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Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion

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Abstract

Monolayers of dipalmitoyl-phosphatidylethanolamine (DPPE) mixing with various mole percentages of distearoyl-phosphatidylethanolamine (DSPE)-conjugated poly-(ethylene glycol) (PEG m.w. 750–5000) were deposited on DPPE-coated glass surfaces by the Langmuir-Blodgett method. Increasing percentages of grafted PEG in these supported lipid surfaces increasingly inhibit the adsorption of bovine serum albumin (BSA), laminin, and fibronectin. Increasing percentages of grafted PEG also inhibit the adhesion of erythrocytes, lymphocytes, and macrophages to these supported lipid surfaces. The adsorption of proteins on lipid coated glass surfaces were assayed by the fluorescence of FITC-labelled proteins. Cell adhesion was measured mainly by microscopic counting. The concentration of PEG-grafted lipids required for the inhibition of erythrocyte adhesion decreases with increasing molecular weight of the grafted PEG. The inhibitory effects are strongly dependent on the graft density of PEG at low concentrations, but weakly dependent on graft density at higher concentrations. For DSPE-PEG5000, the change of graft density dependency occurs approximately at the complete coverage of the lipid surface by the grafted polymer in the mushroom conformation (0.7 mol%), and the transition to partial brush conformation. The change-overs become less distinctive for grafted PEG of lower molecular weights, probably due to the failure of strictly mushroom and brush models of the polymer. The relative inhibitory efficiency is protein or cell dependent. The implication on the function of stealth liposomes is discussed. © 1997 Elsevier Science B.V.

Keywords: Poly-(ethylene glycol); Biopolymer; Stealth liposome; Steric hindrance; Lipid monolayer

1. Introduction

Recently, various PEG derivatives have been used to stabilize liposomes for increasing efficiency in drug or gene delivery. Most ‘stabilized’ liposomes, e.g., the so-called ‘stealth’ liposomes [1], or ‘cryptosomes’ [2], contain a certain percentage of PEG-derivatized phospholipids, which reduce the uptake by mononuclear phagocytic system (MPS), thereof prolonging the circulation times. Unlike conventional liposomes, PEG-liposomes do not show dose-dependent blood clearance kinetics [3]. Vesicles containing PEG-conjugated lipids at various concentrations, molecular weights (MW), or various sizes of PEG-containing vesicles were reported to have different circulation times [2,4–7]. It was shown that the circulation time and the organ distribution of the stabilized lipid vesicles in vivo are very sensitive to the surface

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density of the sterically active head-groups [2]. In vitro, by incubating the vesicles with the components from the mononuclear phagocytotic system (MPS), it was found that the existence of PEG on the surface of the vesicle prevents the adsorption of serum albumin. Furthermore, both the resistance to plasma component adsorption and the resulting longevity in vivo show a maximum at the same lipid/stabilizer molar ratio [2]. In comparison, a large number of plasma components, including serum albumin, immunoglobulin, fibronectin and various apolipoproteins, can bind to the surface of conventional liposomes, affecting their longevity [8].

Because PEG has been shown to have protein-repelling activity when immobilized on a surface, due to its hydrophilicity, chain mobility, and lack of ionic charge [3], several groups have attempted to create a biocompatible but cell repelling surface using PEG as a modifier. A solid support coated with PEG-grafted lipids may provide a biocompatible surface for biosensing and prosthetic devices. It has been recognized that the protection effect of PEG conjugated lipids is dependent on the MW of PEG moieties. In order to produce a significant inhibition of the uptake by macrophages, the ethylene oxide (EO) chains used in block-copolymer-coated polystyrene particles must have at least 50 subunits [9]. A similar result was observed in the study of protection on protein adsorption: the degree of polymerization of PEG on the solid substrate must approach about 100 [10]. A closer simulation of PEG-liposome surfaces is a lipid monolayer or bilayer on a solid support, with PEG only on the water-facing surface. These surfaces have well-defined compositions and structures. The grafted PEG moieties protrude from the surface, while the hydrophobic tails of these molecules are anchored into the surface monolayer. Compared to vesicles, the experimental conditions for supported monolayer or bilayer are easier to control. For example, it is easier to separate free proteins or cells in solution from those deposited on the polymer grafted surfaces; the properties of the polymer-coated surfaces can be determined more accurately, and the system is a convenient one for investigating hypotheses concerning protein adsorption and cell adhesion to the polymer-coated surfaces.

In this paper, we report our findings of the effect of grafted PEG molecules on protein adsorption and cell adhesion to the surface of phospholipid bilayers, which are formed by transferring two successive monomolecular layers on to the same glass coverslip. We chose proteins such as albumin, fibronectin and laminin, and cells such as erythrocyte, lymphocyte, and macrophage for this study. Albumin (MW $\cong$ 66 000) constitutes 50–60% of the total protein in blood and has several important functions, including drug binding and transport. Plasma fibronectin (MW = 220 000–250 000) contains the binding domains for many proteins and cells, and is involved in chemotaxis, phagocytosis, and opsonization. Like fibronectin, laminin (MW $\cong$ 140 000) also facilitates cell-substratum adhesion. Both fibronectin and laminin are involved in wound healing and the clearance of cellular debris; hence, they are important when polymers are used in vivo. The affinity of erythrocytes, lymphocytes and macrophages for the polymers in the bloodstream is another factor which can influence the longevity of the circulation time of the polymers and polymer-coated vesicles. Therefore, the interaction between these proteins and cells with PEG-grafted surfaces is an important issue which we wish to address.

2. Materials and methods

2.1. Chemicals

Dipalmitoyl phosphatidylethanolamine (DPPE) and polyethylene glycol of various molecular weights (designated as PEG + MW) conjugated to distearyl phosphatidylethanolamine (DSPE-PEG), were purchased from Avanti Polar Lipids (Alabaska, AL). DSPE-PEG3000 and DSPE-PEG750 were provided by SEQUUS Pharmaceuticals, Inc. (Menlo Park, CA). The fluorescent dye, fluorescein isothiocyanate (FITC) was purchased from Calbiochem (La Jolla, CA). Bovine serum albumin (BSA), fibronectin (separated from bovine plasma) and laminin (from basement membrane of Engelbreth-Holm-Swarm Mouse Sarcoma) were purchased from Sigma (St. Louis, MO). Lymphoprep™ for lymphocyte separation was purchased from NYCOMED (Oslo, Norway). Macrophages were separated from the peritoneal exudates of stimulated mice.

2.2. Sample preparation

2.2.1. Preparation of FITC-labelled protein solution

FITC labeling is a sensitive method for measuring protein concentration when the protein solution is too diluted to be measured by the optical absorption method [11]. Fluorescein isothiocyanate (FITC) may be covalently bound to the amino group of protein molecules via the N = C = S group. Usually, one protein molecule can bind to several FITC molecules. However, the fluorescence of FITC is quenched when the molar binding ratio of FITC to protein exceeds 6:1 [12]. BSA (powder) was dissolved in phosphate-buffered saline (PBS, pH 7.4). Fibronectin and laminin (solutions) were diluted with the same buffer. The proteins were labeled with FITC at the molar ratio of FITC:protein of 4:1 at room temperature for 1 h. The resulting solution was dialyzed for 48 h to remove the free FITC molecules. The solutions were stored at 4°C.

The fluorescence intensity at various concentrations of FITC in aqueous solution was measured in a fluorometer (8000C, SLM Instrument Inc., Urbana, IL), with the excitation/emission (EX/EM) wavelengths set at 490 nm/520 nm. The fluorescence intensity of FITC is linear with its concentration below 2 μg/ml. The absorbance at 280 nm of BSA, fibronectin, and laminin at various concentrations were measured on a spectrophotometer (DU®-40, Beckman, Columbia, MD). The absorbance at 280 nm is linear with concentrations up to 0.1 mg/ml of these proteins. At these protein and FITC concentrations, the fluorescence intensity and protein absorbance of each FITC-protein were measured. These values were used to determine the protein adsorption to the supported bilayers. The molar ratio of FITC to protein was determined to be 1.70:1 for BSA, 1.08:1 for fibronectin and 1.63:1 for laminin, taking into account that the fluorescence intensity of bound FITC is 10% less than that of free FITC [12]. These molar ratios indicate that, under our experimental conditions, only one or two of the amino groups of each protein molecule reacted with FITC. The protein structure can hardly be affected in this case.

2.2.2. Cell preparation

Fresh rabbit blood was centrifuged at room temperature at 2000 rpm for 10 min. The supernatant was removed, leaving the erythrocyte-rich pellet. The pellet was washed with PBS buffer (pH 7.4, 320 mOs/kg, EDTA as anticoagulant). Cells were washed several times, diluted with the same PBS buffer, and stored at 4°C.

Rabbit lymphocytes were obtained by using the protocol provided with Lymphoprep™ from NYCOMED PHARMA AS. Whole rabbit blood was diluted with an equal volume of 0.9% NaCl. Then 6 ml of the diluted blood was carefully layered over 3 ml of Lymphoprep™ in a 15 ml centrifuge tube. The mixture was centrifuged at $800 \times g$ for 30 min at room temperature. After centrifugation, the distinct band that had formed, mainly by lymphocytes, at the sample/medium interface was harvested. The harvested fraction was diluted with 0.9% NaCl and the cells were pelleted by centrifugation for 10 min at $250 \times g$. The pellet was diluted with 0.9% NaCl and stored at 4°C.

Macrophages were obtained by the method first suggested by Brumfitt [13]. Briefly, 1% glycogen was injected into the peritoneal cavity of a mouse at 3 ml/mouse. After 12–24 h, the exudates, which contained about 1×10^6 cells of which 85–100% were macrophages, were harvested from the peritoneal cavity.

Morphologically, erythrocytes, lymphocytes, and macrophages can easily be distinguished from each other under the microscope. The final concentration of each type of cells was determined by counting the number of cells on the hematology cytometer under the microscope. For experiments using microscopic counting to determine cell adhesion, the concentration was 1.25×10^6/ml for erythrocytes, 1.50×10^6/ml for lymphocytes and 1.0×10^5/ml for macrophages. Alternatively, an erythrocyte concentration of 5.0×10^6/ml was used for optical absorption measurement of cell adhesion.

2.2.3. Deposition of lipid bilayer on glass support by the Langmuir-Blodgett method

Chloroform solutions of DPPE and DSPE-PEG5000 were mixed at molar percentages of 0, 0.25, 0.5, 1.0, 2.5, 5.0% of DSPE-PEG, and the final concentration of the mixtures were 2 mg/ml. The samples were stored at −20°C.

Deposition of phospholipid bilayers on a glass

substrate was similar to that described for depositing on mica surfaces [14]. Glass coverslips were cut to 1.8 cm × 1.3 cm, cleaned in turn with chromic acid and triply distilled water. Before the phospholipid monolayer was spread on the air–water interface, the glass coverslip was positioned under the water surface. 2–5 μl of DPPE for the first monolayer was then spread on the air–water interface with a microsyringe. About 30 min was allowed for the solvent to evaporate at 25°C [15]. The monolayer was compressed by a barrier to a surface pressure of 35 dynes/cm. The surface pressure was kept constant by a servo system in the course of transfer.

At constant surface pressure, the glass coverslip was lifted from beneath the water subphase at the speed of 6 mm/min so that the first layer of phospholipids (DPPE) was deposited on the glass surface. Then, the DPPE monolayer at the air/water interface was removed, and a new monolayer with defined compositions (DPPE or DPPE:DSPE-PEG mixtures) was spread on the water subphase by the same procedure described above. After the glass coverslip covered with a layer of DPPE became completely dry, it was again immersed in the water subphase which was now covered by the newly-formed monolayer. In this process, the second layer of phospholipids was deposited over the first layer of DPPE to form a phospholipid bilayer film on the glass coverslip. The transfer efficiency was usually close to unity. The glass coverslip was collected underneath the water surface in a petri dish. The sample in the petri dish was washed with PBS (pH 7.4) several times, avoiding exposure to the air–water interface.

2.2.4. Attachment of proteins and cells to the lipid bilayer

Each protein was added to separate petri dishes which contained glass coverslip coated with DPPE as the first monolayer, and DPPE:DSPE-PEG mixture, at 0, 0.25, 0.5, 1.0, 2.5, 5.0 mol% of DSPE-PEG, respectively, as the second monolayer. Fibronectin and laminin were used at the concentration of 10 μg/ml, while BSA was used at a concentration of 1 μg/ml because a higher concentration of BSA could result in fluorescence quenching on the glass coverslip surface.

Each type of cell was added to the petri dishes containing the same series of lipid bilayers at the concentrations mentioned before.

For convenience rather than for in vivo simulation, the bilayer samples were allowed to interact with proteins or cells for 2 h at room temperature, at which time the protein adsorption and cell adhesion to the surface of lipid bilayer reached saturation. Afterwards, free proteins or cells were washed off thoroughly with PBS (pH 7.4) without exposing the glass coverslip to the air–water interface. The amount of protein or the number of cells attaching to the lipid bilayer was measured with the fluorometer or light microscope.

2.2.5. Measurement of protein adsorption

The amount of protein attached to the surface of various DPPE:DSPE-PEG5000 combinations was determined by the fluorescence intensity of FITC with the same conditions described above. The sample deposited on a glass coverslip was placed in a cuvette diagonally, and was oriented about 40° back-facing the illumination. By using this procedure along with an emission filter, the reflected light which enters the emission monochromator was minimized. The fluorescence intensity of the individual sample was converted to the number of protein molecules by measuring the fluorescence intensity of proteins, washed off the coverslip surface with 95% alcohol, and using the standard curve of fluorescence intensity of FITC in solution with the same alcohol concentration. The measurements were carried out with the EX/EM at 490 nm/524 nm, where 524 nm is the maximum emission wavelength for FITC in ethanol.

2.2.6. Measurement of cell adhesion

The number of cells adhering to the surface of various combinations of DPPE and DSPE-PEG were counted with the samples immersed in PBS, using an IMT-2 inverted phase-contrast microscope (Olympus Co., Lake Success, NY) with 40 × and 10 × objective lenses.

Erythrocytes contain hemoglobin which has a maximum absorption wavelength at 415 nm. The standard curve of the absorbance vs. the number of erythrocytes was obtained by measuring the absorbance at that wavelength after the cells were lysed by Triton X-100.

3. Results

3.1. Deposition of DSPE-PEG5000-containing phospholipid bilayers on glass

To ensure the optimal conditions for bilayer deposition, we measured the effect of adding a small mol percent of DSPE-PEG5000 to DPPE on the monolayer compression characteristics. Fig. 1 shows the curves of surface pressure vs. the area per molecule (Π-A diagram), at the concentrations of 0, 0.25, 0.5, 1.0, 2.5, 5.0 mol% of DSPE-PEG5000 in the DPPE. As the monolayer is compressed, the area per molecule decreases. At 25°C, the surface pressure of DPPE monolayer shows little change as the area per molecule is reduced from 100 to 80 Å^2. Further compression to 40 Å^2/molecule results in a rapid increase in surface pressure to 60 dyne/cm. At that point, no further pressure changes upon compression, indicating a monolayer collapse. At any given pressure, the areas per molecule of monolayers containing various DSPE-PEG5000 concentrations are larger compared with that of a DPPE monolayer. Generally, the higher the concentration of DSPE-PEG5000, the larger the area per molecule. The pressure of 35 dynes/cm, used in our experiments for transferring the monolayer onto the glass coverslip, is in the range

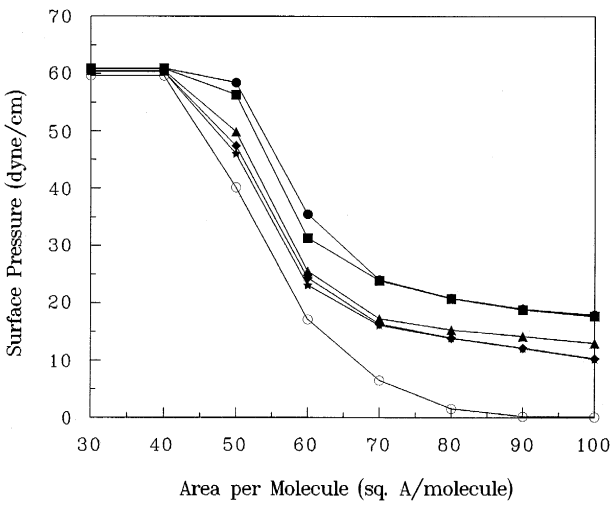

Fig. 1. The pressure-area diagram of monolayers containing mixtures of DPPE and DSPE-PEG5000 at 25°C: the concentrations of DSPE-PEG5000 are 0 mol% (open circle), 0.25 mol% (solid star), 0.5 mol% (solid diamond), 1.0 mol% (solid triangle), 2.5 mol% (solid square) and 5.0 mol% (solid circle).

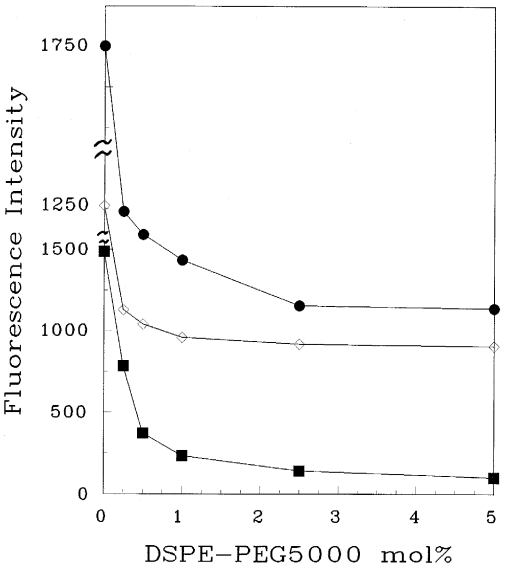

Fig. 2. The fluorescence intensity of FITC-fibronectin, laminin and BSA on glass coverslips at various concentrations of DSPE-PEG5000: fibronectin (solid square), laminin (solid circle) and BSA (open diamond). [Note: Both laminin and BSA curves were shifted vertically for clarity. The highest value for each curve is as marked and scale sub-divisions are uniform throughout.]

where the surface pressure increases steeply as the area per molecule decreases. The areas per molecule at that surface pressure are about 52, 54, 55, 56, 59, and 60 Å^2 for 0, 0.25, 0.5, 1.0, 2.5, and 5.0 mol% of DSPE-PEG5000, respectively.

3.2. Protein adsorption

The fluorescence intensities of FITC-labelled fibronectin, laminin and BSA on the lipid deposited glass coverslip surfaces were plotted against the molar percentage of DSPE-PEG5000 in the DPPE:DSPE-PEG5000 mixtures, (Fig. 2). When the percentage of DSPE-PEG5000 increases, the fluorescence intensities of FITC-BSA, FITC-fibronectin and FITC-laminin adsorbed to the surface of DPPE:DSPE-PEG5000 decrease sharply at DSPE-PEG5000 concentrations from 0–1.0 mol%, and a gradual decrease is shown at higher concentrations. When the concentration of DSPE-PEG5000 is below 0.25 mol%, the fluorescence intensity of fibronectin does not show as much decrease as that of laminin and BSA.

In order to quantitate the adsorption of these three

proteins at various DSPE-PEG5000 concentrations, the fluorescence intensities of FITC-proteins were also measured after they were washed off the glass coverslip with ethanol. The number of protein molecules per cm^2 is calculated from the fluorescence intensities as described in Section 2, and plotted against the molar percentage of DSPE-PEG5000 (Fig. 3). As the molar percentage of DSPE-PEG5000 increases, the number of adsorbed molecules per unit area for all these proteins decreases, more rapidly at DSPE-PEG5000 below 1.0 mol% and gradually at concentrations higher than 1.0 mol%. The trends are similar, but not identical, to the measurements made on glass coverslips before washing (Fig. 2). The surface density of fibronectin molecules is about twice as high as those of BSA or laminin molecules at 0 mol% of DSPE-PEG5000, and remains comparatively higher than those of laminin or BSA molecules at any concentrations of DSPE-PEG5000 below 0.5 mol%. At concentrations higher than 1.0 mol%, the number of molecules adsorbed on the surface for each protein continues to decrease, but more slowly, and BSA shows about twice the adsorption of the other two proteins. The number of molecules/cm^2 at 5.0 mol% is about 21%, 11% and 4% of those at 0 mol% DSPE-PEG5000 for BSA, laminin and fibronectin, respectively.

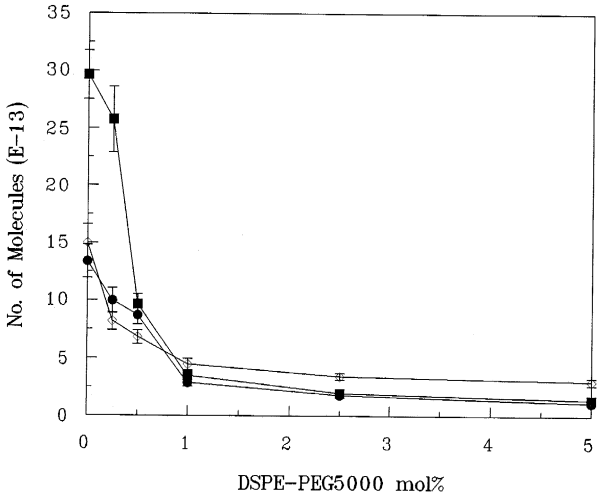

Fig. 3. The densities of protein molecules adsorbed to the lipid bilayer surface at various concentrations of DSPE-PEG5000, as measured by fluorescence intensities of the proteins washed off the coverslips: fibronectin (solid square), laminin (solid circle) and BSA (open diamond).

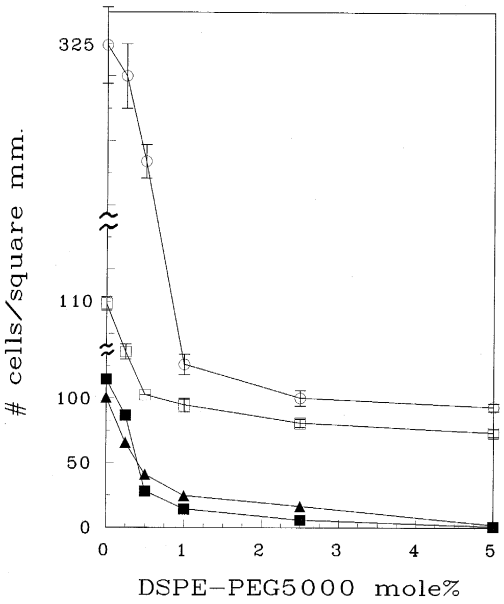

Fig. 4. The density of the adhering erythrocytes, lymphocytes (open square) and macrophages (open circle) at various concentrations of DSPE-PEG5000. For erythrocytes, the values derived from microscopy and hemoglobin absorption measurement are shown as solid squares and solid triangles respectively. [Note: Both lymphocyte and macrophage curves were shifted vertically for clarity. The highest value for each curve is as marked and scale sub-divisions are uniform throughout.]

3.3. Cell adhesion

3.3.1. Erythrocyte

The adhesion of the erythrocyte is measured by the density of cells (number of erythrocytes/mm^2) on the surface of DPPE:DSPE-PEG5000, as shown in Fig. 4. The density of erythrocytes decreases rapidly with the percentage of DSPE-PEG5000 at the 0–1 mol% range, but the decrease slows down until reaching a near zero adhesion at 5.0 mol% of DPPE:DSPE-PEG5000. Typical micrographs of cell adhesion are shown in Fig. 5a–d, corresponding to DSPE-PEG5000 concentration of 0, 1.0, 2.5 and 5.0 mol%, respectively.

Alternatively, the absorbance of hemoglobin at 415 nm from lysed cells washed off the cover slip surface may be used to quantitate the adhesion of erythrocytes. The hemoglobin absorbance from lysed erythrocytes on surfaces containing different concentrations of DSPE-PEG5000 is used to calculate the surface density of erythrocytes. The calculated den-

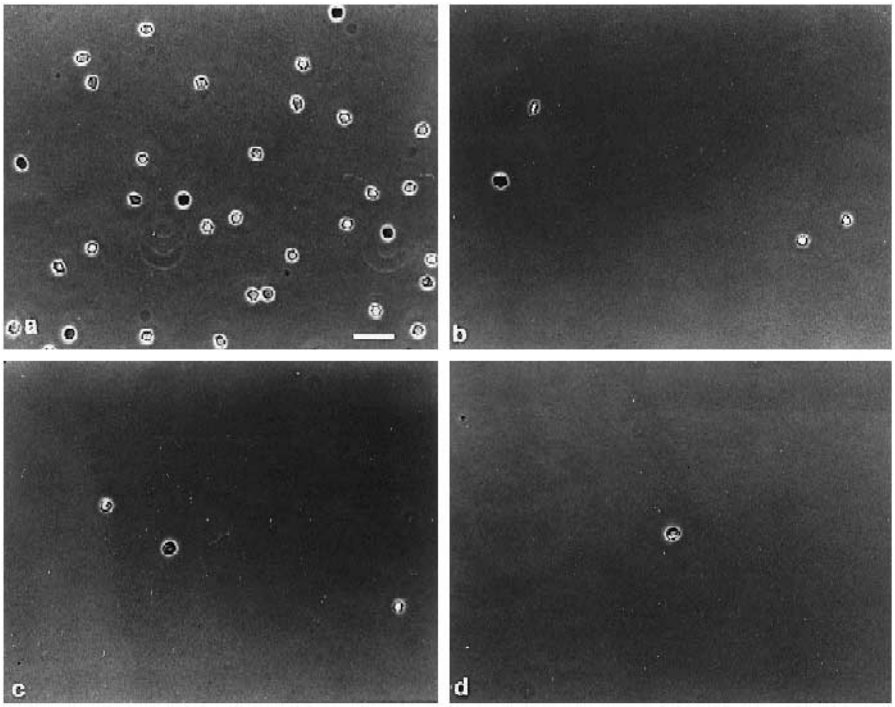

Fig. 5. Micrographs of the adhering erythrocytes at various concentrations of DSPE-PEG5000: (a) 0 mol%, (b) 1.0 mol%, (c) 2.5 mol%, and (d) 5.0 mol%. The bar corresponds to 31.25 μm.

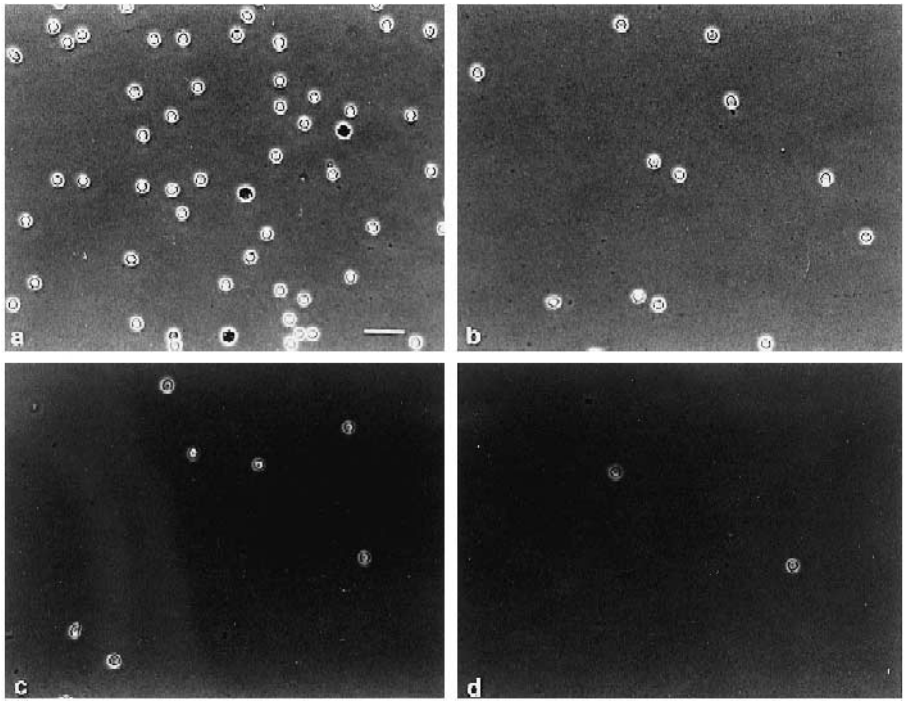

Fig. 6. Micrographs of the adhering lymphocytes at various concentrations of DSPE-PEG5000: (a) 0 mol%, (b) 1.0 mol%, and (c) 2.5 mol%, and (d) 5.0 mol%. The bar corresponds to 31.25 μm.

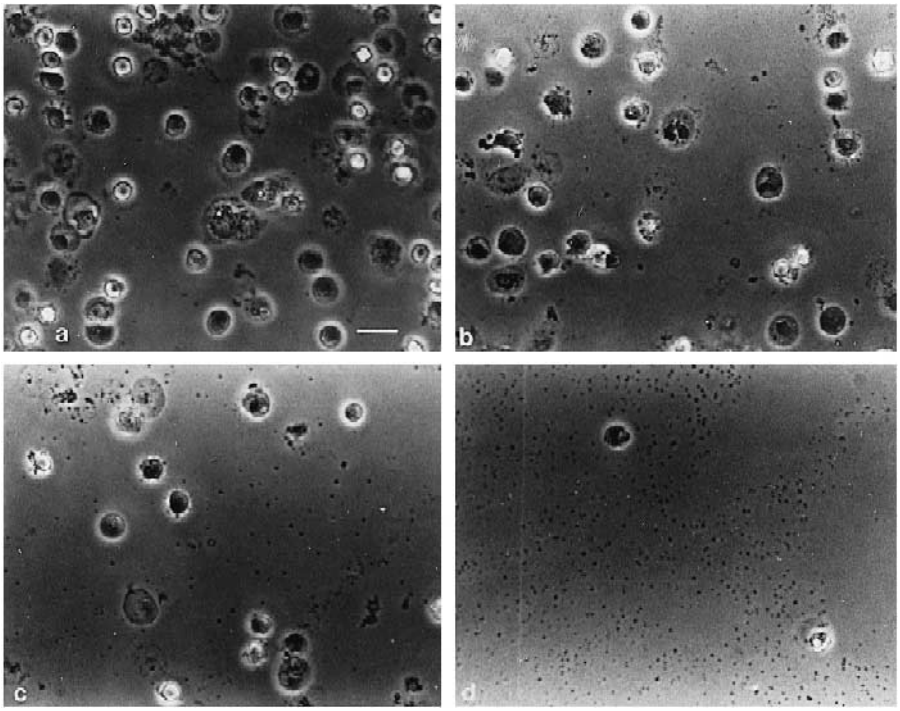

Fig. 7. Micrographs of the adhering macrophages at various concentrations of DSPE-PEG5000: (a) 0 mol%, (b) 1.0 mol%, and (c) 2.5 mol%, and (d) 5.0 mol%. The bar corresponds to 31.25 μm.

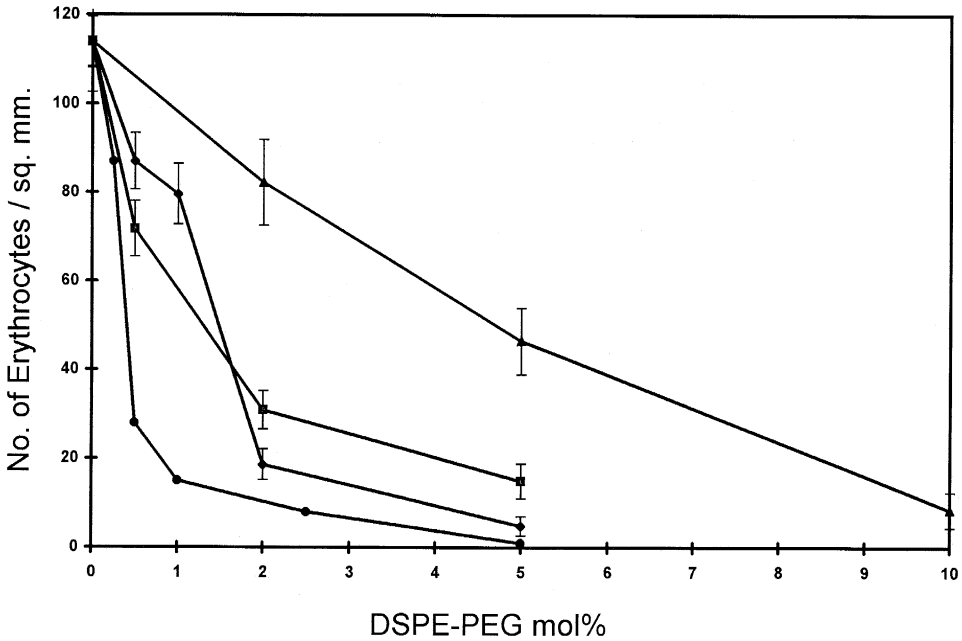

Fig. 8. The density of the adhering erythrocytes at various concentrations of DSPE-PEG of molecular weights: 5000 (solid circle), 3000 (solid diamond), 2000 (solid square) and 750 (solid triangle).

sity vs. DSPE-PEG5000 concentration is also plotted in Fig. 4 for comparison. The agreement is reasonable, given the experimental uncertainties.

3.3.2. Lymphocyte

The density of lymphocytes (number of lymphocytes/mm^2) vs. the concentration of DSPE-PEG5000 is shown in Fig. 4. As the concentration of DSPE-PEG5000 increases from 0–1.0 mol%, the density of lymphocyte decreases rapidly. Again, the decrease slows down at DSPE-PEG5000 concentrations above 1.0 mol%. Corresponding micrographs (Fig. 6a–d) show adhered lymphocytes at concentrations of DSPE-PEG5000 of 0, 1.0, 2.5, and 5.0 mol%, respectively.

3.3.3. Macrophage

The density of macrophages (number of macrophages/mm^2) vs. the concentration of DSPE-PEG5000 is shown in Fig. 4. As in the case of erythrocytes and lymphocytes, the density of macrophages decreases rapidly as the percentage of DSPE-PEG5000 increases from 0–1.0 mol%, and then decreases slowly at higher concentrations. Corresponding micrographs, representing adhered macrophages at concentrations of DSPE-PEG5000 of 0, 1.0, 2.5, and 5.0 mol%, are shown in Fig. 7a–d, respectively. The decrease in the number of macrophages adhering to the phospholipid bilayer surface with the increase of the concentration of DSPE-PEG5000 is demonstrated clearly in the micrographs.

3.4. Effect of PEG molecular weight on erythrocyte adhesion

The effect of different PEG molecular weights on erythrocyte adhesion is shown in Fig. 8. All the four different molecular weights of PEG lipids used show a general trend of decrease in erythrocyte adhesion per unit area with increased mol% of PEG lipid in DPPE. The effect is most pronounced in the case of DSPE-PEG5000 and less pronounced with lower molecular weight PEG moieties. There is a change of decreasing rates at the range of 0.6 mol% in the case of DSPE-PEG5000, but the change becomes less apparent with decreasing molecular weights of the grafted PEG moiety. No apparent change of decreasing rates is observed in the case of DSPE-PEG750.

4. Discussion

4.1. The assembly state of the grafted monolayer

Our Π-A diagram of DPPE approximately agrees with those reported by [16,17], considering the different experimental conditions (i.e., the composition and pH of the subphase, and the temperature). For all Π-A curves, the surface pressure increases as the concentration of DSPE-PEG5000 increases, a result which is approximately in agreement with that of Kuhl et al. [18]. At the same surface pressure, the area occupied by each molecule in the monolayer is larger at higher DSPE-PEG5000 concentrations, as expected. If the concentration of DSPE-PEG5000 exceeds 40 mol%, no stable monolayer can be formed (data not shown) because of micellation caused by the detergent nature of DSPE-PEG5000 molecules with their large head-groups. At 5 mol% or less of DSPE-PEG5000, the area per lipid molecule was 57–59 $Å^2$ at 35 dynes/cm, at which the transfer of monolayer to glass support was made. Since the transfer ratio of the monolayer to the surface of the glass coverslip is roughly 100%, it is likely that the transferred monolayer on the glass coverslip surface retains the composition of the monolayer on the air/water interface.

4.1.1. The effect of DSPE-PEG5000 on protein adsorption

By the theory of steric stabilization, the existence of grafted polymers on the solid surface is expected to protect the surface against adsorption and aggregation of foreign particles [19,20]. In our case, the grafted PEG moiety provides a steric barrier near the surface against the approach and adsorption of other macromolecules [21–23]. Therefore, the concentration of the grafted lipid (grafted density) is the most relevant parameter in relation to the steric stabilization of the monolayer surface. We calculate a priori the percentage of surface area of the monolayer that is covered by the grafted PEG moiety at various concentrations of DSPE-PEG from the theoretical

cross-section areas of the grafted PEG and lipids, as outlined below.

According to the model for the conformation of grafted polymers, there exist two types of grafted surfaces in good solvent conditions [24]. At low grafting densities, the chains of grafted-polymers are displayed like adjacent 'mushrooms'. The area A covered by each molecule is approximately expressed as:

$$A = \pi R_F^2, \quad (1)$$

where the Flory radius R_F [25] is given by:

$$R_F^2 = N^{6/5} a^2, \quad (2)$$

where N is the degree of polymerization, a is the size of a monomer, which is usually in the range 2–5 Å. We calculated the cross-sectional areas of PEG moieties of different molecular weights in the mushroom conformation, using Eq. (1) and Eq. (2). Torchilin and Papisov [23] also derived equivalent radii of mushroom model PEG of different molecular weights on lipid surfaces. Their values are slightly larger than the Flory radii of the corresponding free polymers in solution, as we calculated. The former values are used in our subsequent analysis.

At high grafting densities, the polymer chains prefer to stretch out from the grafted surface in order to avoid overlapping each other, as described by the 'brush' model [24]. We take the cross-sectional area of each polymer chain to be about 40–100 Å^2, assuming PEG5000 is in a fully extended zigzag conformation and extending from the surface. The percentages of the surface area covered by PEG moieties at given mole percentages are then calculated, assuming either the mushroom or the brush model. For either model, the uncovered area decreases linearly with the increase of mol% of PEG-grafted lipids. Only 0.7 mol% of PEG5000 in the mushroom conformation is needed to fully cover the surface, while at least 60 mol% of PEG5000 in the brush conformation is needed to cover the same.

Our experimental results show that the presence of DSPE-PEG5000 at the surface of phospholipid bilayer can reduce the proteins bound to the surface after repeated washing (Fig. 3). This may be a result of either reducing the initial adsorption or facilitating the release of the proteins. The concentration of DSPE-PEG5000 is crucial for the inhibition of the adsorption of these proteins. For the concentration below 1.0 mol%, the protein adsorption decreases steeply as the concentration of DSPE-PEG5000 increases. Higher concentrations of DSPE-PEG5000 still lead to a slight decrease of the protein adsorption which is already very poor. Apparently, neither the mushroom nor the brush model alone can explain the nonlinear adsorption of proteins, if the inhibition of protein adsorption is mainly due to steric repulsion by the grafted polymers. Admittedly, as DSPE-PEG5000 concentrations approaching 0.7 mol%, which corresponds to the concentration when the entire surface is supposedly covered by grafted-PEG in the mushroom conformation, the tighter packing of grafted polymers may lead to their extrusion from the surface to the formation of brush conformation. This may result in gradual increase in inhibition of the protein adsorption, as observed.

For randomly, but not evenly, distributed grafted PEG molecules on the bilayer surface, some PEG in the high PEG surface density areas may be forced to extend into the brush conformation, prior to the complete coverage of the lipid surface by PEG moieties. Our experimental data indicate such a transition prior to the complete surface coverage. We calculated the percentage of surface area covered, based on a simple model that, after a 'transitional' percentage of surface area is covered by PEG in the mushroom conformation, the remaining area would be covered by PEG in the brush conformation, should the grafted PEG concentration exceed this percentage. Several 'transitional' percentages were tested, and the percentage yielding the best fit to our experimental data was selected. The uncovered area percentage, calculated using the best fit 'transition' value of 82.5%, is plotted against the mol percent concentration of DSPE-PEG in Fig. 9A,B. The curve approximates two linear regions corresponding to each model, joined by a transition region. The trend of protein adsorption follows closely the diminishing of uncovered areas as calculated by this method (Fig. 9A). It seems that the mushroom model of the grafted PEG5000 is the more appropriate one at the PEG5000 concentration range of 0–0.5 mol%. Additional concentration of PEG5000 is likely to be in the brush conformation. Some areas in the surface remain uncovered even at high grafting concentration.

Klibanov et al. [5] studied the liposome agglutina-

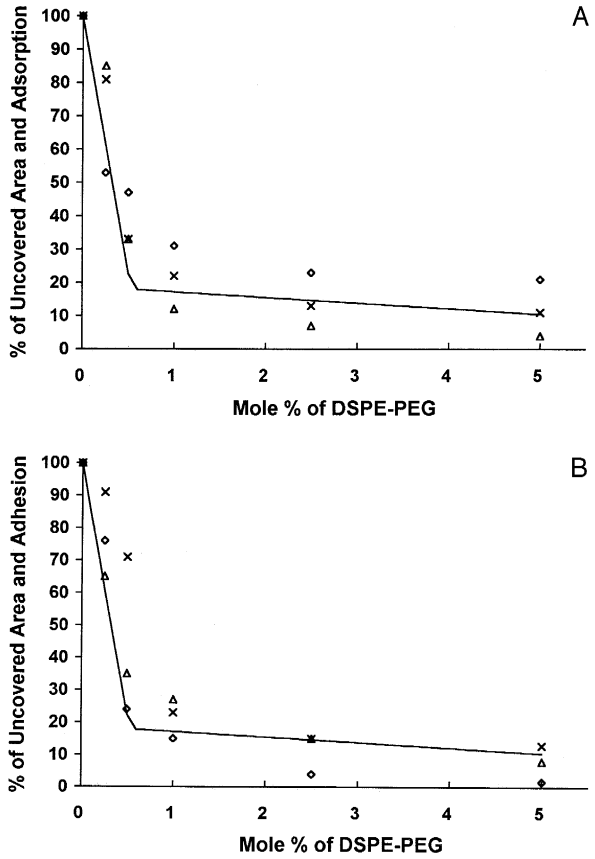

Fig. 9. (A) The relative adsorption of BSA (diamonds), fibronectin (triangles) and laminin (crosses), as percentage of their maximum adsorption at 0 mol% DSPE-PEG5000, and (B) the relative adhesion of erythrocytes (diamonds), lymphocytes (triangles) and macrophages (crosses), as percentage of their maximum adhesion at 0 mol% DSPE-PEG5000, are given at various mole concentrations of DSPE-PEG5000. The calculated percentage of uncovered areas, based on the mushroom/brush model of the grafted polymers, are also drawn in for comparison.

tion induced by the addition of streptavidin to PEG5000-containing liposomes incorporated with a small amount of N-biotin-amino-caproyl-phosphatidylethanolamine, and found that as little as 0.72 mol% PEG5000-PE in these liposomes completely abolished agglutination. The grafting density of 0.72 mol% is similar to the grafting density we calculated for the full coverage of the surface. Noppl-Simson and Needham [26] found that 10 mol% of PEG750 or 4 mol% of PEG2000, when the transition to the brush regime is approached, also blocked out the binding of streptavidin to a surface containing 5 mol% of biotin. Their binding curve for the surface containing PEG750 resembles that shown in Fig. 8 for erythrocyte adhesion. Kuhl et al. [18] also suggested that the surface coverage is an important factor related to the interaction forces between bilayers exposing short-chained PEG such as PEG2000.

The degree of adsorption inhibition differs for each protein we tested. The inhibition effect of PEG5000 on the adsorption of BSA is much less effective than those on the other two proteins. It might be the case that smaller protein molecules can go between the polymer mushrooms. Thus, the inhibitory effect of PEG5000 is weaker for smaller molecules than that for larger ones. The adsorption of BSA may also cause the release of some PEG5000 into the subphase in the micellar form, thereby leaving more room for higher residual protein adsorption. The nature (i.e., the unfolding of proteins upon adsorption, surface hydrophobicity, or other structural properties) of the protein itself also plays a role in determining how tenaciously the protein molecules bind to the substrate. This may explain the discrepancy between fluorescence measurements taken directly from glass coverslips (Fig. 2) and those from ethanol wash (Fig. 3). Furthermore, it is entirely possible that the protein adsorption can only be reduced to a non-zero minimum even when the entire surface is covered by the grafted polymer. In that case, the conformation and distribution of the grafted polymer at high concentration is of little consequence.

The experimental errors on repeating experiments are within 10–15%, which is much smaller than the difference between the data points. The error is largely due to the orientation of the glass coverslip to the incident beam in fluorescence measurements.

4.1.2. The effect of DSPE-PEG on cell adhesion

Our results also give direct evidence that the presence of PEG can reduce cell adhesion to the surface, either by reducing the initial adhesion or by weakening the retention of several types of cells such as erythrocytes, lymphocytes and macrophages during washing.

Cell adhesion is scored mostly by microscopic counting. In this method, an average number is derived from a sampling area on the bilayer-coated glass surface. Because cells do not evenly distribute on the surface, an error of limited sampling (usually

less than 10%) cannot be avoided. For the case of erythrocytes, the cell counts may be checked spectroscopically using cell lysate. In the range of 0–0.5 mol% of grafted PEG5000, the spectroscopic method results in slightly lower counts than that by the microscopic method. At higher grafted polymer concentrations, the trend is reversed (Fig. 4). Again, the deviations are usually smaller compared with the difference between data points.

The comparison of the decrease of cell adhesion with the decrease of exposed area is given in Fig. 9B. Here the decrease of cell adhesion is expressed as the percentages of the maximum adhesion in the absence of grafted polymers. At the 0–0.5 mol% range of grafted lipids, the percentage of erythrocyte and lymphocyte adhesion agrees, within experimental error, with the percentage of the surface area not covered by PEG5000 moieties in the mushroom conformation. For the adhesion of macrophage, the percentage of the cell adhesion is greater than that of the exposed surface area, especially at the 0.25 and 0.5 mol% of DSPE-PEG5000. Since the function of macrophage involves the adhesion to foreign materials, the higher percentages of adhesion than those for erythrocytes and lymphocytes are not unexpected. Our results further show that lipid bilayer surfaces protected by grafted PEG5000 is not entirely immune to macrophage attachment. Therefore, the stealth liposomes containing grafted PEG lipids are not entirely stealth even at optimal composition for surface area protection.

The effect of molecular weight of the PEG moiety on erythrocyte adhesion also reflects the dominating effect of steric repulsion. Fig. 10 shows the decrease of cell adhesion expressed as percentages of the maximum adhesion in the absence of grafted polymers. The percentages of exposed or uncovered areas calculated for mixtures containing PEG moieties of molecular weights from 750, 2000, 3000 and 5000, in the mushroom conformation only, are also plotted for comparison. The inhibition of adhesion is in general less than those predicted by the mushroom model calculation, especially for low molecular weight grafted PEG, but is more than that by the brush model calculation. For low molecular weight grafted PEG, their shorter chains are not as amendable to folding as longer chains, so that the mushroom model becomes less accurate in depicting the actual molecular conformation. In concentrations below confluent coverage, the shorter and straighter chains may wobble at any angles between normal and parallel to the bilayer surface, so that the average area they occupy is between those depicted by the brush and the mushroom models, and vary with surface concentration, without an apparent transition between two models. The reduced thickness of shielding by these shorter chain polymers also reduces their effectiveness as cell adhesion inhibitors. Both reasons may account for the deviation of experimental data from the mushroom model calculation for lower molecular weight grafted PEG. The general concurrence of the decrease of cell adhesion with estimated decreases of uncovered areas for all DSPE-PEG used, supports the proposition that steric repulsion by grafted PEG is largely responsible for the decrease of cell adhesion.

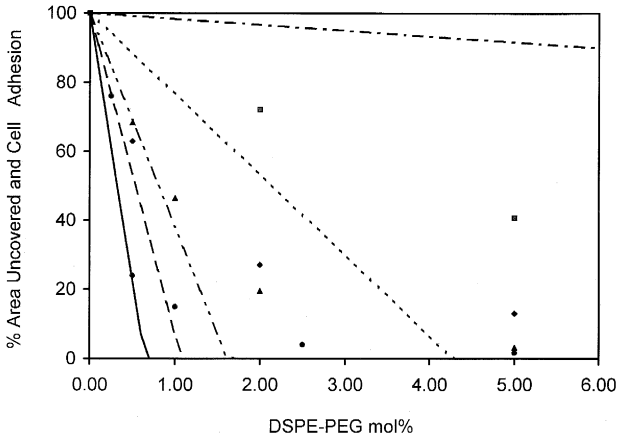

Fig. 10. The relative adhesion of erythrocytes, as percentage of their maximum adhesion at 0 mol% DSPE-PEG5000 of different molecular weights: 5000 (solid circle), 3000 (solid triangle), 2000 (solid diamond) and 750 (solid square), are given at various mol concentrations of DSPE-PEG. The calculated percentage of uncovered areas for different molecular weights: 5000 (unbroken line), 3000 (dashed line), 2000 (dot/dot/dashed line) and 750 (dotted line), based on the mushroom model, and that based on the brush model (dot/dashed line) of the grafted polymers, are also drawn in for comparison.

5. Conclusion

We have established the relationship between covered/protected lipid surface area by grafted PEG and the inhibition of protein adsorption as well as cell

adhesion. The relationship may be interpreted theoretically and quantitatively if a random distribution of the grafted PEG (MW > 3000) in the mushroom conformation is assumed. The random, uneven distribution implies open areas as well as congested areas where the grafted polymers may be forced to assume the brush conformation at high grafting density. Surface heterogeneity, inexact modeling of polymer conformation for low molecular weight polymers, and perturbation of the steric barrier by the attached proteins and cells, among other factors, account for the discrepancy of experimental data from theoretical prediction. The finding provides information in designing stealth liposomes for drug delivery, as well as in designing biocompatible surface to reduce protein adsorption and cell adhesion.

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