doi:10.2533/chimia.2015.207 Chimia 69 (2015) 207–212 

# Mimicking the *in vivo* Environment – The Effect of Crowding on RNA and Biomacromolecular Folding and Activity

Erica Fiorini[§&], Richard Börner[&], and Roland K. O. Sigel*

[§]SCS-DSM Award for best poster presentation

*Abstract: In vitro* studies on macromolecules, like proteins and nucleic acids, are mostly carried out in highly diluted systems where the molecules are studied under artificial conditions. These experimental conditions are optimized for both the system under investigation and the technique used. However, these conditions often do not reflect the *in vivo* situation and are therefore inappropriate for a reliable prediction of the native behavior of the molecules and their interactions under *in vivo* conditions. The intracellular environment is packed with cosolutes (macromolecules, metabolites, *etc*.) that create 'macromolecular crowding'. The addition of natural or synthetic macromolecules to the sample solution enables crowding to be mimicked. In this surrounding most of the studied biomolecules show a more compact structure, an increased activity, and a decrease of salt requirement for structure formation and function. Herein, we refer to a collection of examples for proteins and nucleic acids and their interactions in crowding environments and present in detail the effect of cosolutes on RNA folding and activity using a group II intron ribozyme as an example.

**Keywords:** Excluded volume effect · Molecular crowding · RNA · Single-molecule Förster resonance energy transfer (smFRET)

## 1. Introduction

The cytoplasm of a cell is dense with proteins, nucleic acids, and other biomacromolecules creating a packed milieu. Typically 20–30% of the total cellular volume is occupied by macromolecules (Fig. 1a),[1–3] a situation very different from standard *in vitro* conditions, where usually only a small-molecular-weight buffer is present. Nowadays crowding agents are a widespread tool to mimic the crowded environment in a cell in order to understand the behavior of the system under *in vivo* conditions (see below). The most common molecules used for this purpose are poly(ethylene glycol) (PEG), ethylene glycol (EG), Ficoll (highly branched polysaccharide of sucrose), dextran (polysaccharide) and BSA *(*bovine serum albumin). All of these molecules share characteristics, which make them ideal for the use as crowding agents: i) they are well soluble in water, ii) they do not cause degradation and precipitation of the sample molecules, and iii) they do not prevent water interacting with ions or nucleic acids.[3] PEG and polysaccharides like dextran are the most used due to their neutral charge, which minimizes the direct interaction between the crowder and the molecule of interest. For instance, a 35% PEG 20k solution should correspond to similar conditions inside an *Escherichia coli* cell, where 20% to 30% of the volume is occupied by macromolecules.[4] However, Zhou *et al.* elucidated the importance to use a mixture of different crowding agents to better mimic the *in vivo* situation where macromolecules have a variety of sizes and shapes.[5]

One major consequence of a crowded environment is the so-called excluded volume effect. This effect causes a smaller effective volume available for the macromolecules with a series of consequences (Fig. 1b).[1–3] First, a crowded environment yields a higher effective concentration of all molecules in solution and second, it reduces their spatial movements.[6] Consequently, the interactions between the macromolecules and ligands, metal ions, or others, can be more pronounced in a crowded environment. Spatial movement seems to be strongly dependent on the size of all species present and their concentration.[2,7]

Further, some physical properties of the solution are changed. The dielectric constant of water at 20 °C is 80 while the average value of the relative permittivity of the cellular environment is between 10 and 40.[8] Alcohols, *i.e.* ethylene glycol, and 20% PEG, for example, decrease the value to 50–70.[3] A reduced dielectric constant results in an increase in the electrostatic attraction between charged species, *e.g.* in particular between nucleic acids and metal ions.[9] For example, the addition of 1,4-dioxane decreases the $Mg^{2+}$ requirement for local structure formation of a bulge region within the catalytic core of a group II intron ribozyme.[10] In addition, crowding agents can influence the viscosity of the solution. An elevated viscosity, due to a high amount of background molecules, might rule against the excluded volume effect and influence the diffusion rates.[11–13] Changing the diffusion rate has two effects: the intermolecular diffusion of substrate and its enzyme and the intramolecular diffusion of protein side chains or domains during folding, like the unfolding and refolding of the cold-shock protein CspB, which is strongly slowed down with increased viscosity of the solvent.[12] Taking these effects into account may explain why weak interactions between macromolecules at low salt concentrations are strongly enhanced in the presence of a crowded environment, and thus, explain

*Correspondence: Prof. R. K. O. Sigel
Department of Chemistry
University of Zurich
Winterthurerstrasse 190
CH-8057 Zurich
Tel.: +41 44 635 46 52
E-mail: roland.sigel@chem.uzh.ch

[&]Contributed equally to the manuscript

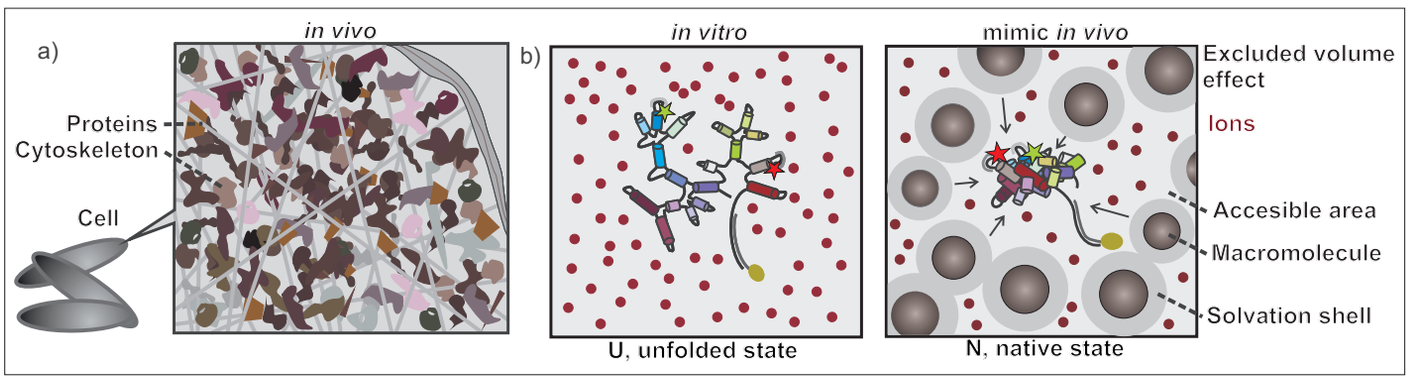


Fig. 1. a) Depiction of the crowded environment within a cell. b) Comparison of the effect of a diluted environment under standard buffer conditions (left) and a crowded one (right) on proteins and/or nucleic acids (*Sc.*D135-L14 ribozyme used as example) folding. Adapted from ref. [3].

the discrepancy between *in vivo* and *in vitro* experiments.

Most of the studies using cosolutes are carried out to study the folding of proteins and nucleic acids and their interactions (see below). The concentration of the crowding molecule is typically indicated as percentage weight/volume and the unit of molecular weight, generally not specified, is Dalton; for example 5% PEG 8000 means a concentration of 5 g of poly(ethylene glycol) with a molecular weight of 8000 Da (or 8 kDa) in 100 mL solution.

## 2. Overview of Molecular Crowding with Biomacromolecules

### 2.1 *Protein Folding and Protein–Protein Interaction*

Many proteins fulfill their function in a crowded, intracellular environment. It has been shown that crowding agents can increase the interaction between proteins, influence their folding, and how this effect depends on the agent used and its concentration.[14–16] The stabilizing effect of a crowded environment was shown by Tokuriki and co-workers.[4] They illustrated the formation of a folded RNase A and the partial recovery of its activity by the addition of PEG 20 k or Ficoll to a solution containing 2.4 M urea, where 70% of the proteins were in the unfolded state. Further, Ladurner and Fersht have shown that the folding rate of the protein chymotrypsin inhibitor 2 (CI2) as well as two types of mutants (representing faster and slower folding) are positively affected by the presence of sucrose and ethylene glycol.[14] They attributed this behavior to the destabilization of the background molecules on CI2 and not to its diffusion inhibition. Regarding the interaction between proteins, the system studied by Kozer and Schreiber does not show any benefit from the presence of a high molecular weight crowding agent.[17] For the complex formed by the two proteins β-lactamase (TEM) and its inhibitor, the β-lactamase inhibitor protein (BLIP), the association rate is slowed down and is inversely related to the viscosity of the solution in presence of a low mass crowding agent (EG and glycerol). In contrast, in high mass cosolutes, like PEG, dextran, and Ficoll 70, the association rate is almost unchanged compared to a diluted solution (buffer). Although they did not attribute their finding to physical properties like a change in the dielectric constant, they considered the structural properties of the cosolutes to explain this result. Solutions of EG and glycerol are homogeneous with respect to those of high molecular weight crowding agents, since polymers have the predisposition to build networks in solution.[18–20] The latter acts as a porous medium, in which proteins can associate reasonably freely due to the larger space between the pores in the case of high molecular weight polymers. This could be the reason for the small effect on the association rate in solutions containing high mass crowding agents.[14,18]

### 2.2 *Protein–DNA Interactions*

A good example of protein–nucleic acid interactions is the replication system of bacteriophage T7 studied by Akabayov and collaborators.[6] In the presence of crowding agents the activity of the DNA helicase was increased and the sensitivity of the DNA polymerase to high salt concentration reduced. With the help of small-angle X-ray scattering (SAXS) analysis, they illustrated how the complex between the DNA helicase and the DNA polymerase/trx is more packed in a crowded environment. They attributed this stabilization of the system to the excluded volume effect. The background molecules cause an increase in the effective concentration of the constituents, a further decrease of the diffusion and therefore the enhancement of binding and catalysis.[6] Sasaki and colleagues showed that molecular crowding has only an effect on the catalytic activity of endonucleases (DNase I and S1 nuclease) but not on exonucleases (exonucleases I and III).[21] However, thermodynamic studies on the stability demonstrate that the structure of both nucleases is stabilized by molecular crowding, resulting in higher melting temperature. Similar results have been obtained on other systems.[22,23] In conclusion, PEG as a crowding agent can have different effects highly depending on the type of enzyme that metabolizes the particular nucleic acid. To the best of our knowledge there are no studies regarding protein–RNA interaction in the presence of background molecules.

### 2.3 *Nucleic Acids*

Nucleic acids are divided into two main classes: DNA and RNA. DNA exists mostly as a double-stranded molecule coiled to form a double helix, while RNA subsists as 'single-stranded' molecule in many of its biological roles.[24] RNA mostly adopts complex tertiary structures and has diverse functions, *e.g.* intermediate (mRNA), adaptor (tRNA), structural (rRNA), regulatory (miRNA), and others. Moreover, RNA can display enzymatic activity as ribonucleic acid enzymes (ribozymes).[25]

One of the most studied nucleic acid structures in the presence of crowding agents is the DNA G-quadruplex, which is stabilized by certain metal ions, most efficiently by $K^+$ and $Na^+$.[26,27] Kan *et al.* showed how PEG reduces the cation dependence of the G-quadruplex formation and how the structure is stabilized in the absence of any cations mimicking the effect of $K^+$.[28] Miyoshi and co-workers have contributed the further information that PEG not only stabilizes the G-quadruplex structure but actually destabilized the duplex conformation. The hydration sites in these two types of structure are different. Thus, the stabilization effect on DNA structures containing Hoogsteen base pairs is much higher than those containing Watson-Crick base pairs.[29,30] Moreover, Heddi and Phan showed that different G-quadruplex topologies that coexist in

solution are converted to parallel-stranded G-quadruplexes in the presence of crowding agents.[31] To the best of our knowledge no studies on RNA G-quadruplexes in the presence of crowding molecules exist.

Folding studies of RNA molecules under molecular crowding conditions mostly concentrate on ribozymes. Ribozymes are catalytic active RNA molecules able to catalyze specific biochemical reactions, similar to protein enzymes. Metal ions are crucial cofactors to facilitate and regulate both folding as well as function of ribozymes.[32] Background molecules can thereby strongly influence the folding, the activity, and requirement of cations.[3,9,33,34] The self-cleaving hammerhead ribozyme is one of the smallest ribozymes and requires $Mg^{2+}$ for both folding and catalysis.[35] Nakano and collaborators showed that in the presence of 20% background molecules the hammerhead ribozyme increases its cleavage activity and even faster reaction rates were reached with higher concentrations of EG and PEG.[3] Thermal studies demonstrated the stabilizing effect of PEG, protecting the ribozyme from inactivation through denaturation.[36] Studies have suggested that the hammerhead ribozyme is active in the absence of $M^{2+}$ ions but in the presence of high concentrations of monovalent metal ions.[33] While such conclusions should be taken with care, because also monovalent salts contain traces of $M^{2+}$ ions, Nakano *et al.* showed that under such conditions as well as at salt concentration close to the physiological range (0.5 mM $MgCl_2$ and 100 mM NaCl) high ribozyme activity can be obtained if PEG is added to the solution, again attributed to the excluded volume effect.[6] Another effect of cosolutes is the destabilization of Watson-Crick base pairs.[29,30] This has a positive effect of helping the dissociation of 'incorrect' base pairing, lowering the energy cost for this rearrangement, and refolding of the ribozyme into the active form.[29]

While some small self-cleaving ribozymes have been suggested to use nucleobases for catalysis, the larger self-splicing ribozymes always directly rely on metal ions as cofactors.[32] The Woodson group focused on the influence of crowding agents on the folding and the activity of a group I intron ribozyme.[9,34,37–39] This ribozyme is derived from the pre-tRNA of the bacterium *Azoarcus* and is the smallest self-splicing group I intron known (197 nt).[9] Compaction of this ribozyme in the presence of PEG and a further decrease in the $Mg^{2+}$ concentration needed for folding was shown by SAXS. They concluded that the major effect of PEG is the excluded volume effect, as the RNA is in an unfolded state at low ionic strength while it folds into a more compact conformation by increasing the $Mg^{2+}$ concentration.[38] The excluded volume in a crowded environment yields a higher effective concentration of $Mg^{2+}$ ions in solution due to the limitation of accessible volume and therefore a shift towards the folded conformation (Fig. 1b).[6] Desai *et al.* further revealed that molecular crowding improves also the self-splicing activity of the *Azoarcus* group I ribozyme.[39] An increase of the $Mg^{2+}$ concentration leads to an enhancement of splicing activity attributed to an increase in the folded fraction of RNA, as both are $Mg^{2+}$ dependent.[37] In the presence of PEG, the measured activity was increased even at physiological $Mg^{2+}$ concentrations (≤ 1 mM). Generally in support of the excluded volume effect hypothesis is the absence of any stabilizing effect in ethylene glycol and in sucrose, in contrast to the effect of PEG and Ficoll.[9,34,39]

Paudel and Rueda used single-molecule Förster resonance energy transfer (smFRET) to investigate the folding of the minimal hairpin ribozyme under molecular crowding conditions.[40] From previous studies it is known that the hairpin ribozyme can assume two conformations, *i.e.* undocked (unfolded) and docked (folded).[41] Cosolutes increase the RNA folded population by a factor of ~10 and decrease the $Mg^{2+}$ requirement for both, folding and catalytic activity of the hairpin ribozyme. In agreement with the finding on the group I intron ribozyme, EG does not affect the docking, while PEG and dextran have a positive effect on the docking rate. In particular, higher molecular weight PEG has a stronger effect, confirming the importance of the size of the background molecule.[2,7]

## 3. Materials and Methods

### 3.1 *Construct Transcription and Purification*

The Sc.D135-L14 ribozyme was obtained by *in vitro* transcription from the plasmid pT7D135-L14.[42] Upon HindIII digestion, the linearized pT7D135-L14 was transcribed with home-made T7 polymerase, purified by denaturing gel electrophoresis, extracted by crush-and-soak and stored at –20 °C in water.[43] DNA strands labelled with Cy3, Cy5 and biotin as well as the substrate 17/7 (5'-CGUGGUGGG ACAUUUUC*GAGCGGU-5') were purchased from IBA, Göttingen, Germany, and adenosine 5'-[γ-$^{32}$P]triphosphate from Perkin-Elmer Switzerland. Poly(ethylene glycol) 1k, 8k, and 35k was purchased from Sigma Aldrich, Buchs, Switzerland.

### 3.2 *Single Molecule Detection*

The smFRET-optimized construct *Sc.*D135-L14 used in our studies contains two additional internal loops that specifically bind DNA-oligonucleotides carrying the fluorophores Cy3/Cy5 and a 3'-end elongation which hybridizes a biotinylated DNA-oligonucleotide for surface immobilization (Fig. 2a).[42] The single molecule FRET experiments were performed in a home-built flow chamber under prism-based TIRF excitation.[44] The labeled group II intron construct was immobilized *via* a biotin-streptavidin linkage to a biotinylated-BSA-passivated quartz slide surface. An oxygen-scavenging system (OSS) consisting of 10% (wt/v) glucose, 2% (v/v) trolox, 50 μg/mL glucose oxidase, and 10 μg/mL catalase was applied to reduce photobleaching.[45,46] All smFRET experiments were carried out at room temperature, 80 mM MOPS pH 6.9, and 500 mM KCl. The desired PEG was dissolved in the OSS imaging buffer and added to the sample by flushing the chamber after immobilization. Individual smFRET measurements were performed under different experimental conditions varying the $Mg^{2+}$ concentration as well as the 8k PEG concentrations. For each condition at least 100 molecule transients are recorded 400s with a frame rate of 10 frames/s with an Andor EMCCD camera, Lot Oirel, Romanel-sur-Morges, Switzerland. Our Matlab (Mathworks) based home-written software package MASH allows for the HMM-based identification of different conformational states to study structural heterogeneities (thermodynamics) as well as the transitions between them (kinetics).[47] The percentage of static molecules was determined to be less than 30% (data not shown) and only dynamic molecules were used for further data processing. In order to determine the sample heterogeneity we applied a bootstrapped-based approach yielding error bars of the FRET states for multiple Gaussian fitting.[48] Transitions between the four observed states (*FRET* initial to *FRET* final, Fig. 2) were plotted as transition density plot (TDP) to characterize the dynamics behavior.

### 3.3 *Activity Assays*

Standard activity assays were performed following the cleavage of a $^{32}$P-labelled RNA substrate (17/7) containing the 17 last nucleotides at the 3'-end of the 5'-exon and the first 7 intronic nucleotides flanking the 5'-splice site.[42,49] The activity of the *Sc.*D135-L14 construct was tested under single turnover conditions (STO) and optimized for activity and cleavage of the substrate (42 °C, 80 mM MOPS pH 6.9, 500 mM KCl).[50] Two mixtures were prepared, one containing the substrate (20 nM) and the second the ribozyme (2 μM). Cy3-DNA, Cy5-DNA and T-biotin were heat-annealed to the ribozyme at 90 °C for 45 sec in reaction buffer (80 mM MOPS pH 6.9, 500 mM KCl) followed by $Mg^{2+}$ addition and incubation

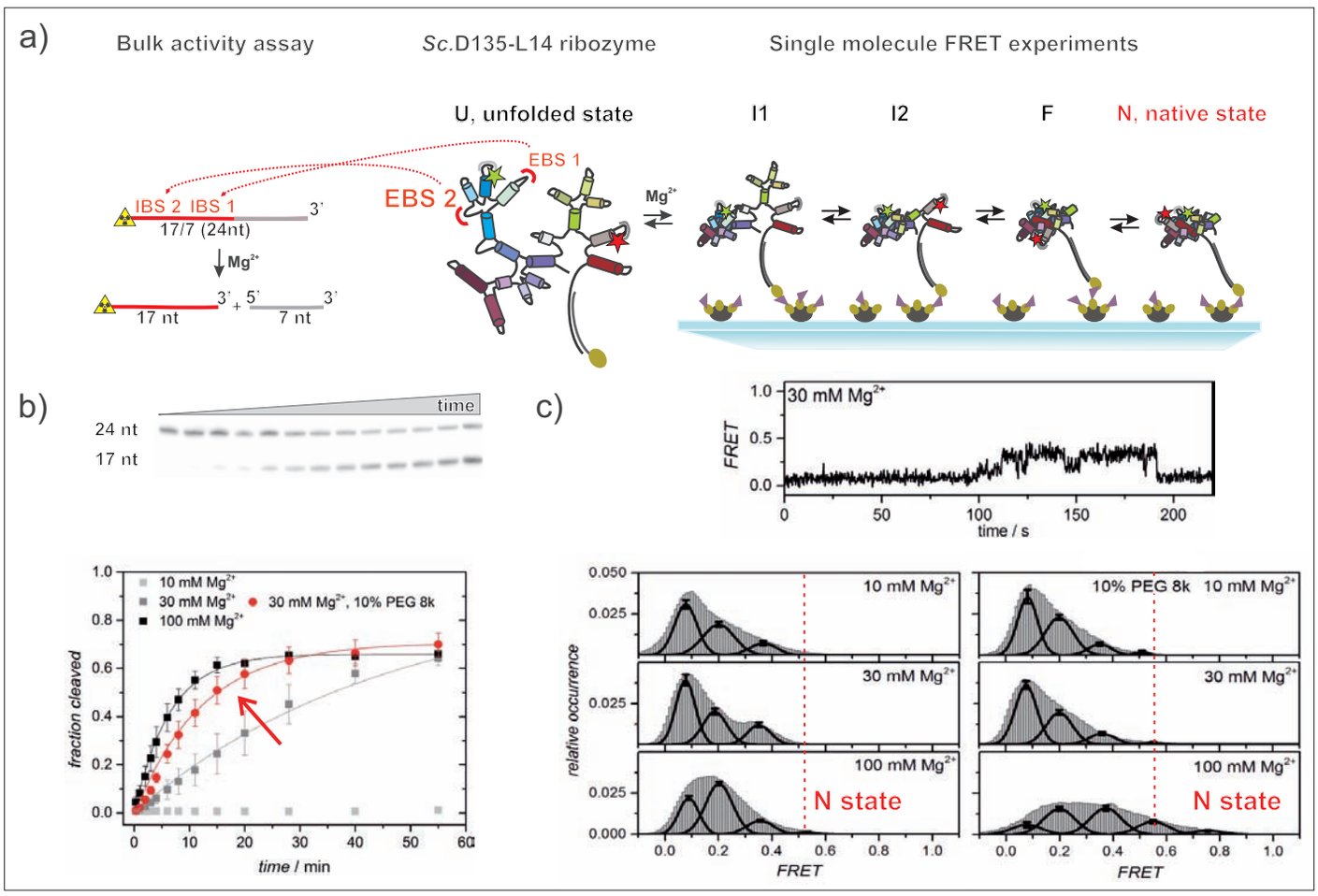


Fig. 2. Bulk and single-molecule experiments to study the activity and folding of the fluorescent labelled *Sc.*D135-L14 ribozyme under crowding conditions. (a) Scheme of the activity assay and linear folding pathway. (b) The fraction cleaved (17nt) can be followed over time *via* denaturing gel electrophoresis. In the presence of 10% PEG 8k the fraction of cleaved substrate strongly increases at low $Mg^{2+}$ (bottom). (c) smFRET experiments reveals five different folding states: unfolded state (U, not seen), first extended intermediate (I1), second extended intermediate (I2), folded intermediate (F) and native state (N). Cumulative histograms at different $Mg^{2+}$ concentrations in the absence and presence of 10% PEG 8k are shown at the bottom. The N state is indicated with a red line. The fraction of the native (N) state increases by increasing the $Mg^{2+}$ concentration. In the presence of 10% PEG 8k the relative occurrence of the native state (N) is enhanced already at low $Mg^{2+}$ concentration.

at 42 °C for 15 min. The substrate was incubated separately and then added to the ribozyme to start the cleavage reaction.[42] Aliquots of 1 µL were removed from the reaction mixture at specific time points and analyzed on a 18% polyacrylamide gel.[50] Product formation was monitored by imaging the radioactive gels on a Molecular Dynamic Typhoon 9400 imager from GE Healthcare and the reaction rate constants ($k_{obs}$) were determined by quantification of the bands using ImageQuant Software Version 8.1 from Molecular Dynamics and fitting a single exponential expression:

$$\text{fraction cleaved} = (1 - A_1) - A_2 x e^{-kt}$$

where $A_1$ is the fraction of uncleaved substrate, $A_2$ the fraction of product formed, and $k$ the first order rate constant.[51] PEG of different molecular weight and mass percentage was added to both mixtures in equal concentration during the sample preparation.

## 4. Results and Discussion

### 4.1 Group II Intron Ribozymes, the Molecule of Interest

Group II introns are among the largest ribozymes known. The group IIB intron ribozyme Sc.ai5γ from *Saccharomices cerevisiae* mitochondria is able to undergo autocatalytic splicing. Some group II introns can also perform reverse splicing into a DNA sequence.[52,53] The structural analysis of the group II intron suggests that they have become fragmented and have evolved into ribonucleoproteins generating the eukaryotic nuclear spliceosome.[54–56] Group II introns are constituted by six domains (D1-D6) radiating from a central wheel.[57–59] The well investigated construct *Sc.*D135 derives from Sc.ai5γ and contains only the domains 1, 3, and 5, fundamental for the first step of splicing, *i.e.* the hydrolytic cleavage of the 5'-splice-site.[50,60,61] Domain 1 is the largest domain, acts as scaffold for the assembly of the other domains and is indispensable for the exonic substrate recognition.[57,62] Domain 3 is an allosteric catalytic effector increasing the reaction rate of splicing.[57,63] Domain 5 is the most phylogenetically conserved domain and catalytic center of the ribozyme.[57,59] We combined activity assays and smFRET spectroscopy experiments in order to test if the presence of a crowding agent can stabilize the folding and increase the activity of this ribozyme together with lower $Mg^{2+}$ requirement (Fig. 2).

### 4.2 Molecular Crowding Increases the Bulk Activity at low $Mg^{2+}$ Concentrations

*Sc.*D135-L14 requires high $Mg^{2+}$ concentrations under *in vitro* conditions to perform cleavage of the 17/7 substrate. At standard *in vitro* conditions (80 mM MOPS pH 6.9, 500 mM KCl, 42 °C) cleavage activity is only observed for concentrations higher than 20 mM $Mg^{2+}$, the midpoint being at $K_d$ = 51.4 ± 2.2 mM $Mg^{2+}$. By introducing a crowding environment, the $Mg^{2+}$ midpoint drastically

decreases to 20.2 ± 0.8 mM. For example at 30 mM $Mg^{2+}$ the determined cleavage rate $k_{obs}$ in the presence of PEG is 10-fold higher than in the absence of PEG (0.082 ± 0.005 $min^{-1}$ and 0.008 ± 0.005 $min^{-1}$, respectively) reaching a value comparable to the wild type construct (*Sc.*D135) at 100 mM $Mg^{2+}$ (Fig. 2b). We tested different concentrations and molecular weights of crowding agent with the aim of identifying the existence of an optimal condition in terms of concentration and size of PEG. We found a maximum in cleaved fraction and cleavage rate around 10% PEG 8k followed by a decrease in cleavage fraction and rate with higher molecular weight and mass percentage of PEG. The cause is likely the increase of the solution viscosity due to concentration and molecular weight of PEG, affecting the substrate diffusion and intra-molecular rearrangements.[11,39] We also showed that the monomer EG has no influence on the activity compared to PEG. Dextran instead of PEG, also showed an increase in activity, but not as pronounced (data not shown). These results can be explained by the excluded volume effects of different PEG sizes (Fig. 1b right). Higher molecular weight cosolutes have a stronger effect on the activity and on the $Mg^{2+}$ requirement. However, we cannot exclude the positive effect on the ribozyme–substrate (RNA–RNA) interaction due to a decrease of the dielectric constant of the solution.[8] An additional outcome of the presence of PEG was the slight increase of activity when lowering the temperature from 42 °C to 37 °C. In the presence of 100 mM $Mg^{2+}$, $k_{obs}$ slightly increases from 0.05 $min^{-1}$ to 0.07 $min^{-1}$ in the presence of 10% PEG 8k. However, at even lower temperatures, the activity was too slow to allow quantification.

### 4.3 *Molecular Crowding Helps Folding into the most Compact State*

We further investigated the effect of PEG on the folding of the *Sc.*D135-L14 ribozyme *via* smFRET experiment at different $Mg^{2+}$ and PEG concentrations. Like the catalytic activity, folding also greatly depends on the $Mg^{2+}$ concentration as only a fully folded ribozyme is able to perform catalysis. An increase of the fraction of folded molecules corresponds to an increase of the number of catalytic competent molecules and consequently to a higher catalytic activity. From the single *FRET* traces we can obtain two types of information: the *FRET* ratio values, *i.e.* the number of conformations of the molecule and the transitions between these conformations.[48] The *FRET* values are accumulated into *FRET* histograms. The analysis of the *Sc.*D135-L14 ribozyme revealed the presence of four reoccurring *FRET* states, which were assigned to four different structural conformations of the folding pathway (Fig. 2a right). From previous results it is known that the highest *FRET* value (~0.6) corresponds to the most compact state corresponding most likely to the native (N) and catalytic active state of the ribozyme.[42,64] The N state is present only at high $Mg^{2+}$ (>40 mM) and is only little populated even at 100 mM $Mg^{2+}$ (Fig. 2c). In the presence of 10% PEG 8k and 30 mM $Mg^{2+}$, some ribozyme molecules already reach the native state. At high $Mg^{2+}$ concentration the general shift to higher FRET states is clearly visible with the fraction of molecules in the conformation I1 being almost gone (Fig. 2c). Furthermore, at 30 mM $Mg^{2+}$ the increase in PEG (0–20%) increases the fraction of folded molecules from completely absent to 7% at 20% PEG 8k (data not shown).

### 4.4 *Molecular Crowding Increases Conformational Dynamics*

The discretization of the single time-trajectories allowed us to obtain information on the transitions between different conformations and to build transition density plots (TDP) (Fig, 3a).[48] The folding of the *Sc.*D135-L14 ribozyme proceeds through five difference conformations, from the fully unfolded state (U) to the native state (N) with three intermediates, the first extended intermediate (I1), a second extended intermediate (I2), and the folded intermediate (F). From the single *FRET* trajectories it is possible to observe the increase in dynamics concomitant with the amount of PEG (Fig. 3a). Our smFRET experiments show that the intradomain dynamics of the ribozyme increase upon addition of crowding agents. In particular, transitions that occur between two or three subsequent conformations increase in the presence of crowding agent. Such off-pathway transitions devoid of an intermediate at our time resolution illustrate nicely the increased dynamics, which also results in a 'cloudier' TDP in the presence of PEG (Fig. 3b). Moreover, the overall number of molecules (single time-trajectories) showing one or more transition increases concomitantly with the percentage of PEG. The concentration of PEG thus strongly influences both folding and dynamics of the ribozyme even at low $Mg^{2+}$ concentrations. We can conclude that the excluded volume effect increases with the amount of crowding agent in solution without any viscosity effect on the RNA domain movements, in contrast to the folding of proteins.[12]

## 5. Conclusion

We investigated the presence of crowding agents on both the folding and catalytic activity of a group II intron ribozyme construct. The presence of PEG decreases the requirement of $Mg^{2+}$ for folding and catalytic activity by increasing the fraction of fully folded ribozyme. The exact action of PEG is unknown and we therefore base the interpretation of our results on previous work with other biomacromolecules and the known characteristics of the crowding agent (see Introduction). We observe that the consequences of excluded volume in our system strongly depend on the concentration and molecular weight of the cosolutes. Moreover, we cannot exclude an effect of the decrease in dielectric constant, which

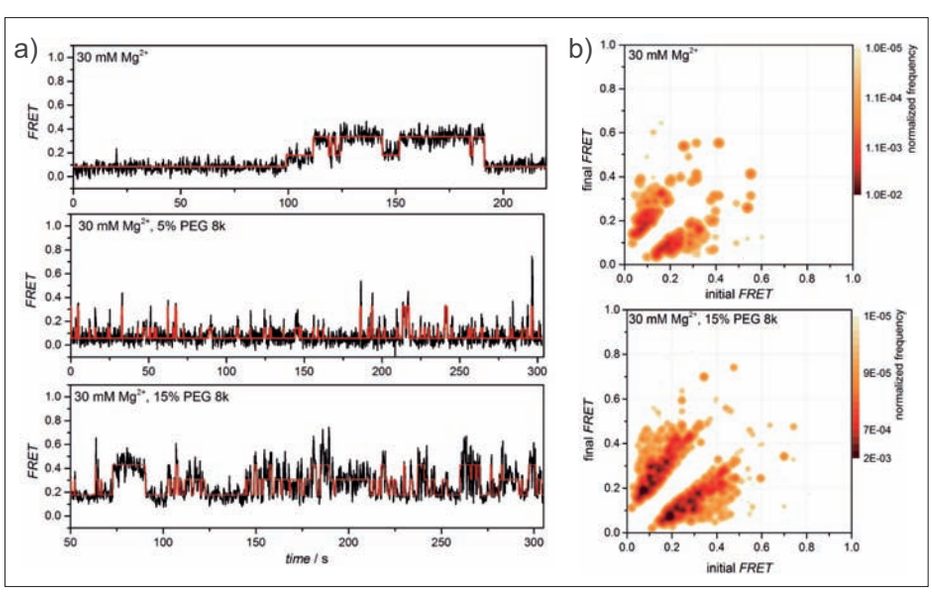


Fig. 3. a) Single *FRET* trajectories showing the effect of different PEG 8k percentage on the dynamics of the ribozyme at 30 mM $Mg^{2+}$. The transitions discretization *via* HMM (4 state model) is depicted in red. b) Transition density plots (TDP) are plotted for 30 mM $Mg^{2+}$ in the absence and presence of 15% PEG 8k.

would not only favor the compaction of the negatively charged backbone of the ribozyme but also the decrease in dynamics. The overall effect of PEG on *Sc*.D135-L14 ribozyme is to favor the folding pathway to a compact state by lowering the energy barriers between conformations similar to the effect of a chaperon protein. Chaperons are proteins that assist the assembly of protein-containing structures.[65] In the case of our group II intron, Mss116, a DEAD-box helicase, acts as natural RNA-chaperon, which facilitates the folding and splicing of all *Saccharomyces cerevisiae* mitochondrial group I and II introns *in vivo*.[65–68] Molecular crowding can partially take over the effect of molecular chaperones thus increasing the ability of large RNAs like the group II intron ribozyme to assemble to the active state *in vitro* (Fig. 1).[2]

***Acknowledgement***

This work was supported by the European Research Council (ERC Starting Grant MIRNA N° 259092, to RKOS) and the Forschungskredit of the University of Zurich (FK-14-096, to RB), which are gratefully acknowledged. We are very thankful for further financial support related to our smFRET studies by the University of Zurich, the Swiss National Science Foundation, and the Swiss State Secretariat for Education and Research (COST Action CM1105).

Received: February 24, 2015

[1] R. J. Ellis, *Curr. Opin. Struc. Biol.* **2001**, *11*, 114.
[2] R. J. Ellis, *Trends in Biochem. Sci.* **2001**, *26*, 597.
[3] S.-i. Nakano, D. Miyoshi, N. Sugimoto, *Chem. Rev.* **2014**, *114*, 2733.
[4] N. Tokuriki, M. Kinjo, S. Negi, M. Hoshino, Y. Goto, I. Urabe, T. Yomo, *Protein Sci.* **2004**, *13*, 125.
[5] H.-X. Zhou, *Proteins* **2008**, *72*, 1109.
[6] B. Akabayov, S. R. Akabayov, S.-J. Lee, G. Wagner, C. C. Richardson, *Nat. Commun.* **2013**, *4*, 1615.
[7] A. P. Minton, *Methods Enzymol.* **1998**, *295*, 127.
[8] S. Tanizaki, J. Clifford, B. D. Connelly, M. Feig, *Biophys. J.* **2008**, *94*, 747.
[9] D. Kilburn, J. H. Roh, L. Guo, R. M. Briber, S. A. Woodson, *J. Am. Chem. Soc.* **2010**, *132*, 8690.
[10] M. Furler, B. Knobloch, R. K. O. Sigel, *Inorg. Chimica Acta* **2009**, *362*, 771.
[11] J. Brillo, A. I. Pommrich, A. Meyer, *Phys. Rev. Lett.* **2011**, 107.
[12] M. Jacob, T. Schindler, J. Balbach, F. X. Schmid, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 5622.
[13] M. K. Kuimova, S. W. Botchway, A. W. Parker, M. Balaz, H. A. Collins, H. L. Anderson, K. Suhling, P. R. Ogilby, *Nature Chem.* **2009**, *1*, 69.
[14] A. G. Ladurner, A. R. Fersht, *Nat. Struct. Biol.* **1999**, *6*, 28.
[15] A. Samiotakis, P. Wittung-Stafshede, M. S. Cheung, *Int. J. Mol. Sci.* **2009**, *10*, 572.
[16] H.-X. Zhou, G. Rivas, A. P. Minton, *Annu. Rev. Biophys. Biomol. Struct.* **2008**, *37*, 375.
[17] N. Kozer, G. Schreiber, *J. Mol. Biol.* **2004**, *336*, 763.
[18] D. L. Ho, B. Hammouda, S. R. Kline, *J. Polym. Sci. B Polym. Phys.* **2003**, *41*, 135.
[19] A. Vergara, L. Paduano, G. D'Errico, R. Sartorio, *Phys. Chem. Chem. Phys.* **1999**, *1*, 4875.
[20] F. Kienberger, V. P. Pastushenko, G. Kada, H. J. Gruber, *Single Molecule* **2000**, *1*, 123.
[21] Y. Sasaki, D. Miyoshi, N. Sugimoto, *Nucleic Acids Res.* **2007**, *35*, 4086.
[22] S. B. Zimmerman, B. H. Pheiffer, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 5852.
[23] J. R. Wenner, V. A. Bloomfield, *Biophys. J.* **1999**, *77*, 3234.
[24] B. Alberts, J. Wilson, T. Hunt, 'Molecular biology of the cell', Garland Science, New York, **2008**.
[25] R. T. Batey, R. P. Rambo, J. A. Doudna, *Angew. Chem. Int. Ed.* **1999**, *38*, 2326.
[26] L.-P. Bai, M. Hagihara, K. Nakatani, Z.-H. Jiang, *Sci. Rep.* **2014**, *4*, 6767.
[27] A. N. Lane, J. B. Chaires, R. D. Gray, J. O. Trent, *Nucleic Acids Res.* **2008**, *36*, 5482.
[28] Z.-Y. Kan, Y. Yao, P. Wang, X.-H. Li, Y.-H. Hao, Z. Tan, *Angew. Chem. Int. Ed.* **2006**, *45*, 1629.
[29] E. N. Nikolova, E. Kim, A. A. Wise, P. J. O'Brien, I. Andricioaei, H. M. Al-Hashimi, *Nature* **2011**, *470*, 498.
[30] D. Miyoshi, H. Karimata, N. Sugimoto, *J. Am. Chem. Soc*. **2006**, *128*, 7957.
[31] B. Heddi, A. T. Phan, *J. Am. Chem. Soc.* **2011**, *133*, 9824.
[32] 'Structural and catalytic roles of metal ions in RNA', Eds. A. Sigel, H. Sigel, R. K. O. Sigel, *Metal Ions in Life Sciences* Vol. 9., Royal Society of Chemistry, Cambridge, UK, **2011**.
[33] J. B. Murray, A. A. Seyhan, N. G. Walter, J. M. Burke, W. G. Scott, *Chem. & Biol.* **1998**, *5*, 587.
[34] D. Kilburn, J. H. Roh, R. Behrouzi, R. M. Briber, S. A. Woodson, *J. Am. Chem. Soc.* **2013**, *135*, 10055.
[35] W. G. Scott, J. T. Finch, A. Klug, *Nucleic Acids Symp. Ser.* **1995**, *81*, 214.
[36] S.-i. Nakano, H. T. Karimata, Y. Kitagawa, N. Sugimoto, *J. Am. Chem. Soc.* **2009**, *131*, 16881.
[37] P. Rangan, S. A. Woodson, *J. Mol. Biol*. **2003**, *329*, 229.
[38] U. A. Perez-Salas, P. Rangan, S. Krueger, R. M. Briber, D. Thirumalai, S. A. Woodson, *Biochemistry* **2004**, *43*, 1746.
[39] R. Desai, D. Kilburn, H.-T. Lee, S. A. Woodson, *J. Biol. Chem.* **2014**, *289*, 2972.
[40] B. P. Paudel, D. Rueda, *J. Am. Chem. Soc.* **2014**, *136*, 16700.
[41] D. Rueda, G. Bokinsky, M. M. Rhodes, M. J. Rust, X. Zhuang, N. G. Walter, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 10066.
[42] M. Steiner, K. S. Karunatilaka, R. K. O. Sigel, D. Rueda, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13853.
[43] S. Gallo, M. Furler, R. K. O. Sigel, *Chimia* **2005**, *59*, 812.
[44] L. Cardo, K. S. Karunatilaka, D. Rueda, R. K. O. Sigel, *Methods Mol. Biol.* **2012**, *848*, 227.
[45] C. E. Aitken, R. A. Marshall, J. D. Puglisi, *Biophys. J.* **2008**, *94*, 1826.
[46] T. Cordes, J. Vogelsang, P. Tinnefeld, *J. Am. Chem. Soc*. **2009**, *131*, 5018.
[47] D. Kowerko, M. C. Hadzic, R. Börner, S. L. B. König, M. Heidernätsch, R. K. O. Sigel, **2015**, to be submitted.
[48] S. L. B. König, M. Hadzic, E. Fiorini, R. Börner, D. Kowerko, W. U. Blanckenhorn, R. K. O. Sigel, *PloS one* **2013**, *8*, e84157.
[49] L. J. Su, M. Brenowitz, A. M. Pyle, *J. Mol. Biol.* **2003**, *334*, 639.
[50] E. A. Jr. Griffin, Q. Zhifeng, W. J. Jr. Michels, A. M. Pyle, *Chem. & Biol.* **1995**, *2*, 761.
[51] A. Nahvi, N. Sudarsan, M. S. Ebert, X. Zou, K. L. Brown, R. R. Breaker, *Chem. & Biol.* **2002**, *9*, 1043.
[52] M. Roitzsch, A. M. Pyle, *RNA* **2009**, *15*, 473.
[53] A. M. Pyle, O. Fedorova, C. Waldsich, *Trends in Biochem. Sci.* **2007**, *32*, 138.
[54] A. M. Pyle, *Crit. Rev. Biochem. Mol. Biol.* **2010**, *45*, 215.
[55] R. Geber, A. Mrowiec, A. Tokarowski, *Chirurgia narzadów ruchu i ortopedia polska* **1991**, *56*, 139.
[56] K. S. Keating, N. Toor, P. S. Perlman, A. M. Pyle, *RNA* **2010**, *16*, 1.
[57] O. Fedorova, N. Zingler, *Biol. Chem.* **2007**, *388*, 665.
[58] S. Somarowthu, M. Legiewicz, K. S. Keating, A. M. Pyle, *Nucleic Acids Res*. **2014**, *42*, 1947.
[59] B. B. Konforti, Q. Liu, A. M. Pyle, *EMBO J.* **1998**, *17*, 7105.
[60] J. F. Swisher, L. J. Su, M. Brenowitz, V. E. Anderson, A. M. Pyle, *J. Mol. Biol.* **2002**, *315*, 297.
[61] M. Podar, V. T. Chu, A. M. Pyle, P. S. Perlman, *Nature* **1998**, *391*, 915.
[62] M. Costa, F. Michel, *EMBO J.* **1999**, *18*, 1025.
[63] O. Fedorova, A. M. Pyle, *EMBO J.* **2005**, *24*, 3906.
[64] M. Steiner, D. Rueda, R. K. O. Sigel, *Angew. Chem. Int. Ed.* **2009**, *48*, 9739.
[65] R. J. Ellis, *Curr. Biol.* **1997**, *7*, R531.
[66] H.-R. Huang, C. E. Rowe, S. Mohr, Y. Jiang, A. M. Lambowitz, P. S. Perlman, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 163.
[67] N. Zingler, A. Solem, A. M. Pyle, *Nucleic Acids Res.* **2010**, *38*, 6602.
[68] K. S. Karunatilaka, A. Solem, A. M. Pyle, D. Rueda, *Nature* **2010**, *467*, 935.