Sequence selective capture, release and analysis of DNA using a magnetic microbead-assisted toehold-mediated DNA strand displacement reaction

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This paper reports on the modification of magnetic beads with oligonucleotide capture probes with a specially designed pendant toehold (overhang) aimed specifically to capture double-stranded PCR products. After capture, the PCR products were selectively released from the magnetic beads by means of a toehold-mediated strand displacement reaction using short artificial oligonucleotide triggers and analysed using capillary electrophoresis. The approach was successfully shown on two genes widely used in human DNA genotyping, namely human c-fms (macrophage colony-stimulating factor) proto-oncogene for the CSF-1 receptor (CSF1PO) and amelogenin.

Selective capture of DNA on two- and three-dimensional surfaces is the underlying principle of a vast majority of nucleic acid molecular analytical techniques such as high density microarrays, nucleic acid biosensors, and personalised point-of-care micro-devices. These approaches use surfaces modified with immobilised capture probes for the hybridisation of nucleic acids, which is regarded as the final point of analysis. Patterned surface modification allows for the localisation of specific DNA probes at different regions, which in turn allows for highly efficient selection, separation and enrichment of complementary DNA molecules (targets) from the initial crude mixture.

More recently however, in light of advances in the field of gene therapy, there has been a great deal of interest in the development of methods for controlled (stimuli-responsive) release of nucleic acids from both 2- and 3-dimensional surfaces. It is envisaged that such systems will facilitate the advent of smart stimuli-responsive gene delivery vehicles. To date, several different methods exploiting such external stimuli have been reported, employing the use of temperature,2 DC electric field,3 light,4 and low molecular weight organic molecules.5 Temperature controlled DNA release relies on thermal denaturation of DNA double helices trapped on a surface via immobilised ligands (capture probes), such as another DNA molecule6 or avidin-biotin complexes.2b This approach possesses low specificity and requires either harsh dehybridisation conditions or fine tuning of the system to be able to operate specifically at physiological temperatures. The application of a DC electric field has also shown potential for the fast and specific dehybridisation of 3’-mismatched DNA duplexes from electro-conductive surfaces.3c Several publications describe the selective release of multiple DNA molecules. These systems are mainly based on the use of gold nanoparticles with varying morphologies, and thus surface plasmon absorbances. Irradiation of gold nanoparticle mixtures, loaded with covalently captured DNA duplexes, at their specific plasmon resonance frequency causes preferential heating of some particles but not others. This consequently leads to melting of DNA duplexes and their subsequent release from the nanoparticle. In order to have specificity of DNA release, there must be no overlapping of the surface plasmon resonances and this is far from trivial.

The discovery of DNA strand-displacement reactions, prompted by the formation of a toehold structure (for references see), has opened a new era in programmable synthetic biology. One of the most attractive features of this reaction is that short artificial DNA strands allow for a specific trigger of different downstream scenarios which include both nucleic acid and protein cascades. Applying these systems to solid phase supports has recently gained much interest.10 For example, 2-dimensional microarrays operated by a strand-displacement reaction were shown to be successful at DNA length polymorphism measurements (short tandem repeat analysis, STR).10 Picuri et al.4e have applied the DNA displacement reaction on sepharose microbeads to translate several specific biologically relevant DNA and RNA molecules (HIV, HCV and smallpox) into an unique unspecific DNA output triggering diagnostic assay. Probst et al.6c covalently immobilised antibody encoded double stranded (ds)-DNA with pendant toeholds onto magnetic beads. After binding with specific antigens, the antibody-antigen complexes were released from the bead surface upon addition of specific displacement probes. Here we report an approach for the capture of multiple ds-DNA molecules on a surface of magnetic beads followed by their sequence selective release using a toehold-mediated strand-displacement reaction. To achieve this we modified magnetic beads with single-stranded oligonucleotides (ss-ODNs) (capture probes). These capture probes were specially designed to capture specific ds-PCR amplification products, tagged with a PEGylated (polyethylene glycol) ss-ODN, of forensically relevant human c-fms (macrophage colony-stimulating factor) proto-oncogene for the CSF-1 receptor (CSF1PO) and amelogenin (AMEL) genes. STR analysis using capillary electrophoresis (CE) is the main form of human
identification used in forensics and considered as the ‘gold standard’ of forensic science. STRs have been used in forensic science since 1994 including the adoption of CSF1PO, which was developed originally by the Promega Corporation. This locus is an example of a simple repeat motif (AGAT)_n and was initially incorporated into the US Combined DNA Index System (CODIS) loci. CSF1PO continues to be one of the loci used in commercial kits by the forensic community worldwide. Amelogenin is one of the few genes with homologues on the X and Y chromosomes and was adopted in 1995 into STR multiplexes and used ever since. The X chromosome version has a 6 bp deletion compared to the Y homologue allowing this size variation to be used to determine the gender of the person from whom the DNA profile was generated.

Each capture probe had a main hybridisation sequence (Fig. 1, domain a) with an extra 8 nucleotide long ss-ODN (toehold) attached at the 5’ termini of the main hybridisation sequence (Fig. 1, domain b). The entire capture probe was immobilised covalently to N-hydroxysuccinimide activated carboxy-terminated magnetic beads (1 µm, Bioclone, USA) using amide coupling through the 3’ end of the amine-modified capture probes.

The synthesis of the ds-PCR products tagged with a PEGylated ss-ODN (Fig. 1, domain a') has been previously described by our group. In brief, the forward primer for amplification of the target genes consisted of the primer’s main sequence (not shown), an additional shorter sequence (Fig. 1, domain a') (for hybridisation to the capture probe (Fig. 1, domain a)) and a PEG spacer linking at the 5’-termini of the primer’s main sequence and the 3’-termini of the hybridisation sequence. At the completion of the PCR all the products of amplification contained a short tail of PEGylated ss-ODN sequences (attached to the amplified ds-PCR product) which could be hybridised (captured) to the surface immobilised capture probes. Importantly, all unreacted PEGylated hairpin primers form a self-complementary intramolecular structure that prevents hybridisation of any unreacted primers with the surface immobilised capture probes.

Figure 1 shows the scheme of the capture and sequence selective release of ds-PCR products with PEGylated ss-ODN on the magnetic beads. The ds-PCR product tagged with PEGylated ss-ODN tail (Fig. 1 domain a') first hybridises with the capture probe covalently immobilised on the surface of the magnetic beads. In particular, during this hybridisation step the ss-ODN (domain a') specifically interacts with domain a of the capture probe thus forming a perfectly matched DNA duplex aa' and at the same time leaving domain b (toehold) free. After several washings of the magnetic beads, now bearing the ds-PCR product, a specific displacing sequence consisting of domains a'b' is added. The domains b and b' then hybridise to each other forming a toehold structure (Fig. 1 complex bb') which then promotes the strand displacement of the domain a’ from the initial hybridisation duplex, resulting in sequence selective release of the ds-PCR product from the magnetic bead surface back into solution. The results of the displacement reactions and the length analysis of the released ds-PCR products were then confirmed using CE.

In order to evaluate the feasibility of the approach for a one-pot sequence selective release of multiple hybridised ds-DNA molecules, we applied this technique for the capture, sequential release, isolation and analysis of two ds-PCR products CSF1PO and AMEL genes. Gene-specific capture probes with corresponding displacing sequences (Table S1, ESI) were designed to capture and release ss-ODN tagged ds-PCR products obtained after amplification with specific modified primers (as described above) of the CSF1PO and AMEL loci in human genomc DNA. Reverse primers for both the CSF1PO and AMEL genes were labelled with a fluorescent dye (fluorescein) at their 5’ termini in order to be observed during CE analysis. Multiplex PCR amplification with the designated primers (Table S1, ESI) was carried out using 5 ng of male genomic DNA isolated from the author’s own blood.

In order to capture the CSF1PO and AMEL ds-PCR amplification products (generated from male genomic DNA) with PEGylated ss-ODN the PCR mixture and hybridisation buffer (final concentration of guanidine thiocyanate (1 M), HEPES (50 mM), pH 7.5, and EDTA (5 mM)) were directly applied to a stoichiometric mixture of the magnetic beads with immobilised CSF1PO and AMEL capture probes. Significantly, this negates laborious and time consuming PCR clean-up procedures. After hybridisation (Fig. 1), the magnetic beads were washed multiple times by removing them from the hybridisation solution with a magnet and washing with a washing buffer (6.5×SSPE buffer pH 7.4, 0.01% Tween 20) and water to remove any non-hybridised ds-PCR products on the bead surface.

Both the extent and stability of hybridisation were then determined by CE analysis. Figure 2A shows the electropherogram of the sequences released after 18 h (overnight) incubation of the magnetic bead mixture in a 1 x TEM (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 12.5 mM MgCl2) displacement buffer with no ss-ODN displacing sequence. Clearly, there has been no release of the ds-PCR products from the magnetic beads indicating that no toehold strand displacement reaction has taken place (Fig. 2A).

Following these results the first round of sequence selective release of the hybridised ds-PCR products was carried out. After washing, the magnetic bead mixture was resuspended in a solution at 30 °C that consisted of a 1 x TEM displacement buffer and a displacing sequence (1 µM) fully complementary to the CSF1PO capture probe (Table S1, ESI). During this stage domain b’ of the CSF1PO displacing sequence binds with the domain b of the CSF1PO capture probe only (Fig. 1), but not the AMEL capture probe. Subsequently, this triggers the release of the CSF1PO ds-PCR product through a 3-way branch migration mechanism. An aliquot was taken from this reaction mixture after 0, 2, 4, 6 and 18 (overnight) h of incubation. However, there was only a noticeable level of ds-PCR product released via CE analysis after 18 h (overnight) of incubation (for 0, 2, 4 and 6 h of displacement data not shown). Slow release kinetics are likely due to sterie hindrance and electrostatic repulsions caused by the long ds-PCR products, ranging from 106 to 346 bp, hybridised on the magnetic bead surface.

Figure 2B shows the CE electropherogram of the ds-PCR products present in solution after a CSF1PO displacing sequence was added to the magnetic bead mixture (18 h of incubation). It can
clearly be seen that after incubation, two peaks with intensities of 1094 and 1021 relative fluorescence units (RFU) of the CSF1PO ds-PCR products were observed (Fig. 2B). The two peaks correspond to two different CSF1PO ds-PCR products (340 and 346 bp) amplified from two alleles of the CSF1PO locus. The length of these amplification products was in full accordance with the values determined by the PowerPlex 21 STR genotyping system (Promega, USA) (Fig. S1, ESI). Unspecific AMEL release was observed at <85 RFU (<8.5 % of the CSF1PO intensity value) (Fig. 2B).

The capability of the system to sequentially release a second hybridised ds-PCR amplification product was then evaluated. The same magnetic bead mixture (after CSF1PO ds-PCR products had been released) was rinsed thoroughly with 1×TE buffer and suspended in an AMEL displacing solution consisting of 1×TE buffer and AMEL displacing sequence (1 μM) (Table S1, ESI). CE analysis was then performed on an aliquot of the solution after 2, 4, 6 and 18 h incubation of the magnetic bead mixture with the washing buffer containing Tween-20 surfactant. To test this, the initial mixture of magnetic beads (with both PCR products hybridised) was washed with 1×TE buffer only (no Tween-20 surfactant). The first round of the release with CSF1PO displacement sequence was then repeated. The CE analysis performed on aliquots taken in the same timeframes (2, 4, 6 and 18 h) showed the successful displacement after 6 h of incubation (c.f 18 h previously) with intensities of the CSF1PO peaks of 216 and 198 RFU (Fig. S3, ESI). The observed increase in the release rate is likely explained by the “shielding” effect of Tween-20 physically adsorbed on the magnetic bead surface. This may hinder the efficient formation of the toehold structure and the subsequent branch migration displacement. The reaction timeframes are not surprising, since similar kinetic release (2 – 72 h) has been observed by Baker et al. who used 15 nucleotide long duplexes immobilised on 5 μm carboxylated latex particles.

Our system was also capable of the simultaneous release of both CSF1PO and AMEL ds-PCR products from the magnetic beads. This was achieved by adding two displacing sequences at once (CSF1PO and AMEL). Figure 2D shows the CE electropherogram of the aliquot taken after 6 h incubation of the magnetic bead mixture (with both PCR products hybridised) in 1×TE buffer containing SCF1PO and AMEL displacing sequences (1 μM, each). As expected, two peaks for each of the PCR products were observed with intensities of 231 and 210 RFU for CSF1PO and 132 and 163 RFU for AMEL. The length of the released PCR products also fits the data shown above (Fig. 2B, Fig. 2C).

A final series of experiments showed the successful release of the surface captured ds-PCR products generated from the author’s female genomic DNA (Fig. S4, ESI). In this case the AMEL ds-PCR product was selectively released first by incubation of the hybridised beads in the AMEL displacing solution. CE analysis of the released product showed only one peak confirming the XX genotype of the female DNA sample (Fig. S4B). Subsequent incubation of the beads in the CSF1PO displacing solution resulted in the release of the CSF1PO ds-PCR product which was then also analysed by CE. The single peak observed (Fig. S4C, ESI) fits the CSF1PO genetic profile previously identified by the PowerPlex 21 STR genotyping system (Fig S2, ESI). Simultaneous release of both PCR products was also shown to be successful (Fig. S4C, ESI).

Conclusions

In summary, we have developed a simple DNA sequence trigger approach to specifically release surface hybridised ds-PCR amplification products. The approach is based on the specific reaction of toehold-mediated DNA strand-displacement. The selective release of one out of two surface hybridised ds-PCR amplification products was manipulated by simple addition of a specific small ss-ODN which played the role of the trigger. The release of the second hybridised ds-PCR product was achieved simply by adding another specific ss-ODN trigger. The release was carried out under mild conditions (low salt Tris-EDTA buffer pH 8) at 30 °C showing that the approach could be readily implemented in in vivo experiments. Isolation and analysis of the released ds-PCR products showed the integrity of the DNA molecules. The method shows great promise for a broad range of other consecutive scenarios such as the development of highly specific stimuli-responsive molecular cargo vehicles operated by DNA or RNA.

Notes and references

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