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Biological and polymeric self-assembled hybrid systems: Structure and properties of thylakoid/polyelectrolyte complexes

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Abstract

A novel hybrid system composed of biological components and synthetic polymer, thylakoid/polycation complex, has been formed and studied. Effects of complex formation on the structure, electrostatics and functioning of thylakoid membranes have been examined. Thylakoids from bean leaves were used to form complexes with polycation polyallylamine hydrochloride (PAAH) in two systems: (i) thylakoid/polycation complexes formed in an aqueous bulk phase, and (ii) immobilized thylakoid/polycation planar complexes. Immobilized on a solid substrate surface, thylakoid/polycation complexes were prepared using layer-by-layer stepwise alternate adsorption technique, i.e., via the sequential alternate adsorption of thylakoids and polycation molecules. The morphology of built up structures was investigated by scanning electron microscopy. Light-induced electron transport in chloroplasts was studied by the electron paramagnetic resonance (EPR) method. Spin probe technique was employed to study the structural and electrostatic characteristics of thylakoid membranes. We have found that efficiency of light-induced electron transport in thylakoid membranes and membrane structure were not changed noticeably by PAAH binding to thylakoids in a wide range of PAAH concentrations. The data obtained indicate the physiologically-soft character of polycation interactions with thylakoid membranes and demonstrate effectiveness of interfacial self-assembly approach to fabrication of complex planar functional nanostructures from biological components and synthetic polymers.

Keywords: Thylakoid membranes; Electron transport; Polyelectrolytes; Layer-by-layer deposition; EPR

1. Introduction

The elucidation of fundamental mechanisms of supramolecular structural organization in biological and artificial molecular systems is an actual task for biophysics and supramolecular science. In particular, the interrelation between structural and functional properties of biological membranes is a point of current interest. Knowledge of mechanisms and peculiarities of structural and functional organization in biological, molecular and colloidal systems is an essential basis for designing new hybrid and synthetic systems which could be used in various fields of biotechnology. Surface properties and interfacial interactions play a key role in development of advanced bioengineered materials [1,2].

Layer-by-layer stepwise alternate adsorption self-assembly technique introduced by Iler [3] has been widely used for surface modification and construction of new inorganic [3], organic [4], hybrid organic/inorganic [5], bio-organic [6] and polymeric [4] multilayer films, capsules [7,8] and nanocomposite structures with nanoscale-controlled structure, composition and properties. Various charged compounds and structures of organic and inorganic nature can be used to form such films [9]. This approach was used earlier to assemble complex films composed by synthetic

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Abbreviations: EPR, electron paramagnetic resonance; CAT_9, *N*,*N*-dimethyl-*N*-nonyl-tempoylammonium bromide; CTAB, cetyltrimethylammonium bromide; PAAH, polyallylamine hydrochloride; PEI, polyethylenimine; PSS, polystyrene sulfonate; PSI and PSII, photosystems I and II, respectively; S.E.M., scanning electron microscopy; 5-SASL, 5-doxylstearate; 16-MeSASL, 16-doxylstearate methyl ester

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polyelectrolyte molecules and biological components including living cells [10], proteins [11–13] and DNA [14,15]. Magnetic nanocomposite multilayer shells of glucose oxidase and inorganic nanoparticles were formed on latex particles and exploited as bio-nanoreactors [16].

Thylakoids are closed membrane vesicles inside chloroplasts—the energy-transducing organelles of plant cell. Key light-induced stages of photosynthetic processes, such as charge separation in photoreaction centres, electron transfer along the chloroplast electron transport chains, generation of transmembrane proton gradient and ATP synthesis, take place in thylakoids. Thylakoids immobilized in polymeric polyvinyl alcohol matrix were used as sensors for herbicides, which inhibited photosynthetic reaction centres in thylakoid membranes [17–19]. Like other biological membranes, under physiological conditions the thylakoid membrane surface is negatively charged [20] due to the presence of negatively charged lipids (for example, phosphatidylglicerol [21]) and other nonlipid membrane components (integral proteins). Therefore, the electrostatic interactions between thylakoid membrane surface and polycation molecules could be exploited for formation of thylakoid/polymer complexes.

In this work, we have fabricated new hybrid biological/polymer system—thylakoid/polycation complexes and have studied effects of complex formation on the structural and functional characteristics of thylakoid membranes. Layer-by-layer self-assembly technique with alternate adsorption of thylakoids and polyallylamine was used to fabricate immobilized complex layers on the solid substrate surface. The morphology of the built-up structures was investigated by scanning electron microscopy. The spin probe method has been proven to be an efficient tool for investigation of biological and model membrane's structure and electrostatics [22–24] and was used in present work for obtaining quantitative characteristics of the thylakoid membranes state. The light-induced electron transport between two photosystems and structural properties of thylakoid membranes were studied by the electron paramagnetic resonance (EPR) method. The data obtained indicate the physiologically-soft character of polycation interactions with thylakoid membranes, demonstrating the efficiency of the self-assembly approach to development of fabrication techniques which enable the assembling of functional hybrid structures from combinations of biological components and synthetic polymers.

2. Materials and methods

2.1. Materials and reagents

Polyallylamine hydrochloride (PAAH), polyethylenimine (PEI), polystyrene sulfonate (PSS) were obtained from Aldrich/Sigma. Chemical formulas of these polyelectrolytes are presented in Fig. 1. Doxylstearic acid spin probes 5-doxylstearate (5-SASL, Fig. 1d) and 16-doxylstearate methyl ester (16-MeSASL, Fig. 1e) were obtained from Sigma. Cationic spin probe *N*,*N*-dimethyl-*N*-nonyl-tempoylammonium bromide (CAT_9, Fig. 1f) was purchased from "Syva" (USA).

Class B chloroplasts (thylakoid membranes) were isolated from bean leaves (Vicia faba) according to the procedure described in [25]. Thylakoids were suspended in a medium containing 0.2 M sucrose, 2 mM $MgCl_2$, and 0.01 M Tris–HCl buffer (pH 7.5). Methyl viologen (20 μM) was used as an artificial mediator of electron transport from PS I to oxygen. A concentration of PS I primary electron donor P_{700} in a chloroplast suspension was determined by recording the EPR signal I from oxidized centers P_{700}^{+} [25].

2.2. Preparation of thylakoid/polyelectrolyte complexes

Thylakoid/polycation complexes in an aqueous bulk solution were formed via adding of PAAH to aqueous suspension of thylakoids. Final concentration of PAAH was varied in the interval 0.1–1.0 mM per monomer, concentration of thylakoids was equivalent to 2–3 μM P_{700}.

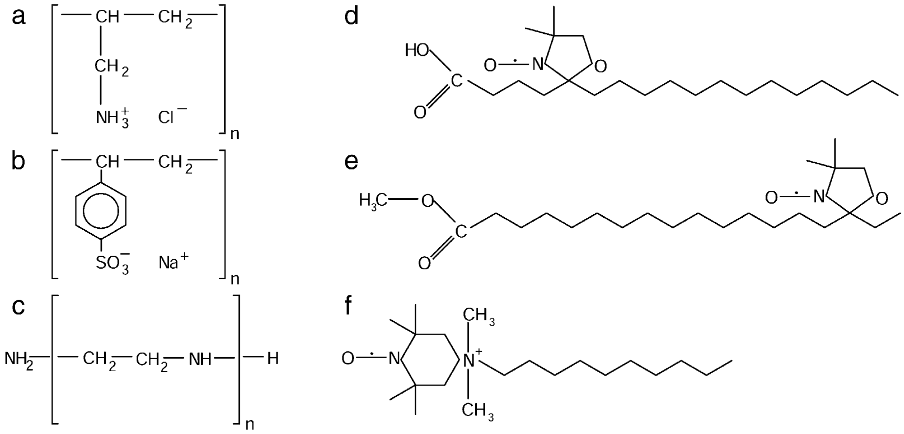

Fig. 1. Chemical structures of PAAH (a), PSS (b), PEI (c) and spin labels used in this work, 5-SASL (d), 16-SASL-Me (e), CAT_9 (f).

Samples for scanning electron microscopy (S.E.M.) measurements were prepared by drying the droplets of chloroplast/PAAH suspension placed on the surface of substrate (polished silicon wafer with natural oxide layer).

To form layers of immobilized thylakoid/polyelectrolyte complex on the surface of a silicon substrate with natural oxide layer, we used layer-by-layer stepwise alternate adsorption technique, which included the following steps. First, a positively charged surface was prepared by the formation of PEI layer on the substrate via PEI adsorption from water solution (1 mg/ml PEI). Second, the positively charged silicon substrate with PEI layer was placed in thylakoids (class B bean chloroplasts) suspension. As a result, thylakoids were adsorbed on the substrate surface. After that, immobilized thylakoids were covered by a layer of PAAH adsorbed from water solution (10 mM PAAH per monomer). Next, polymeric layer was formed by PSS adsorption from water solution (1 mg/ml). Each step of the adsorption procedure described above continued for about 25 min and was separated from the following step by washing the sample in the distilled water for 1 min.

The morphology of immobilized thylakoid/polycation complexes was studied using scanning electron microscopy (S.E.M.). Samples were cooled, exsiccated and coated with Pd/Pt layer using IB-3 Eiko device (Japan). Coating layer thickness was about 20 nm. The Hitachi S-405A Microscope was used to obtain all presented S.E.M. images.

2.3. Investigation of light-induced electron transport in chloroplasts

Electron transport in thylakoid membranes was studied by the EPR method. The light-induced redox transients of oxidized centers P_{700}^{+} were measured at room temperature (20–22 °C) from the kinetics of the EPR signal I according to the protocol described in [25].

2.4. Spin labeling of thylakoid membranes

Lipid-soluble spin probes 5-SASL and 16-Me-SASL were used to investigate structural changes in the lipid matrix of thylakoid membranes. Spin-labeled samples were prepared by incubation of chloroplasts in a suspending medium containing either 5-SASL or 16-Me-SASL. Spin labels were added from concentrated stock solutions of spin labels in ethanol. The final concentration of ethanol in the samples was less than 1–2% by volume. Water-soluble spin probe CAT_9 was added from an aqueous stock solution. Concentrations of spin probes in all samples were chosen to make spin label/lipid stoichiometric ratio not more than 1/50. In order to avoid light-inducted redox reactions, which could effect on nitroxide radicals, all manipulations with spin-labeled samples were carried out in a room illuminated with a dim light. Polycation PAAH was added to suspension of thylakoid membranes after spin-labeling procedure.

EPR spectra were recorded with Varian spectrometer E-4 equipped with IBM-compatible computer operated by original software [26]. All measurements were carried out at the ambient room temperature (20–22 °C).

3. Results and discussion

3.1. S.E.M. images of thylakoid/polyelectrolyte complexes

3.1.1. Thylakoid/PAAH complexes immobilized on the solid substrate surface

We have studied two kinds of thylakoid complexes immobilized on the surface of a silicon substrate: (i) chloroplasts immobilized on the surface of silicon substrate without cationic polymeric coating (“bare chloroplasts”), and (ii) immobilized chloroplasts covered with four self-assembled polyelectrolyte layers formed by PAAH and PSS (“encapsulated chloroplasts”). S.E.M. images of these samples are presented in Fig. 2. Immobilized chloroplasts (Fig. 2) were characterized by typical roundish morphology with mean diameter about 3–6 μm, in contrast to complexes formed in the aqueous phase characterized by large variety of forms and sizes. Surface density of chloroplasts on the substrate surface is approximately 3×10^7 chloroplasts per cm^2. In the images presented in Figs. 2a and b, one can also observe a “fine” structure visible as certain bubbles or granules with linear sizes about 500 nm on the surface of chloroplasts, which likely originate from the piles of grana thylakoids. Similar “bubbles” with sizes about 500–600 nm can be discerned in the thylakoid/PAAH complexes formed in the aqueous phase (Fig. 3). Images presented in Figs. 2a and b (“bare chloroplasts”) and Figs. 2c and d (“encapsulated chloroplasts”) are substantially different. In the latter case, the polyelectrolyte complex PAAH/PSS film covering the thylakoids is readily visible.

It is well known that polymeric films formed of polyelectrolyte molecules are permeable for low molecular weight compounds [27,28], but almost impermeable for large molecules (enzymes) [7,29] and supramolecular structures. Permeability of such polymeric films can vary in a wide range, depending on the composition, thickness and structure of polyelectrolyte molecules. Consequently, the approach used in our work enables creating immobilized chloroplasts (and other functional biological structures such as enzymes, organelles and cells) isolated from environment by thin, robust molecular polymeric layer with controllable permeability. Such structures may be of interest for development of novel biotechnological [10] and biosensor systems, in particular systems for ecological monitoring [17–19,30].

3.1.2. Thylakoid/PAAH complexes in aqueous bulk phase

Fig. 3 demonstrates typical images of chloroplast/PAAH complexes formed in the aqueous bulk phase. These complexes are visualized as oblong objects, which

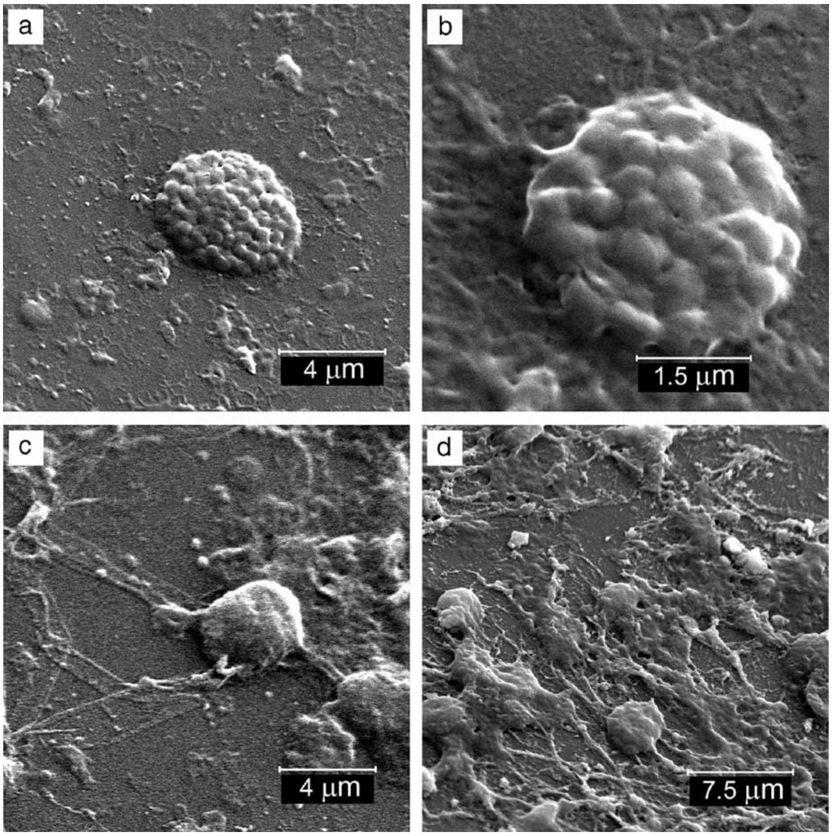

Fig. 2. S.E.M. micrographs of structures from suspension of class B bean chloroplasts immobilized on the surface of oxidized polished silicon substrate (a) 15 × 15 μm^2 (b) 5.2 × 5.2 μm^2, the same structures covered by four self-assembled polyelectrolyte complex layers formed by PAAH and PSS: (c) 16.5 × 16.5 μm^2; (d) 28.5 × 28.5 μm^2.

contain roundish structures (with linear dimensions about of 500–600 nm). Rather large structures presented in Fig. 3 represent chloroplasts or their aggregates glued by polymer, whereas small structures (≈500–600 nm) are typical of native thylakoids under physiological conditions [31,32]. The sizes of polycation/thylakoid aggregates depended on the PAAH concentration in the suspension. In accordance with the literature data on polycation/liposome complexes [33,34], linear sizes of aggregates increased with PAAH concentration. This result can be explained by decrease in negative surface charge of the external surface of thylakoid membranes due to polycation binding and formation of inter-thylakoid bridges by polycation molecules. Thus, it was demonstrated that thylakoid/polycation complexes could be formed in an aqueous bulk phase.

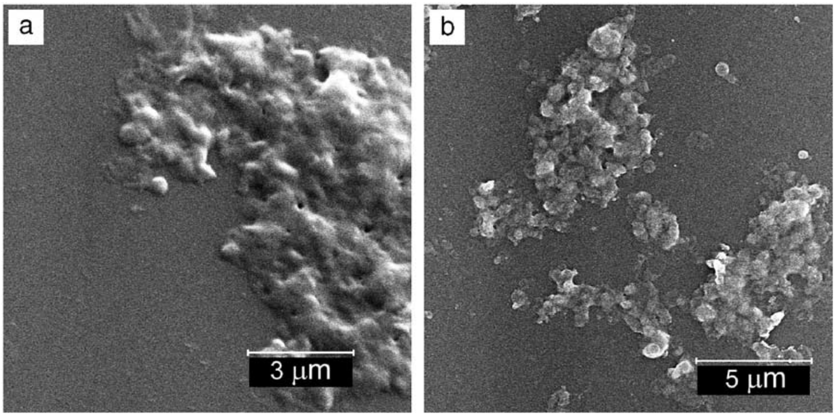

Fig. 3. S.E.M. micrographs of PAAH/thylakoid complexes adsorbed on the surface of oxidized polished silicon substrate: (a) concentration of PAAH in the sample 10^{-3} M PAAH, image size 11 × 11 μm^2; (b) 5 × 10^{-4} M PAAH, 17 × 17 μm^2.

3.2. Kinetics of the light-induced redox transients of P_{700} in thylakoid/polyelectrolyte complexes

Fig. 4 demonstrates the light-induced changes in the amplitude of the EPR signal I from oxidized reaction centers P_{700}^{+}. After switching on the far-red light ($\lambda_{\max}=707$ nm), which activates predominantly photosystem I (PSI), we observed an increase in the EPR signal I amplitude up to the steady state level. This signal is proportional to a number of oxidized centers P_{700}^{+} (parameter A_0). After the far-red light was turned to the red light illumination ($\lambda_{\max}=650$ nm) activating both PSI and PSII, the height of the EPR signal I dropped due to electron flux from PSII to PSI (PS II→plasoquinone pool→b/f-complex→plastocyanine→PS I). An extent of the signal drop (parameter ΔA) can be used as a measure of photochemical activity of PS II [24,25,35]. Light-induced redox transients of P_{700} are reversible-after cutting the illumination off the EPR signal decayed to its initial level. Fig. 4 shows that the ratio $\Delta A/A_0$ is independent (within experimental error) of the concentration of polycation PAAH in a chloroplast suspension. Therefore, we can conclude that PAAH binding to thylakoids does not cause any noticeable inhibition of the light-induced electron transport between two photosystems.

Effects of other cationic compounds on thylakoid membranes can be different. For instance, it is known that divalent cations can markedly affect on the kinetics of redox transients of P_{700}^{+} [35,36]. In particular, the increase in Mg^{2+} concentration from 0 up to 5 mM usually leads to the double increase in the value of parameter $\Delta A/A_0$ [36]. In contrast to Mg^{2+} ions, cationic detergent CTAB added at rather small concentrations (≅0.3 mM) caused substantial inhibition of electron flow from PSII to P_{700}^{+} [37]. The opposite signs of effects produced by different cationic compounds on electron transport in thylakoids can be explained by their specific interactions with the thylakoid membranes. Whereas polycation molecules interact predominantly with membrane surface electrostatically, cationic detergents can embed into the membrane, thus disrupting the membrane integrity. In agreement with the literature data [38,39], the data obtained in our work demonstrate the physiologically-soft character of polycation interactions with thylakoid membranes. The absence of cytotoxic effects of polycations is also characteristic for polyelectrolyte multilayers interactions with endothelial cells [38]. Thus, an approach to the formation of planar polymeric structures with immobilized thylakoids developed in this work can be used for preparation of sensors sensitive to herbicides and other compounds influencing on the photosynthetic processes in thylakoid membranes.

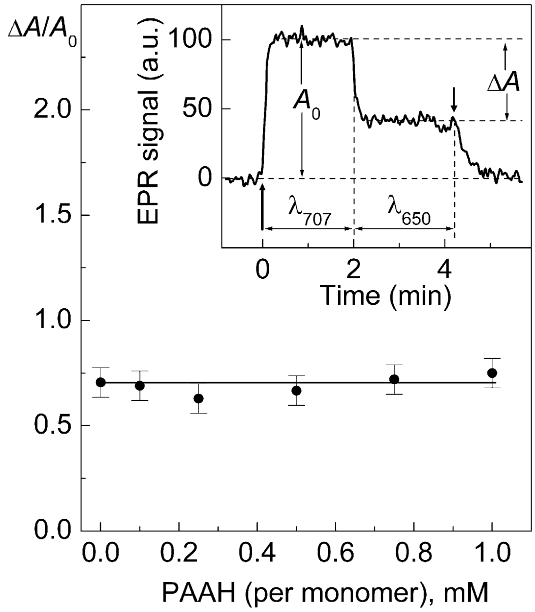

Fig. 4. Typical light-induced changes in the amplitude of the EPR signal I from oxidized centers P_{700}^{+} and the dependence of $\Delta A/A_0$ on the concentration of polycation PAAH.

Addition of high concentrations of PAAH (final concentration in a sample 1 mM) results in the formation of rather large aggregates of chloroplasts in an aqueous medium. It is well known from the literature [20,40,41] that electrostatic interactions between thylakoid membranes play essential role in structural organization of chloroplasts. For instance, the formation of grana (stacked piles of thylakoids) needs the presence of divalent cations (i.e., $\geq$2–5 mM Mg^{2+}). Under these conditions, Mg^{2+} cations screen and bind with negative charges on the outer surface of membrane proteins, resulting in the stacking of thylakoids due to intermembrane ionic and molecular contacts between the membranes of neighboring thylakoids [40].

3.3. Structural and electrostatic characteristics of thylakoid membranes in thylakoid/polyelectrolyte complexes

To study the effects of polycation PAAH on structural and electrostatic properties of thylakoid membranes, we have used the spin-labeling technique. Figs. 5a and b show EPR spectra of lipid soluble spin labels 5-SASL and 16-MeSASL in a chloroplast suspension. These spectra are typical of spin labels localized in the lipid domains of the thylakoid membrane [24]. Amphiphilic spin label 5-SASL intercalates in membranes. Its carboxyl group acts as the anchor that keeps the hydrophilic head of the molecule in the polar headgroup region of the lipid bilayer, while the hydrophobic tail extends downward toward the hydrocarbon core of the membrane. The nitroxide moiety of 5-SASL is localized close (approximately 8 Å) to the surface of the lipid bilayer [24]. The nitroxide radical of lipophylic spin label 16-MeSASL is localized in the internal regions of the membrane bilayer.

To characterize orientation and mobility of the nitroxide radicals in the thylakoid membrane, we used the order parameter S and rotational correlation time τ_c, which were determined according to [23,42] from the EPR spectra of 5-

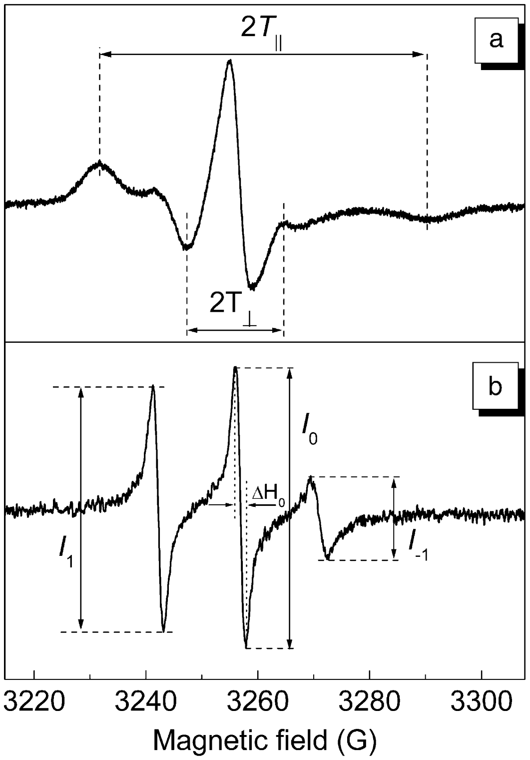

Fig. 5. EPR spectra of the spin probe 5-SASL (a) and 16-Me-SASL (b) in a thylakoid suspension.

SASL and 16-MeSASL, respectively. To estimate the order parameter S, we used formula (1):

$$S = \frac{1}{7}\left(T'_{\|} + 2T'_{\perp}\right) - \sqrt{\left[\frac{1}{7}\left(T'_{\|} + 2T'_{\perp}\right)\right]^2 - 0,46\left(T'_{\|} + T'_{\perp}\right) + 0.6}, \tag{1}$$

where $T'_{\|}$ and $T'_{\perp}$ are the outer and inner splitting parameters of the EPR signal of 5-SASL (Fig. 5a). Rotational correlation time τ_c was determined according to formula (2)

$$\tau_c = K_{0,-1}\Delta H_0\left[\sqrt{\left(\frac{I_0}{I_{-1}}\right)} - 1\right], \tag{2}$$

where $K_{0,-1}$=6,73 × 10^{-10} (s/G), I_0 and I_{-1} are peak-to-peak heights of the central and high-field components of the EPR spectrum of 16-MeSASL, ΔH_0 is the peak-to-peak line width of the central component (parameters I_{-1}; I_0; ΔH_0 are indicated in Fig. 5b).

Fig. 6 shows the dependences of the order parameter S and correlation time τ_c on the concentration of PAAH in chloroplast suspension. Neither significant changes in the order parameter S (Fig. 6b) nor the variations of correlation time τ_c (Fig. 6a) were observed with the rise of PAAH concentrations up to ~1 mM. These findings correlate with the lack of PAAH effect on the light-induced electron transport in thylakoids (Fig. 4).

As we noted above, thylakoid membrane can be affected by cationic compounds. For instance, bivalent cations can induce substantial changes in the lipid bilayer structure of the thylakoid membrane. In particular, as it was shown in [35], Mg^{2+} ions induced changes in the mobility and ordering of lipid hydrocarbon chains in the vicinity of membrane surface. The EPR spectrum of 5-SASL changed noticeably by addition of Mg^{2+} (2 mM) indicating the ordering of the membrane lipids. On the other hand, the addition of $MgCl_2$ did not result in changes of 16-Me-SASL spectrum (data not shown), which means that Mg^{2+} did not influence the ordering and mobility of hydrocarbon chains in the internal areas of thylakoid membrane. Cationic detergent CTAB at concentrations above 0.03% by weight (it is approximately 0.3 mÌ) changed both the order parameter and rotational correlation time of spin probes in thylakoid membranes [37].

To evaluate the changes in the membrane surface potential Ψ_s induced by polycation binding to the thylakoid membrane, we used cationic spin label CAT_9. Typical spectrum of this amphiphilic spin probe dissolved in a suspension of thylakoid membranes is presented in Fig. 7. This spectrum represents a superposition of two spectra. One of them (broadened lines) belongs to immobilized CAT_9 molecules bound to the membrane, while another one (three narrow lines) originates from CAT_9 molecules dissolved in an aqueous bulk phase of a chloroplast suspension. Partitioning of charged spin probe CAT_9 between the membrane and the aqueous phase depends on the surface potential of the thylakoid membrane [43,44]. The ratio of CAT_9 concentration in the aqueous bulk phase to CAT_9 concentration in the membrane, $[SL]_{aq}/[SL]_m$, is proportional to the ratio $\lambda = I_{aq}/I_m$, where I_{aq} and I_m are the amplitudes of well-resolved "aqueous" and "membrane"

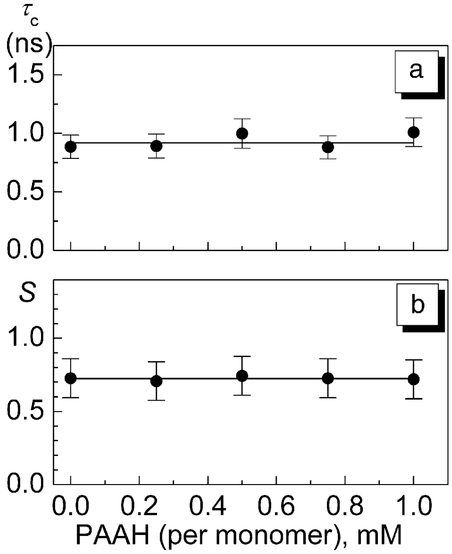

Fig. 6. The dependence of rotational correlation time τ (a) and the order parameter S (b) on the concentration of polycation PAAH in a thylakoid suspension.

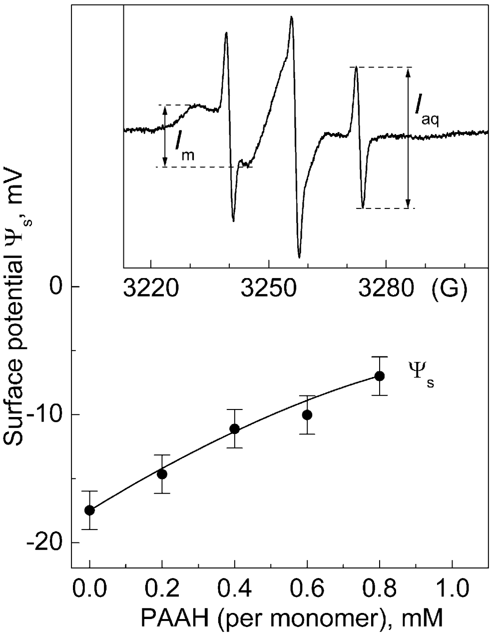

Fig. 7. EPR spectrum of the spin probe CAT_9 in a thylakoid suspension and the dependence of the membrane surface potential on the concentration of polycation PAAH in the thylakoid suspension.

components of the EPR signal, respectively (see for definitions, see Fig. 7). The difference between the membrane surface potentials $\Psi_s(1)$ and $\Psi_s(2)$ related to states 1 and 2 can be calculated using Eq. (3) [43,44]

$$\Psi_s(1) - \Psi_s(2) = \frac{RT}{F} \ln \frac{\lambda_1}{\lambda_2}, \tag{3}$$

where F is the Faraday constant, R is the gas constant, T is the temperature (K).

According to our estimates, the surface potential $\Psi_s(1)$ of the thylakoid membrane in control chloroplasts (i.e., in the absence of added monovalent ions or polycations) was lower than surface potential $\Psi_s(2)$ measured in presence of saturating concentration of monovalent electrolyte (500 mM NaCl), $\Delta\Psi_s = \Psi_s(1) - \Psi_s(2) = -18$ mV. Note that monovalent cations at high concentrations provide efficient screening of the membrane negative charges. According to the data reported by Barber [20], in the presence of 500 mM of monovalent cations (Na^+ or K^+), the absolute value of the surface potential is reduced and equals ≈25% of its initial value. Our estimate of $\Delta\Psi_s$ value is in agreement with the literature data [45]. The increase of PAAH concentration in a chloroplast suspension from 0 to 1 mM (per monomer) resulted in monotonous growth of the surface potential value from −18 up to −7 mV (Fig. 7). This result indicates PAAH binding to the surface of negatively charged thylakoid membrane. The decrease in surface potential absolute value with the addition of PAAH is accompanied with the formation of aggregates–thylakoid/PAAH complexes described above.

4. Conclusions

A novel hybrid system composed of biological components and polymeric molecules – thylakoid/polycation complex – has been fabricated in an aqueous bulk phase and on the solid substrate surface. Planar thylakoid/polyelectrolite film complexes on solid substrate were formed with the use of layer-by-layer stepwise self-assembly technique. The efficiency of photosynthetic processes in thylakoids was not changed noticeably due to the polycation binding and complex formation. We also demonstrated that spectral parameters of lipid soluble spin probes embedded into the thylakoid membrane were not changed substantially after formation of thylakoid/polycation complex. At the same time, thylakoid membrane surface potential value increased monotonously from −18 mV (without polycation) to −7 mV (at 1 mM PAAH) indicating the polycation binding. The data obtained show the physiologically-soft character of synthetic polycations interactions with thylakoid membranes and demonstrate effectiveness of the interfacial self-assembly approach for fabrication of complex planar functional nanostructures from biological components and synthetic polymers.

Acknowledgements

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