
OE-21	$PEO_{40}-PEE_{37}$	3900	0.39

[a] Average molecular weight. [b] Volume fraction of poly(ethylene oxide).

liposomes, or liposomes grafted with poly(ethylene glycol) (PEG) have been used to study the adhesion via receptor−ligand interactions. The adhesion between biotin and avidin has been investigated using phospholipid vesicles; however, the liposome bilayer membrane is not strong enough to sustain the tension required to separate the contact formed by biotin and avidin.[16] Presumably, the liposome membrane undergoes cohesive material failure before the avidin−biotin bond ruptures. The superior material strength of polymer vesicles thus affords us the opportunity to investigate the role of avidin−biotin ligation on adhesive strength.

Previous researchers have used micropipet aspiration to characterize the adhesion strength between strongly and weakly bound cells. The adhesion strength of weakly bound cells was found to follow the Young−Dupre equation, in which adhesion is in thermodynamic equilibrium[10] and spreading and peeling can occur with equal increments of mechanical energy. Strongly adherent cells were found to be "kinetically trapped";[11] in such cells, a negligible tension is generated even when the contact is formed, but a large tension is required to separate the contact zone when it is large. In the kinetically trapped regime, the appropriate metric of adhesion is the critical tension required to peel the membrane from a surface.

Here, we used micropipet aspiration to disengage a biotinylated polymer vesicle from an avidin coated microsphere. We tested the relationship between adhesion strength and the composition of the polymer vesicle. A long polymer of poly(ethylene oxide)−polybutadiene ($PEO_{80}-PBD_{125}$) (Figure 1 and Table 1), with a molecular weight of 10 400 Da and an ethylene oxide volume fraction of 0.29, was functionalized with biotin. The polymer, OB-18b, was mixed with unfunctionalized OB-18 or unfunctionalized OB-2, a smaller polymer with a molecular weight of 3600 Da and an ethylene oxide volume fraction of 0.28. By measuring the adhesion strength of mixtures of these polymers, we systematically assessed the effect of polymer chain length and density on the strength of adhesion. We found that polymer vesicles in which the functionalized polymer is longer than the membrane brush size (OB-18b/OB-2 polymer vesicles) have a vastly superior adhesivity to avidin coated microspheres than the case of when the functionalized polymer is the same size as the background polymer (OB-18b/OB-18). Thus, we have identified the optimal design of functionalized polymer vesicles for maximal adhesion.

Materials and Methods

Materials. Poly(ethylene oxide)−polybutadiene OB2 ($EO_{26}-BD_{46}$) and OB18 ($EO_{80}-BD_{125}$) and poly(ethylene oxide)−polyethylethylene OE-21 ($EO_{40}-EE_{37}$) block copolymers were synthesized according to the protocol from Hillmyer and Bates.[17] Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride); biocytin (free base); avidin−rhodamine isothiocyanate; methyl alcohol (99.8% anhydrous); dichloromethane (99.9% ACS HPLC grade); pyridine (99.9% ACS reagent); phosphate buffered saline (PBS) tablets (pH 7.4 at 25 °C); bovine serum albumin (BSA) (minimum 98% (electrophoresis)); and sucrose (ACS reagent) were purchased from Sigma-Aldrich (St. Louis, MO). A Slide-A-Lyzer dialysis cassette kit (10K molecular weight cutoff) and hydrochloric acid (certified ACS plus) were purchased from Pierce (Rockford, IL) and Fisher Scientific (Pittsburgh, PA), respectively. Texas Red hydrazide, Alexa Fluor 488 streptavidin, and Alexa Fluor 488 biocytin (disodium salt) were purchased from Molecular Probes (Eugene, OR). All materials were used as received.

Synthesis of Tresylated Block Copolymers. The protocol used to tresylate the ethylene oxide block is adopted from Nilsson and Mosbach.[18−20] In brief, block copolymers were dissolved in dichloromethane at 0 °C. A mixture of pyridine and tresyl chloride was added dropwise and allowed to react at room temperature for 90 min. The organic solvent was evaporated, and the product was precipitated using anhydrous methanol and hydrochloric acid until no pyridine was detected under UV spectrophotometry. NMR (chloroform-*d* as solvent) was used to verify the tresylation of block copolymers (data not shown). OE-21 was chosen for the verification of the tresylation protocol because of the large quantity in storage and the NMR spectrum of the OE polymer that is documented in the work of Hillmyer and Bates.[17] The NMR spectra of pure OE-21 has peaks at 0.8 ppm (singlet), 1.1 ppm (singlet), 1.3 ppm (doublet), and 3.65−3.80 ppm (multiple peaks); pure tresyl chloride has peaks at 4.4 ppm (triplet). The NMR spectra of the tresylated OE-21 polymer had peaks at the locations of pure OE-21 and pure tresyl chloride.

Functionalization and Formation of Polymersomes. Biotinylated polymersomes for adhesion experiments were formed by reacting 50 μg of the tresylated polymer with a 2:1 excess of biocytin in anhydrous methanol overnight at 4 °C. A thin film of polymer was deposited on the bottom of a vial by evaporation, and polymersomes were formed by rehydration with 1 mL (300 mOsm) of sucrose solution (osmometer model 3300, Advance Instruments, Norwood, MA). Excess biocytin molecules in the external solution were removed by dialysis using the 10K MW cutoff Slide-a-Lyzer dialysis cassettes purchased from Pierce (Rockford, IL). Vesicle solution was injected into the dialysis cassette with a syringe, and the cassette was immersed in a beaker filled with iso-osmotic PBS solution with soluble neutravidin to reduce the concentration of free biocytin to less than one-thousandth of the dissociation equilibrium constant ($K_D = 10^{-14}$ M) of biotin and avidin. Vesicles were subsequently removed from dialysis and used immediately for adhesion or bulk avidin adsorption measurements.

Biotinylated vesicles for measuring the adsorption of avidin from solution were incubated with an excess of avidin−rhodamine isothiocyanate in PBS overnight. Excess avidin was removed by dialysis in 100K MW cutoff membranes against clean PBS. The resulting samples were imaged with confocal microscopy.

Alexa Fluor biotin polymersomes for confocal experiments were formed by reacting tresylated polymer with a 2:1 excess of Alexa Fluor biocytin in anhydrous methanol overnight at 4 °C. The polymer was dried and redissolved in chloroform, followed by three successive washes with PBS to remove excess Alexa Fluor biocytin. The Alexa Fluor biocytin polymer was mixed in varying ratios with unmodified polymer in chloroform and dried to a uniform film. The film was rehydrated with 300 mOsm of sucrose to form vesicles. This solution was subsequently diluted with iso-osmotic PBS to provide contrast for imaging.

(15) Evans, E.; Leung, A. *J. Cell Biol.* **1984**, *98*, 1201−1208.
(16) Noppl-Simson, D. A.; Needham, D. *Biophys. J.* **1996**, *70*, 1391−1401.
(17) Hillmyer, M. A.; Bates, F. S. *Macromolecules* **1996**, *29*, 6994−7002.
(18) Nilsson, K.; Mosbach, K. *Methods Enzymol.* **1984**, *104*, 56−69.
(19) Delgado, C.; Patel, J. N.; Francis, G. E.; Fisher, D. *Biotechnol. Appl. Biochem.* **1990**, *12*, 119−128.
(20) Delgado, C.; Francis, G. E.; Fisher, D. *Crit. Rev. Ther. Drug Carrier Syst.* **1992**, *9*, 249−304.

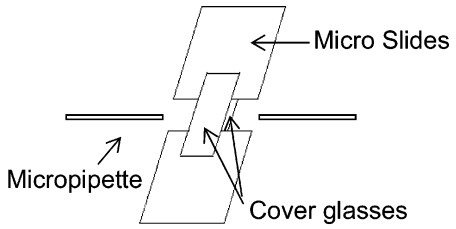

Figure 2. Schematic diagram of an aspiration chamber (not to scale). A reusable chamber frame was made by using a metal paper clip (not shown in the diagram) to connect two microscope slides, 25 mm (width) x 32 mm (length), together. A clean chamber is prepared for each experiment by cutting cover glasses into narrow strips, 5 mm × 18 mm, and fixing them in place with a small amount of vacuum grease. The cover glasses form the ceiling and the floor of the chamber. The volume of the chamber is ∼40 μL. The side of the chamber is open for accessing the micropipets.

Confocal Microscopy. Confocal images of the polymersomes were taken using a Bio-Rad 2000 KR-3MP (Hercules, CA) system equipped with a Nikon TE 300 fluorescent microscope. Images were processed and analyzed using the Scion Image Beta 4.0.2 program from Scion Corporation (Frederick, MD). Quantification of fluorescent edge intensity utilized a 3 × 3 median filter to remove noise before comparison of the fluorescent intensity at the edge of the polymersomes to their interior. Detector linearity and the validity of the processing technique were assessed by the analysis of biotin coated or avidin coated microspheres incubated with serial dilutions of fluorescent ligand with unlabeled ligand. The resulting analysis indicates a linear response in the edge intensity of the beads with the concentration of labeled ligand.

Micropipet Aspiration. Micropipet aspiration is described in detail in many papers.[5−16,21−25] Concisely, micropipets made of borosilicate glass tubing (Friedrich and Dimmock, Milville, NJ) were made using a needle/pipet puller (model 730, David Kopf Instruments, Tujunga, CA) and microforged using a glass bead to give the tip a smooth and flat edge. The inner diameters of the micropipets used ranged from 1 μm to 6 μm. The inner diameter of the micropipets was measured using computer imaging software. Micropipets were used to pick up both polymersomes and microspheres (superavidin coated microspheres, Bangs Laboratories, Inc., Fishers, IN) and apply tension to polymersome membranes. Micropipets were filled with PBS solution and connected to two aspiration stations. Each aspiration station was mounted on a side of a Nikon Diaphot inverted microscope, and each station was equipped with a monometer, two Validyne pressure transducers (models DP 15-32 and DP 103-14, Validyne Engineering Corp., Northridge, CA), two digital pressure read-outs, micromanipulators (model WR-6, Narishige, Tokyo, Japan), and MellesGriot millimanipulators (course *x*,*y*,*z* control). Suction pressure was applied by syringes connected to the monometers. Experiments were performed in PBS solutions that had osmolalities of 350 mOsm in order to make the polymersomes flaccid. The osmolalities of the solutions were measured using an osmometer. Since the sucrose and PBS solutions have different densities and refractive indices, the polymersomes settle and are visible with phase-contrast or Hoffman optics.

Adhesion Experiment. Adhesion experiments were performed inside a chamber (Figure 2) made from microscope cover glasses, 18 mm (width) × 18 mm (length), from Fisher Scientific (Pittsburgh, PA) and microscope slides from Corning Glass Work (Corning, NY). A reusable chamber frame was made by using a metal paper clip to connect two microscope slides (25 mm × 32 mm) together. A clean chamber is prepared for each experiment by cutting cover glasses into narrow strips (5 mm × 18 mm) and fixing them in place with a small amount of vacuum grease. The cover glasses form the ceiling and the floor of the chamber. The volume of the chamber is ∼40 μL. The side of the chamber is open so that micropipets could assay the interior compartment of the chamber. The tip of each micropipet was immersed in 0.5 wt % BSA solution (0.005 g of BSA in 1 mL of distilled water) for ∼30 s to prevent nonspecific adhesion of the aspirated object to the tip of the micropipet. The surfaces of the chamber were coated with 0.5% BSA solution before introducing the microsphere/polymersome solution, preventing vesicles from nonspecifically adhering to the glass walls. A polymersome and a microsphere were picked up using two micropipets that were mounted coaxially and facing each other. After the polymersome and microsphere pair were brought into contact, the tension on the polymersome was decreased gradually to allow the contact zone to form. The surfaces were left undisturbed for 15 min to allow the polymersome surface ligands, biocytin, to bind to the complementary surface groups on the microspheres, superavidin. After 15 min, the tension on the polymersome was increased gradually, and the events were recorded using optical video microscopy. The tension at which the polymersome and microsphere no longer adhered was labeled as the critical tension. Experiments were recorded using a video CCD camera (model 4915, Cohu Inc., San Diego, CA) and a Sony SVO-9500MD VTR (Sony Medical System, Montvale, NJ). Images for analysis were retrieved afterward from the recorded tapes using the IMAQ software from National Instruments for subsequent analysis. Image analyses were done via the computer software Scion Image from Scion Corporation (Frederick, MD).

Results

Formation of Functional Vesicles and Controlling the Surface Concentration of Biocytin. The incorporation of the functionalized polymer into the final vesicular structures is verified in a series of experiments using polymer functionalized with fluorescent biotin. To systematically vary the surface concentration of biotin, the functionalized polymer is mixed with unfunctionalized polymer in chloroform before drying to form a uniform polymer film. Alexa Fluor 488 biocytin functionalized OB-18 (OB-18b) polymer is mixed with either OB-2 or OB-18 at concentrations of 0, 10, 20, 50, 80, and 100% OB-18b (by mass). The resulting polymersomes were imaged using confocal microscopy to assess the relative incorporation of functionalized polymer into the polymersomes (Figure 3). Each series was analyzed sequentially with the same instrumental settings to minimize the effect of laser intensity or detector sensitivity fluctuations. Figure 3A shows four confocal images of labeled OB-18 in an OB-18 membrane at different concentrations. The amount of fluorescence was quantified by measuring the peak intensity of each polymersome above any fluorescence detected in its aqueous core. Figure 3B is the quantification of the images in Figure 3A and an analogous series of OB-18b in OB-2 vesicles. The edge brightness increases proportionally to the amount of modified OB-18 polymer added, indicating the surface concentration of biotin may be effectively varied by changing the concentration of polymer in the film used to make the vesicles. Figure 3 demonstrates that the modification of the OB and OE block copolymers using the protocol of Nilsson and Mosbach[17] is successful. The ability of the block copolymers to self-assemble into polymersomes does not appear to be affected by modification with either biocytin, tresyl chloride, or rhodamine (data not shown).

Adsorption of Avidin onto Functionalized Polymersomes from Bulk Solution. To verify the availability of biotin on the polymersome surface to bind to avidin, biotin labeled polymersomes were incubated with

(21) Kim, D. H.; Klibanov, A. L.; Needham, D. *Langmuir* **2000**, *16*, 2808−2817.
(22) Evans, E.; Klingenberg, D. J.; Rawicz, W.; Szoka, F. *Langmuir* **1996**, *12*, 3031−3037.
(23) Bermudez, H.; Brannan, A. K.; Hammer, D. A.; Bates, F. S.; Discher, D. E. *Macromolecules* **2002**, *35*, 8203−8208.
(24) Lee, J. C.-M.; Santore, M.; Bates, F. S.; Discher, D. E. *Macromolecules* **2002**, *35*, 323−326.
(25) Bermudez, H.; Hammer, D. A.; Discher, D. E. *Langmuir* **2004**, *20*, 540−543.

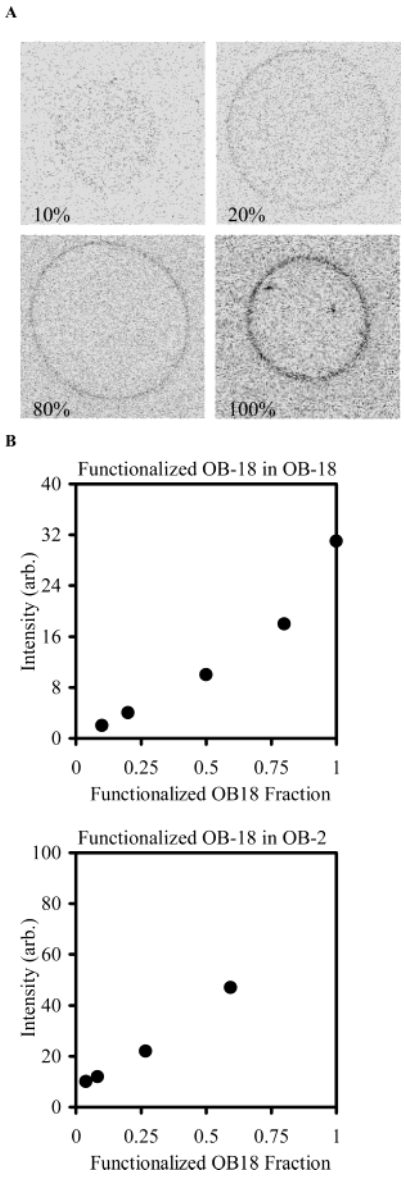

Figure 3. Fluorescent confocal images of functionalized polymersomes and analysis of polymer mixing and surface concentration of biocytin. (A) Confocal images of OB-18 functionalized with Alexa Fluor 488 biotin (OB-18b) mixing in an OB-18 membrane at four different concentrations, 10, 20, 80, and 100% (by mass). (B) The edge brightness increases proportionally to the amount of modified OB-18 polymer added, indicating the surface concentration of biotin may be effectively varied with the bulk mixing ratio. Proportional increases in the surface concentration of Alexa Fluor 488 biocytin are observed in both cases, when modified OB-18 is mixed with OB-2 and modified OB-18 is mixed with OB-18. To the resolution of these experiments, no phase separation or segregation is observed between OB-18 and OB-2, and mixing is in proportion with bulk polymer concentration.

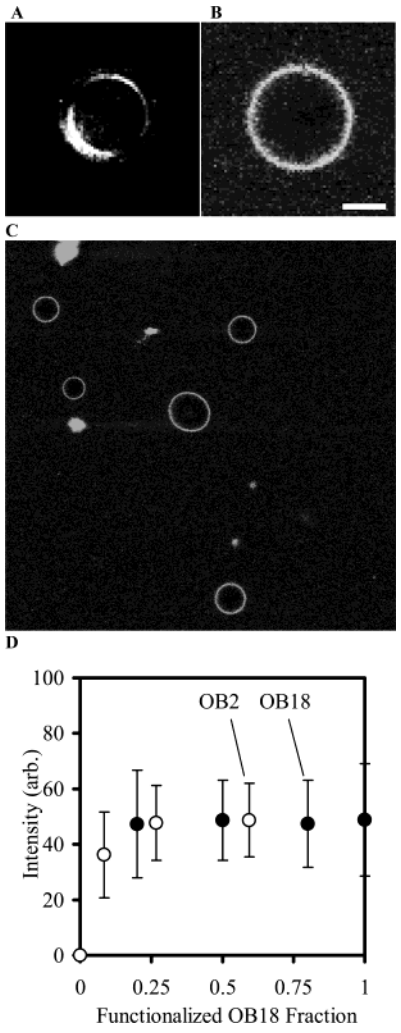

Figure 4. Adsorption of avidin from bulk solution onto biotinylated vesicles. (A) Image of a biocytin coated vesicle with soluble Alexa Fluor 488 conjugated streptavidin under differential interference contrast optics. (B) Fluorescent image of the vesicle from part A after incubation with soluble Alexa Fluor 488 conjugated streptavidin. (C) Field of view showing several avidin coated vesicles. (D) Fluorescent intensity of Alexa Fluor 488 streptavidin adsorption on the surface of polymersomes of different concentrations of OB-18b. In both cases, OB-18b in OB-18 and OB-18b in OB-2 polymersomes, the amount of avidin adsorption saturates at or below 10%. Further addition of biotinylated OB-18 polymer does not result in a significant increase in the avidin surface fluorescent intensity.

soluble Alexa Fluor 488 conjugated streptavidin and then imaged with confocal microscopy (Figure 4A and B). Figure 4A shows a DIC image of an avidin-coated polymersome, while Figure 4B shows the same vesicle under fluorescent microscopy. Figure 4C represents a field of view that clearly indicates the adsorption of fluorescent avidin to the surface of several vesicles. A negative control sample using untresylated polymer indicates that there is no nonspecific adhesion of Alexa Fluor 488 conjugated streptavidin to an unmodified polymersome surface.

Figure 4D is a quantification of the amount of fluorescence at the surface of OB-18 and OB-2 polymersomes as the amount of biotinylated OB-18 is increased. The two series were normalized with respect to one another by the intensity of 100% OB-18b samples. There is little change in the average fluorescent intensity for either series above 10 mol %, although the error bars indicate the variability at each concentration. There is no discernible difference in the amount of fluorescence obtained by adsorption of avidin from the bulk solution onto vesicles made with OB-18b in either OB-18 or OB-2 polymersomes.

Classification of Adhesion. To measure the adhesion between biotinylated polymersomes and superavidin coated microspheres, it is necessary to identify the regime of adhesiveness. Biotin labeled polymersomes bind to superavidin coated microspheres as a kinetically trapped system[11] because the contact area forms spontaneously, and the tension required to peel a contact increases as the contact area decreases. The contact distance, depicted in Figure 5B, was measured as the linear distance between the two edge points of where the polymersome was in

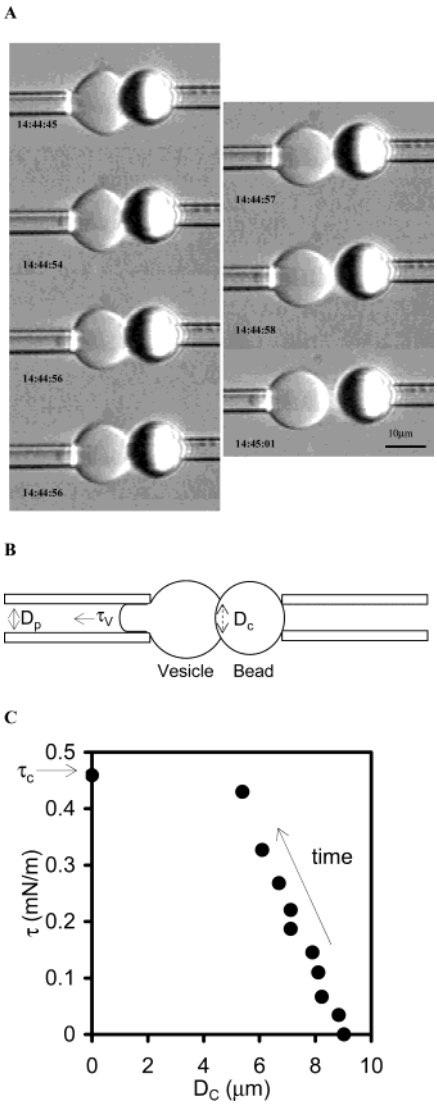

Figure 5. Classification of adhesion. (A) Representative sequence of the peeling experiment. The biocytin surface coated vesicle was flaccid and adhering to the avidin coated microsphere. The micropipet on the right-hand side held the microsphere statically, while the one on the left-hand side came in to just touch the vesicle. As the suction pressure increased, the vesicle was sucked into the micropipet with increasing projected length. The contact distance decreased in a infinitesimal fashion as the tension applied on the vesicle membrane increased gradually until the critical tension was reached, all the biotin−avidin bonds connecting the vesicle and the microsphere were broken, and the contact distance decreased to a single contact point and broke off. (B) Depiction of the contact distance (D_C) the linear distance between the two edge points of where the vesicle was in contact with the microsphere. (C) Graph of tension applied on the polymersome membrane vs the contact distance between biocytin surface coated polymersomes and avidin coated microspheres, analogous to Figure 2 in Evans et al.[13] As the tension increased, the contact distance decreased by a small increment until the point when the connection was broken; this indicated that our system was in the discrete kinetically trapped molecular crossbridge regime.

contact with the microsphere. Figure 5A shows a representative sequence of images from an adhesion experiment performed with 50 mass % OB-18b/OB-2 polymersomes. Prior to the peeling experiment, the biocytin coated polymersome was brought into contact with the superavidin coated microsphere. The tension on the polymersome is decreased gradually to allow the polymersome to adhere to the microsphere; the micropipet is retracted from contacting the polymersome, and the polymersome is allowed to bind to the sphere for 15 min. While one micropipet holds the superavidin coated microsphere statically, the other micropipet is moved to just touch the polymersome. As the suction pressure increases, the polymersome is aspirated into the micropipet with increasing projected length. As the polymersome is initially aspirated, a small portion of the adhering membrane is peeled from contact with the microsphere with a small tension. After this initial decrease in the contact distance, the contact distance decreases slowly as the tension applied on the vesicle membrane increases, until the critical tension is reached. At the critical tension, all the biotin−avidin bonds connecting the polymersome and the microsphere are broken, and the contact distance decreases to a single contact point and breaks. Figure 5C is a graph of the tension applied on the vesicle membrane versus the contact distance between a biotinylated polymersome, 80 mass % OB-18b/OB-2, and a superavidin coated microsphere. This system behaves fundamentally different from an equilibrium system, where the force needed to form the contact is essentially equal to the force required to separate the contact.[10] Thus, the appropriate metric of adhesion is the critical tension, rather than the adhesion energy.

In the current experiment, even though the polymersome initially adheres to the superavidin coated microsphere through spreading, it is the bonds between biocytin and avidin formed within the contact area that are responsible for holding the polymersome to the microsphere. A similar experiment with an unmodified polymersome results in a contact area between the polymersome and the microsphere that decreases rapidly with a negligible applied tension. A similar result is observed if the adhesion experiment is repeated with an excess of soluble biotin, which demonstrates the adhesion is specifically due to avidin−biotin binding.

Measurement of Critical Tensions. Various compositions of biotinylated polymersomes were made by mixing OB-18b with OB-2 or OB-18 in different percentages. Since OB-2 is much shorter than OB-18, it was hypothesized that the two different membranes would result in different molecular surface topographies, depicted in Figure 6A and 6B, that would affect the subsequent adhesion between the polymersome and the microsphere. Figure 6A illustrates functionalized OB-18 (OB-18b) in pure OB-18: there is no advantageous presentation of biotin away from the base membrane. Figure 6B shows functionalized OB-18 in OB-2: the difference in the chain lengths of OB-2 and OB-18 polymers favors a surface topography in which the adhesion molecules on OB-18 are extended beyond the shorter OB-2 surface brushes.

In Figure 6C, the critical tensions needed to separate the polymersomes and microspheres are plotted against the molar percentage of biotinylated OB-18 polymer in the polymer mixture. The critical tensions of polymersomes made from an OB-18 membrane increase with biotinylated polymer concentration up to 10 mol % OB-18b. The critical tension does not increase as OB-18b is increased beyond 10 mol %, suggesting that the avidin binding is saturated.

When biotinylated OB-18 is mixed with OB-2 (closed symbols), the critical tension increases as the percentage of biotinylated OB-18 increases until a maximum is reached near 55 mol % OB-18b. Further increases in biotinylated OB-18 result in lower critical tensions, until the membrane is purely OB-18b. A plateau of adhesion is not observed in this system, and the critical tensions measured when OB-18b is mixed with OB-2 are larger than those observed when OB-18b is mixed with OB-18.

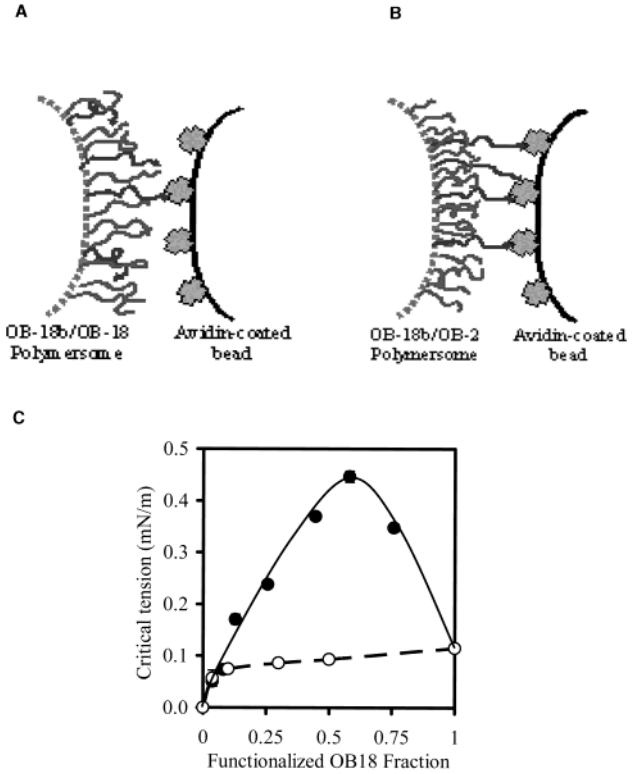

Figure 6. Critical tension measurements. (A) Illustration of functionalized OB-18 in pure OB-18. (B) Illustration of functionalized OB-18 in OB-2. The difference in the chain length between the two polymers, OB-2 and OB-18, leads adhesion molecules presented beyond the surface brushes of the smaller polymer. (C) The dependence of critical tension (mN/m) vs the percentage of functionalized OB-18 polymers. In the case of functionalized OB-18 (OB-18b) in OB-2, the critical tension increased as the percentage of functionalized OB-18 increased to a maximum, after which the adhesiveness decreased with increasing functionalized OB-18 concentration. When functionalized OB-18 was mixed in OB-18, the critical tension increased as the percentage of functionalized OB-18 increased, shown as the dashed line. However, OB-18b/OB-2 adhesion was above that of OB-18b/OB-18 at all OB-18b concentrations. Every point ($n \geq 4$) has an error bar; however, error bars were about the same size as the points.

Several negative control experiments were performed to ensure the measured critical tensions were unique and reproducible. The critical tension of the polymersomes modified with Alexa Fluor 488 biocytin that were used in Figure 3 is indistinguishable from the results in Figure 6. No adhesion was observed between polymersomes made from tresylated polymers and avidin coated microspheres. To exclude the possibility that unreacted tresyl groups participate in or complement the adhesion of the biotinylated vesicles, biotinylated polymersomes were reacted with ethanolamine to cap any remaining tresyl groups. The critical tensions measured from ethanolamine capped vesicles are within a standard deviation of samples at the same concentration without ethanolamine capping. Figure 6 clearly indicates that the surface topology, membrane composition, and presentation of biotin play important roles in the adhesion of polymersomes to a surface.

Tether Formation of Functionalized Polymersomes. Tether formation from the membrane of functionalized polymer vesicles is observed when attempting to measure the critical tension of vesicles with low concentration of OB-18b in OB-2; however, no tether formation is observed from the vesicles with the same concentration of OB-18b in pure OB-18 or from vesicles with higher concentrations of OB-18b polymers regardless of the polymer used as the base membrane. Stable tether formation is highly reproducible at 4 mol % OB-18b in OB-2. Figure 7 shows representative sequential pictures of tether formation. Suction pressure was originally being applied on the polymersome membrane through a micropipet; the suction pressure was subsequently decreased, and the polymersome was released from the micropipet. The polymersome was drawn back to the avidin coated microsphere by the tether formed between the biotinylated polymersome and the superavidin coated microsphere. The tether diameter increases as the polymersome is drawn closer to the superavidin coated microsphere due to conservation of total volume.

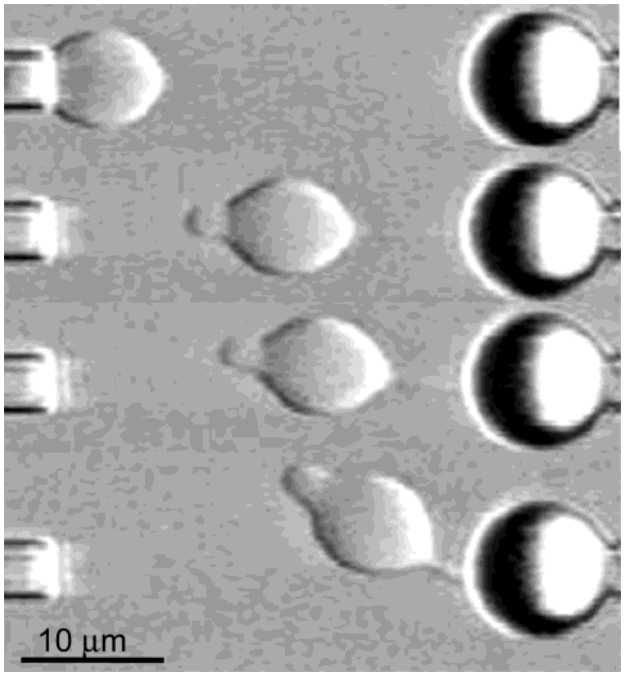

Figure 7. Tether formation pictures in sequential order. Suction pressure was originally being applied on the polymersome membrane through a micropipet; the suction pressure was subsequently decreased, and the polymersome was released from the micropipet. The polymersome was drawn back to the superavidin coated microsphere by the tether formed between the biotin surface coated polymersome and the superavidin coated microsphere. The tether was a very thin tube in the topmost three pictures. The tether diameter increased as the polymersome was drawn closer to the superavidin coated microsphere due to conservation of total volume.

Discussion

Polymersomes are wonderful but complex materials. They are potentially useful materials for numerous applications, but a fundamental understanding of their behavior is needed before their utility may be realized. The larger thickness of polymersome bilayers imparts greater mechanical strength than liposomes, allowing them to withstand greater tensions and stress without cohesive failure. This allows the study of strong adhesion forces between colloids that might disrupt the structure of less stable vesicles. The thickness of the hydrophobic region is the source of the increased stability and provides an interesting bilayer morphology in which many compounds may be stabilized. However, the source of its superior mechanical qualities is also a source of added complexity, since the membrane is constructed from a self-assembled PEO brush. The role of the surface topology of polymer vesicles on adhesion, adsorption, mechanics,

and transport is an open issue that needs clarification before decisive experiments in the medical application of polymersomes may continue.

In this study, the surfaces of polymersomes are modified with various molecules and two surface topographies are created: functionalized OB-18 (OB-18b) in OB-2 and OB-18b in OB-18. Alexa Fluor 488 biocytin and biocytin molecules are linked to the surface of polymersomes to investigate polymer mixing and perform adhesion experiments, respectively. Confocal microscopy and Alexa Fluor 488 biocytin molecules are used to investigate the density of adhesion molecules on the polymersome surface. In both cases, the fluorescent intensity increases linearly as the concentration of OB-18b increases in the membrane and indicates the surface concentration of biotin may be effectively varied with the ratio of polymer used to make the vesicles. This also indicates modification at the hydroxyl terminus of OB-18 polymer does not affect its incorporation into a bilayer of unmodified polymer, or its ability to form vesicles itself. To the resolution of these experiments, no phase separation or segregation of OB-18b is observed in OB-18 or OB-2 polymersomes.

To investigate how an increase in surface concentration of biotin would affect the ability to bind soluble avidin, the attachment of fluorescently labeled avidin to the surfaces of OB-18b/OB-2 and OB-18b/OB18 polymersomes is measured. Figure 4D shows the resulting intensities of fluorescent avidin that adsorbed on vesicles of varying compositions. In both cases, the amount of avidin adsorption saturates at or below 10 mol % OB-18b. Further addition of OB-18b polymer does not result in a significant increase in the surface fluorescent intensity and indicates that the specific adsorption of a large protein (55 KDa) to the PEO surface is limited by the packing density of avidin. The lack of distinction between membranes made from OB-18 and OB-2 indicates that this adsorption process from the bulk does not depend on the surface brush topology or the underlying mechanical properties of the membrane. These results are consistent with the concept that avidin can saturate a biotin surface at low concentrations due to the size mismatch between the molecules and that 10 mol % OB-18b is sufficient to provide enough sites for avidin to coat the membrane.

The critical tension, the tension required to just separate the connection between biotinylated polymersomes and avidin coated microspheres, is measured to investigate the effect of surface topology on the adhesiveness of functionalized polymersomes. When the adhesion molecules are presented beyond the membrane brush, as in OB-18b/OB-2 polymersomes, two adhesion regimes are found. At low concentrations of OB-18b, the critical tension increases with the amount of biocytin molecules presented on the surface of polymersomes. At concentrations of OB-18b > 55%, the critical tension decreases as the concentration of OB-18b polymer increases. However, when OB-18b polymers are mixed with polymers of the same length, as in OB-18b in OB-18 polymersomes, the critical tension reaches a plateau at 10 mol % OB-18b. Patel et al.[26] have shown that P-selectin must extend a sufficient length from the plasma membrane to mediate rolling of neutrophils. Our study has shown that presentation of adhesion molecules away from a base membrane, OB-18b/OB-2 polymersomes, is important and greatly enhances the adhesion. There is some polydispersity of the polymersomes, yet the critical tension did not depend on the dipersity because it is an interfacial effect that acts at the zone of peeling.

A similar saturation of avidin−biotin bonding has been reported in the adhesion between a biotinylated surfactant covered microbubble and an avidin bead.[21] The adhesion of an avidin coated bead to a compressed surface monolayer of PEG and biotinylated PEG modified lipids at the interface of a microbubble was measured as a function of biotinylated PEG content. The compressed monolayer had a topology similar to that of OB-18b/OB-18 polymersomes, where the biotin was presented just slightly beyond the surface PEG layer and indicated a saturation of biotin mediated adhesion after 3−5 molar %. This was attributed to a geometric limit to the number of bonds that are available between avidin and biotin due to the disparity in size (55 KDa vs 1 KDa) and a lack of mobility of avidin on the microbubble or microsphere. Further addition of biotin above this saturation limit would fill in biotin molecules underneath an already bound avidin and would not participate in binding. This is consistent with the bulk avidin adsorption measurements that plateau around 10%.

When OB-18b polymer is mixed with OB-18 to make polymersomes, OB-18b polymer is surrounded by polymers of the same length and the adhesion molecule at the functionalized chain end is not presented away from the surface brush. According to Dan and Tirrell,[27] most of the biotin molecules are buried in the brush interior and there is no control over the small fraction of ends which does extend to the outer brush. However, in the case that OB-18b polymers are surrounded by shorter polymers, the longer polymer should be preferentially driven into the outermost tier of the brush surface. Currie et al.[28] has shown that long chains are stretched in a bimodal brush and the stretching of the long chain will increase as the number of short chains increases. In a bimodal brush, the short chains will reside in the inner brush region[29] near the interface, whereas the long chains will sit at the outer edge of the brush,[30] and the long chains stretched more strongly at a given area in a bimodal brush than a monodisperse brush because of the presence of the short chains.[28] When OB-18b concentration is low, OB-18b is forced into longer extensions and the chain ends are at the surface of the mixed brush layer. In this situation, adding OB-18b directly increases the density of available sites on the surface of the polymersome. With increasing OB-18b concentration, the advantages afforded by mixing the longer OB-18b in OB-2 begin to disappear. Obviously, the critical tension of the mixture must decay to the value obtained by a pure OB-18b membrane. Nevertheless, the concentration of biotin in the membrane is increasing in this region with the concentration of OB-18b, as indicated in Figure 3.

The profile of the polymersome surface brush is complex not only due to its brushiness but also because it is a self-assembled bilayer that can adjust its spacing and area per molecule freely. A change in surface pressure associated with bending or adhesion may cause a local rearrangement of the surface brush, exposing more adhesion molecules for binding. Detailed brush properties have been probed previously using several experimental techniques, including neutron reflectivity, small-angle neutron scattering (SANS),[31] X-ray scattering, and ellipsometry. Theoretical computer simulations have also been done using theories such as scaling and self-consistent field

(26) Patel, K. D.; Nollert, M. U.; McEver, R. P. *J. Cell Biol.* **1995**, *131*, 1893−1902

(27) Dan, N.; Tirrell, M. *Macromolecules* **1993**, *26*, 6467−6473.

(28) Currie, E. P. K.; Wagemaker, M.; Cohen Stuart, M. A.; van Well, A. A. *Macromolecules* **1999**, *32*, 9041−9050.

(29) Levicky, R.; Koneripalli, N.; Tirrell, M.; Satija, S. K. *Macromolecules* **1998**, *31*, 2616−2621.

(30) Terzis, A. F. *Polymer* **2002**, *43*, 2435−2444.

(31) Won, Y.-Y.; Davis, H. T.; Bates, F. S.; Agamalian, M.; Wignall, G. D. *J. Phys. Chem. B* **2000**, *104*, 7134−7143.

theory. A more detailed experimental examination of the surface brush may be necessary to deduce the importance of surface topology.

While previous analyses of bimodal brushes are invaluable for imagining the role of topology on surface ligand presentation, there may be other more mechanical properties of a mixed polymer bilayer that will affect the critical tension needed to separate it from a solid surface. When polymersomes adhere to microspheres in which the location of avidin is fixed, the dynamics of the OB-18b in the bilayer may be crucial. The flexibility of the adhering polymer may play a role in adhesion on the length scale of the membrane contact area, the overlapping region of ligand−receptor coexistence, and lateral diffusion close to the binding pocket.

The actual size of the contact area between a polymer vesicle and a microsphere may be affected by the underlying deformability of the bilayer membrane. Marchi-Artzner et al.[32] illustrate how when a vesicle adheres to a surface, there may be regions of close contact coexisting with regions where the membrane is near the surface but not closely bound. As the membrane of OB-2 vesicles is more flexible than that of OB-18,[25] there may be more regions of close contact within the contact area of the mixed polymer vesicles than with vesicles made solely with OB-18. As a result, the critical tension will be higher in the case of OB-18b/OB-2. As the concentration of biotinylated OB-18 increases in the membrane, the underlying deformability decreases and the ability of the vesicle membrane to accommodate the irregular microsphere surface may be compromised. It is known, from Bermudez et al.,[25] that the bending modulus of the OB-18 polymersome is much bigger, by an order of magnitude, than that of OB-2. Hence, it stands to reason that the bending modulus of OB-18/OB-2 mixtures will be much higher than OB-2 itself. The effect of the higher bending modulus will be that there will be a less intimate contact, from the decrease in the underlying deformability, and that the tension will be focused over a shorter length scale in the contact zone. These effects will make the adhesion strength decrease with the bending modulus.[14]

Also, the irregularity and porosity of the microsphere surface may lead to stratification of avidin within the outer layers of the microsphere instead of being completely immobilized at the surface. In addition, it has been reported that the biotin binding sites on avidin are buried 0.9 nm below the surface of avidin.[33] The longer OB-18b polymer allows greater conformational freedom and may allow the biotin end group to penetrate to deeper surface layers on the avidin microsphere that are unavailable when constrained to a two-dimensional interface. As such, the overlapping volumes between the longer functionalized polymer and the avidin bead lead to a greater effective contact area than what could be deduced from the macroscopic contact area. Hence, the overlapping volume of OB-18b and the avidin on the microsphere surface is greater in OB-18b/OB-2 polymersomes at all concentrations than in OB-18b/OB-18 polymersomes. This effect would explain why the adhesion of the mixed polymersomes does not plateau at lower OB-18b concentration and also why there are larger critical tensions, as the mixed polymer brush layer is exposed to more avidin than a brush which is confined to the bead surface. This effect would decrease as more OB-18b is added, as the effective extension of the polymer end groups will scale with the volume fraction of the smaller polymer. Fluorescent resonance energy transfer (FRET) can be used to get a more accurate measurement of the contact zone.

On a still smaller length scale, the flexibility of the functionalized polymer laterally instead of axially could have an effect on the multiplicity of binding for one avidin molecule. The avidin molecules are covalently attached to the surface of a polystyrene microsphere, resulting in four biotin binding pockets per avidin molecule and a locked orientation of avidin molecules. Due to the vertical segregation of polymer within a bimodal brush, the outermost layer of functionalized polymer has significantly more surface area per chain than the underlying brush. The freedom of movement this affords the chain ends in a mixed polymer bilayer may affect the ability of biotin to diffuse and reach laterally to bind to open binding pockets on an already bound avidin. The degree of multiplicative binding would increase with surface concentration of OB-18 until it begins to behave as a unimodal brush again. A detailed study of the adhesion of polymer vesicles includes aspects of the physics of polymer brushes, the mechanical properties of self-assembled bilayers, and the mechanics of the adhesion event itself. Determination of the most important effects for describing the adhesive behavior of polymer vesicles measured here will be the goal of future studies.

Conclusion

The focus of this report is to gain detailed information about how polymer chain lengths and the surface density of adhesion molecules affect adhesion. Polymersomes made from block copolymers are chosen as a model because of their superior material properties. The current study has succeeded in modifying the hydroxyl terminus of two block copolymers: poly(ethylene oxide)−polyethylethylene (OE-21) and poly(ethylene oxide)−polybutadiene (OB-18). Biotinylated polymersomes are made of mixtures of biotin labeled OB-18 (OB-18b) with OB-18 or OB-2 polymers. We have demonstrated that no phase separation or segregation of OB-18b is observed when OB-18b is mixed with OB-2. Adhesion measurements between biotinylated polymersomes, OB-18b/OB-2 and OB-18b/OB-18, and avidin coated microspheres were performed. Adhesion between biotinylated polymersomes and avidin-coated bends is kinetically trapped and characterized by the critical tension for peeling. The adhesion strengths of OB-18b/OB-2 polymersomes are greater than those of OB-18b/OB-18 polymersomes at all concentrations; a maximum is observed at an intermediate concentration, 55 mol % OB-18b in OB-2, as the presentation of adhesion molecules away from the base membrane is important for effective adhesiveness.

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(32) Marchi-Artzner, V.; Lorz, B.; Gosse, C.; Jullien, L.; Merkel, R.; Kessler, H.; Sackmann, E. *Langmuir* **2003**, *19*, 835−841.

(33) Green, N. M.; Konieczny, L.; Toms, E. J.; Valentine, R. C. *Biochem. J.* **1971**, *125*, 781−791