Molecular properties in cell adhesion: a physical and engineering perspective

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The past several years have seen accelerating growth in research directed towards the understanding and control of cell adhesion processes, from a spectrum of disciplinary approaches including molecular cell biology, biochemistry, biophysics and bioengineering. Consequently, our understanding of the mechanisms involved in cell adhesion has increased substantially. Corresponding quantitative analysis and modeling of the key molecular properties governing their action in regulating dynamic cell attachment and detachment events is crucial for advancing conceptual insight along with technological applications.

The adhesion of cells to the extracellular matrix, other cells or biomaterials is of crucial importance in governing a range of cell functions in physiology, pathology and biotechnological applications. It is a process that uses not only mechanical interactions but also chemical signals as a basis for cell regulation and has therefore attracted growing attention from all disciplines focused on cell-based biotechnology, including biochemistry, cell biology, biophysics and bioengineering. As a consequence of the now well-established role of specific biological macromolecules (cell membrane receptors, extracellular ligands and cytoskeletal components) in mediating adhesion, mechanical analysis of molecular and cellular behavior is needed to fully analyze adhesion processes. Cell adhesion is thus an excellent example of the essential relationship between biochemistry and biophysics. The aim of this article is to outline some significant advances that have occurred since an earlier review in determining how cell adhesion depends on underlying molecular properties, in a manner that can be measured and modeled in terms of quantitative parameters characterizing both biochemical and biophysical aspects of the molecular interactions. Particular issues for which new understandings have been described within the past few years on this topic include: (1) the relationship between receptor–ligand chemical equilibrium binding parameters and cell adhesion; (2) the effects of physical forces on bond association and dissociation; and (3) the locus of disruption of adhesion molecules.

Affinity

Cell adhesion is mediated by the interaction of surface proteins (receptors) with proteins in the extracellular matrix or on the surface of other cells or biomaterials, which are referred to as 'ligands'. The simplest way to model the non-covalent but highly specific interaction of the two molecules is to assume a one step chemical reaction in which receptor (R) and ligand (L) can form a complex (C) as shown in Eqn 1.

\[ [R] + [L] \xrightarrow{k_f} [C] \]

Receptors and ligands associate to form a bond with an intrinsic association rate of \( k_f \), in which '0' refers to the reaction rate in the absence of force. Without an externally applied force, the bond dissociates with a dissociation rate of \( k_r \). The concentration of each species at equilibrium is determined by the equilibrium constant for the interaction \( K_0 \) (also called the affinity); this is the association rate divided by the dissociation rate. Alternatively, the receptor–ligand interaction can be represented by the dissociation constant, \( K_D \) (the inverse of the affinity). The stoichiometry of Eqn 1 is appropriate when both the receptors and ligands are monovalent. However, in many physiological situations, one or both of the molecules can be multivalent, which necessitates a more complex model to describe the chemical reaction.2

Equation 1 can be used to model the different experimental and physiological situations under which receptors and ligands interact. In Table 1, these situations have been sorted according to the spatial restrictions of the reacting proteins. Molecules in solution will be able to diffuse freely in three dimensions, whereas surface-bound molecules will be limited to two dimensions. Typical units for each species are shown, as well as those for the binding constants. This article will deal primarily with the last row of Table 1, in which both receptors and ligands are limited to movement in 2D, such as along the surface of the cell.

To determine values for the rate constants and the affinity, it is essential to know (measure) the number of bonds formed. However, it has not yet been possible to experimentally measure the receptor–ligand bonds when two molecules that are linked to surfaces interact. For cells in contact with surfaces, creative techniques, such as fluorescence...
internal reflection microscopy, are needed to quantify the magnitude of receptor–ligand binding for bound cells. Currently, the number of bonds formed is usually estimated by geometry3,4, inferred from experiments5 or determined from modeling6. A more common approach is to measure the 3D affinity (determined when the receptor, ligand or both are free to diffuse in 3D) and then convert it to a 2D affinity using a parameter, which is in the order of the size of the receptor–ligand pair7,8, ~10 nm (Ref. 7). However, because this parameter represents the ratio of the 3D to 2D affinities and because this relationship has not yet been firmly established, the estimates for this parameter can vary over four orders of magnitude9. Measurements of 2D affinity and rate constants have only recently begun and solid biophysical measurements are required to fully elucidate this relationship. Initial investigations have shown that the 2D affinity can be 40 times that which would be predicted using the 3D constant and the height of the confinement region to deduce a 2D value9–11.

Force
Adhesion is dependent on the magnitude of force applied to the cells. Given that a cell can be adherent under weak forces and not adherent under strong forces, a description of adhesion without a specification of the forces applied to the cell is incomplete. An important property of receptor–ligand bonds that determines adhesion is the bond strength. Drawing from the kinetic theory of the strength of solids, it can be assumed that although the forward rate should not be affected by an applied force, the reverse rate constant (and thus the affinity) will vary exponentially with force9, as shown in Eqn 2.

\[
k_r = k_0 e^{-\frac{f}{k_BT}}
\]

In Eqn 2, \(f\) is a length parameter defined as the bond interaction distance, \(f\) is the applied force, \(N_c\) is the number of receptor–ligand complexes, \(k_0\) is the Boltzmann constant and \(T\) is the temperature. In terms of a chemical reaction, the bond interaction distance can be thought of as a length scale for the transition state of the reaction from a bound to unbound state. To extend this model to the adhesion of cells to surfaces (and other cells), an approximate assumption is that all bonds are stressed equally. By assuming that the association rate is unaffected by applied force, the adhesion strength of an interaction can be determined on a per bond basis, as shown in Eqn 3.

\[
F = 0.7 \left( \frac{k_B T}{\gamma} \right) \ln \left( \frac{[L]}{K^0} \right)
\]

In Eqn 3, \(F\) is the adhesion strength per bond and \([L]\) is the concentration of free ligand. One major unresolved question is, what is the correct model to describe the relationship between force and dissociation for an adhesion molecule? Equation 2 represents one such model, but many other models can be imagined easily. An alternative approach assumes the receptor–ligand bond takes the form of a spring. In this case, the affinity will decrease as the bond length moves away from its equilibrium length according to a Boltzmann distribution12, as shown in Eqn 4.

\[
K = K_0 e^{-\frac{\kappa_0 (s-L)^2}{2k_BT}}
\]

In Eqn 4, \(\kappa_0\) is the mechanical spring constant of the bond, \(L\) is the cell to surface (where the surface could also be another cell) separation distance when the bond is unstressed, and \(s\) is the separation distance when the bond is being stressed. This model can be extended to predict cell detachment using a 1D tape-peeling model12, in which the critical tension can be expressed as a function of receptor and ligand density and molecular affinity8,13.

A third and more recent model accounts for the rate of escape of a ligand from the binding pocket of a receptor, given the energetic landscape of the receptor–ligand pair as well as the rate of application of force to the ligand14. This model reduces to Eqn 2 in limits of fast loading (where forces are applied quickly to the receptor–ligand pair) but can be expressed in more complex forms for different rates of loading. These models auger the possibility that receptor–ligand interactions might be best represented by many different energy wells or transition states, each representing a different functional component of the receptor–ligand complex. New advances in
molecular force spectroscopy, such as the biomembrane force probe\textsuperscript{15} and the atomic force microscope (AFM)\textsuperscript{16} will make it possible to image the force landscape of adhesion molecules with unprecedented precision.

The relationship between applied force and molecular affinity was probed in adhesion experiments using a radial flow chamber\textsuperscript{3}. Experiments using antibody-coated polystyrene beads adhering to a protein-A-coated glass surface showed the necessary force to detach the beads varied logarithmically with the affinity of the bond (as measured with both molecules in solution), which is predicted by Eqn 3. These data provided the experimental verification of Eqn 4. It has also been shown that increased affinity leads to increased strength of adhesion for integrins, a class of cell-surface receptors that mediate interactions between cells and extracellular matrix molecules (such as fibronectin) by recognizing the tripeptide sequence arginine–glycine–aspartate (RGD in single-letter amino acid code)\textsuperscript{17}. In a study on bovine aortic endothelial cells adhering to surfaces of fibronectin, linear RGD or cyclic RGD, the spring constant for all three interactions was determined to be the same (1 dyn cm\textsuperscript{-1}). This could imply that the portion of the bond that functionally acts as the spring might exist in the cytoskeleton.

An important question is, which rheological model is most valid for receptor–ligand binding under force? Recently, a stochastic model relating applied force to the 2D bond affinity was developed for the adherence of tumor cells to recombinant E-selectin in a centrifugation assay\textsuperscript{9}. This model employs a constitutive equation for binding affinity and contained parameters that could be varied to allow several different types of relationships between force and affinity. The best fit was obtained with a model in which the affinity varies as the inverse of the applied force\textsuperscript{10}.

There are caveats to many of these experimental systems. First, although measuring cell or bead adhesion in a flow chamber has provided useful information regarding detachment under stress, it can be difficult to obtain information about a single molecular interaction with confidence. Attempts to characterize the strength of the receptor–ligand pair itself have often used the AFM or biomembrane force probe to study avidin–biotin pairs. Unbinding forces have been measured to be 85 pN for avidin–iminobiotin\textsuperscript{18,19} and 257–350 pN for streptavidin–biotin\textsuperscript{18,20}; however, current concepts of the failure of adhesion molecules suggest that there is no single force for bond breakage, that bond failure might be governed by a series of transition states (not just one) and that these different transition states can be explored by exerting forces on adhesion molecules at different pulling rates (measured in pN sec\textsuperscript{-1} ranging over several orders of magnitude). In addition, similar ‘adhesion strengths’ have been reported for antibody–antigen interactions\textsuperscript{21}. In systems in which multiple receptor–ligand bonds secure contact, numerical simulations can be useful in understanding how individual molecules contribute to the strength of adhesion. This has been demonstrated for detachment of beads from surfaces\textsuperscript{22,23}, and in surface force apparatus experiments\textsuperscript{3}.

Second, many of these systems are designed to study monovalent interactions. Although these are the simplest to study, it has been shown that, in practice, cell adhesion mediated by multivalent interactions can be more difficult to dissociate than monovalent interactions, even when the total number of bonds is the same\textsuperscript{24}. Presumably, this is because multivalency can lead to receptor aggregation on the cell surface, which can trigger a response inside the cell.

Finally, many of these systems use beads rather than cells. The receptors then are cell-surface proteins adsorbed or covalently linked to the surface of the beads. Additionally, ligands are often attached to a surface such as a glass slide and therefore, neither protein is laterally mobile. Although this sort of model system might provide the cleanest way to study adhesion under flow, some important complexities might be lost. Both receptors and ligands might be stuck in a random conformation that either inhibits or enhances binding. Clearly, the molecular interactions will be affected by such limitations. Because the receptors are immobilized to the bead surface it can be difficult to correlate the results to physiological situations. This is because the immobilization will prevent the aggregation of receptors on the cell membrane, thereby possibly preventing a cellular response. However, in support of such work, these cell-free constructs are the best way to elucidate conclusively the role of receptor–ligand physical chemistry in mediating cell adhesion because they eliminate the role of confounding cellular features, such as multiple receptors or heterogeneity, cell roughness, signaling or deformability\textsuperscript{25}.

It is worth noting that a significant amount of work has been done to prevent cell adhesion, or to cause detachment at zero force using competitive inhibition. A soluble form of the ligand can be added to the cells, which prevents them from binding. This has been shown to be effective in preventing bacterial cells from binding epithelial cells, a step in the development of gastroduodenal ulcers and gastric cancer\textsuperscript{26}. Other systems used liposomes that contain ligand on their surface to prevent cells from binding to soluble ligands, which might help modulate the inflammatory response\textsuperscript{27,28}.

Dissociation

A crucial determinant of the dynamics of adhesion is the responsiveness of the dissociation rate to force. In Eqn 2, the bond interaction length γ is a metric of the
leukocytes flow along the vessel wall, they decrease their speed to a slow roll near the site of inflammation or infection. It is now understood that this rolling is a result of bonds formed by proteins called selectins interacting with carbohydrate moieties of proteins. Microfilaments and intracellular domains of receptors are not involved in these initial adhesions, nor are they mediated by integrins, which control firm cell–cell adhesion once the leukocytes have reached the cell. Rolling has been shown to result from the physical chemistry of protein recognition and not from cellular features such as signaling or deformability by recreating rolling using polystyrene spheres coated with receptors rolling over ligand-coated surfaces.

Figure 1 illustrates the different types of arrest a cell might experience as it rolls over a ligand-coated surface. If the shear rate is sufficiently high, the cell surface receptors will not have a chance to bind to the ligand and the cells will flow by without attaching. Transient adhesions are formed at slightly lower shear rates where cells might bind briefly to the surface and translate at a reduced velocity for a short time, then unbind and quickly return to the bulk fluid velocity. Rolling adhesions are formed at still lower shear rates where cells constantly form and break bonds as they translate slowly over the surface. Both transient and rolling adhesions are mediated by selectin–carbohydrate bonds. Finally, when cells are stopped on the surface (by either the absence of flow or the presence of a large amount of selectin bonds) they can bind the surface through integrin receptors, which mediate firm adhesions.

Given that rolling requires the sequential forming and breaking of bonds as the cell moves, it is believed (and has been shown by simulation) that the dissociation rate $k_r$ is more important than the affinity in controlling cell rolling. For this reason, considerable effort has been devoted at characterizing individual protein (selectin)-carbohydrate bond interactions known to mediate rolling. It has been suggested that the protein–carbohydrate interaction is well suited to support rolling under physiological conditions. The P-selectin glycoprotein ligand-1 (PSGL-1)-P-selectin and L-selectin-peripheral node addressin (PNAd) systems are excellent examples of this. In the vascular system P-selectin is expressed on the surface of the endothelial cells lining the blood vessel wall and can interact with PSGL-1-containing leukocytes, whereas L-selectin is expressed on the surface of leukocytes and can interact with carbohydrate ligands expressed on cells in the secondary lymphoid tissues. In studies of neutrophils (with PSGL-1) tethering on a lipid bilayer containing P-selectin in a parallel-wall flow chamber, the protein–carbohydrate pair obeyed first order kinetics as a function of the pause time distribution. The dissociation rate obeyed the rate
Fig. 2. Phase diagram for the states of adhesion. Experimentally determined values for P-selectin (circle); E-selectin (triangle); and antibodies (diamond) are plotted.

law described in Eqn 2. Although the dissociation rate did increase with shear stress as expected, the bond interaction distance (0.5 A) was extremely low, implying only small changes in dissociation rate with applied force. Other studies have revealed that both intrinsic (unstressed) dissociation rate and bond interaction distance have an important role in determining the dissociation rate under flow conditions. For example, the L-selectin interaction has a faster intrinsic off rate than P-selectin interactions (7 s⁻¹ compared with 1 s⁻¹), but is less responsive to force. Because both the intrinsic dissociation rate of the bond and its responsiveness to force can modulate the overall dissociation rate, it is clear that experimental comparisons of bond strengths require the additional knowledge of the stress applied to the bond while making the measurement. If obtained at high force, a bond pair that has a low intrinsic dissociation rate with a high responsiveness to force might have a lower affinity than a high intrinsic dissociation rate pair with a low responsiveness to force.

In contrast, it has been shown that protein–protein bonds are more responsive to force than protein–carbohydrate bonds. Rolling has been shown to occur in systems using an antibody rather than a selectin to bind carbohydrate ligand, suggesting that selectin molecules are not unique in their ability to support this phenomenon. However, studies of cell adhesion under flow mediated by antibody–hapten interactions do not support rolling, even when the intrinsic dissociation rate is nearly that of a selectin–carbohydrate bond. Adhesive dynamics simulations have been used to generate the phase diagram shown in Fig. 2, which succinctly demonstrates the relation of the dynamic state of adhesion (rolling or firm) to the rates of dissociation and the bond interaction length. Indeed, selectins do map to the region of the phase diagram in which rolling states are predicted. The diagram clearly indicates that molecules with fast unstressed dissociation rates (such as L-selectin and its ligand) must have lower values of the bond interaction length to maintain rolling adhesion.

The bond dissociation rate can also be determined using short contact times. One study measured no change in the dissociation rate over a force range from 6–37 pN for mouse anti-rabbit antibodies (attached to beads) reacting with rabbit antibodies (attached to a flat surface). However, another found the initial off rate doubled with a double in force from 11–22 pN when CD48 and CD2 were used as the receptor–ligand pair. Taken together, these data suggest again that knowledge of the responsiveness of a bond to force is important to complete understanding of molecular interactions.

Association

Computer simulations that compare attachment and detachment situations have indicated the increased significance of the affinity for detachment processes in contrast to a greater significance of the association rate constant for transient attachment processes. This delineation of governance into ‘equilibrium-limited’ and ‘kinetics-limited’ regimes is consistent with theoretical predictions. It has also been demonstrated that a system with a high association rate constant can mediate and increase cell detachment compared with a system that might have a higher affinity but a lower association rate constant.

Bond formation is a stochastic process, as was demonstrated in a radial flow chamber study of neutrophils rolling over endothelial cells. In this study, the measured fluctuations in instantaneous rolling velocity about the average velocity were two orders of magnitude greater than what would be predicted by experimental error alone. This feature is similarly observed in vivo as well as in cell-free adhesion experiments. Several studies have shown that the binding efficiency of a cell to a surface was inversely proportional to the cell velocity. This phenomenon was seen in protein–protein, protein–hapten, and protein–carbohydrate interactions, in which the association rate increased tenfold after the initial adhesion. Shear flow can promote rolling of cells over surfaces; this occurs as shear aids in the conversion of transient bonds to rolling adhesions. In fact, rolling velocity increases with increasing shear stress over the physiological range for polystyrene beads coated with sialyl-Lewis-x (a carbohydrate ligand for E-selectin) rolling over E-selectin-IgG chimera-coated surfaces. It has since been shown that the selectin–carbohydrate interaction requires a threshold level of shear to support rolling both in vitro and in vivo.

The stochastic nature of cell rolling and adhesion results not only from fluctuations in receptor–ligand
binding but also can be enhanced by cell and surface topography. Selectin ligands can be clustered on the tips of microvilli of cells, thus the location of the receptors can affect the probability of tethering and rolling\(^{46,47}\); the density of microvilli appears to affect the dynamics of attachment\(^{44}\). For neutrophil adhesion to latex beads under micropipette suction, forces of 45–120 pN failed to detach the cell but rather a long tether formed between the neutrophil and the bead as the cell was sucked back into the pipette\(^{48}\). This rheological response of the microvilli might protect and facilitate selectin-mediated tethering and rolling\(^{49}\).

**The site of adhesion disruption**

Many applications and experimental systems to study cell adhesion involve two or more bonds in series to attach the cell to the surface\(^{48,50,51}\). An example of the contact area of two cells interacting through a linker molecule is shown in Fig. 3 and illustrates the possible mechanisms of bond failure. These bonds can break between receptor-linker, ligand-linker or by receptor or ligand extraction from the cell membrane. It has been shown that intersurface contacts consisting of two receptor–ligand bonds in series are weaker than contacts consisting of either bond separately. For latex beads adhering to glass dishes with different combinations of immobilized proteins in the Radial-Flow Detachment Assay (RFDA), the adhesion strength decreased by \(-85\%\) (Ref. 52).

Although it is often assumed that receptor–ligand bonds break at the point of formation – between the two interacting molecules – in practice this is not the case. It has been shown that an alternative mechanism, receptor extraction from the cell membrane itself, can occur during cell detachment\(^{13}\). Direct comparison studies have been made between cell and cell-free systems to determine how receptor extraction affects cell adhesion. Here, break-up of red cell doublets required lower forces than latex sphere doublets with immobilized ligand, even at the same bond number. Hence, it was concluded that receptor extraction was occurring\(^{52}\). Studies using the surface force apparatus have shown that modulating the affinity of the receptor–ligand bond can affect the mechanism of detachment. It was estimated that the crossover from bond breakage to bond rupture occurred at a bond affinity of \(10^6\) M\(^{-1}\); affinities above this favor receptor extraction. Over the ten orders of magnitudes of affinities studied with the streptavidin–biotin analog system, the average force per bond for receptor extraction was 80 pN (Ref. 54). Others have shown the required force for receptor extraction to be on the order of 10–80 pN (Refs 13,55).

Receptor extraction poses difficulties for immunoaffinity-based cell separation technologies, such as immunomagnetic beads. It has been shown that not just single proteins but entire membrane fragments can be pulled out by the beads\(^{56}\). Indeed, these results could affect the choice of separation technique, as well as the configuration used\(^{67}\).

Cells might overcome the weak link of membrane extraction by receptor clustering. Studies of cells migrating over surfaces coated with extracellular matrix molecules have shown that during locomotion in response to a stimulus, bound integrins aggregate into focal contacts by crosslinking to the cytoskeleton. The steps in migration appear to involve both biomechanical and biochemical processes, ending with the final step of integrin release as the rear of the cell moves forward\(^{58}\). It has been predicted that the cell adhesion strength increases many orders of magnitude when integrins aggregate into focal contacts\(^{59}\).

**Future prospects**

One of the major future directions for adhesion research is to continue to elucidate the relationship between molecular structure, function and the dynamic states of adhesion. First, the structures of adhesion molecules need to be determined using techniques such as X-ray crystallography and 2D-NMR (Ref. 60). Second, the functional properties of adhesion molecules need to be modeled and quantified in parametric terms. This can be accomplished using techniques such as surface plasmon resonance to measure reaction rates and the various molecular force techniques described here to measure the mechanical response of adhesion molecules. Simultaneous elucidation of structure–function relationships can facilitate an understanding of which structural motifs give rise to functional properties. Third, a continuing effort is needed to explore how the dynamics and strength of adhesion are correlated to the underlying structural and functional properties, allowing the incorporation of molecular properties into a full cellular context. For dynamic states of adhesion this is tantamount to mapping a ‘state diagram’, in which the dynamics of adhesion are measured separately from the reaction rates and the bond material properties. The development of state diagrams from both experimental and theoretical bases will probably become a useful paradigm not only for the field of cell adhesion but also in the more general field of receptor-mediated cell processes\(^{61}\).
References