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Bioadhesive polymers as platforms for oral-controlled drug delivery: method to study bioadhesion

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Summary

To overcome the relatively short gastrointestinal time and improve localization for oral-controlled or sustained-release drug delivery systems, it is suggested that bioadhesive polymers which adhere to the mucin/epithelial surface will be effective and lead to significant improvements in oral drug delivery. Improvements would also be expected for other mucus-covered sites of drug administration.

To examine a large number of polymers as to their bioadhesive potential and to derive meaningful information on the structural requirements for bioadhesion, a new, simple experimental technique that can quantitatively measure bioadhesive properties of various polymers has been developed. The technique consists of labeling the lipid bilayer of cultured human conjunctival epithelial cells with the fluorescent probe pyrene. Addition of polymers to this substrate surface compresses the lipid bilayer causing a change in fluorescence as compared to control cells. The fluorescent probe, pyrene, provides information on membrane viscosity, which is proportional to polymer binding. In addition to the use of pyrene, membrane proteins were labeled with fluorescein isothiocyanate, and depolarization of probe-labeled proteins was measured before and after polymer treatment. By using these fluorescent probes, it was possible to compare charge sign, charge type and density, and backbone structure as to their influence on polymer adhesion. Preliminary comments on structural features for polymer binding are that highly charged carboxylated polyanions are good potential bioadhesives for drug delivery.

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Introduction

Interest in controlled and sustained-release drug delivery has increased considerably during the past decade and, in selected areas, it is now possible to employ fairly sophisticated systems which are capable of excellent drug release control. The self-regulating insulin delivery system using lectins (Jeong et al., in press) and oral osmotic tablets (Robinson (Ed.), 1978) are illustrative examples. However, for oral administration, all of these systems are limited to some extent because of gastrointestinal transit. Thus, the duration of most oral sustained-release products is approximately 8–12 h due to the relatively short gastrointestinal transit time, and it is not yet possible to localize a drug delivery system in selected regions of the gastrointestinal tract for purposes of localized drug delivery or to modify the absorbing membrane.

Several approaches have been suggested to increase gastrointestinal transit time without at the same time addressing the issue of localized drug delivery. Both low and high density drug delivery systems have been suggested as possible approaches to extend transit time (Watanabe et al., 1976; Bechgaard and Ladefoged, 1978), but the very premise and results of exploratory studies are equivocal and, in any case, of relatively short duration. In a similar manner, particle size, relative to stomach retropulsion (P. Bass, personal communication), has been suggested as a means to delay stomach-emptying and thereby prolong transit time. This phenomena is also of relatively short duration, particularly when the drug delivery system is administered in the absence of food (P. Bass, personal communication). An alternative approach is to employ bioadhesive polymers that adhere to the mucin/epithelial surface. Such polymers would have application to any mucous membranes and perhaps non-mucous membranes as well. Thus, bioadhesive polymers would find application in the eye, nose, and vaginal cavity as well as the gastrointestinal tract including the buccal cavity and rectum.

The purposes of the present research were to establish procedures to study polymer bioadhesion and to understand structural requirements for bioadhesion in order to design improved bioadhesive polymers for oral use. To accomplish these goals, it was first necessary to develop a technique that could examine the bioadhesive properties of different polymer types and compare them on a quantitative basis. In this paper, we report a new technique that can quantitatively examine the interaction between polymers and cell surfaces, i.e. bioadhesion.

Background

Polymers that adhere to the mucin–epithelial surface can be conveniently divided into 3 broad categories: (1) polymers that become sticky when placed in water and owe their bioadhesion to stickiness; (2) polymers that adhere through non-specific, non-covalent interactions which are primarily electrostatic in nature (although hydrogen and hydrophobic bonding may be significant); and (3) polymers that bind to specific receptor sites on the cell surface. All 3 polymer types can be used for drug

delivery. Any technique to quantitatively assess bioadhesion should be capable of assessing all 3 types of polymer.

The essence of the technique to be described is that a fluorescent probe is added to the cell membrane, and upon addition of an adsorbing polymer, there will be a change in cell membrane property, manifested as a change in fluorescence that is directly proportional to the degree of adsorption. Since the cell membrane is central to the nature of these binding experiments, it may be helpful to provide a topical description of biological membranes.

The original view of the cell membrane (Singer and Nicolson, 1972) as a two-dimensional oriented viscous lipid solution where proteins are freely moving has been continuously modified. Although biological cell membranes are indeed highly dynamic and all components can undergo Brownian motion, proteins are no longer thought of as freely moving. Abnormally low diffusion rates of membrane proteins (Koppel et al., 1981) suggest a possible interaction between membrane proteins and the underlying cytoskeleton. If membrane proteins are anchored to the cell substructure, diffusion of cell membrane proteins will not be determined by membrane lipid viscosity.

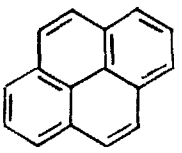
Because mobility of membrane proteins is primarily influenced by the underlying cytoskeleton and not by bilayer lipid viscosity, it was conceptually useful to divide the cell membrane into a protein part and a lipid part to examine polymer binding. Using liposomes as model membranes, it was shown that externally added polymers, such as polylysine (Hammes and Schullery, 1970), polyethylene glycol (Ohno et al., 1981), or polyglutamic acid (Chang and Chan, 1974), reduced mobility of fatty acid groups in the lipid bilayer probably due to induced tighter packing. Thus, it is reasonable to expect increased viscosity of the lipid portion of the cell membrane through polymer binding. A more tenuous expectation is that mobility of membrane proteins will decrease upon polymer binding unless the polymer disrupts the system and detaches proteins.

Theory

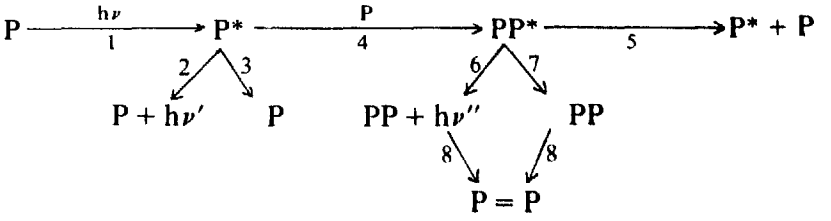
Pyrene fluorescent probe

The E / M ratio. Pyrene molecules, located in biological membranes or lipid vesicles, have been used to monitor the dynamic properties of membranes (Galla and Luisetti, 1980; Vanderkooi and Callis, 1974; Dembo et al., 1979; Galla et al., 1979). The structure of pyrene is shown in Scheme 1. A photoexcited molecule, during its long lifetime of about 300 ns (Thomas, 1980), can react with an unexcited monomer to form a sandwich-like complex called an excimer, as depicted in Scheme 2 (Pownall and Smith, 1973).

As shown in Fig. 1, excimer fluorescence is readily distinguished from monomer fluorescence. Lateral movement of the pyrene molecule in the membrane is closely related to mobility of lipid molecules, and, consequently, excimer formation is a diffusion-controlled process (Galla et al., 1979). This property has been used to measure membrane fluidity (Vanderkooi and Callis, 1974; Dembo et al., 1979; Galla



Scheme 1. Structure of pyrene



	Step	Process
(1)	$P + h\nu \rightarrow P^*$	Excitation
(2)	$P^* \rightarrow P + h\nu'$	Monomer fluorescence
(3)	$P^* \rightarrow P$	Monomer radiationless transition
(4)	$P^* + P \rightarrow PP^*$	Excimer formation
(5)	$PP^* \rightarrow P + P^*$	Excimer decomposition
(6)	$PP^* \rightarrow PP + h\nu''$	Excimer fluorescence
(7)	$PP^* \rightarrow PP$	Excimer radiationless transition
(8)	$PP \rightarrow P = P$	Dimer decomposition

Scheme 2. Ultraviolet excitation and decay pathways for pyrene.

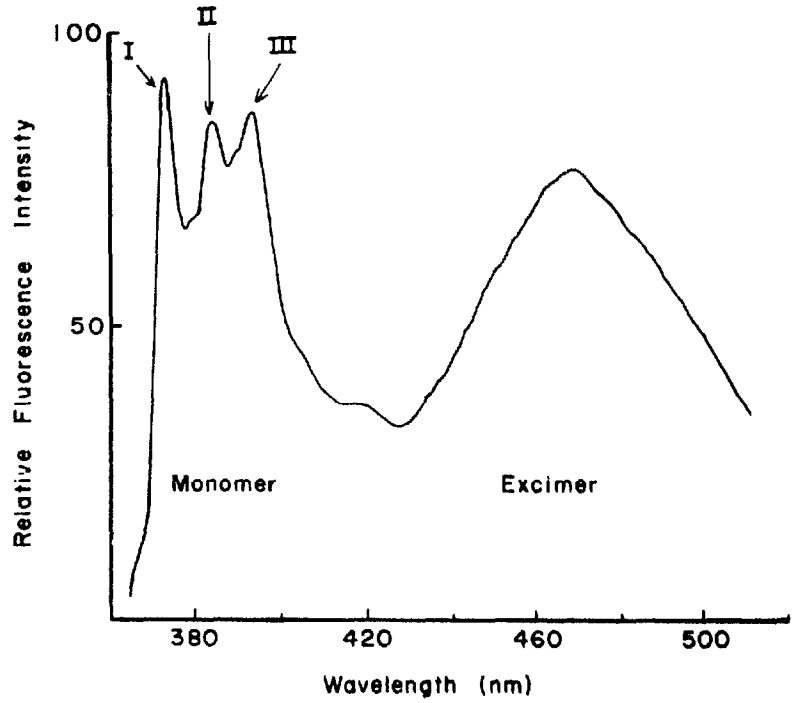


Fig. 1. Fluorescence spectrum of pyrene in cell membrane. Three peaks of monomer (I–III) and one excimer peak are shown.

et al., 1979; Thomas, 1980) or the transition temperatures of lipid vesicles (Galla and Luisetti, 1980). Excimer formation depends on both viscosity and an effective pyrene concentration. For example, high excimer formation can be observed due to phase separation of the membrane bilayer, which will increase the local effective pyrene concentration.

Fluorescence intensities of the monomer (M) and the excimer (E) are related to the diffusion coefficient of pyrene molecules in the lipid bilayer as shown in Eqn. 1 (Galla et al., 1979).

$$D = \left(\frac{1}{4} \langle \eta_x \rangle \lambda^2 \frac{1}{K} \cdot \frac{1}{\tau} \cdot \frac{k_m}{k_E} \right) \frac{E}{M} \tag{1}$$

where $\langle \eta_x \rangle$ is the average step number to prepare excimers and is dependent on the pyrene concentration, λ is the average jump length of the pyrene molecule, K is the proportionality constant characteristic for pyrene, τ is the fluorescence lifetime, and k_m and k_E are transition probabilities for the radioactive decay of excited monomer and excimer, respectively. Although all the parameters in parentheses are measurable quantities, they are of no importance to the conduct of this study, and hence, Eqn. 1 can be simplified to

$$D = c \cdot \frac{E}{M} \tag{2}$$

where c is a constant for a given system. Thus, by measuring and comparing E/M ratios for control and polymer treated cells, it is possible to note the effect of polymer treatment on membrane fluidity, i.e. polymer binding. The reduced E/M ratio by polymer treatment is expressed as Δ(E/M).

$$\Delta(E/M) = (E/M)_{control} - (E/M)_{polymer\ treated} \tag{3}$$

Δ(E/M) can then be used as a parameter to quantitatively compare the ability of polymers to increase viscosity with the assumption that an increase in viscosity is due to polymer binding or adsorption. An example of the change in E/M ratio that occurs upon polymer treatment is shown in Fig. 2.

The P_y value. In addition to information on fluidity of the lipid bilayer, it is also possible to obtain information on polarity of the probe environment from the peak ratio measurement of monomer fluorescence. As shown in Fig. 1, there are 3 clearly defined peaks in the monomer fluorescence. Peak II shows a solvent-dependent change in intensity whereas peak I is insensitive to polarity. Thus, the peak intensity ratio of II/I can be used as a measure of polarity of the probe environment (Thomas, 1980; Pownall and Smith, 1973; Kalyanasundaram and Thomas, 1977). The ratio of II/I will henceforth be referred to as the P_y value. The P_y value is insensitive to the presence of oxygen and most other fluorescence quenchers and is relatively insensitive to hydrogen bonding (Kalyanasundaram and Thomas, 1977). Its value, expressed as a fraction and reproducible to $F = 0.02$, is independent of

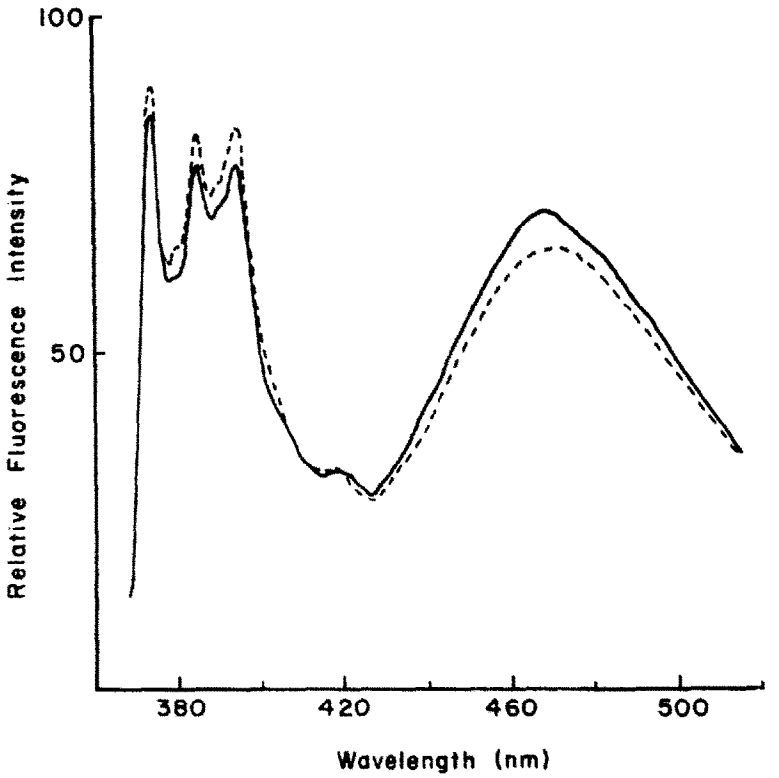


Fig. 2. Fluorescence spectra of pyrene in control cells (———) and cells suspended in 0.1 (w/w)% poly-L-lysine solution (- - - - -).

excitation wavelength and pyrene concentration (Pownall and Smith, 1973). A P_v value of about 0.90 was observed when pyrene was incorporated into cells; and because this value is significantly different from that in water, $P_{v_{water}} = 0.64$, the P_v value serves as an internal check of probe location. The small P_v value in a polar medium is due to a 1 : 1 complex between pyrene and polar solvent (Lianos and Georghiou, 1979).

Cell size measurement. As discussed above, the extent of excimer formation depends on two factors, pyrene–membrane lipid ratio and viscosity of the probe environment. A change in cell size or volume will affect both pyrene concentration in the lipid bilayer and membrane viscosity, both of which can influence the E/M ratio. As cell volume increases, effective pyrene concentration and viscosity decreases. A decrease in effective pyrene concentration has an effect on E/M ratio opposite to that produced by a decrease in viscosity. Thus, the observed net effect will be different depending on the relative contributions of these two effects. In this regard, it is necessary to measure the change in cell size after polymer treatment in order to correctly interpret the Δ(E/M) values.

Fluorescein isothiocyanate probe. Polymer binding to membrane proteins can be measured using fluorescence depolarization, which is a well-established technique (Heyn et al., 1977; Nigg and Cherry, 1980) for measuring rotational diffusion coefficients. In short, it is possible to detect changes in viscosity of the environment

of fluorescent probe-labeled membrane proteins. The Perrin equation for steady-state polarization is shown in Eqn. 4:

$$\frac{1}{\bar{p}} - \frac{1}{3} = \left(\frac{1}{\bar{p}_0} - \frac{1}{3}\right)\left(1 + \frac{\tau kt}{V\eta}\right) \quad (4)$$

where $\bar{p}$ is the steady-state polarization value, $\bar{p}_0$ is $\bar{p}$ at infinite viscosity, τ is fluorescence lifetime, k is Boltzmann constant, T is absolute temperature, V is the hydrated volume of a molecule and η is viscosity. It should be noted that the polarization value is also dependent on several factors such as internal flexibility, shape or volume of proteins, and interaction with other molecules (Heyn et al., 1977).

Rotational movement of protein molecules in cell membranes is expected to be different from that in isotropic solvents, presumably due to a wobbling motion in the membrane (Heyn et al., 1977; Nigg and Cherry, 1980), and thus data treatment to compute an exact viscosity from steady-state polarization measurements is inappropriate. Nevertheless, comparative values were expected to be of value.

Experimental

Materials

The following polymers were studied: essentially fatty acid free bovine albumin [1], λ-carrageenan [1], chondroitin sulfate [1], calf skin gelatin [1], guar gum [1], porcine intestinal mucosal heparin [1], hyaluronic acid [1], bovine submaxillary mucin [1], carboxymethylcellulose [1], dextran [2], dextran sulfate [1], ficoll [1], polyacrylic acid [3], poly-L-aspartic acid [1], polybrene [3], polycarbophil [4], polyethylene glycol [3], poly-L-glutamic acid [1], poly-L-lysine [1], polystyrene-sulfonate [5], polyvinylmethylimidazole [5], psyllium [1], polyvinyl sulfate [1], and polyvinylpyrrolidone [5]. All were used as received. Pyrene [3] was recrystallized in dehydrated ethanol, and 8-anilino-1-naphthalene sulfonate [1] was purified by the method of Weber and Young (1964). Fluorescein isothiocyanate [1] was used without further purification. All other chemicals were either reagent or analytical grade.

Cell culture

Human conjunctival epithelial cells were grown in minimum essential medium [7] containing 10% fetal calf serum [7], gentamycin [6] (50 μg/ml), and Fungizone [7] (250

[1] Sigma Chemicals, St. Louis, MO.
[2] Pharmacia Fine Chem., Piscataway, NJ.
[3] Aldrich Chem., Milwaukee, WI.
[4] A.H. Robins, Richmond, VA.
[5] Eastman Kodak, Rochester, NY.
[6] Elkins-Sinn, Cherry Hill, NJ.
[7] Grand Island Biologicals, Grand Island, NY.

μg/ml) at 37°C with 95% air–5% CO_2. Cells were usually grown in 75 cm^2 tissue culture flasks [8] and passed at a log phase growth state. Trypsin-EDTA solution [7] was added (9 ml/75 cm^2) to the cell monolayer in order to dissociate cells and was incubated at 37°C for 10 min. After dissociation, the same volume of medium containing 10% fetal calf serum [7] was added to inactivate trypsin. Single cells were then used for either subculture or experiments.

Size measurement

The number and average size of single cells were determined by an electronic particle counter [9]. The counter was calibrated with 19.5 μm ragweed pollen [10] using an orifice tube with a diameter of 95 μm [11]. The pollen was dispersed in Sorensen's pH 7.0 isotonic phosphate buffer solution, which was prefiltered through a 0.45 μm filter [12].

Viscosity measurement

Relative viscosity of the polymer solution was calculated using an Ostwald viscometer in 37°C water bath and the equation

$$\eta = \frac{t}{t_0} \cdot \frac{\rho}{\rho_0} \cdot \eta_0 \tag{5}$$

where ρ is the liquid density, t is flow time and the zero subscripts refer to solvent properties. Most of the polymers were studied at low concentration where solutions are close to Newtonian in their rheological behavior. The few polymers that were studied at higher viscosity also employed the Ostwald viscometer and viscosity values are reported for comparative purposes only. Solution density was measured with a 2-ml pycnometer at 37°C.

Labeling with fluorescent probes

Cells were harvested as described in 'Cell Culture', and single cells were cultured at 37°C in a suspended state. After 1 h, cells were separated from the medium containing fetal calf serum and washed with a pH = 7.4 phosphate-buffered saline solution. Cells were again centrifuged and resuspended in minimum essential medium without serum or phosphate-buffered saline solution for labeling with pyrene or fluorescein isothiocyanate. Labeling conditions are shown in Table 1. Incorporation of pyrene into the membrane was accomplished by adding a concentrated pyrene solution to the cell suspension. Pyrene was dissolved in ethanol, and the final ethanol concentration in the cell suspension did not exceed 0.1 v/v%. Fluorescein isothiocyanate (FITC) was dissolved directly in phosphate-buffered saline solution.

[8] Corning, Chicago, IL.
[9] Electrozone/Celloscope, Model III LTS/ADC, S/N 72328, Particle Data, Elmhurst, IL.
[10] Coulter Electronics, Franklin Park, IL.
[11] Particle Data, Elmhurst, IL.
[12] Gelman Sciences, Ann Arbor, MI.

TABLE I

CONDITIONS FOR PROBE LABELING

	Pyrene	Fluorescein isothiocyanate
Concentration	10 μM	20 μM
Cell number	15 million	15 million
Total volume	20 ml	10 ml
Solution	Minimum essential medium	Phosphate-buffered saline solution
Time	20 min	10 min
Temperature	37°C	37°C

After an appropriate incubation time, cells were separated from the labeling solution and rewashed with phosphate-buffered saline solution. Finally, cells were resuspended in phosphate-buffered saline solution, and the cell concentration was adjusted to 2 million cells/ml.

Pyrene fluorescence measurement

Steady-state fluorescence spectra were obtained using a fluorescence spectrophotometer [13]. Samples were maintained at 37°C by circulating water through the cell-holder. Measurements were done in the ratio recording mode to correct for intensity fluctuations of the light source. Spectra were uncorrected for spectral sensitivity of photomultiplier and emission monochromator efficiency.

The excimer : monomer ratio was calculated by comparing the excimer fluorescence intensity at 470 nm to the average intensity of monomer fluorescence, using 338 nm as the excitation wavelength. Bandwidths were 2 and 3 nm for excitation and emission, respectively. A glass filter [14] was used to isolate the wavelength region for excitation, and a 350 nm cut-off filter was used for emission. The excimer/monomer (E/M) ratio was measured for both control and polymer-treated cells at various times following addition of polymer to the cell suspension. There is a small uncertainty in each time point, because intensities of monomer and excimer were not measured simultaneously. This uncertainty, however, is less than one minute, which is the time to complete scanning of the fluorescence spectrum. Corrections for solutions that scatter and depolarize excitation and emission lights are not necessary with pyrene, since this technique is independent of the polarization phenomenon.

Steady-state polarization measurement

Fluorescein covalently linked to cell membrane proteins was excited at 495 nm and the emission intensity was measured at 515 nm with a 490 nm cut-off filter. Slit width was 10 nm for both excitation and emission. Polymer-treated cells were incubated at 37°C for 10 min and then centrifuged to remove excess polymer. Cells

[13] Perkin-Elmer, Model MPF, Norwalk, CT.
[14] Corning, No. 9863, Chicago, IL.
[15] Perkin-Elmer, Norwalk, CT.

were re-suspended in phosphate-buffered saline solution. Polaroid polarizers [15] were used for depolarization measurements. Correction for light scattering due to cells was accomplished by subtracting the intensity of the same number of cells without fluorescein. To analyze fluorescence polarization, the sample was excited by linearly polarized light parallel to the vertical laboratory z-axis. Another polarizer was placed in the emission beam for recording the fluorescence intensity polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the vertical laboratory z-axis. To correct for a wavelength-dependent polarization response due to components of the emission detection system, such as monochromator, optics, and photomultiplier tube, a 'G-factor' was calculated. With linearly polarized excitation perpendicular to the vertical laboratory z-axis, the intensity ratio $I_{\parallel}/I_{\perp}$ is defined as the G-factor of the monochromator. True polarization is then given by:

$$P = \left(\frac{I_{\parallel} - I_{\perp} \cdot G}{I_{\parallel} + I_{\perp} \cdot G} \right)_V \tag{6}$$

where V indicates the vertical direction of polarization for the exciting light.

Results and Discussion

Reproducibility of E / M ratio

Reproducibility of E/M ratios for control and polymer-treated cells is critical in order to compare the effects of different polymers. Excimer formation depends upon the pyrene : membrane lipid ratio, i.e. effective pyrene concentration, and not on the total pyrene concentration in the sample. Thus, it was necessary to measure total lipid content in the cells. For convenience, however, a constant cell number was used instead of measuring the lipid content, with the assumption that cell number reflects lipid content of the cells. By carefully controlling pyrene concentration and cell number in the labeling procedure as shown in Table 1, it was possible to reproduce the E/M values. The normal E/M value of control cells was about 0.84 As shown in Table 2, cell number affects the absolute E/M value while Δ(E/M) is unaffected. This is because the effect of polymer on each single cell is the same, and the

TABLE 2

EFFECT OF CELL NUMBER ON E/M

Cell number	Control cells		Cells in 0.1% polylysine		Δ(E/M)
	E/M	P_v	E/M	P_v	
1,214,000	0.71	0.90	0.60	0.86	0.11
1,786,000	0.84	0.91	0.73	0.91	0.11
2,586,000	0.88	0.92	0.77	0.92	0.11
3,502,000	0.90	0.95	0.80	0.93	0.10

difference in E/M ratio is not a function of the total cell number.

Validity of the technique

With this technique, it has been assumed that pyrene molecules are located in the cell membrane. Even if this assumption is only partially correct, the change of E/M ratio can still be used as an indication of polymer binding to the cell surface since excimers are formed only in the cell membrane. As will be discussed subsequently, treatment of cells with bovine serum albumin resulted in almost complete disappearance of excimer formation (Table 6). This is believed to be due to the transfer of pyrene molecules from the cell membrane to albumin molecules, and the study to be described later in 'Treatment of Neutral Polymers' attests to this assertion. If excimer is also formed inside the cytoplasm, there should be excimer formation even after albumin treatment. It can be argued that pyrene molecules can move out of the cell membrane and interact with polymer molecules to reduce excimer formation. Should this occur, it is easily detected. If the E/M reduction is due to interaction of pyrene with polymer molecules, then the P_y value of pyrene in cells which are suspended in polymer solution should be the same as that of the polymer solution only or at least the P_y value should be lower than that of the control. This is because the P_y values of various polymer solutions are significantly lower than 0.90 as shown in Table 3. In fact, most of the P_y values of polymer-treated cells are not different from that of control cells; and it seems reasonable to conclude that, as long as the P_y values are the same, the change in E/M ratio is due to the change in some property of the cell membrane, such as viscosity. Specific interactions, however, are expected between pyrene molecules and polyvinylpyrrolidone (PVP) ($P_y = 0.56$), polyethylene glycol (PEG) ($P_y = 0.59$), and polybrene ($P_y = 0.57$). The common structural feature in these polymers is that they have a long hydrocarbon chain although polybrene has a charge along the hydrocarbon chain. Due to the nature of the P_y value, one might suspect that polarities of PVP, PEG and polybrene are greater than that of water. This, however, is not true. When the hydrophobic fluorescent probe, 8-anilino-1-naphthalene sulfonate (ANS), was used for PVP and PEG, significant intensity increases, as well as a shift of the fluorescence peak to 470 nm, were observed. This is characteristic of a hydrophobic environment for ANS. Therefore, the very low P_y values of these polymers should be attributed to some other property of the polymers. Strong adsorptive forces between PVP and 3,4-benzopyrene were observed (Chambron and Sadron, 1961), and the strong interaction between pyrene and these polymers is probably responsible for the further reduction in symmetry of the pyrene molecules beyond the reduction due to polar solvent-pyrene complex formation. The high P_y values of bovine submaxillary mucin, gelatin and bovine serum albumin may be due to intercalation of pyrene molecules into the hydrophobic cavities of these proteins.

In addition to an absence of specific interaction for most polymers, no effect of bulk viscosity due to added polymer was observed. Fig. 3 shows a plot of Δ(E/M) as a function of viscosity, and no correlation was found. This eliminates the possibility of a contribution from bulk viscosity to the value of the E/M ratio. Thus, the monitored change in E/M ratio after polymer treatment can be related to binding of

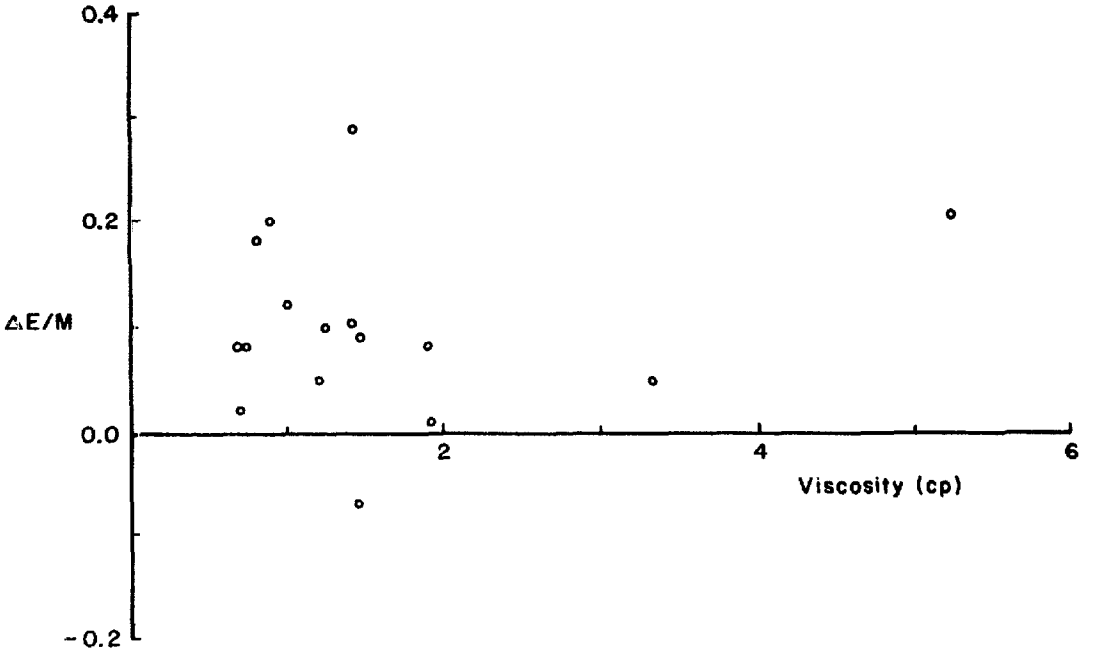


Fig. 3. The E/M ratio change vs viscosity of polymer solution. Polymers which change the P_v value of cells are not included.

TABLE 3

P_v VALUES OF POLYMER SOLUTION (WITHOUT CELLS)

Polymer	P_v value
(1) Cationic	
Polybrene	0.57
Poly-L-lysine	0.61
(2) Anionic	
Carboxymethylcellulose	0.64
Dextran sulfate	0.64
Polyacrylic acid	0.62
Poly-L-aspartic acid	0.64
Polystyrenesulfonic acid	0.64
Polyvinyl sulfate	0.65
Bovine submaxillary mucin	0.75
λ-Carrageenan	0.63
Chondroitin sulfate	0.63
Heparin	0.63
Hyaluronic acid	0.63
(3) Neutral	
Bovine serum albumin	0.86
Dextran	0.67
Ficoll	0.61
Gelatin	0.73
Polyethylene glycol	0.59
Polyvinylpyrrolidone	0.56

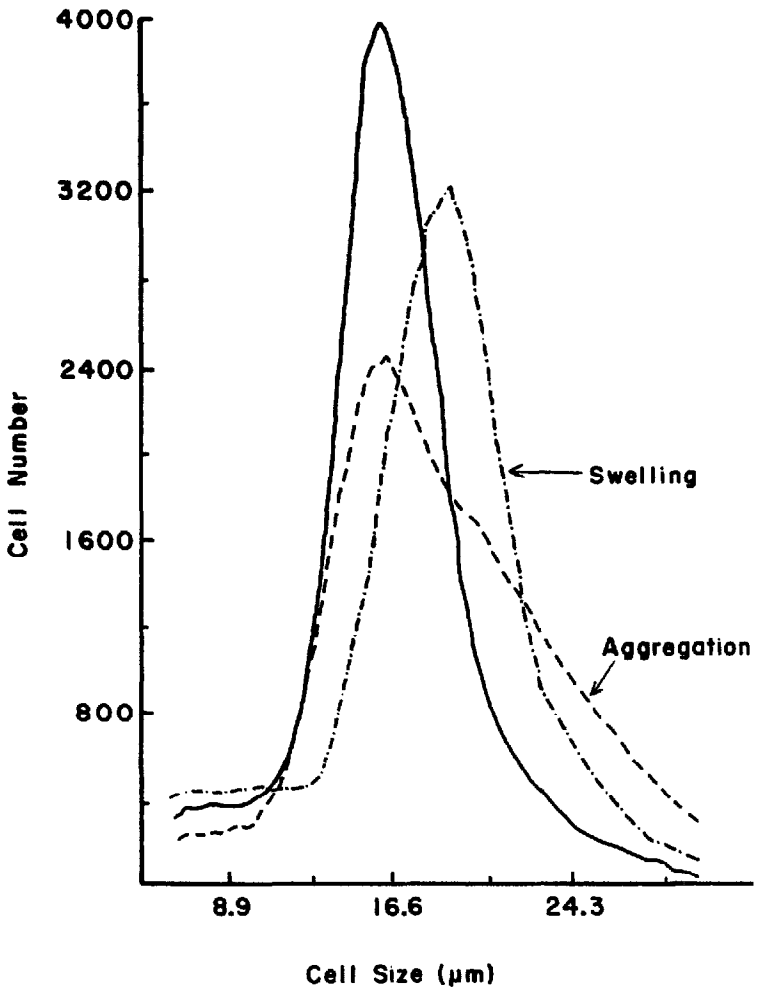


Fig. 4. Size distribution of control cells and polymer-treated cells. Control cells (———), cells in 2.0 (w/w)% polyvinyl sulfate (●–●–●), and cells in 0.1% poly-L-lysin (- - - - - -).

polymers to the cell surface. A high Δ(E/M) represents high binding of polymer to the cell surface. This technique can measure and compare binding of various polymers to the cell surface but cannot measure the amount of polymer bound.

Cell size measurement

Volume changes on a microscopic scale cannot be determined by macroscopic measurement such as those made with electronic particle counters. These macroscopic measurements, however, can give valuable quantitative information on substantial cell size changes, i.e. 20%, or formation of aggregates. Cell swelling or shrinking is easily detected, if the size change is more than 5% of the original. Generally, cell swelling and aggregation produce significantly different size distribution profiles, as shown in Fig. 4. Thus, size measurement is informative in determining the major factors responsible for the Δ(E/M) observed.

Binding of polycations

All of the cationic polymers used in this experiment caused cell aggregation and a decrease in $\Delta(E/M)$, but the observed $\Delta(E/M)$ was not as great as expected. The data for these polycations is shown in Table 4. From the fact that polycations bind non-specifically to net negatively charged cell surfaces, a large decrease in $\Delta(E/M)$ was an a priori expectation. The relatively small change in $\Delta(E/M)$ may be due to either perpendicular binding of these polymers to cell surfaces or to phase changes in the cell membrane. If perpendicular binding occurs, only a small compression of the cell membrane, and hence small change in lipid viscosity, would be anticipated. Phase separation could aid in excimer formation by excluding pyrene molecules from one phase resulting in an increase or decrease in the effective concentration in another phase.

The P_y values of the polycation-treated cells are not different from 0.90, and thus pyrene is considered to be located in the lipid bilayer of the cell. The P_y value of polybrene was 0.57 as shown in Table 3, and this implies a strong interaction of pyrene with the carbon chain of polybrene. However, the observed P_y value of cells after polybrene treatment is not different from that of the control. Thus it can be concluded that pyrene molecules are indeed located in the cell membrane and the reduced $\Delta(E/M)$ is due to reduced membrane fluidity.

All of the cationic polymers at low concentration (0.1%) caused cell aggregation or clump formation, but individual cell size appeared to be somewhat decreased as judged by light microscopy. At higher concentration (2.5%), polybrene did not cause any cell aggregation, and the cell size was clearly decreased as determined by electronic particle counting.

Binding of polyanions

The effects of polyanions were not as simple to interpret as that of polycations. The degree of $\Delta(E/M)$ ranged from very low to extremely high, and the effect was dependent on the nature of the functional group. As shown in Table 5, polymers with carboxyl groups show a large decrease in $\Delta(E/M)$ while polymers with sulfate

TABLE 4

RESULTS OF CATIONIC POLYMER BINDING

Polymer	Mol. wt.	Concentration (w/w%)	P_y value	$\Delta(E/M)$
Polylysine	23,000	0.1	0.94	0.08–0.15
		1.0	0.93	0.08–0.24
	90,000	0.1	0.93	0.08–0.15
		1.0	0.92	0.08–0.24
Polyvinylmethyl imidazole	930,000	0.1	0.93	0.08 ± 0.03
		1.0	0.93	0.13 ± 0.03
Polybrene	5000	0.1	0.94	0.08 ± 0.03
		2.5	0.90	0.18 ± 0.03

or sulfonate groups show a small change, with the exception of polystyrene sulfonate. It was assumed that a large Δ(E/M) indicates high binding potential. In this case, however, caution should be exercised in interpretation of the data because complete cell disruption was observed with sulfated or sulfonated polymers, except for λ-carrageenan, while cell shrinkage was observed with most of carboxylated polymers. In light of the cell damage, the low Δ(E/M) of sulfated or sulfonated polymers does not necessarily mean low binding ability of these polymers.

Upon examination of the P_y values, all were normal except for polyacrylic acid at 2.5% concentration and polystyrenesulfonic acid. The extremely low P_y value for polyacrylic acid at high concentration and the large decrease in cell size due to this polymer suggests movement of pyrene molecules from a hydrophobic to a polar area. Although this explanation may not be complete at this time, the significant binding ability of this polymer is unambiguous from the large Δ(E/M) and decrease in cell size.

TABLE 5

RESULT OF ANIONIC POLYMER TREATMENT

Polymer	Mol. wt.	Concentration (w/w%)	P_y value	ΔE/M
Carboxymethyl cellulose	90,000	1.25	0.93	0.07 ± 0.01
		2.5	0.85	0.21 ± 0.01
Hyaluronic acid		0.1	0.91	0.09 ± 0.02
		0.25	0.92	0.18 ± 0.04
		0.5	0.91	0.27 ± 0.02
Polyacrylic acid	250,000	0.1	0.89	0.0
		2.5	0.77	0.76 ± 0.02
Polyaspartic acid	20,000	2.5	0.90	0.11 ± 0.02
Polyglutamic acid	21,000	2.5	0.86	0.18 ± 0.01
Dextran sulfate	500,000	0.1	0.89	0.08 ± 0.02
		2.5	0.92	0.01 ± 0.04
Polystyrene-sulfonic acid	200,000	0.1	0.76	0.44 ± 0.02
		1.0	0.70	0.74 ± 0.02
		2.5	0.70	0.74
Polyvinyl sulfate	100,000	0.1	0.95	0.0
		2.0	0.91	0.07 ± 0.03
λ-Carrageenan		0.01	0.93	0.05 ± 0.04
		0.5	0.94	0.08 ± 0.03
		1.0	0.92	0.14 ± 0.07
Chondroitin sulfate		2.5	0.91	0.05 ± 0.02
Heparin		0.5	0.94	0.0
		2.5	0.93	0.08 ± 0.01

The P_y value of pyrene in polystyrenesulfonate was found to be 0.64 as shown in Table 3. Therefore, a change in P_y value together with cell disruption upon treatment of cells with this polymer should be interpreted as a significant change in membrane structure. Dextran sulfate and polyvinyl sulfate also disrupted cells, but their P_y values are normal.

A high Δ(E/M) was generally observed for carboxylated polymers. Of great interest is the high binding property of hyaluronic acid. Hyaluronic acid is known to form complexes with cell surface-derived fibronectin in solution (Yamada, 1981), and receptors for hyaluronic acid have been observed on many cell membranes. The binding of hyaluronic acid to membrane proteins was also observed by depolarization measurement (Table 7). From Table 5, the significant difference in Δ(E/M) between polyaspartic acid and polyglutamic acid implies that longer side-chains favor binding to cell surfaces.

The effect of sulfated polymers on the cell membrane is consistent with observations in the literature. Miyazawa et al. (1967) observed rapid lysis of L-cells upon treatment with dextran sulfate. Isolated nuclei were also swollen in the presence of dextran sulfate (Kraemer and Coffey, 1970). The formation of hydrophilic gels was also induced by polystyrenesulfonate as well as polyethylenesulfonate (Tunis and Regelson, 1965). Membrane swelling and eventual disruption by sulfated polyanions suggests that binding of these polymers to reactive groups on cell surfaces may cause mechanical damage which can result in local disruption or pores. Polymers can enter into the cytoplasm through these pores and the nuclear membrane can be disrupted. As a result, formation of a sticky gel is observed. It is also possible that after cell disruption, the membrane viscosity decreases resulting in high excimer formation. An increase in E/M ratio above that of the control, following polyvinyl sulfate treatment (see Table 5), is an example. Therefore, the large Δ(E/M) caused by polystyrenesulfonic acid may be interpreted as an intercalation of this polymer into the membrane bilayer to isolate each pyrene molecule and hence reduce excimer formation. This is consistent with the explanation for the low P_y value of cells after polymer treatment.

Polyanions which have both carboxyl and sulfate groups showed a Δ(E/M) which is intermediate between those with only a carboxyl or sulfate group. Heparin showed very little change in Δ(E/M). This is interesting considering the fact that fibronectin has binding sites for both hyaluronic acid and heparin, and the Δ(E/M) for heparin is far smaller than the Δ(E/M) for hyaluronic acid. Heparin is known to aggregate red blood cells (Jan, 1980) and bind perpendicularly to cell membranes (Chaubal and Lalwani, 1977). The small Δ(E/M) for heparin is therefore probably due to perpendicular binding to the cell membrane. This is reasonable since polylysine which also binds perpendicularly to the cell membrane (Katchalsky et al., 1959) showed a Δ(E/M) of about 0.08 at early times of treatment.

The reason for the difference in behavior between carboxylated and sulfated polymers is not completely understood, and binding affinities may not be compared by simply examining Δ(E/M) values. It has been reported that the interaction of sulfated polymers with proteins is more favorable than carboxylated polymers. Good examples of this are formation of an insoluble complex between sulfated polymers

and negatively charged low density lipoproteins (Bernfeld, 1966) and preferential interaction and consequent inhibition of enzymes by sulfated polyanions (Bernfeld, 1963). Thus, it appears that sulfated polyanions have higher binding affinities to proteins than carboxylated polyanions.

Binding of neutral polymers

Most of the neutral polymers tested resulted in a relatively large decrease in Δ(E/M) compared to charged polymers, as shown in Table 6. The P_y values were also very low except for dextran. In addition, the large increase in fluorescence intensity without any change in cell size suggests extraordinary behavior of neutral polymers on the cell membrane. Dextrans show a Δ(E/M) about the same as that due to polycations or heparin. Although apparent cell aggregation was not observed in this experiment, erythrocyte aggregation using these polymers has been reported in the literature (Sewchand and Bruckschwaiger, 1980; Buxbaum et al., 1982) implying possible binding of these polymers with their long axes perpendicular to the cell surface.

Gelatin is not a neutral polymer and is arbitrarily placed in Table 6 since no attempt was made to favor either an anionic or cationic form of the compound. The large decrease in E/M ratio due to gelatin was expected considering the fact that it

TABLE 6

RESULTS OF NEUTRAL POLYMER TREATMENT

Polymer	Mol. wt.	Concentration (w/w%)	P_y values	ΔE/M
Dextran	70,000	2.5	0.90	0.12 ± 0.02
	151,000	2.5	0.91	0.10 ± 0.02
	252,000	2.5	0.89	0.10 ± 0.02
	500,000	2.5	0.89	0.08 ± 0.04
Gelatin [a]		0.1	0.90	0.02 ± 0.02
		1.0	0.90	0.20 ± 0.02
		2.5	0.91	0.29 ± 0.02
Ficoll	70,000	2.5	0.77	0.17 ± 0.04
	400,000	2.5	0.78	0.17 ± 0.04
Polyethylene glycol	8000	0.5	0.88	0.0
		2.5	0.77	0.16 ± 0.01
		5.0	0.70	0.30 ± 0.01
	100,000	2.5	0.79	0.27 ± 0.02
Polyvinyl-pyrrolidone	40,000	1.0	0.64	0.45 ± 0.01
		2.5	0.61	0.63 ± 0.03
Bovine serum albumin	68,000	0.1	0.89	0.50 ± 0.01
		1.0	0.90	~ 0.80

[a] Arbitrarily placed in the neutral category since no attempt was made to favor either anionic or cationic form.

has been used as a component of a bioadhesive drug system (Gilman and Bernstein) and there is a specific collagen binding site on the cell surface.

To test whether large changes in $\Delta(E/M)$ and P_y values by neutral polymers are due to polymer binding to the cell membrane or to some other factor, an experiment similar to that of Méli-Goubert and Freedman (1980) was conducted. When pyrene-labeled cells were separated from neutral polymers by a dialysis membrane, there was no change in the $\Delta(E/M)$ and P_y value. Thus, by a similar argument made by Méli-Goubert and Freedman (1980), it can be said that pyrene molecules, probably due to close interaction or collision, are transferred from cell membranes to neutral polymer molecules, and a strong interaction between polymer and pyrene results in a large decrease in P_y value and $\Delta(E/M)$. Strong interactions of pyrene with PVP and PEG are also expected from the P_y values given in Table 3, which are smaller than 0.64, the value in water. It is reasonable to assume that neutral polymers may be intercalated into cell membranes to cause this result. Neutral polymers, however, are easy to wash off. The large change in $\Delta(E/M)$ by albumin treatment is consistent with the result of Meli-Goubert and Freedman (1980). Because of this abnormal behavior of neutral polymers, it is not easy to compare binding potentials of these polymers with those of charged polymers. Significant interaction, however, is noted between neutral polymers and cell membranes. PEG is a well-known cell fusing compound, and its effect is through clustering of protein molecules and exposure of lipid molecules (Knutton, 1979). PVP has been used as a cryoprotective agent probably by adsorption and plugging of pores in the cell membrane (Persidksy and Richards, 1962). PVP also converted electronegative red blood cells to an electro-positive particle at concentrations as low as 1.5% (Castanada et al., 1965). Because of wide application of neutral polymers in biological systems, further studies with these polymers are indicated.

Protein labeled polarization measurements

It was observed that polarization values were not reproducible, but a difference between control and polymer-treated cells was always observed. From Table 7, it is clear that an increase in polarization value is accompanied by a decrease in cell size and a decrease in polarization value by an increase in cell size. The increase in polarization value suggests reduced rotational mobility of fluorescein isothiocyanate-labeled proteins. Except for carboxymethylcellulose, carboxylated polyanions showed an increase in polarization value, and this is consistent with the results of $\Delta(E/M)$ measurements. It was discussed earlier that sulfated polyanions have a stronger binding or interaction than carboxylated polyanions, and an increased polarization value by treatment of sulfated polyanions was predicted. It was observed, however, that a decrease in polarization value resulted following sulfated polyanion treatment. The data suggests that most of the fluorescein isothiocyanate-labeled proteins are connected to the cell cytoskeleton. Swelling of cells by treatment with sulfated polyanions caused proteins to detach from the cytoskeleton with a subsequent increase in rotational mobility and a reduced polarization value. If the cell shrinks, rotational motion of proteins in the membrane will be reduced resulting in an increase in polarization value. Neutral polymers tested

TABLE 7

FLUORESCENCE POLARIZATION AND SIZE MEASUREMENTS

Polymer	Concentration (w/w%)	Polarization	Size
(1) Cationic			
Polylysine	0.1	+ +	+ + [a]
(2) Anionic			
Hyaluronic acid	0.5	+	− or NC
Polyacrylic acid	2.5	+ +	− −
λ-Carrageenan	1.0	NC	NC
Polystyrenesulfonic acid	1.0	NC	Disruption
Carboxymethylcellulose	2.5	−	NC
Dextran sulfate	2.5	−	+
Polyvinyl sulfate	2.5	−	+
(3) Neutral			
Dextran	2.5	−	+ or NC
Polyethylene glycol	2.5	−	NC

[a] Size increase is due to cell aggregation. Individual cell size is decreased.
+ + = large increase; + = increase; NC = not changed; − = decrease.

showed a decrease in polarization values. It is probable that neutral polymers disrupt the membrane bilayer to some extent.

Conclusion

The present study provides a technique to study polymer binding to cultured cells. In essence, a lipid-soluble fluorescent probe, pyrene, which localizes in the lipid bilayer portion of the cell membrane, is added to a cell suspension and a fluorescence spectra obtained. Addition of a polymer which binds to the cell membrane compresses the lipid bilayer causing a change in fluorescence. The degree of change in fluorescence is proportional to the degree of binding. Ancillary experiments, such as cell size measurement and perhaps additional fluorescent probe work, are needed in order to accurately interprete the results.

Although it is somewhat premature to describe structural features of the polymers responsible for bioadhesion, there are some preliminary observations that can be made. It appears that charge density is an important element for bioadhesion, and polyanions are preferred over polycations when one considers toxicity in addition to bioadhesion. In addition, carboxylated polyanions appear better than sulfated polyanions when both bioadhesiveness and toxicity are considered.

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