Supporting Information

Structural Analogue of Thioflavin T, DMASEBT, as a Tool for Amyloid Fibrils Study

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# General Experimental Procedures

*Preparation of DMASEBT-Fibrils Tested Solutions by Equilibrium Microdialysis*

The necessity of using the equilibrium microdialysis for sample preparation is due to the fact that the solution of any fluorescent probe with fibrils is an equilibrium system of free and bound to fibrils dye. To determine the affinity and stoichiometry of the dye binding to the fibrils, it is necessary to obtain information on the concentration of each of these dye fractions. Apparently, this problem can be solved only with the use of equilibrium microdialysis, a method that is inherently designed for the determination of dye–receptor binding stoichiometry and binding constants. It was shown early that an alternative method based on the ThT-fibrils samples centrifugation does not lead to correct results.6

Equilibrium microdialysis was performed using a Harvard Apparatus/Amika (USA) device that consists of two chambers (500 μL each) that are separated by a membrane that is impermeable to particles larger than 10 000 Da. DMASEBT in the buffer solution (that was used for amyloid fibrils preparation) was placed in chamber #1 (at an initial concentration $C_0$); lysozyme amyloid fibrils in the buffer solution (at concentration $C_p$) were placed in chamber #2. After equilibration, the dye concentrations in chambers #1 and #2 become equal ($C_f$), and the total DMASEBT concentration in chamber #2 exceeded that in chamber #1 by the concentration of the bound dye ($C_b = C_0 - 2 C_f$). Thereby the use of the equilibrium microdialysis allowed us to obtain a sample solution containing amyloid fibrils and two equilibrium DMASEBT fractions (free and bound dye), as well as a reference solution containing only free dye at a concentration equal to the concentration of free dye in the chamber with fibrils (for details of the experiment, see Scheme 1). Equilibrium microdialysis was performed for a fixed concentration of fibrils (0.4 mg/ml) and a wide range of concentrations of DMASEBT (1.5 - 150 μM). Further spectroscopic study of these sample and reference solutions allowed calculating DMASEBT-lysozyme fibrils affinity and stoichiometry and photophysical characteristics of bound dye with the use of the equations presented in the work.7

In particular, taking into account the calculated molar extinction coefficient of free DMASEBT in the buffer solution ($\varepsilon_{512} = 2.0 \cdot 10^4$ M$^{-1}$cm$^{-1}$), the concentration of dye unbound to fibrils in each experiment was determined (on the basis of the recorded absorption spectra of the dye in the reference solution). Using this concentration and the initial concentration of the dye introduced into the chamber for equilibrium microdialysis the concentration of DMASEBT incorporated into lysozyme amyloid fibrils was calculated.
Scheme S1. Preparation of DMASEBT-fibrils tested solutions by equilibrium microdialysis. (A) Preparation for the equilibrium microdialysis. The Panel shows two chambers with equal volume (chambers #1 and #2) that are separated by a membrane permeable to dye and impermeable to fibrils. DMASEBT solution was placed in chamber #1 (at an initial concentration \(C_0\)); lysozyme amyloid fibrils in the buffer solution (at concentration \(C_p\)) were placed in chamber #2. (B) Samples after equilibration. The Panel shows that the dye concentrations in chambers #1 and #2 become equal (\(C_f\)). (C) Distribution of free and bound to fibrils dye in chamber #2 after equilibration. Panels (B) and (C) shows that the total DMASEBT concentration in chamber #2 exceeded that in chamber #1 by the concentration of the bound dye (\(C_b = C_0 - 2C_f\)).

Electron Microscopy

Micrographs were obtained using a transmission electron microscope Libra 120 (Carl Zeiss, Germany). Amyloid fibrils were placed on nickel grids coated with a formvar films (Electron Microscopy Sciences, USA). To obtain electron micrographs, the method of negative staining with a 1% aqueous solution of uranyl acetate was used.

Spectroscopic Studies

The absorption spectra were recorded using a U-3900H spectrophotometer (Hitachi, Japan). The absorption spectra of amyloid fibrils and DMASEBT in the presence of the fibrils were analyzed along with the light scattering using a standard procedure. The concentration of
DMASEBT in solutions was determined using a molar extinction coefficient of $\varepsilon_{512} = 20000 \text{ M}^{-1}\text{cm}^{-1}$.

Fluorescence spectra and fluorescence excitation spectra were measured using a Cary Eclipse spectrofluorimeter (Varian, Australia). A PBS solution of fluorescent dye ATTO-590, whose fluorescence and absorption spectra are similar to that of DMASEBT, was taken as a reference for determining the fluorescence quantum yield of DMASEBT bound to fibrils. The fluorescence quantum yield of ATTO-590 was taken as 0.8. Fluorescence of ThT and ATTO-590 was excited at a wavelength of 562 nm. The spectral slits width was 5 nm in most of experiments. Changing the slit widths did not influence the experimental results. Recorded fluorescence intensity was corrected on the primary and secondary (see below) inner filter effect with the use of previously elaborated approaches.

Fluorescence anisotropy was determined as: 
$$ r = \left( \frac{F^V_v - GF^H_v}{F^V_v + 2GF^H_v} \right) $$
where $F^V_v$ and $F^H_v$ are vertical and horizontal components of fluorescence intensity excited by vertical polarized light, and $G = \frac{F^H_v}{F^H_v}$ is coefficient which determines the different sensitivity of the registering system for vertical and horizontal components of fluorescence intensity.

CD spectra in the far UV-region were measured using a J-810 spectropolarimeter (Jasco, Japan). Spectra were recorded in a 0.1 cm cell from 260 to 190 nm. For all spectra, an average of three scans was obtained. The CD spectrum of the appropriate buffer was recorded and subtracted from the protein spectra.

**Fluorescence Intensity Correction on the Secondary Inner Filter Effect**

The essence of this approach is to extrapolate the shortwave part of the fluorescence spectrum from its long wave region, where the absorption of the sample is absent and the shape of the fluorescence spectrum is not distorted (i.e., in the case of DMASEBT, it is $\lambda > 630$ nm) using the reference spectrum with the undistorted shape. Since, according to obtained results, the shape of the fluorescence spectra of dye solutions with an optical density of 0.1 - 0.2 coincides both in the long-wavelength and in the short-wavelength regions (Fig. 4 D), their shape (averaged) was used as a reference.

**Time-resolved Fluorescence Measurements**

DMASEBT and ThT fluorescence decay curves were recorded by a spectrometer FluoTime 300 (PicoQuant, Germany) with the Laser Diode Head LDH-C-440 ($\lambda_{ex} = 440$ nm). The fluorescence was registered at $\lambda_{em} = 490$ and 590 nm for ThT and DMASEBT, respectively. DMASEBT and ThT in concentration $2.6 \times 10^{-5}$ M and amyloid fibrils in concentration 0.4 mg/ml were used. The measured emission decays were fit to a multiexponential function using the
standard convolute-and-compare nonlinear least-squares procedure.\textsuperscript{10} In this method, the convolution of the model exponential function with the instrument response function (IRF) was compared to the experimental data until a satisfactory fit was obtained. The fitting routine was based on the nonlinear least-squares method. Minimization was performed according to Marquardt\textsuperscript{11}.

\textit{Dyes Penetration into HELA Cells}

The HeLa cells line was cultured at 37 °C, >80% humidity and 5% CO2 in Modified Eagle’s Medium containing L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 1X non-essential amino acids for minimum essential medium Eagle, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were harvested at 80% confluent monolayer and then reseeded into 12-well plate. ThT and DMASEBT were added to cells in concentration 30 μM and incubated during 1 hour before obtaining the fluorescence images.

\textit{Confocal Microscopy}

For obtaining the fluorescence images of the DMASEBT- and ThT- stained fibrillar structures and evaluation these dyes penetration into the cells the confocal laser scanning microscope Olympus FV 3000 (Olympus, Japan) was used. For each fluorescent probe the fixed excitation laser line was chosen: 405 nm for ThT and 561 nm for DMASEBT (the choice is determined by the available lasers with discrete wavelengths closest to the absorption maxima of the dyes). Registration of fluorescent light was carried out in the range of 420 – 520 nm and 570 – 670 nm for ThT and DMASEBT, respectively.

For obtaining the fluorescence images of the dyes- stained fibrillar structures oil immersion objective with a 60x magnification and numerical aperture NA 1.42 was used. To assess the presence of fibrils in the investigated sample region and show the areas of the dyes accumulation the differential interference contrast (DIC) images was also obtained. For evaluation of the dyes penetration into the cells an objective with a 40x magnification and numerical aperture NA 0.6 was used.

\textit{DMASEBT Properties in the Crowding Conditions}

Solutions of crowding agents Dextran-70 and PEG-12000 were prepared in concentration 380 mg/ml. PEG-400 and glycerol was used in concentration 95%. Concentrations of crowding agents and glycerol were controlled refractometrically using a refractometer IRF-45452M (Russia). The dependence of the refractive index on the glycerol concentration was taken from the work\textsuperscript{12}. For crowding agents the dependences of the refractive index on the concentration of crowding agents obtained in our laboratory were used (data not shown).
Figure S-1. Parameters of DMASEBT binding to lysozyme amyloid fibrils and characteristics of the bound dye. (A) Scatchard plot for DMASEBT interaction with lysozyme amyloid fibrils. Experimental data (circles) and best-fit curve plotted with the use of calculated binding constants ($K_{b1}$, $K_{b2}$) and number of binding sites ($n_1$, $n_2$) to different modes are represented. (B) Concentration of DMASEBT bound to amyloid fibrils ($C_b$) as a superposition of the dye concentrations bound to mode 1 ($C_{b1}$) and mode 2 ($C_{b2}$). (C) 3D dependence of the bound to fibrils dye absorbance ($A_b$) on $C_{b1}$, $C_{b2}$ that was used for determination of the molar extinction coefficient of DMASEBT bound to lysozyme amyloid fibrils. Experimental data, best-fit 3D surface and the values of molar extinction coefficients $\varepsilon_{b1}$ and $\varepsilon_{b2}$ obtained by multiple nonlinear regression are presented. The overlapping region of the absorption and fluorescence spectra is shown.
Figure S-2. Determination of the fluorescence lifetime and anisotropy of ThT (left panels) and DMASEBT (right panels) bound to lysozyme amyloid fibrils. (A), (C). Decay curves of the bound to fibrils dyes fluorescence. The excitation laser impulse profile (1), experimental decay curves of the fluorescence (2), best fit calculated fluorescence decay curves (3), and deviation between the experimental and calculated decay (4) are shown. Obtained values of the dyes fluorescence lifetime are presented. (B), (D). Fluorescence anisotropy of the bound to fibrils dyes. The spectra of vertical (1) and horizontal (2) components of the fluorescence and the dependence of fluorescence anisotropy on the emission wavelength (3) are presented. The excitation wavelengths 440 and 560 nm for ThT and DMASEBT, respectively, were used. Obtained values of the dyes fluorescence anisotropy are presented.
Table S-1. Fluorescence lifetime and anisotropy of DMASEBT and ThT in free and bound to fibrils state

| Dye   | Conditions                  | $\langle \tau \rangle$, ns | r    |
|-------|-----------------------------|-----------------------------|------|
|       | Water$^7$                   | 0.013                       | 0.34 |
| DMASEBT | Lysozyme amyloid fibrils    | 1.1                         | 0.35 |
|       | Insulin amyloid fibrils$^7$ | 2.2                         | 0.37 |
|       | Water$^{13}$                | 0.001                       | 0.38 |
| ThT   | Lysozyme amyloid fibrils    | 1.0                         | 0.36 |
|       | Insulin amyloid fibrils$^{13}$ | 1.8                      | 0.39 |
**Table S-2. An increase of the fluorescence quantum yield of DMASEBT* in different conditions**

| Type of the medium          | Conditions               | $q/q_{water}$ |
|----------------------------|--------------------------|---------------|
| Amyloid fibrils            | Lysozyme fibrils         | 165           |
|                            | (2nd mode)               |               |
|                            | Insulin fibrils          | 162           |
|                            | (2nd mode)               |               |
| Macromiscosity             | PEG-400                  | 28            |
| (molecular crowding)       | PEG-12000                | 6             |
|                            | Dextran-70               | 6             |
| Microviscosity             | Glycerol                 | 67            |
| Diluted medium             | Buffer for lysozyme fibrils | 3       |
|                            | Water                    | 1             |

*Concentration of DMASEBT in experiments was about 10μM.*
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