The biotin–streptavidin interaction can be reversibly broken using water at elevated temperatures
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The biotin-streptavidin system is the strongest noncovalent biological interaction known, having a dissociation constant, \( K_d \), in the order of \( 4 \times 10^{-14} \) M. The strength and specificity of the interaction has led it to be one of the most widely used affinity pairs in molecular, immunological, and cellular assays. However, it has previously been impossible to re-use any streptavidin solid support, since the conditions needed to break the interaction with biotin has led to the denaturation of the streptavidin. Here, we show that a short incubation in nonionic aqueous solutions at temperatures above 70°C can efficiently break the interaction without denaturing the streptavidin tetramer. Both biotin and the streptavidin remain active after dissociation and both molecules can therefore be re-used. The efficiency of the regeneration allowed solid supports with streptavidin to be used many times, here exemplified with the multiple re-use of streptavidin beads used for sample preparation prior to automated DNA sequencing. The results suggest that streptavidin regeneration can be introduced as an improvement in existing methods and assays based on the streptavidin system as well as emerging solid phase applications in fields, such as microfluidics and nanotechnology.

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1 Introduction

The strong interaction between avidin and biotin was discovered as early as 1941 [1]. Avidin is a protein commonly purified from chicken egg white while biotin is a vitamin found in all cells. Streptavidin, a bacterial homologous protein to avidin, isolated from the actinobacterium *Streptomyces avidinii*, is more frequently used than avidin and is commercially available also in a number of engineered forms. The structure of the biotin-streptavidin complex has been described by several groups [2, 3], showing a \( \beta \)-barrel structure of streptavidin binding biotin into its interior. The binding between avidin/streptavidin and biotin has long been regarded as the strongest, non-covalent, biological interaction known, having a dissociation constant, \( K_d \), in the order of \( 4 \times 10^{-14} \) M [4]. The bond forms very rapidly and is stable in wide ranges of pH and temperature [1, 5].

The strong interaction has led to a large number of research and diagnostic applications using avidin-biotin or streptavidin-biotin technology. The strength and reliability of the interaction underlie its importance in biotechnology, but the interaction is also a model for high-affinity receptor ligand binding. In most assays, streptavidin is coupled to a solid phase, such as a magnetic bead, a microtiter plate, or a biosensor chip, while biotin is coupled to the moiety of interest, often a nucleic acid, protein, or antibody. However, harsh conditions, such as formamide treatment combined with high temperatures, have been required to separate biotin from streptavidin, resulting in not only denatured streptavidin molecules [5] but also in limitations of downstream applications due to deterioration of samples during release. Several strategies using various modifications and analogues of both streptavidin and biotin have been tried in order to overcome these problems [6–12]. However, as most of the methods have resulted in either a lowered specificity of the bond or required a more narrow range of conditions to work.

To investigate the possibility of finding better conditions for bond breaking and, ultimately, a reversible dissociation of the biotin-streptavidin complex, we employed a systematic approach using an automated laboratory workstation. A large number of temperatures, times, solvents, and ionic strengths were tested on a model system using streptavidin covalently coupled to the surface of magnetic beads. Surprisingly, the analysis of the results showed that it was possible to find conditions where the biotin-streptavidin interaction is reversibly broken. Here, we show that such conditions can be used to efficiently
re-use streptavidin beads multiple times. This method has the potential to be a valuable tool for many applications both in research and diagnostics.

2 Materials and methods

2.1 Preparation of PCR products

Plasmids (containing inserts ranging from 300 to 1200 bp long) were selected from a shotgun library and PCR-amplified using standard conditions. The PCR reactions contained 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 0.2 mM of each dNTP, 0.5 U AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), 5 pmol biotinylated universal sequencing primer (USP) and 5 pmol reverse sequencing primer (RSP) in a total volume of 50 µL. All primers were obtained from MWG Biotech (Ebersberg, Germany). The USP primer had a biotin molecule covalently coupled to the 5’-end while the RSP primer did not have any modifications. For some experiments, an RSP primer with fluorescein coupled to the 5’-end was used in order to obtain a fluorescent PCR product. The temperature profile used consisted of heating to 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 2 min, followed by a final elongation step of 72°C for 10 min and was performed on a PTC 225 thermostycler (MJ Research, Waltham, MA, USA).

2.2 Capture and elution of dsDNA PCR fragments

All experiments were performed using a Magnatrix 1200 automated workstation (Magnetic Biosolutions, Stockholm, Sweden). This workstation was equipped with an efficient magnetic capture system [13], using a neodymium-iron-boron magnet which is moved to close proximity of the pipette tip. The temperature of the microtiter plate was controlled using a Peltier element. Custom-made protocols were designed for these experiments. M-270 streptavidin-coated paramagnetic beads (Dynal Biotech, Oslo, Norway) were used as a solid phase. These beads are uniform, superparamagnetic, polystyrene beads covered with covalently attached tetrameric streptavidin. The beads were delivered at a concentration of 10 mg/mL in a storage buffer. For each sample, 100 µg beads were magnetically captured and the storage buffer discarded prior to resuspension in 2 x binding buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM NaCl, 0.1% Tween 20) to a concentration of 5 mg/mL. The resuspended beads were then transferred to 20 µL of the biotinylated PCR product. A 15 min incubation at room temperature with three intermittent mixes allowed the PCR products to bind to the beads and the complex was then magnetically collected and rinsed twice with 1 x TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to remove any impurities from the PCR reaction and salts from the binding buffer. Following the wash procedure, the beads were resuspended in 20 µL deionized Milli-Q water and heated to 70°C at a rate of approximately 1°C per 2 s. After a 1 s incubation at 70°C the reaction was allowed to cool to room temperature. Once again the beads were magnetically collected and the supernatant, containing the eluted DNA, was transferred to a clean tube and kept for analysis. After a final wash in 1 x TE the beads were resuspended in 1 x TE to a concentration of 10 mg/mL (neglecting bead loss) and could then be used for capturing new DNA.

2.3 Analysis

Eluates were analyzed using several methods. Initial screenings were performed using agarose gel electrophoresis on 1% agarose gels using the GeneRuler™ DNA Ladder Mix as a marker. Concentrations were determined by measuring absorbance using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) set to the standard DNA50 protocol. Throughout the experiments, released dsDNA was also evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) where the assays used were either DNA7500 or DNA1000. Minimum peak heights for the evaluations were globally set to 2.0 for the DNA7500 chip and 1.0 for the DNA1000 chip. Due to the limitations of the instrument regarding various salt concentrations in the samples, dilutions were carried out when necessary. On the Bioanalyzer all samples are run together with two internal markers, which are used by the Agilent software as both size and concentration standards. These markers can be seen as peaks at approximately 37 and 85 s in the chromatograms obtained from the DNA7500 assay.

2.4 Flow cytometry (FACS)

PCR reactions were performed as described above, with the exception that the RSP primer used was labeled with fluorescein (MWG), using a template resulting in a 300 bp PCR product. The obtained PCR product thus had biotin attached to one primer and fluorescein to the other. DNA was bound to streptavidin-coated beads multiple times with intermittent elutions. In all experiments the same relationship between beads and DNA was kept. The beads were washed and DNA was bound as described above, but following the two washes with 1 x TE aliquots of 25 µg beads were retrieved for FACS analysis. Elution was performed using optimized conditions (20 µL Milli-Q
water, 70°C) and the beads were washed once with 1 x TE. Then bead aliquots of 25 μg were once again retrieved for analysis. Prior to analysis the 25 μg bead aliquots were diluted with 150 μL PBS (1.9 mM NaH2PO4, 8.1 mM Na2HPO4, 154 mM NaCl, pH 7.2). Flow cytometric analysis was performed on a FACS Vantage SE instrument (BD Biosciences, San José, CA, USA). The beads were analyzed at a rate of 500–1000 events/s according to standard procedures and data was recorded from 10,000 beads for each sample.

2.5 Alternative beads

In addition to the M270 streptavidin-coated beads (Bead 1; Dynal Biotech) used in the presented experiments, several other streptavidin-coated beads were tested with comparable results. Bead 2 corresponds to M280 beads, also from Dynal, which are similar to the M270 but have a more hydrophobic and slightly less electronegative surface. Three additional streptavidin-coated magnetic beads from other manufacturers were also tested, Bead 3: Sera Mag (3 μm) Seradyn (Seradyn, Indianapolis, USA), Bead 4: M-PVA SAV2 from Chemagen (Chemagen, Baesweiler, Germany), Bead 5: an experimental bead kindly supplied from Genovision (Oslo, Norway).

2.6 Elution conditions

A number of different elution conditions were investigated in order to determine their effect on elution efficiency. First of all different temperatures and incubation times for the elution were tested. Temperatures ranged from 20°C to 80°C while final incubation at these temperatures ranged from 1 s (i.e., immediately lowering of the temperature after reaching the set point) to 300 s. Different elution buffers (10 mM MgCl2, 10 and 100 mM LiCl and 10, 25, 50, 100, and 500 mM NaCl) were also tested at the standard temperature conditions (70°C and 1 s). In order to determine the elution efficiency of the various conditions, a second elution was performed, using conditions previously found to elute all DNA, any residual DNA still left on the beads were detected and measured. In brief, beads were magnetically separated from the first eluate, resuspended in 20 μL Milli-Q water and again heated to 70°C. Following lowering of the temperature, the beads were again magnetically removed and the eluate containing any residual DNA not released the first time, was transferred to a new tube for analysis.

2.7 Preparation of sequencing reactions

TemplPhi (Phi29; Amersham Biosciences, Uppsala, Sweden) was used to amplify plasmids for the sequencing reactions, and the manufacturer’s protocol was followed. Briefly, 2 μL diluted plasmid solutions from a Poppel library (Uni G09, Poppel Tension wood, clones in pBluescript Sk+ (Stratagene, La Jolla, CA, USA)), were added to 10 μL sample buffer and heated to 95°C for 3 min. After addition of 10 μL phi29 mix, the reactions were incubated at 30°C for at least 16 h. Phi29 was inactivated by heating to 65°C for 10 min. Following dilution of the products with Milli-Q water to decrease the viscosity of the product, 2 μL was used for cycle sequencing reactions, which were performed with 2 μL BigDye (Applied Biosystems, Foster City, CA, USA), 1 μL biotinylated RSP primer (5 pmol/μL) and 15 μL 1 x CS buffer (26 mM Tris-HCl, pH 9, and 6.5 mM MgCl2) in a total volume of 20 μL.

The following cycling conditions were used: an initial denaturing step of 95°C for 30 s, then 35 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min. This procedure was performed for eight parallel plates, each plate was then split into two plates containing 10 μL sequencing products.

3 Results

3.1 Experimental setup

The experimental setup used in this work is shown schematically in Fig. 1. Biotinylated and, in some cases, fluorescently labeled PCR fragments were produced using a plasmid template with various insert lengths as described earlier [14]. A robotic workstation was used for all the experiments in order to automate the large number of tests that were anticipated. The biotinylated products were bound to streptavidin-coated magnetic beads using a high-ionic-strength buffer (1 m NaCl). Various elution conditions were tested using different combinations of temperature, elution time, liquids, and salts. The amount of eluted DNA fragments was screened by agarose gel electrophoresis and measured by capillary electrophoresis and spectrophotometer. Finally, when using optimized conditions (deionized water and 70°C) the amount of immobilized DNA before and after elution for several rounds of binding and elution was measured by flow cytometry (FACS).

3.2 Initial screening

In the initial screening, a limited set of conditions were tested, including temperatures between 20° and 80°C. Temperatures above 80°C were not tested, since streptavidin is denatured at such high temperatures [15]. Monovalent and divalent salts, in concentrations ranging from 0.1 to 1 M, were tested as well as various solvents including water, ethanol, and formamide. As shown by the FACS
Figure 1. The overall principle of the experimental setup. A biotinylated PCR product is mixed with streptavidin-coated magnetic beads. The formed complex is magnetically separated from the supernatant, washed, and mixed with an elution buffer. After gently heating the mixture to allow the dissociation of biotin from streptavidin to occur, the temperature is again lowered, and the beads are separated from the released PCR product. The beads can then be re-used to capture new PCR products.

analysis in Fig. 2A, efficient immobilization and elution could be obtained for samples treated with pure water at elevated temperatures. Analysis by capillary electrophoresis was confirmed by the FACS results. Figure 2B shows that the eluted DNA fragment has the correct size, while Fig. 2C demonstrates that the eluate from the second elution contains no or very little remaining DNA.

3.3 Temperature dependency

The efficiency of the biotin release was further studied at different temperatures and elution times (Fig. 3A). The results show that only a small fraction of the bound DNA was released at 20°C, while the release was more effective as the temperature was increased. At 60°C, approximately half of the DNA was released after a 1-s hold before lowering the temperature. The release was increased to 90% after 30 s and after 60 s most of the DNA was no longer bound to the solid support. At 70°C, the release is further improved with a more than 95% release already after 1 s. To be noted is that the temperatures at all times were raised from 20°C to the test temperature with a slope of approximately 0.5°C per s.

3.4 Effect of ionic strength

The effects of various salt concentrations were further studied using both monovalent and divalent salts. The results, using elution at 70°C for one second, are presented in Fig. 3B. A marked decrease in biotin release was observed when the concentration of NaCl was increased. At 10 mM NaCl, approximately 90% of the bound DNA was released, at 100 mM NaCl only about 30% and higher concentrations decreased the release efficiency even further. LiCl exhibited similar influences as NaCl on the elution efficiency. Divalent salts, here exemplified by MgCl₂, were also tested and, interestingly, the effect was even more pronounced than that of the monovalent salts. Even at relatively low concentrations, such as 10 mM, only about 10% of the bound DNA was released using the same temperature and time (70°C, 1 s) as shown to efficiently release DNA in nonionic solutions.

3.5 Re-use of streptavidin beads

The results above show that the biotin-streptavidin interaction can be dissociated using a nonionic aqueous solution together with gentle heating to 70°C briefly (1 s). The question then arises whether the streptavidin beads can be re-used multiple times after regeneration using these conditions. Figure 4 shows the results of binding and eluting two different PCR fragments of different length (500 and 700 bp, respectively). No contamination or consecutive buildup of contamination was observed during four repeated regenerations of the beads. No detectable contamination from the previous biotinylated product of each round was found, nor any decrease in binding capacity in the subsequent rounds. Similarly, the released biotinylated DNA was also immobilized on new magnetic beads coated with streptavidin and no significant decrease in the amount of bound molecules was seen (data not shown). In summary, these results show that both the biotinylated molecules and the streptavidin beads can be re-used using the elution conditions involving nonionic aqueous solutions at 70°C.

3.6 Regeneration efficiency using streptavidin beads from different sources

The elution strategy was tested on different types of streptavidin-coated magnetic beads from different manufacturers. Four consecutive captures and releases were
Figure 2. Initial elution experiments. The binding and elution of fluorescently labelled DNA fragments to streptavidin-coated magnetic beads were studied. The results from an immobilization in 1 M NaCl and two iterative elution events using water at 70°C are shown. (A) Flow cytometry (FACS) was used to determine the amount of immobilized fluorescently labelled DNA fragments on the beads after immobilization and after elution. The original beads (without DNA) were used as a reference (red line) to show the background fluorescence. The results show that the DNA immobilized on the beads (green) is efficiently eluted (blue) using the conditions described above. (B) The first eluate was studied using capillary electrophoresis, showing a single strong peak corresponding to the same size as the PCR product. Peaks marked M correspond to internal markers. (C) Analysis of the second eluate by capillary electrophoresis revealed that no additional DNA was released.

performed for all of these different beads in a robotic workstation and the amount of released DNA was measured. The results presented in Fig. 5 show that the same efficient regeneration can be observed for all five beads from different manufacturers.

3.7 Capture of DNA-sequencing products for capillary electrophoresis

In order to test the practical use of the regeneration of streptavidin beads, we investigated the efficiency of multiple rounds of capture of DNA sequencing products for capillary electrophoresis. The approach was aimed to eliminate alcohol precipitations or spin column purifications prior to loading a cycle sequencing reaction [16] onto an automated capillary electrophoresis instrument. Here, a biotinylated universal sequencing primer was used during the cycle sequencing to introduce biotin in the extension products. This biotin was then used for capture of the sequencing ladder onto streptavidin-coated magnetic beads, thereby eliminating non-incorporated fluorescent nucleotides and unwanted salts that would otherwise disturb the subsequent electrophoresis analysis. The extension products were eluted with deionized water at 70°C for 1 s, as previously described, and the beads magnetically removed. A convenient
Figure 3. Effect of temperature, time, and salt concentrations on the biotin-streptavidin release. The relative amount (%) of released biotinylated DNA was measured with capillary electrophoresis after eluting immobilized DNA fragments at different conditions. (A) The effects of temperature and time were determined by varying the elution temperatures from room temperature to 80°C using incubation times (at maximum temperature) ranging from 1 s to 5 min. (B) The effect of various salt concentrations during the elution was determined using the same elution temperature (70°C) and time (1 s) for all samples.

Cleaning step was thus obtained, easy to automate using robotic workstations fitted with a magnetic separation system. The elution products were loaded directly on a commercial capillary electrophoresis instrument and the beads were subsequently used in several rounds of capture and analysis. Figures 6A and B show examples of the results from the first and tenth use of the magnetic beads. The average Phred 20 score for 96 individual samples in each sequencing round were used to estimate the quality of the purified sequencing products (Fig. 6C). The results demonstrate that the regeneration of the streptavidin beads can be used for sequencing “cleanup” and the experiments show that the streptavidin beads can be reused at least ten times without adverse effect on the binding capacity or quality of the result.

4 Discussion

Here, we show that the streptavidin-biotin interaction can be reversibly and efficiently broken with nonionic aqueous solutions using a brief exposure to high temperature...
Figure 4. Multiple binding and elution. The repeated binding and elution of fluorescently labelled DNA fragments to streptavidin-coated magnetic beads were studied using DNA fragments to two sizes, 500 bp and 700 bp. Four consecutive immobilization and elution events were performed in a robotic workstation, using 1 M NaCl for the immobilization and water at 70°C for 1 s for the elution. (A) Flow cytometry analysis was used in order to determine the amount of immobilized fluorescently labelled DNA fragments on the beads after binding and elution in each step. The results show no decrease in binding capacity and no buildup of DNA remaining on the beads after elution. (B) Capillary electrophoresis analysis was used to study the eluted DNA from each step in order to detect any possible sample carry-over from the previous DNA-fragment. Only a single peak could be seen in each eluate (peaks marked M correspond to internal markers) indicating very little or no contamination between samples. (C) The amount of eluted DNA from each step was determined using the NanoDrop spectrophotometer and the results confirm that no decrease in binding capacity could be seen when the beads were re-used.
Figure 5. Regeneration efficiency using streptavidin beads from different sources. The elution strategy was tested on different types and brands of streptavidin-coated magnetic beads. Four consecutive captures and releases of DNA fragments were performed, using binding in 1 M NaCl and elution in water at 70°C for 1 s. The amount of released DNA was measured using a NanoDrop spectrophotometer. The types of beads (brands) are further described in Section 2.5.

(70°C). Several reports [1, 5, 6, 8, 17] have earlier failed to find conditions for the reversible dissociation of this strong interaction. The fact that, apart from enabling the release of the biotinylated moiety from streptavidin, both the streptavidin and the biotin moieties can be efficiently re-used, makes the finding particularly interesting for many applications in biotechnology and diagnostics.

The molecular mechanism for the interaction between biotin and streptavidin has been studied extensively. X-ray crystal structure [2, 3], site-directed mutagenesis [18–23], phage display mutagenesis [24], and circularly permutated streptavidin variants [25] suggest three binding features critical to the strength of the streptavidin-biotin interaction: (i) hydrophobic and van der Waals forces, (ii) a large number of hydrogen bonds, and (iii) a flexible surface loop of streptavidin moving to a close conformation when biotin has bound to its site inside the beta-barrel of the streptavidin molecule.

Recent studies have been undertaken by Hyre et al. [26] using a joint computational and crystallographic approach. The three-dimensional structure of the streptavidin mutant D128A shows that the active site is accessible by water molecules through a channel of the streptavidin molecule reaching the biotin in the binding pocket [26]. The study suggests that the bond-breaking events are accompanied by the entry of a single water molecule into the binding pocket, where it serves as a bridge between the Asp-128 carboxylate and the biotin [26]. Here, we show that the dissociation of biotin from the streptavidin-binding pocket can be done in aqueous solutions at elevated temperatures and that salt makes this dissociation less efficient. The fact that the dissociation is efficiently hindered with both monovalent and divalent salts suggests that the interaction is stabilized with ionic molecules and that depletion of salts allows the complex to be dissociated. Interestingly, high salt concentrations are often used to obtain efficient binding of biotin to streptavidin [14], suggesting that the interaction is promoted by the presence of salt molecules. Further studies are needed to obtain a more clear view of the molecular mechanisms in which the salt molecules participate during the streptavidin-biotin association and dissociation.

The possibility to break the interaction without denaturation or destruction of the structure or function of the biotin and the streptavidin opens up a whole array of possible new applications. The fact that the elution buffer is pure water makes the procedure convenient for a multitude of subsequent applications. The sequencing clean-up procedure described here, provides a concentration step combined with removal of salts, nucleotides, and sequencing template. Thus, a convenient protocol suitable for automation is obtained. The fact that the beads can be re-used at least ten times makes the sample preparation highly cost-effective since the reagent costs can be significantly decreased.
Figure 6. Use of the regeneration protocol for automated DNA sequencing. A dye-terminator cycle sequencing protocol was followed using a biotinylated sequencing primer. The cycle sequencing products were captured on streptavidin-coated beads and eluted with water at 70°C for 1 s in a robotic workstation, thereby achieving an efficient purification. The same beads were used for ten consecutive purifications and the eluted material was analyzed on an ABI3700 automated sequencer. (A) Example of a chromatogram obtained after purifying cycle sequencing products on fresh beads. (B) After purifying cycle sequencing products on beads used 10 times the chromatogram still show sequence with the same high quality as in (A). (C) The average Phred20 scores of 96 individual samples (one microtiter plate) from each consecutive use of the beads was calculated. Since no decrease in the number of high quality bases can be seen these results indicate that the beads can be re-used at least 10 times.

The system can also be used for other applications in which streptavidin beads are used to capture biotinylated molecules. Such applications include isotope-coded affinity tag (ICAT) for proteomic applications [27–29] and immunoassays in which biotinylated molecules are immobilized to streptavidin-coated beads. It should also be possible to use the system for affinity purification of biotinylated biomolecules, such as biotin-containing...
fusion proteins [30, 31], to allow the re-use of the solid support. Recently, we have shown that the regeneration can also be used in the preparation of single-stranded DNA using an alkaline elution to separate the two strands, followed by the nonionic elution of the biotinylated single strand to regenerate the beads (Holmberg, unpublished). The single-stranded template can subsequently be used for various applications, including in vitro mutagenesis [32], pyrosequencing [33], or transcript profiling [34–36].

In this study, we have used magnetic beads coated with streptavidin, but it is also possible to use other streptavidin-coated solid supports, such as the walls of microtiter plates or nonmagnetic beads. An interesting application is the use of microfluidic units in which biotinylated molecules can be captured using a streptavidin-coated surface and subsequently regenerated using the conditions described here. In this manner, it is possible to regenerate a solid support containing streptavidin within the microfluidics device.

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5 References