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Optimisation of denaturing ion pair reversed phase HPLC for the purification of ssDNA in SELEX

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ABSTRACT

Aptamers have shown great promise as oligonucleotide-based affinity ligands for various medicinal and industrial applications. A critical step in the production of DNA aptamers via selective enhancement of ligands by exponential enrichment (SELEX) is the generation of ssDNA from dsDNA. There are a number of caveats associated with current methods for ssDNA generation, which can lower success rates of SELEX experiments. They often result in low yields thereby decreasing diversity or fail to eliminate parasitic PCR by-products leading to accumulation of by-products from round to round. Both contribute to the failure of SELEX protocols and therefore potentially limit the impact of aptamers compared to their peptide-based antibody counterparts.

We have developed a novel method using ion pair reversed phase HPLC (IP RP HPLC) employed under denaturing conditions for the ssDNA re-generation stage of SELEX following PCR. We have utilised a range of 5′ chemical modifications on PCR primers to amplify PCR fragments prior to separation and purification of the DNA strands using denaturing IP RP HPLC. We have optimised mobile phases to enable complete denaturation of the dsDNA at moderate temperatures that circumvents the requirement of high temperatures and results in separation of the ssDNA based on differences in their hydrophobicity. Validation of the ssDNA isolation and purity assessment was performed by interfacing the IP RP HPLC with mass spectrometry and fluorescence-based detection.

The results show that using a 5′ Texas Red modification on the reverse primer in the PCR stage enabled purification of the ssDNA from its complimentary strand via IP RP HPLC under denaturing conditions. Additionally, we have confirmed the purity of the ssDNA generated as well as the complete denaturation of the PCR product via the use of mass-spectrometry and fluorescence analysis therefore proving the selective elimination of PCR by-products and the unwanted complementary strand. Following lyophilisation, ssDNA yields of up to 80% were obtained. In comparison the streptavidin biotin affinity chromatography also generates pure ssDNA with a yield of 55%. The application of this method to rapidly generate and purify ssDNA of the correct size, offers the opportunity to improve the development of new aptamers via SELEX.

1. Introduction

In just 30 years, nucleic acid aptamers have gone from a theoretical concept, to the lab bench, to the clinic and beyond [1]. Aptamers can bind to small molecules, metal ions, proteins and even whole cells, as a class of affinity ligands their broad capabilities has led to their utilisation in a range of applications such as drug delivery, diagnosis and biosensing [2]. Their rise as a feasible oligonucleotide counterpart to protein-based antibodies was seen as a new chapter in biomedicine, an inexpensive, non-immunogenic alternative to peptide based antibodies [3]. However, few aptamers have ever been selected for clinical trials. The sparsity of clinically approved aptamers can largely be attributed to in vivo issues, their susceptibility to nuclease degradation causing short half-lives in human serum [4].

Aside from their in vivo problems their wide range of potential targets have led to a plethora of diagnostic and industrial applications. Aptamers have been isolated for a variety of applications ranging from drug detection to affinity chromatography [5,6]. However, there are a number of caveats associated with successful aptamer selection via selective enhancement of ligands via exponential enrichment (SELEX) [7].

A significant problem in SELEX protocols is their low success rate, this is, in part due to complications relating to the generation of ssDNA

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following PCR. This step is crucial as the quality and quantity of ssDNA generated greatly influences the successful evolution of aptamers [8]. Current methods are low yielding or fail to eliminate by-products which can grow exponentially from round to round [9]. The two most common methods, exonuclease digestion and streptavidin/biotin affinity chromatography particularly suffer from this, due to their lack of distinguishing between DNA of different sizes.

Exonuclease digestion involves labelling of the reverse primer in PCR with a 5′ phosphate, which is a substrate for phosphate dependant exonucleases [10]. However, it must be noted that these nucleases are not completely specific and so some of the non-phosphorylated strand can be digested. In addition, any PCR by-products which do not have a 5′ phosphate group will largely be undigested by the enzyme and will be taken through to the selection stage. Furthermore, the presence of any nucleases in the product requires careful removal prior to downstream applications The streptavidin biotin affinity chromatography method involves the labelling of the reverse primer of the PCR reaction with a 5′ biotin modification, this causes the complimentary strand to interact strongly with streptavidin such that the DNA duplex can be denatured, and the ssDNA separated [11]. This method also suffers from the inability to distinguish between products and by-products as well as potentially contaminating the next stage of selection with streptavidin if additional purification is not carried out [12].

Co-polymerisation within an agarose gel using a polymerizable 5′ modification has demonstrated effective separation of the two strands. The gel mixture is loaded into the wells of a pre-set polyacrylamide gel. When it has set, high temperatures are used to denature the duplex and release the strand which is not covalently linked to the acrylamide [13]. This separation is efficient offering yields of over 80%.

Methods such as gel extraction via crush and soak and asymmetric PCR have been demonstrated to yield reasonable quantities of ssDNA. Asymmetric PCR involves biasing primer concentrations so that the concentration of the forward primer exceeds that of the reverse primer. This leads to the generation of a higher quantity of the desired strand making purification more efficient. When coupled with exonuclease digestion, asymmetric PCR affords yields of over 80% [14] However, these methods still do not address the issue of by-product formation, particularly those close in size to the DNA template which are typically not removed using existing methods [15,16]. In addition, re-generation of ssDNA is important for other molecular biology protocols, including sequencing, sample preparation for mass spectrometry and microarray technology [17,14].

In this study we have utilised a range of 5′ chemical modifications on PCR primers to amplify PCR fragments prior to separation and purification of the DNA strands using denaturing IP RP HPLC which offers significant advantages over existing methods. The application of this method to rapidly generate and purify ssDNA of the correct size, offers the opportunity to improve the development of new aptamers via SELEX.

2. Experimental

2.1. Materials

Triethylammonium acetate (TEAA, Sigma-Aldrich), triethylamine (TEA, ThermoFisher), Tributylamine (TBA, Acros Organics), acetonitrile and water (HPLC grade, Fisher Scientific), 1,1,1,3,3,3,-Hexafluoro-2-propanol (HFIP, Sigma-Aldrich). Glacial acetic acid, combined with tributylamine for the preparation of tributylammonium acetate (TBAA) was sourced from VWR. All oligonucleotides were synthesised by Eurofins, SELEX library was sourced from IDT. For MS analysis acetonitrile and water (UHPLC MS grade, ThermoFisher) and 1,1,1,3,3,3,-Hexafluoro-2-propanol LC MS grade (99.9% ThermoFisher) were used.

2.2. Ion pair-reverse phase high performance liquid chromatography (IP-RP HPLC)

Samples were analysed by IP-RP-HPLC on a Vanquish UHPLC (ThermoFisher) or Agilent 1100 HPLC using a DNAPacRP column (100 mm x 2.1 mm I.D. ThermoFisher). Chromatograms were generated using UV detection at a wavelength of 260 nm. Weak IP RP HPLC analysis was performed using the following conditions: Mobile phase A 0.1 M TEAA pH 7.0; Mobile phase B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile or Mobile Phase A: 0.2% TEA, 50 mM HFIP. Mobile Phase B: 0.2% TEA, 50 mM HFIP, 20% acetonitrile. Mobile Phase A: 0.1 M TEAA, 50 mM HFIP. Mobile Phase B: 0.1 M TEAA, 50 mM HFIP, 25% acetonitrile. Strong IP RP HPLC was performed using Mobile Phase A: 5 mM TBAA, 10% acetonitrile. Mobile Phase B: 5 mM TBAA, 80% acetonitrile.

HPLC was performed using the following gradients:

Gradient 1. Mobile phase A 0.1 M TEAA pH 7.0; Mobile phase B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile. Gradient starting at 10% buffer B for 2 min, followed by a non-linear extension (curve 3) to 100% buffer B over 18 min at a flow rate of 0.6 ml/min at 40 °C.

Gradient 2. Mobile Phase A: TBAA 5 mM, 10% acetonitrile. Mobile Phase B: TBAA 5 mM 80% acetonitrile. Gradient starting at 5% buffer B for 2 min, followed by a non-linear extension (curve 3) 40% buffer B over 15 min, then extended to 100% buffer B over 2.5 min at a flow rate of 2.2 µl/min at 50 °C..

Gradient 3. Mobile phase A 0.1 M TEAA pH 7.0; Mobile phase B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile. Gradient starting at 35% buffer B for 2 min, followed by a non-linear extension (curve 2) to 80% buffer B over 18 min, then extended to 100% buffer B over 5 min at a flow rate of 0.6 ml/min at 40 °C.

Gradient 4. Mobile Phase A: 0.2% TEA, 50 mM HFIP. Mobile Phase B: 0.2% TEA, 50 mM HFIP, 20% acetonitrile. Gradient starting at 10% buffer B followed by a non-linear extension (curve 2) to 100% buffer B over 18minutes then extend to 100% Buffer B over 5 min at a flow rate of 0.2 ml/min at 30 or 95 °C.

Gradient 5. Mobile Phase A: 0.1 M TEAA 50 mM HFIP. Mobile Phase B: 0.1 M TEAA, 50 mM HFIP, 25% acetonitrile. Gradient starting at 35% buffer B for 2 min, followed by a linear extension to 80% buffer B over 10 min, then extended to 100% buffer B over 5 min at a flow rate of 0.6 ml/min at 80 °C..

Gradient 6. Mobile Phase A: 0.1 M TEAA 50 mM HFIP. Mobile Phase B: 0.1 M TEAA 50 mM HFIP 25% acetonitrile. Gradient starting at 25% buffer B for 2 min, followed by a linear extension to 80% buffer B over 22 min, then extended to 100% buffer B over 5 min at a flow rate of 0.3 ml/min at 80 °C.

2.3. Library and primers

The library (5′ ATATATGACTCGGGTATATANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNATAT ATGCGCTCAGTATATA) was sourced from Integrated DNA Technologies. The non-randomised template (5′-ATATATGACTCGGGTATATAATGGTCCTAATAGCCCTATAGGTACACTGACTATAGGCCGATATATGCGCTCAGTATATA) along with the forward (5′-ATATATGACTCGGGTATATA) and reverse (5′- TATATACTGAGCGCATATAT) primers (unmodified and modified) were sourced from Eurofins.

2.4. PCR

PCR amplification was carried out by amplifying either the template or the library (50 ng per reaction) with a reverse primer (with the 5′

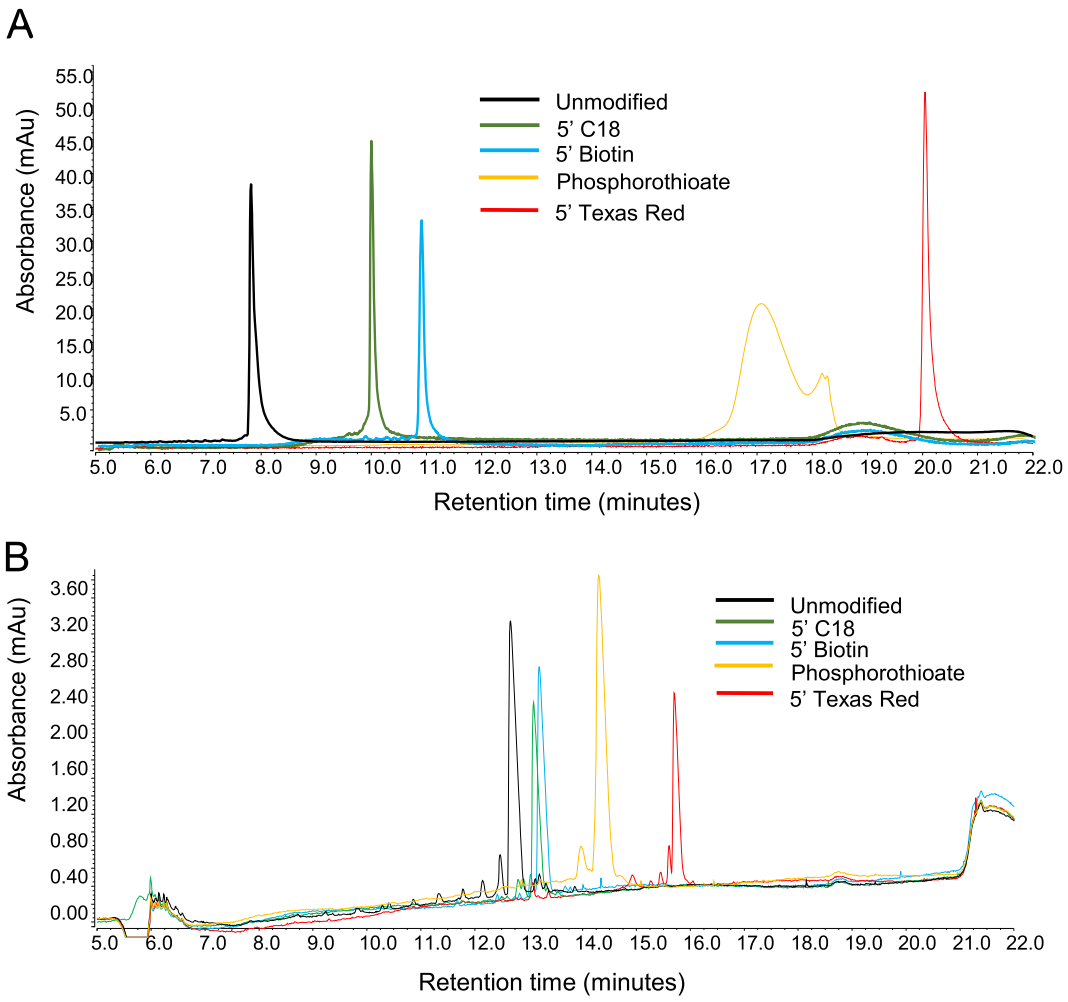

Fig. 1. IP RP HPLC analysis of 5′ modified and phosphorothioate modified primers. A) Weak IP RP HPLC analysis. Mobile phase A 0.1 M TEAA; mobile phase B 0.1 M TEAA, 25% ACN. Gradient 1, flow rate 0.3 ml/min at 40 °C. 5 pmol primer injected. B) Strong IP RP HPLC analysis Mobile phase A 5 mM TBAA; mobile phase B, 5 mM TBAA 80% ACN. Gradient 2, flow rate 2.2 µl/min at 50 °C, 3 pmol primer injected. UV detection at 260 nm.

chemical modification) and an unmodified forward primer. PCRs were performed in 50 µl final volume using 50 ng of a ssDNA template, 1 µl of 10 µM primer, 1 µl of 10 mM dNTPs (New England Biolabs), and 5 units Taq polymerase (New England Biolabs). The PCRs were carried out at 95 °C for 30 s, followed by 47 °C for 30 s and 68 °C for 30 s for 15 cycles. An initial denaturation step was carried out at 95 °C at the start of the PCR for 5 min and a final extension step carried out at the end at 68 °C for 5 min. Following PCR DNA was purified via ethanol precipitation or use of silica magnetic beads (Section 2.5).

2.5. DNA purification and desalting

2.5.1. Ethanol precipitation

Following PCR, dsDNA was re-suspended in a final volume of 300 µl HPLC grade water and 300 µl of phenol-chloroform solution added. The solution was vortexed at 13,000 rpm for 5 min and the aqueous fraction removed and mixed with two volumes of ice-cold ethanol, 1 µl of glycogen was added and the solution was cooled at −20 °C for four hours. The solution was centrifuged at 13,000 rpm for 5 min, and the pellet was washed twice with 300 µl of a 70% ethanol solution prior to re-suspension in H_2O. For the precipitation of ssDNA from the HPLC, the above procedure was performed without the addition phenol-chloroform step.

2.5.2. Magnetic bead clean-up

DNA was mixed with 2X volumes 5 M guanidinium chloride in 90% ethanol (v/v) and 50 µl of silica magnetic beads (Dynabeads, Thermo Scientific) for 15 min on a gentle rocker, beads were removed from solution via magnetic separation and washed twice with 80% ethanol (v/v). The beads were then dried in a 37 °C incubator and the DNA eluted in HPLC grade water.

2.5.3. Freeze drying

The eluted fractions were frozen at −80 °C and freeze-dried overnight at −120 °C. Following lyophilisation the sample was re-suspended in 500 µl of HPLC grade water. DNA fractions were dried in using a vacuum centrifugation at 60 °C until dry and re-suspended in 500 µl of HPLC grade water.

2.5.4. Separation of ssDNA via streptavidin agarose

Biotinylated PCR product were re-suspended in 1x PBS and incubated with 100 µl streptavidin agarose (Thermo Fisher Scientific) for 30 min. Following incubation, the gel was washed with 3x volumes 1x PBS. The ssDNA was eluted with 50 µl 150 mM NaOH (repeated twice for a total of three elutions) and neutralised with 5 µl 1.5 M HCl.

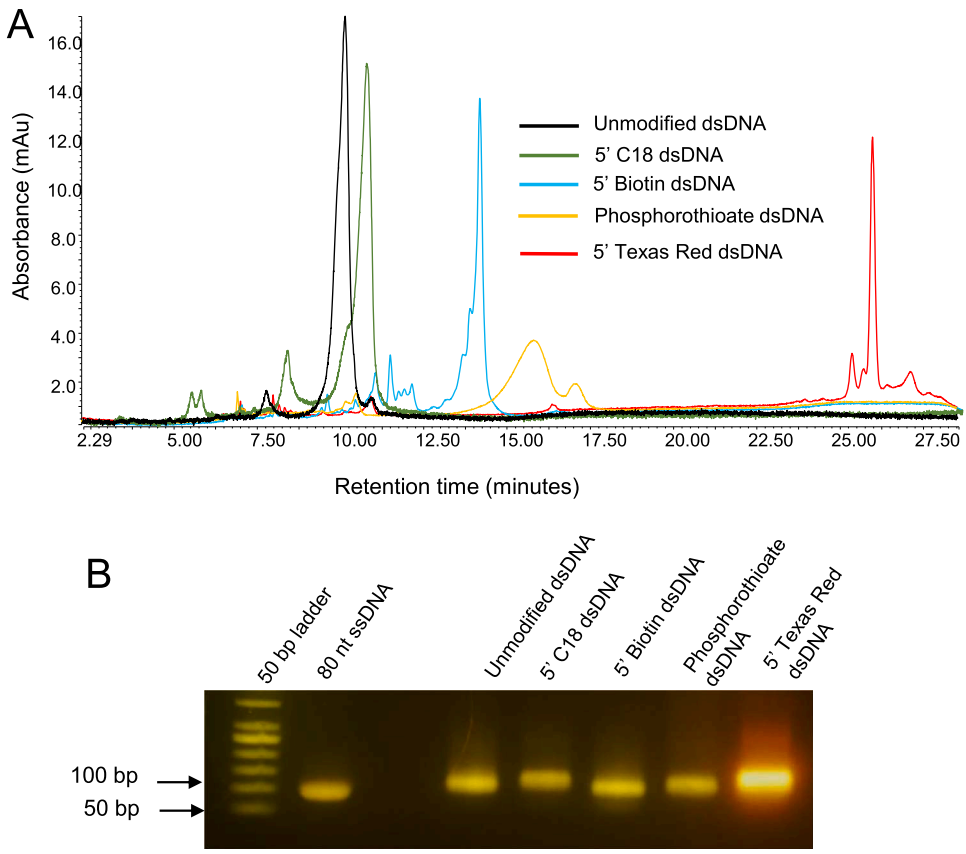

Fig. 2. Analysis of dsDNA PCR products generated using a 5′ modified reverse primer. A) IP RP HPLC chromatogram. Mobile phase A 0.1 M TEAA; mobile phase B 0.1 M TEAA in 25% ACN. Gradient 3, flow rate 0.6 ml/min at 40 °C UV detection at 260 nm. B) Agarose gel electrophoresis analysis, 50 ng PCR product was analysed for each of the dsDNA PCR products. Staining was performed using Midori green direct. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Mass spectrometry

2.6.1. Instrument methods and data acquisition

Mass spectrometry analysis was performed using an Orbitrap Exploris 240 mass spectrometer (ThermoFisher) in negative ion mode. Instrument operation was carried out with Thermo Scientific Tune and XCalibur software. Data acquisition was performed using data-dependant acquisition (DDA) in full-scan negative mode, scanning from 450 to 3000 *m/z*, with an MS1 resolution of 240 000 and a normalized automatic gain control (AGC) target of 300%. Intact mass analysis and deconvolution was performed using the 'Xtract deconvolution" algorithm within FreeStyle software (ThermoFisher). Deconvolution was performed selecting the nucleotide isotope table, negative charge, charge range 5–50 prior to determining the monoisotopic mass. All mass spectra are shown in the supplementary section **(S2–12).**

3. Results and discussion

3.1. Retention comparison of 5′ modified oligonucleotides using IP RP HPLC

In our initial experiments we aimed to assess the effect of a variety of 5′ chemical modifications of oligonucleotides on their retention time when analysed using IP RP HPLC. It was hypothesised that increasing the hydrophobicity of the 5′ modification would result in the ability to separate the two strands of the DNA duplex when analysed under denaturing conditions using IP RP HPLC and therefore enable the separation of the two single strands from an 80 bp dsDNA fragment generated using PCR products via the same principle. Initial work focused on the analysis of a range of 5′ modifications on the PCR primers including C18, Biotin, Texas Red and a 20-base primer with a phosphorothioate backbone which were selected due to the increased hydrophobicity of phosphorothioate oligonucleotides (OGNs) [18–20].

The modified primers were analysed via both weak IP RP HPLC (triethylammonium acetate) and strong IP RP HPLC (tributylammonium acetate) see Fig. 1. The strength of an ion-pair reagent is defined by its overall hydrophobicity. The alkylamine, TEA (short alkyl chains) is termed a "weak" ion-pair reagent and the more hydrophobic TBA (longer alkyl chains) is termed a "strong" ion-pair reagent, which has a stronger interaction with the stationary phase [21]. Comparison of the retention times of the various 5′ chemical modifications demonstrates that the phosphorothioate and 5′ Texas Red result in the largest difference in retention compared to the unmodified OGN. The results show significant shifts in retention time, reflecting the different OGN hydrophobicity in the order unmodified <C18 < biotin < phosphorothioate < Texas Red. These results are consistent with previous IP RP HPLC analysis of oligonucleotides with hydrophobic fluorophores [22–25]. The phosphorothioate OGN is notably broader when analysed under weak IP RP HPLC due to the large number of diastereoisomers present [18,20]. This may have implications for its use in the denaturing HPLC method if fractionating and collecting the phosphorothioate ssDNA for analysis. Collecting much broader peaks may involve splitting the fractions over multiple tubes and potentially decreasing yield.

To further analyse the effects of the 5′ modifications, analysis was also performed using strong IP RP HPLC utilising the larger alkylamine (tributylamine). Under these conditions size-based separations largely dominate and the influence of the 5′ modifications is expected to be reduced [18–20] The results are shown in Fig. 1B and show a reduced difference in retention time of the various 5′ modification compared to weak IP RP HPLC (Fig. 1A). However, the results show that a difference

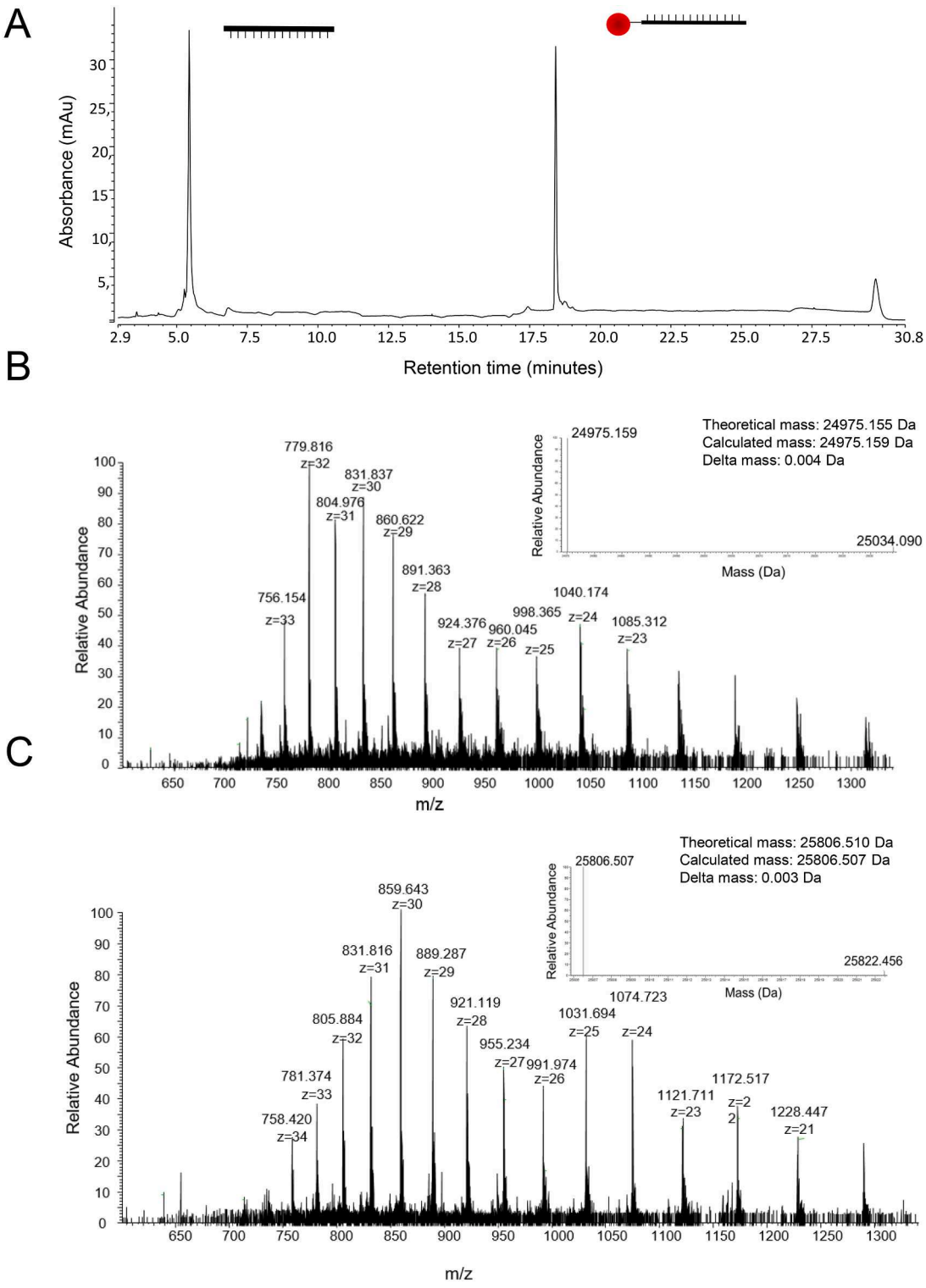

Fig. 3. Denaturing IP RP HPLC analysis. A) Texas Red labelled dsDNA was analysed using weak IP RP HPLC at 95 °C, UV detection 260 nm. Mobile phase A 0.2% TEA, 50 mM HFIP; mobile phase B 0.2% TEA, 50 mM HFIP in 20% ACN. Gradient 4, flow rate 0.2 ml/min. B) ESI MS spectra unlabelled ssDNA. C) ESI MS spectra Texas Red labelled ssDNA. The theoretical and calculated monoisotopic masses are highlighted. Deconvoluted masses are highlighted within the ESI MS spectra shown inset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in overall hydrophobicity of the OGNs is still observed under strong IP RP HPLC conditions, highlighting that fully size based separations are not achieved. The results also show that peak broadening of the phosphorothioate OGN is not seen in the strong ion pair analysis as the strength of interaction between the analyte column is much stronger hence structural features such as base sequence or structural isomerism have less impact on retention time. Consistent with weak IP RP HPLC analysis the phosphorothioate and 5′ Texas Red modifications result in the largest difference in retention compared to the unmodified OGNs. However, strong ion pair reagents are less volatile and therefore less suitable for the purification of ssDNA.

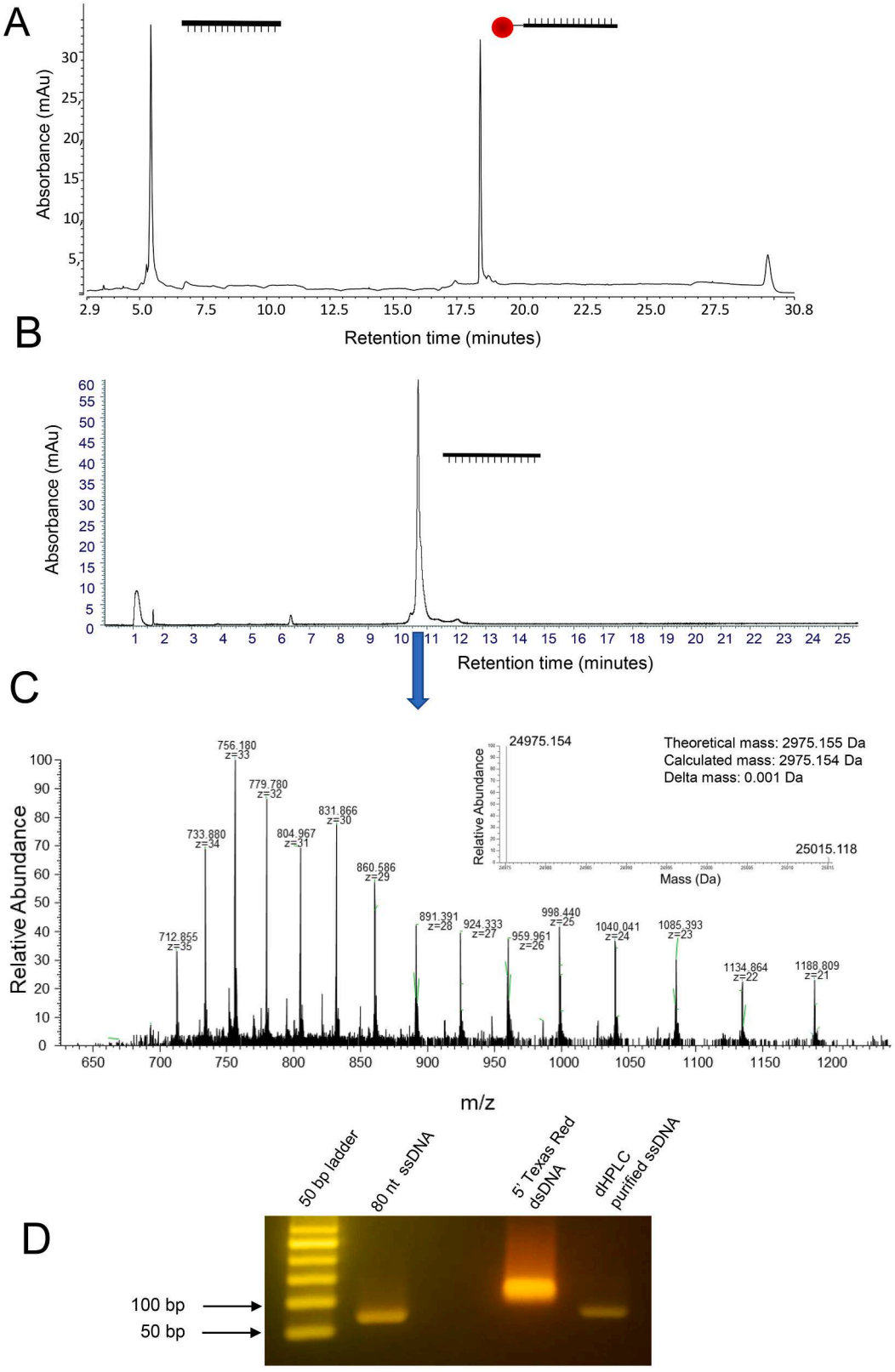

Fig. 4. Denaturing IP RP HPLC purification of ssDNA. A) IP RP chromatogram of the Texas Red labelled dsDNA at 95 °C, UV detection 260 nm. Mobile phase A 0.2% TEA, 50 mM HFIP; mobile phase B 0.2% TEA, 50 mM HFIP in 20% ACN. Gradient 4 flow rate 0.2 ml/min. B) LC UV chromatogram of the purified unlabelled ssDNA at 40 °C, UV detection 260 nm. Gradient 4 flow rate 0.2 ml/min. C) ESI MS spectra of the purified unlabelled ssDNA. The theoretical and calculated monoisotopic masses are highlighted. Deconvoluted masses are highlighted within the ESI MS spectra shown inset. D) Agarose gel electrophoresis analysis of the dsDNA and purified ssDNA. Staining was performed using Midori green direct. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. IP RP HPLC analysis of PCR products amplified using 5′ modified PCR primers

Analysis of the 5′ modified PCR primers via strong and weak IP RP HPLC showed that the phosphorothioate and 5′ Texas Red modifications had the greatest influence on the hydrophobicity of the oligonucleotides and subsequently lead to the greatest shift in retention time relative to the unmodified primer. Further experiments were performed to generate double stranded DNA (dsDNA) to demonstrate that using the 5′ chemically modified primer (reverse) did not decrease the yield of the PCR reaction or generate by-products, as this would prevent it from use in a SELEX protocol. PCR products were generated from each primer and analysed via agarose gel electrophoresis (see Fig. 2A). The results show the successful PCR amplification of the dsDNA using different 5′ modified primers. No significant difference in the yield or purity of the PCR products was observed in comparison to the unmodified control, demonstrating that in principle they are suitable for the PCR amplification stage of SELEX.

In addition, the effect of the 5′ modification on the overall hydrophobicity of the dsDNA PCR product was also investigated using IP RP HPLC (see Fig. 2B). It was expected that the contribution of the 5′ modification on the overall hydrophobicity would be reduced in larger PCR products, compared to shorter primer sequences due to the increased overall hydrophobicity of the longer dsDNA. The results show that consistent with IP RP HPLC analysis of the modified primers (see Fig. 1A) the 5′ phosphorothioate and Texas Red modifications cause the greatest shift in retention time due to their hydrophobicity. A broader peak for the 5′- phosphorothioate dsDNA was also observed due to the presence of the diastereoisomers consistent with previous observations.

3.3. Denaturing IP RP HPLC analysis of 5′ modified dsDNA

Further studies were performed using IP RP HPLC under denaturing conditions to denature the duplex dsDNA with the aim to resolve the single stranded DNA based on differences in their hydrophobicity due to the 5′ chemical modification. Initial work focussed on the analysis using denaturing IP RP HPLC in conjunction with TEAA as the mobile phase (see Fig. 3 and Supplementary Figure S1). The results for the 5′ Texas Red modified dsDNA analysed under denaturing conditions (95 °C) show the separation of the two single strands, with the 5′ Texas Red labelled strand eluting later due to the increased hydrophobicity.

To confirm the identity of the peaks and corresponding ssDNA, IP RP HPLC was interfaced with high resolution accurate mass spectrometry (see Figure 3B/C). The results confirm the 2 peaks are the single stranded DNA and the difference in mass was used to confirm that the later eluting species was the 5′ Texas Red modified ssDNA. Initial mass deconvolution of the two fragments generated accurate masses that did not match the expected theoretical mass of the DNA due to the addition of adenine to the 3′ end of each fragment via *Taq* DNA polymerase in the PCR reaction as described by McCarthy et al. [26]. Taking into account the addition of 3′ adenine to the DNA products led to parity between the theoretical and experimental mass for each ssDNA fragment. LC MS analysis of all the corresponding 5′ modified dsDNA under non-denaturing and denaturing conditions is shown in Supplementary Figures S2–12.

Following separation and validation of the ssDNA using denaturing IP RP HPLC, further work was performed to isolate and purify the ssDNA. 2 µg of 5′modified Texas Red dsDNA was separated and the unmodified ssDNA was fraction collected from the HPLC prior to freeze drying. Following purification of the ssDNA, further analysis was performed using IP RP HPLC and agarose gel electrophoresis (see Fig. 4). The results show the successful isolation and purification of ssDNA from dsDNA using IP RP HPLC under denaturing conditions which was validated using LC MS.

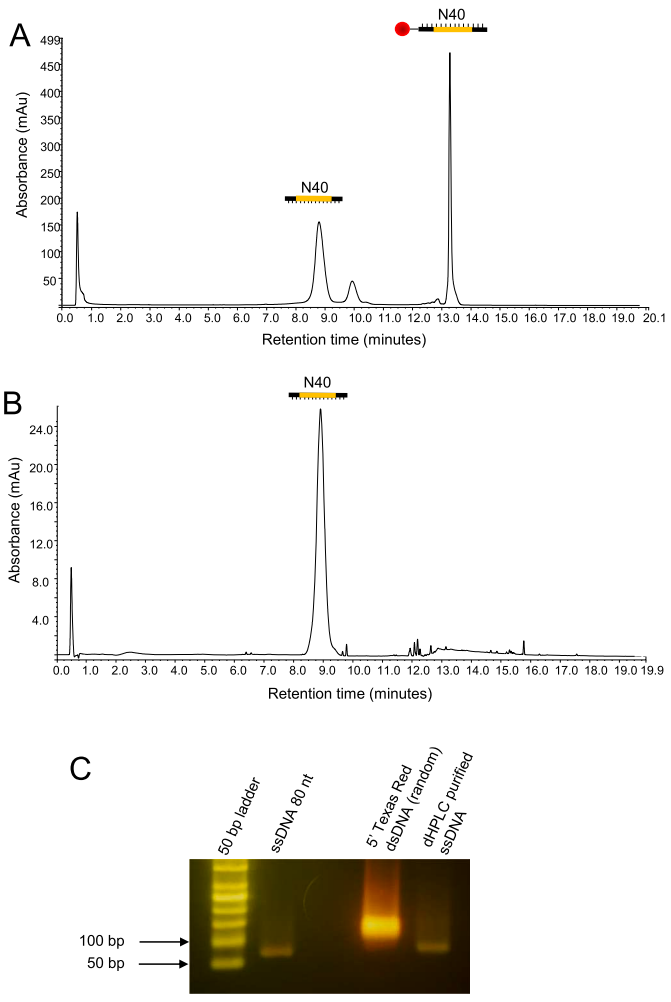

Fig. 5. Purification of ssDNA generated from a SELEX library. A) IP RP HPLC analysis of dsDNA generated from a SELEX library (random region N40) under denaturing conditions (80 °C). B) IP RP HPLC chromatogram of the purified ssDNA (N40). Mobile phase A 0.1 M TEAA, 50 mM HFIP, mobile phase B 0.1 M TEAA, 50 mM HFIP, 25% ACN Gradient 5 flow rate of 0.6 ml/min, UV detection 260 nm. 1500 ng of dsDNA and 100 ng ssDNA injected. C) Agarose gel electrophoresis analysis of the dsDNA and purified ssDNA. Staining was performed using Midori green direct. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Optimisation of denaturing IP RP HPLC conditions for the separation of ssDNA

Previous work had demonstrated that using TEAA as the mobile phase required the use of high temperatures (95 °C) to completely denature the dsDNA PCR product. Furthermore, it was noted that at lower temperatures it was not possible to denature the two DNA strands (see Supplementary Figure S2). G rich sequences are often identified or utilised in aptamer libraries, due to their ability to form G-quadruplexes which are desirable secondary structure elements of aptamers [27]. Therefore, to ensure that efficient denaturation was possible even for potential G rich sequences an alternative mobile phase system was investigated to enable more efficient denaturation of the dsDNA at lower temperatures. Initial work focused on the addition of 1,1,1,3,3,3,-Hexafluoro-2-propanol (HFIP) to the TEAA mobile phases. The addition of HFIP to the mobile phases has been shown to have a denaturing effect on duplexes, while also reducing the effect of oligonucleotide hydrophobicity upon retention[26] The results show that under these conditions effective denaturion was achieved at lower temperatures (80 °C) compared to previous high temperatures required using TEAA mobile

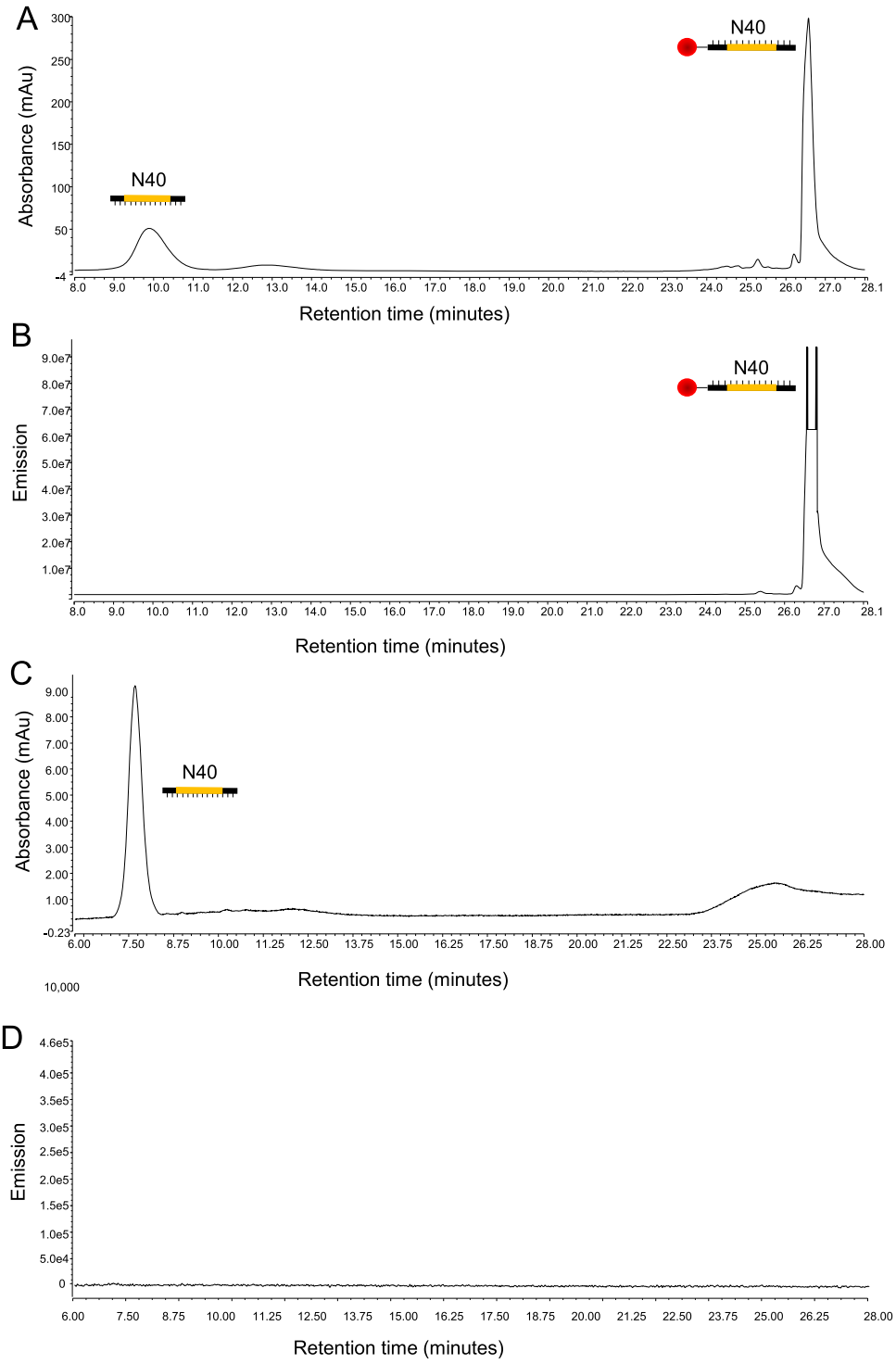

Fig. 6. IP RP HPLC analysis in conjunction with fluorescence detection. Denaturing IP RP chromatogram of the Texas red labelled dsDNA (N40) Gradient 6, 0.3 ml/min A) UV detection 260 nm B) Fluorescence detection: excitation 565 nm, emission 615 nm. Denaturing IP RP HPLC chromatogram of the purified ssDNA (N40). IP RP HPLC chromatogram of the purified ssDNA (N40). A) UV detection 260 nm B) Fluorescence detection: excitation 565 nm, emission 615 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phase (see Supplementary Figure S13). In addition, an alternative mobile phase system was employed using TEA/HFIP in conjunction with denaturing IP RP HPLC. Under these mobile phase conditions it was noted that the PCR products denature at much lower temperatures in comparison to TEAA (see Supplementary Figure S14).

The results show that the duplex dsDNA remains intact only at lower temperatures typically 30 °C and elevated temperature above this effectively denature the dsDNA. Therefore, effective denaturation using this mobile phase was observed at 40 °C (see Supplementary Figure S14). These results demonstrate that employing TEA/HFIP mobile phase or simply the addition of HFIP to the TEAA mobile phase enables effective denaturing of the dsDNA without the requirement for very high temperature and the requirement of specialised columns ovens that can operate at such temperatures. Furthermore, the ability to

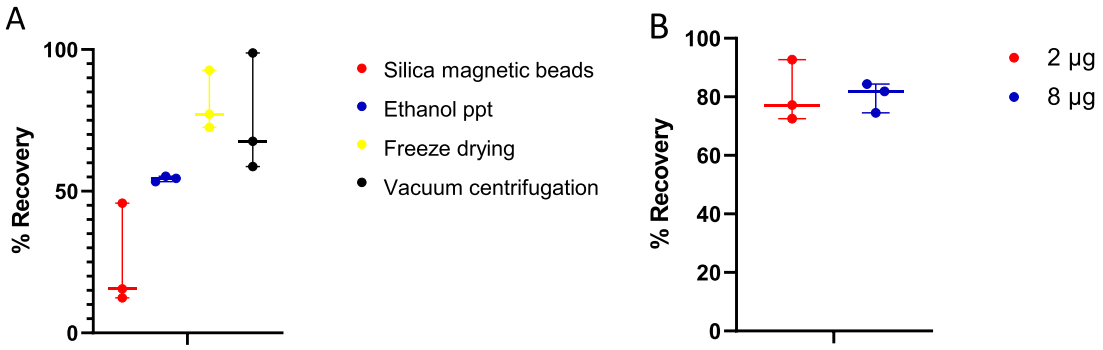

Fig. 7. Comparison of DNA purification methods.% recovery was determined using 2 µg of ssDNA purified using silica coated magnetic beads, ethanol precipitation, freeze drying, and vacuum centrifugation ($n = 3$). B):% recovery of 2 µg and 8 µg ssDNA using freeze-drying ($n = 3$).

denature the dsDNA more effectively at lower temperatures is beneficial for the isolation of ssDNA from PCR products with high melting temperatures and high GC content.

3.5. ssDNA library elution via denaturing HPLC

For application of the method in a SELEX protocol it must be successfully applied to the isolation of ssDNA from the PCR products of a SELEX library containing a random region of DNA. This presents a greater challenge compared to the non-randomised DNA template previously used as the DNA peaks observed on the IP RP HPLC will likely be broader due to the effects of the random region and further complicated by the presence of PCR by-products which are often generated in the PCR of DNA during SELEX.

Following the successful purification of ssDNA, the method was adopted for the analysis and purification of ssDNA generated from a random ssDNA library (N40) used in SELEX protocols. Texas Red labelled PCR product was generated from the random ssDNA library (N40) and analysed via denaturing HPLC (see Fig. 5A). The results show the separation of the two ssDNA strands as previously observed for the non-random DNA. A broader peak for the non-labelled ssDNA was observed as expected due to the presence of the random region (N40) and a large number of different sequences present. Furthermore, a potential PCR impurity (ssDNA of a different size) is also observed in the chromatogram. The non-labelled ssDNA was fraction collected, freeze dried prior to analysis using agarose gel electrophoresis and IP RP HPLC (see Figure 5B/C). The results show the successful purification of the ssDNA (N40) and furthermore demonstrates the purification of the ssDNA from potential larger length impurities that were generated in the PCR, which often hamper SELEX methods. In this case mass spectrometry analysis cannot be used to verify the sequences of the two ssDNA following denaturation of and separation of the ssDNA due to the random sequence nature. However, the presence of the 5′ Texas red group allows for fluorescence analysis of both the denatured PCR product and the purified ssDNA (see Fig. 6). The results show that as expected the later eluting ssDNA gave the corresponding fluorescence consistent with previous analysis. In addition, no fluorescence signal was observed for the purified ssDNA which lacks the Texas Red label.

3.6. Purification and fractionation of ssDNA using IP RP HPLC

Following purification of the ssDNA from SELEX PCR products using denaturing IP RP HPLC it is important to ensure that there is minimal sample loss during the removal of the mobile phase prior to re-suspension and folding of the ssDNA in the SELEX binding buffer. The criteria for the desalting method is that it must effectively remove salts that may interfere with the selection stage, must not be too laborious and must not significantly impact yield. Four alternative methods were assessed and the% recovery of the ssDNA were evaluated, including ethanol precipitation, capture on silica coated magnetic beads [28,29] and removal of the volatile mobile phases using either rotary evaporation or freeze drying. In each case equal mass of the ssDNA was collected via HPLC and the% recovery of the ssDNA determined for each of the different methods (see Fig. 7). The results show that the highest yield recovered was obtained using freeze-drying (80.8 ± 10.6%). The freeze-drying method was taken through to the higher scale, comparison of the yield of the 2 µg and 8 µg scales resulted in similar % yields of the ssDNA. Therefore, these results demonstrate that high recovery and yields of ssDNA can be obtained using denaturing IP RP HPLC in conjunction with freeze drying. Furthermore, such approaches enable purification of the correct size single stranded DNA from potential PCR artefacts commonly generated in SELEX procedures. Verification of the ssDNA obtained was confirmed by both mass spectrometry and fluorescence analysis. A comparison was also performed using streptavidin-biotin affinity chromatography to purify ssDNA. The yield was determined to be 55% consistent with previous reports [30]. Verification of the ssDNA isolated using streptavidin-biotin affinity chromatography was performed using LC MS. Therefore, these results demonstrate that a higher yield of ssDNA is obtained using denaturing IP RP HPLC optimised in this workflow in comparison to streptavidin-biotin affinity chromatography.

4. Conclusions

One of the main challenges when performing successful SELEX procedures to generate ssDNA aptamers, is the ability to generate high-quality ssDNA in high yields from the dsDNA PCR products. In this study, we have developed and optimised denaturing IP RP HPLC for the purification of ssDNA demonstrating significant advantages over existing affinity chromatography and nuclease digestion methods. We have optimised the PCR amplification utilising a PCR primer containing a variety of hydrophobic tags (5′ Texas Red, C18, Biotin and phosphorothioate modifications) which enables the separation of the ssDNA based on differences in their hydrophobicity. Optimal separations were achieved using a 5′ Texas Red labelled PCR primer to amplify DNA containing a random (N40) region. Furthermore, optimisation of the mobile phase conditions was also performed using with the addition of HFIP to the mobile phases which enables complete denaturation of the dsDNA at moderate temperatures and circumvents the requirement of high temperatures often required using TEAA. Validation of the ssDNA obtained was performed using high resolution accurate mass spectrometry analysis and fluorescence based HPLC detection. High yields and recovery (80%) of the ssDNA were obtained using freeze-drying to remove the volatile mobile phase. Furthermore this approach enables purification of the desired ssDNA of the correct size removing potential DNA artefacts often generated in the PCR amplification during SELEX procedures, therefore demonstrating advantages over current affinity chromatography and nuclease digestion methods.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.464699.

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