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BIOPHYSICS AND MEDICAL PHYSICS

Complexes of the ATP-Dependent Lon Protease and DNA Aptamers with G-Quadruplexes as a Model for Developing a Nanosensor Biomagnetic Immunoassay System

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Abstract—The binding to Lon protease through biotinylated aptamers whose structures contain G-quadruplex fragments with magnetic nanoparticles (MNPs) functionalized by streptavidin was investigated. The conditions of binding of target aptamers to MNPs are met. The resulting complexes are proposed for detection of Lon protease in different biological sources and for constructing a novel biomagnetic nanosensor immunoassay system.

Keywords: magnetic nanoparticles, streptavidin-biotin, DNA aptamers, Lon protease.

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INTRODUCTION

The present study is a part of a project that is directed toward the creation of new generation diagnostic medical devices for a separation-free immunoassay scheme [1, 2]. The scheme is based on the use of magnetic nanoparticles (MNPs) with attached oligonucleotides (DNA aptamers) that are capable of specific formation of strong complexes with protein molecules, as markers of diseases of different etiologies. The formation of the complexes of aptamers and proteins in the biomagnetic system that is being developed is performed by a sensitive magnetometer that records changes in the rate of the Brownian relaxation of MNPs upon binding of aptamers immobilized to protein targets as it is carried out for an antigen–antibody pair.

The protein target in this study was the ATP-dependent Lon protease. As an additional marker for the development of a nanosensor biomagnetic system we chose the human interleukin-6 (IL-6) [3–5]. ATP-dependent Lon protease is the key component of a system for quality control of the cell proteome (a complex of cell proteins). At normal concentrations, Lon protease controls the level of several regulatory proteins and destroys nonfunctional (mutant, abnormal, or defective) cellular proteins. At high concentrations, mitochondrial Lon protease accompanies the development of some cancers (Seidel hepatoma and lymphoma; lung adenocarcinoma; oral carcinoma; mammary, colon, or bladder cancers) [6–10]. In this connection, Lon protease, like IL-6, could be a useful target for antitumor drugs [7–9].

It was noted in [11, 12] that Lon protease and other proteins that are connected with the development of cancer can be not only molecular therapeutic targets, but also are clinical biomarkers of tumors of different types. Thus, the evaluation of the content of, e.g., IL-6 and Lon protease, in the cells of cancer-prone organs can be used for detection of cancerogenesis.

The objects of the study were aptamers, small DNA fragments with sizes from 30 to 60 nucleotides that are capable of selective high-affinity binding to different biomolecules that include proteins [13–15]. The investigation of the specificity of the interaction between proteins and nucleic acids (NAs) as an intermolecular complementary interaction served as the basis for the creation of a novel technique, SELEX, using which it is possible to find a complementary partner by using a combinatorial library of NAs. Combinatorics, or the combinatorial approach, has been developed as a field of science not only in mathematics that studies discrete objects, sets, combinations, and permutations, but also in chemical and biological investigations. The use of the basics of combinatorial

chemistry of nucleic acids made it possible to develop a method for the irrational design of aptamers. These fold into complex three-dimensional structures and have high affinity to a protein target, which is comparable with the antigen–antibody affinity. Aptamers are considered as functional analogs of monoclonal antibodies. In contrast to antibodies, whose production requires using living systems, aptamers are obtained in vitro by chemical and enzymatical methods and their production can be automated. Therefore, aptamers are more advanced and promising than conventional antibodies. Aptamers and their properties were described in more detail in [16–21].

Recently we have demonstrated that Lon protease from *E. coli*, an enzyme, which is more than 65% homologous to the human Lon protease [22], forms complexes with aptamers in which duplex domains are combined with a G-quadruplex region [23]. The interest in G-quadruplex structures, in which guanine bases are connected via Hoogsteen type pairing, has significantly increased during the last 20 years, since these G-blocks have been found in biologically active centers of genomic DNA, telomeres, and promotor regions [24, 25]. Proteins that bind these quadruplex regions have been found with some of these proteins being actively involved in the tumor development [26, 27].

This study investigated the possibility of binding biotinylated aptamers that contain G-quadruplexes with Lon protease and magnetic nanoparticles that are functionalized by streptavidin for the purpose of further detection of the presence of Lon protease in biological sources. In connection with this, the goal of the present study was to investigate the conditions of binding of target aptamers to functionalized MNPs to use the obtained complexes in the construction of a nanosensor biomagnetic immunoassay system.

1. MATERIALS AND METHODS

The study employed biotinylated DNA aptamers to Lon protease with the following structures:

ST43biot 5'biotin-GTGACGTATGGTTGGTGTGGTTGGGGCGTCAC

ST43biot5T 5'biotin-TTTTTGTGACGTATGGTTGGTGTGGTTGGGGCGTCAC

RE31biot 5'biotin-GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC

RE31biot5T 5'biotin-TTTTTGTGACGTAGGTTGGTGTGGTTGGGGCGTCAC

Introduction of additional 5T oligonucleotides that are referred to as linkers made it possible to move a biotin label from the main structure of the molecule.

The aptamers and their nonmodified forms were synthesized by Sintol, Russia. The affinity of the unmodified aptamers to Lon protease was described earlier in [23].

1.1. Circular Dichroism (CD) Spectra for Investigation of the Aptamer Structure

The CD method is used for control of the formation and conformational changes of G-quadruplex DNA structures. The CD spectra of antiparallel quadruplexes have typical positive maxima at 294 nm and 248 nm and negative extremum at 265 nm [28, 29]. The spectra were obtained using a Chirascan spectrometer (Applied Photophysics Ltd, United Kingdom) within a wavelength region from 240 to 360 nm at temperature of 25°C in a cell with the optical path length of 1 cm. The rate of the wavelength change was 1 nm/s. The spectrum of buffer that did not contain oligonucleotides was considered as the baseline. Solutions of aptamer DNAs were prepared in PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4, 140 mM NaCl, and 5 mM KCl) in the following concentrations: ST 43biot, 2.3 μM; ST 43biot5T, 2.0 μM; RE31biot, 2.4 μM; and RE31biot5T, 1.9 μM.

1.2. Obtaining Complexes of Lon Protease and Aptamers

To avoid possible self-degradation of Lon protease during prolonged experiments on the physical and chemical characteristics of the enzyme complexes with magnetic nanoparticles, a proteolytically inactive form of the enzyme, Lon-S679A (further Lon), with catalytically active serine being replaced by alanine, was used when the complexes of Lon protease with aptamers were obtained.

Nucleotide samples were heated for 2 min at 100°C, rapidly cooled in an ice bath, and incubated on ice for 1 h with Lon-S679A in 50 mM tris-HCl buffer, pH 7.5, that contained 140 mM NaCl and 5 mM KCl. The volume of the reaction mixture was 20 μL; the aptamer concentration was constant (375 nM) and the ratio between the aptamer and protein varied from 1 : 0.6 to 1 : 4.5. The samples were then supplemented with 10 μL of 20% glycerin and the aliquots of the reaction mixtures were subjected to gel electrophoresis in 8% polyacrylamide gel under nondenaturing conditions. To reveal regions with aptamer DNA, SYBR GREEN fluorescent dye was used. The gel was scanned on a Fluorescent Image Analyzer (FLA-300 series, Fujifilm).

1.3. Immobilization of Biotinylated DNA Aptamers on MNPs Functionalized with Streptavidin

The study employed magnetic nanoparticles with a size of 50 nm (Ocean Corporation, United States). The preparation contained MNPs (1 mg/mL) in 50 mM phosphate buffer, pH 7.4, bovine serum albumin (BSA, 1 mg/mL), and sodium azide (0.02%). To remove BSA from the medium, the particles were washed with PBS several times with further centrifugation (16 000 rpm, 30 min, 4°C).

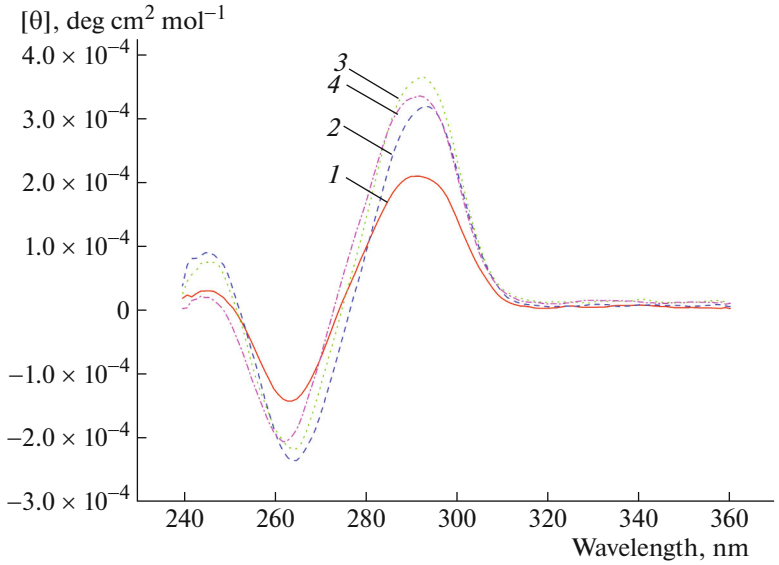

Fig. 1. The CD spectra of the biotinylated aptamers to Lon protease: (*1*) RE31biot5T, (*2*) RE31biot, (*3*) ST43biot, (*4*) ST43biot5T.

To form the MNP–streptavidin–aptamer complex, 200 μL of a standard suspension of the MNPs in working buffer were mixed (25°C) with 5 μL of 100 μM aptamer solution in PBS and diluted with the same buffer to the total volume of 1 mL. The mixture was then incubated for 2 h (4°C) and centrifuged for 30 min (15 000 rpm, 4°C). The amount of the unbound aptamer in the supernatant was determined according to the solution absorption at 260 nm, which made it possible to calculate the portions of the bound biotinylated aptamers on the MNP–streptavidin.

1.4. Binding of Lon Protease to the MNP–Streptavidin–Aptamer Complexes

To estimate the content of Lon protease in an analyte using MNPs 1 mL of the suspension of the MNPs with the aptamer in a buffer that was based on 50 mM tris-HCl, pH 7.5 (140 mM NaCl and 5 mM KCl), was supplemented with 50 μL of 30 μM solution of Lon protease and incubated on ice for 1 h. The aptamer : protein ratio was approximately 1 : 10.

1.5. Dynamic Light Scattering (DLS)

We applied DLS to qualitatively characterize the changes in the size of the MNPs functionalized with streptavidin that resulted from the attachment of the biotinylated aptamer derivatives and, then, protein molecules. The light scattering in the sample was caused by the fluctuations of density and the local concentration of the substance in an individual volume of the sample that occur due to the thermal motion of macromolecules or colloid particles. DLS measurements were carried out using a Zetasizer Nanoinstrument laser light scattering photometer (Malvern Instruments, United Kingdom) with a He–Ne laser with a power of 4 mW and a wavelength of incident light of 633 nm (25°C, cell volume V of 0.5 mL).

2. RESULTS AND DISCUSSION

2.1. The Characteristics of the Aptamers According to the CD Spectra

According to the spectral studies it was found that all the studied aptamers have characteristic positive extrema near 294 and 248 nm and negative extremum in the region of 265 nm, i.e., they form antiparallel G-quadruplexes. As can be seen from Fig. 1, the amplitude of the positive maxima slightly varies depending on the method of attachment of the biotin labels to the aptamers, via the linker (additional 5T) or directly to the oligonucleotide sequence. According to the results of the comparison of the spectra of aptamers that have been and have not been modified with biotin it can be stated that the introduction of the biotin molecule does not disturb the folding of the G-quadruplex structure, which makes it possible to use these DNA oligonucleotide structures in further work.

2.2. Determination of the Affinity of Biotinylated Aptamers to Lon Protease in a Solution

The affinity of the aptamers to the protein was assessed using electrophoresis in a polyacrylamide gel (PAAG) [23]. It can be seen from Fig. 2 that with an increase in the level of the protein in the reaction sample the intensity of the region of the Lon-S679A/aptamer complex significantly increases. The mobility

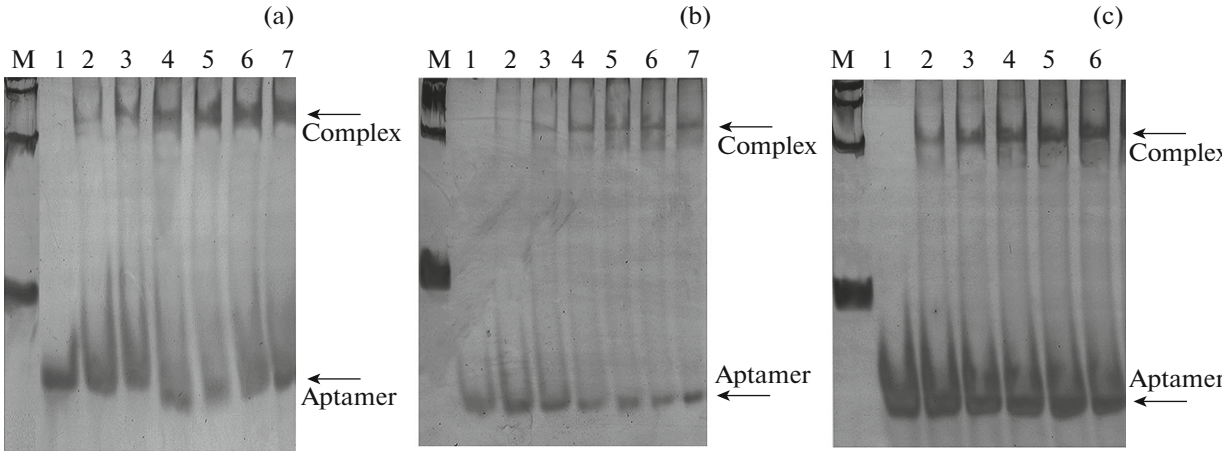

Fig. 2. The formation of the complexes of Lon-S679A and aptamers: (a) ST43biot5T, (b) ST43biot, (c) RE31biot5T (gel electrophoresis in 8% PAAG). Tracks: *M*, nucleotide markers; (*1*) aptamer (control); (*2*)–(*7*) complexes that are obtained by incubation of Lon-S679A and aptamer (4°C, 1 h) at different ratios (see Table 1). The regions of the complexes and individual aptamers are marked.

of the region of the protein complex with the aptamer differs significantly from that of the individual aptamer. This makes it possible to clearly separate the regions of the complex and unbound aptamer and quantitatively assess the intensity of the region.

The measure of the effectiveness of the interaction of the nucleic acid (NA) with the enzyme in each experiment is the portion of the aptamers that bind to Lon protease, which is determined as the ratio of the fluorescence intensity of the complex to the total fluorescence of the complex and free aptamer in the corresponding track of the electrophoregram (Fig. 2). Comparison of these characteristics for different aptamers that form complexes with the enzyme at the same concentrations makes it possible to compare the affinity of the aptamers to Lon protease. The effectiveness of the interaction of the aptamers with Lon-S679A protease is given in Table 1, which demonstrates that the maximum values for the portions of the bound aptamer at any ratio of aptamer : Lon protease are obtained for ST43biot5T, i.e., this aptamer most effectively binds to the enzyme. A close affinity to Lon protease (approximately 90%, when compared to the affinity of ST43biot5T) was also demonstrated by RE31biot5T, whereas the ability of ST43biot to bind the enzyme is noticeably reduced. It follows from these results that the introduction of the linker leads to an increase in the ability of the aptamer to bind the protein (hypothetically, due to overcoming steric hindrances).

2.3. Immobilization of Biotinylated DNA Aptamers on Magnetic Particles Functionalized with Streptavidin

Streptavidin that is fixed on the surface of the MPs is a homotetrameric protein with a molecular weight of 52.8 kDa, which is produced by *Streptomyces avidinii* bacteria. Streptavidin forms a strong complex with the biotin molecule with $Kd \sim 10^{-14}$ M.

In the experiments on the optimization of the attachment of specific aptamer DNA to the magnetic nanoparticle aptamers, solutions of different concentrations were used, which were obtained both in the presence and absence of salts. The reaction was carried out in PBS by controlling the optical absorption of the solution at 260 nm (the wavelength that corresponds to the maximum absorption of the solutions of nucleic acids) before the beginning and after the end of the formation of the complex.

According to the protocol of Rockland Immunochemicals Inc. the optimum conditions for the formation of the complex streptavidin–biotin in a solution are 0.2 M phosphate buffer (pH 7.0–7.5), a temperature of 5°C, and a biotin excess. These conditions were chosen as basic for the attachment of the specific aptamer DNA to the magnetic nanoparticles.

The effect of salts on the effectiveness of the formation of the complexes of MNP–streptavidin–biotin as studied. It was demonstrated that a change in the concentration of sodium chloride in the medium both toward an increase and decrease reduces the attachment of the aptamers to the MPs–streptavidin. The introduction to the buffer of two-valent ions (calcium or nickel) led to a sharp decrease in the aptamer binding to the nanoparticles.

The effect of the size of the oligonucleotide with the biotin label on the aptamer binding to the MNPs also has not been considered earlier. In connection with this, the effectiveness of the binding to the MNP–streptavidin of aptamers that directly bound to biotin or were separated from the label by the linker fragment of five T nucleotides has been compared. Table 2 gives the results of the experiments, which demonstrate that the introduction of the

Table 1. The effectiveness of the interaction of aptamers with Lon-S679A protease

Aptamer, [Apt], nM	Track in Fig. 2	Lon-S679A, nM	[Apt]: [Lon-S679A]	Portionof Apt_{bound}*	Relative binding effectiveness ($a_n : a_n$, $b_n : a_n$, or $c_n : a_n$), %
(a) ST43biot5T, 375 nM	1	–	–	–	–
	2	225.0	1 : 0.6	0.358	100.0
	3	337.5	1 : 0.9	0.460	100.0
	4	562.5	1 : 1.5	0.673	100.0
	5	787.5	1 : 2.1	0.779	100.0
	6	1125.0	1 : 3.0	0.820	100.0
	7	1687.5	1 : 4.5	0.908	100.0
					Mean value: 100.0
(b) ST43biot, 375 nM	1	–	–	–	–
	2	225.0	1 : 0.6	0.034	9.50
	3	337.5	1 : 0.9	0.298	64.8
	4	562.5	1 : 1.5	0.571	84.8
	5	787.5	1 : 2.1	0.714	91.7
	6	1125.0	1 : 3.0	0.800	97.6
					Mean value: 69.7
(c) RE31biot5T, 375 nM	1	–	–	–	–
	2	225.0	1 : 0.6	0.261	72.9
	3	337.5	1 : 0.9	0.433	94.1
	4	562.5	1 : 1.5	0.669	99.4
	5	787.5	1 : 2.1	0.686	88.1
	6	1125.0	1 : 3.0	0.750	91.4
					Mean value: 89.2

*Apt_{bound} is the aptamer bound to the enzyme.

linker weakly affects the aptamer binding to the MNPs: only a slight increase (approximately 10%) in the effectiveness of the binding of the linker-containing aptamers can be seen.

2.4. The Characteristics of the Size of the MNPs–Streptavidin–Biotinylated Aptamer Complexes by Dynamic Light Scattering

Dynamic light scattering was applied to prove the aptamer immobilization on the MNPs, which made it possible to visualize a change in the size of particles that were used. DLS is based on the scattering of a part of the light that passes through a liquid medium and makes it possible to determine the particle size in the solution and zeta potential of their surface. The size of the MNPs with the aptamers increased to 105 nm (Figs. 3a, 3b).

2.5. Binding of Lon Protease to MNPs Functionalized with Biotinylated Aptamers

The interaction of Lon protease with the magnetic particles that carry aptamers was studied in the experiments on light scattering. It was shown that the protein binds to the MNP–aptamer (Figs. 3a, 3b). In this case, the samples were found to have particles of elevated sizes, which is evidence of the formation of complexes on the magnetic particles. As a control experiment, binding of Lon protease to MNPs that were functionalized with streptavidin but not loaded with the aptamers was performed (Fig. 3c). It was found that in the absence of the aptamers the Lon protease does not bind to the MNP–streptavidin preparation. The size of the initial particles was 68 nm in the absence and in the presence of the enzyme.

It should be noted that the interaction with the protein causes a significant increase in the size of the

Table 2. The effectiveness of the immobilization of the biotinylated aptamers to Lon protease on magnetic nanoparticles that are functionalized with streptavidin in PBS

Aptamer	C_{apt}*, μM	Supernatant absorption at 260 nm, A_{260}		Degree of binding, %
		before binding	after binding	
ST43biot	0.31	0.95	0.74	22
ST43biot5T	0.36	1.20	0.90	25
RE31biot	0.35	1.10	0.86	22
RE31biot5T	0.36	1.24	0.94	24

*C_{apt} is the aptamer concentration.

MNPs. Moreover, the comparison of the graphs in Figs. 3a and 3b shows that the presence of the linker leads to a pronounced increase in the diameter of the MNP–streptavidin–aptamer–protein complex. The presence of the linker, when the biotin label was separated from the aptamer structure by five nucleotides (approximately 1.5 nm), appeared to contribute to the size of the MNPs with the protein.

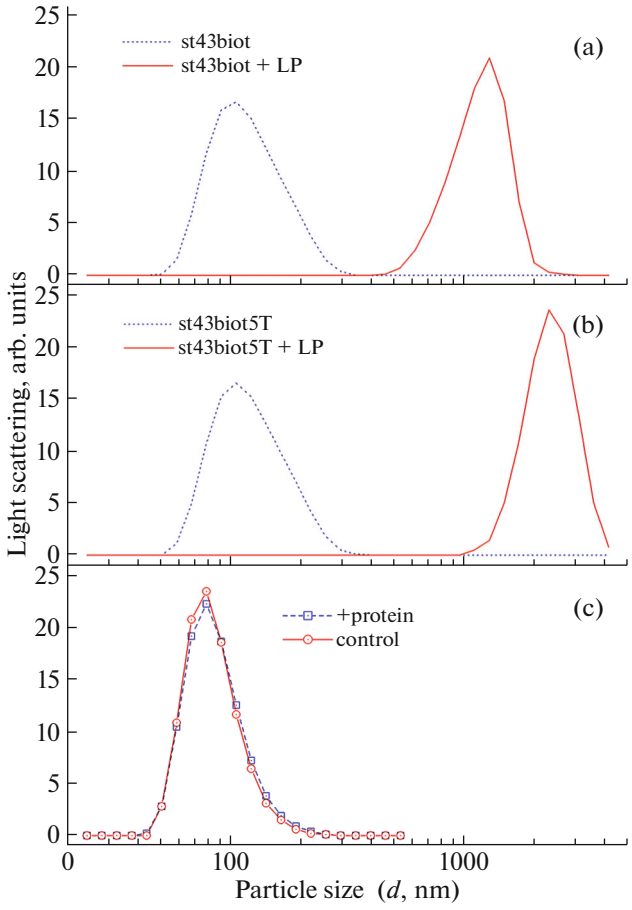

Fig. 3. Binding of Lon protease to aptamers that are immobilized on the MNP–streptavidin preparation: (a) ST43biot5T and (b) ST43biot. The dashed lines show the graphs of the volumes that are occupied by the MNP–aptamer preparations. Solid lines demonstrate the distribution graphs of aptamer–containing MNPs that bound Lon protease. (c) Binding of Lon protease to MNPs that are functionalized with streptavidin (50 nm) without aptamers is given.

In the binding reaction the same amounts of the protein and particles per an aptamer were taken. The amount of the bound protein was significantly greater when the aptamer with the linker was used. This once more emphasizes the important role of the steric availability of the total volume of the aptamer molecule for protein binding.

CONCLUSIONS

Carrying out the experiments on the binding of the magnetic particles that were functionalized with streptavidin with the protein targets via the biotinylated aptamers revealed that the proteins actively bind to their aptamers and MNPs of large sizes (68 nm for the initial MNPs, 105 nm for the MNP–aptamer and more than 1000 nm for the MNP–aptamer–protein) occurred, which indicates the formation of specific complexes of the protein with the aptamer in the solution.

Thus, we found conditions for the formation of the complexes between the MNP–aptamers and the protein, Lon protease, which makes it possible to use these objects for tuning and characterizing the biomagnetic test system that is based on superconducting quantum interferometers [30] and performs a separation-free immunoassay.

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REFERENCES

1. R. M. Ferguson, A. P. Khandhar, et al., IEEE Trans. Magn. **49**, 3441 (2013).
2. V. A. Spiridonova, T. M. Novikova, and O. V. Snigirev, Moscow Univ. Phys. Bull. **71**, 135 (2016). doi 10.3103/S002713491601015X

3. M. Kawano, T. Hirano, T. Matsuda, T. Taga, et al., Nature **332**, 83 (1988).
4. P. Ataie-Kachoie, M. H. Pourgholami, et al., J. Clin. Pathol. **67**, 932 (2014).
5. M. Ghandadi and A. Sahebkar, Curr. Pharm. Des. **22**, 518 (2016).
6. K. Luciakova, B. Sokolikova, et al., FEBS Lett. **444**, 186 (1999).
7. S. H. Bernstein, S. Venkatesh, et al., Blood **119**, 3321 (2012).
8. H. M. Wang, K. C. Cheng, et al., Cancer Sci. **101**, 2612 (2012).
9. Y. Liu, L. Lan, K. Huang, et al., Oncotarget **5**, 11209 (2014).
10. C. W. Cheng, C. Y. Kuo, C. C. Fan, W. C. Fang, et al., Cell Death Discovery **4**, e681 (2013).
11. A. A. Khalil, N. F. Kabapy, et al., Biochim. Biophys. Acta **1816**, 89 (2011).
12. A. L. Bulteau and A. Bayot, Biochim. Biophys. Acta **1807**, 595 (2011).
13. C. Tuerk and L. Gold, Science **249**, 505 (1990).
14. A. D. Ellington and J. W. Szoztak, Nature **346**, 818 (1990).
15. R. Stoltenburg, C. Reinemann, and B. Strehlitz, Biomol. Eng. **24**, 381 (2007).
16. S. Missailidis and A. Hardy, Expert Opin. Ther. Pat. **19**, 1073 (2009).
17. V. A. Spiridonova, Biochemistry (Moscow) Suppl. Ser. B: Biomed. Chem. **4**, 138 (2010).
18. B. Santosh and P. K. Yadava, Biomed. Res. Int. **540**, 451 (2014). doi doi 10.1155/2014/540451
19. D. Xiang, S. Shigdar, G. Qiao, T. Wang, et al., Theranostics **5**, 23 (2015).
20. X. Wu, J. Chen, M. Wu, and J. X. Zhao, Theranostics **5**, 322 (2015).
21. L. I. Hernandez, I. Machado, et al., Curr. Top Med. Chem. **15**, 1066 (2015).
22. T. V. Rotanova, N. I. Dergousova, and A. D. Morozkin, Russ. J. Bioorg. Chem. **39**, 268 (2013).
23. V. A. Spiridonova, A. M. Kudzhaev, A. V. Melnichuk, A. A. Gainutdinov, A. G. Andrianova, and T. V. Rotanova, Russ. J. Bioorg. Chem. **41**, 626 (2015).
24. A. De Cian, L. Lacroix, et al., Biochimie **90**, 131 (2008).
25. A. Siddiqui-Jain, C. L. Grand, et al., Proc. Natl. Acad. Sci. U. S. A. **99**, 11593 (2002).
26. S. Cogoi and L. E. Xodo, Nucleic Acids Res. **34**, 2536 (2006).
27. F. E. Indig, I. Rybanska, P. Karmaker, et al., PLoS One **7** (6), e35229 (2012).
28. N. Kumar and S. Maiti, Biochem. Biophys. Res. Commun. **319**, 759 (2004).
29. B. I. Kankia and L. A. Marky, J. Am. Chem. Soc. **123**, 10799 (2001).
30. I. I. Soloviev, N. V. Klenov, A. E. Schegolev, et al., Supercond. Sci. Technol. **29**, 094005 (2016).

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