# Relaxation of ultralarge VWF bundles in a microfluidic–AFM hybrid reactor

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The formation of clots in our vascular system under high-shear flow conditions is strongly dependent on the correct functioning of a protein, the so-called von Willebrand Factor (VWF). This protein self-assembles into multimers (biopolymers) which, when stretched, can reach sizes as long as 100 μm. The repeat unit of this fibres is a dimer comprising two VWF proteins held together by a disulphide bond, each composed of 2050 amino acids [1]. The shape of the repeat unit in the bulk has been recently found to be ellipsoidal with a major axis of ~70 nm and a minor axis of ~10 nm [2]. Although much is known about its structure, the functional behaviour of VWF in the blood stream is rather counter-intuitive: it becomes active at high-shear rates, and thus, common mechanisms of adhesion fail to explain its increased adhesion potency. Following current ideas that VWF multimers are presumably in a compact (globular) form due to self-association, hydrogen bonding and hydrophobic interactions, we have recently shown both experimentally [3] and theoretically [4] that high-shear stresses can trigger a globule-stretch transition in VWF fibres. This transition in turn leads to an enhanced adhesion potential of the molecules since more binding sites become exposed. Furthermore, when VWF is immobilized on a surface and exposed to an elevated shear flow, self-association of multiple VWF fibres into ULVWF networks can take place [5,6]. This association eventually

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causes the formation of large protein networks, being haemostatically extremely active and are believed to finally lead to the seal of vessel wall lesions.

Recently, we started to investigate the shear stress triggered formation of ULVWF networks and found that solely mechanical forces are able to trigger the formation of haemostatically active networks. In the same study, we were able to demonstrate that acidic pH (6.6) drastically decreases the critical shear $\dot{\gamma}_{\mathrm{crit}}^{\mathrm{ULVWF}}$ for ULVWF-network formation. However, even though the connection between physiological function and mechanical stress has been realized for some time, very little is known on the actual response of ULVWF bundles to mechanical stress (relaxation).

In order to provide a first insight into the relaxation of ULVWF bundles we combined a planar microfluidic reactor, mimicking the hydrodynamic conditions in the microcirculatory system of our body [10] with an atomic force microscope (AFM) for stretching and length determination experiments. The completely planar reactor layout and fluid actuation technology gives us the technological freedom to create a continuous flow in any desired geometry. No drain and hence no supply is necessary, allowing to monitor the identical protein solution without any loss of material for long periods of time ($\sim$1 h). Using this hybrid setup we were able to study the formation of ULVWF networks due to hydrodynamic stress and investigate the relaxation of bundles of different lengths pulled from VWF networks by an AFM cantilever tip.

Theoretical analysis of our experiments indicates that the relaxation of these long bundles proceeds through a multitude of energy minima, similar to the case of protein folding [8,9]. In particular, the relaxation process can be well described by an extended exponential response with two characteristic time scales. The longest relaxation time is dominated by internal reconfiguration events and does not show a clear dependence on the length of the originally stretched bundle. The fastest relaxation time, on the other hand, does in fact depend on the length and on the viscosity of the solvent. Our findings clearly exhibit the relaxation of these bundles to be completely different from the one of single polymers with attractive interactions, displaying exponential or supra-exponential relaxation kinetics [10]. Our results thus demonstrate that the relaxation of VWF bundles can be viewed as a mixture between (entropically driven) polymer relaxation and simple folding or complexation of two or more fibres. Moreover, VWF bundles provide a simple yet complex system where theories on polymer dynamics and protein folding might be tested.

## Materials and methods

*SAW—microfluidic and surface chemistry.* In Fig. 1, we sketch our newly developed hybrid system, consisting of a SAW driven microfluidic reactor combined with an AFM for minute protein manipulation. The flow pattern and therefore the shear forces in the reactor is controlled by SAW technology, which has been developed and used in the past [11–13]. In brief, a SAW of roughly 1 nm in amplitude (nanopump) is excited on a $LiNbO_3$ substrate. The interaction of the SAW with the liquid on the chip surface causes a considerable amount of energy to be diffracted which induces an internal streaming referred to as acoustic streaming. By means of optical lithography a broad variety of different hydrophobic/hydrophilic channel structures and with it also a broad variety of different flow patterns can be processed onto the reactor substrate.

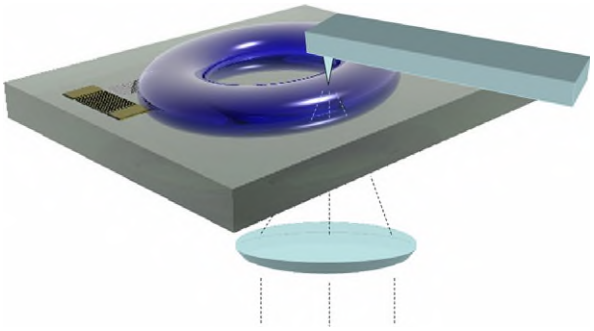

Fig. 1. The microfluidic set up. The unique combination of a microscope, an AFM tip and a SAW driven pumping system enables both studying the formation of macromolecular VWF networks and conglomerates in life time and the mechanical manipulation of VWF aggregates and bundle pulling in particular.

*AFM with SAW.* This SAW-chip is combined with an AFM setup (JPK, Berlin, Germany). In the work presented here, the AFM is solely used as an anchor to manoeuvre the bundle and not for quantifying rupture forces. Therefore, in the course of an experiment the AFM cantilever tip is approached to a protein network which was created by shear flow induced VWF conglomeration beforehand. The attached VWF bundles are gently pulled by the AFM tip until they finally rupture. The planar, optical transparent setup is completed by an inverted microscope for recording the microfluidic channel, the AFM tip and the bundle relaxation simultaneously. Acquired data of the time course of the relaxation process were analysed using standard imaging software.

## Results

### *Ultra large VWF assemblies*

Recently, we were able to demonstrate the controlled formation of ULVWF networks using a microfluidic reactor. Following these studies, a VWF-buffer solution (concentration $\sim 0.2$ mg/ml) was placed onto the acoustic path of our microfluidic device and exposed to shear stress for a few minutes. Under high-shear flow conditions the formation of tight VWF networks and conglomerates from single VWF polymers could be observed. From these networks ULVWF bundles will be picked to study their relaxation spectrum.

### *Relaxation of ULVWF*

One of the key features of the dynamics of (bio-)polymer systems is its relaxation spectrum, since it contains the relevant time scales for the polymer to reach its equilibrium configuration from an imposed non-equilibrium state [14]. By using the appropriate model, one can in principle extract the relaxation time $\tau$, and relate it to the internal chain dynamics being controlled through the monomer–monomer interactions. Therefore, the investigating of the

relaxation dynamics as a function of the different variables represents an important step towards a deeper understanding of the mechanical properties of ULVWF bundles. Moreover it provides the basis to test theoretical models describing the relaxation of these assemblies. Here, our setup has the advantage that it provides simultaneous mechanical manipulation and optical detection. In Fig. 2A, we present an ULVWF being immobilized onto the tip of our AFM and exposed to mechanical stress. The protein binding to the AFM tip is achieved due to a thin collagen coating of the cantilever. To have a strong grip on the bundle, the tip was allowed to equilibrate for several minutes just in contact with the protein assembly. A thick multi-fibre bundle was then formed by gently retracting the AFM tip, whereby only bundles, which appeared similar in thickness, were analysed. The relaxation of the bundle, governed by strong attractive interactions and the entropic penalties associated with the stretched conformation was followed after it ruptured from the ULWVF assembly (Fig. 2B). From the sequence shown in the figure, it is important to note that the filament remains fairly straight during the contraction process. The stretching and retraction proceed by an inhomogeneous coarsening of the bundle in the apparent form of pearls.

On a more quantitative basis, we monitored the dynamics of the system by measuring the end-to-end length of the bundle as a function of time. The maximum length $L_0$ was determined by the distance between the AFM tip and the point where rupture occurred. A typical trace of the end-to-end distance (i.e. the distance from the AFM tip to the free end of the ULVWF) as a function of time is presented

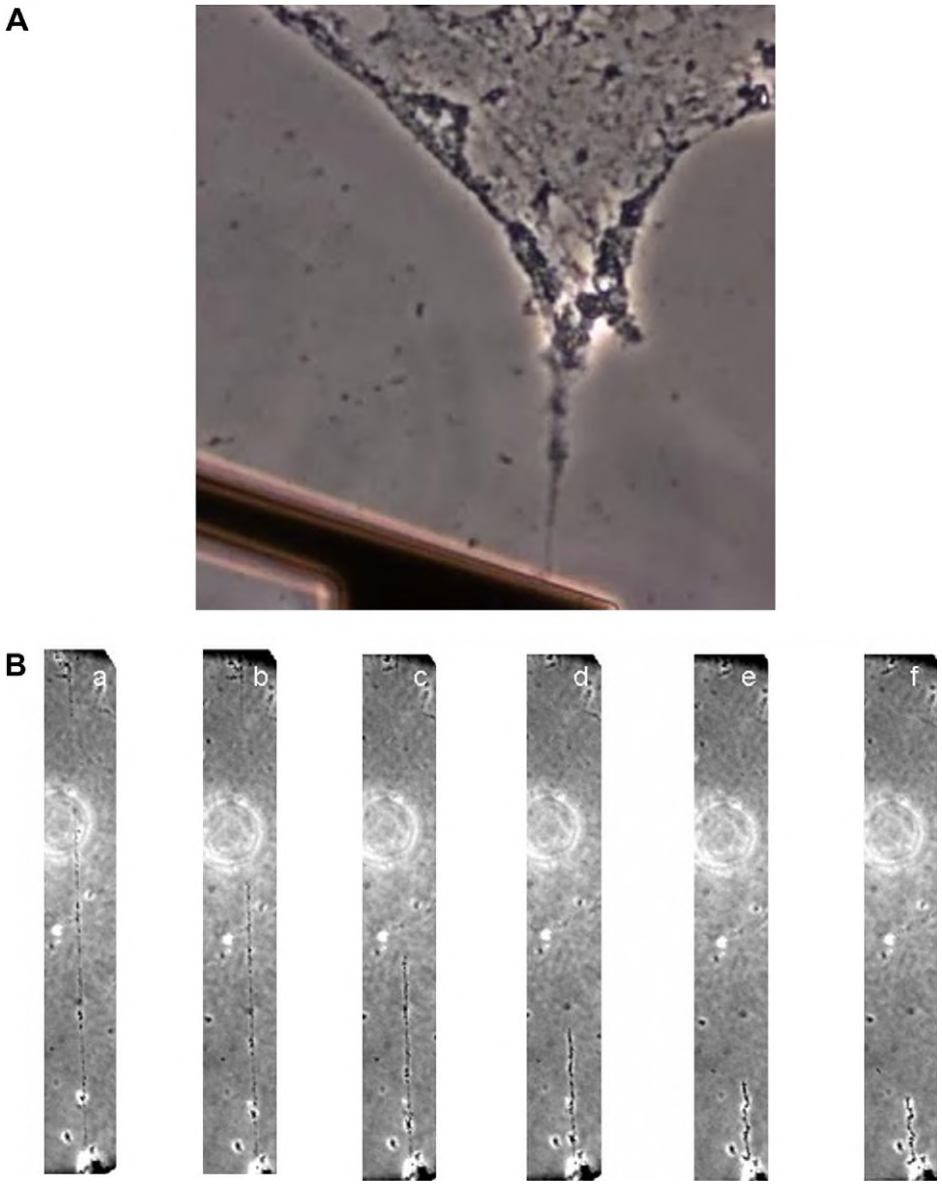


Fig. 2. (A) ULVWF immobilized on AFM tip. (B) Relaxation after rupture (each image of the sequence is approximately 840 μm in height and 120 μm in width).

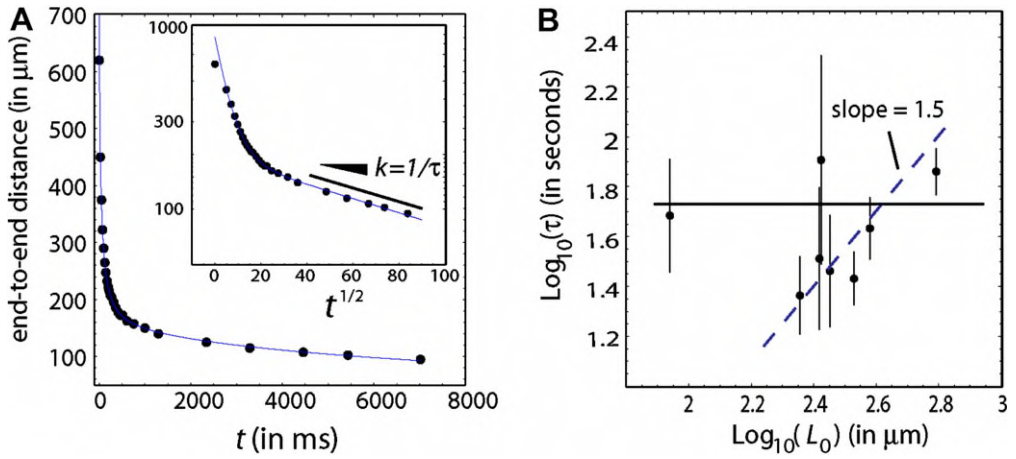


Fig. 3. (A) End-to-end distance as a function of time. The data correspond to that of Fig. 4B. (Inset) The same data plotted in a log-linear scale, and the $x$ axis is taken to be $\sqrt{t}$. The continuous line is a fit to the data using: $L_1 \exp(-t/\tau_1)^{0.5} + L_2 \exp(-t/\tau_2)^{0.5}$, where $L_1 = 676$ μm, $L_2$, $= 194$ μm, $\tau_1 = 156$ ms, $\tau_2 = 67465$ ms. In principle one would need to consider also the final length in the previous expression, but one can estimate that this number is rather small (<20 μm), and thus it will not affect the parameters nor the behaviour detailed here. (B) Longest relaxation time $\tau = 1/k$ as a function of the initial length of the bundle $L_0$ for all of the runs performed. The viscosity in all these experiments was held fixed at 1 cP. The dashed line has a slope = 1.5, and corresponds to the slope one would obtain for the longest relaxation time of single polymers, i.e. it corresponds to Zimm scaling.

in Fig. 3A. We note that the relaxation has two characteristic time scales (see inset), and both of these periods are well described by stretched exponentials of the form: $L \propto e^{(t/\tau)^{\beta}}$ with $\beta \sim 0.5$. This is corroborated by observing the nice fit to the data over the full range using a sum of two extended exponentials denoted by the continuous curve (for details of the fitting parameters see caption). The fact that the relaxation process follows a stretched exponential implies that the underlying dynamics is governed by hopping events between random minima in a rough energy landscape. Equivalently, the relaxation spectrum is composed of a broad (random) distribution of exponential processes [15]. Such relaxation behaviour is very common in nature, and has been found in different contexts. In particular, it has been observed in the electric birefringence relaxation of ensembles of synthetic polyelectrolytes [16], in the relaxation of DNA [17], and in proteins [18], to name a few. In these studies, the origin of the relaxation spectrum arises from the averaging over a multitude of parallel non-interacting (presumably exponential) relaxation processes. Theoretically, stretched exponential and power-law behaviour have been extensively studied in the area of critical dynamics and glasses [19]. In the context of single polymer relaxation, Cherayil have shown [20] that after inclusion of memory effects to the dynamics of the system, the relaxation is a stretched exponential with a exponent of $\beta \sim 0.5$. These memory effects included in this work accounted for the fact that a given conformation at earlier times affects the distribution of conformations at a later time along the relaxation process.

The system we are considering here presents new features compared to those described previously: (i) it has two clear relaxation time scales and (ii) the relaxation of the individual molecules is coupled to the one of all others in a non-trivial fashion. In this sense, we can say that VWF bundle relaxation is a more complex problem than those studied before. One of the striking features of our findings is that the longest relaxation rate $k$, corresponding to the inverse of the longest relaxation time $\tau$, seems not to be dependent of the initial length of the bundle, as can be seen in Fig. 3B. This implies that the dynamics of relaxation is dominated by conformational constraints, as well as effective internal friction limiting the rate of deformation of the object itself. For reference, we included the Zimm scaling of the longest relaxation time of the end-to-end distance for a single polymer (dashed curve) [21].

To further unravel the physical origin of the observed phenomenon we studied the effect of viscosity of the solvent on the long and short timescales of the bundle relaxation by adding a thickening agent, such as glycerol. In Fig. 4 we show two different traces of the end-to-end distance at different viscosities: 1 cP and 40 cP. As can be seen, the initial temporal decay is slowed down such that the full relaxation can be well described by a single stretched exponential. Interestingly, the slowest relaxation rate is now faster than the one for pure buffer solution (with lower viscosity). Further discussion on the origin of the double-time relaxation spectrum, and on the apparent enhancement of the long-time relaxation rate is given below.

## Discussion

VWF multimerization and self-association via the A1 domain, but not at its A3 domain [22] has been discussed previously. However, so far the molecular origin (binding sites and interaction forces) for the association process remains unclear. Recently we presented a model explaining the hydrodynamic stretching of single VWF fibres under flow. Based on our results, we found a critical shear rate $\dot{\gamma}_{crit} \sim 5000\ s^{-1}$ under normal puffer conditions (pH 7.4).

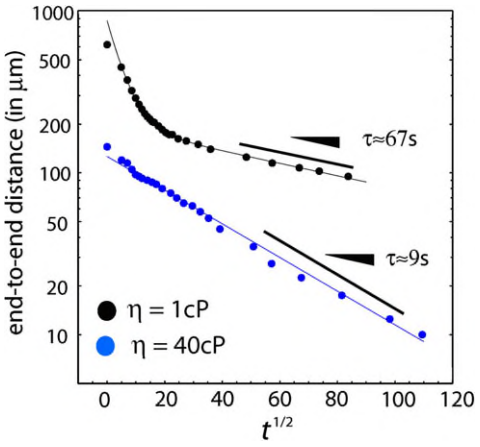


Fig. 4. End-to-end distance as a function of $\sqrt{t}$ plotted in a log-linear scale for different viscosities. The upper (black) curve corresponds to a viscosity of 1 cP, and the lower (blue) curve corresponds to a viscosity of 40 cP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our theoretical findings enabled us to prove that the origin of VWFs stretching behaviour can be traced back to attractive interactions among VWF monomers that maintain the multimer in a compact globular conformation when the shear rate is below its critical value. Moreover, we were also able to predict that the size of the repeating unit must be unusually large (~80 nm) for it to be haemostatically active at physiological shear rates. This is also confirmed by the findings of Singh et al. [2]. Our model is further able to explain the effect of hydrodynamic stress and pH changes on the formation of ULVWF.

*Relaxation of ULVWF bundles*

To gain a deeper insight into the mechanical properties and the relaxation behaviour of the ULVWF bundles, we employed our microfluidic/AFM hybrid setup. As shown in Fig. 3, the relaxation of the bundles does not follow a simply exponential law, but rather is well described by stretched exponentials with an exponent $\beta = 0.5$ throughout all our relaxation experiments. In the case where the viscosity of the solvent is low (essentially that of water), we find two characteristic decay rates. In a high viscous environment, surprisingly, only a single relaxation time is observed. However, the relaxation spectrum found here is still different from the one of a single collapsing polymer. There, the relaxation of the end-to-end distance is supra-exponential, or in other words, the rate of compactification grows as the polymer relaxes [23]. If at all, we only find evidence of such a relaxation type during the first few 100 ms after rupture. This observation is not too surprising, as the ULVWF consists of many single fibres of different lengths relaxing at the same time in a complex fashion. Comparing it to the dynamics of a single polymer seems to be at least to be doubted. The complexity of our system arises because the relaxation of one fibre affects the relaxation of the other fibres, and vice versa. For a better feeling of why the observed relaxation is a stretched exponential, it is probably better to describe the bundle as a single entity relaxing in a rough potential landscape determined by the configurations of the VWF single fibre constituents. The manifold of configurations is presumably strongly dependent on the number of self-association contacts, and hopping from one state to the other can only be done through thermally activated processes. The average barrier height will be reduced in the highly stretched state because of entropic forces, and thus the rate of relaxation should be faster at the beginning. Nonetheless, this argument would imply that one would see a continuous change in the relaxation of the bundle, and not two clear relaxation times as it is found here. An alternative explanation is that there are two attractive interactions of different origins, which in this case would presumably correspond to self-association and hydrophobic attraction among monomers, driving the relaxation in each of these two regimes. To this date it is still unclear which of these interactions is the dominant one in the collapse of VWF, but we might argue that at long times, the hydrophobic interactions must become important since in principle the majority of the possible self-association contacts within the bundle should be present and only unfavourable surface interactions with the solvent and entropy can drive the further decrease in size. Nevertheless, hydrogen bonding might also play a crucial role and a complete description should include the three different interactions. The entropic contribution, on the other hand, is presumably negligible at the latter stages, since the thickness of the bundle (as measured with optical microscopy) is about ~10 µm, a value that exceeds that of an individual molecule of a VWF (~5 µm, assuming that a single fibre is 100 µm long and obeys random walk statistics). Nevertheless, one should be careful since entropy might still play a role if the polymers remain stretched within the bundle due to strong constraints on its mobility, a fact that can be indirectly assumed by seeing the snapshots of the relaxation where the filament remained rather straight (Fig. 2B).

From another perspective, we note that the fast relaxation at short times can lead to jamming, or highly constrained dynamics (corresponding to deep metastable minima) because it does not have enough time to sample the local energy landscape. If one decelerates the initial relaxation by using a highly viscous solvent, the system will be closer to equilibrium as it relaxes and will not longer get trapped in deep minima. In fact, one might be lead to think that the longest relaxation time should be shorter for this system. This is beautifully illustrated in Fig. 4 where the rate of compactification at long times is larger for the bundle in the higher viscosity medium, while the initial relaxation is strongly slowed down.

In conclusion we have presented the first study on the relaxation of VWF bundles using a newly designed microfluidic/AFM hybrid, and found that their behaviour is well characterized by a stretched exponential relaxation. Moreover, we have found that at low viscosities the system has

two characteristic relaxation times, while for larger viscosities internal friction effects dominate the relaxation, and only a single time scale is necessary. Interestingly, the long time relaxation is faster for the system at higher viscosities, implying that reorganization within the bundle at the initial stages of relaxation leads to faster relaxation at long times. Further experiments with partially labelled fibres, as well as self-association blocking drugs should provide more details about the role of entropy, and that of specific interactions in the relaxation behaviour and will give more insight in the activation-independent platelet aggregation as observed by Ruggeri et al. [24].

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## References

[1] J.E. Sadler, Biochemistry and genetics of von Willebrand factor, Annu. Rev. Biochem. 67 (1998) 395–424.
[2] I. Singh, H. Shankaran, M.E. Beauharnois, Z. Xiao, P. Alexandridis, S. Neelamegham, Solution structure of human von Willebrand factor studied using small angle neutron scattering, J. Biol. Chem. 281 (50) (2006) 38266–38275, Epub. 2006 October 19.
[3] S.W. Schneider, S. Nuschele, A. Wixforth, C. Gorzelanny, A. Alexander-Katz, R.R. Netz, M.F. Schneider, Shear-induced unfolding triggers adhesion of von Willebrand factor fibers, Proc. Natl. Acad. Sci. USA 104 (19) (2007) 7899–7903.
[4] A. Alexander-Katz, M.F. Schneider, S.W. Schneider, A. Wixforth, R.R. Netz, Shear-flow-induced unfolding of polymeric globules, Phys. Rev. Lett. 97 (13) (2006) 138101/1–138101/4.
[5] A. Barg, R. Ossig, T. Goerge, M.F. Schneider, H. Schillers, H. Oberleithner, S.W. Schneider, Soluble plasma-derived von Willebrand factor assembles to a hemostatically active filamentous network, Thromb. Haemost. 97 (4) (2007) 514–526.
[6] H. Shankaran, P. Alexandridis, S. Neelamegham, Aspects of hydrodynamic shear regulating shear-induced platelet activation and self-association of von Willebrand factor in suspension, Blood 101 (7) (2003) 2637–2645, Epub. 2002 November 27.
[8] D. Joseph, J.N.O.N.D.S.P.G.W. Bryngelson, Funnels, pathways, and the energy landscape of protein folding: a synthesis, Proteins Struct. Funct. Genet. 21 (3) (1995) 167–195.
[9] K.A.D. Hue Sun Chan, Protein folding in the landscape perspective: chevron plots and non-arrhenius kinetics, Proteins Struct. Funct. Genet. 30 (1) (1998) 2–33.
[10] T. Frisch, A. Verga, Slow relaxation and solvent effects in the collapse of a polymer, Phys. Rev. E Stat. Nonlin. Soft Matter Phys. 66 (4 Pt. 1) (2002) 041807, Epub. 2002 October 28.
[11] Z. Guttenberg, A. Rathgeber, S. Keller, J.O. Radler, A. Wixforth, M. Kostur, M. Schindler, P. Talkner, Flow profiling of a surface-acoustic-wave nanopump, Phys. Rev. E Stat. Nonlin. Soft Matter Phys. 70 (5 Pt. 2) (2004) 056311, Epub. 2004 November 23.
[12] K. Sritharan, C.J. Strobl, M.F. Schneider, A. Wixforth, Z. Guttenberg, Acoustic mixing at low Reynold's numbers, Appl. Phys. Lett. 88 (5) (2006).
[13] A. Wixforth, C. Strobl, C. Gauer, A. Toegl, J. Scriba, Z. Guttenberg, Acoustic manipulation of small droplets, Anal. Bioanal. Chem. 379 (7–8) (2004) 982–991, Epub. 2004 July 15.
[14] T.T. Perkins, D.E. Smith, S. Chu, Single polymer dynamics in an elongational flow, Science 276 (5321) (1997) 2016–2021.
[15] J.C. Phillips, Rep. Prog. Phys. 59 (1996) 1133.
[16] V. Degiorgio, T. Bellini, R. Piazza, F. Mantegazza, R.E. Goldstein, Stretched-exponential relaxation of electric birefringence in polymer solutions, Phys. Rev. Lett. 64 (9) (1990) 1043.
[17] M.K. Hong, O. Narayan, R.E. Goldstein, E. Shyamsunder, R.H. Austin, D.S. Fisher, M. Hogan, Internal dynamics of DNA probed by transient electric birefringence, Phys. Rev. Lett. 68 (9) (1992) 1430.
[18] S.J. Hagen, W.A. Eaton, J. Chem. Phys. 104 (9) (1996).
[19] R. Metzler, J. Klafter, The random walk's guide to anomalous diffusion: a fractional dynamics approach, Phys. Rep. 339 (2000) 1–77.
[20] B.J. Cherayil, Stretched exponential relaxation in polymer dynamics, J. Chem. Phys. 97 (3) (1992) 2090–2094.
[21] M. Doi, S. Edwards, The Theory of Polymer Dynamics, Clarend Press, Oxford, 1988.
[22] B. Savage, J.J. Sixma, Z.M. Ruggeri, Functional self-association of von Willebrand factor during platelet adhesion under flow, Proc. Natl. Acad. Sci. USA 99 (1) (2002) 425–430, Epub. 2001 December 26.
[23] T. Frisch, A. Verga, Unwinding globules under tension and polymer collapse, Phys. Rev. E 65 (4) (2002) 041801.
[24] Z.M. Ruggeri, J.N. Orje, R. Habermann, A.B. Federici, A.J. Reininger, Activation-independent platelet adhesion and aggregation under elevated shear stress, Blood 108 (6) (2006) 1903–1910, Epub. 2006 June 13.

# Update


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Corrigendum

# Corrigendum to "Relaxation of ultralarge VWF bundles in a microfluidic–AFM hybrid reactor" [Biochem. Biophys. Res. Commun. 369 (2008) 507–512]

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