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ORIGINAL PAPER

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FluMag-SELEX as an advantageous method for DNA aptamer selection

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Abstract Aptamers are ssDNA or RNA oligonucleotides with very high affinity for their target. They bind to the target with high selectivity and specificity because of their specific three-dimensional shape. They are developed by the so-called **S**ystematic **E**volution of **L**igands by **Ex**ponential Enrichment (SELEX) process. We have modified this method in two steps—use of fluorescent labels for DNA quantification and use of magnetic beads for target immobilization. Thus, radioactive labelling is avoided. Immobilization on magnetic beads enables easy handling, use of very small amounts of target for the aptamer selection, rapid and efficient separation of bound and unbound molecules, and stringent washing steps. We have called this modified SELEX technology FluMag-SELEX. With FluMag-SELEX we have provided a methodological background for our objective of being able to select DNA aptamers for targets with very different properties and size. These aptamers will be applied as new biosensor receptors. In this work selection of streptavidin-specific aptamers by FluMag-SELEX is described. The streptavidin-specific aptamers will be used to check the surface occupancy of streptavidin-coated magnetic beads with biotinylated molecules after immobilization procedures.

Keywords Aptamer · SELEX · Streptavidin · Magnetic beads

Introduction

Since the first appearance of aptamers in the literature in 1990 [1, 2] they have been selected for a multitude of different substances, thus the fields of application of aptamers are very wide-ranging [3]. Aptamers have great potential to function as molecular recognition elements in analytical systems for detection, separation, or purification of target molecules. Use of aptamers in capillary electrophoresis, affinity chromatography, flow cytometry, image analysis, laser-scanning microscopy, and biosensors has been described [4–13]. Aptamers can substitute antibodies or be used in conjunction with antibodies in these assays and play a increasingly important role in medical diagnosis and therapy [14], in cytomic research [13], and also in the wide area of environmental analysis.

Aptamers have several advantages that offer the possibility of overcoming limitations of antibodies, for example an in-vitro selection process with no need for animals or cell cultures. Therefore, aptamers can be selected for toxic or non-immunogenic targets. When selected, aptamers can be produced by chemical synthesis at any time, in large amounts, and reproducibly. Aptamers also bind their target with high affinity and specificity, comparable with that of monoclonal antibodies. Most important, aptamers can be chemically modified depending on their application, e.g. to enhance their stability in biological fluids by use of modified nucleotides or to introduce reporter molecules and functional groups for their detection and immobilization. Because of their low molecular weight, aptamers have become a useful tool for in-vitro and in-vivo imaging analysis [13].

Aptamers are generated by an in-vitro selection and amplification technology called SELEX [1, 2]. The starting point of the SELEX process is a combinatorial library of synthetic oligonucleotides consisting of a multitude of ssDNA fragments (approximately 10^{15}) with different sequences. Aptamer selection is characterized by the repetition of successive steps of target binding and removal of unbound oligonucleotides, followed by elution, amplification, and purification of the selected oligonucleotides [1, 2]. Radioactive labels are usually used to quantify the bound and unbound fraction of oligonucleotides during the SELEX process. This

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is a rather sensitive method, which enables detection of low concentrations of DNA. The disadvantage of this method is that the whole SELEX has to be managed within an isotope laboratory. Therefore, the method is expensive and not environmentally compatible. To avoid this additional expenditure and enable work in an ordinary biomolecular laboratory fluorescence labels, for example, can also be used. The separation of target bound and unbound oligonucleotides during the SELEX process is the crucial step for successful aptamer selection. A conventional separation method is affinity chromatography wherein the targets are usually immobilized on column materials, e.g. sepharose or agarose [5]. Substantial amounts of target molecules are therefore necessary to achieve sufficiently high loading of the column. Another kind of separation method is filtration using, for example, nitrocellulose filters [5]. In contrast, using magnetic beads for target immobilization requires only very small amounts of target and enables comfortable handling. The magnetic separation technology is a much faster, easier, and more effective method. Our objective is to develop ssDNA aptamers, especially for application as molecular recognition elements in biosensors for environmental analysis and other applications. The first step was to establish a modified, easy to handle, selection procedure, which we called FluMag-SELEX. This SELEX process combines the advantages of fluorescence labelling of the DNA with magnetic separation technology.

As an example of this SELEX process, enrichment of DNA aptamers for streptavidin as target, immobilized on magnetic beads, is described. These aptamers will be used in further aptamer developments as a tool to characterize the occupancy of streptavidin-modified surfaces with biotinylated target molecules.

Experimental

Materials and instruments

All chemicals for preparing buffers and solutions were purchased from VWR International, Germany. PCR components, for example buffer, $MgCl_2$, and Taq DNA polymerase were purchased from Promega, Germany, and stock solutions of dNTPs were from Hybaid, Germany. Biotin was purchased from Sigma, Germany.

The fluorescence measurements of the fluorescein-labelled ssDNA were performed in 96-well black microtiter plates (NUNC) using the Wallac Multilabel Counter 1420 Victor^2 V (Perkin–Elmer, Germany) under the conditions: prompt fluorimetry, excitation filter 485 nm, emission filter 535 nm, time 1 s, CW-lamp energy 15,000. The sample volume was 100 µL/well. The calibration curve was prepared with fluorescein-labelled ssDNA of the random oligonucleotide library "BANK" and ranged from 10 ng mL^{-1} to 4 µg mL^{-1}.

Random oligonucleotide library and primers

The random oligonucleotide library, also referred to as "BANK", was synthesized on the 1 µmol scale and purified by PAGE (Invitrogen, UK). This library consisted of a multitude of ssDNA fragments ($\sim 10^{15}$ molecules) comprising a central random region of 60 nt flanked by different specific sequences of 18 nt which function as primer binding sites in the PCR: 5′-ATACC AGCTTATTCAATT–N_{60}–AGATAGTAAGTGCAAT CT-3′ [15]. The following primers that anneal to the 5′-end and 3′-end of the library fragments were used in the aptamer selection process for amplification and cloning of the oligonucleotides: primer "AP10" 5′-ATACCA GCTTATTCAATT-3′ and primer "AP20" 5′-AGATT GCACTTACTATCT-3′ (Invitrogen). Two special modified PCR primers "AP60" (equals "AP10" with 5′-fluorescein; Invitrogen) and "TER-AP20" (equals "AP20" with 5′-poly-dA_{20}-HEGL; IBA, Germany) were used for fluorescent labelling and quantification of oligonucleotides during the SELEX process and for separation of ssDNA from the double-stranded PCR products [16].

Target protein for the aptamer selection

Magnetic beads are a very useful matrix for immobilization of targets during aptamer selection and enable easy and efficient processing. Streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin from Dynal Biotech, Norway) were used as a defined model target, after testing non-magnetic micromer-Streptavidin beads (micromod Partikeltechnologie, Germany) for the first three rounds. The magnetic beads have the advantage of well-known surface properties, very appropriate for the establishment of the FluMag-SELEX. The streptavidin-coated target beads were suspended in PBS pH 7.4 containing 0.1% BSA and 0.02% NaN_3 and washed in appropriate buffer before use. The washing procedure was facilitated by using a magnetic separation stand.

In-vitro selection of DNA aptamers (FluMag-SELEX)

A fresh aliquot of 1×10^8 streptavidin-coated magnetic beads was washed eight times with binding buffer (100 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris-HCl pH 7.6, 2 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} KCl, 1 mmol L^{-1} $CaCl_2$, 0.02% Tween 20) before each selection round. The bead aliquots contained 1×10^7 beads in rounds 1–5. Each ssDNA pool was heated to 90°C for 10 min, immediately cooled, and kept at 4°C for 15 min, followed by a short incubation (5–8 min) at room temperature before its application in the binding reaction.

In the initial selection round, the washed streptavidin-coated beads were re-suspended in 500 µL binding

buffer containing 2–3 nmol of the random oligonucleotide library "BANK". After an incubation of this mixture at 21°C for 30 min with mild shaking, the unbound oligonucleotides were removed by five washing steps with 500 µL binding buffer. Subsequently, the bound oligonucleotides were eluted by incubating the binding complex twice with 200 µL elution buffer (40 mmol L^{-1} Tris-HCl pH 8, 10 mmol L^{-1} EDTA, 3.5 mol L^{-1} urea, 0.02% Tween 20) at 80°C for 7 min with mild shaking. The pooled oligonucleotides were precipitated with ethanol in presence of 6 µL 0.25% linear polyacrylamide. The ssDNA was then re-suspended in a smaller volume of binding buffer. The entire selected ssDNA was amplified in 15 parallel PCR reactions. Each contained 50 mmol L^{-1} KCl, 100 mmol L^{-1} Tris-HCl pH 9, 1% Triton X-100, 1.9 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs each, 1 µmol L^{-1} of each primer (AP60 and TER-AP20), and 5 U Taq DNA Polymerase in a volume of 100 µL. The amplification conditions were 5 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 47°C, 1 min at 72°C, then 10 min at 72°C after the last cycle. In this way it was possible to produce dsDNA fragments with a fluorescein modification at the 5′-end of the relevant strand and a poly-dA_{20} extension at the 5′-end of the complementary strand. Electrophoresis on 2.5% agarose gel was used to monitor successful amplification and the correct size of the DNA fragments.

It was necessary to separate the relevant DNA strands from the double-stranded PCR products after the amplification step. For this purpose, all PCR products were pooled, precipitated with ethanol, and re-suspended in 100 µL TE pH 7.4. Both different sized DNA strands of the PCR products were separated by a preparative denaturing PAGE in a 10% PAA gel containing 7 mol L^{-1} urea and 20% formamide in TBE. The fluorescein-labelled, relevant DNA strands could be identified in the gel by using a UV transilluminator. The corresponding DNA bands were cut out and the DNA was eluted from the gel with 2 mmol L^{-1} Na-EDTA, 300 mmol L^{-1} sodium acetate, pH 7.8, at 80°C for 45 min, with mild shaking. After precipitation of the DNA with ethanol and re-suspension in binding buffer, a new selected and fluorescein-labelled ssDNA pool was ready for the next selection round of the SELEX process.

After the first selection round 200 pmol, or rather the entire ssDNA from the previous round was used in the next round starting with the binding reaction to the target beads. The fluorescein labelling of the selected DNA enabled its quantification by fluorescence measurement during the further SELEX process. The fluorescence measurement was performed directly without addition of solutions or chemicals, which is beneficial for further utilization of the sample after measurement. The DNA in the different SELEX fractions (binding solution, solution of unbound DNA, washing solutions, and solution of DNA eluted from target beads after ethanol precipitation) of each round was measured and quantified by use of a calibration plot as described above.

Altogether 13 selection rounds were performed to enrich target-specific oligonucleotides (aptamers). To enhance the specificity of the selected oligonucleotides, counter selections with tosylactivated magnetic beads (Dynabeads M-280 Tosylactivated, Dynal Biotech, Norway) used as matrix for the immobilization of streptavidin were performed in rounds 11, 12, and 13. In these three rounds the DNA binding solution was first incubated with tosylactivated magnetic beads at 21°C for 30 min, with mild shaking, applied to a magnetic separation stand, and subsequently transferred to streptavidin-coated magnetic beads for target binding.

Cloning, sequencing and structure analysis of selected aptamers

The selected oligonucleotides (aptamers) from SELEX round 13 were amplified with the unmodified primers AP10 and AP20 and cloned into pCR2.1-TOPO vector. *Escherichia coli* TOP10 (TOPO TA Cloning Kit; Invitrogen) were transformed by up-taking this vector construct. The pDNAs of several clones were prepared by means of the NucleoSpin Plasmid Kit (Macherey–Nagel, Germany) and the inserted aptamer DNA of each clone was sequenced (Agowa, Germany). The sequences were analysed and aligned by using Vector NTI Suite 8 (Invitrogen). The secondary structure analysis of several aptamers was performed by means of the free-energy minimization algorithm according to Zuker [17] using the internet tool *mfold* [18].

Binding assays

The selected individual aptamer clones were characterized by several binding assays choosing SELEX conditions. The fluorescein-labelled aptamer was heated to 90°C for 10 min and rapidly cooled to 4°C, then incubated briefly at room temperature. Approximately 8–12 pmol of this aptamer was incubated with washed 1×10^7 streptavidin-coated magnetic beads at 21°C for 30 min in a total volume of 200 µL of binding buffer. After the binding reaction, the unbound aptamers were removed by several washing steps with binding buffer. Elution of bead-bound aptamers was then performed by incubation of the aptamer–target complex twice with 100 µL binding buffer at 80°C for 7 min, with shaking. The amount of aptamer DNA eluted from the beads was determined by fluorescence measurement and calculation using a calibration plot.

Determination of the dissociation constant K_D

The affinity of the selected aptamers for their target was determined by performing binding assays as described above but with increasing amounts of a single aptamer clone and a constant amount of streptavidin-coated

magnetic beads for each assay. The solution in each binding reaction contained 1×10^7 target beads and an increasing concentration of fluorescein-labelled aptamer DNA in the range of 0.25–190 nmol L^{-1}. The amount of bead-bound aptamers was calculated after elution from the beads, followed by fluorescence measurement. On the basis of these data a saturation curve was obtained and the dissociation constant K_D was calculated by non-linear regression analysis.

Effect of biotin on the binding of the aptamer to its target

Biotin is the strongest natural binding partner of streptavidin. To investigate its effect on the binding of the aptamers to streptavidin-coated magnetic beads, elution experiments were performed. For this purpose, binding reactions of fluorescein-labelled aptamer (8–12 pmol) and target beads (1×10^7) were carried out as described above on an enlarged scale dependent on the number of subsequent elution experiments. The washed target beads with the bound aptamers were aliquoted to 1×10^7 beads and re-suspended in 200 µL binding buffer, containing an increasing amount of biotin (0, 40, 120, 500 pmol biotin). After incubation at 21°C for 15 min while shaking, the tubes were set into a magnetic separation stand. The supernatant containing the eluted aptamers (after biotin treatment of the beads) was removed and the beads were subsequently washed three times with 200 µL binding buffer. Finally, twofold heat treatment of the beads at 80°C for 7 min in 100 µL binding buffer was performed to elute all the remaining aptamers from the beads. The amounts of DNA in the fraction of biotin elution, washing fraction, and fraction from heat elution were determined by fluorescence measurement and calculation using a calibration plot as described above.

Results and discussion

An in-vitro DNA aptamer selection procedure, FluMag-SELEX, based on magnetic beads as immobilization matrix for the target molecules and utilizing a fluorescence label for quantification of the DNA during the SELEX process was optimized (Fig. 1).

After the first round of the FluMag-SELEX the selected DNA was labelled with fluorescein by PCR using a 5′-modified primer. Thus, it was possible to quantify

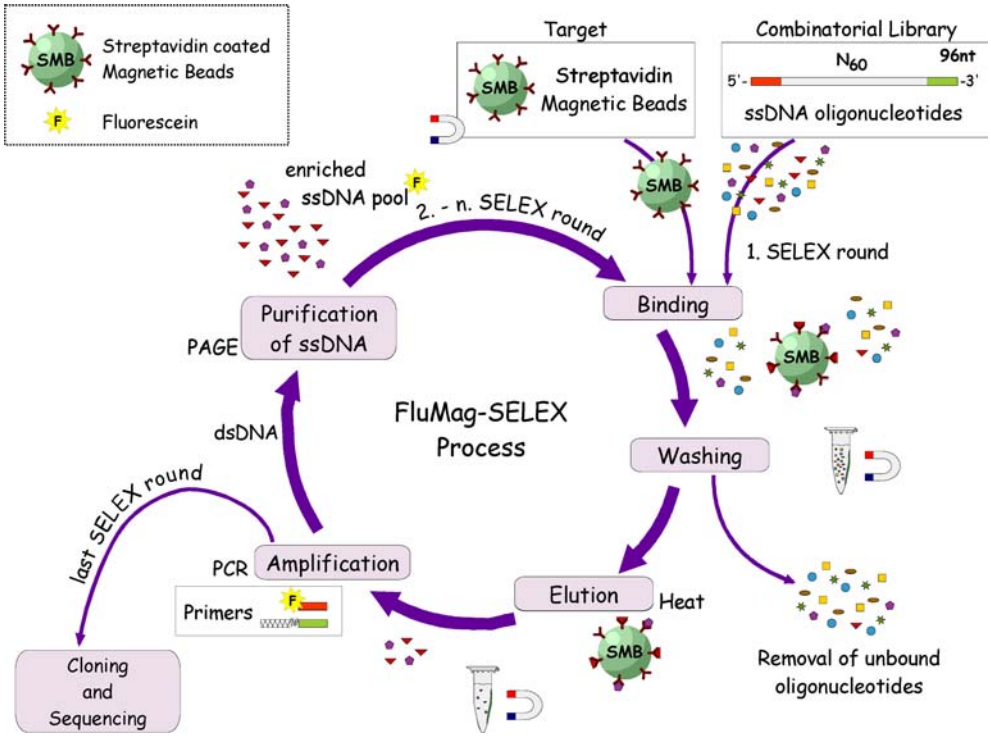

Fig. 1 Schematic representation of the fluorescence-monitored in-vitro selection procedure, the so-called FluMag-SELEX, for generating DNA aptamers for specific target molecules (here streptavidin) immobilized on magnetic beads. The starting ssDNA combinatorial library consisting of oligonucleotides with a centrally randomized region of 60 nucleotides flanked by two specific regions was incubated with the target beads (streptavidin-coated magnetic beads, *SMB*) for binding. Unbound oligonucleotides were washed away and the bound oligonucleotides were eluted from the target beads by heat treatment. The selected oligonucleotides were amplified by PCR using specially modified primers. The relevant ssDNA was subsequently purified from the PCR products and the resulting enriched DNA pool was used in the next SELEX round for binding with target beads. The fluorescein (*F*) labelling of the selected oligonucleotides after the first SELEX round enabled their quantification in further rounds and thus monitoring of the enrichment of target-specific aptamers. In the last round the selected aptamers were cloned and several monoclonal aptamers were characterized

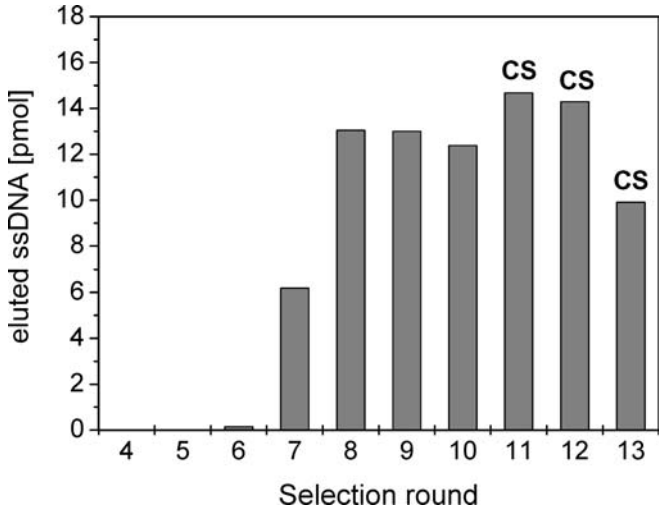

Fig. 2 Enrichment of streptavidin-specific aptamers during the FluMag-SELEX. The *bar graph* shows the amounts of ssDNA eluted from streptavidin-coated magnetic beads in each selection round. In rounds 11, 12, and 13 a counter selection step (*CS*) was introduced to remove ssDNA unspecifically bound to the bead matrix

the ssDNA in the further selection rounds in each SELEX fraction directly, without addition of chemicals or solutions. The ssDNA was, moreover, subsequently amplified and purified without further treatment. Fluorescein labelling was very convenient and its sensitivity was adequate for ssDNA quantification during the SELEX procedure. In this manner, radioactive labelling of the DNA could be avoided.

In the first three selection rounds of the described FluMag-SELEX process non-magnetic micromer-streptavidin beads were tested as target beads. By use of these beads, separation of bead-bound ssDNA (binding complex) from unbound ssDNA in the buffer solution was possible by centrifugation. Separation by centrifugation was, however, less efficient than magnetic separation by use of magnetic beads. Because this separation step is the crucial step of a SELEX process to obtain target-specific aptamers, streptavidin-coated magnetic beads were used as target beads in the further selection rounds to achieve highly efficient separation steps.

The enrichment of target-specific aptamers was monitored during the selection process and an increasing amount of ssDNA bound to streptavidin-coated magnetic beads was observed after rounds 6, 7, and 8 (Fig. 2). The amount of eluted ssDNA remained nearly constant in further rounds, possibly because of full occupation of binding sites on the beads. In the last three selection rounds a counter selection step was introduced against tosylactivated magnetic beads, which were used as a matrix for the immobilization of streptavidin. Enrichment of unspecifically bound ssDNA to the bead matrix was not detected. After round 13 the selection process was complete and the selected aptamer pool was cloned and characterized. Five groups of sequences were identified containing a different number of aptamer clones with mostly identical sequences within each group (Table 1).

One representative from each group was chosen at random, aptamer clones 2, 26, 30, 31 and 33, and used for further characterization. First they were tested in individual binding assays which enable rapid and comparative determination of the relative binding capabilities of different aptamers generated in a SELEX experiment. The conditions for the assays were applied according to the FluMag-SELEX conditions using streptavidin-coated magnetic beads as target and one of the selected and fluorescein-labelled aptamers. All of the tested aptamers had affinity for streptavidin immobilized on magnetic beads, as shown in Fig. 3. But the results also revealed clearly marked differences between their binding capability.

The best results were obtained with aptamers 26 and 31; binding capability was slightly lower for aptamers 30 and 33. In contrast aptamer 2 had very low binding capability to the streptavidin-coated magnetic beads. The unselected ssDNA pool of the library "BANK" was

Table 1 Five groups of streptavidin-specific aptamers selected by the FluMag-SELEX were identified. The sequences of the aptamer core regions (5′-3′) of one representative from each group are shown. Two conserved regions (*grey shaded*) were found, one in aptamer 26 and 31 and a different one in aptamer 30 and 33. Both regions are able to form a stem-loop structure in which the stem is *underlined*. The number of almost identical aptamer clones within one group and the dissociation constants are listed on the right

Group with a representative aptamer clone		Core region of the selected aptamers	No.	K_D [nM]
I	26	GATGCAGCCACCTACATTGCAGGTTTTATAATACAACTACGCGCCGTTCGCAGGCTAGTT	3	85.2 ±5.3
II	31	CTATACTCCACTTTGCTATTTCTCGGTTCCTTCACGCGCCGATCGCAGGCTGATGAATTG	1	56.7 ±8.2
III	30	CGGGGGGTACGTGCCCGTGTTGCTCGGCGGCCCCCTCGCTTATTTTTGTGTGCCCTTT	1	65.5 ±8.6
IV	33	ACAATGTCGCTCTCCGCCGCAGGAGCATTGTCTGTCTTTATGCTTCTCTTTTTTGTTGC	2	59.0 ±7.5
V	2	TCGCGTGATTTGGTCACAAATTGTATGATCCGGTGTCCTGCAAGTGTTGGTGTTCTCTCA	3	n.d.

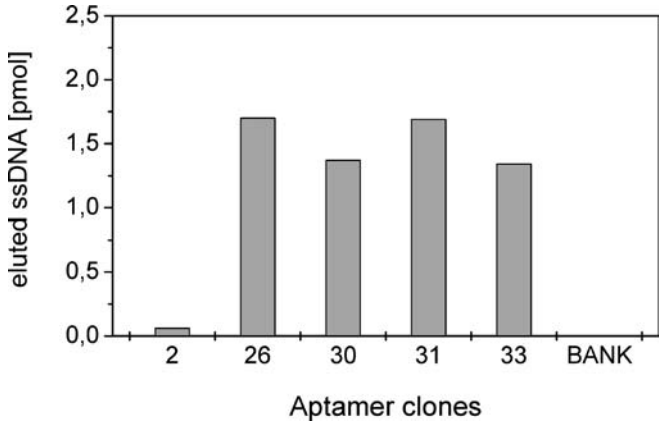

Fig. 3 Binding assays with the individual aptamer clones 2, 26, 30, 31 and 33, to test their ability to bind to streptavidin-coated magnetic beads. In each assay 1×10^7 streptavidin-coated magnetic beads were incubated with 8–12 pmol fluorescence-labelled aptamer in a volume of 200 µL according to the SELEX conditions. The unselected, fluorescence labelled ssDNA library ("BANK") was used as the negative control. After several washing steps the bead-bound aptamers were eluted by heat treatment and quantified by fluorescence analysis. The results are shown as a *bar graph*

used as the negative control in these assays. As expected, no binding of "BANK" to the target beads was found.

The sequence alignments of the core regions of the selected aptamers and their secondary structure prediction confirmed the correspondences and differences among the five aptamers derived from the binding assays. Aptamers 26 and 31 contained a consensus sequence of 17 nucleotides near the 3′-end of the core region (Table 1). This conserved motif formed a stem-loop structure in which the stem consisted of three guanosine–cytidine base pairs (Figs. 4a, b). The sequences of aptamers 30 and 33 were completely different from those of aptamers 26 and 31. There was a thymidine-rich sequence at the 3′-end of the core region with short homologous sections (Table 1). The secondary structure analysis also predicted the formation of a stem-loop in this motif. The stem consisted of two guanosine–cytidine base pairs and the loop was characterized by five or six successive thymidines (Figs. 4c, d). It is conceivable that both stem-loop structures play an important role in the target recognition and binding of the four aptamers to streptavidin-coated magnetic beads. The stems could have a stabilizing function whereas the bases in the loops could be responsible for binding. In contrast, none of the identified sequence motifs was found in aptamer 2.

The successful application of the optimized FluMag-SELEX process for generation of target specific ssDNA aptamers was thus shown. This fluorescence-monitored selection procedure is universal and applicable to a wide variety of target molecules. Moreover, it is characterized by use of magnetic beads, which provide a homogenous surface with different functional groups for the immobilization of the target molecules. The combination of target-coated magnetic beads and fluorescence-labelled aptamers can also be used in different binding assays to characterize the selected aptamers.

To determine the affinities of individual aptamers 26, 30, 31, and 33 for their target, the dissociation constants

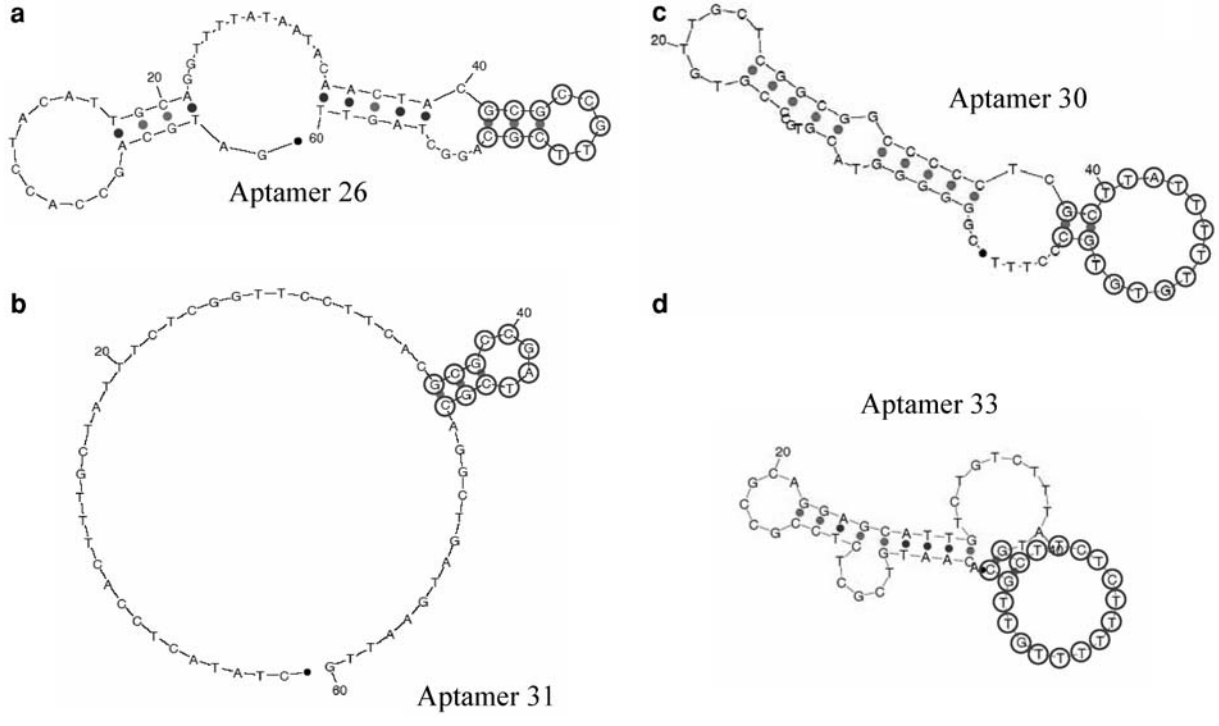

Fig. 4 Secondary structure prediction for the core regions of the aptamers 26 (**a**), 31 (**b**), 30 (**c**), and 33 (**d**). The stem-loop structures in the conserved regions of these aptamers are marked by *circles*

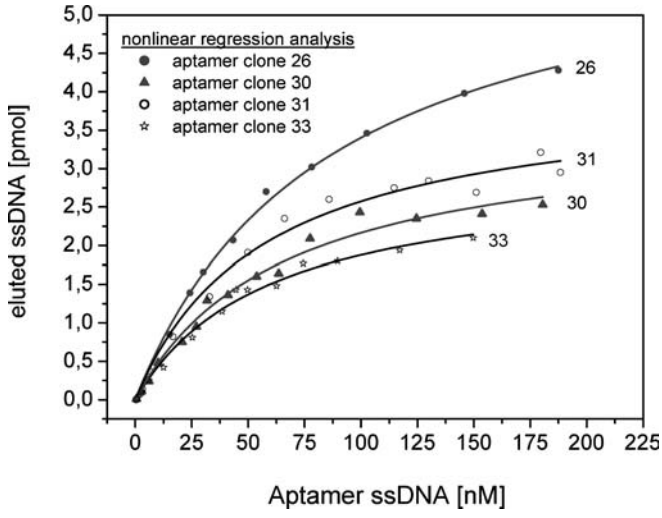

Fig. 5 Determination of the dissociation constants (K_D) for aptamers 26, 30, 31, and 33. Binding assays were performed using a constant number of streptavidin-coated magnetic beads (1×10^7) in combination with a concentration series (0.25–190 nmol L^{-1}) of the fluorescence labelled aptamers in a volume of 200 µL. After the binding reaction the beads were washed several times and the bead-bound aptamers were eluted by heat treatment. On the basis of the amount of aptamers eluted, saturation curves were obtained and the dissociation constants (K_D) were calculated by non-linear regression analysis

(K_D values) were ascertained. Binding reactions with a constant number of streptavidin-coated magnetic beads (1×10^7) as target beads and a concentration series of each fluorescein-labelled aptamer (0–188 nmol L^{-1}) were conducted. After several washing steps the bead-bound aptamers were eluted and the amount was calculated by fluorescence measurement and plotted against the concentration of the aptamers used in the binding reaction. Figure 5 shows the non-linear regression analysis of the binding data of the selected aptamers.

The dissociation constants of the four aptamers were in the low nanomolar range. The lowest K_D of 56.7 ± 8.2 nmol L^{-1} was obtained for aptamer 31. The K_D values of the other three aptamers were slightly higher: 59.0 ± 7.5 nmol L^{-1} for aptamer 33, 65.5 ± 8.6 nmol L^{-1} for aptamer 30, and 85.2 ± 5.3 nmol L^{-1} for aptamer 26. The dissociation constants obtained in this work are comparable with those previously published for streptavidin-specific aptamers, which range from 7 to 153 nmol L^{-1} [19–21]. These aptamers are mainly RNA aptamers, which were used as affinity tags embedded within RNA sequences for study of special RNAs and riboproteins or incorporated into bi-specific capture ligands [19, 20]. In this work, the DNA aptamers selected as a result of the establishment of the FluMag-SELEX are a new variant of streptavidin-specific aptamer. Comparison of the sequences and structural features showed no significant similarities with known aptamers. This is not unusual, because streptavidin is a complex molecule and different aptamers could recognize different parts of this protein. Furthermore the chosen SELEX conditions have a strong influence on the resulting aptamers, like the design of the random oligonucleotide library or the buffer composition. Moreover, different results are expected among the selection of RNA or DNA aptamers for a special target.

Further experiments were performed with aptamers 26, 30, 31, and 33 to investigate the effect of biotin on the interaction of these aptamers with streptavidin, because biotin is the natural binding partner of streptavidin with extraordinary high affinity ($\sim10^{-15}$ mol L^{-1}). These experiments were performed in two steps. The first was the binding of one of the individual, fluorescein-labelled aptamers to streptavidin-coated magnetic beads. In the second step, after washing, the aptamer-saturated beads were incubated with different amounts of biotin. The beads were then washed in binding buffer and heat treatment was used to remove all remaining aptamers from the beads. The results are summarized in Fig. 6.

All four aptamer clones bound to streptavidin-coated magnetic beads were released by biotin in a concentration dependent manner. The strongest effect was found for aptamers 30 and 33. For both aptamers 85–90% was rapidly removed from the target beads even by the presence of 40 pmol biotin (200 nmol L^{-1}). They were eluted completely by 120–500 pmol biotin (600–2500 nmol L^{-1}), a concentration, which is equimolar with or higher than that of biotin-binding sites of the streptavidin-coated magnetic beads (1×10^7) used in the assay. In contrast, biotin had less effect on binding of aptamers 26 and 31 to the target beads. Both of these aptamers were released much more slowly. Complete elution in the presence of the highest amount of biotin (500 pmol) in the assays was not observed, especially for aptamer 31. Other experiments showed that binding of the aptamers to streptavidin-coated magnetic beads was blocked if the beads were pre-incubated with sufficient biotin to saturate all biotin-binding sites (data not shown). The separation or blocking effect of biotin on the aptamer bond to the target beads might be because of their competition to bind to streptavidin. It is possible that the aptamer-binding site is near to or at the biotin-binding site of streptavidin. On the other hand, the aptamers described in this work were selected for unmodified streptavidin-coated magnetic beads. The binding of biotin could cause a conformational change of streptavidin so that the aptamers are no longer able to recognize and bind to it.

One possible application of streptavidin-specific aptamers is use as an affinity tag or as a linker element to immobilize a target molecule of interest on a surface or connect it to other molecules [19, 20]. Furthermore, the aptamers described in this work could be a useful tool to check the surface occupancy of streptavidin-coated magnetic beads with biotinylated molecules after immobilization procedures. The binding of the selected aptamers to the streptavidin-coated magnetic beads was

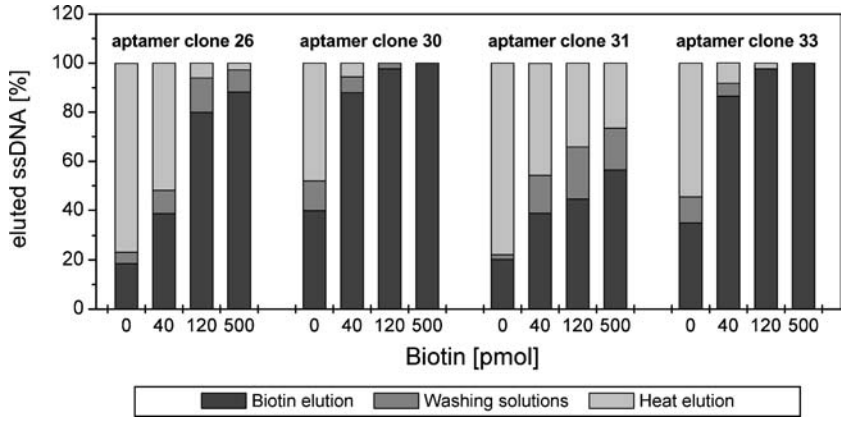

Fig. 6 Effect of biotin on the interaction of aptamers 26, 30, 31, and 33 with streptavidin. The aptamers were bound to streptavidin magnetic beads, washed, and 1×10^7 of these aptamer-saturated beads were then incubated with different amounts of biotin (0, 40, 120, or 500 pmol) in a volume of 200 µL binding buffer for 15 min. After three washing steps with binding buffer all remaining aptamers were eluted from the beads by heat treatment. The amount of aptamers eluted by biotin, washing steps, and heat are shown as percentages of the total amount of aptamers formerly bound to the beads in a *stacked bar graph*

affected by biotin or biotinylated molecules. Therefore, saturation of the bead surfaces with biotinylated molecules completely blocked the binding of the aptamers to these beads. But if the bead surfaces were occupied only partly with biotinylated molecules, binding of aptamers to the streptavidin-coated magnetic beads was possible. The detection an aptamer binding to streptavidin-coated magnetic beads after immobilization of biotinylated molecules was indicative of incomplete surface coverage with biotinylated molecules. This is shown in Fig. 7. A primary amount (p.a.) and a tenfold higher amount (10×p.a.) of two biotinylated peptides were immobilized on streptavidin-coated magnetic beads and these modified beads (1×10^7) were used in binding assays with aptamer 31 to check the extent of surface coverage. After the first immobilization of biotinylated peptides on streptavidin-coated magnetic beads the aptamers were able to bind to these peptide-modified beads. This led to the conclusion that the bead surfaces were only partially occupied with biotinylated peptides, because the amount of peptides used for their immobilization was too low. But the use of a tenfold larger amount of peptides for the second immobilization resulted in saturation of the bead surfaces with biotinylated peptides and binding of aptamers to these beads was no longer observed. In this manner, the aptamers served to check the surface coverage after immobilization. This is of interest for experiments in which it is not possible to determine the immobilization efficiency of biotinylated molecules on streptavidin-coated surfaces by other means. This test method is applied in further aptamer selection experiments by use of streptavidin-coated magnetic beads for target immobilization. The presence of unmodified streptavidin, because of partial occupancy of the bead surfaces with biotinylated target molecules, leads to rapid enrichment of target-unspecific oligonucleotides. Thus, saturation of the bead surfaces with target molecules, in addition to the customarily used counter-selection steps, should prevent this problem. The aptamer binding test provides a simple method for checking the surface occupancy of streptavidin-coated magnetic beads after immobilization of biotinylated molecules.

Conclusions

FluMag-SELEX has been established as an optimized in-vitro selection procedure for generating ssDNA aptamers. This SELEX process is characterized by use of fluorescein-labelled ssDNA to monitor the enrichment

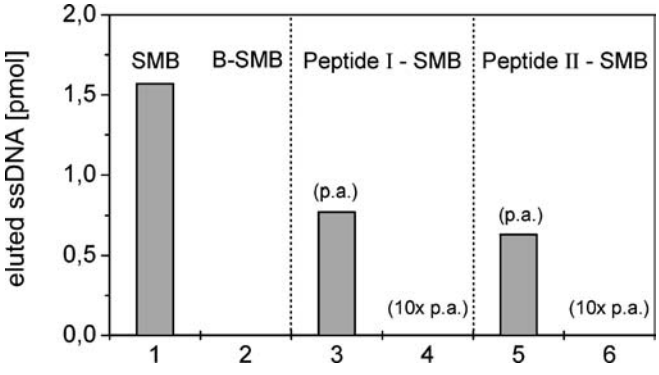

Fig. 7 Binding assays with aptamer clone 31 to check the surface occupancy of streptavidin-coated magnetic beads with biotin or biotinylated molecules. Beads: (*1*) *SMB* streptavidin-coated magnetic beads, (*2*) *B-SMB* biotin-saturated SMB, (*3*, *4*) *Peptide I—SMB* SMB modified with biotinylated peptide I, using different amounts of peptide I [primary amount (*p.a.*) and tenfold primary amount (10×p.a.)] for immobilization, (*5*, *6*) *Peptide II—SMB* SMB modified with biotinylated peptide II, using different amounts of peptide II [primary amount (*p.a.*) and tenfold primary amount (10×p.a.)] for immobilization. In each binding assay 1×10^7 of these different modified beads were incubated with 10–11 pmol fluorescein-labelled aptamer 31 in a volume of 200 µL according to the SELEX conditions. After several washing steps, the bead-bound aptamers were eluted by heat treatment and quantified by fluorescence analysis. The results are shown as a *bar graph*

of target-specific aptamers during selection. Magnetic beads were used as matrix for the immobilization of the target molecules. Magnetic separation technology enables easy handling and efficient separation of selected and unselected ssDNA. It offers a potential for parallel but manual processing of multiple targets without the need of expensive robotics. FluMag-SELEX is a universal selection procedure and adaptable to different kinds of target. Successful selection of streptavidin-specific aptamers using this FluMag-SELEX was shown. The dissociation constants of the selected aptamers were in the low nanomolar range. In the presence of biotin, aptamers bound to streptavidin were either released or binding was blocked.

The aptamers developed for unmodified streptavidin serve as a tool for checking the surface occupancy of streptavidin-coated magnetic beads with biotinylated molecules. If the bead surface is only partly occupied with biotinylated molecules, fluorescein-labelled streptavidin aptamers are also able to bind to these beads. The test has been applied in further aptamer selections for other targets immobilized on streptavidin-coated magnetic beads.

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