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Sensing and inhibition of amyloid-β based on the simple luminescent aptamer–ruthenium complex system

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ABSTRACT

Aggregation of amyloid-β (Aβ) peptide has been known to be pathologically associated with Alzheimer and dementia diseases. Amyloid-β fibrils serve as an important target for the drugs development and diagnosis of neurodegenerative diseases. Herein, we report a new [Ru(dmbpy)(dcbpy)dppz)] complex (dmbpy; 4,4′-dimethyl-2,2′-bipyridine, dcbpy; 4,4′-dicorboxy-2,2′-bipyridine, dppz; dipyridophenazine) intercalated aptamer based recognition of amyloid-β. Interestingly, aforementioned Ru(II) complex shows weak luminescence intensity in the aqueous medium but it shows strong luminescence intensity in the presence of RNA aptamer. Upon addition of amyloid-β monomers, the luminescence intensity of Ru(II) complex is reduced due to the strong interaction of aptamer with amyloid-β monomer/small oligomers. Furthermore, present study implies that our system has ability to inhibit the formation of amyloid-β fibrils, which is confirmed from the AFM morphological structures in the absence and presence of aptamer. This work may contribute the rapid diagnosis and inhibition of amyloid-β aggregation in the clinical applications.

1. Introduction

Amyloid-β, an extracellular protein fragment, is self-assembled to form aggregates (amyloid fibrils), which is implicated in many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and type II diabetes [1,2]. Alzheimer's disease is a common amyloidosis disease, characterized by severe cognitive dysfunction and memory impairment leads to complete loss of independent living and self-carrying capacity [3,4]. In addition, amyloid-β fibrils are potential reservoir for low molecular weight oligomers and form amyloid plaque, which is the primary neurotoxic agent and responsible for neuronal cell death [5]. However, the recent investigations clearly indicate that soluble amyloid oligomers are the most cytotoxic aggregates, as compared with amyloid fibrils [6,7]. Further, amyloid oligomers form channel-like structures, which consequently bind to cell membranes [8] leading to the destruction of synaptic functions [6,9] and disruption of the ionic gradient in cells [10]. Generally the oligomers are soluble in aqueous medium and become insoluble if they are attached to the cell membrane or other macromolecules owing to the formation of amyloid plaque [11,12].

Nowadays, according to the World Alzheimer Report more than 35 million people are suffering in worldwide with dementia and it is expected to double in 2030 [13]. Until now, there is no effective treatment to delay or slow down the progression of Alzheimer's disease. Therefore highly efficient sensor and inhibitor for amyloid fibrils are urgent in healthcare. Numerous organic molecules have been identified as inhibitors and sensor for amyloid-β fibrils [14–19]. Switching of amyloid-β peptide to fibrils is mostly exploited through thioflavin T and Congo Red through luminescence readout for many years due to its specific binding with amyloid-β fibrils [20,21]. Of late, the epigallocatechin-3-gallate (EGCG), a potential antioxidant polyphenol present in green tea, has been reported to modulate the misfolding of prion proteins [22]. Recently, fluorescence self-quenching of HiLyte Fluor 555 labeled amyloid-β peptides has been demonstrated to monitor the self-assembly process of amyloid aggregation in real-time analysis [23]. Apart from the organic luminophores, some metal complexes are also bind with amyloid-β and inhibit the fibril formation. Lim et al. [24] reported that the amyloid-β fibrils have been recognized by luminescence enhancement using bis (thiosemicarbazonato)copper(II) complex. In addition, Jozsa et al. [25] have demonstrated the utility of Cu(II), Ni(II) and Zu(II) complexes for the recognition of amyloid fibrils through spectroscopic measurements. Moreover, the light switch dipyridophenazine ruthenium(II) complex $[Ru(bpy)_2(dppz)]^{2+}$ has been used as sensor for amyloid

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aggregation [26]. Latterly our group reported the Re(I) complex as sensor for amyloid fibrils through the luminescence method [27]. However, the identification of amyloid small oligomers remains challenging owing to the formation of different morphological structures [28].

Single-domain antibody and B10 antibody have been developed for the detection of amyloid oligomer selectively and inhibit the amyloid fibril formation [29,30]. In this manuscript, we elucidate the application of the RNA aptamer–Ru(II) complex system for specific recognition of amyloid monomer and inhibit the oligomer/fibril formation. Aptamers are DNA or RNA molecules that can specifically bind to a wide range of targets from small molecules to whole cells [31] that can be selected through an in vitro selection method known as systematic evolution of ligands by exponential enrichment (SELEX). Aptamers are more opportune than antibodies, owing to the ease of modification, viable labeling with useful small probe molecules (dye, drug and biotin), renaturing and designing structural changes. The binding efficacy and specificity of aptamers are comparable to those of antibodies [32]. Recently, Takashi et al. [33] have reported that the aptamer selectively binds with monomer and soluble oligomers of amyloid-β; however, it requires long preparation time, high cost, skilled technicians and complex mechanism. Our recent research interest is on the protein binding properties and design of biosensors using luminescent metal complexes [34–39]. This prompted us to develop sensor for amyloid-β aggregation. To eradicate these issues, we demonstrate a simple and selective detection of amyloid-β monomers and inhibit the formation of amyloid fibrils through the aptamer–[Ru(dmbpy)(dcbpy)dppz)] complex system without any modification/labeling. The aptamer–Ru(II) complex system has the capability to bind with amyloid-β monomer and small oligomers as compared to other proteins. To the best of our knowledge, this is the first report on simple label free [Ru(dmbpy)(dcbpy)dppz)]–aptamer system as sensor and inhibitor for amyloid-β.

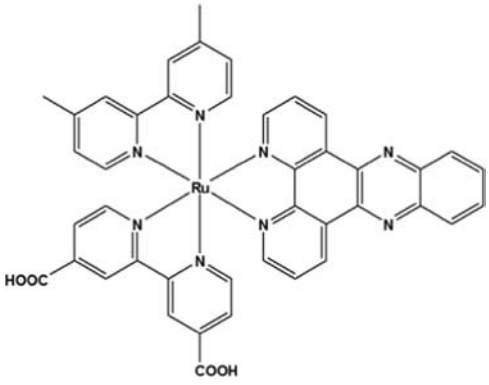


Chart 1. Structure of Ru(II) complex used.

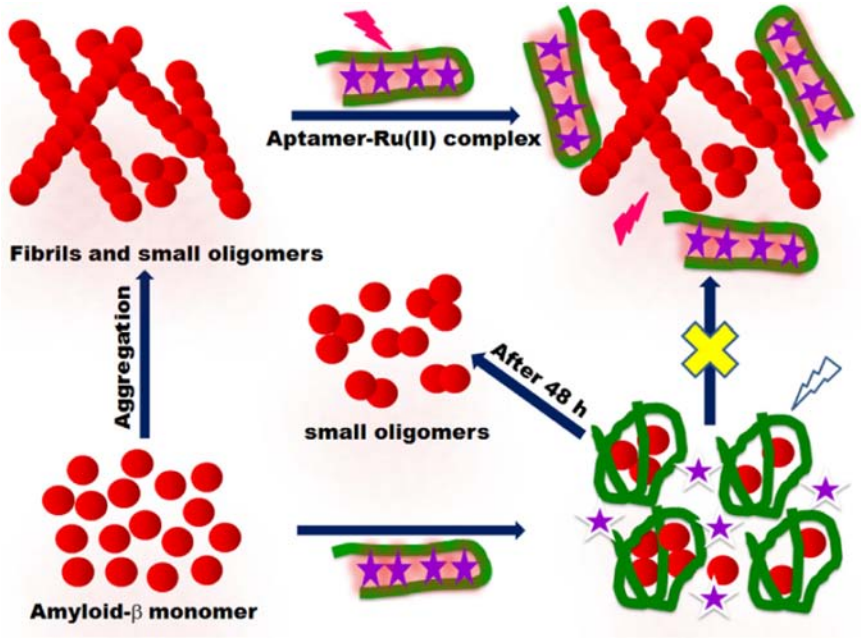
2. Experimental

2.1. Chemicals and materials

Thrombin, BSA (bovine serum albumin), lysozyme, myoglobin, cytochrome C and PDGF (platelet derived growth factor) were purchased from Sigma-Aldrich and amyloid-β was obtained from Cal Bio Chem, USA. Sodium chloride, potassium chloride, disodium phosphate, sodium phosphate, ammonia solution and all the solvents purchased from Merck were used as such. The [Ru(dmbpy)(dcbpy)dppz)] (Chart 1) complex was synthesized using the previous literature [40–42] and characterized by ^{1}H NMR and mass spectral techniques, the data are shown in Supporting information (Fig. S1, S2). The amyloid binding aptamer [33], 5′-GGGAUGUUCU AGGCGUUGAUGAUAGC-GUAUGCAACUCUCCUGGGACCCCCGCCGGAUGGCCACAU CCAGAGUGG-CAUAUUGAUCCGA-3′, was purchased from Ocimum Biosolutions Ltd. (Hyderabad, India). All the samples were prepared using 10 mM PBS buffer pH 7.4 (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 800 mL of Milli-Q water).

3. Methods

The concentration of aptamer was verified spectrophotometrically by monitoring the absorbance at 260 nm, on an Analtikjena Specord S100 diode-array spectrophotometer. The luminescence



Scheme 1. Schematic representation of the amyloid-β peptide fibril formation and inhibition of aggregation using aptamer–Ru(II) complex.

spectra were recorded using a JASCO FP6300 Spectrofluorimeter in ambient temperature (298 K) and the 1 cm path length cuvette. All the fluorescence quenching measurements were carried out under aerated condition. The atomic force microscopy (AFM) image was obtained by A100 SGS AFM instrument operating in the non-contact mode working at 100 kV. Circular dichroism (CD) measurements were performed on a JASCO J810 spectropolarimeter at RT over the wavelength 200–400 nm. Parameters were set as: path length, 50 mm; resolution, 0.5 nm; scan speed, 50 nm min^{-1}; band width, 1 nm; response 1 s.

Atomic force microscopy (AFM): Freshly cleaved mica sheet was washed two to three times with 600 μL Milli-Q water. A sample of 20 μL of diluted amyloid sample adsorbed on the mica sheet for 20 min was washed with deionized water (3 × 30 μL), and dried overnight. The mica sheet is glued to a microscope slide; AFM images were recorded under non-contact mode. Similar experiments were performed for amyloid-β in the presence of aptamer and the details of sample preparation are given below.

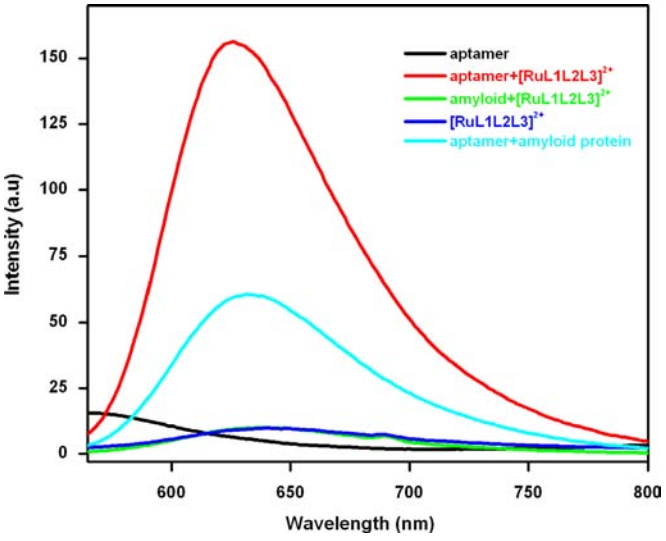


Fig. 1. Luminescence spectra of Ru(II) complex in the absence and presence of amyloid. The concentration of Ru(II) complex, aptamer and amyloid-β are 1×10^{-7}, 1×10^{-10} and 80×10^{-6} M respectively.

Sample preparation and amyloid aggregates: The Ru(II) complex was prepared in 5% acetonitrile/PBS buffer and all the proteins were prepared in 10 mM PBS buffer. The amyloid-β monomers were incubated for 1, 4, and 48 h at room temperature with the aptamer–Ru(II) complex system, then the luminescence experiments and AFM measurements were performed. Amyloid-β fibrils were obtained after 4 h agitation of amyloid-β monomer solution and the above experiments were performed.

4. Results and discussion

In accordance with the previous reports, the $[Ru(bpy)_2dppz]^{2+}$ complex is used as a promising fluorogenic moiety for DNA sequence analysis [26]. Ru(II) complexes containing dppz ligand are well known DNA light switching probes. Recently, Cook et al. [26] demonstrated $[Ru(bpy)_2dppz]^{2+}$ complex as sensor for amyloid aggregation *via* luminescence property. This light switch probe shows strong interaction with the fibril framework and a poor interaction to amyloid monomers. Therefore we aimed to introduce steric/electron withdrawing group into the Ru(II) complex in order to absorb low energy radiation and increase the dissociation constant with aptamer to enhance the sensitivity of the sensor strategy. The steric factor may decrease the binding efficacy with aptamer or easily dissociate upon the addition of targets.

Accordingly, we have attempted to recognize and inhibit the amyloid-β peptide through the simple combination of RNA aptamer with light switching [Ru(dmbpy)(dcbpy)dppz)] complex by luminescence signal. The structure of the [Ru(dmbpy)(dcbpy) dppz)] complex and the schematic representation of amyloid sensor are shown in Scheme 1. The luminescence intensity is very low for free [Ru(dmbpy)(dcbpy)dppz)] complex in the aqueous buffer medium owing to the triplet MLCT (metal-to-ligand charge transfer) excited state is effectively quenched by hydrogen binding between water and the phenazine nitrogen moiety of the ligand. But, the significant luminescence enhancement is observed along with 10 nm blue-shift when it is bound with RNA aptamer due to the protection of probe from the solvent environment [43]. On the other hand, upon the addition of amyloid-β the luminescence intensity is decreased due to the stronger binding of aptamer

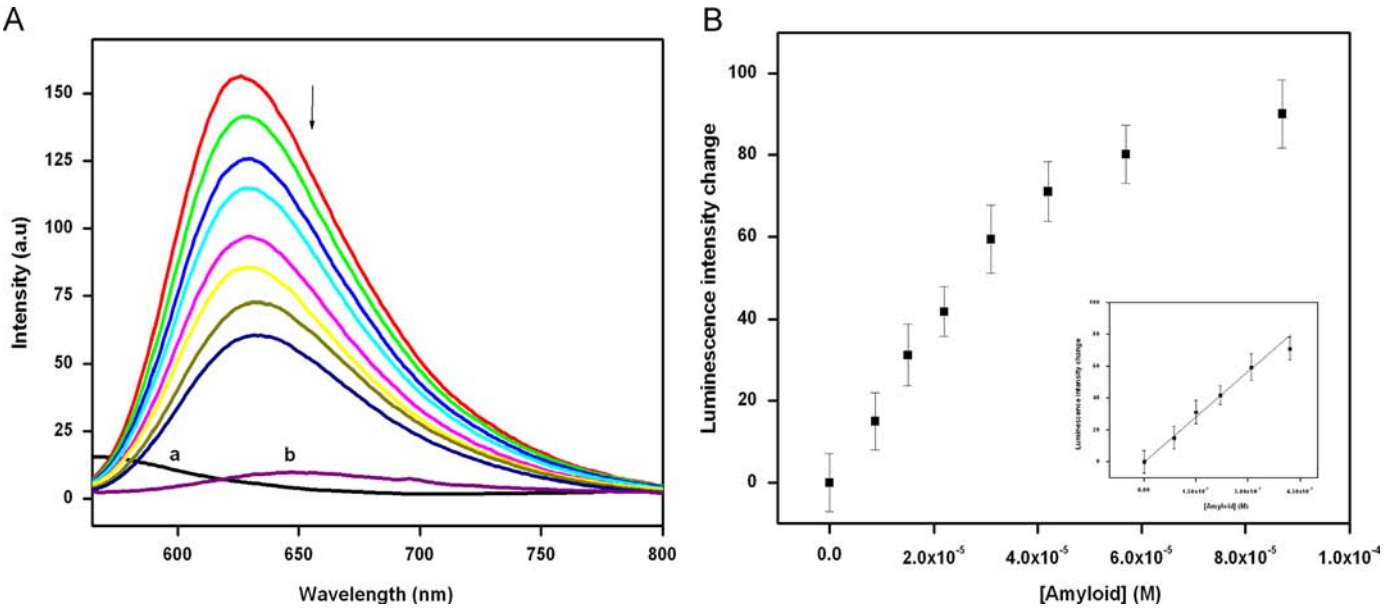


Fig. 2. Luminescence titration spectra of Ru(II) complex (A) towards amyloid protein. (a) Aptamer alone, (b) complex alone. (B) Calibration plot amyloid sensor. The concentration of Ru(II) complex, aptamer and amyloid-β are 1×10^{-7} M, 1×10^{-10} M and 0–80 μM respectively.

with amyloid proteins that leads to the dissociation of aptamer–[Ru(dmbpy)(dcbpy)dppz)] complex.

4.1. Luminescence studies

A simple luminescence assay is performed to determine the interaction of RNA aptamer with amyloid-β peptide by utilizing Ru (II) complex. A sample of 100 μL (100 pM) of RNA aptamer is incubated with a series of amyloid-β peptide sample (0–80 μM) for 1 h followed by excitation of the probe at 460 nm and the luminescence intensity change is recorded. The luminescence spectra of the aptamer–[Ru(dmbpy)(dcbpy)dppz)] complex system in the presence and absence of amyloid-β are shown in Fig. 1.

Luminescence intensity is decreased upon the addition of amyloid-β into the aptamer–Ru(II) complex system due to the strong binding of amyloid-β monomer and soluble oligomers with aptamers [33]. Now the [Ru(dmbpy)(dcbpy)dppz)] complex is displaced from the aptamer double helix structure and the accessibility of solvent water molecule is increased leading to the quenching of luminescence intensity. The interaction between amyloid monomer and [Ru(dmbpy)(dcbpy)dppz)] complex is also examined by adding the Ru(II) complex with amyloid monomer. It shows the negligible change in the luminescence intensity of Ru(II) complex in the presence of amyloid monomer which indicates the weak interaction between probe and amyloid monomer [26]. On the hand, the light switching dppz complex binds with fibrils and increases the luminescence intensity [26]. Initially amyloid-β solution contains major amount of monomers and minor amount of small oligomers. The amyloid aggregation starts after a few minutes; the small fibril aggregates act as seeding for the further assembly of Aβ fibrillar structures. Therefore, the above discussion indicates the absence of amyloid fibrils in the monomer solution. Fig. 2a shows the luminescence titration spectra of successive addition of amyloid protein into the aptamer–Ru(II) complex solution. Upon increasing the concentration of amyloid-β protein, the luminescence intensity is gradually decreased due to the strong binding of aptamer with amyloid protein. The detection limit (50 nM) is calculated from the linear range of the luminescence titration curve shown in Fig. 2b. This sensor strategy shows better sensitivity than the previous report [32] and the binding constant value is $4.1 \times 10^6\ M^{-1}$ (Fig. S3).

4.2. AFM analysis

We have also analyzed the formation and inhibition of amyloid-β aggregation by atomic force microscopy (AFM). AFM is a powerful technique to determine the morphological parameters of amyloid fibrils [45]. AFM facilitates the comparison of amyloid structure in a size-dependent manner in the absence and presence of aptamer. After the incubation period the samples of amyloid-β are taken on freshly cleaved mica surfaces and scanned at different μm areas of amyloid fibrils using the AFM technique. The formation of amyloid fibrils is confirmed before the incubation of aptamer through structural morphology of AFM image shown in Fig. 3A. The AFM images show fibrils of identical morphology with varying length of amyloid fibrils. All the images are taken from the aliquots of amyloid samples after 4 h incubation. From these images we can clearly observe the length and the height of the individual fibrils. These results confirmed the transformation of α-helical to β-sheet structure of amyloid fibrils with various width sizes 20, 23 and 27 nm and various lengths ~500 nm–1 μm (Fig. S4). Many groups have reported the similar type of results with various size of amyloid fibrils [46].

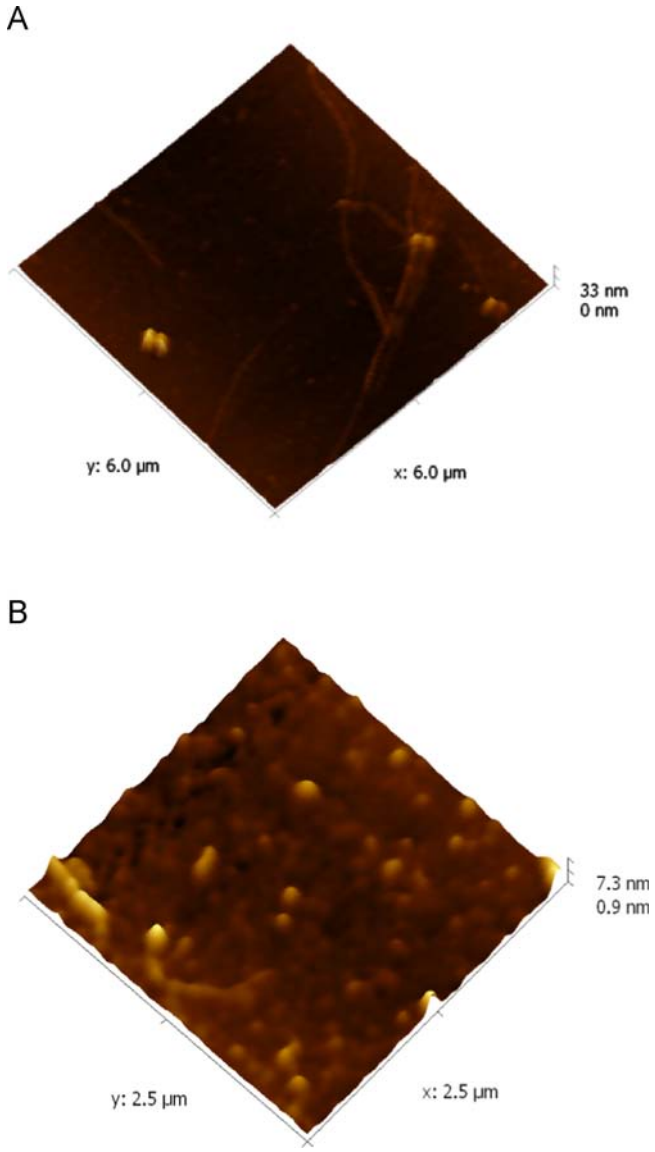


Fig. 3. AFM image of amyloid in the absence and presence of aptamer after 4 h incubation.

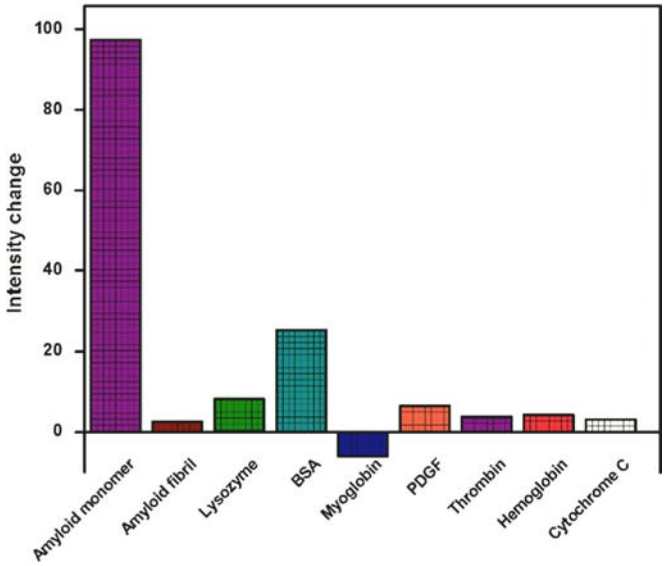


Fig. 4. Luminescence decrease of 1×10^{-7} M Ru(II) complex upon the additions of various proteins and the concentration of proteins are 80×10^{-6} M. Fluorescence intensities were recorded at 613 nm with an excitation wavelength of 460 nm.

Further the amyloid-β peptide is incubated with aptamer for 4 and 48 h and then the samples are taken for AFM analysis. It clearly indicates that the aggregation is inhibited in the presence of aptamer as shown in Fig. 3B. A significant number of amyloid fibrils and small oligomers are observed in the incubation of amyloid-β peptide alone. In contrast, considerable reduction in the size of amyloid fibrils is observed after incubation of aptamer with amyloid-β (~10 nm). These results indicate that the RNA aptamers can inhibit the amyloid-β fibril formation. Consequently, the aptamer can bind not only to the monomeric form of amyloid-β peptide but also to the small oligomers, which controls the further elongation/aggregation. This suggests that nearly complete inhibition of amyloid-β aggregation is exhibited in the presence of aptamer (Fig. 3B). The observed results are matched well with previous reports on inhibition of amyloid aggregation in the presence of aptamer [47]. However, the amyloid oligomers of average size of 10–20 nm were observed after 48 h incubation of amyloid-β with aptamer (Fig. S5). The matured amyloid fibrils are rarely observed in the AFM image in the presence of aptamer. The AFM analysis clearly shows the effective reduction of amyloid fibrils in the presence of RNA aptamer.

4.3. Interference and selectivity

To confirm the selectivity of amyloid-β sensor strategy towards amyloid monomer, we added 80 μM of each sample of some common extracellular proteins such as lysozyme, BSA, PDGF, thrombin, myoglobin, hemoglobin and cytochrome C into the aptamer-probe solution. There is no considerable luminescence intensity change in the presence of other proteins, which indicates that these proteins do not interfere with the detection of amyloid-β. An enormous luminescence intensity change is observed only in the presence of amyloid-β monomer compared to fibrils and other extracellular proteins. These results suggest that this aptamer–Ru(II) complex system selectively binds with the amyloid monomer (Fig. 4).

4.4. CD spectral study

To determine whether the amyloid-β influences the conformation of aptamer, we have measured CD spectra of aptamer in the absence and presence of amyloid-β. The CD spectrum of RNA aptamer in the absence of amyloid-β is shown in Fig. S6, which indicates the positive band at 217 nm and 270 nm and the negative peak at 240 nm respectively. In the presence of amyloid-β the conformational change of aptamer is shown in Fig. S6 resulting in the disappearance of positive peak at 217 nm. This result clearly demonstrates that the aptamer binds with amyloid-β protein and undergoes conformational change. The above results are in good agreement with previous report [48].

5. Conclusions

In this investigation, we have employed the new aptamer–Ru(II) complex system toward amyloid-β sensor. This system shows the stronger inhibitory activity toward aggregation of the amyloid-β peptide, because of its specificity and strong association with amyloid-β monomer. The formation and inhibition of amyloid aggregates are clearly investigated through the AFM analysis. From the AFM images, we believe that the morphology of amyloid aggregation is controlled in the presence of aptamer. This strategy serves as simple, selective sensor and inhibitor for amyloid-β aggregates.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.11.020.

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