PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2020, **54**(1), p. 38–45

Biology

STUDY OF BINDING PECULIARITIES OF ETHIDIUM BROMIDE AND METHYLENE BLUE WITH DOUBLE-STRANDED RNA

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The paper presents the results of studies on the interaction of intercalators – ethidium bromide (EtBr) and methylene blue (MB) with synthetic double-stranded (ds) ribonucleotide ds-poly(rA)-poly(rU) at a solution ionic strengths of 0.02, 0.04 and 0.1 *M*. These results revealed that poly(rA)-poly(rU) has a relatively unstable structure at the ionic strengths $\mu \leq 0.02$ *M*, which leads to the interaction of the mentioned ligands with this polynucleotide. EtBr affinity for ds-poly(rA)-poly(rU) was revealed not to depend on the ionic strength of the solution, while the affinity of MB for this polynucleotide depends on this factor, as well as on the structural state of the polynucleotide.

***Keywords*:** synthetic polyribonucleotide, intercalator, ethidium bromide, methylene blue, complex, melting parameters.

Introduction. The development of the genosensor technologies, applied as elements of molecular recognition of the nucleic acids, synthetic and natural oligonucleotides, is an urgent topic, since it lies at the basis of the elaboration of new methods in bioanalysis. In these technologies, high attention is paid to the interaction processes of nucleic acids (NA) with high- and low-molecular compounds of natural and anthropogenic origin, which can be explained by several reasons. First of all, it is important for resolution of wide spectrum of biomedical problems connected to oncological and autoimmune diseases, as well as for the solution of the tasks of genetic diagnosis. In genosensors or genochips an interaction of short-stranded (ss) oligonucleotide (NA-probe), fixed on the surface of signal transducer, with complementary regions of sample oligonucleotides is detected. There occurs binding of nucleotides into stable complementary base pairs, forming helical double-stranded (ds) NA, i.e., so called hybridization process takes place. Such interactions possess a high specificity. They make it possible not only the complementary binding, but also the effect of various factors on it [1–7].

Nucleic acids are targets for many low-molecular compounds – ligands that binding specifically to DNA determine the action of anti-cancerous drug preparations, the consequences of oxidative and thermic shock, the damage processes and the reparation efficacy of NA [8–11]. The number of ligands-intercatators belongs to these preparations, and considering that the intercalation is possible only into ds-DNA, the

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intercalators can serve as markers for the hybridization processes between DNA–DNA, DNA–RNA and RNA–RNA [12–16]. Meanwhile, in comparison with DNA, the role of RNA in this aspect has not yet been adequately studied and evaluated, despite the fact that different types of RNA have been revealed in living cells during the recent decades. These RNAs can decrease the expression degree of the gene in the case of complementary binding to *m*-RNA or genome. These RNAs are called micro-RNAs (containing 21–22 nucleotides) and are found practically in all eukaryotes. Micro-RNAs affect through the mechanism of RNA-interference, though the complex of micro-RNAs and enzymes can result in methylation of nucleotides in the promoter region of the gene, which serves as a signal for decreasing of its activity. In the case of another type of *m*-RNA regulation, the complementary micro-RNA is degraded [17–21]. Moreover, there is a class of micro-RNAs that increase, but does not decrease the expression of genes [22–27].

Given the above the questions become interesting: if dyes are specific to DNA ds-structure, which are the various intercalators, including ethidium bromide (EtBr), methylene blue (MB), or groove binding ligands, can they bind to RNA, and which peculiarities can they perform in this case? Response to these questions can be obtained based on the model experiments of interaction of such ligands with ds-RNA or oligo- and polyribonucleotides. Appropriate methods for such experiments are UV-melting, absorption spectroscopy, fluorescence spectroscopy, as well as calorimetry. This work is aimed at the summarizing experimental studies on EtBr and MB interaction with poly(rA)-poly(rU), which serves as a model of ds-RNA.

Materials and Methods. Synthetic polynucleotide poly(rA)-poly(rU), MB (“Sigma”, USA, ultrapure), EtBr (“Serva”, Germany), EDTA (ethylenediaminetetra-acetate), NaCl, trisodium citrate (chemically pure) were used in experiments. All preparations were used without additional purification. The concentrations of the preparations used were determined spectrophotometrically, using the following coefficients of extinction: ε_{260}=7140 $M^{-1}cm^{-1}$ for poly(rA)-poly(rU), ε_{668}=76000 $M^{-1}cm^{-1}$ for MB, ε_{480}=5800 $M^{-1}cm^{-1}$ for EtBr. The experiments were carried out at the ionic strengths of the solution of 0.02, 0.04 and 0.1 *M*, containing only monovalent Na^+ cations.

UV-melting of the complexes of poly(rA)-poly(rU) with MB and EtBr was carried out on UV/VIS PYE Unicam-SP8-100 (England) spectrophotometer, heating of thermostating cells was realized in hermetically sealed, quartz cuvettes with volume of 3 *mL* and optic pathway length of 1 *cm*. UV-melting was carried out as described in [28]. Melting of poly(rA)-poly(rU) and its complexes with ligands was carried out at the wavelength of maximal absorption of polynucleotide – 260 *nm*. The concentration ratio r = [ligand] / [RNA] was varied in the interval $0 \le r \le 0.33$ (per nucleotide). During melting, the heating rate was 0.5°*C*/*min*, registration was made automatically every 60 *s*. Data were displayed on PC monitor in LabVIEW software. Data on temperature and absorption values of the samples were transformed and saved using Microsoft Excel, Office 13 software. All calculations of experimental data and melting curves were obtained in Excel software. The values of the melting parameters (T_m, ΔT, δT_m and $\delta\Delta T$) were determined on the basis of the melting curves of the poly(rA)-poly(rU)–ligand complexes. The dependency curves of δT_m and $\delta\Delta T$ versus r were plotted in Excel software.

Spectrophotometric study of complexation of the ligand with poly(rA)-poly(rU) was carried out on UV/VIS PYE Unicam-SP-8-100 spectrophotometer, fluorometric studies – on Varian Cary Eclipse Fluorescence Spectrophotometer (Australia), as described in [29]. Spectroscopic measurements were performed in quartz cuvettes with volume of 3 *mL*, optic pathway length of 1 *cm*.

Results and Discussion. The melting method is based on the hyperchromic effect of NA at the transformation from ds- to ss-state, which is possible to register in ultraviolet region at the wavelength of NA maximal absorption (UV-melting). At the same time, the melting parameters (melting temperature, melting interval width) are possible to measure and based on them to carry out the thermodynamic analysis as well as to reveal the modes of the given ligand binding to NA. Thus, there exists a sufficient number of theoretical and experimental works, in which the interaction of EtBr, MB and other ligands with DNA was studied and various binding modes were revealed [28–30].

Research data by the aforementioned method revealed that DNA ds-structure is more stable at low ionic strengths of the solution (lower compared to physiological ionic strength), compared to RNA ds-structure. It is indicated by studies on the melting of ds-poly(rA)-poly(rU) and its complexes with EtBr and MB in the interval of the ionic strength change 0.02–0.1 *M*, in the presence of only monovalent Na^+ cations. These studies revealed that poly(rA)-poly(rU) at $\mu = 0.02$ *M* has an unstable ds-structure (it is peculiar for RNA as well), while ds-structure of DNA or poly(dA)-poly(dT) is stable even at the ten-fold decrease of the ionic strength of the solution. An increase of the ionic strength by a factor of two or more results in stabilization of ds-structure of poly(rA)-poly(rU). Poly(rA)-poly(rU) was also revealed to have a wider melting interval, as compared to its deoxy-analogue, which is usually peculiar for DNA with a quasi-random sequence of nucleotides.

The formation of complexes of this polynucleotide with EtBr and MB leads to an increase in the stability of its ds-structure, depending on increase of the ligand concentrations. This fact indicates the binding of EtBr and MB with poly(rA)-poly(rU) (most apparently with RNA as well), while the melting interval width of the complexes increases as well. Based on the data of the melting of poly(rA)-poly(rU) and its complexes with EtBr and MB, the values of the melting parameters were determined – the melting temperature T_m and the melting interval width ΔT at the mentioned ionic strengths, as well as their change: $\delta(\Delta T/T_m^2) = \Delta T/T_m^2 - \Delta_0 T/T_0^2$, where T_0 and T_m are the melting points, $\Delta_0 T$ and ΔT are the melting interval widths of poly(rA)-poly(rU) and its complexes with ligands, respectively; $\delta(1/T_m) = 1/T_0 - 1/T_m$ depending on *r*. The characteristic dependence curves of $\delta(\Delta T/T_m^2)$ and $\delta(1/T_m)$ on *r* for the complexes of EtBr (a) and MB (b) with ds-poly(rA)-poly(rU) are presented in Fig. 1 and 2, respectively. The experimental points were obtained at $\mu = 0.02$ *M* (blue), 0.04 *M* (red) and 0.1 *M* (green). As can be clearly seen from the Figures, the experimental points repeat the form of the curves: in the case of EtBr, the dependence of $\delta(\Delta T/T_m^2)$ on *r* has a bell-like shape at the mentioned three ionic strengths of the solution. This fact permits concluding that EtBr binds to ds-poly(rA)-poly(rU) by several modes simultaneously, as to DNA [28–30]. In the case of MB at the ionic strengths of 0.04 and 0.1 *M*, the dependencies of $\delta(\Delta T/T_m^2)$ on *r* are similar, despite the fact that their values differ significantly. At $\mu = 0.02$ *M* the experimental points at high

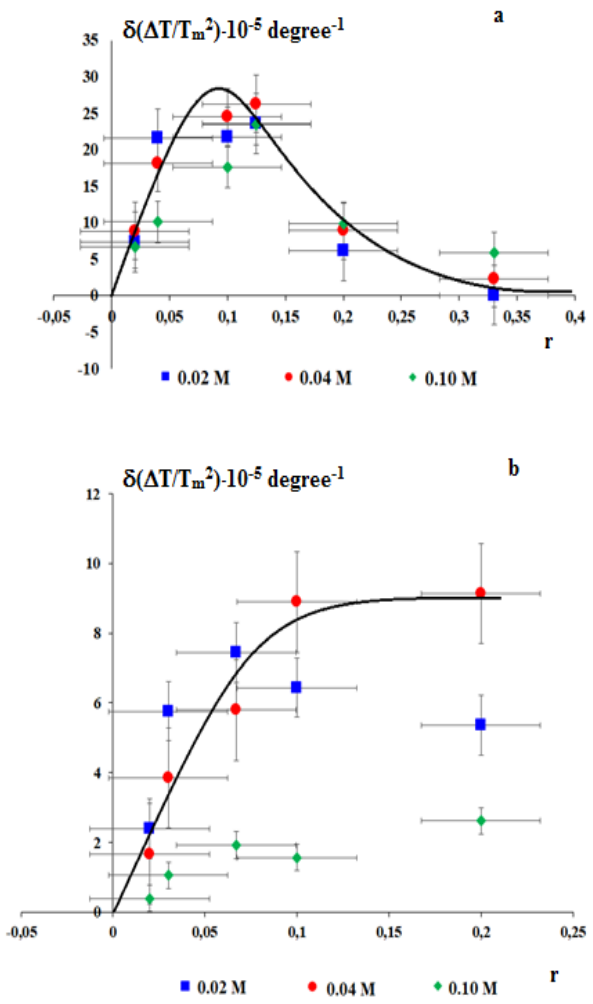

Fig. 1. Characteristic dependence curve of $\delta(\Delta T/T_m^2)$ on r of the complexes of EtBr (a) and MB (b) with ds-poly(rA)-poly(rU).

values of r deviate from the characteristic curve and tend to bell-like changing, which was revealed in the case of MB interaction with ds-DNA as well [30].

The obtained data also reveal that the effect of widening of the melting interval of the complexes of poly(rA)-poly(rU) with the mentioned ligands is higher at $\mu = 0.04$ *M*. Obviously, at this ionic strength of the solution poly(rA)-poly(rU) is in stable ds-form, though, the structural state is the most favorable for the binding of EtBr and MB by their intrinsic modes [30]. Along with this, in the case of EtBr, the multimodality is preserved regardless of the ionic strength of the solution, while in the case of MB the interaction relevantly weakens with the increase of the ionic strength. This effect is reflected most expressively on the melting temperature of the complexes; the change in T_m significantly depends on the ionic strength of the solution and at $\mu = 0.1$ *M* T_m practically does not change depending on the rising concentration of MB, at the same time the change in $\delta(\Delta T/T_m^2)$ is the highest.

Based on these data, a thermodynamic analysis of the interaction of EtBr and MB with ds-poly(rA)-poly(rU) was carried out and it was revealed that the values of ΔH and ΔS of the EtBr–poly(rA)-poly(rU) complexes increase with the rising of the ionic strength of the solution: ΔH increases from ~5.5 to 13.2 *kcal*/*mol*, ΔS increases from 18 to 41 *cal*/*mol*·*K*. In the case of MB these values do not show a tendency to monotonous change, since at the transition of ds-poly(rA)-poly(rU) from unstable to more stable ds-state ΔH and ΔS increase: ΔH increases from 3.5 to 14.1 *kcal*/*mol* and ΔS increases from 11 to 45 *cal*/*mol*·*K*.

With an increase in ionic strength, a decrease in the values of these parameters is observed: the value of ΔH is equal to 8.7 *kcal*/*mol*, ΔS is equal to 27 *cal*/*mol*·*K*. It

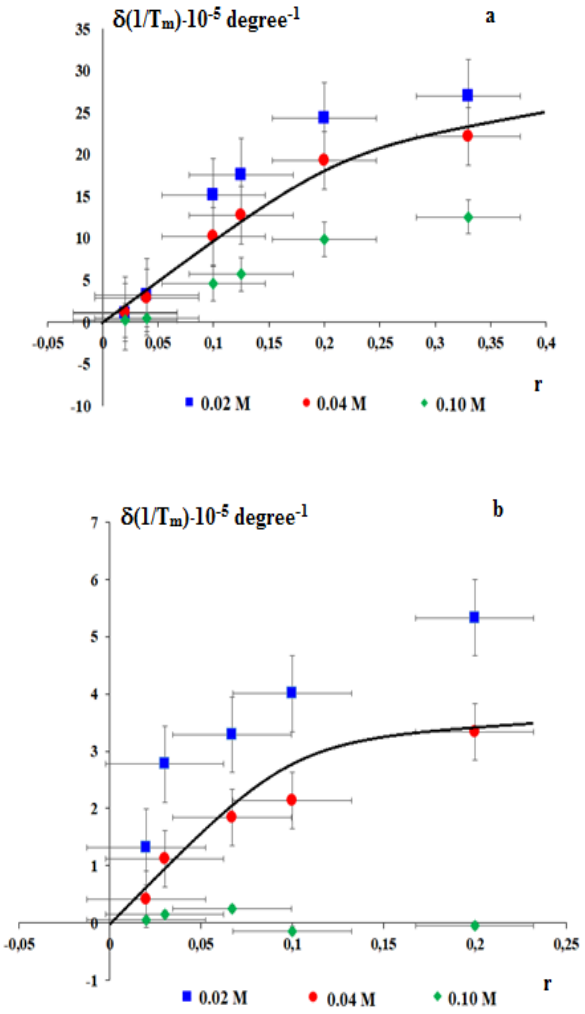

Fig. 2. Characteristic dependence curve of $\delta(1/T_m)$ on r of the complexes of EtBr (a) and MB (b) with ds-poly(rA)–poly(rU).

is obvious that in the case of EtBr the preference for unstable or stable ds-form is not so expressed as in the case of MB, which indicates that this ligand can show selectivity for the structural forms of NA.

The mentioned peculiarities of the complexes of ds-poly(rA)-poly(rU) with EtBr and MB are revealed from spectroscopic studies, despite the fact that the changes of spectral and fluorescent properties of these ligands upon binding to ds-DNA and ds-poly(rA)-poly(rU) are practically similar. This fact, apparently, indicates the universality of the optical or fluorescent properties of EtBr and MB in the bound state, which, even though, do not depend on NA type.

Meanwhile, in the case of EtBr the spectral changes make it possible to carry out a quantitative analysis, calculate the part of bound and free molecules of the ligand and based on them construct the binding curves at the afore-mentioned ionic strengths of the solution. These data are summarized in the work [30] (and are not presented here). From the obtained results, it was revealed that one of the binding modes of EtBr to poly(rA)-poly(rU) is sufficiently strong and significantly prevails over the second one: $K_1/K_2 \approx 50 \div 60$. If to compare the results of UV-melting, spectroscopic studies, and thermodynamic analysis, as well as the literature data [28–30], one can conclude that EtBr can interact with poly(rA)-poly(rU) by more than two modes, although one or more types of the binding are hidden under the strong one.

In the case of MB, although the changes of the absorption and fluorescent spectra upon complexation with poly(rA)-poly(rU) are similar with the analogous changes, taking place at the interaction of this ligand with DNA, their quantitative analysis does not allow to calculate the fraction of bound and free molecules of the ligand, therefore, to determine the binding parameters. That is why, based on the

results of UV-melting of the MB–ds-poly(rA)-poly(rU) complexes and the thermodynamic values, we conclude that MB more expressively shows selectivity for the RNA and DNA (preferably binds to deoxyribonucleotides), which also depends on the ionic strength of the solution.

Conclusion. Thus, the data obtained reveal that poly(rA)-poly(rU) has a relatively unstable structure at the ionic strengths $\mu \leq 0.02\ M$; this fact conditions the affinity of various ligands for this polynucleotide. Particularly, ligands-intercalators, binding to ds-DNA with high affinity, can also bind to poly(rA)-poly(rU), though their interaction depends on the structural state of this polynucleotide and is more preferable in conditions, at which poly(rA)-poly(rU) is not only in ds-state, but also is available for their intercalation. This fact is mostly pronounced for MB, which being an intercalator does not always bind to NA by this mode. Particularly, the affinity of MB for both poly(rA)-poly(rU) and DNA decreases at relatively high ionic strengths, at which the molecules of NA transform to more densely-packed state. Obviously, this ligand can totally intercalate into DNA or RNA in the case, when their helix is untwisted. This fact permits us to conclude that MB can be a good marker in genosensor technologies, since the change in ionic strength can be applied as a measure to modulate the binding peculiarities of this ligand to NA. On the other hand, in the case of EtBr, the aforementioned effect is more expressed, which makes it more universal both as a probe in genosensor technologies and as a modulator of the interaction peculiarities of multimodal ligands with NA.

Received 22.10.2019
Reviewed 05.02.2020
Accepted 10.04.2020

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Մ. Ա. ՓԱՐՍԱԴԱՆՅԱՆ

ԷԹԻԴԻՈՒՄԻ ԲՐՈՄԻԴԻ ԵՎ ՄԵԹԻԼԵՆԱՅԻՆ ԿԱՊՈՒՅՏԻ ԿԱՊՄԱՆ ԱՌԱՆՁՆԱՀԱՏԿՈՒԹՅՈՒՆՆԵՐԻ ՈՒՍՈՒՄՆԱՍԻՐՈՒԹՅՈՒՆԸ ԵՐԿՇՂԹԱ ՌՆԹ-Ի ՀԵՏ

Ամփոփում

Աշխատանքում ընդհանրացվել են սինթետիկ երկշղթա ռիբոնուկլեոտիդ եշ-poly(rA)-poly(rU) ի հ-ետ ինտերկալյատորներ էթիդիումի բրոմիդի (ԷԲ) և մեթիլենային կապույտի (ՄԿ) փոխազդեցության ուսումնասիրությունների արդյունքները լուծույթի 0,02; 0,04 և 0,1 *Մ* իոնական ուժի պայմաններում: Այս արդյունքները բացահայտել են, որ poly(rA)-poly(rU)-ն ունի հարաբերականորեն անկայուն կառուցվածք $\mu \leq 0{,}02$ *Մ* իոնական ուժերի դեպքում, ինչը պայմանավորում է նշված լիգանդների փոխազդեցությունը այս պոլինուկլեոտիդի հետ: Ցույց է տրվել, որ ԷԲ-ի խնամակցությունը եշ-poly(rA)-poly(rU)-ի նկատմամբ կախված չէ լուծույթի իոնական ուժից, այն դեպքում, երբ ՄԿ-ի խնամակցությունը այս պոլինուկլեոտիդի նկատմամբ կախված է այդ գործոնից, ինչպես նաև պոլինուկլեոտիդի կառուցվածքային վիճակից:

М. А. ПАРСАДАНЯН

ИЗУЧЕНИЕ ОСОБЕННОСТЕЙ СВЯЗЫВАНИЯ БРОМИСТОГО ЭТИДИЯ И МЕТИЛЕНОВОГО СИНЕГО С ДВУХЦЕПОЧЕЧНОЙ РНК

Резюме

В работе обобщены результаты исследований по взаимодействию интеркаляторов бромистого этидия (БЭ) и метиленового синего (МС) с синтетическим двухцепочечным рибонуклеотидом дц-poly(rA)-poly(rU) при ионных силах раствора 0,02; 0,04 и 0,1 *М*. Выявлено, что poly(rA)-poly(rU) имеет относительно нестабильную структуру при ионных силах $\mu \leq 0{,}02$ *М*, что обуславливает взаимодействие указанных лигандов с этим полинуклеотидом. Показано, что сродство БЭ к дц-poly(rA)-poly(rU) не зависит от ионной силы раствора, в то время как сродство МС к этому полинуклеотиду зависит от этого фактора, а также от структурного состояния полинуклеотида.