Less is more: removing membrane attachments 

stiffens the RBC cytoskeleton

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Abstract. The polymerized network of the cytoskeleton of the red-blood cell (RBC) contains different protein components that maintain its overall integrity and attachment to the lipid bilayer. One of these key components is the band 3–ankyrin complex that attaches the spectrin filaments to the fluid bilayer. Defects in this particular component result in the shape transformation called spherocytosis, through the shedding of membrane nano-vesicles. We show here that this transition and membrane shedding can be explained through the increased stiffness of the network when the band 3–ankyrin complexes are removed. ATP-induced transient dissociations lead to network softening, which offsets the stiffening to some extent, and causes increased fragility of these mutant cells, as is observed.

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1. Introduction

The two-dimensional (2D) network of proteins that composes the cytoskeleton of the red-blood cell (RBC), is attached to the fluid bilayer to form a composite membrane [1, 2]. The elastic properties of this composite membrane have been studied extensively: the spectrin filaments that form the links of the network, behave as entropic springs and give the cytoskeleton an elastic shear modulus [3]: \( \mu \simeq k_B T / r_g^2 \sim 2 \times 10^{-6} \text{J m}^{-2} \), where \( r_g \sim 45 \text{ nm} \) is the radius of gyration of the spectrin filament in water. These filaments are attached at their ends to the bilayer through a complex of proteins that include band-4 and actin (figure 1(a)). These complexes serve as the nodes of the network, and are each linked by 4–7 spectrin filaments to the neighboring nodes. The network has on average a hexagonal symmetry (with many defects) [1], and the end-to-end distance is \( r \sim 80–100 \text{ nm} \). The attachment of the spectrin at its ends goes through a transient dissociation process, driven by ATP hydrolysis and phosphorylation [4], and maintains the cytoskeleton’s ability to modify and adapt in response to externally imposed deformations [5, 6].

In addition to the attachment of the spectrin filaments to the membrane at their end, there are protein complexes that link an individual filament to the bilayer at a random point along its length, on average at its midpoint (figure 1(a)) [7]. These complexes contain band-3 and ankyrin proteins, and may also go through a dynamic process of dissociation and reassociation [8].

In a previous work [4], we showed that the ATP-induced dissociations at the network nodes lower the overall number of connected filaments in the network, and decrease the stiffness \( \mu \). In a normal RBC, with ample ATP, the network is therefore softer than in ATP-depleted cells. Furthermore, depleting the ATP and increasing the stiffness also increases the tension that the cytoskeleton is applying to the lipid bilayer, and can drive the cell into the echinocyte (spiny and shrunken) shape [4]. We later showed [9] that increased cytoskeleton tension, upon ATP-depletion or cell aging or \( \text{Ca}^{2+} \) loading, can drive the shedding of membrane vesicles that bud from the membrane between the nodes of the network and carry away the excess bilayer area. These vesicles therefore do not contain any spectrin filaments, and have a typical diameter of \( \sim 100 \text{ nm} \). When enough excess membrane has been shed the cell is left with a spherical shape called a spherocyte.

2. Mid-point attachments soften the network

Here, we wish to describe the role of the mid-point attachments of the spectrin filaments to the bilayer, through the band-3 and ankyrin complex (figure 1(a)). When this complex is missing, as occurs in different anemic mutations, the RBC exhibit spherocytosis [7, 10, 11], i.e. the cells become spherical and smaller through the shedding of membrane vesicles [12]. When the band-3 and ankyrin complexes are removed from a normal RBC using a special drug, the cells are observed to form membrane blebs (which are cytoskeleton-free membrane protrusions), lose membrane and shrink [8]. These cells also become stiffer and much less deformable [8]. Note that removal of these attachment mid-points does not alter the overall connectivity of the network, which is determined by the attachments at the filaments’ ends. The experimental data therefore indicate that removing the mid-point attachments increases the overall stiffness of the cytoskeleton. Such an increase in the cytoskeleton stiffness means a lower deformability and drives the shape-changes and vesicle shedding, through mechanisms we proposed before [4, 9]. What drives this paradoxical stiffening behavior upon removal of attachments?
Figure 1. Schematic side view of the spectrin filaments in the RBC cytoskeleton. (a) In the normal RBC the spectrin filament (solid wavy line) is attached at its ends to the fluid bilayer (thick black line) through band-4 and actin complexes (black ellipses), and at random mid-points (band-3 and ankyrin complexes, red ellipses). The ATP-induced transient dissociation of the left end of the filament is indicated. The spring end-to-end distance is $r/2$, lowering the entropic restoring force, and decreasing the time for the ends to reassociate. In mutants (b) the mid-point attachments are missing and the filament has larger entropy and stronger entropic restoring force, and longer reassociation time at the ends.

We can try and understand this behavior from the basic nature of the spectrin filaments as entropic springs. Imagine a flexible polymer which is free in bulk solution. In the Gaussian approximation, the probability distribution function for the end-to-end distance $r$ of such a polymer is given by

$$P(r) = \left(\frac{2\pi \sigma^2}{3}\right)^{3/2} e^{-r^2/(2\sigma^2)},$$

where $\sigma^2 = Nb^2/3$, $N$ is the number of monomers in the polymer, $b$ is the monomer length (which for the spectrin is the repeat unit of $\sim 5$ nm) and $L = Nb \sim 200$ nm is the length of fully stretched spectrin filament. The average mean-square end-to-end distance is: $\langle r^2 \rangle = N\sigma^2$. The end-to-end distance behaves like the position of a particle inside a harmonic potential at finite temperature: $P(r) \propto \exp(-k_r r^2/k_BT)$. If we now hold on to the two ends of such a polymer at some fixed separation, we’ll feel some tension due to the fact that by fixing the two ends we have decreased the entropy of the filaments (constrained its possible conformations). If this distance is of order $r_g$, then this resting tension is negligible. If we now stretch the filament by increasing the separation between the two end points, we will feel a restoring force of the form of a simple (Hookean) spring with spring constant: $k_s = 3k_BT/Nb^2$. The restoring force is entropic in nature, and therefore proportional to the temperature. At separations that are large compared to $r_g$ (but still smaller than $L$) the restoring force increases faster and there is a well-known strain-stiffening response.
Now what happens if such a polymer has in addition to its two attached ends a further fixed attachment at its mid-point? We now have two springs connected in series, with the end-to-end distance and the number of monomers in each of them halved compared to the original filament: \( r \rightarrow r/2 \) and \( N \rightarrow N/2 \Rightarrow \sigma^2 \rightarrow \sigma^2/2 \). The distribution of the separation between the ends of the original whole filament is now proportional to (equation (1)): \( P(r) \propto \exp(-r^2/4\sigma^2) \). Comparing to the harmonic oscillator distribution we now have a reduced effective spring constant for the new system: \( k_s \rightarrow k_s/2 \). The physics behind this effect is clear: adding another attachment adds a geometric constraint on the possible filament configurations, reducing the entropy of the filament and therefore reducing its entropic restoring force (figure 1). Two entropic springs in series, with a total extension \( r \), which is now divided between them, give a weaker restoring force than the original full-length spring. This result follows from the scaling relation \( r_g \propto N^v \), and is valid as long as \( v < 1 \) (\( v = 3/5 \) for chains with excluded volume).

Above, we described the spectrin as a flexible polymer that is very close to its free conformation in the bulk. In the context of the cytoskeleton network of the RBC, the spectrin has its ends stretched compared to the bulk mean end-to-end separation (figure 1): the mean separation of the ends in the network is: \( \langle r \rangle \sim 80 \text{ nm} \), while the mean diameter of gyration in the bulk is (in the Guassian approximation): \( r_g = \sqrt{2Lb} \sim 45 \text{ nm} \). The connected network of the RBC cytoskeleton is therefore under tension, which is partially relieved by the ATP-induced transient dissociations [4]. This tension that the cytoskeleton applies to the membrane determines the overall cell shape and the rate of vesicle shedding, according to our models [4, 9]. This tension can be estimated as: \( F \sim (k_B T/r_g)(r/r_g - 1) \), in the linear approximation. When the spectrin has a mid-point attachment, this tension is lowered, since now we have: \( r \rightarrow r/2 \), while \( r_g \rightarrow r_g/\sqrt{2} \). If we use the values for \( r \) and \( r_g \) given above, the reduction in the tension can be estimated to be of order 2. From this relation we can estimate the critical number of attachment points \( N_c = (r/r_g)^{1/v} \), where the tension in the cytoskeleton would vanish.

We expect that for a given number of attachment points that is smaller than \( N \), the filament has the shape shown in figure 2(a), which is a combination of 3D and 2D conformations. Constraining the filaments to \( N_b \) independent blobs [13], is equivalent to stretching it, such that each blob has size: \( \xi \approx k_B T/F' \) (figure 2(a)). The number of blobs is given by \( N_b = r/\xi \), so that the overall equivalent stretching force due to the attachments is: \( F' \approx (k_B T/r)N_b \), increasing with the number of blobs. Adding mid-point attachments is therefore equivalent to an additional spontaneous stretching force, which balances the compressive force \( F \) that acts to shrink the filament to the bulk size of \( r_g \) (see previous paragraph). Again we conclude that adding mid-point attachments results in softening of the entropic-springs. The treatment given above is very similar to the study of the effects of sliding-links on the force–extension response of polymers [14]. As in our treatment, they find that the additional geometric constraints soften the elastic response of the polymer.

So far we have assumed that both the ends and mid-point attachments are fixed, while the mid-point attachment could be mobile inside the plane of the fluid membrane, and diffuse around (figure 2). This means that such an attachment point does not reduce the entropy of the filament as much as we have assumed above. One way to estimate the effect of such mobile attachment points of a bulk filament to a flat fluid membrane is by looking at the following limiting cases: no mid-point attachments (treated above) and \( N \) attachment points such that the entire filament is now in the plane of the membrane. In the second case, we transformed the
Figure 2. Schematic picture of the spectrin filament in the RBC network. (a) A filament attached to a fluid bilayer has 3D blobs (black dashed circles) along a 2D chain (red dashed line). (b) When there are no band-3/ankyrin attachments the filament can have any conformation in 3D, indicated by the dashed line. (c) In the limit of many mobile band-3/ankyrin attachments (red circles), the filament can only have 2D conformations inside the plane of the membrane (arrow and dashed line).

original 3D filament (figure 2(b)) to an effectively 2D filament (figure 2(c)). Using the Gaussian approximation of equation (1) in 2D, we find that the effective spring constant is softer compared to 3D by a factor of: \( k_{s,2D} = (2/3)k_{s,3D} \). This is the maximal softening that we can get, and is a weaker effect than for a fixed attachment point, as can be expected, since the conformational entropy inside the plane is maintained.

In the normal RBC, with a given proportion of mid-point attachments, the cytoskeleton is therefore overall softer than in mutants that are missing these attachments. Our simple analysis shows that this effect can increase the overall stiffness \( \mu \) by a factor of up to 1.5–2, comparable to the observed reduction in cell deformability [8] by factors of \(~2\). This is a large
effect, comparable to the stiffening effect of ATP-depletion, and can therefore drive both the echinocyte-like shape changes [4] and the membrane shedding [9].

3. Dynamical effects of mid-point attachments

Let us now describe the changes to the filament’s dynamics due to the mid-point attachments. We have a system that mixes 3D and 2D behavior; the conformations of the filament between anchoring points are in 3D blobs of size \( \xi \) (bounded by the membrane), while the motion of the attachment points is confined to the two dimensional plane of the membrane (neglecting the bilayer out-of-plane fluctuations), figure 2(a). There are huge differences in the viscosity of the bulk fluid \( \eta_b \) and that of the membrane \( \eta_m \), which means that there are large differences between the typical conformation times within the three-dimensional blobs \( \tau_{3D} \) (figure 2(a)) and of the conformation times of the larger-scale two-dimensional chain within the membrane \( \tau_{2D} \) (figure 2(a)). These timescales can be estimated as [15]

\[
\tau_{3D} \approx \frac{\eta_b}{k_B T} \approx \frac{r^3}{N_b k_B T} \quad \tau_{2D} \approx \frac{\eta_m}{k_B T} \quad \Rightarrow \quad \tau_{2D}/\tau_{3D} \approx \frac{\eta_m N_b^3}{\eta_b} \gg 1.
\]

For timescales of external perturbations that are short compared to \( \tau_{2D} \), the filament behaves as if the mid-point attachments are fixed and each blob is an independent entropic spring. For timescales that are long compared to \( \tau_{2D} \) the filament has an additional entropic restoring force (stiffness) related to the conformations inside the plane of the membrane. For a spectrin filament inside the RBC we can estimate these timescales to be: \( \tau_{3D} \sim (1/N_b^3) \text{msec} \) using \( \eta_b \sim 6\eta_{\text{water}} \), which means that \( \tau_{2D} \) can be of order seconds, since \( \eta_m \sim 10^2-10^3\eta_b \). Note that the longitudinal fluctuations of the end of a semi-flexible filament may follow an exponent that is smaller than the value of 3 given in equation (2), such as 16/7 [16].

There is another effect of the mid-point attachment, which plays an opposite role to the entropic stiffening we described above, and is unique to an active network, such as the cytoskeleton of the RBC: as ATP induces transient dissociations of the spectrin end attachments, the overall stiffness is proportional on average to the fraction of time that each spectrin link (a spectrin filament that links two nodes of the cytoskeleton network) is associated with the network [4, 5]. Since the reassociation time increases for longer filaments, we expect that removal of the midpoint attachment will increase \( \xi \) and the random ‘search’ time of the dissociated end \( \tau_{3D} \) (equation (2)). This will result in an increase in the proportion of dissociated filaments and lead to a decrease in the overall stiffness, according to

\[
\mu = \mu_0 \left(1 + \frac{\tau_{\text{reass}}}{\tau_{\text{dis}}}\right)^{-2},
\]

where \( \tau_{\text{dis}} \) is the average time between dissociation event, and \( \tau_{\text{reass}} \) is the average time for a dissociated filament to have its end reattached. We do not expect the mid-point attachment to change the chemical reaction rates at the spectrin ends, so \( \tau_{\text{dis}} \) is a constant, while \( \tau_{\text{reass}} \) should depend on the number of attachment points.

If the reassociation occurs due to the thermal motion inside the three-dimensional blob (figure 2(a)), then we have \( \tau_{\text{reass}} \sim \tau_{3D} \propto N_b^{-3} \) (equation 2), i.e. removing the mid-point attachments increases the reassociation time, and leads to softening of the network. If on the other hand the reassociation is limited by the thermal motion of the attachment points inside the
plane of the membrane, i.e. by $\tau_{2D}$, then we expect it not to depend much on $N_b$, and to be very large (of order of seconds).

Since we do not know the microscopic parameters of the ATP-induced dissociations we resort to experiment in order to determine which of the two effects described above is dominant. The evidence from the mutant RBC [7, 8, 10, 12] indicates that the entropic stiffening effect is dominant in determining the overall deformability of these cells. Nevertheless, under dynamic conditions of shear-flow, the increase in reassociation time when the mid-point attachments are lost, can lead to faster failure and ‘tearing’ of the dynamic cytoskeleton, as was recently shown to happen in computer simulations [6]. This can explain the larger fragility and lower fragmentation times found for RBC mutants with band-3/ankyrin attachments missing, in shear-flow experiments [8].

Note that the softening effect of the removal of the mid-point attachments is induced entirely by the activity of the ATP, and is therefore unique to an active network (similar to the ‘active strain softening’ described in [5]). In general, any source of transient dissociations will give the same behavior, including thermally-induced dissociations, but in the particular case of the RBC cytoskeleton the attachments have an adsorption energy of $\sim 7k_B T$, which makes thermal dissociations negligible [4].

4. Conclusion

To conclude, we showed here that the physics of a network of entropic springs leads to curious effects, even more so when this network is being dynamically remodeled as an active process. These effects should be important whenever such networks form, inside cells or in synthetic structures. Such networks appear in many cellular membranes and organelles [5]. We further show that these mechanical changes in the cytoskeleton can provide a possible explanation of the observed phenomena in some types of mutant RBC (spherocytosis). Future theoretical studies of these effects need to include the intricate coupling between the spectrin filaments and the flexible and fluctuating membrane [17].

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