# Hidden Dynamics of Vesicle Adhesion Induced by Specific Stickers

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We experimentally studied the adhesion dynamics of vesicles decorated with specific stickers onto bioactive surfaces. The growth laws were found to strongly depend upon the chemical preparation of the substrate and were rationalized with theoretical descriptions based on scaling law arguments. By using a micropipette-free approach, we demonstrate that two-dimensional binding rates between receptor and ligand can be lower than three dimensional on rates by orders of magnitude due to reduced accessibility of the immobilized protein.

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The adhesion of cells to the extracellular matrix, to other cells, or to biomaterials is of crucial importance in governing a range of cell functions in physiology (embryology), pathology (cancer metastasis), and biotechnological applications (prothesis design). Cell adhesion is initiated by weak noncovalent interactions between surface proteins called receptor and ligand. In solution, the biochemical properties of these adhesion molecules have been well characterized in terms of dissociation rates and affinity constants [1]. Recent studies on individual specific bonds tethered to solid surfaces have also shown that the dissociation rate of such bonds is drastically increased upon force application [2]. At the cellular scale, the static shape of adhering model cells (vesicles) [3] or living cells has been extensively studied [4].

Although the very nature of cell adhesion is intrinsically dynamic, the kinetics of cell adhesion has been poorly explored to date. Spreading of cells onto the extracellular matrix may be anticipated to be more difficult to interpret than wetting of surfaces by liquid droplets [5]. Among other differences, the membrane tension of cells is likely to vary during spreading, as well as the surface density of stickers since adhesion molecules can diffuse along the cell surface and be recruited towards the adhesion patch [6]. Very little information is available about binding and rupture kinetics of a collection of receptor-ligand bonds when both partners are linked to apposing cells or surfaces. By micropipette manipulation, Prechtel *et al.* [7] were able to measure unbinding forces of living cells adhering to vesicles as a function of loading rate. Here, we used giant unilamellar lipid vesicles decorated with receptors as test cells and glass substrates functionalized with ligands as target tissue and studied the kinetics of the spreading of individual vesicles onto biomimetic substrates in a direct, micropipette-free manner.

Vesicles of egg phosphatidylcholine doped with 0.05% to 5% in weight of biotinylated lipid (Dioleoylphosphoethanolamine-PEG$^{2000}$-biotin from Avanti Polar Lipids) were prepared in a sucrose solution by electroformation [8]. Streptavidin was added to the suspension of vesicles and the excess was washed off by gentle centrifugation. Finally the vesicle suspension was diluted in a glucose solution in order to provide a slight gravity gradient between the vesicle interior and the bath solution. As target surfaces, we generated two kinds of biotinylated substrates referred to as casein-biotin and PEG-biotin surfaces. The former was obtained by adsorption of casein pretagged with a short biotin derivative, NHS-biotin (Pierce), following a standard procedure [9]. The latter was obtained by grafting a long and flexible SBA-PEG$^{3400}$-biotin cross-linker (Nektar) to an aminosilanized glass slide [2]. Using fluorescent Cy3-Extravidin (Sigma), and following the calibration procedure reported in [10], we found that casein-biotin and PEG-biotin surfaces used here bear a similar surface density of biotin groups available to streptavidin, equal to $2.8(\pm 0.5) \times 10^{16}$ m$^{-2}$. As controls for nonspecific adhesion, we prepared surfaces covered with the nonbiotinylated analogs, namely SBA-PEG$^{5000}$-methoxy (Nektar) and $\beta$-casein.

The observation chambers were made of one biotinylated cover slip and one naked slide assembled using a parafilm spacer, and they were placed on the stage of an inverted microscope (Axiovert 200, Zeiss) equipped with interference and fluorescence filters and a 100× immersion oil objective. Two kinds of experiments were then performed.

First, the equilibrium static shape of vesicles was observed by light microscopy using a 90° tipped microscope working at low-angle incidence [11]. Figure 1 shows side-view micrographs of streptavidin vesicles in contact with both methoxy-PEG and PEG-biotin substrates. Whereas vesicles simply sit on PEG passivated surfaces, they look like truncated spheres on PEG-biotin surfaces. The equilibrium contact angle, $\theta_e = 45 \pm 7°$, suggests that adhesion is strong, i.e., $WA \gg \kappa$, with $W$ the adhesion energy per unit area, $A$ the area of the adhesion disk, and $\kappa$ the bending rigidity of the membrane [3]. Similar experiments carried out on casein-biotin surfaces yielded similar values for $\theta_e = 43 \pm 9°$.

In a second set of experiments, the spreading kinetics of streptavidin vesicles onto biotin substrates was moni-

 

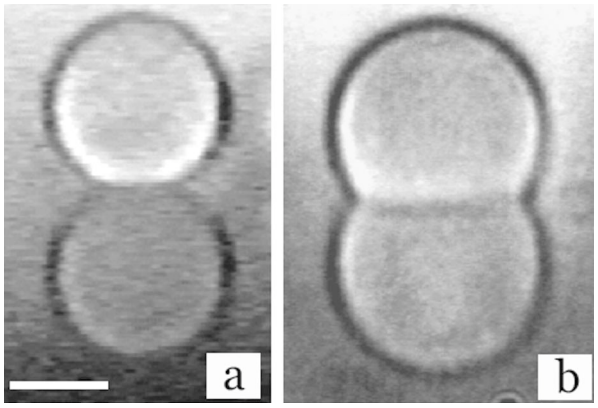


FIG. 1. Side-view images of a streptavidin vesicle on (a) a passivated methoxy-PEG substrate and (b) a bioactive PEG-biotin substrate. Scale bar, 10 $\mu$m.

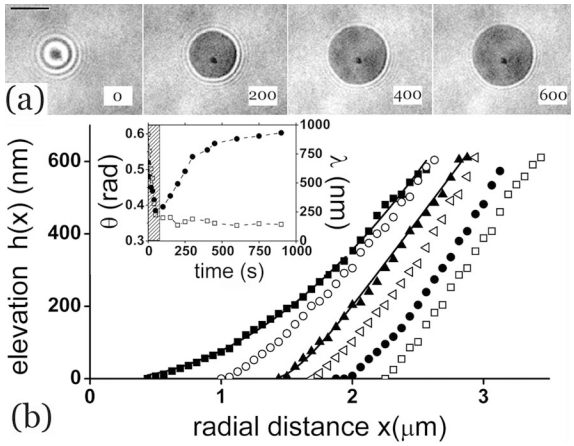


FIG. 2. (a) RICM snapshots of a 14 $\mu$m diameter streptavidin vesicle spreading on a casein-biotin substrate. Time between successive images is $\Delta t = 200$ s. Scale bar, 2 $\mu$m. (b) Typical reconstructed surface profiles over a time interval of 900 s. The height profiles $h(x)$ were fitted with $h(x) = \theta(x - R - \lambda) + \theta\lambda \exp[-(x - R)/\lambda]$ for $x > R$, and $h(x) = 0$ for $x < R$ [13]. The inset shows the time evolution of $\theta$ (closed circles) and $\lambda$ (open squares). The shaded region corresponding to short times will be disregarded (see the text).

tored by reflection interference contrast microscopy (RICM). RICM is a suitable interferometric technique that permits one to visualize adhesion patches as dark spots and to reconstruct the membrane profile close to the surface from Newton ring patterns with a vertical resolution of ~5 nm up to a maximal elevation of ~1 $\mu$m. Reconstructing the profiles in time provides the main two geometric parameters: (i) the contact angle $\theta(t)$ which obeys Young's equation $W \approx \sigma(1 - \cos\theta)$, with $\sigma$ the membrane tension, and (ii) the capillary length, $\lambda(t) = (\kappa/\sigma)^{1/2}$ [12]. All images were grabbed using a digital charge-coupled device (CCD) camera (Ropper, CoolSnap HQ) at a time resolution of 100 ms. Figure 2(a) displays four RICM snapshots of a vesicle saturated in streptavidin ($\Gamma_0 = \Gamma_{sat} \approx 3 \times 10^{16}$ m$^{-2}$) spreading on a casein-biotin substrate. The adhesion patch grew from a single nucleation point and retained a circular shape during the whole growth (indicating that the vesicles were initially quite tense), before reaching a saturation size. Figure 2(b) shows typical surface profiles. Fitting the height profile yields the time evolution of $\theta$ and $\lambda$ [inset in Fig. 2(b)]. Here, two remarks are important. First, the radius of curvature of the membrane near the contact line, and thus $\lambda$, decreased during the initial times from ~1 $\mu$m down to ~200 nm. However, this decrease was correlated with the initial reduction of $\theta$, which has been interpreted as an out-of-equilibrium spreading process in wetting experiments [5]. Our analysis being based on quasistatic equilibrium assumptions, we discarded the short times following nucleation of the patch. Over the authorized time interval, $\lambda$ was found to be roughly constant, in the 100–200 nm range. This means that spreading occurs at constant surface tension, typically 1–5 $\mu$N/m by taking $\kappa \simeq 12 k_B T$ (measured independently—data not shown). Second, it is noteworthy that $\theta$ can be alternatively derived from the radius of the adhesion disk: $\sin\theta(t) = R/R_v$, with $R_v$ the equatorial radius and $R(t)$ the radius of the adhesion zone plus a first-order correction in $\lambda$. Figures 3 and 4 show typical time evolutions of $R/R_v$ for spreading onto PEG-biotin and casein-biotin surfaces. For each surface, three different densities in streptavidin on the vesicles, $\Gamma_0$, were investigated. Quite surprisingly, the adhesion kinetics strongly depends upon the chemical preparation of the substrate. Vesicle adhesion was completed within a few seconds on PEG-biotin substrates and was slowing down with decreasing $\Gamma_0$, whereas spreading took more than 10 min and was not affected by $\Gamma_0$ on casein-biotin substrates. Since these differences cannot be attributed to a difference in biotin density on the surface,

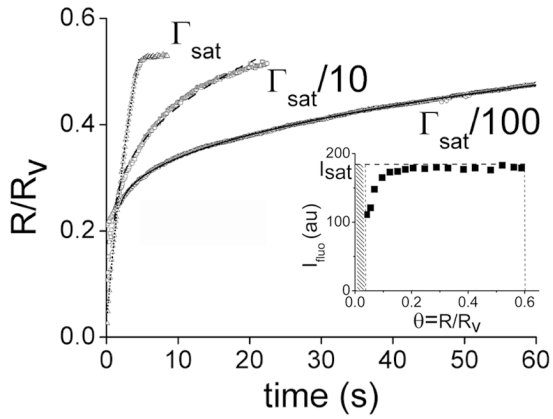


FIG. 3. Typical time evolution of the patch radius normalized by the radius of the streptavidin vesicle adhering to a PEG-biotin substrate for different streptavidin densities $\Gamma_0$. The superimposed dashed lines are fits using, respectively, (2) for fully covered vesicles ($\Gamma_{sat}$) and (1) for sparsely covered vesicles ($\Gamma_{sat}/10$ and 100). The inset shows the radial fluorescence intensity inside the patch when Cy3-Extravidin was used ($\Gamma_{sat}/10$).

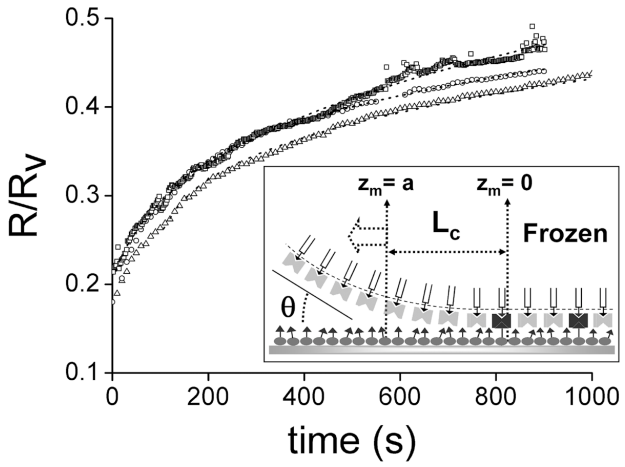


FIG. 4. Typical time evolution of the patch radius normalized by the radius of the streptavidin vesicle adhering to a casein-biotin substrate for different streptavidin densities $\Gamma_0$ (same as in Fig. 3). The superimposed dashed lines are fits using (5). Inset: hypothetical sketch of the adhesive interface between streptavidin membrane and casein surface.

our results have to be interpreted by taking care of the details of the chemical procedure.

de Gennes *et al.* [14] proposed several scenarios for the dynamics of vesicle adhesion. Although none of these scenarios exactly matches our experimental conditions, we use similar scaling law arguments. Let us first examine the case of PEG-biotin surfaces. For vesicles with low coverage in streptavidin ($\Gamma_0 < \Gamma_{\rm sat}$), we may write the transport equation, which expresses that the accumulation of stickers in the patch zone is supplied by the inward diffusion current: $(\Gamma_{\rm sat} - \Gamma_0)R^2 \approx \Gamma_0 Dt$. For $\Gamma_0 \ll \Gamma_{\rm sat}$, the growth law of the adhesion patch is

$$\theta(t) \cong \left(\frac{\Gamma_0}{\Gamma_{\rm sat}}\right)^{1/2}\left(\frac{D}{R_v^2}\right)^{1/2} t^{1/2}. \qquad (1)$$

Here, the underlying assumption is that the density of bound stickers is $\Gamma_{\rm sat}$ through the whole patch. This hypothesis was confirmed by monitoring the density profile of Cy3-Extravidin molecules in the patch by fluorescence microscopy. The inset in Fig. 3 shows that the density of stickers inside the patch quickly reaches $\Gamma_{\rm sat}$ after nucleation, as soon as $\theta \geq 0.1$. As displayed in Fig. 3, the data for spreading of vesicles sparsely coated with streptavidin on PEG-biotin substrates can be well fitted by a power law, with an exponent $n = 0.48 \pm 0.06$ (dashed lines) averaged over about ten events for each concentration, which is in good agreement with (1). Additionally, we can check that the prefactor is reduced threefold when the coverage density is diluted tenfold, as expected from the dependence in $\Gamma_0^{1/2}$. The diffusion coefficient derived from (1) is then found to be $\sim 5\ \mu{\rm m}^2\,{\rm s}^{-1}$, in good agreement with values reported for lipid diffusion [13]. When vesicles fully covered with streptavidin are considered, the transport equation is not valid anymore. By analogy with the spreading of a liquid drop, the Young force is balanced by the viscous force [15]: $\theta^{-1}R_v d\theta/dt = \sigma(\theta_e^2 - \theta^2)/3\ell\eta$, where $\ell$ is a logarithmic factor of order 10 and $\eta$ is the viscosity of water. For $\theta$ approaching $\theta_e$, we obtain

$$\theta = \theta_e\left[1 - \exp\left(-\frac{\sigma\theta_e^2}{3\ell\eta R_v}t\right)\right]. \qquad (2)$$

As seen in Fig. 3, the adhesion dynamics of saturated vesicles is well fitted by (2). The rise time $3\ell\eta R_v/\theta_e^2\sigma$ is expected to be of order 3 s for $R_v = 10\ \mu$m, $\sigma = 2.5\ \mu$N/m, and $\theta_e = 0.6$, as indeed observed. Finally, we may notice that $\theta$ levels off at 0.5–0.7 in the very late stages of spreading. This saturation has been explained in [14] as the result of a surface tension increase associated with the relative increase in area, which blocks the growth around $\theta_c = (2\kappa_B T/\pi\kappa)1/4 \simeq 0.5$.

On casein-biotin substrates, the kinetics of adhesion is significantly slower. We revise the previous basic assumptions by taking into account that the accessibility of the biotin molecules is reduced. Since the kinetics is not sensitive to $\Gamma_0$ over 2 orders of magnitude, we may introduce a binding efficiency factor, $0 < \alpha(\Gamma_0) < 1$, which takes into account that crowding of streptavidin molecules on the vesicle surface may hinder molecular binding. For instance, our data suggest that, at $\Gamma_{\rm sat}$, a fraction of streptavidin molecules lower than $10^{-2}$ found a biotinylated partner, i.e., $\alpha(\Gamma_{\rm sat}) < 0.01$. The second hypothesis is that binding rate between grafted biotin and streptavidin is no longer much faster than diffusion times but occurs with a characteristic time, $\tau_r$. Note that $\tau_r$ is not *per se* a molecular on rate, as classically defined in bulk solution, but mainly accounts for orientational and steric constraints due to 2D geometry. Hence, by assuming a first-order rate equation, the effective density of bound stickers is given by $\Gamma_b^{\rm eff} = \alpha\Gamma_0 t_c/\tau_r$, where $t_c$ is the time scale at which binding occurs. We might be tempted to identify $t_c$ with the time interval during which the stickers have been exposed to the support. However, because of steric hindrance and reduced degrees of freedom, unbound streptavidin molecules [fraction $1 - \alpha(\Gamma_0)$] that are tightly intercalated between the substrate and the membrane in contact are unlikely to find a biotin group in the time course of the experiment. We then assume that the fraction of bound stickers is mostly built up when the fluctuating decorated membrane comes in contact with the surface at the edge of the patch and that all free receptors inside the patch will remain in a frozen unbound configuration. As sketched in Fig. 4 (inset), the corresponding capture length $L_c = t_c(dR/dt)$ (with $dR/dt$ the velocity of the adhesion front) is set by the distance between the substrate-membrane contact line and an elevation of molecular size, $a$. Taking an approximated parabolic shape for the membrane, $z = x^2\theta/2\lambda$, we find $L_c^2 = 2\lambda a/\theta$. Defining $k = (2\lambda a)^{1/2}$, the density of bound stickers is then given by

$$\Gamma_b^{\mathrm{eff}} = \alpha\Gamma_0 \frac{k}{R_v \tau_r \theta^{1/2}(d\theta/dt)}. \quad (3)$$

The balance between the Young force (including a correction due to small nonspecific attraction $\sigma\theta^2/2$ in order to account for the initial steps of spreading) and the chemical energy due to specific adhesion, $W_s = \Gamma_b^{\mathrm{eff}} U$ (with $U$ the binding energy per receptor-ligand pair), gives $\sigma(\theta^2 - \theta_0^2)/2 = \Gamma_b^{\mathrm{eff}} U$. From (3) we obtain

$$(\theta^2 - \theta_0^2)\theta^{1/2}(d\theta/dt) = 1/t^* \quad (4)$$

with $t^* = \tau_r R_v/(k\varepsilon_u)$ and $\varepsilon_u = 2\alpha\Gamma_0 U/\sigma$. At short times, the growth law scales as $\theta \sim (t/t^*)^{1/2}$. At times exceeding the crossover time given by $t^*\theta_0^{7/2}$, (4) yields a power law:

$$\theta \approx R/R_v \cong (t/t^*)^{2/7}. \quad (5)$$

The curves in Fig. 4 are well described by a power law $(t/t^*)^n$ with $n = 0.27 \pm 0.04$, which is close to $2/7$ and $t^* \approx 5 \times 10^4$ s. This sets the crossover time around $65 \pm 20$ s (with $\theta_0 \approx 0.10$). Remember that we had to disregard the initial times up to $\sim$100 s. For that reason, only the second regime could be reliably adjusted. Finally, with $a \approx 5$ Å, $\lambda \approx 200$ nm, $\alpha\Gamma_0 = 3 \times 10^{14}$ m$^{-2}$, and $U \approx 30 k_B T$ for streptavidin-biotin, we finally estimate the molecular binding time $\tau_r = 1700 \pm 500$ s. In order to test further the origin of this surprisingly long binding time, we performed another experiment. Instead of linking a short NHS-biotin derivative to casein, we tagged the adsorbed layer of casein with a PEG$^{3400}$-biotin. Doing so, the adhesion kinetics observed for the grafted PEG-biotin surfaces was recovered (data not shown). Consequently, our measurements unambiguously demonstrate that the 2D binding rate between receptors and ligands tethered to surfaces is not an intrinsic feature of receptor-ligand pairs but is strongly governed by the accessibility of linkers. This finding is thus in disagreement with Bell's proposal [16], according to which bulk forward rates could be converted into 2D binding times, $\tau$, via a molecular length, $d_r$, and an appropriate geometric factor, $\gamma \sim$ 1–100: $\tau = d_r/(\gamma k_{\mathrm{on}}^{3\mathrm{D}}\Gamma_0)$. Taking $d_r \approx$ 1–10 nm, $k_{\mathrm{on}}^{3\mathrm{D}} = 10^8$ M$^{-1}$ s$^{-1} \approx 10^{-19}$ m$^3$ s$^1$ [2], and $\Gamma_0 \approx 10^{16}$ m$^{-2}$, one obtains $\tau = 1$ ms for streptavidin-biotin, i.e., 6 orders of magnitude smaller than our measured value for $\tau_r$.

To our knowledge, there is only one set of careful experimental data similar in spirit to ours. Boulbitch *et al.* [17] monitored the spreading of vesicles functionalized with RGD peptides on substrates decorated with integrins by physical adsorption. The authors found two growth laws $R \sim t$ and $R \sim t^{1/2}$ depending on the density of arginine-glycine-aspartate sequence (RGD) ligands, which is far from what we observed. However, their vesicles were very floppy and their analysis was based on the knowledge of the binding time for integrin-RGD (that they derived from 3D data). From our viewpoint, this assumption is questionable since tethering ligands to surfaces may slow down the apparent binding rate by more than a factor $\gamma < 100$.

To conclude, we have studied the adhesion dynamics of streptavidin-decorated vesicles onto biotin substrates and shown that the kinetics was strongly dependent on the chemical preparation of the bioactive substrate, and more precisely on the accessibility of the surface ligands. From a biotechnological point of view, this finding highlights the difficulty of designing real biomimetic substrates, since minor changes in the surface modification can drastically alter the dynamics of cell adhesion. From a biological point of view, although vesicles are devoid of cytoskeleton, which is an important ingredient in cell mechanics, our study suggests that passive diffusion of adhesion molecules at the cell surface may play an important role, beside any active cytoskeletal rearrangement or intracellular signals.

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