Analytical Biochemistry 389 (2009) 52–62

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Characterization of a fluorophore binding RNA aptamer by fluorescence correlation spectroscopy and small angle X-ray scattering

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ARTICLE INFO

Article history:
Received 24 January 2009
Available online 20 March 2009

Keywords:
Fluorescence correlation spectroscopy
Fluorescence cross-correlation spectroscopy
Small angle X-ray scattering
Dynamic light scattering
RNA aptamer
Sulforhodamine B
Patent blue V

ABSTRACT

Using fluorescence correlation spectroscopy (FCS), we have established an in vitro assay to study RNA dynamics by analyzing fluorophore binding RNA aptamers at the single molecule level. The RNA aptamer SRB2m, a minimized variant of the initially selected aptamer SRB-2, has a high affinity to the disulfonated triphenylmethane dye sulforhodamine B. A mobility shift of sulforhodamine B after binding to SRB2m was measured. In contrast, patent blue V (PBV) is visible only if complexed with SRB2m due to increased molecular brightness and minimal background. With small angle X-ray scattering (SAXS), the three-dimensional structure of the RNA aptamer was characterized at low resolution to analyze the effect of fluorophore binding. The aptamer and sulforhodamine B–aptamer complex was found to be predominantly dimeric in solution. Interaction of PBV with SRB2m led to a dissociation of SRB2m dimers into monomers. Radii of gyration and hydrodynamic radii, gained from dynamic light scattering, FCS, and fluorescence cross-correlation experiments, led to comparable conclusions. Our study demonstrates how RNA–aptamer fluorophore complexes can be simultaneously structurally and photophysically characterized by FCS. Furthermore, fluorophore binding RNA aptamers provide a tool for visualizing single RNA molecules.

Fluorescence-based techniques provide insight into the characteristics of proteins and nucleic acids in vitro and in vivo. Green fluorescent protein (GFP)[1] and its derivatives are nearly ideal tools to investigate, for example, the expression and function of proteins, but an equivalent approach for the investigation of nucleic acids is not yet available. Apart from chemical labeling with a dye, two other techniques are mainly used to visualize RNA in fluorescence spectroscopy and microscopy. In one case, the RNA is hybridized to a molecular beacon, a fluorescence-tagged complementary probe [1–3]. In the other case, the RNA is genetechnologically fused to an RNA sequence, binding to a bacteriophage MS2 coat protein, which is fused to GFP itself [4,5]. A similar approach provided by fluorophore binding RNA aptamers would reduce the tag size, similar to the development of biarsenical dyes in protein labeling [6], avoiding distortions of RNA behavior [7–9]. To establish this, fluorophore binding RNA aptamers could be genetechnologically fused to a second RNA of interest. This would also lead to reversible labeling by membrane-permeable dyes and enable RNA detection independent of protein synthesis. Changes in fluorescence emission of fluorophores due to binding to RNA aptamers were examined in previous studies [9–11]. The aim of the current work was to broaden the spectrum of RNA aptamer applications in exploring RNA dynamics at the single molecule level. Therefore, we studied fluorophore–RNA aptamer interaction by fluorescence correlation spectroscopy (FCS) [12,13]. With this method, dynamics in the range of diffusion coefficients between 10^{-6} and 10^{-9} $cm^2\,s^{-1}$ can be measured, thereby allowing one to characterize short RNAs as well as messenger RNAs (mRNAs) of several thousand nucleotides. Although ensemble measurement methods, such as fluorescence anisotropy and lifetime, could characterize fluorophore–RNA complexes, we used the single molecule technique FCS to enable the detection of single RNA molecules (e.g., single gene transcripts) in vivo. In addition, structural information could be gained by FCS. Besides translational diffusion, rotational diffusion [14] also allows one to monitor conformational changes more exactly, especially in living cells. However, because rotational diffusion time scales of small molecules, such as the RNA aptamers investigated in this study, may interfere with

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[1] *Abbreviations used:* GFP, green fluorescent protein; FCS, fluorescence correlation spectroscopy; mRNA, messenger RNA; SRB2m, minimized sulforhodamine B binding RNA aptamer; PBV, patent blue V; FCCS, fluorescence cross-correlation spectroscopy; SAXS, small angle X-ray scattering; AOTF, acousto-optic tunable filter; R6G, rhodamine 6 green; PCR, polymerase chain reaction; DEPC, diethylpyrocarbonate; DLS, dynamic light scattering; DTT, dithiothreitol; tRNA, transfer RNA; MW, molecular weight; cpm, counts per molecule; rRNA, ribosomal RNA.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.03.018

antibunching time, the dead time of the detector, or the triplet relaxation time, translational diffusion seems to be more easily accessible to reveal the hydrodynamic radius [15].

For our study we chose the minimized sulforhodamine B binding RNA aptamer, SRB2m, which binds the disulfonated xanthene dye sulforhodamine B with high affinity and specificity (Fig. 1A) [9]. Sulforhodamine B is a photostable dye of high molecular brightness (quantum yield of 0.70) with absorption and emission maxima at 565 and 586 nm, respectively [16,17]. It is known that the nonminimized RNA aptamer SRB-2 is able to recognize a second dye, patent blue V (PBV) [10]. PBV absorbs and emits photons at higher absorption and emission wavelengths with maxima at 638 and 666 nm, respectively. On binding to SRB-2, the fluorescence emission of PBV is strongly increased. The chemical structure of PBV is similar to that of sulforhodamine B, lacking only the linking oxygen atom between two phenyl rings and carrying an additional hydroxyl group at the phthalein ring (Fig. 1B). Our results show that, despite the low quantum yield of PBV of 3.9×10^{-4}, it is a suitable dye for single molecule spectroscopic studies in combination with SRB2m. To analyze possible conformational changes induced by target binding, the molecular size, oligomerization state, and three-dimensional structure of SRB2m, unbound and bound to the fluorophores, were characterized by FCS, fluorescence cross-correlation spectroscopy (FCCS), and small angle X-ray scattering (SAXS).

Materials and methods

Fluorescence correlation spectroscopy

Using FCS, the residence time τ_{Diff} of a fluorescent molecule in a detection volume of a confocal microscope is determined [12,13]. In the intensity-normalized autocorrelation function $G(\tau) = \langle \delta F(t+\tau)\delta F(t)\rangle / \langle F(t)\rangle^2$, the similarity of fluorescence intensities over a time trace is calculated.

With

$$\tau_{Diff} \approx \sqrt[3]{m}, \tag{1}$$

diffusing species can be identified separately if the molecular masses m of the species differ by a factor of 8 or more [13,18]. FCS experiments were carried out using ConfoCor2 (Carl Zeiss, Jena, Germany) [19]. The fluorophore sulforhodamine B was excited by a HeNe laser at 543 nm with 1.2 mW maximum output and was regulated by an acousto-optic tunable filter (AOTF) to a value of 15%. A C-Apochromat 40×, 1.2 NA water immersion objective (Carl Zeiss) focused the excitation light into the object plane. The emitted light from the detection volume was separated by both a dicroic mirror (HFT 543 nm) and an emission filter (BP 585–615 nm). The pinhole diameter at the image plane in front of the photodiode was adjusted to 78 μm. The dye PBV was excited with 3% AOTF of a HeNe laser at 633 nm with 5.0 mW maximum power. For FCS measurements with PBV, HFT 633 and the emission filter LP 650 were used, setting the pinhole to 90 μm. FCCS experiments were performed with an HFT 488/633 and the emission filters LP 605 and BP 505–530 nm. An avalanche photodiode detected the fluorescence signal (SPCM-AQR-13-FC, Perkin Elmer, Wiesbaden, Germany).

With the help of Carl Zeiss software, the measured intensity-normalized fluorescence autocorrelation function $G(\tau)$ was fit to a model describing free three-dimensional diffusion and triplet excited state as

$$G(\tau) = 1 + \frac{1}{N}\cdot\left(\frac{1 - T + Te^{-\frac{\tau}{\tau_T}}}{1-T}\right) \cdot\left\{\sum_{i=1}^{n}\frac{f_1}{\left(1+\frac{\tau}{\tau_{Diff;i}}\right)\sqrt{1+\frac{\tau}{\tau_{Diff;i}}S^{-2}}}\right\} \tag{2}$$

to determine the number of particles N, the diffusion time $\tau_{Diff;\,i}$, and the fractional population f_i of n different diffusion species using the correlation or lag time τ [19]. The structure parameter S describes the ratio of the radial and axial distances between the maximum and $1/e^2$ laser intensities, $S = z_0/r_0$. Fluctuating intensities of the fluorescence, originating from the triplet excited state, were included in the fitting model by τ_T, the decay time of the triplet state, and T_T, the fractional population of the triplet state. The parameters were derived from a Levenberg–Marquardt algorithm [19]. The reliability of the chosen model was determined by χ^2 tests and the shape of the residual deviation curve of the fit from the autocorrelation function. To define the optical setup more accurately, the autocorrelation function of a standard fluorophore was measured using the same laser and filter system as in the planned experiment. The structure parameter S was determined with rhodamine 6 green (R6G), Alexa488, or Cy5 and was kept fixed during the experiment.

The radial distance to the center of the laser beam focus r_0 was calculated as

$$D_i = r_0^2/4\tau_{Diff,i} \tag{3}$$

with the known diffusion coefficient D_i of R6G ($D = 2.8 \times 10^{-10}$ m^2 s^{-1} [19]), Alexa488 ($D = 2.8 \times 10^{-10}$ m^2 s^{-1} [20]), or Cy5 ($D = 3.16 \times 10^{-10}$ m^2 s^{-1} [19]) and the diffusion time $\tau_{Diff;i}$ to the value of 169, 189, or 250 nm for excitation at 488, 543, or 633 nm, respectively.

To calculate the complex fraction Y, the concentrations of complex [C] and free fluorophore [F] were used in the equation

$$Y = \frac{[C]}{[C]+[F]}. \tag{4}$$

A correction of Y into Y' based on changes of molecular brightness occurred in the equation

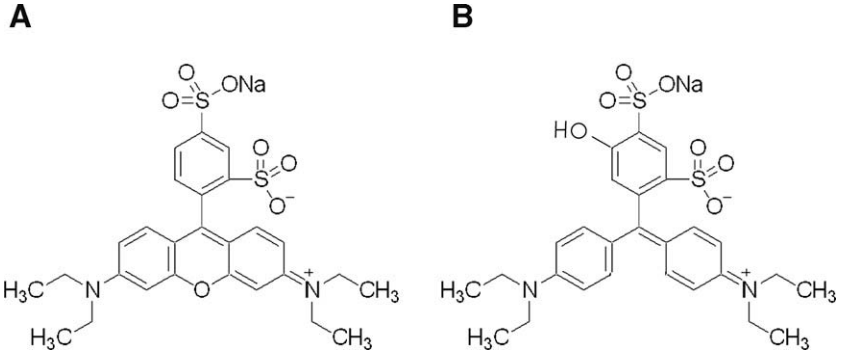

Fig. 1. Chemical structures of sulforhodamine B (A) and patent blue V (B).