ChemPubSoc Europe

DOI: 10.1002/cphc.201801126

CHEMPHYSCHEM Articles

DNA Aptamers in the Detection of Leukemia Cells by the Thickness Shear Mode Acoustics Method

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By using the thickness shear mode acoustics method (TSM) and single-molecule force spectroscopy (SMFS) we studied the interactions between DNA aptamers (sgc8c) specific to the protein tyrosine kinase 7 (PTK7), which is localized in the membranes of leukemia lymphoblastics (MOLT-4), and lymphocyte (Jurkat) cell lines, as well with PTK7-negative U266 myeloid leukemia cells. The TSM method allowed the development of a highly sensitive, label-free biosensor for the detection leukemia cells with a limit of detection of (195 ± 20) cells/mL. SMFS approved the high selectivity of the sgc8c aptamers to the PTK7 receptors at the cell surface and allowed determining the binding probability of the aptamers to the PTK7 receptors at different cell lines.

1. Introduction

Cancer belongs to the most serious diseases. The success in the therapy depends on early diagnosis of various types of the cancer. The leukemia belongs to the most aggressive type of cancer.[1] Currently the diagnosis of this disease is based on rather expensive and time consuming methods of determination leukemia cells such as flow cytometry,[2] polymerase chain reaction[3] or fluorescence assay.[4,5] These methods, however, require qualified staffs and can be performed only in specialized hospital laboratories. In contrast, the biosensor technology may improve the current situation in proposing much easier and user friendly methods of cancer cell detection. It is based on sensing layer that contains specific receptors that recognize certain cancer markers that are incorporated in plasma membranes of cancer cells. Among them protein tyrosine kinase 7 (PTK7) is of special interest due to its over expression in various cancer cells including leukemia. This membrane protein is responsible for molecular recognition, ion regulation and other functions in cell metabolism.[6,7] PTK7 is an important cancer marker of leukemia, solid tumors including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), stomach, colon and lung cancer.[8–11] PTK7 has been for the first time identified in melanocytes.[12,13] Subsequently, the role of these receptors in regulation of signals generated by neurons, polarity of mammalian cells and in morphogenetic cell movement during embryonic development has been established.[14–16] PTK7 is also responsible for development of metastases.[17] The leukemia cells can be therefore detected by affinity biosensors that can recognize PTK7. Among recognizing elements, those based of DNA aptamers are of special interest.[18,19] Aptamers are synthetics DNA or RNA molecules that in a solution fold into 3D structures forming binding site to the analyte of interest. Aptamers can be in principle developed for any substances, cells or tissues. They are also considered as artificial antibodies. However, in contrast with antibodies they are selected by *in vitro* SELEX method (Systematic Evolution of Ligands by EXponential enrichment) that is based on combinatorial chemistry.[20,21] Aptamers are more stable than antibodies, can be chemically modified that protect them by nuclease cleavages. In addition, sensors based on aptamers can be regenerated without lost of sensitivity and specificity. Using the cell-SELEX it has been developed the DNA aptamer (sgc8c) which can recognize T-cells of acute lymphoblastics leukemia.[22–24] Sgc8c aptamer is selective to the PTK7 with a high affinity ($K_D \sim 1$ nM). This aptamer can be used for development biosensor for detection of various leukemia cell lines.[22,23]

Electrochemical and acoustics aptasensors for leukemia diagnostics are in focus due to possibility of label-free detection of the cells. This is great advantage for early diagnostics of cancer due to low cost and easy to use. The existing approaches in development electrochemical and acoustics aptasensors for detection leukemia cells have been reviewed in our recent paper.[24] In particularly we developed label-free aptasensor for detection leukemia Jurkat cells using thiolated sgc8c aptamers immobilized by chemisorption at gold surface. Using electrochemical impedance spectroscopy we were able to detect these cells with a limit of detection (LOD) of 105 ± 10 cells/mL.

The mass-sensitive aptasensors are very convenient for label-free monitoring of binding the cells to the receptors immobilized at piezocrystal surface as well as due to easy evaluation of the sensor response. These properties make them very convenient for practical applications. This technique, known as quartz crystal microbalance (QCM), is rather popular method, that allowing studying binding events at the surface

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cphc.201801126

with high sensitivity by measuring the changes of the series resonant frequency, f_s ,that corresponding to the changes in mass or thickness at the surface of piezocrystal. More sophisticated thickness shear mode method (TSM) determines in addition to the f_s value also motional resistance, R_m, which evaluation of viscosity contribution in the binding event. So far the TSM method has been used in development of various affinity biosensors based on antibodies, DNA aptamers or calixarenes.[25–27] However, aptamer-based acoustics biosensors for detection cancer cells including leukemia cell lines were up to now only very little explored. So far only 3 papers focused on detection leukemia cells by QCM/TSM aptasensors were published. Among first was the paper by Pan et al.[28] where aptamers selective to PTK7 conjugated with magnetic beads were used for detection leukemia cell lines CCRF-CEM. The magnetic beads allowed better separation of the cells with adsorbed aptamers. After injection of the cells the resonant frequency decreased. The LOD of detection of leukemia cells was $8x10^3$ cells/mL. Better sensitivity (LOD 1160 cells/mL) of detection of CCRF-CEM cells has been achieved by QCM aptasensor with amplified detection using silver enhancement method.[5] In our recent paper we used TSM method for detection of leukemia Jurkat cells with LOD of 463 ± 50 cells/mL using the sgc8c aptamers chemisorbed at gold layer of quartz crystal.[24] However, so far developed mass-sensitive sensors for detection leukemia cells were not validated in real blood samples. Therefore in this work we applied TSM method for detection of Jurkat as well as MOLT-4 leukemia cell lines that containing PTK7 receptors in their membranes. We used also another method of aptamer immobilization. Biotinylated sgc8c aptamers were attached onto the neutravidin layer self assembled at TSM transducer. Jurkat cells are good model for studying acute T-cell leukemia. MOLT-4 T-cells have also been isolated from a patient with T-cell acute lymphoblastic leukemia.[29] As a control we used PTK7-negative U266 cell line.[22] We also used atomic force microscopy (AFM) for study the topography of the cells and single molecule force spectroscopy (SMFS) for comparative analysis of the binding probability of sgc8c aptamers to PTK7 receptors in various cell lines. In this case the DNA aptamers were immobilized on the AFM tip. SMFS method allowed study of molecular interactions between aptamers and PTK7 receptors. This approach has been applied in our recent work for study the interactions of sgc8c aptamers with Jurkat and U266 cells.[30] However, interaction of aptamers with MOLT-4 cells was not studied so far by SMFS and not compared with acoustics method.

2. Results and Discussion

2.1. Viability of Cell Lines

Determination of the cell viability is important for many biological and biomedical experiments in cell biology, pharmacology and oncology. We tested the viability of cells using trypan blue method.[31] The test is based on differences in the penetration of this day into the cytoplasm of the cells. The trypan blue is accumulated in the cells with damaged plasmatic membrane. These cells are blue in contrast with not damaged living cells. If the cells are in stress conditions the process of apoptosis or necrosis is initiated. Damaged cells bind various reagents and antibodies and have tendency to initiate various non-specific interactions. Therefore it was important to analyze whether the cell lines used in experiments (Jurkat, MOLT-4 and U266) are not damaged at PBS, pH 7.4 during all experiments with duration of 4–6 hours at standard laboratory conditions. The experiments were performed in PBS (pH 7.4) at $T = 23\,^{\circ}C$ without CO_2. After centrifugation and removing of cultivation media the cell pellet has been resuspended in 5 mL of PBS. The cell integrity was tested by direct cell counting in Bürker cell by mixing of trypan blue and cell suspension in ratio 1:1. According to our analysis all cell lines were viable. Only after 12 hours a low decay of viability was observed. The results of cell viability within 24 hours are presented in Figure SI-1 of Supporting information. This means that all cell lines can be used in further experiments.

2.2. Determination of Leukemia Cells by the TSM Method

Thickness shear mode (TSM) is a sensitive method for studying the affinity interactions at surfaces. We prepared aptasensors based on immobilization of sgc8c aptamers at TSM transducer either by chemisorption of thiolated aptamers or based on high affinity of biotin to neutravidin using biotinylated aptamers. We analyzed the changes of series resonant frequency, f_s, and motional resistance, R_m, following addition of Jurkat, MOLT-4 and U266 cell lines. TSM method allows also monitoring of all steps of sensing surface preparation. Figure 1 shows the changes of f_s and R_m values after addition of neutravidin (NA) and biotinylated sgc8c aptamers.

It can be seen, that addition of neutravidin (concentration: 250 μg/mL) induced decrease of resonant frequency and increase of motional resistance. Washing of the surface by water was accompanied by slight increase of f_s and increase of R_m

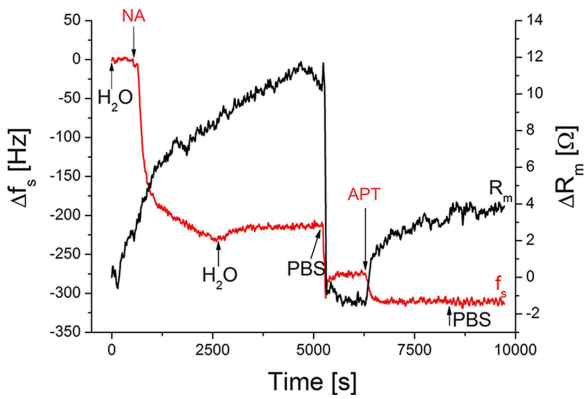

Figure 1. Changes in the series resonant frequency, f_s, and motional resistance, R_m, after addition of neutravidin (NA) dissolved in water at a concentration of 250 μg/mL and biotinylated sgc8c aptamers (APT) dissolved in PBS at a concentration of 0.5 μM. Addition of neutravidin, aptamers, and washing of the surface with water or PBS is shown by arrows.

values which correspond to removal of the physically adsorbed neutravidin molecules from the surface. This is evidence of formation stable neutravidin layer. The final changes of measured values after washing steps were: $\Delta f_s = -185.5 \pm 17.4$ Hz and $\Delta R_m = 3.3 \pm 1.6\ \Omega$ (mean ± S.D., 3 independent experiments). This is in agreement with previously published work.[33,34] Because the aptamers were dissolved in PBS, prior their addition to the neutravidin layer it has been necessary to wash the surface by PBS. This step is also shown at Figure 1. The flow of PBS resulted in sharp decrease of both f_s and R_m values. This can be connected with changes of molecular slip between the neutravidin layer and the buffer. The sensitivity of acoustics parameters to the changes of ionic conditions has been previously reported.[32,34] Addition of biotinylated sgc8c aptamers dissolved in PBS in a concentration of 0.5 μM resulted in decrease of f_s and in increase of R_m values. This is due to the well known strong affinity of biotin to the neutravidin (constant of dissociation for this bond is $K_D = 10^{-15}$ M). Washing of the sensor by PBS resulted in only slight changes of the acoustics parameters. This is evidence on a weak non-specific adsorption of aptamers. Thus, the aptamers form stable array at the neutravidin surface. The final changes of the acoustics parameters after addition of aptamers and washing step by PBS were: $\Delta f_s = -42.7 \pm 3.0$ Hz, $\Delta R_m = 4.6 \pm 1.7\ \Omega$. The increase of the R_m value is evidence of certain viscosity contribution due to the interaction of DNA strands with surrounding PBS. We should note that the ratio between Δf_s and ΔR_m values are different when NA and APT are added at the TSM transducer surface. This ratio depends on the surface properties, such as rigidity of the sensing layers. It has been shown in our previous work that NA layer is more rigid in comparison with aptamer arrays.[33] Therefore in later case we can observe larger contribution of surface viscosity to the changes of frequency and motional resistance. In analogy with our previous work[33] from the frequency changes it is possible to estimate surface density of neutravidin and aptamers using Sauerbrey equation:[35]

$$\Delta f_s = -2.26 \times 10^6 f_0^2\ \Delta m/A \qquad (1)$$

where f_0 is the fundamental frequency of piezocrystal (8 MHz), Δm is the mass change in grams and $A = 0.2\ cm^2$ is the area of the gold layer sputtered at the surface of piezocrystal onto which the sensing layer is formed. It should be noted that Sauerbrey equation is strongly valid for a rigid layer in vacuum. However, for small changes in the R_m values it might be also used for a layer in a liquid. In analogy with ref.[33] and using Eq. (1) we obtained following values of surface density for NA and sgc8c aptamers: 1.14×10^{12} and 4.8×10^{13} molecules cm^{-2}, respectively. NA has 4 binding sites for biotin. However, due to immobilization at TSM transducer only 2 binding sites are available. Therefore maximum binding sites available for aptamers are $2{,}28 \times 10^{12}\ cm^{-2}$, which is substantially lower in comparison with estimated surface density of aptamers. This may be due to viscosity effect that also contribute to the mass changes. For example increase of R_m value after addition of aptamers can contribute to decrease of resonant frequency, which is higher than the real value that is due to only mass changes. This effect can explain overestimation of aptamer surface density. Similar result has been obtained in our recent paper focused on detection of cytochrome c by acoustics aptasensor.[33]

We analyzed two types of biosensors based on 1) biotinylated or 2) thiolated sgc8c aptamers. The scheme of the sensors is shown on Figure 2. Addition of the leukemia cells (Jurkat

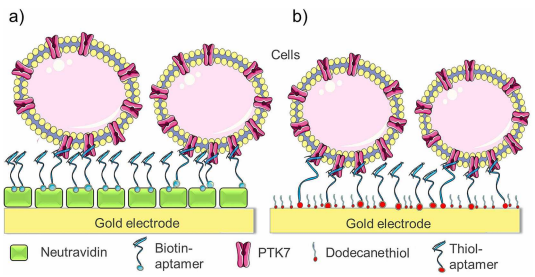

Figure 2. Scheme of the sensor surface based on the neutravidin and biotinylated aptamers (a) and those based on thiolated aptamers and dodecanethiols (b).

and/or MOLT-4) to the different sensing surfaces in a concentration range $50–5 \times 10^5$ cells/mL induced decrease of f_s and increase of R_m values. This can be seen on Figure 3 where changes of these values are presented following addition of MOLT-4 and Jurkat cells on the sensing surface formed by NA-biotinylated aptamers (a) or by chemisorbed aptamers on a gold surface (b).

The decrease of resonant frequency demonstrates the binding of the cells at the sensing surface. Similarly to the analysis performed above, the viscosity effect indicated by increase of R_m values can also contribute to the decrease of resonant frequency. The obtained results does not allow determination of the surface density of the cells at the sensing surface. However, they might be useful for diagnostics purposes. In contrast with MOLT-4 and Jurkat cells the control U266 cells with no PTK7 receptors, did not affect the acoustics parameters. Based on the kinetics experiments we constructed calibration curves that represent the plot of Δf_s and ΔR_m vs. concentration of the cells (Figure 4). The plots for Jurkat and MOLT-4 cell lines represent typical isotherms with saturation at higher cell concentrations. Frequency changes for MOLT-4 cell lines are more remarkable in comparison with those for Jurkat cells. Practically no changes of frequency were observed for control U266 cells. It can be seen that sensing layer formed by thiolated aptamers (Figure 3B) is very sensitive for low concentration of MOLT-4 and Jurkat cells. Already at the concentration of 50 cells/mL of MOLT-4 cells we observed significant changes of the acoustics parameters. Addition of the MOLT-4 cells in a maximal concentration of 5×10^5 cells/mL resulted in higher sensor response ($\Delta f_s = -121.2 \pm 12.0$ Hz and $\Delta R_m = 6.9 \pm 1.1\ \Omega$) in comparison with Jurkat cells at the same concentration ($\Delta f_s = -97.6 \pm 9.4$ Hz and $\Delta R_m = 2.5 \pm 0.8\ \Omega$). We assume that higher response for MOLT-4 cells is due to better access and higher surface concentration of PTK7 receptors for this cell line in

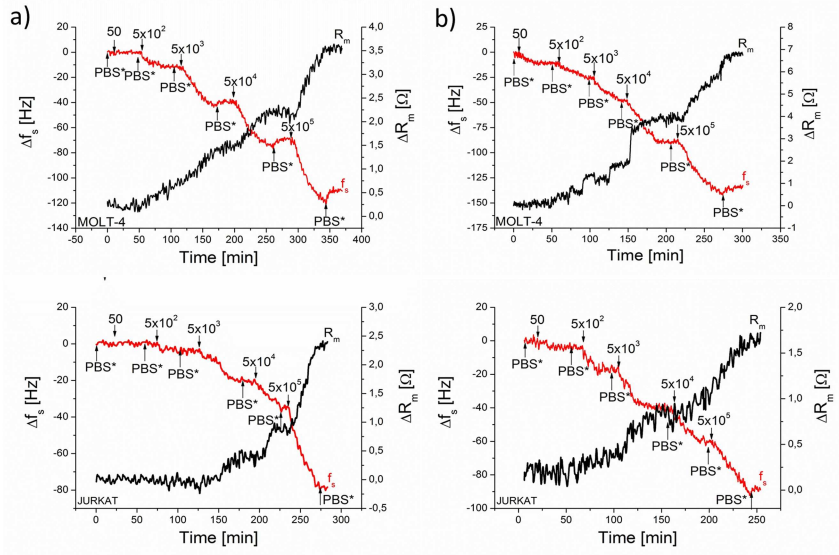

Figure 3. Kinetics of the changes in the f_s and R_m values following the addition of MOLT-4 and Jurkat cells (in cells/mL). Addition of cells and the washing step in RPMI media (PBS*) are shown by arrows. The sensor was formed by a) neutravidin and biotinylated aptamers and b) thiolated aptamers chemisorbed on the gold layer of the TSM transducer.

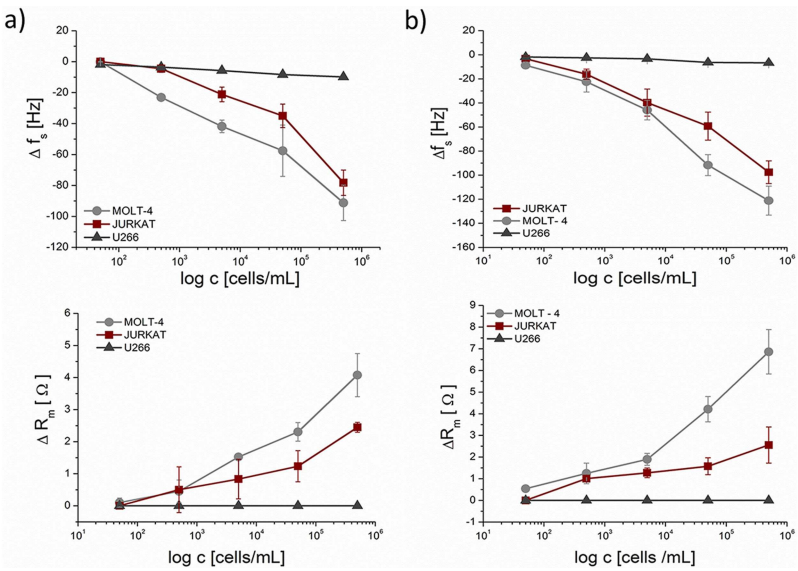

Figure 4. Plot of the changes in f_s and R_m versus the concentration of the cells. The sensor was formed by: a) neutravidin and biotinylated aptamers and b) thiolated aptamers chemisorbed at gold layer of TSM transducer. The results represent the (mean ± S.D.) from three independent experiments.

comparison with Jurkat cells. When the sensor was formed by neutravidin layer and biotinylated aptamers the response was less sensitive. In this case the lowest concentration of MOLT-4 cells that was possible to detect has been 500 cells/mL.

In order to exclude possible non-specific interactions we tested the response of TSM transducer following addition of MOLT-4 and Jurkat cell lines onto the neutravidin layer without aptamers. In both cases very low non-specific interaction was observed. For example addition of MOLT-4 at the concentration of 5×10^4 cells/mL resulted only in small changes of acoustics parameters: $\Delta f_s = -7.4 \pm 4.0$ Hz a $\Delta R_m = 1.3 \pm 0.2\ \Omega$, which is less than 10% of the total specific response for this concentration of the cells. The calibration curves allowed determination of the limit of detection (LOD) based on signal to noise ratio (S/N = 3) rule. LOD for MOLT-4 and Jurkat cells in a most sensitive sensor response (thiolated sgc8c aptamers at the TSM transducers) was: 195 ± 20 cells/mL and 463 ± 50 cells/mL, respectively.

As we mentioned above the changes in resonant frequency at maximal MOLT-4 cell concentration used for aptasensor formed by thiolated aptamers were approx. -121.2 ± 12.0 Hz. Lower frequency changes were observed for other cell lines

Table 1. Comparison of the properties of the acoustics aptasensors based on sgc8c aptamers for detection leukemia cells.

Cell line	Aptamer immobilization	Method of detection	Linear range cells [mL]	LOD, cells [mL]	Ref.
CCRF-CEM	Magnetic beads conjugated with aptamers	QCM	1×10^4–1.5×10^5	8×10^3	[28]
CCRF-CEM	Chemisorption on Au layer and amplification by nanoparticles	QCM	2×10^3–10^5	1160	[5]
Jurkat	Chemisorption on Au layer	TSM	$50–5\times10^5$	463 ± 50	[24]
MOLT-4	Chemisorption on Au layer	TSM	$50–5\times10^5$	195 ± 20	This work
Jurkat	Biotinylated aptamers on neutravidin layers	TSM	$50–5\times10^5$	510 ± 55	This work
MOLT-4	Biotinylated aptamers on neutravidin layers	TSM	$50–5\times10^5$	570 ± 60	This work

including CCRF-CEM that were reported by Shan et al.[5] It should be, however, noted that these changes corresponding to label-free acoustics sensors without additional amplification. These changes are, however, more remarkable when nanoparticles are used for signal amplification although this make the cell detection more complicated.

Table 1 shows the basic properties of acoustics aptasensors for leukemia cells detection published so far. It can be seen that aptasensors presented in our work revealed rather high sensitivity. The sensor response for MOLT-4 cells was reported in our work for the first time.

Thus, the best LOD (195 ± 20 cells/mL) has been obtained by TSM sensor based on chemisorbed sgc8c aptamers. This is comparable with the LOD for impedimetric aptasensor reported in our recent work (105 ± 10 cells/mL).[24] We should also point out that as it can be seen from the Table 1 there are differences in LOD for MOLT-4 cells at different sensing surfaces – NA and biotinylated aptamers as well as those for thiolated aptamers. The purpose of this work was particularly in analysis of the interaction of two cell lines containing PTK7 receptors with two different sensing layers. Certainly, in the case of Jurkat cells we did not observed significant changes in the LOD for sensing layers prepared either by NA layers and biotinylated aptamers and by the layers formed by thiolated aptamers. However, Jurkat cells induced less expressed changes of measured values in comparison with MOLT-4 cells. Therefore different LOD in detection of MOLT-4 cells at different sensing surfaces can be in particularly due to the fact of higher response of the sensors in this case and thus in better resolution of induced changes. We should also mention, that using amperometric detection with aptasensors it is possible to reach detection limit of 8–10 cells/mL. This sensitivity has been reported for detection CCRF-CEM leukemia cells.[36,37] However, for such a high sensitivity more complicated approach is required that include sandwich assay or indirect detection (see Ref.[24,36,37] for more details). The advantage of acoustic TSM sensor is in easy handling and analysis of the sensor response. By means of amplification of detection using for example nanoparticles it would be possible substantially improve the sensitivity of leukemia cell detections by mass-sensitive aptasensors. The principles of cell detection can be applied also for pathogen determination, see for example paper by Ozalp et al.[38] in which the aptamer-based QCM sensor has been used for detection of *Salmonella* cells in food samples.

2.3. Regeneration of the Aptamer Surface

The advantage of the aptasensors is in possibility of regeneration of sensing surface due to high stability of DNA aptamers. The regeneration can be performed by various methods for example by immersion of the sensor in a solution of high salt concentration, by temperature heating or by immersion in glycine solution. In our work the best results were achieved by regeneration of the sensors in 0.2 M glycine, pH 2. In regeneration experiments we used MOLT-4 cells that demonstrated strongest binding to the sensing surface. The incubation of aptasensor with bound MOLT-4 cells in a concentration of $5x10^3$ cells/mL in the glycine for 10–15 min. resulted in removal of the cells from the surface. After washing the biosensor with PBS it was reusable at least for 3 times. This is shown on Figure SI-2 (see Supporting information) for 3 regeneration cycles. Thus, at least after 3 times of sensor regeneration the sensor revealed similar response for the same concentration of the MOLT-4 cells. This make aptasensors most useful for diagnostics purposes in comparison with those based on antibodies because minimize the costs for sensor preparation. In the case of antibody based biosensors the regeneration is practically impossible and various attempts resulted in lost of binding affinity of antibodies.[25]

2.4. Analysis of a Spiked Human Plasma Sample

To validate developed aptasensors in a complex matrix, the sensors were used for determination leukemia cells in a spiked human blood plasma diluted 20×. The obtained recoveries for various concentrations of MOLT-4 and Jurkat cells are shown in Table 2. The changes of resonant frequency, f_s, after reacting of sgc8c aptamer with different concentrations of cells in PBS buffer divided on the frequency response of sgc8c aptamer to PTK7 positive cells in plasma ×100% have been taken for calculation of sensor recovery. The obtained results suggest that the aptasensors exhibit satisfactory analytical response towards PTK7 positive leukemia cells in plasma samples.

2.5. Single-Molecule Force Spectroscopy of the Cells

Single molecule force spectroscopy (SMFS) is unique tool for study of different type of intra- and inter- molecular interactions. Information obtained by SMFS is significant for under-

Table 2. Recovery of the aptasensor in a blood plasma spiked by various concentrations of Jurkat and/or MOLT-4 cells. Recovery is calculated as: $[(\Delta f_s)_{PBS}/(\Delta f_s)_{plasma}]\times 100\,\%$,where $(\Delta f_s)_{PBS}$ are changes of resonant frequency following addition of cells at certain concentration in a PBS and $(\Delta f_s)_{plasma}$ those in a blood plasma, respectively. Results represent mean ± SD obtained from 3 experiments in each series.

Concentration of cells in a blood plasma	Δfs [Hz]	Recovery [%]
Jurkat cells		
5×10^2	−15.3 ± 3.5	95.7 ± 21.5
5×10^3	−36.0 ± 8.5	92.3 ± 21.6
5×10^4	−54.7 ± 9.2	92.7 ± 15.5
MOLT-4 cells		
5×10^2	−19.0 ± 4.6	86.0 ± 20.9
5×10^3	−46.3 ± 9.1	99.0 ± 23.8
5×10^4	−86.7 ± 9.0	94.7 ± 9.1

standing of the relations between molecular structure and functions of various substances.[39] SMFS is used for determination of very small forces in a range of pN, provides information about sample topography, their mechanical properties and is useful for study the thermodynamics and kinetics of molecular interactions.[40] SMFS is excellent tool for direct measurements of intermolecular bonds, for obtaining information on infrequent molecular states and on mechanical properties of molecules that are important for protein functions.[41] For this purpose it is necessary to measure forces between the AFM tip modified by certain ligand and receptor that is immobilized on the corresponding surface. Due to existence of repulsive forces the interaction between the ligand and receptor is interrupted which is accompanied by cleavage of the intermolecular bonds. The force corresponding to the bond cleavage, f_U, is measured.[42] SMFS in our experiments has been realized using AFM tips modified by sgc8c aptamers that recognized PTK7 proteins at the surface of leukemia cells. The scheme of basic principles of SMFS is presented on Figure 5.

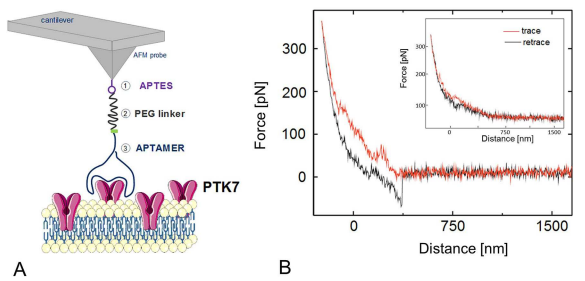

Figure 5. Scheme of the principles of SMFS. A) AFM tip chemistry. Inert silicon nitride tip is reacted with APTES (1) resulting in reactive amino-groups on the surface. The heterobifunctional NHS-PEG-Acetal linker (2) is coupled and deprotected, resulting in an aldehyde residue on the outer PEG end. Finally, the sgc8c aptamer is covalently bound (3) via an amino-group. B) Typical force-distance cycle. The ligand functionalized cantilever is approached to the surface (red line) until a bending force limit is reached followed by a retraction period (black line). In the case of a sgc8c aptamer – PTK7 complex formation a downwards bending is observable followed by a sudden rupture of the complex. This effect is not observable when the same tip is used but no PTK7 is present in the cells (B, inset).

Steric conditions for complex formations are crucial for SMFS measurements. In order to obtain strongest possible bonds between aptamers and PTK7 receptors, good orientation and optimal density of aptamers at the tip are necessary. At the moment of the contact the aptamer at the AFM tip binds to the PTK7 receptor at the cell surface. First, the attractive forces until certain critical values are present. Then the repulsive forces take place. During the action of repulsive forces the bonds between aptamer and receptors interrupt. This event is recorded as a force-distance curve (Figure 5B).[41] Parabolic shape of the force-distance curve is the result of specific interaction between the aptamer and receptor. The force is increased until certain critical value. Then the interaction is interrupted and the force as well as the cantilever is zeroed. The height of the jump into the initial (zero) position reflexes the disruption force of the bond at certain degree of loading. The principle of this measurement is based on the direct relation between voltage of photodiode and the bending of the cantilever, which can be considered as a spring. Using the Hooke's law it is possible to transform this bending into the force.[30] For each AFM tip we have measured 5 retraction velocities in the range from 300 to 7500 nm/s. This corresponds to 10000–14000 force-distance curves. In the analysis we have to consider each force-distance curve that corresponded to the specific interaction. Corresponding part of this force-distance curve that described stretching of PEG linker has been fitted by first degree polynomial. From available data we obtained information on the most probable interactions and force. Obtained values of "binding probability" represent the ratio of the number of curves of specific interaction to the totally measured force-distance curves for certain retraction velocity. Figure 6 shows the results of analysis of SMFS for Jurkat (left) and MOLT-4 (right) cells, respectively before and after blocking of PTK7 receptors. The blocking was provided by incubation of the cells with sgc8c aptamers. We have tested 9 AFM tips that were modified by sgc8c aptamers through flexible acetal-PEG-NHS linker.

To distinguish the specific interactions between aptamers and PTK7 receptors from non-specific for example at adhesion, it has been necessary to perform experiments that can approve this specificity. For this purpose we performed blocking experiments in which the PTK7 receptors at the cells were blocked overnight by incubation with sgc8c aptamers. The aptamers bind to the receptors, and thus blocked them from interaction with aptamers immobilized at the cantilever. The blocking experiments were performed with the same cantilever (C) and with the same retraction velocity for which we obtained maximal probability of interactions. The blocking of the receptors resulted in substantial decrease of binding probability from 48.6 ± 3.9% to 4.2 ± 1.4% for MOLT-4 cells and from 37.5 ± 5.0% to 4.3 ± 3.1% for Jurkat cells (Figure 6). Donoghue et al.[43] compared interaction of HeLa cells with sgc8c aptamers and with anti-PTK7 antibodies using SMFS. They performed also blocking experiment by addition of surplus amount of the sgc8c aptamers. This resulted in substantial decrease of binding probability from 13.2% to 4.7%. The binding probability obtained for MOLT-4 cells in our experiments was higher in comparison with results published in Ref.[43] This can be due to

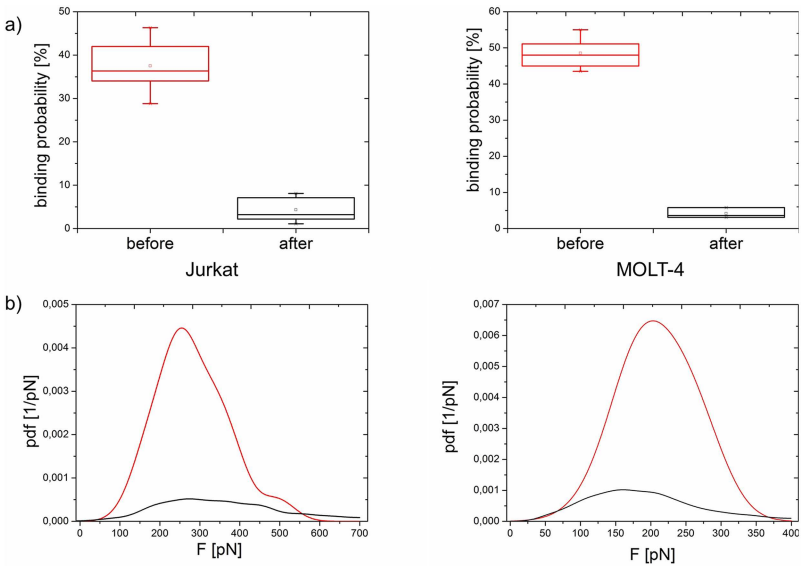

Figure 6. The results of the SMFS analysis expressed by means of the binding probability (a) before and after blocking the PTK7 receptors at the surface of Jurkat (left) and MOLT-4 (right) cells, respectively. The values represent (mean ± SD) obtained from 9 experiments in each series. b) The plot of probability density function (pdf) vs. force, F. The red curve represents a typical pdf of the unbinding forces measured on cell surface with expressed PTK7 at constant pulling velocity (1500 nm/s).

the higher surface density of PTK7 receptors at the surface of MOLT-4 cells. Additional reason that resulted in higher binding probability was due to the flexible linker that allowed free rotation of the aptamer and thus it was able to find more receptors at the cell surface.[44] So far discussed results have been realized on the MOLT-4 and Jurkat cell lines. For complete understanding of the mechanisms of the specific interactions of sgc8c aptamers with the cell surface it has been necessary to test the interactions also using the cells that do not contain PTK7 receptors. It was important to use the same cantilever with the same retraction velocity 1500 nm/s like in previous experiments. Our experiments showed that the binding probability was highest for MOLT-4 cells (48.6 ± 3.9%) and lowest for U266 cells (3.3 ± 1.5). The binding probability for Jurkat cells were 37.5 ± 5.0% and this is in good agreement with work by Leitner at al.[30] Figure SI-3 in Supporting information summarizes all experiments on the cells including U266 cells that do not contain PTK7 receptors. Different values of binding probability for MOLT-4 and Jurkat cells with those for HeLa cells obtained in Ref. [43] is evidence of different surface density of PTK7 receptors at above mentioned cell lines. The experiments on MOLT-4 cells have been realized by us for the first time. The sensitivity of sgc8c aptamers to the MOLT-4 cells is, however, rather high as it is evident from paper by Shangguan et al.[22] Therefore we assume that higher binding probability is connected with higher surface density of PTK7 receptors at MOLT-4 cells. Taghdisi et al.[45] used flow cytometry for targeted delivery of daunorubicin into the MOLT-4 cells using sgc8c aptamers. For comparison of non-specific interactions they used U266 cells and showed that sgc8c-daunorubicin complex was effective for MOLT-4 but not for U266 cells. This experiment does not evaluate probability of the availability of PTK7 receptors at the surface of MOLT-4 cells, but approve existence of these receptors, which agree well with our work.

2.6. AFM Topography of the Cells

To obtain complete information about the cells used in experiments we also applied AFM topography method. This allowed us to obtain information about the size and distribution of the cells at the surface. The samples were prepared according to the method described in Experimental section below. The topography has been investigated at 3 to 5 different sites at the sample surface with the size of the scans 80 × 80, 50 × 50, 20 × 20 μm. The typical topography of the cells with height profiles are presented on Figure 7. Based on obtained height profiles it can be seen that the size of MOLT-4 cells is similar to those of Jurkat cells: the height was approx. 3 μm and width of 11–13 μm. These cell lines are suspended cells of spherical shape in cultivation media. However, after immobilization on the surface the cells partially lost their spherical shape. After immobilization at the surface the MOLT-4 cells formed certain groups close to each others (Figure 7a). It can be also seen certain branching at the cell surface. The average size of the MOLT-4 cells was 11 μm (Figure 7b) which is in good agreement with paper by Lin et al.[46] At the surface the cell shape was, however, elliptic. Average size of Jurkat cells was 13–15 μm (Figure 7e) which agree well with Ref. [47] U266 cells are partially semi adherent. Their behavior at the surface was similar to the epithelial cells. After immobilization at the surface they changed the shape from spherical to flatten of a different shape and formed certain aggregates. Average height of these cells was 1.5–2 μm (Fig-

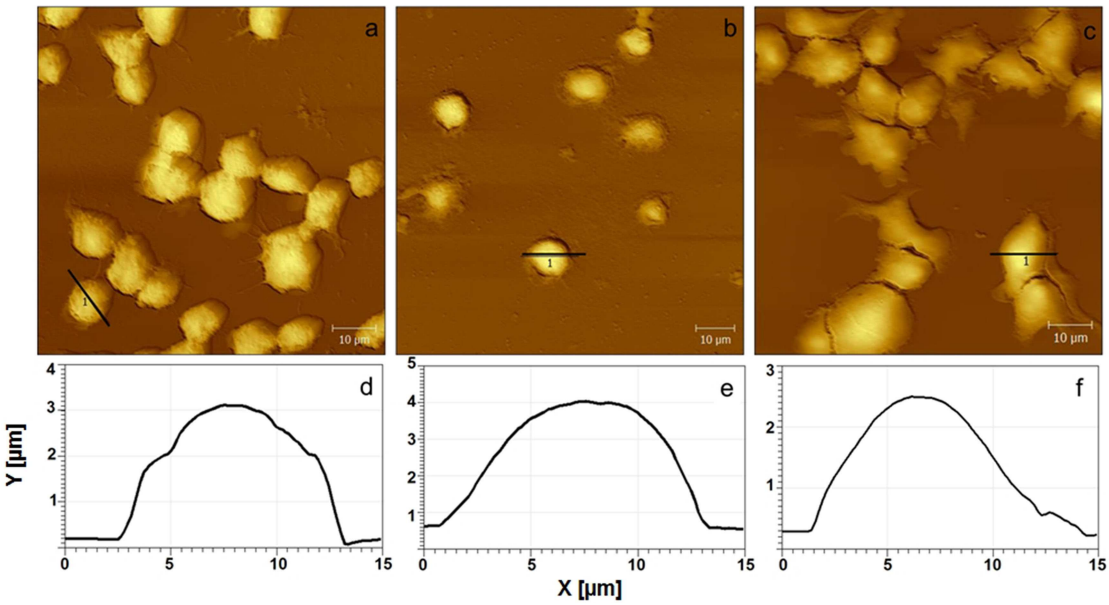

Figure 7. AFM topography of the cells immobilized at the glass surface by the Cell-Tak method. a) MOLT-4, b) Jurkat, c) U266 cell lines. d)–f) height profiles in marked sites. The size of AFM pictures is 80×80 µm, the height scale is z = 4 µm.

ure 7f). It should be noted that planar cells are more suitable for measuring topography and SMFS.

3. Conclusions

Early diagnostics of cancer is nowadays a big challenge that substantially improves positive prognosis in the therapy of this disease. The development of highly sensitive and easy-to-use diagnostics methods is therefore a rather urgent issue. The biosensor technology can substantially help in solving this problem. It is based on using specific receptors that recognize cancer markers in the biological liquids (blood or urea) or in a surface of cancer cells; for example, protein tyrosine kinase 7 (PTK7) is a well-known cancer marker that is in particularly localized at several leukemia cell lines including MOLT-4 and Jurkat. Among biosensors those based on label-free approach are of special interest, because minimize the cost for labeling the receptors and make the assay easier. The acoustics TSM method fulfills these requirements. Another novelty in cancer diagnostics consisting in application of DNA/RNA aptamers that are rather selective to the cancer markers and can replace more expensive and less stable antibodies. Using TSM method and DNA aptamers (sgc8c) specific to PTK7 receptors we developed high sensitive label-free biosensor for detection of leukemia cells with limit of detection (LOD) 195 ± 20 cells/mL. We have shown that this method most sensitive determine MOLT-4 cell lines, which is probably due to higher density of PTK7 receptors in their membranes. Control experiments using U266 cells that does not contain PTK7 receptors approved high selectivity of the sgc8c aptamers. This has been shown also by additional method that we used in experiments – single molecule force spectroscopy (SMFS). This method allowed us to determine probability of binding the aptamers to PTK7 receptors, which was highest for MOLT-4 cell lines. Thus, the obtained results are rather promising for improvement of cancer diagnostics.

Experimental Section

Cell Lines

Jurkat (TIB-152, human acute leukemia T cells), MOLT-4 (CRL-1582, human T lymphoblast cell line), U266 (TIB-196, B lymphocyte, human myeloma) cell lines were incubated in RPMI 1640 (PAN Biotech, Germany) supplemented with 10% fetal bovine serum (FBS, PAN Biotech) and 1% penicillin-streptomycin (Sigma Aldrich, Germany). Cell cultures were maintained at 37 °C in humidity 5% CO_2 atmosphere. As a buffer we used PBS, pH 7.4.

Reagents and Materials

(3-Aminopropyl)triethoxysilane (APTES), sodium cyanoborohydride ($NaCNBH_3$), ethylenediaminetetraacetic acid (EDTA), Tris(hydroxylmethyl)aminomethane (Tris), triethylamin (TEA), 1-dodecanethiol (DDT), paraformaldehyde, HBSS buffer (all were of analytical grade) were supplied by Sigma Aldrich (Germany). Inorganic salts, chloroform, ethanol, isopropanol, citric acid (p.a.) were from Slavus (Slovakia). Human plasma norm-Trol 1 samples were acquired from Helena BioSciences, UK. Neutravidin (NA) was purchased from Thermo Fischer Scientific GmbH (Germany). The sgc8c DNA aptamers (5′ ATC TAA CTG CTG CGC CGC GAA AAT ACT GTA CGG TTA GA-3′) modified at its 5′-end by thiol, biotin or amino groups were synthesized by Eurogentec (Belgium). This sequence was taken from Ref..[22] The aptamer stock solutions were prepared by dissolving lyophilized oligonucleotides in TE buffer (1 mM EDTA, 10 mM Tris, pH 8). As a buffer we used PBS (10 mM phosphate buffer saline: 10 mM NaH_2PO_4,1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Cell-Tak was obtained from BD Bioscience (Belgium). The heterobifunctional crosslinker NHS-PEG-Acetal were

obtained from JKU Linz (Austria). The deionised water was prepared by PureLab Classic UV (Elga Water Systems, UK).

Preparation of Acoustics Aptasensors and Cell Detection

The thickness shear mode (TSM) acoustic method has been described in details in our previous paper.[25] Briefly it is based on determination of series resonant frequency, f_s, and motional resistance, R_m, of AT-cut quartz crystal with immobilized DNA aptamers. The f_s value is sensitive to the mass or thickness changes, while R_m represents viscosity contribution to the frequency changes of the molecular slip between biosensing layer and surrounding liquid.[48] The f_s and R_m values were determined using a network analyzer 8712ES (Agilent Technologies, USA) by analysis of complex impedance spectra of quartz crystal oscillation. In experiment the quartz crystal of fundamental frequency 8 MHz and working area of 0.2 cm^2 (Total Frequency Control Ltd, UK) has been placed into the acryl flow cell with a working volume of approx. 100 μL. The cell was generous gift from prof. M. Thompson (University of Toronto, Canada) and its construction was published in Ref. [34]. The cells studied were allowed to flow with a rate of 50 μL/min on the surface of the aptasensor using syringe pump Genie Plus (Kent Scientific, USA). We used two types of biosensors based on 1) biotinylated or 2) thiolated sgc8c aptamers. Biotinylated aptamers were immobilized on neutravidin (NA) layer self assembled at clear gold surface of quartz crystal (for cleaning procedure of quartz crystal, see Ref. [33]). NA layers were formed by flow of neutravidin dissolved in a deionised water in a concentration of 250 μg/mL. Aptamer array was formed by flowing sgc8c aptamers dissolved in PBS in a concentration of 0.5 μM. In a second method, the mixture of 0.5 μM thiolated aptamers and 1 mM dodecanethiol has been added on the surface of clean quartz crystal and incubated overnight. After rinsing the sensing surface by PBS buffer it was ready for experiments with cells. All experiments were performed at ambient temperature (T = 23 °C). We should note that the concentration of aptamers 0.5 μM has been chosen based on the special experiments in which we analyzed the response of the sensors based on both biotinylated and thiolated aptamers for various concentrations of aptamers (0.5–2 μM) and showed that surface formed by 0.5 μM aptamers revealed most remarkable changes of the resonant frequency. This is illustrated on Figure SI-4 of Supporting information.

Detection of Cells in Spiked Human Plasma

The experiments were performed using aptasensors based on thiolated aptamers that revealed higher response to the cells. The samples were prepared by addition of corresponding cells into the 20 times diluted blood plasma. The response of the sensor after addition of the cells in a plasma and those in PBS was compared and the sensor recovery was determined as follows: $[(\Delta f_s)\text{PBS}/(\Delta f_s)\text{plasma}] \times 100\,\%$, where (Δf_s)PBS are changes of resonant frequency following addition of cells at certain concentration in a PBS and (Δf_s)plasma those in a blood plasma, respectively.

Sample Preparation for the AFM Measurements

Cells were adsorbed on a glass slide under sterile conditions. Prior cell adsorption the glass slides were washed in isopropanol and in sterile Millipore water several times. BD Cell-Tak in 5% acetic acid was hand spreaded on the glass surface. As soon as the acetic acid evaporates (about 5 min.), a coating of BD Cell-Tak is left behind. The glass slide was washed with ethanol and sterile Millipore water. The cells were centrifuged and cell pellet was resuspended in RPMI media (without FBS). 1000 μl of cell suspension was added on the glass slide and incubated under air atmosphere with 5% CO_2 at 37 °C for 30 min. Before fixation the glass surface with adsorbed cells was rinsed three times in PBS. Cells were then fixed with 4% paraformaldehyde in HBSS over a period of 60 min. at room temperature and washed again in PBS for three times. Afterwards the cells were left in PBS and used immediately for force measurement or stored in the fridge for a maximum 2 days.[30]

AFM-Tip Modification by Aptamers

Functionalization of AFM tips often requires a three-step protocol (Figure 5A). First, amino-functionalization of the inert tip material is necessary, followed by the connection of a crosslinker and ending up in the coupling of the aptamer molecule. AFM probes were functionalized with 3-aminopropyl triethoxysilane (APTES) by the method described in Ref. [49]. Briefly, the freshly APTES coated tips were immersed in NHS-PEG-Acetal crosslinker for 2 h, at presence of triethylamine. After 120 minutes, the tips were washed three-times with chloroform and ethanol and dried in a nitrogen stream. Finally, the AFM tips were immersed in 40 μL PBS solution of 0.5 μM amino-sgc8c aptamer and 2 μL freshly prepared aqueous solution of 1 M $NaCNBH_3$ was added and allowed to react for 1 hour. The unreacted aldehyde groups were blocked by addition of 5 μL of 1 M ethanolamine to the tip surface. In the last step, the tips were washed by PBS and used immediately for force spectroscopy experiments or stored in PBS buffer in fridge until use. All washing steps were performed three times.

Single-Molecule Force Spectroscopy and AFM Topography

AFM measurements were performed in PBS buffer using an Agilent 5500 AFM (Agilent Technologies, USA). Silicon AFM cantilevers (model MSCT, Bruker AFM probes, Germany) with a nominal spring constant of 0.01 to 0.03 N/m were used. The imaging scan speed was adjusted to 1 line/s at 512 data points/line. AFM topography measurements were performed in tapping mode. The data analysis was performed using freeware Gwyddion software (http://gwyddion.net). SMFS method was used for study the interaction between amino-terminated aptamers sgc8c and PTK7 receptors. For this purpose the force-distance cycles were recorded using sgc8c functionalized MSCT cantilevers. The MOLT-4, Jurkat or U266 cells were fixed on a glass slides. In SMFS experiments different pulling velocities were used and the loading rate dependence of the unbinding force was determined. At each pulling velocity, 1000 to 2000 force distance cycles (FDCs) were recorded. To ensure position-independent results, the location on the cell was shifted by 200 nm after 200 FDCs. MATLAB R2008a (The MathWorks, Natick, MA) was used for force curve analysis. Finally, in order to show the specificity of the results the cell surface was blocked by sgc8c aptamers and compared to unblocked measurement at the same pulling velocity. The data analysis used in this work was described in details in our previous paper.[50]

Acknowledgements

We are grateful to the Slovak Research and Development Agency (Project No. APVV-14-0267) and to Science Grant Agency VEGA (project No. 1/0152/15) for financial support. This contribution is also the result of the project implementation (ITMS 26240120027) supported by the OPRaD funded by the ERDF.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: atomic force microscopy · DNA aptamers · leukemia · PTK7 · thickness shear mode

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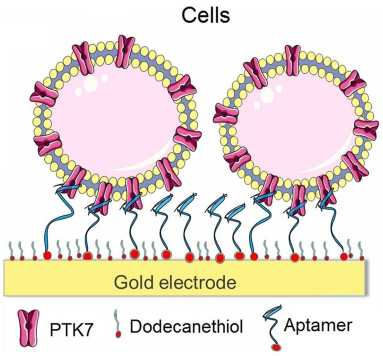
Manuscript received: December 4, 2018
Revised manuscript received: December 13, 2018
Accepted manuscript online: December 15, 2018
Version of record online: ■■, ■■■

ARTICLES

DNA aptamers that are specific to the protein tyrosine kinase 7 (PTK7), which is localized in the membranes of various leukemia cell lines, were studied. The acoustics thickness shear mode method allowed the development of a highly sensitive biosensor for the detection of leukemia cells with a detection limit of (195 ± 20) cells/mL.



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