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Purification of a highly modified RNA-aptamer Effect of complete denaturation during chromatography on product recovery and specific activity

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Abstract

To evaluate RNA-aptamers as potential drug candidates, efficient and scaleable purification protocols are needed. Because aptamers are highly structured and rigid molecules, denaturation during the purification process is a critical aspect to obtain a pure and active product. A two-step chromatographic procedure was developed to purify a synthetic anti-VEGF aptamer at the preparative scale. A reversed-phase chromatographic step was optimized with a highly hydrophobic ion pairing reagent, followed by ion-exchange chromatography in which heat and a chaotropic salt were used. Because of the presence of 2′-modified ribose, denaturation conditions had to be optimized in both chromatographic steps to achieve a fully active molecule.

Keywords: RNA-aptamers

1. Introduction

One of the most recent approaches to developing nucleic acid based diagnostics or therapeutic drugs utilizes a process, known as the SELEX process (Systematic Evolution of Ligands by EXponential enrichment) [1], that can identity specific RNA and DNA molecules that bind uniquely and with high affinity to protein targets [2]. Targets have included small molecules [3,4] as well as proteins such as basic fibroblast growth factor [5], vascular endothelial growth factor [6], thrombin [7], elastase [8], L-selectin [9], keratinocyte growth factor [10], platelet-derived growth factor [11], antibodies [12,13] interferon γ [14], human phospholipase A_2 [15] and wheat germ agglutinin [16]. Some of these nucleic acids ligands, known as aptamers, bind to their target with a picomolar dissociation constant and many of them show inhibitory activity in cell assays [9,10,14]. Like peptides, the specific binding observed with single stranded nucleic acids is due to the ability of these molecules to adopt very sophisticated three dimensional conformations. In contrast to peptides, where in addition to the backbone, the side

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chains have numerous conformational states, oligonucleotides have a tendency toward greater rigidity. This rigidity is due to two characteristics of nucleic acids: first, only the backbone (sugar ring, phosphodiester and glycosidic bond) has torsional freedom and second, base pairing can restrict the allowed conformations of the molecule [17]. From a purification prospective, such intra- and inter-molecular associations may be a problem when a high yield of pure product is required. Therefore a better understanding of the conditions required for a complete denaturation of the aptamer can be very useful when new purification procedures are developed.

One potentially troubling aspect of nucleic acids as therapeutics is that nucleic acid polymers are known to be highly susceptible to enzymatic degradation. In serum, the half life of an unmodified RNA molecule is less than a minute. It has been shown that the stability of such molecules can be greatly improved by chemical modification of the ribose ring at the 2′-position [18] or by changing the phosphodiester backbone into a phosphorothioate backbone [19].

These very diverse chemical modifications present new challenges for the synthesis and the purification of such 2′-modified nucleic acid molecules. In order to better understand the chemical mechanisms involved during the purification step of 2′-modified RNA molecules, we have deliberately used a starting material that contained an increased ratio of truncated molecules versus full length molecules. Using this approach, we were able to determine the kinds of interactions that exist between the different length molecules, and how denaturation conditions can modify these inter-molecular interactions. Furthermore, since the ultimate goal of aptamer production is to make compounds that are active in vivo, we have chosen for this study an aptamer against vascular endothelium growth factor (VEGF). This aptamer has shown inhibitory activity against VEGF [20], which is believed to be involved in tumorogenesis [21].

The anti-VEGF aptamer used in this study (NX213) has been designed to be highly resistant to nucleases [20,22]. This molecule is a 36-mer RNA with a phosphodiester backbone except for four thymine nucleosides at the 5′-terminus and four thymine nucleosides at the 3′terminus that have a phosphorothioate backbone. NX213 has ten 2′-amino-pyrimidine, ten 2′*O*-methyl purines and four 2′-hydroxy purines.

Therapeutic development of aptamers requires that methods be developed and optimized to manufacture these highly rigid and highly modified nucleic acid ligands at large scale and at high purity. In this article we describe a purification methodology, performed under denaturing conditions, that allowed the production of several hundred milligrams of NX213. The effects of different denaturation conditions used during the reversed-phase and the ion-exchange chromatographic step on the product recovery and on the specific activity of the aptamer are reported. The final product was 99% pure based on analyses by anion-exchange chromatography and 95% pure based on capillary electrophoresis. Mass spectroscopy analyses showed that the purified aptamer had the correct molecular weight. An in vitro competition VEGF-binding assay showed the purified aptamer to be fully active.

2. Material and methods

2.1. Chemical compounds

Triethylamine (TEA), tetrabutylammoniumn bromide (TBABr), acetic acid, lithium chloride (LiCl), sodium chloride (NaCl), sodium perchlorate ($NaClO_4$), urea, HEPES, sodium thiocyanate (NaSCN), Trizma Acetate, EDTA, acetonitrile (ACN), were reagent grade or better and supplied by Aldrich (Milkwaukee, WI, USA). 2′*O*-Methyl ribose, 2′-hydroxy-ribose and 2′amino-ribose phosphoramidites were supplied by Glenn Research, Cruachem (Dulles, VA, USA) and Millipore (Bedford, MA, USA).

2.2. Aptamer sequence and structure

Although we have shown recently that optimized large scale synthesis could yield to a high ratio of full length product in the crude synthesis solution by utilizing DCI as an activator instead of tetrazole [23], we have used, in our study a non-optimized synthesis protocol. The idea behind this approach was that a

starting material with a high ratio of short molecules over full length product would be more adequate to show differences between the different purification protocols that were investigated.

The following sequence was synthesized: 5′-TsTsTsTs mAaCaCa aCaUrG rA aUmGrG aUmAmG rAaCmG aCaCmG mGmGmG aUmG TsTsTsTsT-3′, where aU and aC stand for 2′-aminouridine and 2′-aminocytidine, mG and mA stand for 2′*O*-methylguanosine and 2′-*O*-methyladenosine, rG and rA stand for guanosine and adenosine, T stands for thymidine and s stands for a phosphorothioate internucleotide linkage. The underlined bases represent the bases involved in the presumed secondary structure of NX213, a bulged hairpin. The Tm of the molecule was 46°C.

2.3. Analytical HPLC

Full length oligonucleotide ratios in the different samples were estimated by HPLC analysis on a weak anion-exchange column (Gen-Pak FAX, 4.6 mm× 100 mm, Waters corporation, Milford, Massachusetts, USA) heated at 80°C. Absorbance was monitored at 260 nm. HPLC equipment: solvent delivery system with a column heater, a 717 autosampler, a 496 single wavelength detector and Millennium 2010 chromatography manager software (Waters). Buffer A: 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5 with 10% ACN. Buffer B: 10 m*M* Tris–HCl, 1 m*M* EDTA, 1*M* NaCl, pH 7.5 with 10% ACN. The addition of 10% ACN to the chromatographic buffers as well as chromatography performed at elevated temperature was utilized to minimize the presence of secondary structure in the oligonucleotide [24]. Flow rate was 1 ml/min.

After the injection (10–30 μg), the column was washed for 5 min with buffer A+10% buffer B. A linear gradient from 10% to 90% buffer B was run through the column over 30 min. The column was washed with 100% buffer B for 5 min and re-equilibrated with buffer A+10% buffer B.

2.4. Aptamer/VEGF competitive binding assay

All assay procedures were performed at ambient temperature (19°C). Microtiter plates (White breakable combiplate, Labsystems, Needham Heights, MA, USA) were prepared by overnight incubation with 50 μl/well of a 50 m*M* sodium bicarbonate buffer, pH=9.6, containing 0.4 mg/ml of $VEGF_{165}$ (Research and Diagnostic Systems; Minneapolis, MN, USA). Plates were blocked for 1 h with TBS buffer (25 m*M* Tris–HCl pH 7.5, 140 m*M* NaCl) containing 0.5% fish skin gelatin and were then washed with binding buffer (TBS containing 0.5% gelatin and 0.05% Tween-20).

For each aptamer tested, serial dilutions were made with binding buffer such that the each dilution contained a 10 n*M* final concentration of the biotinylated VEGF-binding aptamer NX-0121 [10]. Dilutions (100 μl) were added to duplicate wells of the VEGF plate and allowed to incubate for 2 h. Wells were washed three times with binding buffer and then each well received 50 μl of a 1:1000 dilution of alkaline phosphatase conjugated streptavidin (Boehringer Mannheim Corp., Indianapolis, IN, USA). Following a 30 min. incubation, the plate was washed and substrate added. After a 20 min. incubation, chemiluminescence was determined by measuring light levels over 1 s. Data were fit to a sigmoidal dose-response curve.

2.5. Purification method development

Reversed-phase conditions were optimized on a 10 μm particle size, 75 Å porosity, nonfunctionalized polystyrene-divinyl-benzene column (4.1 mm×250 mm, PRP1, Hamilton, Reno Nevada, USA). Flow rate was 1 ml/min. Different ion-pairing reagents were tested: triethylamine (TEA, 100 m*M*) buffered at pH 7.5 with acetic acid and tetrabutyl ammonium bromide (TBABr) at various concentrations (10, 50 and 100 m*M*), in 1 m*M* Tris–HCl buffered at pH 7.5. ACN was used as the eluant.

Conditions for the ion-exchange chromatographic step were optimized on a 15 μm particle size, quaternary amine column (Resource Q, 6.4 mm×30 mm, Pharmacia). Flow rate was 1 ml/min. For the temperature effect study, the column was equilibrated for 30 min at the desired temperature and a blank run was done to prevent any carry over between runs. Several salts were tested for their effect on resolution and denaturation: NaCl, LiCl, $NaClO_4$ and NaSCN. Solutions were buffered with

25 m*M* Tris–HCl, pH 7, except when LiCl was used (25 m*M* HEPES, pH 7). Because of the temperature dependence of the p*K*a of Tris–HCl (dp*K*a/dT= −0.028/°C) and HEPES (dp*K*a/dT=−0.014/°C), solutions were buffered at room temperature to account for the effect of the temperature on the pH during column elution. Absorbance was monitored at 280 nm for NaSCN buffers and at 260 nm for all other buffers.

2.6. Preparative reversed-phase chromatography

The preparative chromatography system included: a Delta Prep 4000 solvent delivery system, a single wavelength detector, a preparative fraction collector and Millennium 2010 chromatography manager software (Waters). The absorbance was monitored at 285 nm. The purification was performed at room temperature on a 12–20 μm particle size, 75 Å column (PRP1, 50 mm×250 mm, 491 ml, Hamilton). Flow rate was 150 ml/min (458 cm/h).

Organic solvent contained in the crude synthesis solution was evaporated and the solution was filtered. The column was washed with 10 column volumes (cv) of water at 150 ml/min and equilibrated with 5 cv of 50 m*M* TBABr, 1 m*M* Tris–HCl pH 7.5. After loading of the crude synthesis solution, the column was washed with 2 cv of buffer A followed by 5 cv of 40% ACN in buffer A. A 20 cv gradient was run through the column from 40% ACN to 70% ACN. The column was cleaned with 100% ACN for 5 cv. Fractions (600 ml) were taken along the gradient and analyzed (OD reading at 260 nm and ion-exchange chromatography analysis). Selected fractions were pooled and ACN was evaporated.

For desalting of the purified material obtained after the ion-exchange chromatographic step, a 500 ml reversed-phase column (Amberchrom GC-300md, Toso Haas, Montgomeryville, PA, USA), 35 μm particle size, polystyrene-divinyl-benzene matrix was packed in a glass column (300 mm×50 mm, AP glass column, Waters) according to the manufacturer's recommendations. The column was equilibrated with 1 *M* NaCl for 5 cv and the material was loaded. The media was washed with 5 cv of salt solution and 10 cv of deonized water. The material to be desalted was eluted in one step with 20% ACN in water. The final product was then aliquoted and lyophilized.

2.7. Preparative ion-exchange chromatography

The preparative equipment was as stated above except for the pump delivery system (Prep LC 2000, Waters). One litre of Source 15Q (Pharmacia) was packed in a stainless steel column (Fineline 100, Pharmacia) according to the manufacturer's recommendations.

The separation was run at elevated temperature and absorbance was monitored at 285 nm. The buffers were brought to 88°C by single pass stainless steel heat exchangers. With a flow rate of 250 ml/min, the column temperature settled at 70°C in 90 min. As soon as the temperature of the outlet reached 70°C, the column was equilibrated with buffer A for 5 cv.

The reversed-phase purified pool was filtered on a 0.45 μm filter, heated to 70°C and loaded on the column. The column was washed with 5 cv of buffer A (25 m*M* HEPES, pH 7.8) at 250 ml/min. A first gradient from 0 to 30% buffer B (25 m*M* HEPES+1 *M* NaSCN, pH 7) was run over 5 cv. Then, a more shallow gradient, from 30% to 40% buffer B was applied over 10 cv and 1 cv fractions were taken.

3. Results

3.1. Outline of the purification scheme

Ion pairing reversed-phase chromatography was used as the initial step both to purify and to eliminate most of the free salts present in the crude oligonucleotide solution. Following this step, the bulk of the purification was accomplished by ion-exchange chromatography. Finally, the purified oligonucleotide was desalted by reversed-phase chromatography and the final product was lyophilized.

3.2. Analysis of the starting material

After synthesis and deprotection, the RNA sample was analyzed by ion-exchange chromatography to

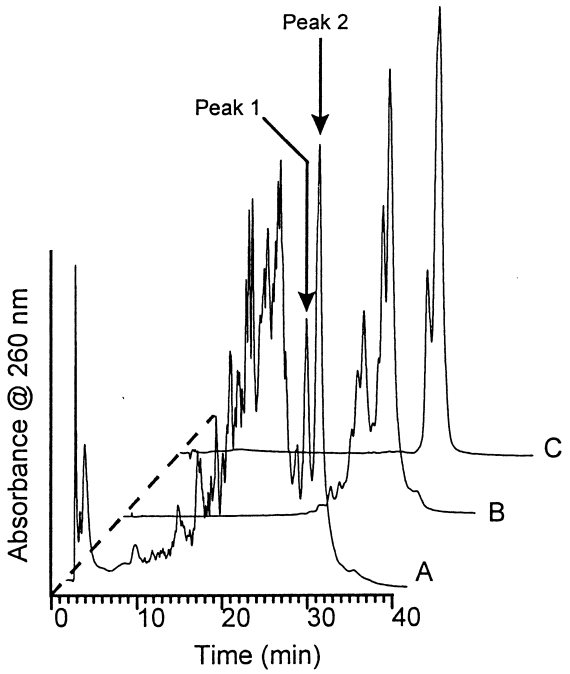

Fig. 1. Ion exchange chromatographic profiles of the crude synthesis before purification (A), the reversed-phase purified pool (B) and the final purified product (C).

assess the starting purity (Fig. 1A). Because of their chemical characteristics, the phosphorothioate oligonucleotides have longer retention times on anion-exchange and reversed-phase chromatography than the corresponding phosphodiester oligonucleotide [25,26]. Peaks 1 and 2 (Fig. 1A) were isolated at the analytical scale and subjected to mass spectroscopy analyses. They showed masses of 10744 AMU and 10758 AMU respectively (data not shown). The theoretical MW of NX213 is 10759 Daltons. Thus the mass difference between these two peaks is 14 AMU and it is likely that peak 2 represents the aptamer NX213 with eight phosphorothioate internucleotide linkages whereas peak 1 represents the aptamer NX213 with one sulfur (MW 32.066) replaced with an oxygen molecule (MW 15.9994), leading to a molecule with seven phosphorothioate internucleotide linkages.

Both peaks contain the complete sequence and have shown the same activity in the in vitro inhibition assay. Considering both peaks as product, the starting material has an estimated purity of 38%.

3.3. Chromatographic methods development

3.3.1. Effect of the nature of the ion-pairing reagent on reversed-phase chromatography

We have focused our work on two ion-pairing salts with different hydrophobicity characteristics: TEA and TBA. TEA is less hydrophobic than TBA. Selection criteria for the optimum ion pairing reagent were its ability to resolve the full length product from the failure sequences and its effect on the overall recovery of the full length product.

When the purification was performed with 100 m*M* TEA as ion pairing reagent, all oligonucleotides eluted between 15% and 25% ACN (Fig. 2A). However when 100 m*M* TBABr was used, the elution of the oligonucleotides began with 35% ACN and 75% ACN was required for complete elution of the full length product (Fig. 2B).

Comparison of Fig. 2A and B shows that, even though the ACN gradient was shallower with TEA (0.3%/min) than with TBABr (1%/min), TBABr salt allowed a much higher resolution than TEA, particularly within the range of ACN where the short sequences were eluted.

Analyses of the peak eluting at 72′ (Fig. 2A, TEA) indicated 67% full length recovery and was 75% pure whereas, analyses of the peak eluting at 79′ (Fig. 2B, TBABr) indicated a 68% full length recovery and was 86% pure. Interestingly, when TEA salt was used, there was a significant amount of material eluting later than the full length oligonucleotide. This late peak, eluting at 82′ (Fig. 2A), was found to be a mixture of full length oligonucleotides and failure sequences.

To further optimize the ion pairing reversed-phase purification of NX213, we have tested different concentrations of TBABr (10, 50 and 100 m*M*). As the concentration of the salt increased from 10 m*M* to 50 m*M* the separation of the full length oligonucleotide from related impurities was improved but no difference was observed between 50 m*M* and 100 m*M* TBABr. As expected, as the ion pairing reagent concentration increased, higher concentrations of ACN were required to elute the product: 70% and

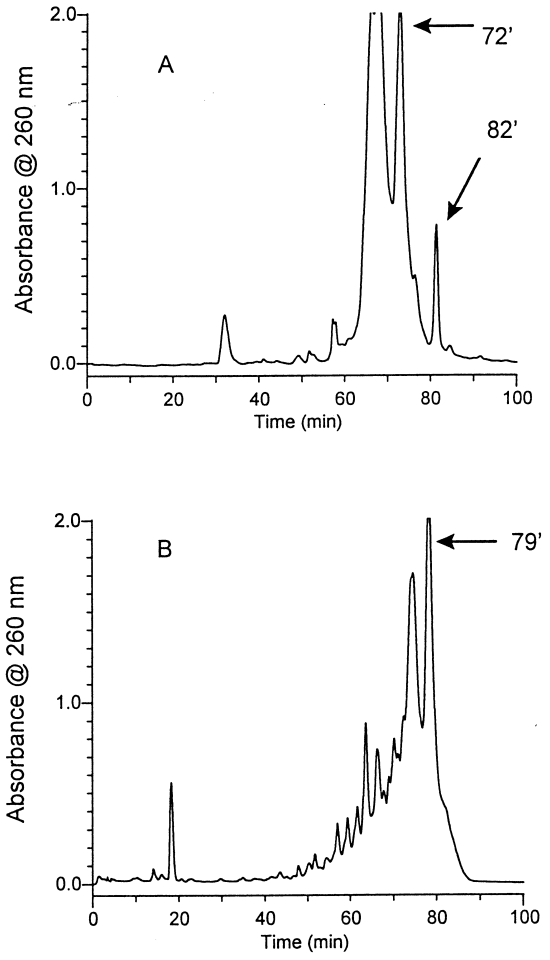

Fig. 2. Effect of the nature of the ion pairing reagent on the separation profile of the crude aptamer solution. Ion pairing reagent: 100 m*M* TEA buffered at pH 7.5 with acetic acid (A) or 100 m*M* TBABr buffered at pH 7.5 with 1 m*M* Tris–HCl (B). Peak at 82′ (A) is a mixture of full length oligonucleotide and failure sequences, indicating an incomplete denaturation with TEA. B shows a better resolution and a better denaturation with TBABr. Conditions: The PRP1 column (see Material and method) was equilibrated with 10 cv of the ion pairing solution at 1 ml/min. 21 OD's (0.8 mg) of the crude synthesis solution were loaded on the column. Absorbance was monitored at 260 nm. A: ACN gradient run over 30 cv from 0% to 30% ACN (0.3%/min). B: ACN gradient run over 30 cv from 0% to 90% ACN (0.9%/min).

76% ACN with 10 m*M* and 50 m*M* TBABr respectively (data not shown). We chose a concentration of 50 m*M* TBABr for subsequent optimization of the reversed-phase purification step.

The shape of the gradient was then optimized and we found that a gradient from 40% to 70% ACN gave the best full length recovery and the highest purity

3.3.2. Optimization of the ion-exchange chromatographic step

To achieve a greater purity of full length product, an anion-exchange chromatographic step was developed. Because of the ability of the NX213 molecule to form stable secondary and tertiary structures, denaturing conditions had to be optimized in order to improve the separation.

A common method to purify oligonucleotides under denaturing conditions is to use elevated temperature and buffers with high concentrations of polar denaturants that have a high polar moment like urea [27] or formamide [28,29,30]. When such conditions were tested, the purified NX213 molecule showed a dramatic reduction in target binding activity as determined by the VEGF in vitro competition binding assay (Fig. 3). Analyses of the base composition of the purified material obtained with urea or formamide at elevated temperature showed more peaks than the ones expected from the NX213

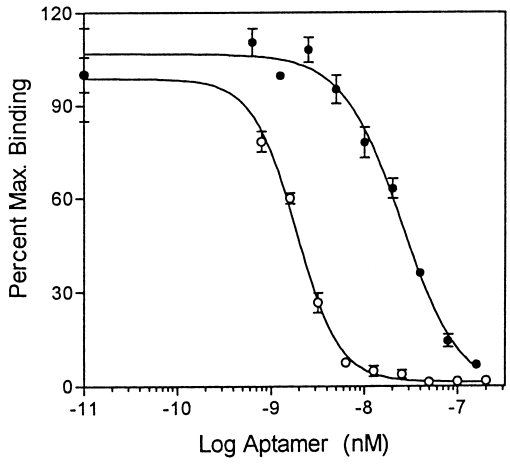

Fig. 3. In vitro VEGF-aptamer inhibition assay. The relative VEGF-binding affinities of the RNA-aptamer purified using urea (●) or NaSCN (○) were compared by competition analyses. Each point represents the mean of three determinations and error bars indicate the standard error of the mean. Data were fit to a four-parameter logistic equation. IC_{50} of the urea purified aptamer (IC_{50}: 23.7 n*M*) was decreased by 15-fold as compared to the NaSCN purified aptamer (IC_{50}: 1.57 n*M*).

sequence (data not shown). Using HPLC nucleoside standard, analyzed before or after incubation with urea or formamide at 60°C for 18 h, we were able to determine that urea and formamide were reacting with the amine moiety at the 2′ position of the ribose. NMR studies corroborated these hypotheses (data not shown). Attempts to purify NX213 with buffers containing urea at room temperature did not result in sufficient resolution for a practical separation step. Thus, utilization of urea or formamide as a denaturant to purify an active 2′-amino pyrimidine RNA aptamer was not feasible. Consequently, we chose to investigate using chaotropic salts denaturation.

For this study, the reversed-phase purified NX213 was used as the starting material. The effect of the temperature on the chromatographic profile in presence of a non-denaturing salt (NaCl) is shown in Fig. 4. As the temperature was increased from 60°C to 90°C (by 10°C step increment), the peak that eluted between 40 min and 45 min tended to diminish until it completely disappeared when the column was heated at 90°C. The peak obtained at 60°C was collected and analyzed. It was a mixture of full length and shorter length molecules, indicating a possible hybridization between these different species. With non-denaturing salt, the column had to be heated at 90°C in order to totally denature the oligonucleotide. Under these conditions, a salt concentration of 900 m*M* was necessary to elute the full length product.

Denaturing salts (NaSCN, LiCl and $NaClO_4$) were also tested. At 60°C and below, the same phenomenon was observed: the last peak to be eluted was not the full length oligonucleotide but a mixture of short molecules and product. With each of these salts, complete denaturation was achieved at 70°C. At this temperature, the concentration of salt required to elute the full length molecule was 770 m*M* with LiCl and 400 m*M* with NaSCN or $NaClO_4$. It is interesting to note that even with a high concentration of chaotropic salt, high temperature was still necessary to achieve complete denaturation of the oligonucleotide molecules.

These three salts behaved very similarly but they have different physical and chemical properties. $NaClO_4$ can be an explosion hazard, especially when heated, and is, therefore, not suitable for large scale purification. LiCl was not suitable either because, at elevated temperatures, chloride ions attack stainless steel particularly if the stainless steel has not been passivated. Therefore, we further optimized the chromatographic conditions using NaSCN although it strongly adsorbs at 260 nm. Salt concentration as well as shape of the gradient and column temperature were extensively investigated and optimized at small scale until the resolution and the recovery of the full length product were satisfactory.

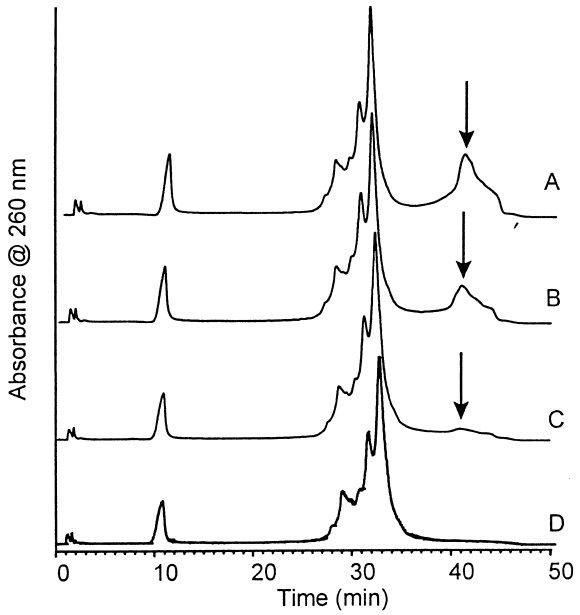

Fig. 4. Effect of the temperature on the ion-exchange chromatographic profile, in presence of non denaturing salt (NaCl). 60°C (A), 70°C (B), 80°C (C), 90°C (D). The arrows indicate the oligonucleotide fraction that was not totally denatured. This fraction diminishes as the temperature increases. Conditions: See Material and Methods. After loading (2 OD's), the column was washed with 10% buffer B (25 m*M* Tris–HCl+1 *M* NaCl) for 10 min (1 ml/min). Then, a gradient was run over 25 cv from 10% buffer B to 90% buffer B. Column regenerated with 100% buffer B for 10 cv before equilibration with 100% buffer A (25 m*M* Tris–HCl). A blank run was done before each experiment.

3.4. Preparative scale purification

3.4.1. Reversed-phase step

In order to purify several hundred milligrams of product, the method was scaled using TBABr. A 150 fold increase in scale was carried out on a 500 ml

column. We found that a larger particle diameter (20 μm) did not adversely affect the column resolution and therefore decided to use a larger particle size during preparative chromatography to both reduce backpressure and cost (data not shown).

The crude material was loaded on the preparative PRP1 column and fractions were collected along the ACN gradient. Fractions that contained a percent purity greater than 60% were pooled and the ACN was evaporated. The results of this purification are summarized in Table 1. The final percent purity of the reversed-phase purified pool was 73% (Fig. 1). Recovery of the full length product was greater than 95%. A purification factor of 1.9 was obtained.

3.4.2. Ion exchange step

The reversed-phase pooled fraction was filtered and 21 000 A_{260} units (777 mg) were loaded on the anion-exchange column. The purification was performed with NaSCN at elevated temperature as described in Material and Methods. Fractions with full length to failure ratio greater than 9:1 were pooled and the solution was desalted. The full length recovery achieved by the anion-exchange chromatographic step was 67%.

After desalting by reversed-phase chromatography (see Material and methods), 390 mg of product were obtained. The overall purification process yield of the full length oligonucleotide was 65%.

Table 1
Summary of the preparative reversed-phase chromatographic step[a]

	Total amount (mg)	Purity	Full length recovery
Starting material	1700	38%[b]	–
Reversed-phase pool	870	73%[c]	>95%

[a] Conditions: Column (PRP1, 50 mm×250 mm) equilibrated with Buffer A (1 m*M* Tris–HCl+50 m*M* TBABr, pH 7.5) for 5 cv. After quenching and evaporation of THF, the NX213 crude solution was filtered through a 0.45 μm sterile filter. The solution (500 ml) was loaded on the column (92 OD's/ml or 3.4 mg/ml). After loading, the column was washed with 5 cv of 40% ACN in buffer A. Flow rate: 150 ml/min (460 cm/h). Gradient from 40% to 70% ACN in buffer A over 15 cv. 4 min. Fractions (600 ml) were collected along the gradient. The column was cleaned with 5 cv ACN, depyrogenated and stored in 30% ACN in water.

[b] See Fig. 1A.

[c] See Fig. 1B.

3.5. Characterization of the purified material

As expected, ion-exchange HPLC analyses of the final product (Fig. 1C) showed two peaks. Mass spectroscopy analyses of the final product indicated two masses. The main peak (69%) had the higher mass (10 758 AMU) and represented the full length oligonucleotide with eight phosphorothioate internucleotide linkages (theoretical MW: 10 759) whereas the lower mass peak (30%, 10 744 AMU) represented the same sequence, but with seven phosphorothioate internucleotide linkages instead of eight. Assuming that these two peaks are full length oligonucleotides, the purity of the final material was 99% as determined by analytical ion-exchange HPLC.

The final product was also analyzed by capillary electrophoresis and appeared as two peaks representing the molecule with eight (65%) and seven (30%) phosphorothioate internucleotide linkages respectively (data not shown). By this technique, the final product was 95% pure.

Finally, the purified product was tested for its biological activity in vitro, using the VEGF competition binding assay and showed the expected activity: $EC_{50}=9\times10^{-10}$ M.

4. Discussion

Nucleic acids have emerged as promising new therapeutic drug candidates. The strategy of using antisense nucleic acids to prevent the expression of an undesired protein is currently being developed by several pharmaceutical companies and clinical trials are ongoing [31]. Other approaches have been taken in which the catalytic properties of certain kinds of nucleic acids have been utilized to specifically target and cleave intermediate molecules (mRNA) involved in protein expression [32]. More recently, the SELEX technology [1] has expanded the use of nucleic acid based therapeutics to protein targets [2]. As gram scale of nucleic acid are needed, methods of increasing the quality of large scale synthesis and purification processes need to be developed.

The overall production yield as well as the activity of the final product will depend upon both the synthesis and the purification quality. It is therefore

critical to optimize large scale purification schemes that will give the highest purity product (no $n-1$ or $n+1$ molecules), with good activity (no incomplete deprotection and no chemical modification).

There are few articles describing the purification of RNA molecules and most of them deal with purification of small RNA oligonucleotides (<25-mers) [33] or RNA molecules with the dimethoxy trityl (DMT) group attached at the 5′-terminus [34,35]. The hydrophobic nature of the DMT group allows separation by reversed-phase chromatography of the DMT-on full length molecule from the DMT-off failure sequences. The DMT group is then cleaved under acidic conditions and the oligonucleotide is subsequently purified, if necessary, by anion-exchange chromatography. Although common, this technique has several disadvantages upon scale-up. First, capping failures are co-purified with the full length material and, second, acidic detritylation of the DMT-on purified material can generate 2′, 3′-cyclic phosphate intermediates that can lead either to the cleavage of the molecule or to a 2′–5′ phosphoryl migration [36,37]. However, it is fair to say that this last point is subject to controversy and there are groups who have claimed no significant 2′–5′ migration during detritylation [38].

The high affinity and high specificity of aptamers obtained with the SELEX technology is mainly due to the highly structured shapes that nucleic acids molecules can adopt because of Watson–Crick as well as non Watson–Crick interactions [17]. Consequently, short sequences generated during the synthesis of the oligonucleotide, during the cleavage from the solid support or during the deprotection of the synthetic oligonucleotides, may hybridize with the full length product. On a chromatographic column some of these complexes can behave as longer oligonucleotides and thus they are eluted after the full length molecule. This phenomenon appears if the conditions used to purify the full length oligonucleotide are not denaturing enough to disrupt all possible secondary structure interactions. Possible secondary structures of the molecule are therefore important characteristics to understand when developing a purification protocol. The more structured the molecule, the more difficult the separation between the full length product and the abortive synthetic products.

In this article, a purification protocol is described in which method optimization was based upon conditions that allowed each separation step to be performed under conditions that completely denature the aptamer. We first optimized a reversed-phase step to both purify and desalt the full length aptamer from the crude synthesis. This step also eliminates any aptamers that are not completely deprotected. We have focused our work on the effect of the nature and the concentration of the ion-pairing reagent and have found that TBABr was a more efficient ion at separating the full length molecule from the failure sequences than a less hydrophobic and widely used ion pairing reagent, TEA. It is likely that the enhanced separation obtained with TBABr was due to the strong hydrophobicity of this reagent and therefore to the higher concentration of ACN required to elute the full length molecule. When the concentration of organic solvent increases, the dielectric constant of the mixture is lowered and the hydrogen bond network is disturbed. Concomitantly, as the dielectric constant is reduced, the electrostatic interactions between ions increase [39]. Therefore, addition of ACN to the aqueous buffer will cause the phosphate groups to repel one another and to induce the denaturation of the oligonucleotide. When intra- and inter-molecular interactions are disrupted, we expect a well behaved chromatographic system in which the nucleic acids are eluted strictly according to their length.

To further purify the reversed-phase product, a denaturing anion-exchange chromatographic step was developed. Even though the T_m of the full length molecule is relatively low (46°C) [22], heat was necessary to fully denature the oligonucleotides even in the presence of a strong denaturant. When classical denaturation conditions were used, such as high concentration of urea or formamide along with heat, the purified 2′-modified aptamer was no longer active. This was due to the chemical modification of the amine at the 2′ position of the ribose. Therefore, other denaturation conditions had to be developed.

According to the Hofmeister series, salts can be described as antichaotropic if they promote the ordering of water or as chaotropic if they have the opposite effect [40]. The former will force the hydrophobic residues to get closer to each other and are non-denaturing salts whereas the latter will force

the hydrophobic residues to be more exposed to the solvent and ultimately will cause denaturation of the macromolecule. Consequently, different salts were tested for their ability to break any secondary structure within the oligonucleotide and ultimately to increase resolution and recovery of product. By performing the column chromatography at elevated temperature and using the appropriate denaturing buffers, the overall full length molecule recovery and the final purity of the product was improved.

The purified product is a mixture of two major products. Although these two products are full length molecules, they differ in their phosphorothioate content. As described by Bergot and Egan [41], during the generation of phosphorothioates by oxidative sulfurization, some oxygenated phosphodiesters are produced concomitantly. In our case, we co-purified the full length aptamer containing either seven or eight phosphorothioate internucleotide linkages. Both molecules were shown to be active in an in vitro binding inhibition assay (data not shown).

Using this purification procedure we have obtained 390 mg of a pure and active anti-VEGF synthetic aptamer. This amount of high quality product will allow us to further characterize the biological properties of the NX213 aptamer using a variety of in vitro and in vivo models.

References

[1] C. Tuerk, L. Gold, Science 249 (1990) 505.
[2] L. Gold, B. Polisky, O. Ulhlenbeck, M. Yarus, Annu. Rev. Biochem. 64 (1995) 763.
[3] A.D. Ellington, J.W. Szostak, Nature 346 (1990) 818.
[4] R.D. Jennison, S.C. Gill, A. Pardi, B. Polisky, High-resolution molecular discrimination by RNA, Science 263 (1994) 1425.
[5] D. Jellinek, C.K. Lynott, B.D. Rifkin, N. Janjic, Proc. Natl. Acad. Sci. 90 (1993) 11227.
[6] D. Jellinek, L.S. Green, C. Bell, N. Janjic, Biochemistry 33 (1994) 10450.
[7] M. Kubik, A.W. Stephens, D. Schneider, R.A. Marlat, D. Tasset, Nucl. Acids Res. 22 (1994) 2619.
[8] Y. Lin, A. Padmapriya, K.M. Morden, S.D. Jayasena, Proc. Natl. Acad. Sci. 92 (1995) 11044.
[9] D. O'Connell, A. Koenig, S. Jennings, B. Hicke, H. Han, T. Fitzwater, Y. Chang, N. Varki, D. Parma, A. Varki, Proc. Natl. Acad. Sci. 93 (1996) 5883.
[10] N. Pagratis, C. Bell, Y. Chang, S. Jennings, T. Fitzwater, D. Jellinek, C. Dang, Nature Biotech. 15 (1997) 68.
[11] L.S. Green, D. Jellinek, R. Jennison, A. Ostman, C. Heldin, N. Janjic, Biochemistry 35 (1996) 14413.
[12] T.W. Wiegand, P.B. Williams, S.C. Dreskin, M.H. Jouvin, J.P. Kinet, D. Tasset, J. Immunol. 157 (1996) 231.
[13] S. Lee, B.A. Sullenger, Nature Biotech. 15 (1997) 41.
[14] M. Kubik, C. Bell, T. Fitwater, S.R. Watson, D. Tasset, J. Immunol. 159 (1997) 259.
[15] P. Bridonneau, Y. Chang, D. O'Connell, S.C. Gill, D.W. Snyder, L. Johnson, T. Goodson Jr., D.K. Herron, D.H. Parma, J. Med. Chem. 41 (1998) 778.
[16] P. Bridonneau, Y. Chang, A. Velati-Bellini Buvoli, D.H. Parma. Antisense & Drug Dev., in press.
[17] B. Eaton, L. Gold, D. Zichi, Chem. & Biol. 2 (1995) 633.
[18] W. Pieken, D.B. Olsen, F. Benseler, H. Aurup, F. Eckstein, Science 253 (1991) 314.
[19] G. Zon, W. Stec, in: F. Eckstein (Ed.), Oligonucleotides and Analogues. A Practical Approach, IRL Press, Oxford, 1991, p. 87.
[20] D. Jellinek, L.S. Green, C. Bell, N. Janjic, Biochemistry 33 (1994) 10450.
[21] K.J. Kim, B. Li, J. Winer, M. Armanini, N. Gillett, H.S. Phillips, N. Ferrara, Nature 362 (1993) 841.
[22] L.S. Green, D. Jellinek, C. Bell, L.A. Beebe, B.D. Feistner, S. Gill, F.M. Jucker, N. Janjic, Chem. & Biol. 2 (1995) 683.
[23] C. Vargeese, J. Carter, J. Yegge, S. Krivjansky, A. Settle, E. Kropp, K. Peterson, W. Pieken, Nucl. Acids Res. 26 (1998) 1046.
[24] W. Warren, J. Vella, in: S. Agrawal (Ed.), Methods in Molecular Biology, Vol. 26, Humana Press Inc, Totowa, NJ, 1994, p. 233.
[25] W.J. Stec, G. Zon, J. Chromatogr. 326 (1985) 263.
[26] S. Agrawal, J.Y. Tank, D.M. Brown, J. Chromatogr. 509 (1990) 396.
[27] T.R. Jonhson, J. Ilan, Anal. Biochem. 132 (1983) 20.
[28] M.J. Gait, C. Pritchard, G. Slim, in: F. Eckstein (Ed.), Oligonucleotides and Analogues. A Practical Approach, IRL Press, Oxford, 1991, p. 25.
[29] L. McLaughlin, N. Piel, in: M.J. Gait (Ed.), Oligonucleotides Synthesis. A Practical Approach, IRL Press, Oxford, 1990, p. 117.
[30] J. Duhamel, D.M. Liu, C. Evilla, N. Fleysh, G. Dinter-Gottlieb, Nucl. Acids Res. 24 (1996) 3911.
[31] M.D. Matteucci, R.W. Wagner, Nature 384 (1996) 20.
[32] R.E. Christoffersen, J.J. Marr, J. Med. Chem. 38 (1995) 2023.
[33] B. Sproat, F. Colonna, B. Millah, D. Tsou, A. Andrus, A. Hampel, R. Vinayak, Nucleosides and Nucleotides 14 (1995) 255.
[34] F. Wincott, A. DiRenzo, C. Shaffer, S. Grimm, D. Tracz, C. Workman, D. Weedler, C. Gonzalez, C. Scaringe, N. Usman, Nucl. Acids Res. 23 (1995) 2677.
[35] A. DiRenzo, S. Grimm, K. Levy, P. Haeberli, L. Maloney, N. Usman, F. Wincott, Nucleosides and Nucleotides 15 (1996) 1687.
[36] D.C. Capaldi, C.B. Reese, Nucl. Acids Res. 22 (1994) 2209.
[37] M.A. Morgan, S.A. Kazakov, D.M. Hecht, Nucl. Acids Res. 23 (1995) 3949.

[38] B. Mullah, A. Andrus, Nucleosides and Nucleotides 15 (1996) 419.
[39] A. Streitwieser, C.H. Jr, in: Introduction to Organic Chemistry, Macmillan Publishing Co., Inc, 1976, p. 164.
[40] R.E. Shansky, S. Wu, A. Figueroa, B.L. Karger, in: K.M. Gooding, F. Regnier (Eds.), HPLC of Biological Macromolecules, Marcel Dekker, Inc, NY, 1990, p. 95.
[41] B.J. Bergot, W. Egan, J. Chromatogr. 599 (1992) 35.