Probing messenger RNA conformational heterogeneity using single-molecule fluorescence anisotropy

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In this letter we describe a method to probe biomolecular conformations and their dynamics at the single molecule level. We show, using fluorescence anisotropy based methods, that the hydrodynamic volume of biomolecules captures the intrinsic heterogeneity within a population. Population distributions of conformations and their dynamics are studied by making anisotropy measurements on one molecule at a time within a confocal volume. The mean anisotropy of mRNA is lowered on addition of salt while the spread remains the same. The intrinsic heterogeneity is revealed when conformational transitions are frozen, resulting in a drastic increase in the spread of the anisotropy. These studies reveal that mRNA samples a broad range of conformations. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2183358]

RNA is an important biopolymer and its structure is closely related to its function.[1] Measurement of the conformational distribution of an RNA molecule requires a noninvasive mapping of all the contact points of RNA over a small time interval that is becoming accessible with existing technology.[2] Single molecule RNA FRET experiments have established the presence of multiple conformational states in small RNA molecules.[3] However, distribution and fluctuation of conformations of long RNA molecules such as messenger RNA (mRNA) have remained elusive. Stem loops in mRNA control the ribosome binding, serving as kinetic barriers for the ribosome during translation elongation. They also control the overall translation efficiency and mRNA stability.[4,5] The structure-function relationship therefore makes it important to explore the conformational distribution of mRNA. This could provide a basis for understanding the dynamics of molecular motors like the ribosome on mRNA.

In this letter we describe a method to measure the ensemble distribution of mRNA conformations. Rotational mobility of mRNA, obtained from single molecule fluorescence anisotropy, is used as an index of macromolecular conformation. For this, a diffraction-limited confocal volume of diameter $\sim 0.4\ \mu$m is set up with a polarized excitation laser on an inverted microscope using a 100×, 1.4 numerical aperture (NA) objective lens. In this experiment, we use fluorescently tagged and purified mRNA with a concentration of less than one molecule per confocal volume. This reduces the probability of two or more mRNA molecules occupying the observation volume simultaneously. The fluorescence emission from individual mRNA molecules diffusing through the confocal volume is detected using a 50 μm pinhole, an analyzer and two avalanche photodiodes (APD-EG&G) as shown in Fig. 1(a). The TTL pulse generated by the avalanche photodiodes (APDs) are synchronously counted using counters (PCI 6602; National Instruments) with 1 ms gate width. The measured intensities, $I_{\parallel}$ and $I_{\perp}$, are then used to quantify the fluorescence anisotropy.[6] These measurements show that the probability distribution of fluorescence anisotropy directly captures the ensemble distribution of mRNA conformations and their dynamics.

mRNA molecules are synthesized, using *in vitro* transcription, such that 5% of the rUTP are labeled with Alexa-488.[7] The fluorophore can rotate about its linker (local rotation) as well as around the axis of the mRNA molecule (global rotation). The local and global rotation of the probe (Alexa-488 r-UTP) about both the axes causes fluorescence

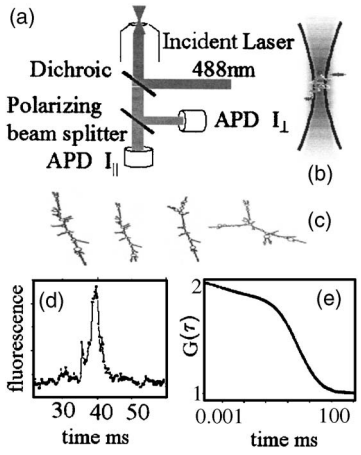

FIG. 1. (a) Schematic of the experimental setup. (b) Profile of laser intensity and the diffusion of a fluorescently marked mRNA molecule. (c) Four representative secondary structures obtained by simulation. (d) Typical intensity profile of a fluorescent mRNA diffusing through the confocal volume. (e) Corresponding autocorrelation function of the time series of fluorescence intensity indicating the associated diffusion coefficient.

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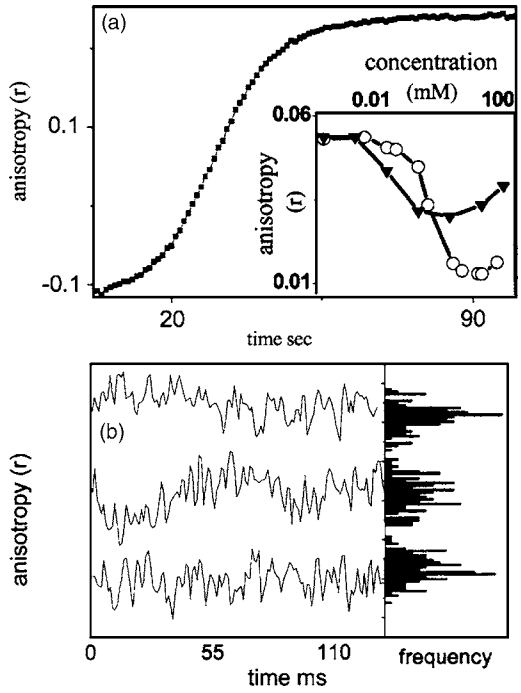

FIG. 2. (a) Polymerization kinetics of mRNA measured using fluorescence anisotropy. Inset: Bulk anisotropy of labeled mRNA as a function of salt concentration: (○) open circles represent NaCl and filled triangles represent $MgCl_2$. (b) Fluorescence anisotropy time series of three representative mRNA molecules. Inset: corresponding distributions of individual mRNA anisotropy.

depolarization of the probe, which is quantified by fluorescence anisotropy of labeled mRNA. The hydrodynamic volume dependence on anisotropy is given by the Perrin equation, $r=r_0/1+(\tau RT/\eta V)$ where r is anisotropy of the fluorophores, r_0 is anisotropy of the immobilized fluorophores, η is viscosity of the medium, τ is fluorescence lifetime, R is gas constant, T is temperature, and V is hydrodynamic volume of the mRNA molecule.[8] The effective hydrodynamic volume of mRNA depends on the secondary and higher order contacts formed as a result of mRNA folding. This determines its translational and rotational dynamics[9] in solution as well as its conformational states as shown in the schematic of Figs. 1(b) and 1(c). Figure 1(d) shows the signal corresponding to the passage of a single mRNA molecule diffusing through the confocal volume. The corresponding autocorrelation function of the fluorescence time series, depicted in Fig. 1(e), indicates the time taken by mRNA molecule to cross the observation volume. The correlation time, $\tau_D \sim 10$ ms, is the average interval of time available for the observation of the molecule.[6] The measured value of fluorescence anisotropy is therefore averaged over the conformations that fluctuate in less than $\tau_D \sim 10$ ms interval of time.

Fluorescence anisotropy measurements of single mRNA molecules were performed under different conditions of monovalent (NaCl) and divalent ($MgCl_2$) salt concentrations. The distribution of anisotropy values thus obtained is compared to give a measure of the conformational heterogeneity. The polymerization of the mRNA molecule can be directly mapped using mean anisotropy as a function of time as shown in Fig. 2(a). As the polymerization reaction proceeds, the Alexa-488 fluorescent labeled rUTP monomers incorporates into the growing mRNA polymer chain resulting in the increase in mean anisotropy value as a function of time. Under these reaction conditions, the mRNA synthesis is complete in about 60 s and was then purified as described earlier.[6] The effect of monovalent and divalent salts on the mRNA molecules was assessed from bulk anisotropy experiments performed using the confocal detection setup. The inset shows the mean value of fluorescence anisotropy as a function of both monovalent (NaCl) and divalent ($MgCl_2$) salt concentrations. The hydrodynamic volume of mRNA reflects the degree of compaction of the mRNA molecules. In the presence of NaCl, the hydrodynamic volume of mRNA decreases by 47.5% and in the presence of $MgCl_2$ the degree of compaction is 30.6%. Therefore, the observed decrease in anisotropy could result from either compaction of the mRNA molecule or a change in the fluorescence lifetime of the probe. However lifetime measurements of Alexa488-rUTP and labeled mRNA under different salt concentrations proved to be invariant in the regimes of salt concentration used in the experiment. Furthermore, control experiments on the free probe showed no change in fluorescence anisotropy with different ($NaCl/MgCl_2$) concentrations. We can therefore conclude that the observed decrease in fluorescence anisotropy under different salt concentrations is unambiguously due to the compaction of the mRNA molecule alone. At salt concentrations higher than the physiological levels, the anisotropy was found to increase, due to nonspecific aggregation of mRNA.

In our measurements it is possible to track the anisotropy of single mRNA molecules, since the concentration of mRNA can be reduced so that only a single molecule is present at a time in the observation volume. We exploit this ability to probe the conformational heterogeneity, by measuring the anisotropy of single mRNA molecules diffusing through the confocal volume. Three representative time series of single molecule anisotropy trace, obtained from the analysis of fluorescence burst due to three different mRNA molecules, are shown in Fig. 2(b). The corresponding histograms of the respective anisotropy time series are shown in the inset. We see that in all cases the mRNA conformational distribution shows a high degree of heterogeneity compared with protein molecules.

Having measured the conformational fluctuations of a single mRNA molecule, we proceeded to carry out ensemble fluorescence anisotropy measurements. This was done by observing a dilute collection of mRNA molecules, one molecule at a time, while they diffused through the confocal volume.[10] The distribution of anisotropy values obtained for mRNA in water and in 25 mM NaCl [Fig. 3(a)] indicates that the effective hydrodynamic volume is less in the latter than in the former due to electrostatic screening. Importantly single molecule experiments indicated a 54.3% compaction as opposed to 47.5% compaction in the bulk experiments suggesting a statistically significant greater sensitivity in the single molecule method. The mean and standard deviation of the conformational distribution as a function of salt concentration is shown in Fig. 3(b). The mean anisotropy, which is averaged over many molecules, decreases with increase in salt concentration due to the observed compaction. On the other hand, the standard deviation (SD) of the anisotropy, which is a quantity proportional to the conformational heterogeneity, showed only minor deviation. This is likely to be due to the sampling of several conformations accessible to the molecule within the observation timescale. The SD

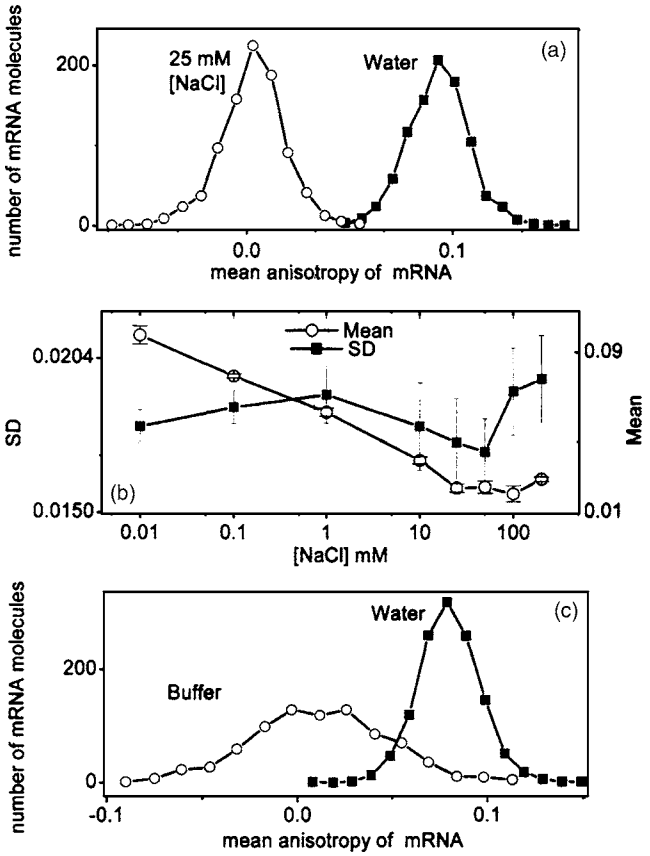

FIG. 3. (a) Histogram of fluorescence anisotropy values of mRNA in water (open circles) and 25 mM NaCl (filled circles). (b). Mean anisotropy of mRNA as a function of NaCl concentration. The associated standard deviation is also shown. (c) Anisotropy histogram for mRNA in water (open circles) and in RNA hybridization buffer (RHB) (filled squares).

shows an interesting initial increase followed by a decrease as a function of the salt concentration. This might be because, at low salt concentrations, the mRNA acts like a random coil leading to a smaller SD. This results in different mRNA molecules appearing identical. However, the incremental increase of salt concentration could result in a fraction of mRNA molecules forming secondary structures, thereby increasing the SD. A further increase in salt concentration leads to compaction of all mRNA molecules, leading to a decrease in the observed heterogeneity. The marginal changes in SD suggest two possibilities: (i) the conformational heterogeneity is minimal when compacted or (ii) the conformational fluctuations are fast on the observation timescales. To test this, experiments were carried out in RNA hybridization buffer (RHB), which is known to conformationally lock RNA secondary structure, thus trapping the mRNA molecules in their existing conformational states. The distribution of anisotropy value for mRNA in water and in RHB [Fig. 3(c)] reveals a decrease of the mean anisotropy in RHB along with a remarkable increase in SD. While the decrease in anisotropy precludes any aggregation of mRNA, the increase in SD confirms that the existence of many possible mRNA conformations. This reaffirms that mRNA structures are much more conformationally dynamic, at least within the order of the 10 ms measurement timescales, in salt solutions as compared to RHB.

In conclusion, we have developed a methodology using fluorescence anisotropy to detect the hydrodynamic volume of individual mRNA molecules. Using this approach we show that mRNA exist in a broad range of conformations. The mean anisotropy of mRNA is lowered on addition of salt without affecting the spread. The intrinsic heterogeneity of conformations is revealed by freezing conformational transitions by the addition of hybridization buffer, which results in a drastic increase in the spread of the anisotropy. In our earlier study,[5] we have shown that antisense targeting of luciferase mRNA to a site other than the ribosome-binding site reduces the rate of translation, as a result of secondary structure formation. Stabilizing or destabilizing the secondary structure using mutations showed a clear correlation in ribosomal motor movement.[5] Thus, an assessment of the conformational distribution in mRNA could prove valuable in understanding regulation in translational control that could result in differential protein expression. In addition, evaluation of temporal conformational fluctuations in mRNA could find implications as a molecular switch controlling protein synthesis. Despite degeneracy in anisotropy values between conformations, the present technique can be used to elucidate conformational heterogeneity in a variety of RNA assemblies.

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[7] A transcription reaction (100 μl) was carried out using T7 Luciferase control DNA (Bangalore genei) and T7 transcription kit (Promega). The mRNA was labeled by mixing Alexa488-labelled rUTP (molecular probes) with unlabeled rUTP supplied with the kit in a 1:20 ratio. The labeled mRNA transcripts were purified using RNeasy kit (Qiagen). The transcripts were then diluted 50-fold in nuclease-free de-ionized water for subsequent measurement of anisotropy time series. Critical dimensional experiments confirmed that introduction of the fluorescent label did not perturb mRNA structure.
[8] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy* (Kluwer Academic/Plenum, New York, 1999).
[9] Translation diffusion related measurements using fluorescence correlation spectroscopy (FCS) on mRNA as a function of salt concentration proved to be less sensitive to conformational heterogeneity than rotation related measurements outlined in this letter.
[10] Data analysis: Two different but synchronized counters are used to acquire the fluorescence intensity of the parallel ($I_{\|}$) and perpendicular ($I_{\perp}$) components of fluorescence time series and is passed through a filter (computer program written in *LabView*) to separate the mRNA signal from the background fluorescence. The filtered data yields a series of fluorescence peaks, which is obtained from different labeled mRNA molecules. Each fluorescence peak is averaged to find the mean $I_{\|}$ and $I_{\perp}$ and are subsequently used to calculate the fluorescence anisotropy of the given mRNA molecule.