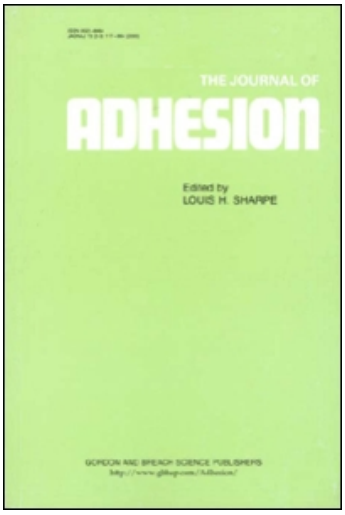

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An *In Situ* Investigation of the Adhesion of Glutaraldehyde-Fixed Human Erythrocytes to Polymer Surfaces

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An *In Situ* Investigation of the Adhesion of Glutaraldehyde-Fixed Human Erythrocytes to Polymer Surfaces*

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An *in situ* methodology has been developed for the study of cell adhesion at the individual cell level. Our investigation involved the static sedimentation of glutaraldehyde-fixed human erythrocytes onto flat, horizontal, and transparent polymer surfaces. An inverted microscope was utilized for observations. Cells which did not adhere to the surface displayed small oscillations due to Brownian motion, and thus could be distinguished from adherent cells. Individual cells either adhered instantaneously upon contact with the surface or achieved adhesion gradually. In general, the percentage of adherent cells for a given system increased with time to a characteristic plateau value. The process of cell adhesion was modeled by thermodynamic consideration of the relevant interfacial tensions. Factors under consideration were the liquid medium's surface tension (γ_{LV}), the cell membrane surface tension (γ_{CV}), and the substrate surface tension (γ_{SV}). The model predicts that for $\gamma_{LV} > \gamma_{CV}$, adhesion increases with decreasing γ_{SV}. For $\gamma_{LV} < \gamma_{CV}$, the opposite trend is expected. In cases where $\gamma_{LV} = \gamma_{CV}$, adhesion is expected to be independent of γ_{SV}. The liquid mediums used in this study were 10 mM NaCl ($\gamma_{LV} = 72.9\,\text{mJ m}^{-2}$), 10 mM NaCl containing 1% (v/v) 1-propanol ($\gamma_{LV} = 64.1\,\text{mJ m}^{-2}$), and 10 mM NaCl containing 2% (v/v) 1-propanol ($\gamma_{LV} = 58.3\,\text{mJ m}^{-2}$). The surfaces used were fluorinated ethylene propylene (FEP) ($\gamma_{SV} = 15.2\,\text{mJ m}^{-2}$), polystyrene (PS) ($\gamma_{SV} = 29.8\,\text{mJ m}^{-2}$), polyethylene terephthalate (PET) ($\gamma_{SV} = 39.5\,\text{mJ m}^{-2}$), and acetal ($\gamma_{SV} = 43.5\,\text{mJ m}^{-2}$). The value of γ_{CV} has been established in the past to be approximately 65 mJ m^{-2} by solidification front and droplet sedimentation experiments. FEP and PET showed complete agreement with the model. On the other hand, PS and acetal showed partial agreement; any discrepancy was due to a higher level of adhesion than expected. A two-way analysis of variance indicated that a highly significant ($> 99.9\%$ confidence level) degree of interaction exists between γ_{SV} and γ_{LV} in the adhesion of fixed human erythrocytes. This may indicate that preferential adsorptions at the solid-liquid interface lead to deviation of the results from the predictions of the thermodynamic model.

KEY WORDS Cell-surface interactions; erythrocyte adhesion; particle adhesion; effect of surface tension on adhesion; adhesion kinetics; Brownian motion.

1. INTRODUCTION

The significance of cell adhesion, and particle adhesion in general, has been well established in many biomedical and biotechnological processes.[1-3] The role of interfacial tensions in cell adhesion was recognized as early as the 1920s.[4] Since then, Baier

* One of a Collection of papers honoring James P. Wightman, who received the 13th Adhesive and Sealant Council Award at the ASC's 1993 Fall Convention in St. Louis, Missouri, USA, in October 1993.

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has characterized surfaces in terms of their critical surface tension, an experimentally-derived parameter, and has offered a range of surface tensions (20–30 mJ m^{-2}) in which adhesion is hindered.[5] McIver and Schürch related their contact angle measurements and work of adhesion, using the aqueous phase as a reference, to the structure of adhering surfaces.[6] Criteria for compatibility of blood contacting materials have been proposed by expression of surface energies in terms of polar and dispersive components.[7] Schakenraad *et al.* have shown a sigmoidal relationship between the extent of cell spreading and the substrate surface tension; polymers with surface energies greater than 57 mJ m^{-2} were found to facilitate spreading of human skin fibroblasts.[8,9] Determination of surface tensions of cells and proteins by contact angle measurements has been used to interpret engulfment (phagocytosis) and adhesion phenomena.[10,11]

We wish to report on the utilization of an *in situ* methodology for investigation of the role of interfacial tensions in cell adhesion. The technique involves the static sedimentation of cells, in this case glutaraldehyde-fixed human erythrocytes, onto flat, horizontal, and transparent polymer surfaces. These cells are expected to act essentially like stable, monodisperse, hydrophilic polymer particles. An inverted microscope provides a direct view of the cell-suface interface as cells settle onto the surface. Not all the cells which arrive at the surface adhere. Cells which do not adhere to the surface undergo small oscillations due to Brownian motion, and thus can be distinguished from adherent cells. Therefore, the extent of adhesion, based on individual cells, may be expressed in terms of an adhesion percentage. Moreover, the variation of this percentage with time may be investigated. The results are interpreted within the framework of a thermodynamic model for particle adhesion based on the relevant interfacial tensions. Factors under consideration are the cell membrane surface tension (γ_{CV}), the liquid medium's surface tension (γ_{LV}), and the solid substrate surface tension (γ_{SV}).

2. THEORETICAL CONSIDERATIONS

In order to make quantitative predictions regarding the trends in extent of cell adhesion, a thermodynamic model is adopted.[11,12] This approach indicates that a properly identified thermodynamic potential, namely the grand canonical potential which we will simply refer to as the free energy, will be minimized at equilibrium. Consider a particle, such as a cell (C), suspended in a liquid medium (L), sedimenting and adhering to a solid substrate (S) immersed in the liquid medium. This process involves the generation of a cell-substrate interface and the removal or annihilation of a cell-liquid and substrate-liquid interface. It follows that the change in the free energy due to the process of adhesion per unit area, ΔF^{adh}, may be expressed in the following manner

$$\Delta F^{adh} = \gamma_{CS} - \gamma_{CL} - \gamma_{SL} \tag{1}$$

where γ_{CS}, γ_{CL}, and γ_{SL} are the cell-substrate, cell-liquid, and substrate-liquid interfacial tensions, respectively.

Effective utilization of this model requires determination of values for the relevant interfacial tensions. This is accomplished by the equation of state approach[13] which expresses the solid-liquid interfacial tension, γ_{SL}, in the following form

$$\gamma_{SL} = f(\gamma_{SV}, \gamma_{LV}) \tag{2}$$

where γ_{SV} and γ_{LV} are the surface tensions of the solid and liquid in contact with their vapour, respectively. The existence of an equation of state of interfacial tensions has been validated by interfacial Gibbs-Duhem equations[14] and the modified phase rule for systems possessing moderately curved surfaces.[15] The explicit form of the equation of state has been semi-empirically determined to be

$$\gamma_{SL} = \gamma_{LV} + \gamma_{SV} - 2[\gamma_{LV}\gamma_{SV}]^{1/2} \exp[-0.0001247(\gamma_{LV} - \gamma_{SV})^2] \quad (3)$$

If Eq. (3) is used in a generic form, *i.e.* $\gamma_{AB} = f(\gamma_{AV}, \gamma_{BV})$, where A and B are two different components and V signifies the vapour phase, each of the interfacial tensions in Eq. (1) may be expressed in terms of the surface tensions of the components in contact with their vapour

$$\Delta F^{adh} = f(\gamma_{CV}, \gamma_{SV}) - f(\gamma_{CV}, \gamma_{LV}) - f(\gamma_{SV}, \gamma_{LV}) \quad (4)$$

Thus, knowledge of γ_{CV}, γ_{SV}, and γ_{LV} in combination with the equation of state for interfacial tensions allow determination of the change in the free energy of adhesion. The sign of ΔF^{adh} indicates whether or not adhesion is favoured, *i.e.* the directionality of the process is elucidated. A process is favoured if it results in a decrease in the free energy of the system. Thus, a negative value of ΔF^{adh} indicates that adhesion is favoured and, conversely, a positive value of ΔF^{adh} indicates that adhesion is not favoured. Moreover, the magnitude of ΔF^{adh} provides a means of gauging the extent of the thermodynamic driving force for the process. Substitution of a range of γ_{LV} and γ_{SV} values, for a given γ_{CV}, into Eq. (4) results in identification of three distinct trends for particle adhesion (Fig. 1). For conditions where γ_{LV} is greater than γ_{CV}, the net change in the free energy becomes more negative with decreasing γ_{SV} and, thus, an increase in cell adhesion is favoured. For conditions where γ_{LV} is less than γ_{CV}, the opposite trend is predicted, *i.e.* hydrophilic substrates support adhesion more readily. For the limiting case where γ_{LV} is equal to γ_{CV}, ΔF^{adh} becomes equal to zero implying that the extent of cell adhesion is independent of the substrate surface properties. It is apparent that cell adhesion measurements require consideration of all the relevant interfacial tensions and can not merely involve examination of the substrate properties. The thermodynamic model may be modified for cell spreading by consideration of cell geometry and the relevant interfacial areas involved.[16] Incidentally, thermodynamic predictions based on expression of surface energies in terms of polar and dispersive components follow the same trends as outlined above if the polar components of γ_{LV} and γ_{CV} are compared.[9]

Experimentally, γ_{LV} is readily measured by methods such as Axisymmetric Drop Shape Analysis (ADSA)[17] and the Wilhelmy plate technique. The substrate surface tension, γ_{SV}, can be determined by contact angle data and the equation of state approach. For particles such as cells, various methods such as the freezing front, sedimentation volume, and droplet stability have been devised for determination of γ_{CV}.[11] Thus, the means exist for experimental evaluation of the model.[18-21] However, a few precautions must be borne in mind. The model neglects any conformational changes of the species due to adhesion. Also, it excludes the role of gravity, electrostatic forces, and specific biochemical interactions. Thus, it considers the process of non-specific particle-substrate adhesion to be governed by the interfacial tensions; these parameters have been related to van der Waals interactions through calculation of

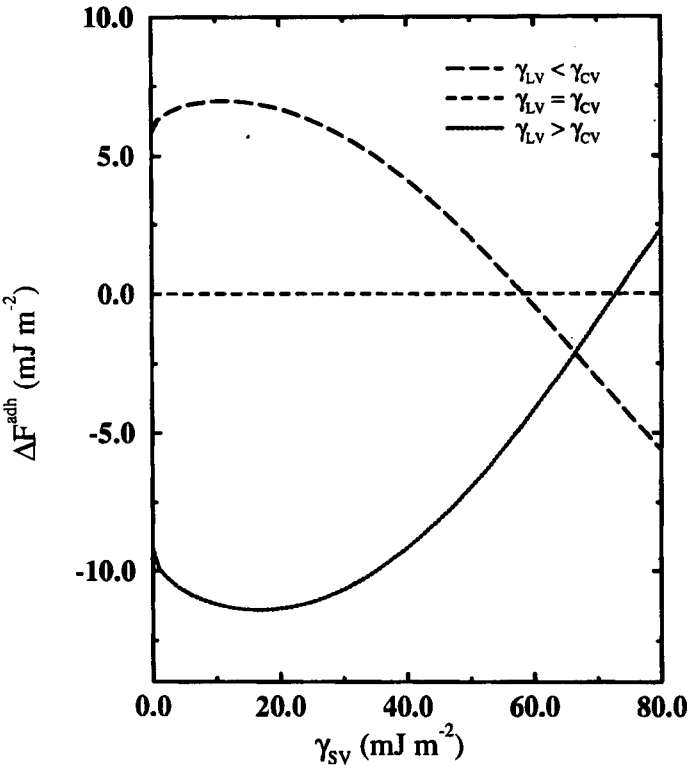

FIGURE 1 Thermodynamic model for particle adhesion based on the relevant interfacial tensions. The change in the free energy of adhesion, ΔF^{adh}, for fixed erythrocytes is given as a function of the substrate surface tension, γ_{SV}. Adhesion is favoured when ΔF^{adh} holds a negative value. The following values are used for the calculations: $\gamma_{LV} = 72.9\,\text{mJ m}^{-2}$ ($\gamma_{LV} > \gamma_{CV}$), $\gamma_{LV} = 58.3\,\text{mJ m}^{-2}$ ($\gamma_{LV} < \gamma_{CV}$), $\gamma_{CV} = 65.0\,\text{mJ m}^{-2}$.

Hamaker constants.[22,23] Moreover, it utilizes the equation of state in a generic form, although this equation was specifically derived from consideration of a two-component, three-phase, solid-liquid, system. However, partial agreement and particularly the pattern in which the model fails may provide valuable information, for example, by identifying other forces or interactions which may play a role in cell adhesion.

3. MATERIALS AND METHODS

3.1. Cell Suspensions

Our system consisted of glutaraldehyde-fixed human erythrocytes suspended in a 10 mM NaCl solution deposited over transparent substrate surfaces. A 10 mM NaCl solution, in comparison with distilled water, increases the kinetics of adhesion and facilitates the measurement of the steady state adhesion percentage; increasing ionic strength accelerates the rate of adhesion due to a reduction of repulsive electrostatic forces.[20] Glutaraldehyde-fixed human erythrocytes offer several distinct advantages:

(a) erythrocytes are available in large quantities and display uniform properties with respect to size, shape, and chemical composition from batch to batch; (b) glutaraldehyde fixation produces cells which remain stable outside the blood plasma environment while maintaining some of the cell's characteristics; (c) fixation prevents the loss of membrane proteins and glycoproteins which may otherwise preadsorb onto carefully prepared substrates rendering interpretation of the experimental data more difficult; (d) fixation stabilizes the contact area between the cell and the subtrate by increasing cell rigidity; (e) fixation has a small effect on the net surface charge of the cells[24] or on the local distribution of their charge-bearing glycoproteins in the membrane lipid bilayer;[25] (f) the surface tension of fixed human erythrocytes has been established by different methods (solidification front technique and droplet sedimentation) to be approximately 65 mJ m^{-2}.[11,26,27] The solidification front technique[26] and droplet sedimentation[27] experiments showed that glutaraldehyde-fixed human erythrocytes may be modeled as stable, monodisperse, hydrophilic polymer particles obeying thermodynamic predictions based on the relevant interfacial tensions.

Human erythrocytes were isolated from whole blood collected by venipuncture from a healthy male donor in vacutainers precoated with EDTA. The cells were subsequently washed with 150 mM NaCl and mixed with 1% (v/v) glutaraldehyde (BDH, Toronto, ON, Canada) in phosphate-buffered saline (PBS). After mixing, drop-wise addition of glutaraldehyde was undertaken to ensure that an excess of the fixing agent was present in the suspension. The suspension was subsequently stored at 4 °C in a refrigerator. All adhesion experiments were completed within three months after fixation of the cells, although similar adhesion percentages were obtained for the saline-FEP system twelve months after fixation of the cells (data not shown). Prior to use, the cells were washed with distilled and deionized water (Corning Water Purifier LD-5a) by six successive cycles of centrifuging and decanting to remove any free glutaraldehyde; centrifugation beyond six cycles made re-suspension of the erythrocyte pellet difficult. To allow for uniform distribution of the cells on the substrate, such that ample opportunities are provided for the observation of cell-surface interactions without saturating the surface, the cells were diluted to an appropriate concentration which was found to be approximately 2×10^6 cells mL^{-1} for our particular set-up. The concentration of the washed cells was determined by a hemacytometer counting chamber (American Optical, Buffalo, NY, USA). Based on this concentration, the appropriate dilution was made by mixing the cells with a saline solution producing a 10 mM NaCl suspension containing the appropriate amount of 1-propanol. The variation in the liquid medium's surface tension was achieved by the addition of 1-propanol (Caledon, Georgetown, ON, Canada) to the suspension. Propanol was used because very low concentrations produce drastic lowering of γ_{LV}. The liquid mediums utilized in our experiments were 10 mM NaCl ($\gamma_{LV} = 72.9$ mJ m^{-2}), 10 mM NaCl containing 1% (v/v) 1-propanol ($\gamma_{LV} = 64.1$ mJ m^{-2}), and 10 mM NaCl containing 2% (v/v) 1-propanol ($\gamma_{LV} = 58.3$ mJ m^{-2}); the γ_{LV} measurements were made by the Wilhelmy plate technique.

3.2. Solid Substrates

An inverted microscope was utilized for observation of cells on the polymer surfaces. Since transmission light microscopy was used, a transparent surface was required. The

polymer surfaces used in our investigation were: polyethylene terephthalate (PET) (Hoechst Canada, Willowdale, ON, Canada), polystyrene (PS) (Dow Chemical Company, Midland, MI, USA), and fluorinated ethylene propylene (FEP) (Warehoused Plastic, Toronto, ON, Canada). PET and PS are commercially available as thin films. Thin and smooth films of FEP and acetal were made in a hydraulic heat press (Dake model 44–273, Wabash Metal Products, IN, USA) by pressing strips of the polymer between chromic-acid-cleaned glass slides at their glass transition temperature. Siliconized glass slides were used for acetal. The surface tension of the polymers was calculated from contact angle measurements by Axisymmetric Drop Shape Analysis-Diameter (ADSA-D)[28,29] and the equation of state approach.[13] The results are summarized in Table I. Note that the surfaces utilized provide a wide range of γ_{SV}. To minimize the disturbance on sedimented cells, flat and horizontal surfaces were used. The polymer surfaces were cleaned in anhydrous ethanol (Commercial Alcohols, Brampton, ON, Canada), air dried, and secured onto glass slides with tape.

3.3. *In Situ* Adhesion Test

A stagnant suspension for the sedimentation of cells onto the surface was utilized. This eliminates complications introduced by a dynamic system where the effects of turbulence, shear fields, cell rotation, and diffusion must be considered. To apply a fixed volume of the liquid medium to the substrate surface, a well 7 mm in diameter formed in a Teflon mold was used. To seal the suspension within the well, an ethylene propylene diene monomer O-ring (Bearings and Beltings, Toronto, ON, Canada) was placed between the mold and the surface. The Teflon mold and the O-ring were cleaned by immersion in a clean beaker of ethanol and application of ultrasound for 10 min. They were subsequently air dried. Tape was used to secure the mold onto the glass slides bearing the polymer surfaces. The inverted microscope (Olympus IMT-2) used for observation of cells arriving at the surface, was linked to a CCD camera (Cohu 4800 monochrome). The image from the camera was fed to a monitor. The monitor allows visual recording of the adhesive behaviour of cells. The inverted microscope was placed on a vibration-free table (Technical Manufacturing Corporation, Peabody, MA, USA) to isolate the system from external disturbances.

Once the Teflon mold was cleaned and assembled with the desired substrate surface, 35–40 μL of the erythrocyte suspension (depending on the hydrophilicity of the surface) was applied to the well. This provides a thin layer of suspension (approximately 1 mm

TABLE I

Water contact angle (θ) measurements by Axisymmetric Drop Shape Analysis-Diameter (ADSA-D).and surface tension of the polymer sufaces (γ_{SV}) determined by the equation of state approach.

Polymer Surface	$\theta \pm 95\%$ CL[1] (degrees)	γ_{SV} (mJ m^{-2})	Number of Drops
FEP	112.9 ± 0.6	15.2	9
PS	88.5 ± 0.3	29.8	8
PET	73.0 ± 0.9	39.5	8
Acetal	66.5 ± 0.7	43.5	7

[1] CL ≡ confidence limits

in height) over the surface, thus reducing lateral cell motion due to convection. The assembly was placed on the microscope stage and a region (130 μm × 170 μm) on the substrate was selected for observations at a magnification of 20 ×; in order to avoid wall effects, only the central portion of the well was examined. The most noteworthy observation regarding the behaviour of a suspension of fixed erythrocytes is that some cells undergo small oscillations once they have settled onto the surface. These oscillations are a manifestation of Brownian motion and result in small random changes in the position of the settled cells without causing large displacements or a loss in microscopic focus. Cells which exhibit Brownian motion are considered to be non-adherent. Conversely, cells which have adhered to the surface are distinctly stationary and do not exhibit any oscillatory behaviour. Thus, Brownian motion can be used to distinguish between adherent and non-adherent cells. The time of arrival and time of adhesion (if it occurs) for every cell were determined at 1 min intervals for 40 min. Three to five trials were performed for each liquid medium-substrate system. The accumulated time data for the trials were transformed to reflect the change in the percentage of adherent cells with time.

Subsequent to the kinetic studies, ten additional areas surrounding the central portion of the well, each containing approximately 10–15 cells, were observed on each substrate at a magnification of 40 ×. Within each area, the number of adherent cells and the total number of cells were noted. These data were combined with the results of the kinetic studies for a given trial to obtain an adhesion percentage. The adhesion percentage for the trials were combined to determine a mean adhesion percentage for every liquid medium-substrate combination. The area observations were completed within 1–3 h after application of the suspension to the surface. This provided ample time for the system to reach a constant level of adhesion. All experiments were performed at controlled room temperature (23 ± 1 °C). Steps have also been taken for automation of the methodology based on digital image analysis.[30] Instantaneous images of cells resting on the surface may be recorded in a given period. Subsequently, pair-wise subtraction of the images is performed. This operation involves subtracting the grey level of every pixel in one image from the grey level of the corresponding pixel in another image. Adherent cells occupy the same position in both images and are deleted from the subtraction image. Conversely, non-adherent cells appear in the subtraction image since they occupy different positions in the instantaneous images due to Brownian motion.

4. RESULTS

4.1. Extent of Adhesion

The total number of cells (n_t) examined for each liquid medium-substrate system is given in Table II. Also, the number of trials (N) performed and the number of cells observed for kinetics of adhesion (n_k) are provided; n_t consists of n_k and the number of cells used solely for determination of extent of adhesion, n_e, in the area observations. It is apparent that the technique is not limited by the number of cells which may be observed. Thus, the following data for the various systems are based on representative sample sizes.

TABLE II

Number of trials (N) for various liquid medium-substrate systems, the number of observed cells under kinetic studies (n_k), and the total number of cells examined for extent of adhesion (n_t)

	Saline			1% 1-Propanol			2% 1-Propanol		
	N	n_k	n_t	N	n_k	n_t	N	n_k	n_t
FEP	3	59	390	3	59	435	3	44	402
PS	4	81	498	3	74	443	3	46	407
PET	3	46	391	3	72	447	4	62	500
Acetal	5	93	650	3	45	398	5	72	610

The adhesion percentages obtained for all the trials are given in Table III. The mean adhesion percentage and its standard deviation for each of the liquid medium-substrate systems are summarized in Table IV. The tables also include the changes in the free energy of adhesion predicted by the thermodynamic model. Recall that a negative value

TABLE III

Adhesion percentages of glutaraldehyde-fixed human erythrocytes for various liquid medium-substrate systems

	FEP	PS	PET	Acetal	
Saline	68.2	66.7	38.8	28.8	Trial 1
	71.3	78.7	42.4	30.1	Trial 2
	67.4	65.4	40.8	35.5	Trial 3
		59.0		24.3	Trial 4
				18.5	Trial 5
1% 1-Propanol	48.3	50.0	35.1	30.4	Trial 1
	47.3	56.8	32.4	32.6	Trial 2
	50.4	50.7	31.5	29.6	Trial 3
2% 1-Propanol	21.5	34.8	32.8	32.8	Trial 1
	17.6	44.1	22.0	27.6	Trial 2
	22.8	41.9	23.3	43.5	Trial 3
			25.6	36.8	Trial 4
				27.1	Trial 5

TABLE IV

Adhesion percentages and ΔF^{adh} for various liquid medium-substrate combinations utilizing 1-propanol in the liquid medium. ($\gamma_{CV} \approx 65\,mJ\,m^{-2}$)

Solid Substrate		Saline ($\gamma_{LV} = 72.9\,mJ\,m^{-2}$)			Saline/1% 1-Propanol ($\gamma_{LV} = 64.1\,mJ\,m^{-2}$)			Saline/2% 1-Propanol ($\gamma_{LV} = 58.3\,mJ\,m^{-2}$)		
	γ_{SV} ($mJ\,m^{-2}$)	Adhesion (%)	SD^1	ΔF^{adh2}	Adhesion (%)	SD^1	ΔF^{adh2}	Adhesion (%)	SD^1	ΔF^{adh2}
FEP	15.2	69.0	2.1	−11.53	48.7	1.6	+1.10	20.6	2.7	+6.91
PS	29.8	67.4	8.2	−10.82	52.5	3.8	+0.97	40.3	4.9	+5.74
PET	39.5	40.7	1.8	−9.39	33.0	1.9	+0.77	25.9	4.8	+4.22
Acetal	43.5	27.4	6.4	−8.37	30.9	1.5	+0.65	33.6	6.8	+3.23

[1] SD ≡ standard deviation of the adhesion percentage.
[2] ΔF^{adh} is expressed in units of $mJ\,m^{-2}$.

signifies that adhesion is favoured. The magnitude of ΔF^{adh} may be utilized to make predictions regarding trends of adhesion. In saline, the adhesion percentage increases with decreasing substrate surface tension, γ_{SV}. There exists a marked difference in the adhesion percentage for the most hydrophilic surface (acetal 27.4%) and the most hydrophobic surface (FEP 69.0%). With the addition of 1% (v/v) 1-propanol to the suspension and lowering of γ_{LV} from 72.9 mJ m^{-2} to 64.1 mJ m^{-2}, FEP, PS, and PET experience a reduction in their extent of adhesion, whereas acetal's adhesion percentage increases. In this liquid medium PS shows the highest extent of adhesion (52.5%) followed FEP (48.7%), PET (33.0%), and acetal (30.9%). With a further reduction of γ_{LV} to 58.3 mJ m^{-2} by the addition of 2% (v/v) 1-propanol to the suspension, FEP, PS, and PET experience a further reduction in their adhesiveness. Acetal's adhesion percentage increases slightly. PS shows the highest adhesiveness. The remaining surfaces show increasing levels of adhesion with increasing γ_{SV} in contrast to the trend observed in saline alone.

4.2. Kinetics of Adhesion

Varying degrees of Brownian motion are displayed by non-adherent cells. Cells which display minute motions may be assumed to be in a transition state and in the process of adhering.[30] The mean durations of Brownian motion for adherent cells after arrival at the surface and prior to adhesion are reported in Table V. These values are determined by noting the time of arrival and time of adhesion of adherent cells. The estimated precision for these measurements is 30 s for times less than or equal to 3 min and 60 s for times greater than 3 min. The ability to measure such values indicates that adhesion of individual cells under our experimental conditions is not on average instantaneous. Upon comparison of the times obtained in saline with liquid mediums containing 1-propanol, the following trends are observed. For FEP, with decreasing γ_{LV}, and thus a decrease in extent of adhesion (Table IV), the mean duration of oscillations decreases from 5.5 min to 3.4 min. Cells interacting with PET display a similar decrease in duration of Brownian motion, although the value at 1% 1-propanol (1.7 min) is less than the value at 2% 1-propanol (3.6 min). Although the times for acetal also decrease with decreasing γ_{LV}, the extent of adhesion increases (Table IV). Durations of motion displayed on PS decrease with increasing γ_{LV} and increasing extent of adhesion.

The data for times of arrival and adhesion may be transformed to elucidate the variation of the adhesion percentage with time. Figures 2 and 3 illustrate this variation

TABLE V

Mean duration (minutes) of Brownian motion for glutaraldehyde-fixed human erythrocytes after arrival at the surface and prior to adhesion in various liquid medium-substrate systems. The number of cells observed in each system is given in brackets

	Saline	1% 1-Propanol	2% 1-Propanol
FEP	5.5 (33)	3.4 (30)	3.4 (10)
PS	0.88 (56)	1.1 (37)	3.9 (17)
PET	4.1 (17)	1.7 (22)	3.6 (16)
Acetal	1.5 (24)	1.1 (17)	0.45 (29)

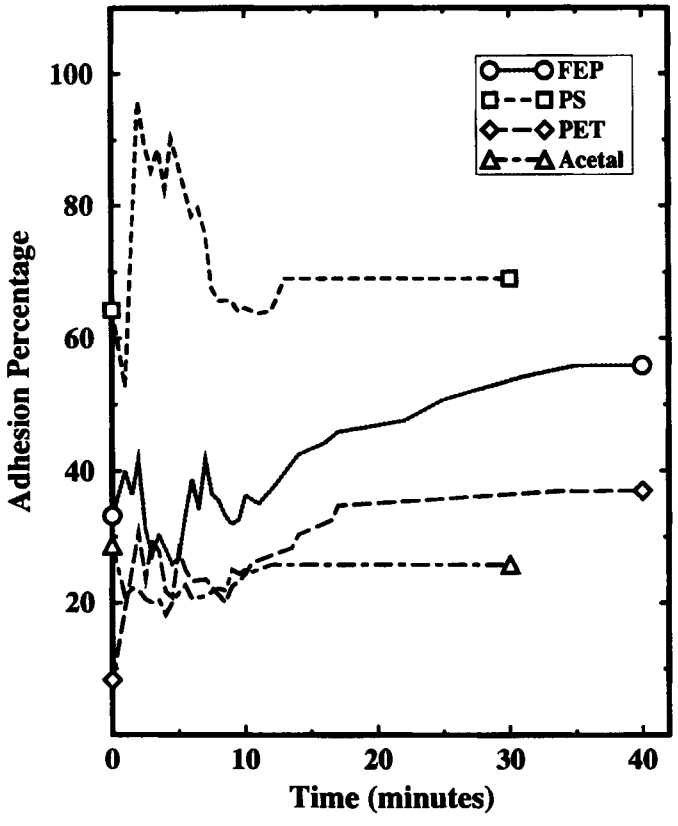

FIGURE 2 Time dependence of adhesion of glutaraldehyde-fixed human erythrocytes suspended in 10 mM NaCl to various polymer surfaces.

for saline and liquid mediums containing 1-propanol, respectively. The initial fluctuations in the graphs are due to the relatively rapid increase in the sample size as cells sediment onto the surface. Any rise in the plots is due to increased adhesion. Any decrease in the plots results from an increase in the sample size and not from the cells becoming unstuck; *i.e.* cell adhesion is irreversible. Note that once the sample size stabilizes and the fluctuations are attenuated, in general, the plot for every surface occupies a distinctly different position in the graphs. Furthermore, although they are based on a much smaller sample size, the final value attained by every liquid-substrate system is in good agreement with the adhesion percentages reported in Table IV. Most liquid medium-substrate systems display a period of rise followed by a plateau which is maintained for over 10 min prior to termination of time-dependence observations; in certain cases, such as 1% propanol-PS, and 2% propanol-acetal, the plateau is reached following a period of decline in the adhesion percentage. The largest deviation between the final kinetic adhesion percentage and the adhesion percentage obtained from the additional area studies occurs in the saline-FEP system. The adhesion percentage attained after 40 min is 55.9% compared with the value of 69.0% obtained in the area

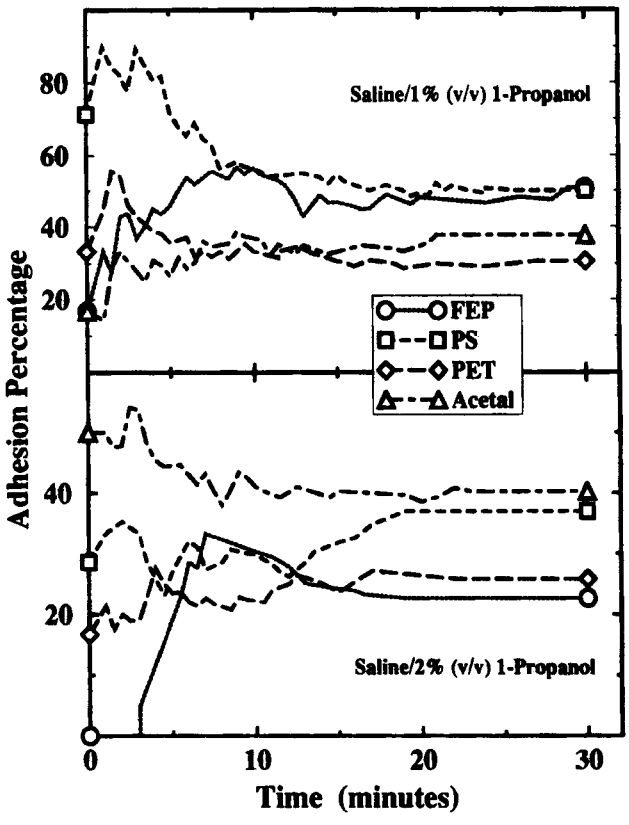

FIGURE 3 Time dependence of adhesion of glutaraldehyde-fixed human erythrocytes suspended in 10 mM NaCl containing 1-propanol to various polymer surfaces.

studies at approximately 120 min with a larger sample size ($n_t = 390$, $n_k = 59$; Table II). Also, the plateau for this curve is not as pronounced as the plateaus for the other curves. These findings indicate that this system requires in excess of 40 min to reach a constant state of adhesiveness.

5. DISCUSSION

Our investigation of cell adhesion essentially involved alteration of two factors, namely γ_{LV} and γ_{SV}, for a given γ_{CV}. Several trials or replications were performed for each liquid medium-substrate system. This experimental design facilitates the evaluation of the interaction between these factors by a two-way analysis of variance (ANOVA) with replication.[31] Two factors are deemed as interacting if the behaviour of one factor depends on the particular level of the other factor. In other words, if interaction exists, the effects of γ_{LV} and γ_{SV} on the adhesion percentage are not independent. To facilitate the performance of such analysis, and avoid mathematical complexities, it is necessary

for all liquid medium-substrate systems to possess the same number of replications. A review of Table III reveals that the following systems involve more than three trials: saline-PS, saline-acetal, 2% propanol-PET, and 2% propanol-acetal. Trial 1 for saline-PS, trials 1 and 2 for saline-acetal, trial 4 for 2% propanol-PET, and trials 1 and 4 for 2% propanol-acetal are ignored in this analysis. These trials hold values which most closely resemble the trial means for their respective liquid medium-substrate systems. Therefore, their removal causes the least amount of alteration to the mean and variance of the systems under consideration. In fact, their inclusion would lower the variance for a given system (*i.e.* the residual error) and facilitate the detection of a significant contribution from a given factor or interaction of factors more readily. Thus, if a significant level of interaction is found without their inclusion, this conclusion would most likely hold true with their inclusion. The results of the two-way ANOVA with replication are given in Table VI. The second-last column in the table is the calculated F distribution value, and the last column is the probability of obtaining a higher F value than the calculated F. The level of interaction between γ_{LV} and γ_{SV} is found to be highly significant and surpasses the 99.9% ($Pr > F = 1.2 \times 10^{-6}$) confidence level. Thus, it is concluded that, so far as extent of adhesion of glutaraldehyde-fixed human erythrocytes is concerned, γ_{LV} and γ_{SV} do not act independently, and that the level of one factor affects the behaviour of the other. Since interaction exists, the difference in the adhesion percentages due to γ_{LV} and/or γ_{SV} alone is of importance only if their effects are large compared with the interaction. It is seen that the effect of γ_{LV} is significant at a 89.0% ($Pr > F = 0.110$) confidence level and the effect of γ_{SV} is significant at a 86.2% ($Pr > F = 0.138$) confidence level relative to their interaction.

In cases where a significant interaction is present, the significance of the main factors, *i.e.* γ_{LV} and γ_{SV}, must be studied separately using a one-way ANOVA which involves evaluation of a factor while the other is fixed at one level.[31] The results of the one-way ANOVA are provided in Table VII. It is apparent that the adhesion percentages are significantly affected at a confidence level of $\geq 99.5\%$ by γ_{LV} for a given γ_{SV}, and vice versa. The exception to this finding is the effect of γ_{LV} for systems involving propanol-acetal (69.2% confidence level). Once the effect of a single factor is deemed significant, in order to assess exactly which adhesion percentages are different, Fisher's protected least significant difference (LSD) may be used.[31] Pair-wise comparison of adhesion percentage means is performed by determining whether their difference is significantly different from zero. If the following relation holds, the difference between the means is

TABLE VI
Two-way analysis of variance (ANOVA) with replication

Source of Variation	Sum of Squares SS	Degrees of Freedom	Mean Square MS	F	$Pr > F$
γ_{SV}	3286.3287	3	1095.4429	2.7127*	0.138
γ_{LV}	2637.0594	2	1318.5297	3.2651*	0.110
Interaction	2422.9194	6	403.8199	13.4754	1.2×10^{-6}
Residual Error	719.2123	24	29.9672		
Total	9065.5198	35			

* ≡ calculated relative to MS_I; *i.e.* $F_{SV} = MS_{SV}/MS_I$ and $F_{LV} = MS_{LV}/MS_I$

TABLE VII

One-way analysis of variance (ANOVA)

A. Confidence level (%) at which γ_{SV} significantly alters the adhesion percentage

Source of Variation	Saline	1% 1-Propanol	2% 1-Propanol
γ_{SV}	> 99.9	> 99.9	99.6

B. Confidence level (%) at which γ_{LV} significantly alters the adhesion percentage

Source of Variation (γ_{LV})	FEP	PS	PET	Acetal
Saline/1-propanol	> 99.9	99.8	99.7	69.2

deemed statistically significant

$$|X_i - X_j| > t\left(\frac{\alpha}{2}, v_e\right)\sqrt{MS_e}\sqrt{\frac{1}{n_i}+\frac{1}{n_j}} = LSD \tag{5}$$

where X_i and X_j are the two sample means, v_e is the number of degrees of freedom associated with the mean square of the residual error MS_e, and n_i and n_j are the sample sizes for means under consideration. The value of α is selected as 0.05 for cases where a confidence level $\geq$ 99.5% is obtained and as 0.35 for the propanol-acetal case where a confidence level of 69.2% was obtained. The adhesion percentages for one factor at a fixed level of the other factor may then be ranked from largest to smallest using the LSD values calculated in Eq. (5). Table VIII provides a comparison of the results of this

TABLE VIII

Comparison of trends in extent of adhesion predicted by the thermodynamic model and experimentally observed trends. A rank of (1.) signifies the largest adhesion percentage and a rank of (4.) signifies the lowest adhesion percentage. (unless noted otherwise the experimentally determined trends are significant at a 95% confidence level)

One and the same liquid medium		
	Thermodynamic Model	Experimental Observations
Saline	1. FEP, 2. PS, 3. PET, 4. Acetal	1. FEP, 1. PS, 3. PET, 4. Acetal
Saline/1% 1-Propanol	1. FEP, 1. PS, 1. PET, 1. Acetal	1. PS, 1. FEP, 3. PET, 3. Acetal
Saline/2% 1-Propanol	1. Acetal, 2. PET, 3. PS, 4. FEP	1. PS, 1. Acetal, 3. PET, 3. FEP
One and the same substrate (liquid medium containing 0%, 1%, or 2% 1-propanol)		
	Thermodynamic Model	Experimental Observations
FEP	1. 0%, 2. 1%,3.2%	1. 0%, 2. 1%, 3. 2%
PS	1. 0%, 2.1%, 3.2%	1. 0%, 2. 1%, 3. 2%
PET	1. 0%, 2. 1%,3.2%	1. 0%, 2. 1%, 3. 2%
Acetal[1]	1. 0%, 2. 1%,3.2%	1. 2%, 1/3.1%,3.0%

[1] 65% confidence level

ranking with the rankings predicted by the thermodynamic model for particle adhesion. In this table, a ranking of (1.) signifies the largest mean adhesion percentage value and a ranking of (4.) signifies the lowest adhesion percentage value. If the means for two liquids or surfaces are not found to be statistically different, they are assigned the same ranking. The mean adhesion percentage for 1% 1-propanol over acetal holds an intermediate value between the means for saline and 2% 1-propanol; however, it does not differ significantly from the mean adhesion percentage for either liquid medium. Thus, two rankings are allotted for 1% 1-propanol over acetal.

In saline, where $\gamma_{LV} > \gamma_{CV}$, the thermodynamic model predicts that the largest extent of adhesion occurs on the most hydrophobic surface and the smallest on the most hydrophilic surface. This prediction is corroborated by the experimental results. FEP shows the highest adhesion percentage followed by PS, PET, and acetal. However, the difference between FEP and PS is not found to be statistically significant. PS displays a higher affinity for adhesion than expected. In a study of plant cell (*Catharanthus roseus*, $\gamma_{CV} = 54$ mJ m^{-2}) adhesion to polymer and glass surfaces suspended in distilled water containing various percentages of 1-propanol, similar trends were observed for cases where $\gamma_{LV} > \gamma_{CV}$, *i.e.* decreasing adhesion with increasing γ_{SV}.[20] This investigation involved static adhesion tests followed by rinsing, and the extent of adhesion was expressed as the percent area of the substrate covered by adherent cells. The results were interpreted with the same thermodynamic model presented here. The observed trends were in agreement with the predictions of the model. The results for FEP and PS were in close agreement in this study also. The reported area coverages in water alone were $30 \pm 1.5\%$ for FEP and $27 \pm 1.5\%$ for PS, where the errors are the 95% confidence limits. In fact, in a suspension containing 2% 1-propanol ($\gamma_{LV} = 58.1$ mJ m^{-2}, $\gamma_{LV} > \gamma_{CV}$), PS showed a higher adhesiveness than FEP. Also, Chang *et al.* reported decreasing cell adhesion with increasing γ_{SV} for glutaraldehyde-fixed human erythrocytes suspended in Hank's balanced salt solution under flow conditions.[18] The surfaces studied, in decreasing order of hydrophobicity, were siliconized glass, polystyrene, low-density polyethylene, acetal, sulphonated polystyrene (SPS), and glass.

In saline containing 1% 1-propanol, where γ_{LV}(64.1 mJ m^{-2}) is approximately equal to γ_{CV} (65 mJ m^{-2}), the thermodynamic model predicts independence of adhesion with respect to γ_{SV}. Our findings reveal that adhesion is independent of the substrate for hydrophobic (FEP and PS) and hydrophilic (PET and acetal) surfaces; however, a significant difference exists between these two groups. The hydrophobic surfaces display a higher extent of adhesion. Stewart *et al.* report an independence of granulocyte spreading on γ_{SV} of polymer surfaces (FEP, PS, PET, and SPS), although this independence was not limited to the case where $\gamma_{LV} = \gamma_{CV}$; this study involved static sedimentation followed by a rinse.[16] *C. roseus* cells showed a slope of + 0.1 for a plot of percentage area coverage *versus* γ_{SV} for a case where $\gamma_{LV} = \gamma_{CV}$;[20] a slope of zero signifies that adhesion is independent of γ_{SV}. Of course, ΔF^{adh} is equal to zero only if the surface tensions of the liquid medium and the cell are exactly equal. However, if a slight deviation from this equality exists with γ_{LV} holding a greater value than γ_{CV}, then adhesion on hydrophobic surfaces is favoured over hydrophilic surfaces.

In saline containing 2% 1-propanol, where $\gamma_{LV} < \gamma_{CV}$, the thermodynamic model predicts increasing adhesion with increasing γ_{SV}, *i.e.* a trend opposite to that observed in saline. For FEP, PET, and acetal such a reversal in trend is observed; however, the

difference between FEP and PET is not statistically significant at a 95% confidence level according to the LSD analysis; in fact, the difference is found to be significant at a 74% cofidence level. However, a Student t-test of the means for the two samples, with the assumption of unequal variances, reveals a statistically-significant difference at a 93.6% confidence level. PS shows a higher adhesion percentage than expected. A reversal in trend for $\gamma_{LV} > \gamma_{CV}$ was also seen for *C. roseus* cells; however, the reported area coverages did not cover a wide range: FEP (3%), PS (4%), PET (8%), SPS (9%), and glass (11%).[20]

The foregoing discussion considered the effect of variation of γ_{SV} on the adhesion percentage for one and the same liquid medium. Alternatively, the data may be assessed for the effect of γ_{LV} for one and the same substrate (Table VIII). For FEP, with a reduction of γ_{LV}, the thermodynamic model predicts a reduction in extent of adhesion as ΔF^{adh}, which holds a negative value for saline, becomes positive for mediums containing 1-propanol. This reduction in extent of adhesion is, in fact, observed experimentally and is found to be statistically significant at a 95% confidence level. A similar scenario applies to PS and PET. However, acetal does not conform with the predictions of the model. Its adhesion percentage increases with decreasing γ_{LV} and a significant difference exists between its values in saline and saline containing 2% 1-propanol. In overview, systems involving FEP and PET conform completely with the predictions of the thermodynamic model, whereas partial agreement is found for systems involving PS and acetal. Disagreement occurs due to increased levels of adhesion on these surfaces. Also, only partial independence of adhesion relative to γ_{SV} is found for saline containing 1% 1-propanol.

Thus, it appears that factors other than interfacial tensions, such as surface charge or surface chemistry, may play significant roles in determination of the adhesiveness of systems involving PS and acetal. Consideration of the duration of Brownian motion of adherent cells on the various polymer surfaces (Table V) may provide insight into the mechanism involved in their adhesion. A pair-wise comparison of the durations of Brownian motion in saline and suspensions containing propanol for a given polymer surface using the one-tail Student t-test, with the assumption of unequal variances for the means, was performed and the confidence levels at which differences were detected are given in Table IX. Note that in a majority of the cases highly significant differences exist amongst the durations of oscillation for a given surface. For FEP and PET, the duration of cell oscillations increases in liquid mediums which support a higher extent of adhesion. On the other hand, PS and acetal, surfaces which are in partial agreement

TABLE IX

Confidence levels (%) determined by the Student *t*-test at which the duration of Brownian oscillations in one liquid medium is greater than the duration in another liquid medium for a given substrate. The following three mediums are considered: saline, 1% propanol, and 2% propanol. The mean value for each liquid medium-substrate system is given in Table V

	Saline-1% Propanol	Saline-2% Propanol	1% Propanol-2% Propanol
FEP	90.7	92.6	51.1
PS	74.6	98.9	98.3
PET	93.8	59.9	91.9
Acetal	82.7	99.4	99.7

with the model, show decreased periods of cell motion for systems which are more conducive to adhesion. The following scenario is proposed to explain these trends. Upon approaching a surface, a cell encounters an energy barrier similar to the barrier in the DLVO theory.[32] In order to achieve adhesion the cell must overcome this barrier. On surfaces such as FEP and PET, if the system properties, such as interfacial tensions, are changed such that adhesion is rendered more favourable, with the passage of time a larger proportion of cells are able to overcome this barrier. This manifests itself in increased durations of Brownian motion prior to adhesion. However, if, in addition to the greater ability of cells to adhere, the energy barrier is reduced in magnitude, shorter durations of oscillation will result. This may, in fact, be the case for PS and acetal. Thus, for these polymers additional factors may promote adhesion to higher levels than predicted from thermodynamic considerations.

Figures 2 and 3 indicate that a time-dependence exists for adhesion of populations of cells. In most cases, an initially erratic period, due to a combination of changing sample size and increase in the number of adherent cells, is followed by a rise and a subsequent levelling of the adhesion percentage. The graphs indicate that each liquid medium-substrate system reaches a characteristic plateau and occupies a distinctly different position within the graph. However, adhesion of individual cells is not necessarily time dependent and may occur instantaneously upon contact. Furthermore, the degree of oscillation is not the same for all non-adherent cells. Some cells undergo a reduction in the extent of their oscillation prior to adhering to the surface. This may indicate that cells which undergo only minute oscillations may be in a state of transition and are in the process of adhering. It is possible that a cell which is only loosely bound still displays minute oscillations. Note that such cells, along with other more strongly bound cells, may be removed from the surface by a conventional rinsing methodology. One of the advantages to the *in situ* method presented here is that it may be combined with another technique, such as micropipette aspiration,[33,34] to determine the force of adhesion (detachment) of individual cells. Table V summarizes the duration of Brownian motion prior to adhesion for adherent cells. It would also be interesting to track and quantify, perhaps by image analysis schemes, the change in the degree of oscillation of individual cells prior to adhesion.

In an attempt to identify other forces responsible for cell adhesion, surface electric charges were investigated, Zeta potential measurements were carried out on FEP, PET, and PS.[35] Readings could only be recorded at low levels of ionic strength, such as 1 mM NaCl, signifying that these surfaces are weakly charged. At pH = 5, corresponding approximately to the pH of saline and liquid mediums containing 1-propanol (measured by pH indicator strips), all three surfaces recorded similar ζ-potentials. A greater degree of deviation was found amongst the surfaces at higher pH values with PS holding the most negative surface charge. From electrophoretic studies, it is well known that glutaraldehyde-fixed human erythrocytes hold a negative surface charge.[24] Thus, it is expected that a more negatively charged surface would impose a larger repulsive force upon fixed erythrocytes, and thus display decreased levels of adhesion. This is not the case for PS; in fact, higher levels of adhesion than expected occur on this surface. Therefore, at the present time, electric charges do not provide a satisfactory explanation for the anomalous behaviour of PS with respect to the thermodynamic model for particle adhesion.

In discussing the discrepancies between the model and experimental observations, the following points must be borne in mind. We have utilized the equation of state in a generic form for calculation of the relevant interfacial tensions for particle adhesion. However, the equation of state has been developed for contact angle systems consisting of three bulk phases (solid, liquid, and the liquid's vapour), two components (solid and liquid), and three surface phases (solid-liquid, solid-vapour, and liquid-vapour). With the development of a phase rule for moderately curved surface systems, Li *et al.* show that the above system possesses two degrees of freedom, thereby justifying the use of a relation in the form of the equation of state.[15] Furthermore, contact angle measurements of various binary liquids, mixtures of 1-propanol and water, on smooth FEP surfaces, have revealed that the equation of state is not applicable to systems involving binary liquids due to the presence of an additional degree of freedom, namely the mole fraction of the binary liquid mixture.[36] For example, the γ_{SV} calculated with a water solution containing 2% 1-propanol is 4 mJ m^{-2} less than the value obtained with water alone (17 mJ m^{-2}). This analysis illustrated that the equation of state is not presently equipped to handle systems involving binary liquids, *i.e.* an equation in the form of $\gamma_{AB} = f(\gamma_{AV}, \gamma_{BV}, x)$, where x is the mole fraction of the binary liquid mixture, is required.

The thermodynamic model provides qualitative predictions with respect to the trends in adhesion. It is postulated that in systems consisting of a binary liquid in contact with a solid surface, preferential adsorption at the solid-liquid interface may have a major effect. The fact that ANOVA reveals a significant level of interaction between γ_{SV} and γ_{LV} may indicate that preferential adsorption is present in our systems. Such preferential adsorption may change the solid-liquid and cell-liquid interfacial tension significantly from the predictions of the equation of state for interfacial tensions. Thus, the effective γ_{SL} in systems involving binary liquids may not be equivalent to the values determined by water contact angle measurements as outlined in Table I. Recall that the thermodynamic model was developed with the inherent assumption that γ_{SV} and γ_{CV} are constant and independent of the liquid mediums with which they come into contact, *i.e.* there is no allowance made for adsorption. Moreover, the degree to which this effect is accentuated varies from one system to another depending upon the properties, such as polarity and surface tension, of the interacting components. Thus, we did not *a priori* know to what extent the preferential adsorption would affect the results. Apparently, for as of yet unknown reasons, it is not an issue for systems involving FEP and PET. However, adsorption on PS and acetal are significant enough to cause deviations. Nevertheless, partial agreement for two of the polymers and complete agreement for the others indicate that in our system interfacial tensions indeed play a significant role in determining the extent of particle adhesion. A viable option at this point is to repeat the experiments with properly selected single liquids for variation of γ_{LV}.

CONCLUSIONS

(1) Interfacial tensions play a significant role in adhesion of glutaraldehyde-fixed human erythrocytes to various polymer surfaces suspended in mixtures of 10 mM NaCl and 1-propanol.

(2) A statistically-significant level of interaction exists between γ_{SV} and γ_{LV} at a confidence level of 99.9% in the extent of adhesion of glutaraldehyde-fixed human erythrocytes.
(3) In general, the liquid medium-substrate systems achieve characteristic adhesion levels.
(4) Complete adhesion (*i.e.* 100% adhesion) is not observed in any of the systems under investigation.
(5) Populations of cells display time-dependence of adhesion: A period of rise is followed by a characteristic plateau.
(6) Individual cells may adhere instantaneously upon contact with the surface or achieve adhesion gradually with the passage of time.
(7) Varying degrees of Brownian motion are displayed by non-adherent cells.
(8) FEP and PET comply completely with the predictions of the thermodynamic model for particle adhesion.
(9) PS and acetal show partial agreement with the predictions of the thermodynamic model. The discrepancies are due to higher levels of adhesion on these surfaces than expected.
(10) In saline containing 1% (v/v) 1-propanol, where $\Delta F^{adh} \approx 0$, partial independence of adhesion from γ_{SV} is found.
(11) Partial agreement with the model and data on duration of Brownian motion of adherent cells prior to adhesion indicate that factors other than interfacial tensions may be operative as well.

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