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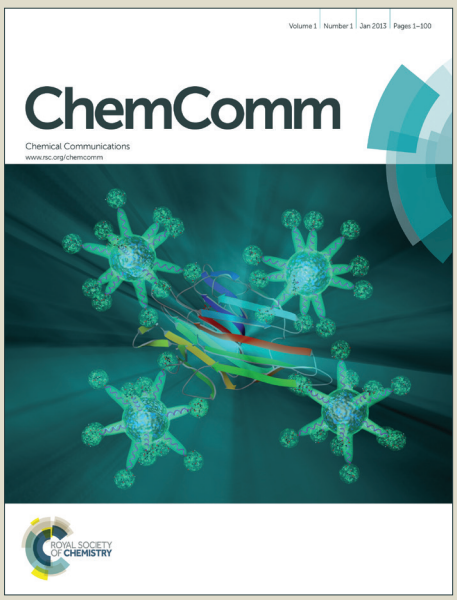

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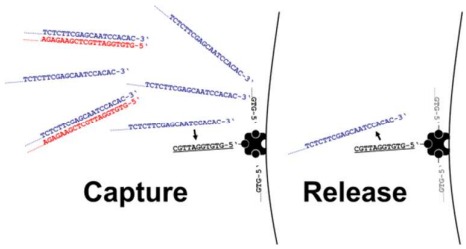

Streptavidin-coated magnetic beads loaded with biotinylated oligodeoxynucleotides allow for convenient purification of ssDNA from asymmetric PCR mixtures.

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Capture and Release (CaR): A simplified procedure for one-tube isolation and concentration of single-stranded DNA during SELEX.

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Short biotinylated oligodeoxynucleotides immobilized on streptavidin-coated magnetic beads allow for convenient and rapid purification of single-stranded oligodeoxynucleotides from crude asymmetric PCR mixtures, facilitating the selection of DNA aptamers.

The preparation of single-stranded DNA (ssDNA) after PCR-based amplification is a crucial step during the selection of DNA-aptamers, a process also termed as systematic evolution of ligands by exponential enrichment (SELEX).[1] Currently applied strategies range from asymmetric PCR and enzymatic strand digestion to the most commonly used alkaline-based denaturation of biotinylated PCR-products after immobilization onto streptavidin-coated magnetic beads (SMB).[2]

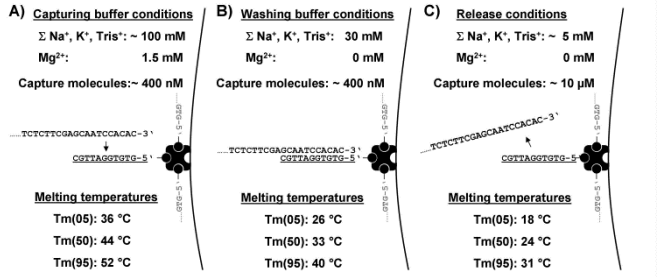
The possibility to directly purify PCR-products even from crude reaction-mixtures might be the main reason for the common utilization of the SMB-based method. Separation of strands is usually done by alkaline-treatment rather than heat-denaturation, because of the massive release of streptavidin into solution at elevated temperatures.[2a, 2c] However, in this case final neutralization or solvent exchange of the product is required to proceed with the selection cycle. Furthermore, due to the strong association between biotin and streptavidin, SMBs are usually used once, significantly increasing costs.

In principle, asymmetric PCR represents the method of choice for direct generation of ssDNA,[3] followed by gel-electrophoresis for the selective extraction and purification of ssDNA from dsDNA and reaction components.[2a] Not least due to the short length of random libraries used for SELEX (< 100 nts), however, loss of ssDNA during the purification processes is high. Moreover, gel components and conceivably applied nucleic-acid dyes are additional sources of potential contamination.[4]

In order to combine the advantages of asymmetric PCR with SMB-based concentration and purification, we established a novel approach, named capture and release (CaR) that facilitates purification of ssDNA from crude PCR mixtures. During CaR, short biotinylated oligodeoxynucleotides (capture molecules), complementary to a defined site at the 3'-end of the target-ssDNA are employed. After their immobilization onto SMBs they are added to (pooled) crude asymmetric PCR mixtures. During this step the capture molecules bind to the target-ssDNA and subsequently the beads are washed and finally taken up in a small volume of purified water (Aqua ad iniectabilia). Due to the strongly decreased concentration of monovalent and magnesium ions, the melting temperature (Tm) of the immobilized capture-molecules bound to the targets decreases,[5] allowing the release of pure ssDNA at only moderately increased temperatures, whereby the integrity of the SMBs is preserved (Scheme 1).

The CaR method was successfully applied during capillary-electrophoresis (CE)-based SELEX (CE-SELEX).[6] We employed two distinct ssDNA-libraries (IHT1 and IHT2N) and selected aptamers for two different protein targets, namely activated protein C (APC) and the activated A-subunit of factor XIII (FXIIIAa), two key enzymes of the coagulation cascade.[7] For the design of corresponding capture molecules, an online tool for the prediction of DNA thermodynamics

was used [5] (see ESI† for methodological details). As shown in Scheme 1 (IHT1) and Fig. S1 (IHT2N, ESI†), predicted melting temperatures between the library- and capture molecules mainly depend on the concentration of salt-ions but were also influenced by the concentration of capture molecules present during the different steps of CaR. Due to the inevitable presence of residual amounts of washing buffer associated with the SMB-pellet and the tube, a concentration of 5 mM of monovalent cations was assumed to be present during the final release step.



Scheme 1. Principle of CaR and predicted melting temperatures of the IHT1-capture molecules at different buffer conditions during the different steps. A, Capture; B, Wash; C. Release. Tm-values represent the melting temperature at which 5% [Tm(05)], 50% [Tm(50)], or 95% [Tm(95)] of captured ssDNA molecules are predicted to be released from the capture molecules.

We first assessed the basic functionality of CaR using asymmetrically amplified DNA-library IHT1 (ESI†). For capturing of ssDNA molecules, 5'-biotinylated IHT1 capture molecules were bound to SMB (200 pmole/ mg SMB; Fig. S2, ESI†). Subsequently, 500 µl of pooled crude asymmetric PCR mixtures (Figs. S3 and S4, ESI†) were added to 1 mg of SMB that were loaded with capture-molecules (SMB+). After incubation for 30 min at RT, SMB+ were washed and finally resuspended in 20 µl of purified water. The release of captured ssDNA was assessed at RT and three elevated temperatures (37°C, 43°C, and 50°C). After two minutes of incubation, SMB+ were separated by magnetic force and supernatants collected. Three consecutive elution steps using new batches of purified water at each temperature were conducted.

Comparable yields of ssDNA in the low pmole range (low µM concentrations) were released at elevated temperatures with about 90% of the ssDNA being released during the first elution step as determined by UV-measurements (Fig. 1A). Gel analysis and A260/A280 ratios revealed high purity of isolated single-strands (Fig. S3, ESI†).

In order to prove the specificity of CaR, non-loaded SMB or SMB loaded with non-complementary IHT2N capture molecules were introduced to the described assay with captured ssDNA released at 43°C (ESI†). As shown in Figs. 1B and S4 (ESI†), only the application of SMB+ enabled the isolation of IHT1-ssDNA from crude asymmetric reaction mixtures, demonstrating the sequence specificity of the assay.

To assess the potential rate of contamination of produced ssDNA with streptavidin, 1 mg of non-loaded SMB in 20 µl of purified water were incubated for 2 min at RT, 37°C, 43°C, 50°C, 70°C or 90°C. After the separation of beads, supernatants were tested for the presence of streptavidin-(subunits) (streptavidin) by ELISA (ESI†). Three consecutive experiments using the same SMBs were performed at each temperature. As shown in Fig. 1C, even at RT, a detectable amount of streptavidin (~6 fmole [300 pM concentration]) leaked from the SMB during the first elution step. Up to a temperature of 43°C, leakage only marginally increased to a total of ~7 fmole (~350 pM) while a further apparently exponential increase in leakage was observed at temperatures of 50°C or higher.

These data demonstrate that the amount of streptavidin contamination depends on the elution temperature used, the amount of SMB used, and the total yield of ssDNA. For instance, when considering an elution temperature of 43°C and a yield of 20 pmol of ssDNA (1 µM concentration), the degree of contamination on a molar basis would be around 0.04% (400 ppm) when using 1 mg of SMB.

It has been previously described that the interaction between biotin and streptavidin gets weakened in deionized aqueous solutions in a temperature-dependent manner.[8] Thus, besides adverse release of streptavidin from the SMB, also the leakage of biotinylated capture molecules from streptavidin into the used purified water was considered. To study this effect, SMB were loaded with 3'-fluorescence-labelled capture molecules (200 pmole/ mg) and treated as described for the streptavidin release experiments. The concentration of capture molecules in the supernatants was determined by fluorescence measurements (ESI†). Indeed, it was found that significant amounts of capture molecules leaked into solution. Up to an elution temperature of 43°C, however, leaked amounts were limited to a maximum of ~2 pmole (100 nM concentration) during the three consecutive experiments, corresponding to a maximum loss of ~1% of SMB-bound capture molecules per incubation (Fig. 1D). Nevertheless, at a yield of 20 pmole of ssDNA (1 µM concentration), the degree of contamination would be up to 10% under these conditions.

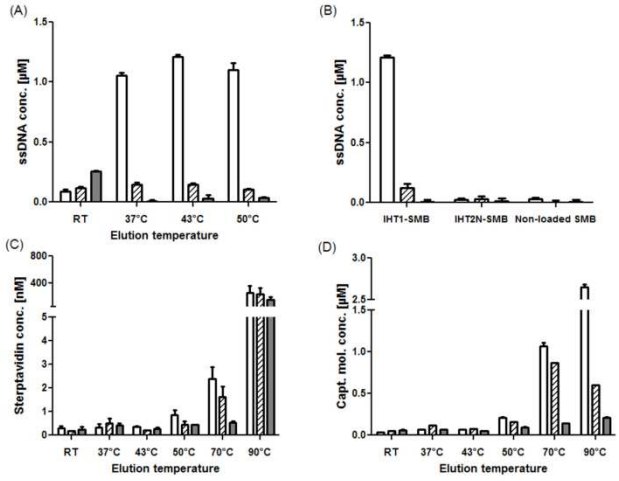


Fig. 1. Key-characteristics of the CaR procedure. Open bars show results of the first elution, striped bars: 2nd, gray bars: 3rd. A, Yield of ssDNA at different elution temperatures. B, Prove of sequence-specificity. C, Degree of streptavidin-contamination at different elution temperatures. D, Leakage of capture molecules from SMB.

In summary, these results demonstrate that ssDNA prepared by SMB-based CaR will be contaminated with streptavidin and biotinylated antisense molecules.

The contamination of ssDNA-preparations with SMB-derived streptavidin has also been described for the, in comparison to heat denaturation, more gentle alkaline (NaOH)-based denaturation of immobilized PCR-products.[2c, 2e, 9] For example, Civit et al. achieved ssDNA-concentrations of ~40 nM while the contaminating concentration of streptavidin, as also measured by ELISA, was reported to be ~ 150 pM.[2c] This corresponds to ~ 3,750 ppm, an approximately 10-fold higher rate than observed in the present study. However, due to the use of different SMB and/ or different (mainly qualitative) methods for the detection of streptavidin in different studies, a more comprehensive comparison of contamination rates appears to be difficult. The same is true for the following adverse effect.

Since alkaline conditions also negatively affect the interaction between biotin and streptavidin, the NaOH-based denaturation of SMB-bound PCR-products also leads to the contamination of ssDNA with released double-stranded PCR-products and/ or (re-annealing) complementary strands, [2a, 2e] while biotinylated capture molecules exclusively bind to their corresponding target-region at the 3'-end of the isolated aptamers. Thus, in comparison to NaOH-denaturation of full-length complementary strands, binding of released capture molecules will, if at all, lead to considerably lower interference with the tertiary structure of the selected aptamers.

In contrast to the previously described methods for ssDNA-generation, the characteristics of CaR determine that potential aptamers that find their 3'end involved in intra-molecular folding patterns may not be efficiently captured and therefore be sorted out during selection. Indeed, one might argue that, at least during the first cycle of CaR-based SELEX, aptamers are not solely selected for target binding but also with respect to proper annealing to the applied capture molecules. Due to the presence of high overall sequence diversity at coincidently high frequency of identical or similar sequence patterns during the first selection cycles, however, this appears to be more of a fact than a problem.

Using the IHT1 and IHT2N ssDNA libraries, asymmetric PCR/ CaR was applied during CE-SELEX to obtain DNA-aptamers recognizing activated protein C (APC) and the activated A-subunit of factor XIII (FXIIIAa).[7] Since APC was used successfully during previous selections,[10] this enzyme was mainly considered as a model target for evaluation purposes. In contrast, the selection of aptamers against FXIIIAa has not been described so far. In total, 6 selection cycles were performed during 3 independent selections (APC targeted by IHT1 and IHT2N, FXIIIAa targeted by IHT1, ESI†). The yield and purity of ssDNA obtained during the different cycles of IHT1- and IHT2N-based selections was found to be comparable (Tables S1-S3, ESI†). After selection, the starting- and enriched libraries were radioactively labelled in order to (i) sensitively assess the quality of prepared ssDNA by PAGE and (ii) to determine the binding affinity by filter retention analysis (ESI†).

As shown in Figs. 2A and 2B, distinct bands as determined by denaturing PAGE confirmed the high quality of CaR-prepared ssDNA. More importantly, filter retention analysis revealed successful enrichment of DNA aptamers by CaR-SELEX binding to APC or FXIIIAa. Highest apparent binding affinities of gathered pools were obtained after 4 to 6 cycles of selection (Figs. 2C and 2D).

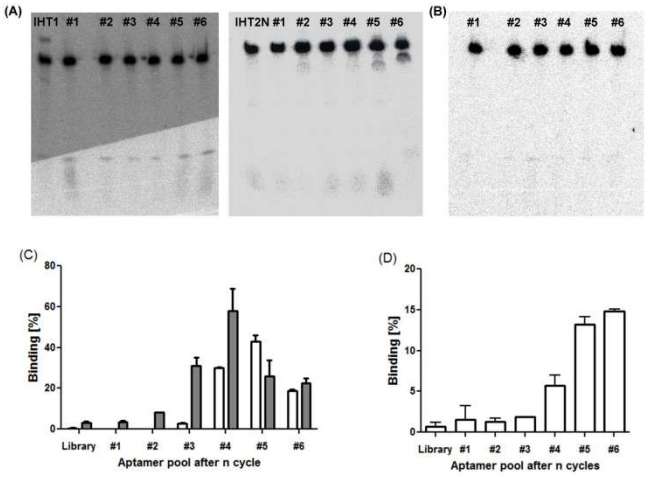


Fig. 2. Quality of CaR-produced ssDNA (aptamers) and target binding patterns. A, B, PAGE analysis of radioactively labelled original IHT1 and IHT2N libraries and aptamer pools obtained after 1 to 6 selection cycles (A, APC-SELEX; B, FXIIIAa-SELEX). The distinct background levels on the screen on the left were caused by an artefact during exposure. C, D, Filter retention analysis. C, Percentage of binding of IHT1- (open bars) and IHT2N- (closed bars) derived aptamer pools to APC (100 nM). D, Binding of IHT1-derived aptamer pools to FXIIIAa (100 nM).

Cloning and sequencing of the selected aptamers led to the identification of individual sequence clones from which ssDNA was produced by CaR (Fig. S5, ESI†) and tested by filter retention analysis. As shown in Fig. S6 (ESI†), determined binding affinities of individual sequences were within the low nanomolar range. As expected, in silico two-dimensional DNA folding analysis indicated that the 3'-ends of found aptamers are accessible to the used capture molecules (Fig. S7, ESI†).

Having shown the usefulness of CaR during aptamer selection and single clone ssDNA-production, we next focused on the reusability of the applied SMB+ for ssDNA-production. For this purpose, asymmetrically amplified ssDNA (clone #1 of the IHT1-based selection against APC, Fig. S5, ESI†) was pooled and aliquotes of 500 µl each introduced to repetitive CaR using the same batch of SMB+ (ESI†). As shown in Fig. S8 and Table S4 (ESI†), comparable yields of highly pure ssDNA were achieved during four consecutive experiments. In order to avoid contaminations, however, it must be mentioned that SMB+ should only be reused for the additional generation of ssDNA from the same species of asymetrically amplified source material.

In conclusion, we introduced a novel fast and convenient principle for the purification of ssDNA. In contrast to previously described applications, CaR allows isolation and concentration of ssDNA from crude reaction mixtures in a single tube without harsh conditions and without the need for any post-processing steps like pH-adjustment or sample purification. Thus, we believe that, also due to its scalable nature, the combination of asymmetric PCR and CaR will also be implemented in other applications that require the generation of ssDNA in the future.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental section, agarose gel analysis, details on results of UV- and fluorescence-measurements. See DOI: 10.1039/b000000x/

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