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REVIEW

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Development of aptamers against unpurified proteins

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Abstract

SELEX (Systematic Evolution of Ligands by EXponential enrichment) has been widely used for the generation of aptamers against target proteins. However, its requirement for pure target proteins remains a major problem in aptamer selection, as procedures for protein purification from crude bio-samples are not only complicated but also time and labor consuming. This is because native proteins can be found in a large number of diverse forms because of posttranslational modifications and their complicated molecular conformations. Moreover, several proteins are difficult to purify owing to their chemical fragility and/or rarity in native samples. An alternative route is the use of recombinant proteins for aptamer selection, because they are homogenous and easily purified. However, aptamers generated against recombinant proteins produced in prokaryotic cells may not interact with the same proteins expressed in eukaryotic cells because of posttranslational modifications. Moreover, to date recombinant proteins have been constructed for only a fraction of proteins expressed in the human body. Therefore, the demand for advanced SELEX methods not relying on complicated purification processes from native samples or recombinant proteins is growing. This review article describes several such techniques that allow researchers to directly develop an aptamer from various unpurified samples, such as whole cells, tissues, serum, and cell lysates. The key advantages of advanced SELEX are that it does not require a purification process from a crude bio-sample, maintains the functional states of target proteins, and facilitates the development of aptamers against unidentified and uncharacterized proteins in unpurified biological samples.

KEYWORDS
aptamer generation, crude bio-sample, electrophoresis, proteomics, SELEX

1 | INTRODUCTION

Molecular-targeted drugs that can bind to pre-selected target molecules with high affinity and specificity play an essential role in the development of therapeutic drugs and detection reagents for diagnostic applications. Various types of molecular-targeted drugs have been developed, such as antibody-, peptide-, and aptamer-based drugs. Aptamers are single-stranded oligonucleotides that bind their target molecule with high affinity and specificity. The advantages of aptamers in comparison to large protein-based antibodies are their small size and the ease of molecular design, development, chemical modification, functional group addition, handling, and adaptation to analytical methods and nanostructures. Proteins play significant roles in the physiological and pathological mechanisms of living organisms including human beings. Hence, many cell membrane proteins and proteins dissolved in body fluids have been used as both medical drug

targets and biomarkers. Several aptamers against proteins have been developed and are considered candidates for the development of medical drugs or diagnostic reagents (Ahmad Raston & Gu, 2015; Ahmad Raston, Nguyen, & Gu, 2016; Sun et al., 2014). One of the most successful aptamers is Macugen, the first aptamer-based drug for treating age-related macular degeneration. Macugen® asserts its therapeutic action based on the molecular mechanism through which proteins of the vascular endothelial growth factor (VEGF) family participate in the formation of the abnormal blood vessels that damage the retina in wet macular degeneration. Macugen® is an anti-vascular endothelial growth factor (VEGF)-165 RNA aptamer with substituted modified nucleotides (Ng et al., 2006).

Aptamers are generated by SELEX (Systematic Evolution of Ligands by EXponential enrichment), a process that allows the extraction of oligomers with binding affinity for a given molecular target from an initially random oligonucleotide library of oligonucleotides (Elington & Szostak, 1990; Tuerk & Gold, 1990). The procedure can be used to select the strongest binders for a given protein. Although SELEX is a useful method because of its accuracy and the reproducibility of aptamer generation, there is plenty of room for improvement with respect to the methods used for the purification of target proteins from crude samples, as current procedures are not only complicated but also time and labor consuming. A native protein may exist in a large number of diverse forms because of its complicated conformation and the posttranslational modifications. Moreover, some proteins are difficult to purify due to their chemical properties, small quantity in available samples, or molecular fragility. Therefore, complicated multistep purification methods requiring careful biochemical operations are required, such as gel filtration chromatography, hydrophobic chromatography, and affinity chromatography. Although a high degree of automation and optimization has been achieved in the field of chemical biology (Blind & Blank, 2015), major parts of protein purification protocols remain manual processes depending on the character of the targeted protein, sample condition, and user skill and are thus often characterized by poor accuracy and reproducibility.

Owing to the ability of recombinant proteins, which are constructed through genetic engineering, to be produced in a homogenous state and to be easily purified through tagged affinity assays, they have been widely used in aptamer technology as a source of target proteins for SELEX. However, there are two important disadvantages in using recombinant proteins. First, aptamers generated against recombinant proteins produced in prokaryotic cells may not interact with the respective native proteins owing to the differences between the posttranslational modification systems of eukaryotes and prokaryotes. Moreover, to date, recombinant proteins have been synthesized for a relatively small portion of the approximately 20,000 proteins expressed in the human body (Sun & Zu, 2015; Uhlén et al., 2015). Therefore, the demand for the development of advanced SELEX techniques, which do not rely on complicated processes for the purification of target proteins from native samples or recombinant proteins, is growing.

In this review, we describe several advanced SELEX techniques that can screen aptamers against unpurified protein samples. These "non-purification" SELEX methods are categorized into three groups: (1) aptamer selection for cells or tissues; (2) aptamer selection by gel electrophoresis; and (3) aptamer selection by a proteomics approach.

The key advantages of advanced SELEX are that it (a) does not require a purification process from a crude bio-sample; (b) allows for screening the functional states of target proteins; (c) facilitates the development of aptamers against unidentified and uncharacterized proteins in unpurified biological samples; and (d) enables the efficient screening of aptamers with a low SLELX cycle by subtractive selection or competitive selection. The selected aptamers obtained by advanced SELEX can be used for various applications such as biomarker identification, protein purification, imaging probes, activation/inhibition of signaling pathways in cells, targeted delivery for diagnostics, and in therapeutic applications as molecular targeted drugs.

2 | APTAMER GENERATION AGAINST CELLS AND TISSUES

Aptamer selection against unpurified proteins must be adapted to native or solubilization conditions. In this chapter, we introduce three similar techniques: Cell-SELEX against native cells, in vivo SELEX, and tissue slide-based SELEX against specific tissues.

2.1 | Cell-SELEX

Cell-SELEX is widely used for aptamer generation against proteins located on the surfaces of whole cells. The original Cell-SELEX was reported by Hicke et al. (2001) for selection against an overexpressed tenascin-C protein in its native functional state on a cell membrane. Subsequently, Wang et al. (2003) reported application of Cell-SELEX for an unknown target against fixed PC12 cells through introducing a subtractive selection strategy. Shangguan et al. (2006) used Cell-SELEX for developing aptamers that can recognize molecular differences in live cells. Moreover, several advanced Cell-SELEX methods have been developed in recent years. Target expressed on cell surface (TECS)-SELEX can be used to obtain aptamers against target recombinant proteins on the cell membrane without a purification process (Ohuchi, Ohtsu, & Nakamura, 2006). In addition, fluorescence-activated cell sorting (FACS)-based SELEX can separate cells that are bound to fluorescence-labeled aptamers, which is an efficient and high-throughput method employing a FACS device (Mayer et al., 2010). Transmembrane proteins on the surfaces of whole cells play important roles in various physiological functions. For example, transmembrane receptors trigger cell signalling pathways by receiving/binding extracellular molecules. Therefore, many transmembrane proteins, including G protein-coupled receptors, receptor kinases, and ion channels, are important targets for the development of molecular-targeted drugs (Rask-Andersen, Almén, & Schiöth, 2011). However, amphiphilic nature of transmembrane proteins makes them difficult to be solubilize and purify. Therefore, Cell-SELEX is often utilized for

selection of aptamers against transmembrane proteins, as it does not require purified proteins.

There were a lot of researches using Cell-SELEX against membrane proteins has already been used in a significant number of studies, and the developed aptamers have proven useful for identification of various target proteins, such as TGF-Beta-1-Binding Protein CD109 (Jia et al., 2016), B-lymphocyte antigen CD20 (Al-Youssef, Ghobadloo, & Berezovski, 2016), RET receptor tyrosine kinase (Cerchia et al., 2005), haemagglutinin on the surface of human influenza viruses (Gopinath et al., 2006), growth factor-β type III receptor (Ohuchi et al., 2006), and protein tyrosine phosphatase 1B (Townshend, Aubry, Marcellus, Gehring, & Tremblay, 2010). In addition, aptamers against cell surface proteins are useful as biomarkers. Biomarkers indicate changes in the expression or state of proteins that take place under changing physiological conditions or during pathogenesis, thus providing an important tool for clinical diagnosis, monitoring, and treatment.

The key process of Cell-SELEX is the direct addition of a nucleotide library or wash buffer for the bound/free (B/F) selection of the nucleotide to whole cells. The first stage of Cell-SELEX is the preparation of a synthesized random oligonucleotide library and the collection of target cells of interest. These are used in iterative cycles consisting of the following steps: (1) incubation of target cells with the randomized DNA library; (2) collection of cells bound to oligonucleotides; (3) elution of oligonucleotides bound on target cells; (4) amplification of eluted oligonucleotides and preparation of an enriched oligonucleotide pool; (5) counter selection using non-target cells (Figure 1). Counter selection, also named subtraction selection, plays an important role in Cell-SELEX. Counter selection using cells not displaying the target protein on their surface enables the generation of an aptamer with high affinity in a lower number of Cell-SELEX cycles, because of the elimination of unbound oligonucleotides.

Aptamers generated against target transmembrane proteins expressed in prokaryotic cells do not interact with the same proteins expressed in eukaryotic cells, as the latter undergo different posttranslational modifications that render their epitopes inaccessible to aptamers generated against proteins expressed in prokaryotic cells. In contrast, aptamers generated by Cell-SELEX are functional and can recognize the native conformation of transmembrane proteins. In recent years, this technique has allowed the identification of a large number of novel transmembrane proteins that represent potential therapeutic drug targets. To sum up, Cell-SELEX is a technique that allows the development of aptamers against native transmembrane proteins. However, there is still plenty of room for improvement in this technology, given that Cell-SELEX is still not able to target certain proteins. Moreover, Cell-SELEX can be further developed for use in the collection of homogeneous target cells from crude samples using cell fraction technologies such as flow cytometry, cell centrifugation, and adsorption.

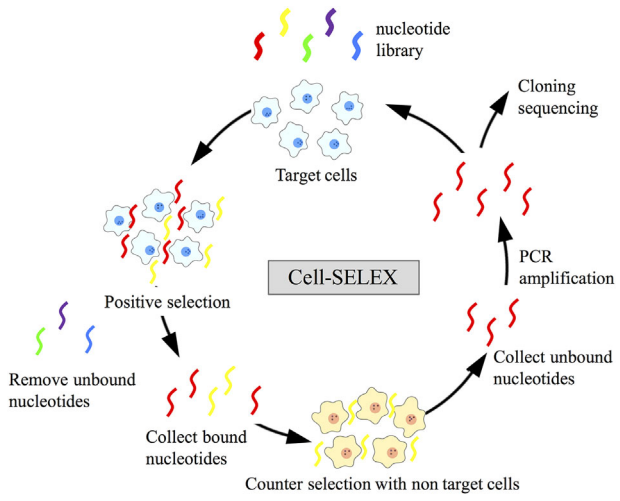

FIGURE 1 Schematic representation of Cell-SELEX for aptamer selection against whole cells

2.2 | In vivo SELEX

In vivo SELEX was developed to select aptamers capable of specifically localizing inside tumors of living animals (Mi et al., 2008). The overall selection process in in vivo SELEX is similar to that in conventional SELEX, except for that living animals are used for the selection process instead of purified target proteins. The key process of in vivo SELEX is direct injection of the nucleotide library into the animal. After binding to the target tissue via the blood circulation in the body, the target tissue is then surgically removed for the collection of bound nucleotides.

Mi et al. (2010) used mice with intrahepatic colorectal metastases as a selection model for in vivo SELEX. First, RNA oligonucleotide libraries with 2′-fluoro-pyrimidine substitutions were injected into the tail vein of mice. Liver samples containing intrahepatic tumors were collected 20–30 min after injection. RNA aptamers were extracted from the samples, amplified by PCR, and injected into other mice bearing the same type of tumors. Fourteen selection rounds were performed in the same manner (Figure 2). As a result, an aptamer with high affinity for p68 was selected. P68 is an RNA helicase that had been shown to be up-regulated in colorectal cancer and was already considered as a candidate therapeutic drug target. Mi et al. (2010) showed that in vivo SELEX was able to generate tissue-specific aptamers without requiring a purification process. Additionally, in vivo SELEX enjoyed the significant advantage that the generated aptamers did not bind to blood cell-surface proteins because blood cell-binding oligonucleotides were dispersed into blood circulation.

Recently, Mi et al. (2016) successfully used in vivo SELEX to develop an RNA aptamer against DHX9, an RNA helicase known to be up-regulated in colorectal cancer. They first established intrahepatic xenografts in immunodeficient mice using cell lines derived from human patients undergoing liver resection for colorectal liver metastases, and then used in vivo SELEX to select aptamers specific for the injected cell lines. After 12 rounds of in vivo SELEX, they identified aptamers that recognized the induced xenografts.

Cheng, Chen, Lennox, Behlke, and Davidson (2013) reported the selection of aptamers capable of penetrating the blood-brain barrier in

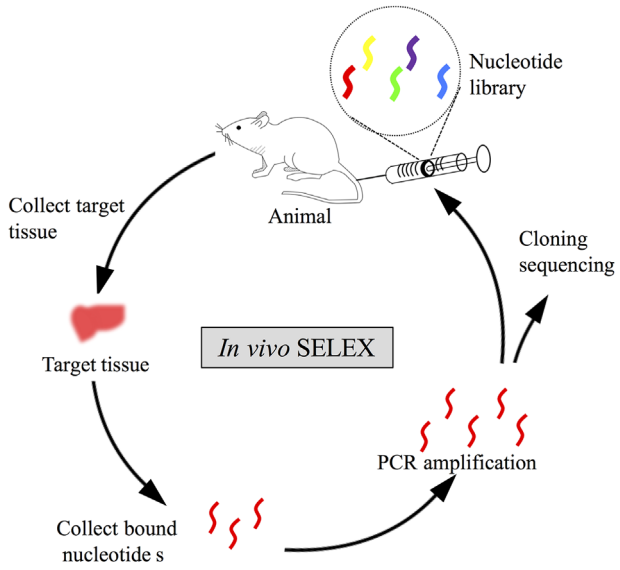

FIGURE 2 Schematic representation of In vivo SELEX for aptamer selection against tissue in living animal

wild-type mice employing in vivo SELEX. The selection scheme resembled the one described above. After 12 rounds of SELEX, the resulting RNA oligonucleotide library underwent iterative counter selection rounds with mouse serum to increase specificity for brain tissue. After 22 rounds of counter selection, in situ hybridization was carried out to determine the distribution of the obtained RNA aptamers, and confirmed that the selected aptamers localize in various brain compartments, namely the cortex, hippocampus, cerebellum, and striatum. The detection of the injected aptamers in brain tissues indicated that these oligonucleotides were able to penetrate the blood-brain barrier. In vivo SELEX can be used as an alternative to in vitro SELEX in order to produce aptamers free of the problems characterizing the in vitro obtained aptamers.

Using in vivo SELEX, it is possible to directly select an aptamer against a specific tissue without cell separation or collection. However, in vivo SELEX is a highly invasive method and has thus far only been achieved in mice. Moreover, it is difficult to perform in vivo SELEX in a medium- or large-sized animal because of the requirement of a larger nucleotide library, and the longer time in the blood circulation after injection.

2.3 | Tissue slide-based SELEX

Tissue slide-based SELEX is used for the selection of aptamers against clinical specimens (Li et al., 2009; Wang et al., 2016; Zhang et al., 2015). Tissue sections with a thickness of a few micrometers placed on glass slides are routinely used for pathological diagnosis. Li et al. (2009) developed the tissue slide-based SELEX method using sections of neoplastic tissues from breast cancer patients. The key process of tissue slide-based SELEX is that it allows for directly adding the nucleotide library or wash buffer for B/F selection of the nucleotide to the tissue slide. Their method included the following steps: First, DNA oligonucleotide libraries were incubated with slices of cancerous tissue. After incubation, oligonucleotides were scraped from the cancer tissue and amplified by PCR. This was followed by a counter selection step in which the PCR products were incubated with normal tissue slices. The unbound oligonucleotides were again incubated with cancer tissue slices. After 12 rounds of selection, the obtained oligonucleotides were cloned and their affinity was tested against various cancer types (Figure 3). Results showed that the selected DNA aptamers could successfully bind to various types of clinical specimens, such as those from lobular carcinoma, comedo and non-comedo ductal carcinomas, and metastatic lymph node.

The slide-based SELEX method that Li et al. (2009) developed has several advantages. First, it selects aptamers against clinical specimen sections, which are more representative of the underlying disease than cultured cell lines. Second, tissue slide-based SELEX can select aptamers against all fractions of a tissue, including the extracellular matrix, membrane components, and intracellular targets, while the obtained aptamers may be used as novel molecular probes for pathological diagnosis. Regarding the latter, of special interest is the fact that this method can select aptamers against intracellular targets such as lysosomal enzymes, apoptotic factors, and transcription factors, in unpurified samples (Mitragotri, Burke, & Langer, 2014; Yeh ennifer, Toniolo, & Frank, 2013). However, the main disadvantage of tissue slide-based SELEX is that the target proteins on the tissue slide might denature during the process of tissue slicing, immobilization, and staining with chemical reagents. In addition, similar to Cell-SELEX and in vivo SELEX, slide-based SELEX cannot target certain proteins.

3 | APTAMER SELECTION USING GEL ELECTROPHORESIS

Advanced methods combining gel electrophoresis and SELEX aptamer selection achieved have been reported as effective against unpurified proteins such as those contained in serum, urine, and cell lysates.

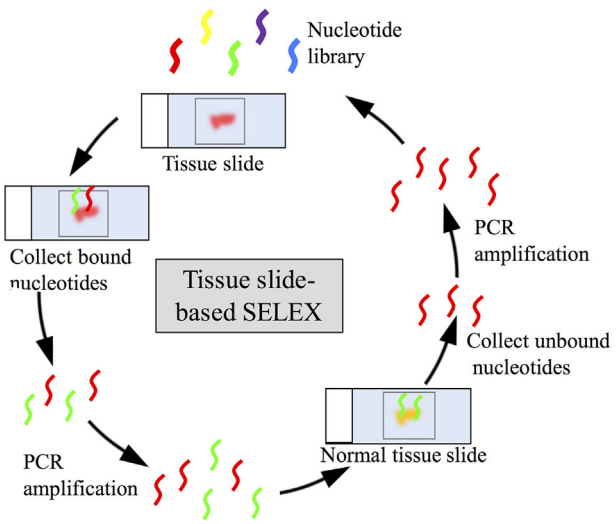

FIGURE 3 Schematic representation of tissue slide-based SELEX for aptamer selecton against specific tissue

Gel electrophoresis is a popular method for the separation of proteins based on the speed with which they migrate through a proper medium under the influence of an electric field; in turn, this mobility is determined by factors such as the size of the protein. There are various types of gel electrophoresis, and these are chosen according to the desired application or purpose. For example, in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), proteins are treated with SDS. The latter binds to proteins and bestows a strong negative charge upon them, neutralizing the effect of the protein inherent charge. As a result, when these proteins are electrophoresed, they all migrate toward the positive pole with a mobility that is only dependent on their size. On the other hand, capillary gel electrophoresis is an electrophoretic separation method performed in submillimetre diameter capillaries and in micro- and nanofluidic channels. The narrow tubes used in capillary gel electrophoresis allow high resolution separation of minimum volume samples. Electrophoretic Mobility Shift Assay (EMSA) is used to study oligonucleotide-protein interactions, and is based on the changes that the negative charge of the oligonucleotides causes to the electrophoretic mobility of a protein. This method that was initially developed for the analysis of nucleotide binding proteins such as transcription factor can also be used for the separation of oligonucleotide libraries and target proteins.

These gel electrophoresis techniques are widely used in the field of molecular biology because of their ease of use and high reproducibility. Therefore, combination of gel electrophoresis and SELEX may provide powerful tools for the generation of aptamers in unpurified protein samples, enabling the simultaneous separation of unpurified proteins and B/F separation of oligonucleotides. Below, we describe two techniques deriving from such combinations.

3.1 | Aptamer-facilitated protein isolation from cells

Javaherian, Musheev, Kanoatov, Berezovski, and Krylov (2009) introduced AptaPIC (Aptamer-facilitated Protein Isolation from Cells), a technique that allows selection of aptamers against target proteins in unpurified cell lysates. The defining features of AptaPIC are the use of gel capillary electrophoresis and the fact that it does not require any prior purification of the target protein. Javaherian et al. (2009) demonstrated AptaPIC by using it to select aptamers against recombinant MutS protein. MutS is a thermostable DNA mismatch-binding protein from *Thermus aquaticus*. The key process of AptaPIC is the separation of bound nucleotides and unbound nucleotides against the target protein using capillary electrophoresis. The process is described below.

In the first step, a library of fluorescently labelled oligonucleotides consisting of a very large number of individual aptamers was incubated with *E. coli* cell lysate containing a large amount of recombinant MutS protein (54 mg/ml of MutS in 972 mg/ml total protein). Gel capillary electrophoresis was employed for separation of free oligonucleotides and MutS protein-oligonucleotide complexes, whose electrophoretic mobility depends on the negative charge of oligonucleotides and the molecular weight of the protein. Fluorescence was used to detect the complexes, from which the bound oligonucleotides were then collected. These were amplified by PCR, and then used in the next round of selection. After five rounds of AptaPIC, a DNA aptamer was selected with specificity and affinity for MutS (Figure 4). To evaluate the performance of the generated aptamer, they used magnetic beads conjugated to the aptamer to collect MutS from *E. coli*. SDS–PAGE analysis showed that the collected protein was pure MutS, confirming both the specificity and the affinity of the generated aptamer for this protein.

This report showed that AptaPIC could be used for the generation of aptamers against a large amount of soluble MutS, a DNA binding protein. Later, the same research group successfully generated aptamers against the human platelet-derived growth factor chain B (PDGF-B) protein after only three rounds of selection, demonstrating that their method can also be applied to proteins that do not bind DNA (Kanoatov, Javaherian, & Krylov, 2010).

AptaPIC is an integrated method that allows the development of aptamers against soluble target proteins in complex cell lysates without purification. However, the main disadvantage of this method is that it requires a large amount of the target protein and the use of easy-to-operate and high-sensitivity fluorescent labeling technology. Recently, researchers developed a biochemical protocol for quick and reliable protein labeling using click chemistry (Nikić et al., 2015). For example, some functional molecules can rapidly and selectively mark the target protein in one or two simple steps (Horisawa, 2014). We believe that the combination of high-throughput AptaPIC and click chemistry would provide a powerful system for aptamer selection.

3.2 | Subtractive-EMSA SELEX

Many aptamers have been successfully developed by EMSA. Examples are the aptamers against the transactivating Tax protein from human T-cell leukaemia virus (Tian et al., 1995), serum protein C5 (Lee, Kim, Ko, & Lee, 2002), *Plasmodium lactate* dehydrogenase (Lee et al., 2012)

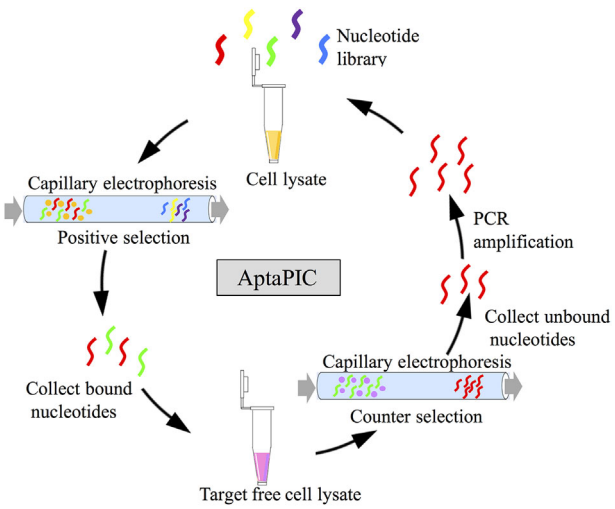

FIGURE 4 Schematic representation of AptaPIC (Aptamer-facilitated Protein Isolation from Cells) foraptaemer selection against cell lysate

which is used as a diagnostic biomarker, and the transcriptional factor AP1 (Walters, McSwiggen, Goodrich, & Kugel, 2014). Moreover, we previously generated a DNA aptamer that binds to a-synuclein using EMSA (Tsukakoshi, Harada, Sode, & Ikebukuro, 2010).

Li et al. (2015) introduced subtractive-EMSA SELEX as a screening method for biomarkers in serum. Serum is a key sample for drug or biomarker discovery because it contains several proteins that are candidate targets for drug discovery, such as cytokines, endocrine factors, and other functional molecules (Liu, Fang, Guo, Mei, & Zhang, 2013; Pritchard, 2013; Siebert, Tsoukas, Robertson, & Mcinnes, 2015). EMSA is based on the fact that the electrophoretic mobility of a protein-nucleic acid complex is typically lower than that of the free nucleic acid (Hellman & Fried, 2007). Moreover, EMSA is commonly used in aptamer generation because it allows for high-resolution B/F separation, that is, the separation of unbound oligonucleotides from the oligonucleotide-protein complex (Saito, Yoshida, Yokoyama, Abe, & Ikebukuro, 2015; Sosic, Meneghello, Cretaio, & Gatto, 2011; Tsukakoshi et al., 2010). The key process of subtractive-EMSA SELEX is the separation of bound nucleotides and unbound nucleotides against the target protein using EMSA.

Li et al. (2015) used subtractive-EMSA SELEX to select aptamers against AFP (alpha-fetoprotein)-negative serum samples of hepatocellular carcinoma (HCC) patients with extrahepatic metastases. First, a counter selection step was performed by incubating an oligonucleotide library with serum from healthy subjects. EMSA was used for the B/F separation of oligonucleotides. The free oligonucleotides were used in the following positive selection step, in which they were incubated with serum from hepatocellular carcinoma (HCC) patients. After B/F separation by EMSA, bound oligonucleotides were isolated and amplified by PCR, then used in iterative cycles. After five rounds of subtractive-EMSA SELEX, Li et al. (2015) successfully identified the protein vasorin as a biomarker of HCC (Figure 5). There was a statistically significant increase in vasorin concentration in sera from HCC patients compared to that in sera from healthy individuals. Therefore, Li et al. (2015) showed that subtractive-EMSA SELEX can be used to compare the sera of patients and healthy subjects in order to identify biomarkers that are mainly found in the sera of patients. Subtractive-EMSA SELEX might prove a valuable method for the development of aptamers against soluble unpurified proteins such as those found in serum. However, the main disadvantage of this method is the limited ability for the selection of an aptamer against a negatively charged or high-molecular-weight protein, because negatively charged proteins would not bind to nucleotides and larger proteins would not be shifted by gel electrophoresis.

4 | APTAMER SELECTION USING THE PROTEIN-TRANSFERRED MEMBRANE AFTER ELECTROPHORESIS

Proteomics is the large-scale study of proteins, particularly with respect to their composition, structure, function, and interaction with other proteins directing cellular activities. Although various new and improved proteomic techniques are available today, traditional gel-based proteomics, such as western blotting and two-dimensional electrophoresis, are still widely used (Chandrasekhar, Dileep, Lebonah, & Kumari, 2014; Magdeldin et al., 2014). Western blotting is a semi-quantitative technique used extensively in research to detect specific proteins and determine their levels (Sanders, Kim, & Dunn, 2016). Proteins in crude samples are separated based on size using gel electrophoresis, transferred to membranes, and detected by antibodies specific for the protein of interest (Sanders et al., 2016).

Two-dimensional gel electrophoresis (2DE) is widely used in the field of proteome research for the analysis of complex protein mixtures extracted from cells, tissues, or a variety of unpurified biological samples. This technique separates proteins in two steps (dimensions), each of which relies on a different property of the protein. In the first-dimension, isoelectric focusing (IEF) is used to separate proteins according to their isoelectric points (pI). The second step is SDS–PAGE, which separates proteins according to their molecular weight (MW). Unpurified mixtures consisting of thousands of different proteins can be resolved and the relative amount of each protein determined. In this way, gel-based proteomics play a significant role in focused or comprehensive analyses of proteins.

Advanced methods combining gel-based proteomics and SELEX have been introduced for effective aptamer generation. The membrane to which proteins are transferred after electrophoretic separation, usually made of nitrocellulose membrane, is suitable for aptamer selection. The fact that proteins are immobilized on the membrane allows easy treatment with reagents, such as nucleotide library solutions or washing buffers, ensuring reproducibility. Additionally, the membrane-immobilized proteins can be easily detected by multiple imaging procedures, such as fluorescent and chemiluminescent imaging. Moreover, the band/spot of the membrane containing the target protein can be located based on the protein's PI and MW, then easily cut off and picked up.

4.1 | Aptamer selection against proteins immobilized on membrane after SDS–PAGE

Bianchini et al. (2001) originally developed aptamer selection on a protein-transfer membrane. They selected DNA aptamers against ERK2, an extracellular signal-regulated kinase, by two rounds of SELEX. Dot blots on a membrane and proteins transferred to a membrane after SDS–PAGE were used in the first and second round, respectively. The key aspect of this method is the use of a protein-immobilized membrane. Analytically, the process was as follows: A peptide representing a part of ERK2 was synthesized and blotted on a nitrocellulose membrane. In parallel, lysates of T84 cells, a human colon carcinoma cell line expressing ERK2 (42 kDa), were separated by SDS–PAGE, and the proteins were transferred onto a nitrocellulose membrane. The dot-blot membrane was treated with an oligonucleotide library. Bound oligonucleotides were collected, amplified by PCR, and incubated with the membrane on which the SDS–PAGE-separated proteins had been transferred. Oligonucleotides bound to ERK2 were cloned and sequenced (Figure 6). DNA aptamers against ERK2 were

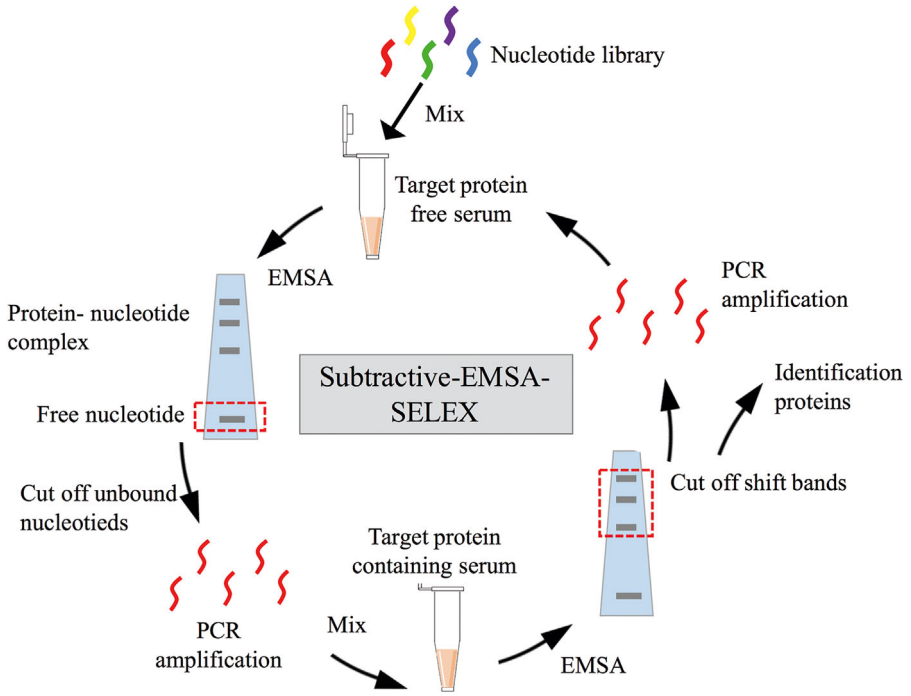

FIGURE 5 Schematic representation of subtractive-EMSA-SELEX for aptamer selection and protein identification in serum

obtained as the result of these two steps of on-membrane screening. Bianchini et al. (2001) showed that a combination of SDS-PAGE and incubation/isolation on a protein-transfer membrane can be effectively applied for the selection of aptamers against identified target proteins in unpurified samples.

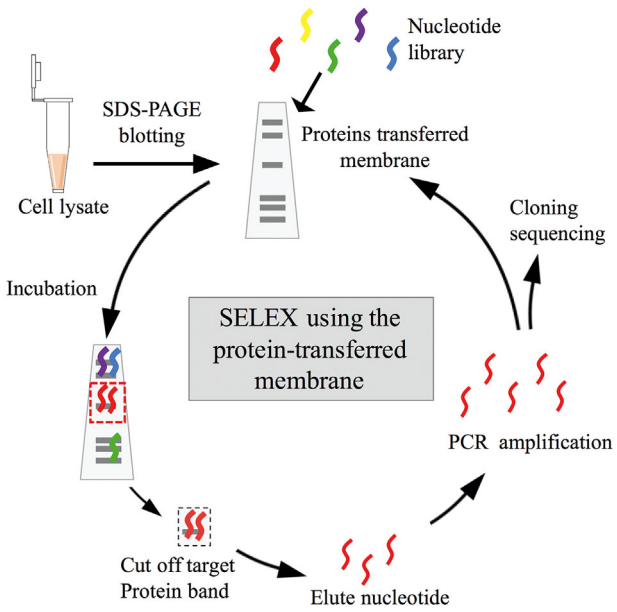

FIGURE 6 Schematic representation of SELEX using the protein-transferred membrane after SDS–PAGE foraptamer selection against cell lysate

On the other hand, we were able to develop DNA aptamers against an unidentified protein following a similar process including SDS–PAGE and transfer onto membranes (Noma et al., 2006). In contrast to Bianchini's method, which required pre-determined and identified target proteins, our research aimed to develop aptamers against unidentified proteins in unpurified samples through a proteomics approach. Aptamer selection against unidentified proteins whose functions are not well characterized is important for research on pharmaceutical drugs or biomarkers because aptamers bound to unidentified target proteins may become powerful tools for the identification of these proteins (Blank, Weinschenk, Priemer, & Schluesener, 2001). Therefore, we developed a protocol for generation of aptamers against unidentified proteins using chicken skeletal muscle as a model sample. Target proteins in the mixture were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Subsequently, a DNA oligonucleotide library was added onto the membrane. Aptamers bound to the target protein were visualized by chemiluminescence and collected by cutting out the visualized band of an unidentified protein near 35 kDa. To characterize and identify target-specific aptamers, the obtained oligonucleotides were again incubated on a membrane with transferred SDS–PAGE-separated cell lysate proteins. Specific aptamers against the target protein were selected after only one round of selection using this SDS–PAGE/membrane-based screening method. Thus, we confirmed that the employed method allows the selection of aptamers against unidentified proteins in unpurified samples. The main disadvantage of this method is that the target proteins might become denatured during SDS–PAGE and electroblotting onto the membrane, and in many cases, the target protein cannot be clearly separated with SDS–PAGE alone.

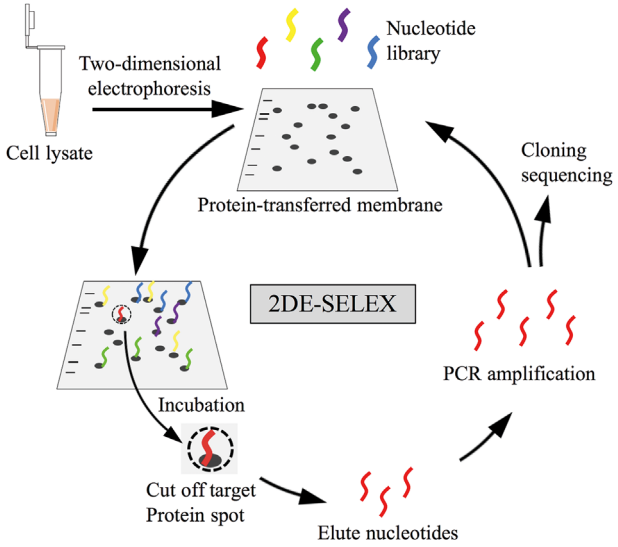

FIGURE 7 Schematic representation of two-dimensional electrophoresis-based SELEX for aptamer selection against a cell lysate

4.2 | Two-dimensional electrophoresis-based SELEX

We previously introduced two-dimensional electrophoresis-based SELEX (2DE-SELEX), a technique combining 2DE and SELEX (Savory et al., 2013). 2DE allows the comprehensive analysis of proteins and enables us to investigate uncharacterized and unidentified proteins in unpurified samples. Similar to SDS–PAGE, 2DE is followed by transfer of proteins to a membrane. Membrane-immobilized proteins can easily undergo treatment with reagents such as oligonucleotide library solutions and washing buffers, as well as chemiluminescent or fluorescent detection. Moreover, in the field of proteome study, combination of 2DE and western blotting is a useful tool for the analysis of posttranslational modifications of specific proteins, such as in the study of protein glycosylation and phosphorylation patterns (Hirayama et al., 2013; Magdeldin et al., 2014) because Western blotting with highly sensitive antibodies allows the detection of even subtle alternations in protein structure. For all these reasons, we considered the possibility that separation of crude protein samples with 2DE may be useful for aptamer selection against uncharacterized and unidentified proteins in complex protein samples.

In our previous study (Savory et al., 2013), we set out to examine this possibility by performing the following procedure (Figure 7). The main aspect of this method is the direct addition of a nucleotide library to the membrane after two-dimensional electrophoresis and B/F separation on the membrane. (1) Crude protein was solubilized in an appropriate buffer (solubilization buffer), tagged with a fluorescent labelling marker, and separated by 2DE. (2) The separated proteins in the acrylamide gel were transferred onto a nitrocellulose membrane. (3) The membrane was incubated with an oligonucleotide library solution. (4) A spot containing an unidentified target protein was cut off and the bound nucleotides were eluted giving a mix of enriched nucleotides. (5) The enriched oligonucleotides were amplified with PCR and used in the next selection round. (6) After several rounds of selection, aptamers against the target protein were cloned and sequenced. Mouse liver lysate labeled with fluorescent marker was separated by 2DE with isoelectric focusing in the pH range of 4–7 and SDS–PAGE in 48 mm-long gels. After transfer, a single spot corresponding to a PI of 4.5 and a MW of 35 kDa containing an unidentified target protein was cut off, followed by four rounds of selection. The secondary structures of

TABLE 1 Summary of aptamer selection against unpurified proteins

Name of method	Description	References
Cell-SELEX	Use of whole cells; Aptamer generation against membrane proteins on cell surface	Daniels, Chen, Hicke, Swiderek & Gold (2003), Jia et al. (2016), Al-Youssef et al. (2016)
In vivo SELEX	Use of live animals and injection of oligonucleotides library into the tail vein; Aptamer generation against unpurified proteins of a specific organ or tissue	Mi et al. (2010), Cheng et al. (2013), Mi et al. (2016)
Tissue slide-based SELEX	Use of tissue slices on glass slides; Aptamer generation against unpurified proteins of a specific tissue	Li et al. (2009), Zhang et al. (2015), Wang et al. (2016)
Aptamer-facilitated Protein Isolation from Cells (AptaPIC)	Use of gel capillary electrophoresis for separation; Aptamer generation against unpurified soluble proteins	Javaherian et al. (2009), Kanoatov et al. (2010)
Subtractive-EMSA SELEX	Use of gel mobility shift assay (EMSA) for separation; Aptamer generation against unpurified soluble proteins	Li et al. (2015)
Aptamer selection using protein-transfer membrane after electrophoresis	Use of proteins transferred to a membrane after polyacrylamide electrophoresis; Aptamer generation against unpurified soluble proteins	Bianchini et al. (2001), Noma et al. (2006), Van Simaeys et al. (2014)
Two-dimensional electrophoresis-based SELEX (2DE-SELEX)	Use of proteins transferred to a membrane after two-dimensional electrophoresis; Aptamer generation against unpurified soluble proteins	Savory et al. (2013)

the selected sequences were predicted and their melting temperatures were calculated using the m-fold software (Zuker, 2003). The binding affinity and specificity of the selected oligonucleotide sequences for the unidentified protein were assessed by incubating them with transfer membrane containing cell lysate proteins. Specificity and affinity were confirmed as the obtained DNA aptamer only bound to the PI 4.5/Mw 35 kDa spot.z.

2DE-SELEX may facilitate the development of aptamers against unidentified and uncharacterized proteins in unpurified biological samples for proteomic analysis and biomarker discovery. In addition, on-blot protein digestion and LC-MS (liquid chromatography–mass spectrometry) analysis have been used for the identification of proteins separated by 2DE (Jiang, Jia, Zhou, & Jarrett, 2009). A combination of 2DE-SELEX and mass spectroscopic identification of target proteins would ultimately provide a novel strategy for simultaneous development of multiple biomarkers and corresponding aptamers by directly targeting tissue samples. On the other hand, the practical limitation is the disadvantage of using a proteomics technique for aptamer selection since these processes are complex and time-consuming (Rabilloud & Lelong, 2011). Automation providing both greater speed and high reproducibility is required in order to develop and expand proteomics-based aptamer generation into practical applications. Recently, an automated trans-blotting system that can carry out all procedures from SDS–PAGE to blotting in 60 min (Goto, Savory, Abe, Kinoshita, & Ikebukuro, 2015) and an automated 2DE system that performs all steps from isoelectric focusing electrophoresis to SDS–PAGE in 100 min (Hiratsuka et al., 2007; Tani et al., 2014) were reported. These automated systems might be applied to 2DE-SELEX and grant it both accuracy and high reproducibility.

5 | CONCLUSIONS

We reviewed advanced SELEX techniques driven by the fact that conventional SELEX cannot directly generate aptamers against unpurified proteins. This disadvantage becomes especially important in the following frequent cases: (A) the amount of the target protein is very small; (B) the protein is so fragile that it is difficult to be purified from native samples; or (C) the protein is unidentified or uncharacterized. Therefore, this review article aimed to describe several methods that allow us to directly develop an aptamer against proteins from various unpurified samples such as whole cells, tissues, serum, and cell lysates (Table 1).

In summary, the main advantage of advanced SELEX is that (a) it does not require a purification process from a crude bio-sample such as tissue, whole cell, serum, or cell lysate; (b) it can be used for screening the functional states of the target protein, especially in the case of membrane proteins, which is a particularly important advantage; (c) it facilitates obtaining aptamers against unidentified and uncharacterized proteins in unpurified biological samples; and (d) it can be used to efficiently select an aptamer with a low SLELX cycle by subtractive selection or competitive selection. Nevertheless, there is still plenty of room for improvement of these advanced SELEX techniques. First, Cell-SELEX, in vivo SELEX, and slide-based SELEX cannot target certain proteins. Second, the abilities of electrophoresis-based SELEX for the selection of an aptamer against certain characteristic proteins such as lowly expressed proteins, negatively charged proteins, and high-molecular-weight proteins are limited. Finally, denaturation of target proteins on the protein-transferred membrane after electrophoresis during SDS–PAGE and electroblotting would prohibit effective aptamer selection.

The selected aptamers obtained by unpurified protein SELEX can be used for various applications, including as a biological research tool such as for protein purification, imaging probes, and biomarker identification, as well as for therapeutic/diagnostic drug development such as activation/inhibition of cellular signaling pathways and molecular targeted delivery.

Recombinant protein technology is currently widely used for aptamer generation despite its drawbacks. In this review, we focused on several methods that can be used to generate aptamers directly from crude proteins, without the need for recombinant proteins. In particular, we consider that Cell-SELEX and similar methods will play an increasingly important role in the generation of aptamers against membrane proteins located on the cell surface. Electrophoresis-based SELEX and proteomics-based SELEX have vast potential for improvement, and it is expected that certain advanced technologies such as click chemistry and automation will help to promote their practical application.

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