

## Short Communication

## Polyanion induced circular dichroism of Thioflavin T \*

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**Abstract.** Thioflavin T (ThT) is amyloid specific fluorescence dye possessing the properties of molecular rotor. We have shown that Thioflavin T forms complexes with non-peptide polyanions heparin, polyadenylate and polystyrene sulphonate by means of absorption spectroscopy. In the presence of chiral polyanions – heparin and polyadenylate – induced optical activity of ThT occurs whereas interaction with achiral polystyrene sulphonate (PSS) does not lead to production of induced circular dichroism signal. The positively charged ThT forms centre for ordered binding of chiral polyanion. Similarly, complexation of structurally different chromophore 9-aminoacridine with polyanions has led to induction of optical activity only in the presence of chiral ones. We suggest that, primarily, the optical activity of environment plays important role in inducing optical activity of achiral compounds.

**Key words:** Thioflavin T — Induced circular dichroism — Polyanion

Thioflavin T (ThT) – 3,6-dimethyl-2-(4-dimethylaminophenyl)-benzothiazolium cation – is a fluorescence dye, generally used for staining amyloid tissues or detection of amyloid fibrils in solutions (Waldrop et al. 1984). Scheme of chemical structure of ThT is on the Fig. 1. The binding of ThT to amyloid aggregates causes the occurrence of fluorescence emission in the 475–600 nm region after excitation in the 440–450 nm region. In contrast, very low emission quantum yields of ThT are observed in the presence of native proteins as well as unfolded or partially folded monomeric protein conformations (Naiki et al. 1989). Since ThT binding only slightly affects the early stages of fibrillization (Fodera et al. 2008), this method is also suitable for *in situ* fibril detection and monitoring of kinetics of fibril formation.

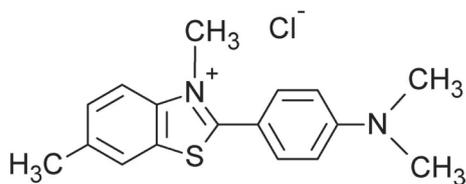
In spite of extensive study the precise mechanism of ThT interactions with biosystems is not understood in detail yet. Several studies on the mechanism of ThT-fibril interactions have been reported (Khurana et al. 2005; Groenning et al.

2007a; Fodera et al. 2008).  $\beta$ -sheets, as major components of amyloid fibrils, contain cavities and channels, which can serve as possible ThT binding sites. ThT emission changes after binding to amyloids were attributed to formation of highly fluorescent dimers (Raj and Ramaraj 2001; Groenning et al. 2007b), excimers (Raj and Ramaraj 1997), or micelles (Khurana et al. 2005). Recent findings suggest another mechanism based on ThT behavior as molecular rotor (Stsiapura et al. 2008). For the molecular rotors, fluorescence quantum yield significantly increases with increasing viscosity or rigidity of microenvironment due to the decreased torsional relaxation in the molecule. Thus, free rotation of benzothiazole and benzaminic rings around shared C–C bond is blocked in ThT-amyloid complexes, leading to significant increase of fluorescence yield (Stsiapura et al. 2008). The fluorescent conformer was also observed in complex of ThT with an  $\alpha$ -helical protein acetylcholinesterase, in which planarised conformation of ThT were formed due to the  $\pi$ - $\pi$  stacking interactions with protein aromatic residues (Harel et al. 2008). The fluorescent conformer with lower quantum yield was also observed after binding to  $\beta$ -sheet structure of monomeric non-amyloid protein  $\beta$ 2-microglobulin (Wolfe et al. 2010). The entrapment of ThT molecules in amyloid fibrils leads to formation not only fluorescent conformers, but also to optically active conformers (Dzwolak and Pecul 2005; Lokszejn and Dzwolak 2008). This induced circular dichroism was for example observed for ThT complex with

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**Figure 1.** Scheme of chemical structure of Thioflavin T.

insulin amyloid fibrils (Loksztejn and Dzwolak 2008). However, the induced optical activity of ThT was also found after binding to  $\alpha$ -helical polyglutamic acid lacking the  $\beta$ -sheet structure or aromatic side-chains (Babenko and Dzwolak 2011). Moreover, the polyglutamic acid binds ThT in conditions even before the polypeptide becomes fully  $\alpha$ -helical. The authors (Babenko and Dzwolak 2011) suggest that docking interaction of ThT with different protein targets may be more unspecific as previously assumed.

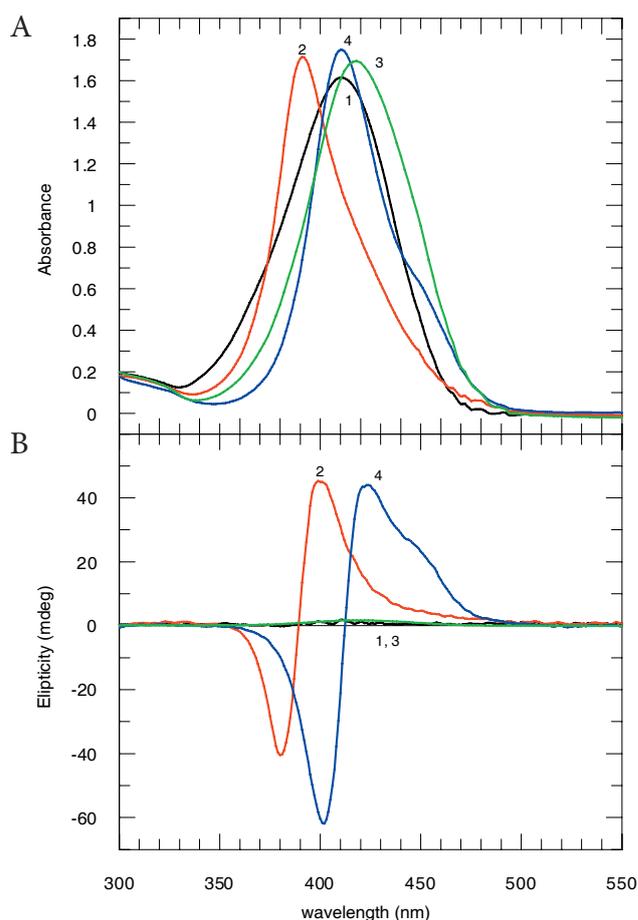
In order to test the possible variability of ThT interactions with other than regular secondary structures, we have studied the complexes of ThT with non-peptide chiral polyanions – heparin and polyadenylate and nonchiral – polystyrene sulphonate (PSS). These polyanions possess different charged groups, hydrophobicity and charge density. Heparin is glycosaminoglycan, consisting of sulphate and carboxylate groups and it is one of the most negatively charged biomacromolecules without regular tertiary structure (Rabenstein 2002). The structurally related glycosaminoglycans such as heparan sulphates are part of blood coagulation pathways (Rosenberg et al. 1997). They form stable complexes with thrombin and antithrombin protease systems and other proteins. True physiological role of heparin in the body remains still unclear. The potential role of heparin in transport of positively charged drugs or other biologically active compounds has been studied.

Nucleic acids represent another voluminous group of polyanions capable to form complexes with small positively charged compounds. One of the simplest polynucleotide is polyadenylate containing negatively charged phosphate groups. The polyadenylate tail is important for the nuclear export, translation, and stability of mRNA (Zhao et al. 1999). Polyadenylate forms high affinity complexes with positively charged isoquinoline alkaloids such as coralyne, berberine, palmatine and sanguinarine (Bhadra and Kumar 2011). Many studies have shown the beneficial effects of berberine on the cardiovascular system and its antiinflammatory potential (Kuo et al. 2004).

Polystyrene sulphonate possess together with negatively charged sulphonate groups also hydrophobic styrene rings. This macromolecule is used as potassium binder for patients suffering from hyperkalaemia – abnormal high blood serum potassium levels (Kessler 2011). It has been shown that PSS

forms complexes with ThT and enhances the formation of ThT excimer (Li et al. 1985).

The interaction of ThT with these selected polyanions has been studied by absorption and CD spectroscopy. The measurements were carried out on JASCO-815 spectropolarimeter. ThT and polyanions were purchased from Sigma-Aldrich, 9-aminoacridine hydrochloride from Serva and other chemicals were of analytical grade and were purchased from Fisher. All chemicals were used without further purification. Fig. 2A illustrates the absorption spectra of ThT at the presence of various polyanions. The absorption band of free ThT at 412 nm corresponds to ThT monomer (Naik et al. 2009). In the complex with polyadenylate, narrower absorption peak occurs with maximum at 412 nm and shoulder at 455 nm. This shoulder probably corresponds to the formation of ThT homodimer as it was previously shown for ordered binding of ThT to the



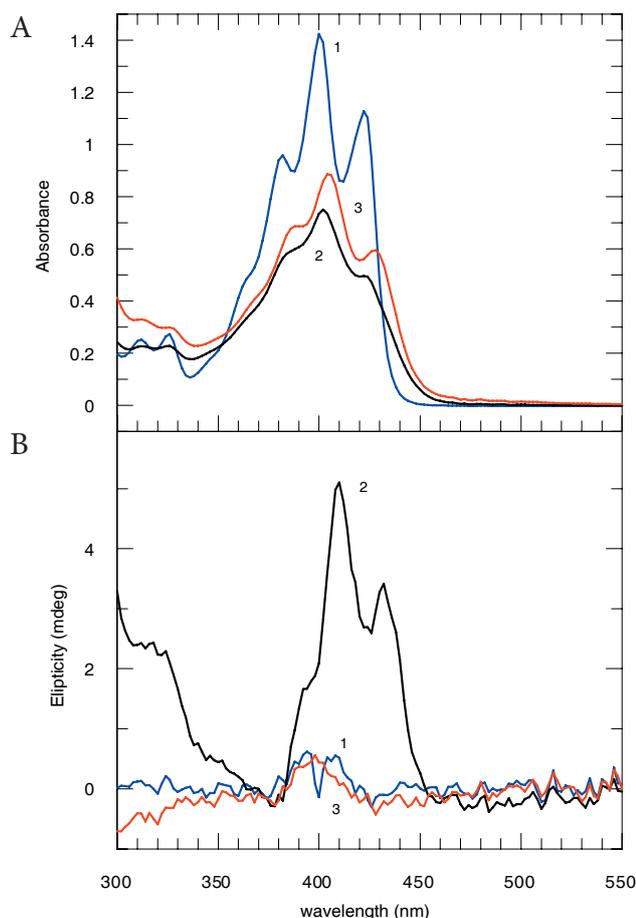
**Figure 2.** Absorption (A) and circular dichroism (B) spectra of free ThT (1) and ThT in the presence of heparin (2), PSS (3) and polyadenylate (4) in 10 mM phosphate buffer (pH 6.5). The concentrations of used compounds: ThT – 700  $\mu$ M, polyanions – 1.0 mg/ml except for PSS – 0.27 mg/ml.

macrocyclic molecules of curcubit [8]uril (Mohanty et al. 2009) or as for partially forbidden transition of dimerized molecule of freezing aqueous solutions (Schirra 1985). In the presence of PSS, the absorption maximum is shifted to 418 nm. Similar shift was observed for ThT spectra in less polar environment (DMSO) (Naik et al. 2009). PSS contains hydrophobic benzene group which can be responsible for maximum position shift. Absorption maximum of ThT is shifted to 392 nm in the presence of heparin. The red shift of absorption maximum and increase in the intensity has also been observed for ThT binding with SDS (sodium dodecyl sulfate) suggesting the intermolecular interaction between bound ThT molecules (Kumar et al. 2008). These results reveal the formation of ThT complexes with all studied polyanions. Fig. 2B shows corresponding ICD (induced circular dichroism) spectra of ThT at the same conditions. The presence of heparin or polyadenylate leads to occurrence of ICD spectra of ThT. The spectrum of ThT-heparin complex consists of negative band at 380 nm, positive bands at 402 nm. At the presence of polyadenylate, positive band at 422 nm with shoulder at 450 nm and negative band at 402 nm is observed. For ThT-PSS complex no ICD signal occurs even if the absorption spectra change indicates the interaction of PSS with ThT.

It has been previously implied that the entrapment of ThT molecules in amyloid fibrils and polyglutamate is responsible for formation of optically active conformers of ThT, because frozen ThT conformers with nonzero angle between benzothiazole and benzaminic rings are chiral except for those perfectly planar or twisted at the right angle (Dzwolak and Pecul 2005; Lokszejn and Dzwolak 2008). We suggest that the optical activity of environment plays also important role in inducing optical activity of potentially achiral compound. As follows from Fig. 2B, the ICD signals of ThT occurs only in complex with chiral polyanions (heparin, polyadenylate), whereas at the presence of achiral PSS no ICD signal is observed, despite significant changes in absorption spectrum. We have not observed any ICD signal for ThT-PSS complex in wide concentration range (from  $\mu\text{M}$  to  $\text{mM}$  – not shown). Similarly, no ICD signals were present for ThT complex with another achiral polyanions such as polyacrylate, polyvinyl-sulfate (not shown). Heparin contains asymmetric centres of the sugar residues providing chiral templates for binding of positively charged small molecules. This binding is responsible for inducing strong CD (circular dichroism) bands in UV or visible absorption region of bound compounds (Salter et al. 1976; Zhang et al. 2002). Zsila and Gedeon (2006) observed ICD spectra for heparin-quinacrine complexes. They suggested that the origin of ICD signal comes from the exciton splitting between small helically arranged units of 2–3 quinacrine molecules bound to the adjacent anionic sites of heparin template (Salter et al. 1976). However, binding of quinacrine to chondroitin sulfates did not induce any

CD bands, which was referred to the different conformation of chondroitin sulfate not allowing definite chiral organization of quinacrine molecules (Zsila and Gedeon 2006). Another fluorescent dye phenosafranin has been found to bind to both, heparin and chondroitin sulfate polyanions in dimeric binding mode but with different orientation of phenosafranin molecules (Zhang et al. 2002). The occurrence of strong ICD signals was also confirmed for the heparin complexes with another triacyclic aromatic ring systems such as acridine orange (Salter et al. 1976), methylene blue (Stone and Moss 1967) as well as complexes with bicyclic aromatic drug chloroquine (Stanley et al. 2009). Formation of induced CD bands of achiral dyes has been observed not only for their complexes with glycosaminoglycans. The occurrence of induced CD bands was reported also for achiral alkaloids with planar and rigid structures bound to chiral environments of polyadenylate (Bhadra and Kumar 2011). These studies have shown that coralyne and sanguinarine induced the formation of polyadenylate self-structure (Xing et al. 2005; Giri and Kumar 2007; Bhadra and Kumar 2011). As a conclusion, in complexes with flexible polyanions with random structure the dominant reason for formation of optical activity is chirality of environment and not only the ligand itself. We suggest that the positively charged ligand ThT forms centre for ordered binding of chiral polyanion. The absorption spectroscopic measurements indicate the possible formation of ThT dimer bound to polyanion template. For better understanding of ThT arrangement alongside polyanion chain more extensive study is required.

In order to support the hypothesis concerning the importance of chirality of interacting partner of achiral chromophore we measured the ICD spectra of achiral molecule 9-aminoacridine hydrochloride in the presence of heparin and PSS. This molecule is rigid and planar without any potential spatial chiral arrangement. As is shown at Fig. 3A, in the presence of both polyanions, the significant changes in absorption spectra of 9-aminoacridine are observed manifesting the formation of the complex. The position of aminoacridine absorption band at 402 nm is shifted to 405 nm and its intensity together with intensities of absorption bands at 383 nm and 425 nm are decreased in the presence of heparin. In the presence of PSS, position of all absorption bands is shifted (383 nm to 390 nm, 402 to 408 nm and 425 to 430) and their intensities are decreased. Similar decrease of the absorbance intensities and bathochromic shift was observed for 9-aminoacridine in the presence of various small molecule derivatives of uracil indicating the formation of the complex (Manivannan and Renganathan 2011). As is seen in Fig. 3B, the induced positive CD signals at 392 nm, 411 nm and 432 nm occur only for complex of 9-aminoacridine with chiral heparin. 9-aminoacridine is optically inactive molecule with no intrinsic CD, therefore the occurrence of ICD signals points out strong binding



**Figure 3.** Absorption (A) and circular dichroism (B) spectra of free 9-aminoacridine (1) and 9-aminoacridine in the presence of heparin (2) and PSS (3) in 10 mM phosphate buffer (pH 6.5). The concentrations of used compounds: 9-aminoacridine – 140  $\mu$ M, polyanions – 0.1 mg/ml.

in polyanion chain and the role of polyanion chirality as origin for CD signal. It is in agreement with finding that in complex with achiral PSS no CD signal is found (Fig. 3B). The complexation of 9-aminoacridine with polyanions has induced more pronounced decrease of absorption intensities and only slight shift of maxima positions, whereas for ThT-polyanions absorption characteristic opposite changes are observed. The CD spectra characteristics of both chromophores with polyanions are also different, however for both dyes CD signals occur only in the presence of chiral polyanions. More detailed analysis of induced CD spectra of larger numbers of various chromophores in the presence of polyanions and amyloids will be needed for better understanding the origin of induced CD signals and utilization of induced CD spectroscopy for determination of structural characteristic of interacting achiral compounds with chiral environments.

As a conclusion, our study of ThT binding to polyanions has shown that ThT forms complexes with non-peptide polyanions heparin, polyadenylate and PSS. From ICD studies follows that induction of optical activity depends mainly on chirality of polyanion since for achiral PSS no signal was observed. The positively charged ThT acts as centre for ordered binding of chiral polyanion. In the case of ThT binding to amyloid fibrils, the cavities with  $\beta$ -sheets provides relatively rigid pattern for ThT entrapment leading to formation of chiral ThT conformers. We suppose that also chirality of  $\beta$ -sheets could play important role in induction of optical activity of this molecular rotor dye.

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