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Development and Characterization of Novel DNA Aptamers Specific to Heart-Type Fatty Acid Binding Protein (hFABP)

Vasilisa V. Krasitskaya[a], Tatiana A. Vereshchagina[b], Tatiana E. Smoliarova[c], Alexey E. Sokolov[c], Alexey E. Tupikin[d], Vicente Dominguez-Arca[e] and Ludmila A. Frank[a]*

[a]Institute of Biophysics SB RAS
Federal Research Center "Krasnoyarsk Science Center SB RAS"
Krasnoyarsk, Russian Federation
[b]Institute of Chemistry and Chemical Technology
Federal Research Center "Krasnoyarsk Science Center SB RAS"
Krasnoyarsk, Russian Federation
[c]Kirensky Institute of Physics
Federal Research Center "Krasnoyarsk Science Center SB RAS"
Krasnoyarsk, Russian Federation
[d]Institute of Chemical Biology and Fundamental Medicine SB RAS
Novosibirsk, Russian Federation
[e]University of Santiago de Compostela
Santiago de Compostela, Spain

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Abstract. Heart-type fatty acid-binding protein (hFABP) has been proposed as a new biochemical marker for the early diagnosis of acute myocardial infarction (AMI). The work describes the development of high-affinity and specific DNA aptamers as sensor elements of analytical systems for the rapid detection of this marker. Several novel DNA aptamers to hFABP were selected by using hFABP-activated magnetic microparticles as a target. The DNA library enrichment, affinity and specificity of candidate aptamers as well their truncated variants, were examined by solid-phase obelin-based bioluminescent assay. High binding ability was shown for the aptamer FABPAp4 by applying isothermal titration calorimetry (ITC) technique. The developed aptamers suggest to contain G-quadruplex (GQ) forming motifs that play a key

* Corresponding author E-mail address: lfrank@yandex.ru
ORCID: 0000-0001-7018-2581 (Krasitskaya V.); 0000-0003-4538-8902 (Vereshchagina T.); 0000-0002-0123-1965 (Smoliarova T.); 0000-0002-8194-0322 (Tupikin A.); 0000-0002-3500-5915 (Dominguez-Arca V.); 0000-0003-4462-1944 (Frank L.)

role in binding the target. Demonstrated high affinity and specificity for hFABP determine the prospects of the obtained aptamers as sensor elements of analytical systems intended for AMI early diagnosis.

Keywords: DNA aptamers, heart-type fatty acid-binding protein (hFABP), Ca^{2+}-regulated photoprotein obelin, isothermal titration calorimetry (ITC).

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Получение и свойства ДНК-аптамеров к сердечному белку, связывающему жирные кислоты (кардиоБСЖК)

В. В. Красицкая[а], Т. А. Верещагина[б], Т. Е. Смолярова[в], А. Э. Соколов[в], А. Е. Тупикин[г], В. Домингес-Арка[д], Л. А. Франк[а]

[а]Институт биофизики СО РАН
ФИЦ «Красноярский научный центр СО РАН»
Российская Федерация, Красноярск
[б]Институт химии и химической технологии СО РАН
ФИЦ «Красноярский научный центр СО РАН»
Российская Федерация, Красноярск
[в]Институт физики им. Л. В. Киренского СО РАН
ФИЦ «Красноярский научный центр СО РАН»
Российская Федерация, Красноярск
[г]Институт химической биологии
и фундаментальной медицины СО РАН
Российская Федерация, Новосибирск
[д]Университет Сантьяго-де-Компостела
Испания, Сантьяго-де-Компостела

Аннотация. Сердечный белок, связывающий жирные кислоты (кардиоБСЖК), рассматривают как один из новых биохимических маркеров ранней диагностики острого инфаркта миокарда (ОИМ). В настоящей работе описано получение высокоаффинных и специфичных ДНК-аптамеров как сенсорных элементов аналитических систем, предназначенных для быстрого выявления этого кардиомаркера. Обогащение ДНК-библиотек при направленном отборе кандидатных

олигонуклеотидов, их относительные аффинность и специфичность определяли с помощью твердофазного биолюминесцентного микроанализа на основе фотопротеина обелина. Для одного из полученных аптамеров FABPAp4 константа связывания с мишенью была определена с помощью метода изотермической титрационной калориметрии. Молекулы полученных аптамеров предположительно формируют G-квадруплексные мотивы, которые играют ключевую роль в формировании комплексов с целевой кардиомишенью. Высокая аффинность и специфичность к кардиоБСЖК определяют перспективность полученных аптамеров в качестве сенсорных элементов аналитических систем для ранней диагностики ОИМ.

Ключевые слова: ДНК-аптамеры, сердечный белок, связывающий жирные кислоты (кардиоБСЖК), Ca^{2+}-регулируемый фотопротеин обелин, метод изотермической титрационной калориметрии.

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Introduction

Aptamers are single-stranded DNA or RNA oligonucleotides of 25–90 bases length capable of being specifically bound to a wide variety of targets (from metal ions to whole cells) with high affinity due to their particular three-dimensional shape. They have been shown to have a number of advantages and to be a promising alternative to antibodies at devising analytical systems (aptasensors) (Liu et al., 2021). Aptamers can be selected “in vitro” from large random oligonucleotide libraries to almost any target without immunization of animals, which significantly reduces the production costs. The chemical synthesis of aptamers allowed various chemical modifications, including the design of “chimeric” structures (Davydova et al., 2020). Usually, the aptamer capability of binding to the target is not reduced by chemical modification at the 5’ or 3’residues, which for example may be required at fixing the aptamer on the carrier. And besides, aptamers are not second to antibodies in terms of sensitivity. All this makes the development of aptasensors of various design possible – from enzyme-linked apta-sorbent assay (ELASA) (Mukherjee et al., 2018; Aira et al., 2021), aptamer-based lateral flow assay (Majdinasab et al., 2022), different kinds of label-free systems (based on electrochemical, electrical signals etc.) (Grabowska et al., 2018; Sharma, Jang, 2019) to homogeneous assays based on conformational switchable design strategies (Wang et al., 2021; Seok et al., 2015) or aptamer assisted CRISPR-Cas12a strategy (Yang et al., 2023).

Acute myocardial infarction (AMI) is one of the worldwide leading causes of death and disability (WHO). The effectiveness of AMI treatment is crucially dependent on the time

between the onset of acute heart failure and the start of therapeutic measures. Myocardial infarction is diagnosed when the level of sensitive and specific biomarkers in blood increases in the presence of clinical signs of acute ischemia. The main markers of myocardial damage are cardiac troponins (I and T), creatine kinase (KK-MB) and myoglobin (Aydin et al., 2019). Heart-type fatty acid-binding protein (hFABP) has been proposed as a new biochemical marker for the early diagnosis of AMI. The concentration of hFABP in blood at myocardial injury was shown to significantly increase within 3 hours after the onset of AMI symptoms due to the small size of the protein (15 kDa) and to return to normal level in 12–24 hours (Lescuyer et al., 2005). This fact, as well as good tissue specificity in comparison with myoglobin, make hFABP a promising marker for the early diagnosis of AMI (Moon et al., 2021).

We are currently developing aptamer-based biosensors for the rapid detection of myocardial damage early markers. In the present study, we report the development of DNA aptamers against human hFABP. Application of Ca^{2+}-regulated photoprotein obelin and its genetically modified variants as an effective analytical instrument effectively promoted the research. Photoprotein obelin like other Ca^{2+}-regulated photoproteins is a stable noncovalent complex comprised of apoprotein (22.2 kDa) and substrate 2-hydroperoxycoelenterazine. The binding of calcium ions causes small conformational rearrangement of the protein and instantaneous decarboxylation of the substrate with the emission of light (fast blue flash, λ_{max} = 482 nm). Obelin-based labels are convenient for the use in research and biomedical application due to some obelin advantages over the other reporter systems. Among such merits are: high quantum yield, virtual absence of the background, simple reaction (no dependence on a substrate, oxygen or any cofactor), no toxicity and protein stability under storage and modifications (Krasitskaya et al., 2020). Recently a special type of bioluminescent solid-phase analysis involving obelin as a reporter has been suggested by us to monitor the selection process and to evaluate binding capacity and target specificity of the candidate aptamer (Krasitskaya et al., 2020a). Here, using this approach, several aptamers were selected, size-optimized, and characterized with regard to affinity and specificity to hFABP.

Materials and methods

Materials and reagents

The following proteins were used in the work: human serum albumin (HSA; Reanal); human myoglobin (Myo) and creatine kinase MB (CKMB) (Abcam); bovine serum albumin (BSA; Sigma-Aldrich); recombinant human cardiac troponin I (cTnI), skeletal troponin I (skTnI), monoclonal anti heart-type fatty acid-binding protein antibody (anti-hFABP) clone 28 and clone 10E 1 (HyTest, Russia). Recombinant cardiac fatty acid-binding protein was expressed and purified as described in (Krasitskaya et al., 2023). Streptavidin was produced as described in (Bashmakova et al., 2020). The variant of Ca^{2+}-regulated photoprotein obelin with a unique cysteine residue (Obe D 12C) and its conjugate with anti-hFABP antibody (clone 28, anti-hFABP IgG-Obe) or oligothymidine bearing the 5'-hexamethylamino group [5'-NH_2-$(CH_2)_6$-p-T_{30}, (NH_2-T_{30})] were produced according to (Krasitskaya et al., 2017). Streptavidin-obelin fusion protein (Stavi-Obe) was obtained according to (Bashmakova et al., 2017).

Artificial diagnostic human serum was purchased from Microgen (Russia). Dimethyl suberimidate dihydrochloride (DMS) was from Sigma-Aldrich; coelenterazine – from NanoLight Technologies (USA); Hot Start Taq DNA polymerase, dNTPs – from SibEnzyme (Russia).

All other reagents purchased from Sigma-Aldrich (USA), unless otherwise stated, were of analytical reagent grade or better.

Single-stranded DNA library consisting of a randomized region of 40 nucleotides (N_{40}) flanked by two constant primer-hybridization regions for PCR (5'-GGCAGCAGGAAGACAAGACA–N_{40}–TGGTTCTGTGGTTGCTCTGT-3') was purchased from Sangon Biotech (China).

All oligonucleotides used in the work were synthesized in the Laboratory of synthetic biology (ICBFM SB RAS, Russia).

Before use, each DNA aptamer was refolded by heating to 90 °C for 5 min in binding buffer (0.15 M NaCl, 50 mM K-Na phosphate buffer pH 7.0 (PBS), 1 mM $MgCl_2$) followed by cooling at room temperature (RT) for 15 min.

A solid-phase bioluminescent assay was carried out in white Low Volume Stripwell TM Microplate (Corning, USA) and white microtiter assay plates (Costar, USA).

Aminated magnetic particles were kindly provided by Ya. Chernykh (ICCT SB RAS, Krasnoyarsk). Magnetic microparticles (MPs) were derived from magnetic hollow glass-crystalline microspheres (fraction size 100–163 μm, bulk = 0.41 g/cm^3) (Anshits et al., 2005) and aminated using 3-aminopropyltriethoxysilan, the concentration of NH2-groups was 0.26 m-eq/g (Voroshilova et al., 1980). Measurement of specific magnetization of the particles were perfomed by Dr. V. Dudnikov (IP SB RAS, Krasnoyarsk).

Covalent immobilization of hFABP on magnetic particles

The mixture of MPs (5 mg in 50 mM Bicine pH 8.5), DMS (10 mg dissolved in dimethyl sulfoxide) and hFABP (0.45 mg in 50 mM Bicine pH 8.5) was incubated for 2 h at RT, then at 4 °C during the night and washed successively with the following buffers: 20 mM Tris-HCl pH 7.0 (3 times); PBS, 1 mM $MgCl_2$, 0.1 % Tween (3 times) and then PBS, 1 mM $MgCl_2$ (3 times). Suspension of produced particles carrying hFABP (hFABP-MPs) was stored with 0.01 % sodium azide. The density of hFABP on the surface of the final particles was estimated using obelin-based bioluminescent analysis. Briefly: 50 μL aliquote containing hFABP-MPs were incubated with anti-hFABP IgG–Obe, then washed and bioluminescence of the complexes, formed on the surface was measured with LB 940 Multimode Reader Mithras (Berthold, Germany) by rapid injection of 0.1 M $CaCl_2$ in 0.1 M Tris-HCl, pH 8.8. The signal was integrated for 5 s. To avoid nonspecific sorption of the label, the particles were preincubated with a 1 % BSA solution. Signals from the corresponding control samples (suspension of the initial aminated particles) were taken as the background and subtracted. The density of the hFABP immobilized on the particles was assessed using bioluminescence binding assay, with an anti-hFABP IgG-Obe conjugate as a label. Initial NH_2-particles were used as a control. Calculation of the immobilized hFABP amount was made using the dependence of the D 12C obelin bioluminescent signal on its content as a calibration curve.

In vitro selection of the DNA aptamers specific to hFABP

Proteins used for negative selection – HSA, cTnI, skTnI, Myo, CKMB, BSA and artificial diagnostic human serum were immobilized on the well surface of microplate by incubations (100 μL per well, 5 μg/mL each in PBS) at 4 °C overnight. After washing, 1 nmol (1 round) or 0.5–0.24 nmol (2–12 rounds) of ssDNA was added to hFABP-MPs (2–3 mg) and incubated at RT for 1 h (1–2 rounds), 45 min (3–7 rounds) or 30 min (8–12 rounds) with shaking. The particles were washed two-five times (0.1 % Tween 20 in

binding buffer) and the bound ssDNA pool was eluted three times with 50 μL of TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA) at 95 °C for 10 min. Each round of positive selection was performed using a fresh sample of hFABP-MPs.

Negative selection was performed with intact aminated magnetic particles before each round of selection and with HSA (rounds 3 and 8), cTnI (rounds 4 and 12), skTnI (round 9), BSA (round 7), Myo (rounds 5 and 10), CKMB (rounds 6 and 11) and artificial diagnostic human serum (after rounds 10 and 11). The selection was strengthened by decreasing incubation time and increasing the number of washing steps.

The SELEX libraries were amplified using a) symmetric and b) asymmetric PCR. Briefly: (a) 5 μL of the aptamer pool (5–15 ng) in TE was mixed with 45 μL of symmetric PCR mixture containing: PCR buffer, 0.2 mM dNTPs, forward (5'-GGCAGCAGGAAGACAAGACA-3') and reverse (5'-ACAGAGCAACCACAGAACCA-3') primers, 0.3 μM each, and 0.05 U/μL Hot Start DNA polymerase. Amplification was performed as follows: 2 min at 95 °C, 15 cycles of 30 s at 95 °C, 15 s at 56 °C and 15 s at 72 °C. (b) 5 μL of the symmetric PCR product in TE buffer was mixed with 45 μL of the asymmetric PCR mixture containing: PCR buffer, 0.2 mM dNTPs, 1 μM forward primer, 50 nM reverse primer and 0.05 U/μL Hot Start DNA polymerase. Amplification was performed as described in (a).

For every next round of selection, ssDNA was purified using Amicon Ultra-0.5 10K Centrifugal Filter Devices (Merck Millipore, Germany) to remove low-molecular weight compounds. Then the solution over the filter was incubated at 95 °C for 10 min, and the polymerase precipitate was separated by centrifugation. ssDNA was quantified spectrophotometrically by NanoDrop Lite Spectrophotometer (Thermo Scientific, USA). In total, 12 rounds of selection were carried out.

Biotinylated enriched DNA libraries were synthesized after 8, 10 and 12 selection rounds by amplification as described above using biotinylated forward primer (Bio-5'-GGCAGCAGGAAGACAAGACA-3').

Illumina high-throughput sequencing and data analysis

About 10 ng of the 12th round enriched dsDNA library were ligated with adapters from NEBNext Multiplex Oligos using NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB). The library was sequenced on a MiSeq platform using 2x300 bp paired-ends sequencing kit (Illumina) in SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia) with coverage >100000. Raw reads were analyzed with UPARSE pipeline (Edgar, 2013) using Usearch v11.0.667. The UPARSE pipeline included: merging of paired reads; read quality filtering; length trimming; merging of identical reads (dereplication); discarding singleton reads; removing chimeras and OTU clustering.

DNA aptamers' binding affinity studies

a) Binding affinities of enriched DNA libraries and individual DNA aptamers were evaluated by the previously developed bioluminescent solid-phase assay (Krasitskaya et al., 2020a; Davydova et al., 2019). Specifically: the 50 nM solution of biotinylated aptamer or biotinylated enriched DNA library (50 μL) in binding buffer was added into the streptavidin-activated well, incubated with shaking for 30 min at RT and washed (three times, PBS, 1 mM $MgCl_2$, 0.1 % Tween 20). Then 50 μL aliquots of hFABP (18 μM – 2.7 nM in binding buffer) or 50 μL binding buffer (control wells) were added and incubated for 1 h at RT. After washing, 50 μL aliquots of anti-hFABP IgG – Obe (0.1 μg/mL in binding buffer, containing 0.1 % BSA) were added, incubated for 40 min at RT

and washed thereafter. Obelin bioluminescence was measured with LB 940 Multimode Reader Mithras (Berthold, Germany) by rapid injection of 0.1 M $CaCl_2$ in 0.1 M Tris-HCl, pH 8.8. The signal was integrated for 5 s. Signals from the corresponding control wells were taken as the background and subtracted during dose–response curve plotting. All measurements were performed in duplicate. The affinity constant was calculated according to (Krasitskaya et al., 2020a).

b) Isothermal titration calorimetry (ITC) measurements were performed using Affinity ITC instrument (Waters, USA). The aptamer FABPAp4 (1 μM) solution was loaded into the syringe (100 μL), while the hFABP solution (0.5 μM) was loaded into the sample cell (183 μL). The syringe was discharged for 20 injections of 5 μL each, the range of H-FABP concentration in the cell has been diluted from 0.50 μM to 0.32 μM. The sample cell was continuously stirred at 125 rpm throughout the experiment that was conducted at 40, 45 and 50 °C with an initial delay of 300 seconds and a spacing of 240 seconds between the injections. To ensure the quality of the data, control experiments were performed in which the FABPAp4 solution was titrated into PBS buffer alone. The data obtained were subtracted from those of the corresponding protein-ligand titration experiments.

The raw ITC data were analyzed using the AFFINImeter ITC software package provided by the manufacturer (Piñeiro et al., 2019). The heat of injection (Q) was calculated for each injection, and the integrated heat values were plotted against the molar ratio of ligand to protein. The data were fitted using the single-set of identical sites model, applying a Langmuir isotherm model to obtain the binding constant (K_D) and the enthalpy change (ΔH) was calculated from the binding constants using the following equation:

$\Delta H = \Delta H° + \Delta Cp\ (T - T°)$, where ΔH is the enthalpy change at temperature T; ΔH° is the enthalpy change at a reference temperature T°; ΔCp is the change in heat capacity upon binding; T, T° – temperature in Kelvin.

DNA aptamers' specificity studies

The samples of hFABP, cardiac troponin I (cTnI), myoglobin (Myo), human serum albumin (HSA), human immunoglobulin G (hIgG) and human serum (100 μL, 10 μg/mL in PBS, for each) was adsorbed on surface of the microtiter plates, overnight at 4 °C. After washing, the solution of biotinylated aptamer (FABPAp1c, FABPAp3c or FABPAp4) in binding buffer was placed into the wells, incubated for 1 h at RT, and washed (PBS, 1 mM $MgCl_2$, 0.1 % Tween 20). Then 50 μL hybrid protein Stavi-Obe solutions (50 ng/mL in binding buffer) were incubated for 40 min at RT. After washing, obelin bioluminescence was measured with LB 940 Multimode Reader Mithras (Berthold, Germany) by rapid injection of 0.1 M $CaCl_2$ in 0.1 M Tris-HCl, pH 8.8. The signal was integrated for 5 s.

Analysis of predicted structures of individual anti-hFABP DNA aptamers

Predicted secondary structures of aptamers were calculated using Vienna RNAfold algorithm (Gruber et al., 2008). According to the selection conditions, we applied a rescaling of energy parameters to 25 °C and included G-quadruplex formation into the structure prediction algorithm.

Results and discussion

In vitro selection of the DNA aptamers specific to hFABP

hFABP were covalently immobilized on NH_2-functionalized magnetic particles with the density of 0.2±0.01 μg of hFABP per 1 mg, calculated using obelin-based technique, as described in "Materials and Methods". The obtained hFABP-activated magnetic particles

were used for selection of specific aptamers by conventional SELEX technology as is shown in Figure 1A. Randomized oligonucleotide pool (10^{15} different sequences) was incubated with hFABP-activated magnetic particles, unbound DNAs were washed out and the bound aptamers were eluted by denaturation. Negative selection was performed with the initially aminated MPs before each round of selection. To enhance the aptamer specificity we used human serum protein, cardiac troponins I, myoglobin, creatine kinase MB and artificial diagnostic human serum for negative selection steps.

The selection was monitored by bioluminescent solid-phase assay developed earlier (Krasitskaya et al., 2020a). The enriched libraries after the 8, 10 and 12 SELEX rounds were re-amplified with biotinylated forward primer and the obtained biotinylated derivatives were analyzed by bioluminescent assay (Fig. 1B). Significant DNA library enrichment with the aptamers, affine to hFABP is observed only after 12 SELEX rounds.

After the 12th round of SELEX the library was sequenced with the Illumina NGS. To select aptamer sequences most potentially affine to target, raw NGS data were analyzed as described in "Materials and Methods". The sequences of most frequently aptamers and some their properties are represented in Table 1.

The aptamer sequences that occurred most frequently in enriched library were found to be shortened (less than 80 nt). One possible reason for this could be attributed to the polymerase skipping of the areas with a high content of guanine and cytosine nucleotides.

Analysis of the DNA aptamers affinity to hFABP

All aptamers were tested in the bioluminescent solid-phase assay of hFABP and compared with regard to their ability to bind target. For this, their derivatives, bearing biotin at the 5' terminus were chemically synthesized and analyzed. The analytical complex formed on the surface and the results for 5 aptamers are

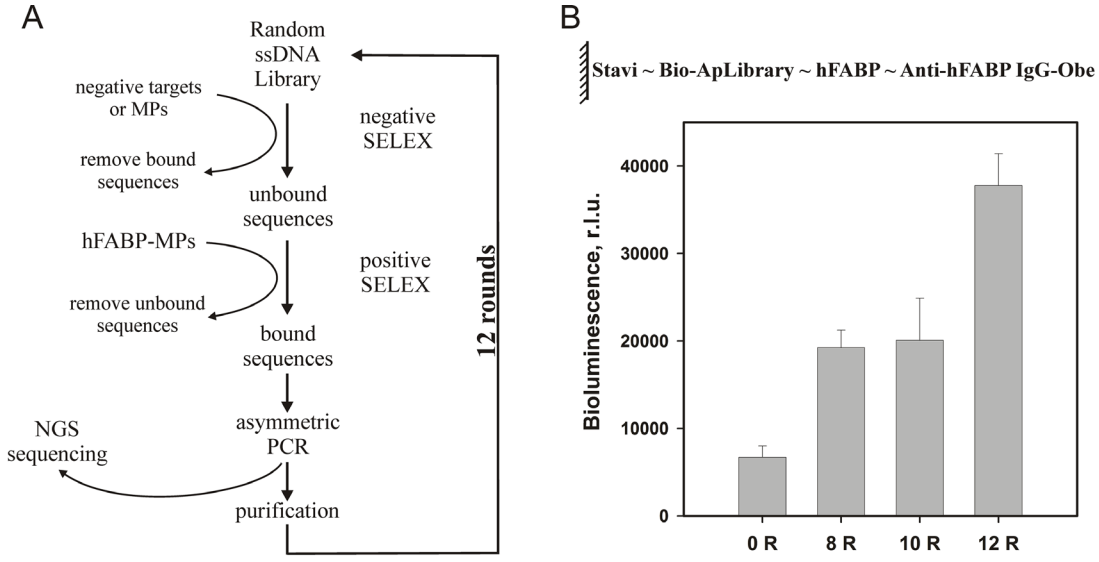

Fig. 1. A) In vitro selection of the DNA aptamers specific to hFABP using magnetic particles. B) Bioluminescent solid-phase assay to monitor the library enrichment: signals from the initial DNA library (0R) and after SELEX 8, 10 and 12 (8R, 10R, 12R) rounds. Composition of complexes formed on the surface is given in figure above. Stavi – streptavidin, Bio-ApLibrary – biotinylated aptamer library, Obe – Ca^{2+}-regulated photoprotein obelin

Table 1. Nucleotide sequences of most frequently DNA aptamers and some of their properties

Aptamer, size	Nucleotide sequences 5'-Bio → 3'	frequency, %	G%; GQ motif† (+ or –)	apparent K_D,* μM
FABPAp1n, 54 nt	GGCAGCAGGAAGACAAGACACCGTGT GGGGAGTGTGGTTCTGTGGTTGCTCTGT	1.3	57, -	1.53
FABPAp2n, 53 nt	GGCAGCAGGAAGACAAGACACGACAT CCCGGGGTGGTTCTGTGGTTGCTCTGT	1.2	58, -	n.d.
FABPAp3n, 47 nt	GGCAGCAGGAAGACAAGACAGGGGG CGTGGTTCTGTGGTTGCTCTGT	1.1	60, -	n.d.
FABPAp5n, 60 nt	GGCAGCAGGAAGACAAGACATGTGGCC AGTATCGTGCGTGTGGTTCTGTGGTTGCTCTGT	0.6	55, -	0.61
FABPAp1c, 80 nt	GGCAGCAGGAAGACAAGACATCGGGAGGGAGGG AGGGCAGTCTAGTCTCATGTGTTTCCATGGTTCT GTGGTTGCTCTGT	0.6	55, +	0.21
FABPAp3c, 80 nt	GGCAGCAGGAAGACAAGACATGGGTGGCGGGGA CGGGGCGATGGGAACTTAGATTGCTAGTGGTTC TGTGGTTGCTCTGT	0.7	58, +	0.56
FABPAp4, 80 nt	GGCAGCAGGAAGACAAGACACACGGGAGGGAGGG TAGGGTGTGTGTCGAATCACTGCGCATGGTTCTGT GGTTGCTCTGT	0.5	58, +	0.52

† The secondary structures of aptamers predicted by VIENNA RNA fold.
*K_D was calculated by bioluminescent solid-phase assay.
n.d. – not determined

shown in Figure 2. Based on the assay data, the saturation curves were obtained and apparent dissociation constants of aptamer-hFABP complexes (K_D) were calculated (Table 1). The full-length aptamers FABPAp1c, FABPAp3c and FABPAp4 demonstrate better target affinity as compared that to shortened ones, and were taken for the further study.

It should be remarked that the K_D values calculated by bioluminescence-based approach are apparent and may be taken rather as a comparative characterization of affinity than as its absolute value. It must be borne in mind that the bioluminescence of photoproteins occurs with a quantum yield of about 20 % (Malikova et al., 2022) and the value obtained is somewhat an estimate. Besides, aptamer–target complexes on the surface were detected indirectly, using a sandwich-type complex with the antibody. As hFABP is a relatively small protein (14.8 kDa) the affinity molecules can compete for a poor binding surface when forming multilayer complexes, which can interfere this process and distort constant values.

To test this assumption, we studied the affinity of one of the aptamers, FABPAp4 (apparent K_D = 0.52 μM) by isothermal titration calorimetry (ITC). It is a powerful technique used to measure the strength and thermodynamics of interactions between two or more molecules (proteins, nucleic acids, small molecules, etc.) in their native state under label-free and non-immobilized condition. ITC measures the heat released or absorbed during a binding event, which provides information about the binding affinity, stoichiometry, and thermodynamics of the interaction. It is widely used to investigate aptamer-protein interactions (see, e.g., Sakamoto et al., 2018; Amano et al., 2019; Slavkovic et al., 2020). Figure 3 (a, c, e) shows the titration of FABPAp4 aptamer into protein solution at 40, 45, and 50 °C

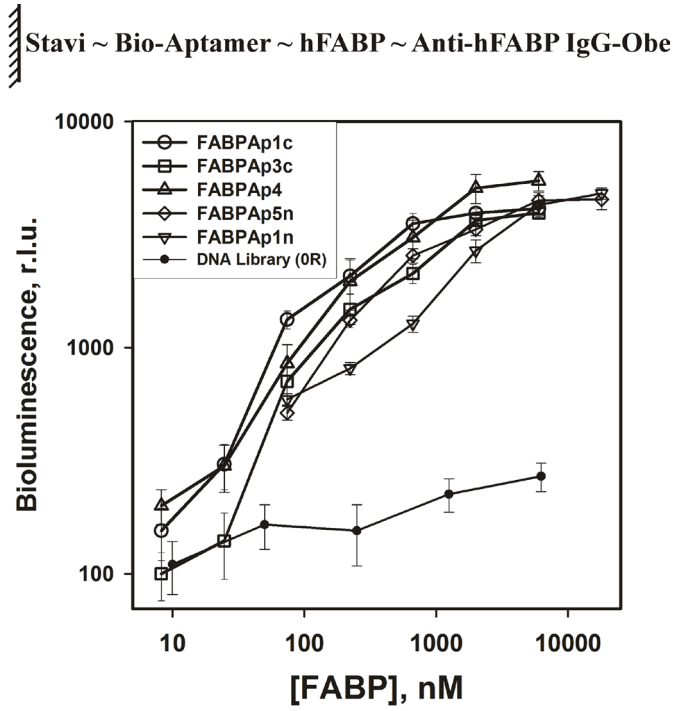

Fig. 2. Saturation curves for the corresponding aptamers and initial randomized DNA libraries. Upper panels – composition of complexes formed during solid-phase bioluminescent assay; lower panels – results of the corresponding assay (N=3)

Table 2. Thermodynamic parameters of the FABPAp4 titrations at different temperatures

Temperature, °C	K_D[M]	ΔH, [kcal/mol]	Response time, s
40	$0.83 \cdot 10^{-9}$	-0.26	1
45	$0.11 \cdot 10^{-8}$	-0.78	1
50	$0.22 \cdot 10^{-8}$	-0.59	4

correspondingly. The graph consists of a series of peaks reflecting the absorbed heat at each injection of FABPAp4, represented as the heat of binding in microcalories per second (μcal/s) as a function of time (min). The initial peak corresponds to the first injection of FABPAp4, and the subsequent peaks decrease in amplitude as aptamers become increasingly saturated with target. The integrated area under each peak is proportional to the amount of the absorbed heat at the corresponding injection, and the resulting data relates to a binding isotherm equation to obtain the thermodynamic parameters of the interaction, including the dissociation constant (K_D) and the enthalpy change (ΔH) (Fig. 3 b, d, f). Figure 3 (a, c, e) graphs show a clear and specific binding interaction between FABPAp4 aptamers and hFABP, with a K_D = 0.83 nM at 40 °C with ΔH = –0.26 kcal/mol (Fig. 3a). The data points are fitted to single-set of identical sites binding model and the solid line represents the fitted curve. Table 2 compares the thermodynamic parameters obtained from titrations performed at different

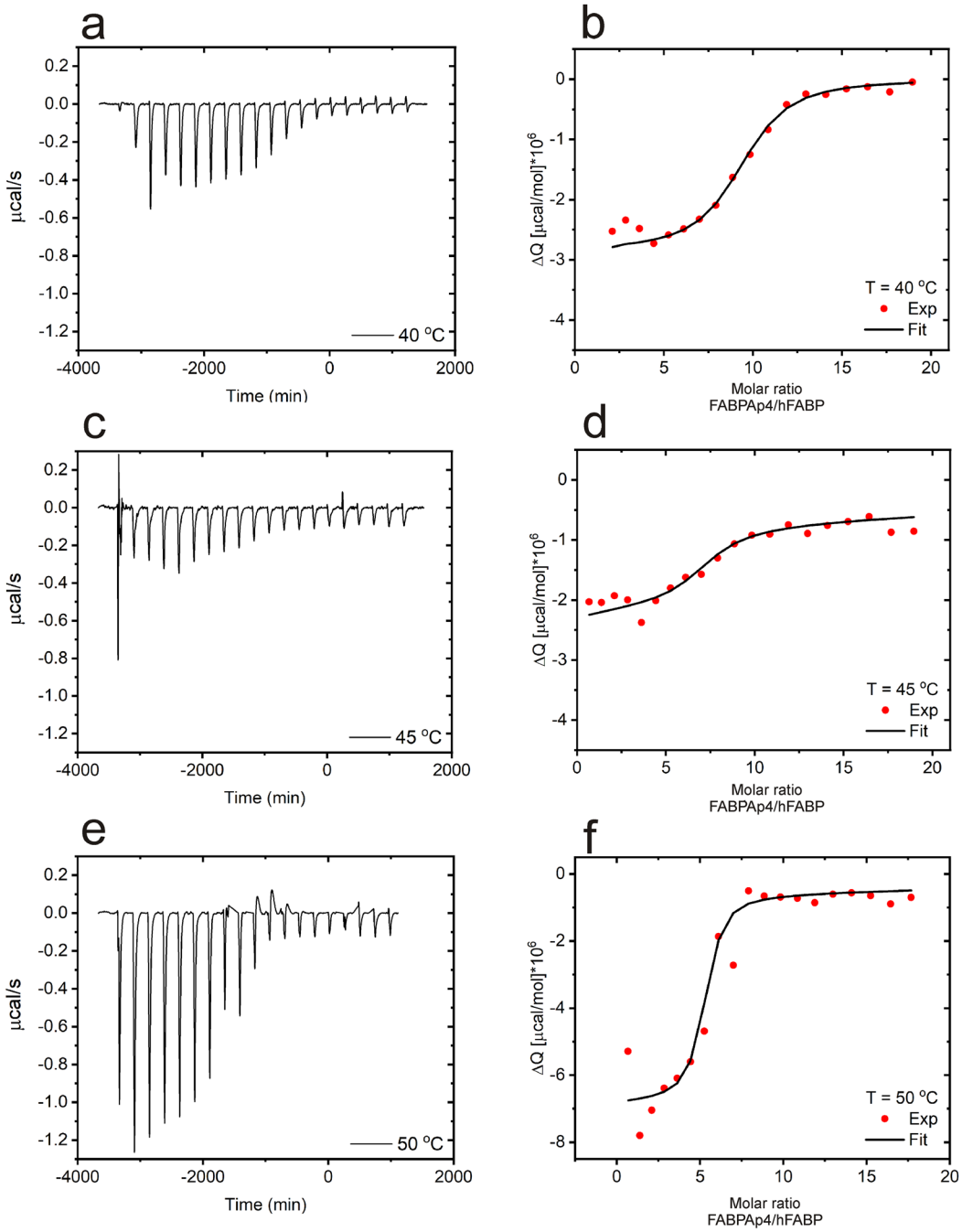

Fig. 3. ITC data of titration of FABPAp4 to hFABP for thermodynamic analysis and integrated heat values respectively at 40 °C (a, b); 45 °C (c, d); 50 °C (e, f)

temperatures. By comparing the values of ΔH and K_D obtained at different temperatures, it was determined that the binding of the ligand to the target molecule is entropy-driven, since the values of ΔH and K_D change with temperature range studied. The negative ΔH values indicate that the binding process is exothermic, meaning that heat is released during the binding event. The response time is the time required for the instrument to achieve thermal equilibrium after

the injection of a ligand into a protein sample. A faster response time can increase the speed of the experiment, but it should not be done at the expense of accuracy and precision. The response time was calculated by analyzing the injection heat pulses during a titration experiment using AFFINImeter. A linear relationship between the enthalpy change and temperature in a reaction indicates that ΔH is dependent on temperature, which corresponds to the Dulong-Petit law in classical thermodynamics.

Thus, according to ITC data, the FABPAp4 affinity to hFABP essentially exceeds the apparent one (K_D values 0.8 via 520 nM). This offers that the affinity of the remaining selected aptamers may also be high. Recently, two anti-hFABP aptamers were obtained by Kakoti and Goswami (2017) and studied for their binding interactions using circular dichroism (CD) technique. They found that the aptamers N 13 and N 53 exhibited affinity for hFABP with K_D = 74.3 ± 14.2 nM and 333.7 ± 148.5 nM, correspondingly.

Specificity of DNA aptamers to hFABP

FABPAp1c, FABPAp3c and FABPAp4 were evaluated for binding with hFABP, cTnI, Myo, HAS, hIgG and standard human serum by bioluminescent solid-phase assay (Fig. 4). Proteins under study were immobilized on the plate and incubated with biotinylated aptamer. The surface-formed complex was detected by using streptavidin–obelin hybrid protein. As is displayed in Figure 4, bioluminescent signals from the group of non-target proteins are reliably lower than that from hFABP (p=0.009, Mann-Whitney *U*-test), that clearly demonstrates significant differences between the aptamers specificity to hFABP and to the group of the other substances under

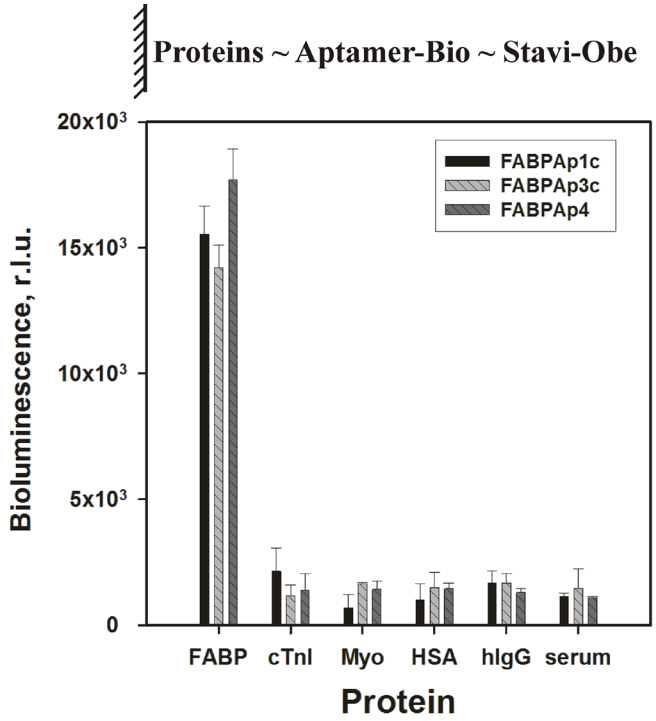

Fig. 4. Specificity analysis for the corresponding aptamers. Upper panels – composition of complexes formed during solid-phase bioluminescent assay; lower panels – results of the corresponding assay. Stavi-Obe – streptavidin-obelin fusion protein (N=3)

examination. This attests to a high specificity of the obtained aptamers to hFABP relative to other cardiomarkers and blood proteins.

Individual aptamers: structure opimization

All of the three 80-n.o. aptamers are G-rich oligonucleotides (55–58 %, Table 1). Circular dichroism (CD) spectroscopy was used to examine the possibility of guanine quadruplexes (GQ) formation (Kypr et al., 2009). CD-spectra contain a dominant positive band at 260 nm and additional characteristic positive peak at 210 nm, that clearly shows parallel G-quadruplexes formation (Fig. 5).

The truncated variants FABPAp1c, FABPAp3c and FABPAp4 were proposed basing on RNA fold-predicted secondary structure 80 nt DNA aptamer (Fig. 5). As is seen, this program predicted the presence of G-quadruplex (GQ) motif in DNA aptamers. G-quadruplex structures are believed to connect a common scaffold with various loop motifs that operate in target recognition (Tucker et al., 2012). When shortening these aptamers the GQ motif was kept in all variants. The truncated aptamers FABPAp1c-t53, FABPAp3c-t58 and FABPAp4-t57 consist of 53, 58 and 57 nucleotides, correspondingly and contain all loops with shortened stems. Aptamers FABPAp1c-t38, FABPAp1c-t28, FABPAp3c-t40 and FABPAp4-t31 consist of 38, 28, 40 and 31 nucleotides, correspondingly and contain a loop presumably forming the GQ with shortened stems. The resulting aptamers were chemically synthesized and supplied with 3′-biotin residues for subsequent binding experiments using the solid-phase bioluminescent assay according to the scheme shown in Figure 2.

The binding ability of truncated aptamers has not improved but has not deteriorated essentially either (Table 3). So, these 80 nt long aptamers can be shortened down to 27, 40 and 31 nt, correspondingly, without loss of binding ability to hFABP.

The CD spectra data (Fig. 5) clearly offer similar configuration of the GQ motif for truncated and full-size aptamers. This may indicate its key role in FABP binding. However, of note is that the cost of the truncated aptamers manufacturing is significantly lower, which is important from the point of view of the potential practical application of these aptamers.

Screening of aptamers to different epitopes of hFABP

Further, among the obtained truncated aptamers, a search was carried out for binding various hFABP epitopes applicable for sandwich-type analysis. The competitive binding of FABPAp4-t31, FABPAp3c-t40 or FABPAp1c-t38 with other obtained anti-hFABP aptamers was analyzed according to the scheme presented in Figure 6. Biotinylated aptamer under study (16 in total) was immobilized on streptavidin-activated surface and incubated with hFABP. Truncated FABPAp4-t31, FABPAp3c-t40 or FABPAp1c-t38 was chemically synthesized with oligoadenylate at 3’ terminus (FABPAp4-t31dA, FABPAp3c-t40dA, FABPAp1c-t38dA) forming a sandwich-type complex aptamer under study only if aptamers are affine to different epitopes. This complex is detected by bioluminescent signal of the conjugate obelin-oligothymidylate (Obe-dT_{30}). The results are shown in Figure 6. Low bioluminescent response leads to the conclusion that most aptamers under study join the same epitope of hFABP.

Thus, the attempts to find a pair of aptamers that would bind different hFABP epitopes were not successful, which may be due to this protein small size and (or) peculiarities of spatial structure. Since all studied aptamers presumably contain GQ motif, one may speculate that it makes the main contribution to binding at the same site of hFABP. Identification of hFABP key amino acid residues is planned at the next stage

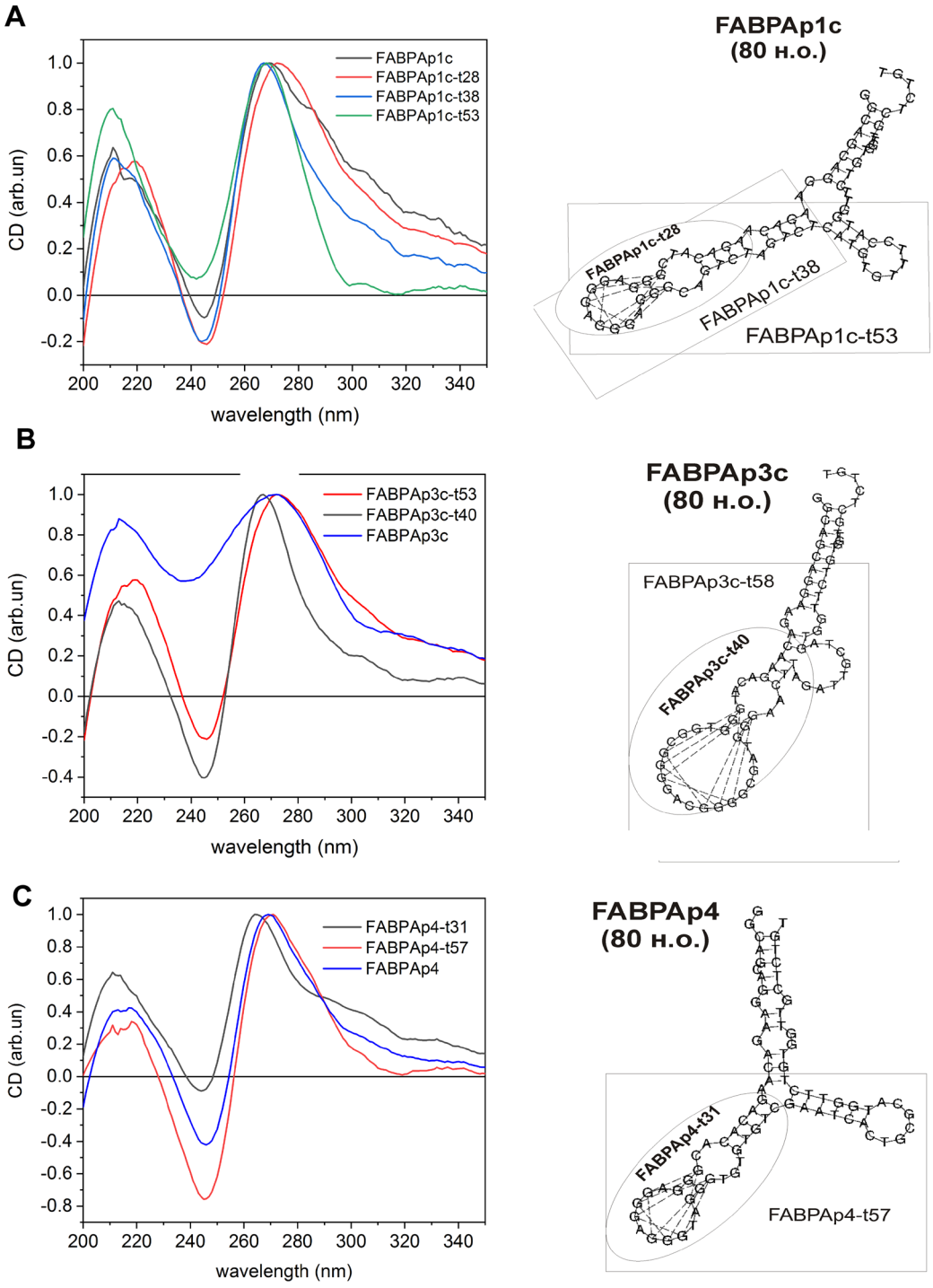

Fig. 5. CD spectra of FABPAp1c (A), FABPAp3c (B) and FABPAp4 (C) and their truncated variants. On the right – predicted secondary structures of the full-size aptamers and their truncated variants (RNAfold web server). Plausible formation route for GQ motifs is shown by a dashed line

Table 3. Nucleotide sequences of truncated anti-FABP DNA aptamers and some of their properties

Aptamer	Nucleotide sequences 5'-Bio → 3'	KD, μM	G%; GQ motif (+ or -)
FABPAp1c-t53	AGACAAGACATCGGGAGGGAGGGAGGGCAGTCTAGTCT CATGTGTTTCCATGG	0.24	55, +
FABPAp1c-t38	AGACAAGACATCGGGAGGGAGGGAGGGCAGTCTAGTCT	0.26	58, +
FABPAp1c-t28	AGACATCGGGAGGGAGGGAGGGCAGTCT	0.24	64, +
FABPAp3c-t58	GAAGACAAGACATGGGTGGCGGGGACGGGGCGATGGG AACTTAGATTGCTAGTGGTTC	1.02	57, +
FABPAp3c-t40	ACAAGACATGGGTGGCGGGGACGGGGCGATGGGAAC TTAG	0.61	63, +
FABPAp4-t57	ACAAGACACACGGGAGGGAGGGTAGGGTGTGTGTCGAA TCACTGCGCATGGTTCTGT	0.52	56, +
FABPAp4-t31	GACACACGGGAGGGAGGGTAGGGTGTGTGTC	0.61	65, +

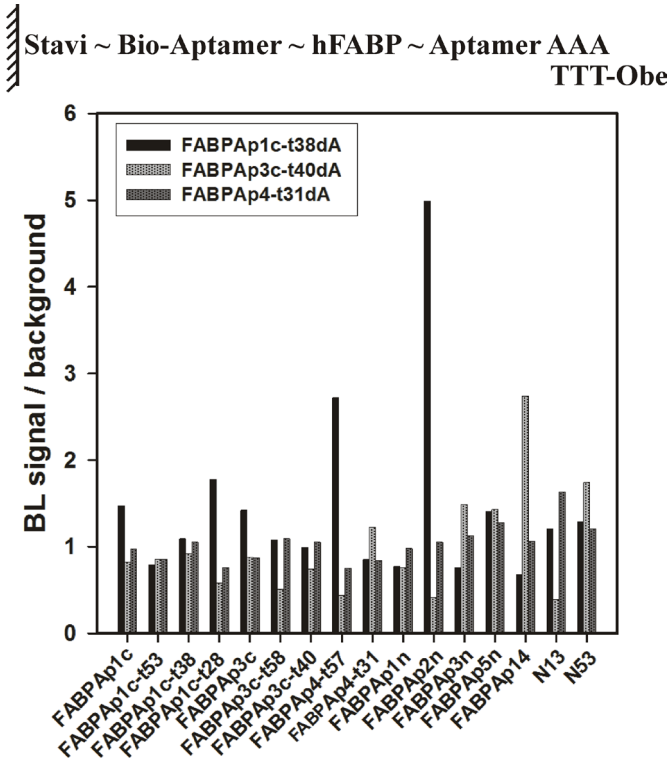

Fig. 6. Composition of the complexes formed on the surface (upper panel) and results (lower panel) of the bioluminescence assay for screening aptamers non-competitively pairing aptamers FABPAp4-t31, FABPAp3c-t40 and FABPAp1c-t38 (N=3)

of our research. This knowledge will promote more rational design of aptamer molecules to the targets of interest.

Conclusions

We selected several new DNA aptamers specific to heart fatty acid-binding protein. To our

knowledge, the only attempt to obtain aptamers to this important early marker of AMI is described in the article by Kakoti et al., 2017, mentioned above. Ca^{2+}-regulated photoprotein obelin shown to be a reporter of choice: its bioluminescence is bright, fast and easy to trigger, allowing to solve numerous analytical tasks. Here we applied it to characterize the microparticles surface capacitance as well as for SELEX monitoring and DNA aptamer affinity evaluation. Its application allows to determine the apparent values of the dissociation constants of the resulting intermolecular complexes, however, it allows you quickly compare aptamers' affinities and to select promising ones. Isothermal titration calorimetry (ITC) makes it possible to study the aptamer-target protein interaction in solution in native state and calculate the corresponding K_D value.

The CD spectra and the predicted secondary structure of the selected aptamers suggest the presence of G-quadruplex (GQ) forming motifs. The nucleotide sequences of the aptamers were minimized while retaining the probable GQ motif. The truncated aptamers demonstrated almost the same affinities to hFABP, which may indicate a key role of GQ motif in binding of the target. Moreover, it seems that all the resulting aptamers are directed to the same hFABP site on the protein: we were unable to find a pair of aptamers suitable for the development of a sandwich-type assay. But obviously, the resulting aptamers are applicable in the biosensors that exclude the formation of such complexes (see, e.g., Weaver et al., 2023) or apply mixed aptamer-antibody complexes (Kim et al., 2022).

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