EDN: UYVQZC
УДК 577.271.34; 538.955

Magnetic Particles as a Carrier to Select Aptamer for Survivin (BIRC5), a Low Molecular Weight Protein Tumor Marker

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Received 10.08.2024, received in revised form 16.09.2024, accepted 24.10.2024

Abstract. Magnetic iron oxide nanoparticles modified with aminosiloxane (γ-Fe_2O_3@SiO_2-NH_2) were used as a carrier to select DNA aptamers for a small protein tumor biomarker survivin (BIRC5, 16.5 kDa) from a randomized synthetic DNA library by the SELEX method. Survivin was covalently immobilized on the nanoparticles' surface. The library enrichment was monitored by solid-phase bioluminescent immunoassay and the DNA melting method. The prospects are outlined for the use of the obtained nanoparticles for the effective selection of aptamers specific to a given target.

Keywords: DNA aptamers, survivin (BIRC5), magnetic nanoparticles.

Citation: N.S. Panamarev, A.V. Tyumentseva, V.V. Krasitskaya, L.A. Frank, Magnetic Particles as a Carrier to Select Aptamer for Survivin (BIRC5), a Low Molecular Weight Protein Tumor Marker, J. Sib. Fed. Univ. Math. Phys., 2024, 17(6), 769–775.
EDN: UYVQZC.

Introduction

Aptamers are synthetic single-stranded DNA or RNA molecules (20÷80 b.p.) that, due to their specific spatial structure, are capable of specifically recognizing and binding target molecules [1]. In essence, aptamers are nucleotide analogues of antibodies and lack such disadvantages as difficulties of production, batch-to-batch variability, and instability. Selection and identification of the oligonucleotides, affine to the target is a key step in producing the aptamers. The technology for selecting individual sequences from a large randomized oligonucleotide pool is called

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SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [2]. Traditionally, nitrocellulose membranes, microplates' surface and various affinity sorbents are employed as target carriers at the stage of selection. Covalent immobilization of the target on magnetic particles allows for multiple washes without risking of losing immobilized complexes, while the magnetic properties of the carrier significantly simplify manipulations, making the SELEX process less labor-intensive [3]. In the case of immobilization on particles, the probability of preserving the native conformation of the target molecule and the accessibility of epitopes on the protein surface can be higher than, for example, with the adsorption immobilization on the surface of a plate. In this work, we selected DNA aptamers for the tumor biomarker protein survivin (BIRC5), covalently immobilized on magnetic iron oxide nanoparticles coated with a double siloxane layer and containing functional amino groups (γ-Fe_2O_3@SiO_2-NH_2).

1. Materials and methods

1.1. Materials and reagents

Highly-purified recombinant proteins: survivin (Surv), luciferase NanoLuc(C164S)LCTPSR (NLuc), Ca^{2+}-regulated photoprotein obelin (OL), streptavidin (Stavi) were obtained as described in [4–7]. We used: bovine serum albumin (BSA), dimethyl suberimidate dihydrochloride (DMS) (Sigma-Aldritch, USA); human serum albumin (HSA) (Panreac, Russia); anti-survivin antibodies (AntiSurv) (CloudClone, China); artificial human serum (Microgen, Russia), furimazine (TargetMol, USA); Hot Start Taq DNA polymerase and other PCR reagents (SibEnzyme, Russia). AntiSurv conjugates with bioluminescent proteins (AntiSurv-OL and AntiSurv-NLuc) were synthesized as described previously [5–6].

The single-stranded (ssDNA) library consisting of 40 nucleotides (N40) randomized region, flanked by the two constant primer-hybridization regions (5'-GGCAGCAGGAAGACAAGACA–N40–TGGTTCTGTGGTT GCTCTGT-3'), was from Sangon Biotech (China). All the other reagents were of high purity grade.

Magnetic iron oxide nanoparticles (MNP) coated with a double siloxane layer and containing functional amino groups (γ-Fe_2O_3@SiO_2-NH_2) were obtained as described in [8].

1.2. Protein covalent immobilization on the magnetic particles (MNP)

Solutions of 0.2 mg protein and 3 mg DMS were added successively to a MNP suspension (1 mg in 50 mM Bicine, pH 8.5). The mixture was incubated with shaking at room temperature (RT) for 2 h and then at 4°C overnight. Then the particles were washed five times with PBS (0.1 M phosphate buffer, 0.15 M NaCl pH 7.0), 1 mM $MgCl_2$, 0.01% Tween-20.

The amount of protein immobilized on the particles was estimated spectrophotometrically (NanoDrop, Thermo Fisher Scientific, USA) by its decrease in protein after the reaction. Magnetic particles with immobilized survivin (MNP-Surv) were used for a week, with BSA (MNP-BSA) — for two weeks. Survivin availability was assessed by a bioluminescence assay: MNP-Surv and MNP-BSA (control) were incubated with 1% BSA (PBS, 1 h, RT), washed and incubated with AntiSurv-OL (1 h, RT, 50 r.l.u.). After washing, bioluminescence of obelin immobilized on the surface was initiated by injecting $CaCl_2$ (0.1 M in 0.1 M Tris-HCl pH 8.8, 50 μl per well) and the signal was integrated for 5 s using a LB 940 Multimode Reader Mithras plate luminometer (Berthold, Germany).

1.3. Anti-survivin DNA aptamers selection

Before each round of selection (R), the DNA library was heated at 90°C for 5 min in binding buffer (PBS, 1 mM $MgCl_2$, 0.01% Tween-20) followed by cooling at RT for 15 min.

The library solution was added to 30 μg of MNP-BSA for negative selection and incubated for 30 min with shaking. The library was placed into a tube with MNP-Surv (30 μg for R1; 20 μg for R2-4; 15 μg for R5-8; 10 μg for R8+ were used) and incubated at RT for 1 h , 45 min (R2–R6) or 30 min (R8+). MNP were thoroughly washed (3–7 times, depending on the round) with a washing solution (PBS pH 7.0, 1 mM $MgCl_2$, 0.1% Tween-20). The bounded DNA sequences were eluted with 40 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), at 90°C for 5 min.

Starting from the 2-nd round, an additional stage of negative selection was introduced with targets immobilized on the surface of a well of the plate (Costar, USA). These were streptavidin (rounds 2 and 12), HSA (3), AntiSurv (4 and 9), OL (5), NLuc (6, 10 and 14), healthy human urine (7 and 11), artificial serum (8), and nonfat dry milk (13). The negative selection targets were immobilized by incubation in a well of the plate (20 μg/ml (except serum and urine), 1 h, 37°C).

The DNA library eluted from the particles was amplified by symmetric and asymmetric PCR as described earlier [9]. The number of cycles for symmetric PCR for each round depended on the amount of PCR by-products. The purity of the PCR product was assessed by using horizontal DNA electrophoresis in 2% agarose gel. The asymmetric PCR product was purified from primers and free dNTPs in Amicon Ultra-0.5 10K cells (Merck Millipore, Germany). The purified DNA library was heated (95°C, 10 min), and denatured polymerase was removed by centrifugation.

1.4. Monitoring of the library enrichment with the molecules affine to survivin

a) *Bioluminescent immunoassay:* 100 μl aliquots of the 50 nM biotinylated library were placed into the streptavidin-activated wells, incubated with shaking at RT for 1 h, and washed. Then 100 μl survivin solution (5 μg/ml) was placed into the wells, incubated at RT for 1 h; control wells contained buffer solution. After washing, 100 μl aliquots of the AntiSurv-NLuc (50 ng/ml) were placed into the wells, incubated for 40 min, and washed. Bioluminescence was initiated with freshly prepared furimazine solution (1 μM in 20 mM Tris-HCl pH 8.0, 0.15 M NaCl) and measured as described above.

b) *DNA melting method:* mixture of 0.5 μM DNA library and 1x SYBR Green I (20 μl, PBS pH 7.0, 1 mM $MgCl_2$) was used for melting (Rotor-Gene Q, Qiagen, Germany). Melt curve analysis protocol: hybridization — from 95°C to 25°C, 1°C/min step; incubation — 25°C, 30 min; melting — from 25°C to 95°C, 0.5°C/min step, with fluorescence registration. Melt curves were presented as fluorescence–temperature dependence (dF/ldT).

2. Results and discussion

2.1. Survivin covalent immobilization on the MNP

Magnetic iron oxide nanoparticles obtained by $FeCl_2$ and $FeCl_3$ co-precipitation [8] were used as a carrier for targets in the selection of aptamers. The surface was functionalized sequentially using TEOS and APTES, forming a siloxane layer containing functional aminopropyl groups. At that, a γ-Fe_2O_3@SiO_2-NH_2 composite of the "raisin bun" type was formed. The diameter of the

resulting agglomerate ("bun") was about 100–400 nm, the size of the embedded γ-Fe_2O_3 particles ("raisins") in the γ-Fe_2O_3@SiO_2 composite was 6 nm (Fig. 1A). The concentration of NH_2 group (calculated according to [10]) was 0.7–1.2 mmol/g of the particles (19–33 molecules/nm^2), which exceeds the content of NH_2 groups in aminosilanized superparamagnetic nanoparticles obtained by T. Sen et al. (0.24 mmol/g, 4.8 molecules/nm^2) [11] and S. Thalhauser et al. (1–4 μmol/g) [12].

The protein of interest was covalently immobilized in an alkaline medium using DMS as homobifunctional coupling agent. Fig. 1B shows the structure of the final complex, formed on the particles' surface. The amount of immobilized survivin was 62 μg per 1 mg of MNP, BSA — 28 μg. The availability for binding of survivin covalently immobilized on the particle surface was assessed by bioluminescent immunoassay using a conjugate of antibodies to survivin with the photoprotein obelin (AntiSurv-OL) (Fig. 1C).

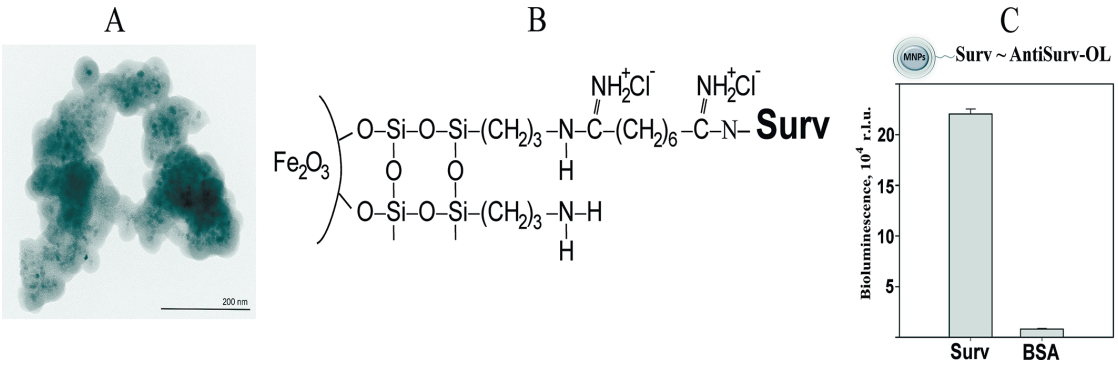

Fig. 1. HRTEM images of γ-Fe_2O_3@SiO_2-NH_2 nanoparticles, obtained using a Hitachi FE-SEM S-5500 microscope (Japan) (A). Survivin immobilization on the particles' surface (B). Bioluminescence immunoassay of survivin immobilized on the particles' surface; BSA-activated particles analysis is shown for comparison. The scheme of the molecular complex formed on the surface is shown on the top (C)

2.2. Selection of aptamers specifically binding survivin

The selection of an enriched DNA aptamer library specific to the survivin was performed as a result of 14 SELEX rounds. The molar ratio of oligonucleotides to the target during positive selection of the first round was 10:1. To increase the pressure during selection, the number of magnetic particles and incubation time were successively reduced, and the number of washes was increased. To exclude sequences that are non-specific to survivin, double negative selection was done by using various proteins immobilized on a well of a microplate and the magnetic particles with immobilized BSA.

The eluted sequences were amplified using symmetric PCR, and then the ssDNA library was restored by using asymmetric PCR. Due to the high capacity of the MNP used, the quantity of eluted sequences after the selection round is quite large, especially starting from the middle rounds, when aptamers affine to the target begin to be accumulated. In order to minimize the production of by-products, we varied the number of symmetric PCR cycles during library amplification. Fig. 2A exemplifies the choice of the symmetric PCR cycles. Thus, after the 12th round of selection, six PCR cycles turned out to be optimal for DNA production.

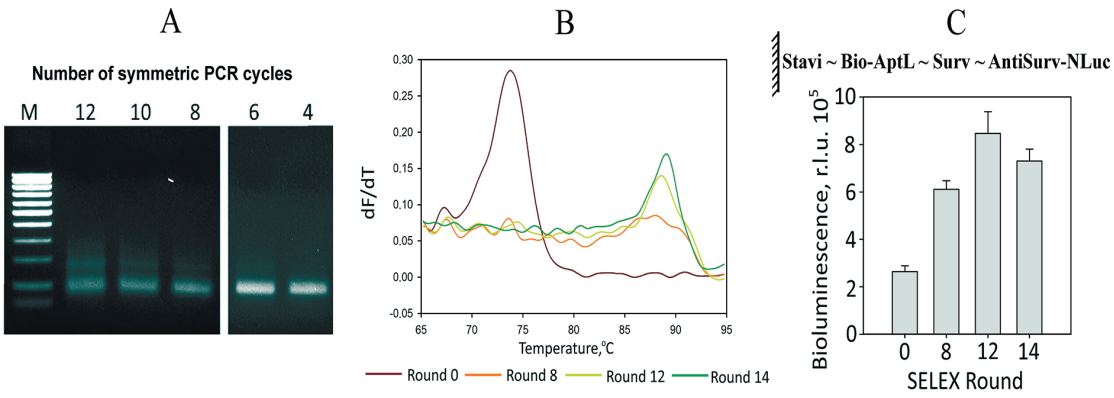

Fig. 2. Variation of the PCR cycles number during DNA library amplification for the 13-th round selection. M — DNA marker 100 bp + 50 bp (SibEnzyme) (A). Melting profile of the original and enriched dsDNA libraries (B). Bioluminescent solid-phase analysis of library enrichment. Scheme of the complex formed on the plate surface is shown on the top (B). BioAptL — biotinylated library

2.3. Monitoring the enrichment of libraries with survivin-affinity sequences

The enrichment monitoring during SELEX was performed using the method of thermal denaturation of dsDNA libraries (DiStRO method) [13]. Melting profiles of the original and enriched libraries show a high-temperature shift (Fig. 2B) after 8, 12 and 14 rounds in the presence of the intercalating dye SYBR Green I, indicating thereby a decrease in the diversity of nucleotide sequences and enrichment of sequences affine to the target.

The selection specificity was monitored by bioluminescent solid-phase analysis of libraries, applying the method we developed earlier [9]. The results obtained show the bioluminescent signal from enriched libraries to increase as the number of rounds grows, and to cease after the 12th round (Fig. 2B). Thus, in the course of our selection, the sequences that specifically bind survivin are being accumulated.

Conclusion

It is shown that aminated magnetic iron oxide nanoparticles modified with aminosiloxane (γ-Fe_2O_3@SiO_2-NH_2) can be used as a convenient target carrier in the selection of aptamers by the SELEX method. As a result of the work, during 12 rounds of SELEX, a DNA library enriched with sequences affinity to tumor biomarker survivin was obtained. The efficiency and specificity of the selection were demonstrated by the DNA melting method and by bioluminescent solid-phase immunoassay of the target protein.

This study was supported by the Russian science foundation and the Krasnoyarsk regional fund of science and technology support, project no. 22-14-20020.

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Магнитные частицы как носитель для отбора аптамеров к низкомолекулярному белку-онкомаркеру сурвивину (BIRC5)

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Аннотация. Магнитные наночастицы оксида железа модифицированные аминосилоксаном (γ-Fe_2O_3@SiO_2-NH_2) использовали как носитель для проведения отбора ДНК–аптамеров к низкомолекулярному белку-онкомаркеру сурвивину (BIRC5, 16,5 кДа) из рандомизированной синтетической ДНК–библиотеки методом SELEX. Сурвивин ковалентно иммобилизовали на поверхность наночастиц. Мониторинг обогащения библиотек проводили с помощью твердофазного биолюминесцентного иммуноанализа и методом плавления ДНК. Показана перспективность применения полученных наночастиц для эффективного отбора аптамеров, специфичных к заданной мишени.

Ключевые слова: ДНК-аптамеры, сурвивин (BIRC5), магнитные наночастицы.