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Design of Aptamer Sandwich Pairs for Colorimetric Detection of TNFa, Interleukin-17A and Dickkopf-1 Proteins Associated with Ankylosing Spondylitis

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Abstract. We employed DNA and 2'-fluoro RNA aptamers to assembly novel systems for colorimetric microplate detection of TNFa, interleukin-17A and Dickkopf-1 proteins as biomarkers associated with ankylosing spondylitis. For each protein, we chose an optimal sandwich pair made of plate-immobilized capture aptamer and biotinylated reporter aptamer recruiting streptavidin-peroxidase conjugate. A feasibility of these test systems was demonstrated in model protein solutions.

Keywords: oligonucleotide aptamers, biosensors, colorimetric detection, ankylosing spondylitis.

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Introduction

Ankylosing spondylitis (AS) or Bekhterev's disease is a chronic autoimmune pathology which involves sacroiliac joints and spine. AS is quite widespread in the population (9–30 cases on

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10000 people) and affects young persons of active working age [1]. Insufficient detection rate together with late diagnosis remains one of the main obstacles for effective treatment of AS. Early diagnosis, choice of therapeutic strategy and prognosis of the disease course demands not only general clinical laboratory analyses and radiological control, but also the measuring of specific biomarkers in the patients' blood and search of their associations with the activity of the disease and type of its progression [2]. Nowadays, levels of biomarkers are generally measured by enzyme-linked immunosorbent assay (ELISA) which employs specific antibodies for target recognizing. Unique properties of oligonucleotide aptamers make them a good alternative for antibodies [3]. Key advantages of aptamers include the availability of the digital twin and possibility of an automatic chemical synthesis providing high reproducibility of characteristics, tolerance to different chemical modifications and stability under a wide range of transportation and storage conditions. Aptamer-based test systems are especially attractive for long-term studies, such as monitoring of the course of chronic rheumatic diseases [4].

In a present work, we investigated a possibility of creating colorimetrical test systems on the basis of aptamers for detection of AS serological markers: pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin17-A (IL-17A) [5], and a WNT pathway antagonist Dickkopf-1 (DKK-1), one of the key regulators of bone tissue metabolism [6]. AS is accompanied by a non-controllable excessive ossification with the development of structural changes, therefore the tools for quantitative and dynamic estimation of this process are of high demand. Engineering aptamer-based test systems for clinical laboratory diagnostics have to account such criteria as high detection sensitivity and specificity, high reproducibility, minimal sample processing, and robust protocol of analysis compatible with a routine laboratory equipment. To meet these requirements, we have chosen a microplate colorimetric assay.

1. Materials and methods

1.1. Chemicals and reagents

In the present study, we used protected phosphoramidites and modified supports for oligonucleotide synthesis from Glen Research (USA), 1-methylimidazole, N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC), Tween-20, polyethylenglycol 8000 (Sigma-Aldrich, USA), biotin N-hydroxysuccinimide ester (Lumiprobe, Russia), phosphate-buffered saline (PBS) tablets (VWR, USA), recombinant proteins TNFα, IL-17A, DKK-1 (Cloud Clone Corp., China), streptavidin-peroxidase conjugate (Imtek, Russia), 3,3',5,5'-tetramethylbenzidine (TMB) (Fluka, USA).

1.2. Synthesis of oligonucleotide aptamers and their biotinylated conjugates

Oligonucleotides were synthesized on an automatic DNA/RNA synthesizer ASM-800 (Biosset, Russia), using the protocols specially optimized for the instrument and β-cyanoethyl-N,N-diisopropyl phosporamidites of 5',N-protected 2'-deoxyribonucleosides/2'-fluorodeoxyribonucleosides/2'-O-tret-butyldimethylsilylribonucleosides. Aptamers that contained at the 3'-end an aminohexanol residue and hexaethylenglycol phosphate were synthesized with the use of a modified support 3'-PT-Amino-Modifier C6 and hexaethylenglycol phosphoramidite (Spacer 18). The aptamer TD10 bearing 5'-aminohexanol was obtained using 6-(trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl) phosphoramidite at the last syn-

thetic cycle. Oligonucleotides were deprotected as described in [7], fully deblocked aptamers were purified by the preparative electrophoresis in denaturing 15% acrylamide gel. Oligonucleotide conjugates with biotin attached to the terminal aliphatic amino group were synthesized according to [8].

1.3. Microplate colorimetric analysis with the use of aptamer sandwich pairs

The aptamers bearing aliphatic amino groups at the 3'- or 5'-end were immobilized in the wells of 96-well plate with carboxy-modified surface NucleoLink^{TM} (Thermo Fisher Scientific, USA): 100 μL of 250 nM aptamer solution containing 10 mM 1-methylimidazole and 10 mM EDC were vortexed at 50°C for 16 h. Then the solution was discarded and the wells were washed thrice by the 300 μL of PBS supplied with 5 mM $MgCl_2$ and 0,05% Tween-20 (here and after, PBSMT buffer). After immobilization, the surface was blocked by the 250 μL of 1% bovine serum albumin in PBS or 0.1% of polyethtleneglycol-8000 in PBS for 1,5 h at 25°C and vortexing, and washed thrice by 300 μL of PBSMT. The solution of a protein with a certain concentration in PBSMT was added to the wells and incubated for 1 h at 25°C, then the solution was discarded and the wells were washed thrice by 300 μL of PBSMT.

As a second component of a sandwich pair, we used a biotinylated aptamer. Each well was supplied with 100 μL of 100 nM biotinylated aptamer in PBSMT, vortexed for 1 h at 25°C and washed four times by 300 μL of PBSMT. Then, 100 μL of 0,2 μg/mL streptavidin-peroxidase conjugate were added and the plate was vortexed for 1 h at 25°C. The solution was discarded and the wells were washed by PBSMT (5×300 μL). To get a colorimetric signal, we added the 0,2 mg/mL TMB solution in a 0.1 M Na acetate (pH 5,2) with 0,03% H_2O_2 and incubated for 20 min at 25°C. Then 100 μL of 10% H_2SO_4 were added, vortexed for 1 min at 25°C for obtaining a uniform yellow color, and the 450 nm absorbance was measured at the microplate reader AMR-100 (All-Sheng, China). The obtained data was approximated by a linear function in the program package GraphPad Prism 8.

2. Results and discussion

Based on the literature analysis, we chose previously published nucleotide sequences of DNA aptamers specific to TNFα [9–11] and 2'-fluoro modified aptamers to the IL-17A [12]. As candidate aptamers for forming a sandwich pair for the DKK-1 detection, we employed the DNA aptamer TD10 published in 2019 [13] and the DNA aptamer DK4_41t recently developed by our research team [14]. Nucleotide sequences and chemical modifications of the aptamers are listed in the Tab. 1. During the solid-phase phosphoramidite synthesis, we introduced a hexaethyleneglycol phosphate linker and aliphatic amino group to the 3'-end of the aptamers. This way of modification allows both for immobilizing the aptamer as a capture agent on a carboxy modified plate surface and for attaching a biotin residue which recruit a reporter streptavidin-peroxidase conjugate. In the case of TD10 aptamer, the aliphatic amino group was introduced to the 5'-end of the oligonucleotide chain, since in the original article [13] this particular type of functionalization was successfully used for surface immobilizing of the aptamer.

To form a system for colorimetric detection, one of the amino-modified aptamers was covalently immobilized on the surface of carboxy modified NucleoLink plate via carbodiimide condensation. Biotinylated aptamer acted as a second component of a sandwich pair. Colorimatrical

Table 1. Nucleotide sequences of DNA and 2'-F-RNA aptamers employed in the study

Target protein	Aptamer	Nucleotide sequence, 5'→3'	Reference
TNFα	T1	GGTCAGTGCATGTGACC-L1-NH_2	[9]
	T4	TCCGATCGGTATATCCGTCGGA-L1-NH_2	
	VR11	TGGTGGATGGCGCAGTCGGCGACAAp-L1-NH_2	[10]
	AptTNFα	GCGCCACTACAGGGGAGCTGCCATTCGAATAGGTGGG-CCGC-L1-NH_2	[11]
IL-17A	Apt3-4	GGAU^{F}AGC^{F}GAAGU^{F}C^{F}AU^{F}U^{F}GAGC^{F}GC^{F}C^{F}p-L1-NH_2	[12]
	Apt21-2	GGU^{F}C^{F}U^{F}AGC^{F}C^{F}GGAGGAGU^{F}C^{F}AGU^{F}AAU^{F}C^{F}GGU^{F}AG-AC^{F}C^{F}p-L1-NH_2	
DKK-1	TD10	NH_2-L2CATATGATTAGGCTGTAACGGGGCTAGGCG-GGGATCATT	[13]
	DK4_41t	CCTCGGAGTGCGTACAGCAAGCACATATGTGCTCT-CCCAGG-L1-NH_2	[14]
L1: -p-$(OCH_2CH_2)_6$-p-O$(CH_2)_6NH_2$ L2: $NH_2(CH_2)_6$-O-p- U^F - 2'-fluoro-2'-deoxyuridine, C^F - 2'-fluoro-2'-deoxycytidine			

signal was generated by means of TMB oxidation by the horse raddish peroxidase in the presence of a hydrogen peroxide. The detection scheme is presented in the Fig. 1A. Each of the aptamers was examined both as an immobilized capture component and as biotinylated reporter component of a sandwich system.

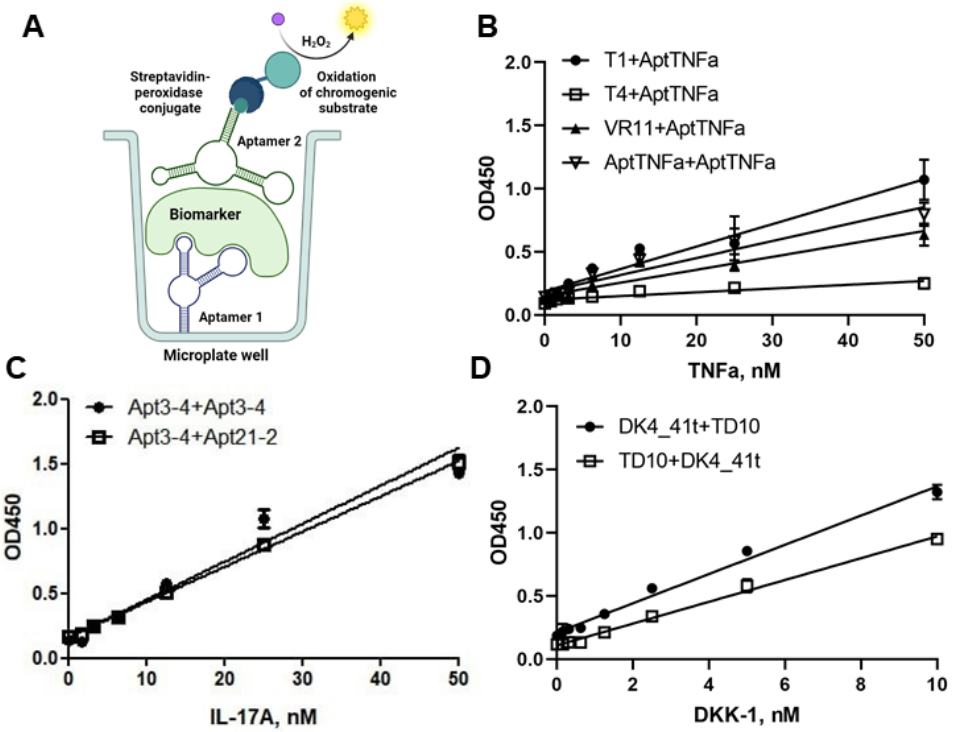

Fig. 1. A schematic representation of colorimetrical microplate analysis in a sandwich format with the use of two aptamers (A) and the results of colorimetrical analysis of target proteins TNFα (B), IL-17A (C), DKK-1 (D). OD450 — optical density of the solution at 450 nm

In the case of TNFα, all four DNA aptamers demonstrated the ability to function as an analyte-capturing component. Nevertheless, only AptTNFα suited as a reporter component and provided the appearance of colorimetrical signal proportional to the concentration of the target protein (Fig. 1B). We found the most prominent signal-concentration dependency for the system with isurface-immobilized T1 aptamer. The minimal detected concentration (the quantity of analyte with a signal-to-background ratio of 2) was found to be 5 nM.

For detection of the IL-17A, we examined sandwich microplate systems on the basis of two 2'-F-RNA aptamers, Apt3-4 and Apt21-2. The use of a standard blocking solution (1% BSA in PBS) during the system assembly led to rather high background signal. This problem was solved by the use of another blocking molecule, namely a 0.1% polyethyleneglycol-8000 in PBS, that decreased the background signal and retained sufficient absorbance in the wells with IL-17A. The highest magnitude of absorbance together with minimal background signal were observed for the variants with immobilized Apt3-4, at that either Apt 21-2 or Apt3-4 provided virtually the same efficacy as reporter components. A possibility to use the same aptamer both as a capture and reporter molecule is probably explained by the fact that IL-17A is a homodimeric protein [15]. Minimal IL-17A concentration detected in these systems was 6.25 nM.

DNA aptamers TD10 and DK4_41t as components of DKK-1 detection system also provided a linear growth of signal upon the raise of the target concentration. The magnitude of a signal was higher for the variant with plate-immobilized DK4_41t and biotinylated TD10 as a reporter, a minimal detecting DKK-1 concentration in this system was 2.5 nM.

To summarize, for all three biomarkers of interest we obtained aptamer-based colorimetrical systems potentially suitable for a microplate analysis with an optical detection. It should be noted that typical levels of these proteins in human blood serum are about 10 pg/mL (which corresponds to 1 pmole/L) for the TNFα and IL-17A [16] and about 3 ng/mL (0.1 nmole/L) for the DKK-1 [17], therefore in future studies we have to improve the sensitivity of the detection. This task can be solved, for example, by replacing a colorimetric signal by chemiluminescent, or the use of bioluminescent reporter proteins that have already shown their advantages in aptamer-containing test systems [18]. Further development of this work includes an optimization of the analysis methodology in the created sandwich systems for improving the sensitivity of detection, and examining of test-systems for their workability in the real serum samples form patients with the ankylosing spondylitis.

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Формирование сэндвич-пар олигонуклеотидных аптамеров для колориметрической детекции ассоциированных с анкилозирующим спондилитом белков ФНОа, интерлейкина-17А, Dickkopf-1

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Аннотация. На основе ДНК- и 2'-фтор-РНК-аптамеров сконструированы новые системы колориметрической микропланшетной детекции белковых биомаркеров ФНОа, интерлейкина-17А, Dickkopf-1, ассоциированных с анкилозирующим спондилитом. Для каждого белка выбрана оптимальная сэндвич-пара иммобилизованного на планшете улавливающего аптамера и биотинилированного репортерного аптамера, который взаимодействует с конъюгатом стрептавидин-щелочная фосфатаза. Показана принципиальная работоспособность таких тест-систем в модельных растворах белка.

Ключевые слова: олигонуклеотидные аптамеры, биосенсоры, колориметрическая детекция, анкилозирующий спондилит.