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Characterization of small interfering RNA by non-denaturing ion-pair reversed-phase liquid chromatography

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ABSTRACT

Small interfering RNAs (siRNA) are emerging as a novel therapeutic modality for the specific inhibition of target gene expression. siRNA are typically formed by annealing of two complementary single stranded oligoribonucleotides. Compared to purity determination of non-hybridized single strands by denaturing chromatographic methods, characterization of the hybridized duplex is challenging. Here we are reporting a non-denaturing ion pairing-reversed phase (IP-RP) chromatography method capable of separating optimal duplex (full-length single strands only) from non-optimal duplex variants (containing shortmers, longmers and 2′,5′-isomers) using ultraviolet- and mass spectrometric detection. The impact of different annealing conditions on siRNA composition was investigated. Optimized annealing conditions lead to a significant increase in optimal duplex, while total duplex content remained constant. The non-denaturing method reported herein showed high mass spectrometric sensitivity and superior separation efficiencies compared to other IP-RP buffer systems. The method is useful for *in-process* control and release testing of therapeutic double stranded nucleic acids such as siRNA.

1. Introduction

RNA interference (RNAi) is a natural process to regulate gene expression that has initially been discovered in worm [1]. The same cellular mechanism has been described in plants [2] and fungi [3] and for almost all eukaryotic species including humans [4]. In a stepwise order of cellular events involving sequence dependent target recognition and enzymatic cleavage, RNAi leads to the down modulation of a specific messenger RNA. The natural RNAi mechanism can be triggered by so-called small interfering RNA (siRNA), short helical RNA molecules which are generated in the cell from larger double stranded RNA precursors by enzymatic nucleolysis [5]. RNAi can also be triggered very effectively by introducing chemically synthesized siRNA into living cells, circumventing several upstream RNA processing steps of the natural pathway [4]. RNAi has quickly become an important research tool in molecular biology to analyze protein function and to identify novel therapeutic targets. Various siRNA molecules are currently under clinical investigation as promising therapeutic agents to treat diseases including cancer, viral infection, and ocular disease [6–10].

siRNA molecules are formed by two at least partially complementary RNA single strands, namely the passenger strand and the guide strand. Typical strand lengths are 19–23 nucleotides. Single strands are chemically synthesized step-wise from the 3′ to the 5′ terminus employing conventional solid phase phosphoramidite chemistry [11,12]. After solid phase synthesis, the strands are cleaved from the support and deprotected. Subsequent chromatographic purification and desalting yield the purified single strands [13,14]. Among the expected impurities resulting from RNA synthesis are (a) shortmer sequences (N − x; e.g. N − 1 or N − 2) [15–17], (b) longmer sequences (N + x; e.g. N + G or N + A) [18] and (c) sequences containing phosphodieser linkages (PO) instead of phosphorothioate linkages [19]. In addition, 2′,5′-isomers can be generated when ribonucleicacids are subjected to high temperature and/or basic pH [20,21]. The final siRNA duplex is formed by mixing the two purified single strands in near equimolar ratio in aqueous solution, typically followed by a heating and cooling phase (i.e. annealing). Strand association is mediated non-covalently *via* base-pairing and stacking interactions. The resulting siRNA typically contains a duplex fraction, including optimal duplex (two full-length single strands) and non-optimal duplex variants (containing at least one single strand impurity and/or mismatched sequences) as well as a single strand fraction, which comprises all non-hybridized single strands.

Numerous chromatographic methods have been reported for the physicochemical characterization of single stranded oligonucleotides as well as the hybridized duplex. The employed tech-

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niques include denaturing anion exchange (AEX) HPLC [22–25], denaturing ion-pairing reversed-phase (IP RP) HPLC [17,26–29], non-denaturing AEX HPLC [30–32]; and non-denaturing IP RP-HPLC [33–37].

One important advantage of IP RP chromatography over AEX chromatography is that this technique can be combined with mass spectrometric (MS) detection. IP RP chromatography with electrospray ionisation mass spectrometry (ESI-MS) has been used extensively to study single and double stranded oligonucleotides and a wide range of ion-pairing reagents have been used for the interfacing of IP RP HPLC to ESI MS (reviewed in: [38]). Buffer systems that have been successfully used for separations of nucleic acids include triethylammonium bicarbonate (TEAB) [34], butyldimethylammonium bicarbonate (BDMAB)/butyldimethylammonium acetate [39,40] and cyclohexyldimethylammonium acetate (CycHDMAA) [41]. Buffers containing triethylammonium acetate (TEAA), triethylammonium (TEA)/1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) [29,26] and hexylammonium acetate (HAA) [42] in particular have been reported as giving excellent separation for single and double stranded oligonucleotides [27,42]. Because of its unique ion-pairing properties, high separation efficiency and high mass-sensitivity, TEA/HFIP is now used as widely as TEAA in the analysis of oligonucleotides. The use of HFIP in ion-pairing buffers is particularly attractive because it is compatible with ESI-MS and often provides excellent separation [26,29]. Another important feature of HFIP appears to be that it reduces the impact of oligonucleotide hydrophobicity upon retention [43].

In the analysis of siRNA, non-denaturing ion-pairing techniques have yet to achieve the level of peak separation that is commonly achieved using denaturing chromatography of single strands. Due to almost identical physico-chemical properties of optimal duplex and non-optimal duplex variants, chromatographic separation between these species is difficult to achieve. Insufficient chromatographic resolution is currently the main reason why non-denaturing techniques do not permit detection and quantification of individual duplex impurities. To our knowledge, two IP-RP HPLC methods have been reported which allow high resolution separations of siRNA or its metabolites under non-denaturing conditions: (a) IP-RP HPLC using a HAA buffer system [42] and (b) IP-RP HPLC based on a TEA/HFIP buffer system [33,44,45]. Both methods are compatible with ESI-MS detection. However, a method that combines the benefits of high duplex peak resolution with high mass spectrometric sensitivity has not yet been established.

In this work we describe the development of a highly efficient non-denaturing chromatographic method in combination with sensitive ESI-MS detection. The novel method was used to characterize duplex composition of siRNA samples before and after annealing. The method is useful for *in-process* control and release testing of therapeutic double stranded nucleic acids such as siRNA.

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN) (LC–MS grade), HFIP (puriss., p.a.), hexylamine (puriss., GC grade), acetic acid (puriss., p.a.) and triethylamine (puriss., p.a.) were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). 10× phosphate buffered saline (PBS) pH 7.4 was purchased from GIBCO (Life Technologies, Carlsbad, CA, USA). A Milli-Q 185plus apparatus (Millipore, Bedford, MA, USA) was used to prepare deionized water (>18 MΩ cm) for all solutions and mobile phases.

2.2. RNA

All single-stranded oligonucleotides were synthesized by conventional solid-phase synthesis. Sequences of full-length passenger strand (PS-Luc), full-length guide strand (GS-Luc) and their major impurity markers are listed in Table 1. siRNA-Luc is a double stranded RNA formed by PS-Luc and GS-Luc. The guide strand sequence is complementary to a region in the mRNA of firefly luciferase. All oligonucleotides were manufactured at Roche Kulmbach GmbH.

2.3. Annealing conditions

If not stated otherwise, 50 μM RNA solutions were annealed in 1× PBS by heating to 85 °C for 5 min followed by cooling to room temperature (RT) over a period of 3 h. Until analysis all solutions were stored at +2 to +8 °C. For annealing experiments 1000 μM siRNA solutions were either (i) heated to 85 °C for 5 min and snap cooled on ice (snap cooling), (ii) heated to 85 °C for 5 min and allowed to cool to RT over a period of 30 min (fast cooling), or (iii) heated to 85 °C for 5 min and allowed to cool to RT over a period of 3 h (slow cooling).

2.4. Size exclusion chromatography (SEC)

SEC was carried out on a Dionex Ultimate 3000 series HPLC system (Dionex, Sunnyvale, CA, USA) using UV-detection at 260 nm. Oligonucleotides were separated on a Superdex 75 column, 10/300 GL (GE Healthcare, Pollars Wood, UK) at room temperature. Mobile phase consisted of 1× PBS containing 10% ACN. The flow rate was 0.75 ml/min. Injection volume was 5 μl of a 50 μM RNA solution in 1× PBS.

2.5. IP-RP HPLC

For IP-RP HPLC separations, samples were applied to an Ultimate 3000 RS series HPLC system (Dionex, Sunnyvale, CA, USA) using UV-detection at 260 nm. Oligonucleotides were separated by ACN-gradient elution on an Acquity UPLC OST C18 2.1 × 100 mm column with a 1.7 μm bead-size (Waters, Milford, MA, USA). In the triethylamine (TEA)/HFIP system, mobile phase A consisted of 100 mM HFIP, 16.3 mM TEA and 1% methanol. Mobile phase B consisted of 100 mM HFIP, 16.3 mM TEA and 95% methanol. In the HAA system, mobile phase A consisted of 25 mM HA/acetate and 5% ACN. Mobile phase B was 80% ACN. During the scouting process, mobile phase A contained HA at concentrations of 5–30 mM and HFIP at concentrations of 50 mM, 100 mM or 150 mM. Mobile phase B was 80% ACN. The gradient was run from 5% to 35% of mobile phase B in 25 min at a flow rate of 250 μl/min. In the optimized system, mobile phase A consisted of 15 mM HA, 50 mM HFIP and 5% ACN. Mobile phase B consisted of 15 mM HA, 50 mM HFIP and 50% ACN. The gradient was run from 30% to 62% of mobile phase B in 25 min at a flow rate of 250 μl/min. Injection volume was 2 μl of a 50 μM RNA solution in 1× PBS. If not stated otherwise, column temperature was 20 °C.

2.6. Mass spectrometry

For mass-spectrometric analysis, an LCQ Deca XP+ Ion-Trap mass spectrometer equipped with a ESI interface (ThermoFisher Scientific, Waltham, MA, USA) was used in line with the IP-RP HPLC system. The mass spectrometer was run in negative ion mode, spray voltage was 4.5 V, capillary temperature was set to 315 °C, capillary voltage was set to −100 V and scanning mass range was from 600 to 2000 *m*/*z*. Data acquisition and analysis was performed using the

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Table 1
Single strands and impurity markers of siRNA-Luc. Upper case letters indicate 2′-OH RNA nucleotides, lower case letters indicate 2′-*O*-methyl nucleotides, TT in italic case indicate two 2′-deoxy-thymidine nucleotides connected *via* a phosphodiester linkage, TT in bold case indicate two 2′-deoxy-thymidine nucleotides connected *via* a phosphorothioate linkage. G in bold case indicate the additional guanine-nucleotides. Bold italic letters indicate the position of the 2′,5′-linkage.

	Sequence	Orientation	Description	Calc. mass
PS-Luc	cuuAcGcuGAGuAcuucGA**TT**	Passenger	Full-length strand	6778.5
PS-PO	cuuAcGcuGAGuAcuucGA*TT*	Passenger	PO variant	6762.4
Short-P1	uuAcGcuGAGuAcuucGA**TT**	Passenger	5′($n-1$) shortmer	6459.2
Short-P2	uAcGcuGAGuAcuucGA**TT**	Passenger	5′($n-2$) shortmer	6139.0
Long-P1	cuuAc**G**cuGAGuAcuucGA**TT**	Passenger	N+G (Pos1)	7123.7
Long-P2	cuuAcGcu**G**AGuAcuucGA**TT**	Passenger	N+G (Pos2)	7123.7
Long-P3	cuuAcGcuGAGuAcuucG**GA**TT	Passenger	N+G (Pos4)	7123.7
ISO-P1	cuu***A*** cGcuGAGuAcuucGA**TT**	Passenger	2′,5′-isomer	6778.5
Iso-P2	cuuAcGcu***G***AGuAcuucGA**TT**	Passenger	2′,5′-isomer	6778.5
GS-Luc	UCGAAGuACUcAGCGuAAG**TT**	Guide	Full-length strand	6752.4
GS-PO	UCGAAGuACUcAGCGuAAG*TT*	Guide	PO variant	6736.3
Short-G1	CGAAGuACUcAGCGuAAG**TT**	Guide	5′($n-1$) shortmer	6446.2
Short-G2	GAAGuACUcAGCGuAAG**TT**	Guide	5′($n-2$) shortmer	6141.0
Long-G1	UCG**G**AAGuACUcAGCGuAAG**TT**	Guide	N+G (Pos1)	7097.6
Long-G2	UCGAAGuACUcAGCGuAAG**GTT**	Guide	N+G (Pos5)	7097.6
Iso-G1	UCG***A***AGuACUcAGCGuAAG**TT**	Guide	2′,5′-isomer	6752.4
Iso-G2	UCGAAGuAC***U***cAGCGuAAG**TT**	Guide	2′,5′-isomer	6752.4
Iso-G3	UCGAAGu***A***CUcAGC***G***uAAG**TT**	Guide	2′,5′-isomer	6752.4

Xcalibur software as well as the ProMass deconvolution software (ThermoFisher Scientific, Waltham, MA, USA).

3. Results and discussion

3.1. Comparison of non-denaturing SEC and IP-RP HPLC methods

The aim of our study was to develop a chromatographic method that is capable of characterizing a typical siRNA (siRNA-Luc, 21mer strands, 3′-two nucleotide-overhangs, 2′OMe-modifications) by separation of duplex variants from optimal duplex. Ion-pairing buffer composition should be compatible with sensitive MS detection, to allow identification of the different duplex variants. Chromatographic methods based on TEA/HFIP or HAA [42] buffer systems, did not achieve sufficient separation of shortmer variants V1 and V2 from optimal duplex in the initial experiments (Fig. 1, traces 2 and 3). In addition, mass sensitivity of the HAA system was significantly lower than in buffer systems containing HFIP. Since the main reason for the low signal in the HAA system was considered to lie with the use of acetate as the buffering acid [29,46], a buffer system was employed that used HA as the ion-pairing agent in combination with HFIP. HFIP lends itself better to ESI-MS, primarily because it is more volatile than acetate and readily evaporates from the droplets produced during the electrospray process [38].

In a buffer system containing 25 mM HA and 100 mM HFIP a significant improvement of peak separation was observed compared to the TEA/HFIP and the HAA systems. Duplex variants V1–V3 were well separated from optimal duplex (Fig. 1, trace 1). Peak resolution (R_s) for V1 to optimal duplex was $R_s = 1.34$ in the HA/HFIP buffer system compared to $R_s = 0.82$ in the HAA system (Fig. 1, trace 2, Table 3). No separation was observed for the TEA/HFIP system (Fig. 1, trace 3). This effect was attributed primarily to the increased alkyl chain length and hydrophobicity of the ion-pairing reagent HA compared to TEA and its impact on the retention of the oligonucleotides [47]. An analogue improvement in duplex separation has been reported by McCarthy et al. In the chromatographic separation of siRNAs the weaker ion-pair reagent TEA/acetate was replaced by the stronger ion-pair reagent HA/acetate, resulting in higher separation efficiency [42]. In addition to the beneficial effect of HA on duplex separation, the hydrophobic character of HFIP itself may have lead to a higher adsorbed concentration of HA on the stationary phase, resulting in improved ion-pairing efficiency and, in turn, higher separation selectivity [26,29,38].

The duplex content values obtained with the IP-RP methods were compared to values obtained with SEC (Fig. 2). For a sample containing equimolar amounts of guide and passenger strand a total duplex content of 99.9% was recorded by all three methods. Total duplex content describes the sum of the relative peak areas of optimal duplex and all detected duplex variants. For a sample containing a small excess of guide strand, total duplex content was 97.2% as measured by SEC, 97.4% using HA/HFIP and 97.6% using HAA chromatography. For a sample containing a small excess of passenger strand, total duplex content was 98.5% by SEC, 98.5% using HA/HFIP chromatography and 98.7% using HAA chromatography. In all analyses, the duplex values obtained by the IP-RP HPLC methods were similar to the values obtained by SEC, indicating

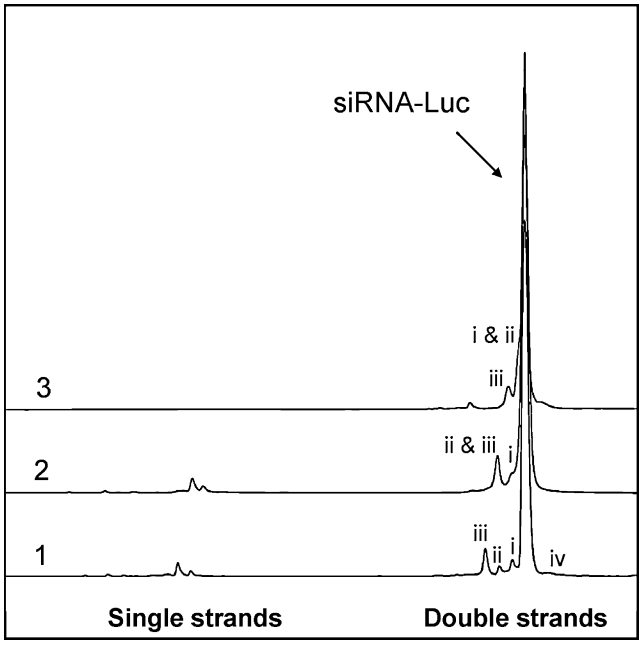

Fig. 1. Comparison of non-denaturing IP-RP HPLC methods. UV-traces of buffer systems containing 25 mM HA and 100 mM HFIP (trace 1), 25 mM HAA (trace 2) or 16.5 mM TEA and 100 mM HFIP (trace 3) are shown. The analyzed samples were siRNA-Luc spiked with 10% Short-P1. Non-hybridized single strands eluted earlier than the duplex (single strands). Peak i indicates duplex variant V1; peak ii indicates duplex variant V2, peak iii indicates duplex variant V3 and peak iv indicates late eluters.

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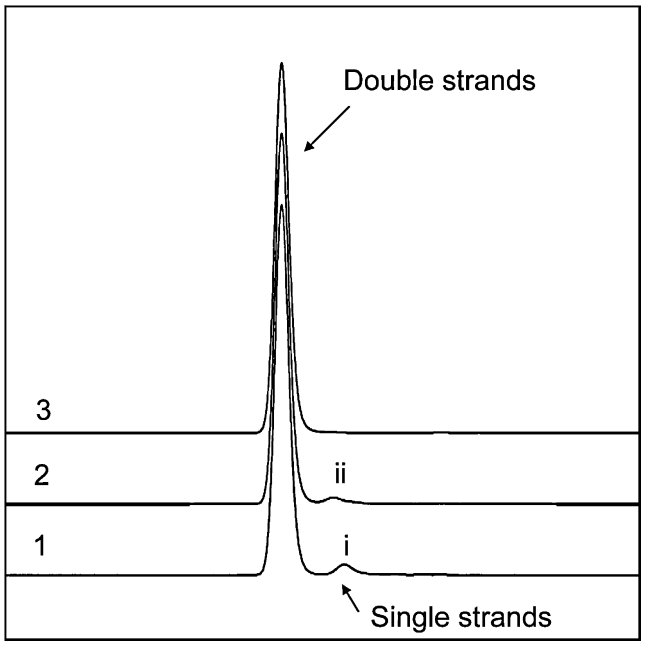

Fig. 2. SEC of samples containing a small excess of passenger strand (trace 1), a small excess of guide strand (trace 2) or equimolar amounts of guide and passenger strand (trace 3). Peak i indicates non-hybridized passenger strand; peak ii indicates non-hybridized guide strand.

that both IP-RP buffer systems were completely non-denaturing for pre-formed duplexes.

3.2. Duplex separation in IP-RP HPLC

Buffer optimization of the HA/HFIP system was performed using four duplex samples containing N – 1 and N – 2 shortmers (variants V1–V4; Table 2). Where possible, peak resolutions between optimal duplex and duplex variants were determined. Mobile phase A was prepared in steps of 5 mM HA up to a concentration of 30 mM HA. Acetonitrile was used as eluting agent (mobile phase B). In the 25 mM HA system peak resolution between V1 and optimal duplex was lower at 50 mM HFIP (R_s = 1.01) compared to 100 mM HFIP (R_s = 1.34). Increasing the HFIP content to 150 mM caused strong fluctuations of the operating pressure especially at higher acetonitrile concentrations during gradient elution (data not shown). This observation indicated limited miscibility of HA in aqueous solutions containing HFIP and ACN. Furthermore, increasing the concentration of HFIP was reported to impose a denaturing effect on duplexes [42], which would be undesired in non-denaturing applications. Hence, 100 mM HFIP was used for all further buffer preparations.

Table 2
Passenger and guide strands of siRNA-Luc duplex variants V1–V16 and respective RRT.

Duplex ID	Passenger ID	Guide ID	RRT
siRNA-Luc	PS-Luc	GS-Luc	1
V1	PS-Luc	Short-G1	0.984
V2	PS-Luc	Short-G2	0.967
V3	Short-P1	GS-Luc	0.950
V4	Short-P2	GS-Luc	0.945
V5	PS-Luc	GS-PO	0.974
V6	PS-PO	GS-Luc	0.984
V7	Long-P1	GS-Luc	0.952
V8	Long-P2	GS-Luc	1.045
V9	Long-P3	GS-Luc	nd
V10	PS-Luc	Long-G1	0.977
V11	PS-Luc	Long-G2	0.985
V12	ISO-P1	GS-Luc	0.975
V13	Iso-P2	GS-Luc	1.025
V14	PS-Luc	Iso-G1	nd
V15	PS-Luc	Iso-G2	nd
V16	PS-Luc	Iso-G3	0.939

Table 3
Effect of buffer composition on peak resolution. Average peak resolutions of optimal duplex, V1, V2 and V3 peaks in non-denaturing IP-RP HPLC using different HA concentrations and 100 mM HFIP in mobile phase A.

Peak	25 mM	5 mM	10 mM	15 mM	20 mM	25 mM	30 mM
Resolution	HAA	HA	HA	HA	HA	HA	HA
siRNA-Luc/V1	0.82	nd	0.93	1.33	1.46	1.34	1.12
V1/V2	1.03	nd	0.31	1.03	1.44	1.51	1.46
V2/V3[a]	0,685[a]	nd	0.75	1.17	1.54	1.68	1.66
Average	0.85	nd	0.66	1.18	1.48	1.51	1.41

nd = low peak separation, peak resolution values were not determined.
[a] Peak resolutions were calculated using V1, V2 and V4 peaks, since V2 and V3 peaks were co-eluting.

Separation efficiency of the different buffer systems was determined by comparing the averages of the peak resolutions. Resolution R_s for every neighbouring pair of peaks is defined as $R_s = (t_r, 2 - t_r,1)/(\omega_2 + \omega_1)/2$. t_r and ω stand for retention time and peak width at half peak height respectively. For better comparability, average peak resolutions were determined for each buffer system (Table 3). In the HA/HFIP system duplexes eluted in the order (a) variant V4 (relative retention time (RRT) = 0.945); (b) variant V3 (RRT = 0.950); (c) variant V2 (RRT = 0.967), (d) variant V1 (RRT = 0.984), and (e) optimal duplex (RRT = 1). Since RRT of V3 and V4 were too similar to determine peak resolution, only resolutions between optimal duplex and variants V1–V3 were determined. Average peak resolution values were calculated and plotted (Table 3 and Fig. 3). No or low resolutions were observed for optimal duplex and duplex variants V1 and V2 at HA concentrations of 5 mM and 10 mM respectively. A significant increase in resolution was recorded for buffer containing 15 mM HA. Resolution for optimal duplex and variant V1 was R_s = 1.33. The best separations were achieved with a buffer system containing 20 mM HA in mobile phase A (R_s = 1.46). When mobile phase A contained 25 mM HA, resolution was slightly lower (R_s = 1.34). Increasing HA concentration over 25 mM resulted in a decrease of peak resolutions. At the higher concentrations of ion-pairing agent, sor-

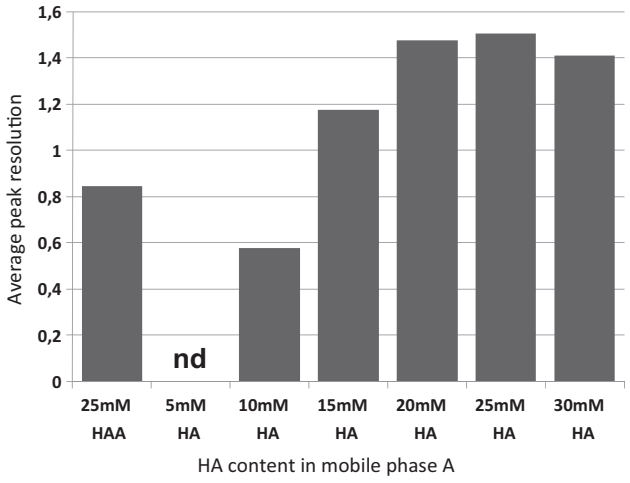

Fig. 3. Effect of buffer composition on peak resolution. Average peak resolutions of optimal duplex, V1, V2 and V3 peaks in non-denaturing IP-RP HPLC using different HA concentrations and 100 mM HFIP in mobile phase A. For the 25 mM HAA system, peak resolutions were calculated using optimal duplex, V1, V2 and V4 peaks, since V2 and V3 peaks were co-eluting. nd = due to low peak separation, peak resolution values could not be determined.

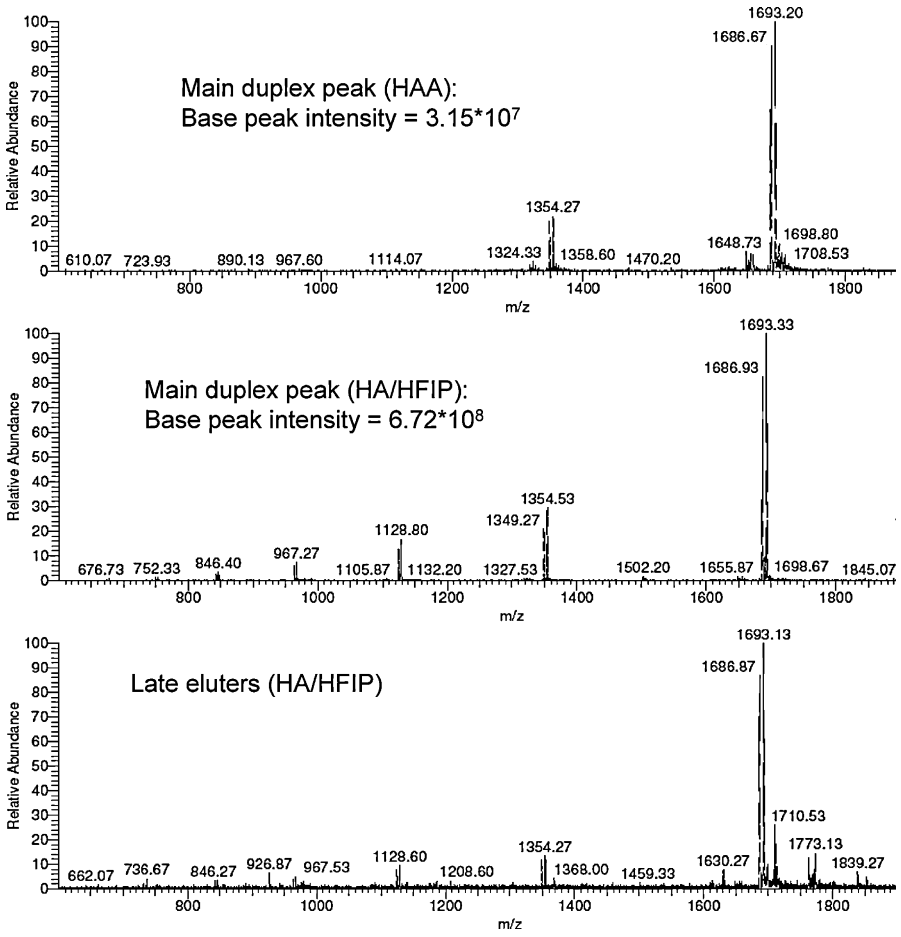

Fig. 4. MS spectra of siRNA-Luc after non-denaturing IP-RP HPLC. Upper panel: main peak in the buffer system containing 25 mM HAA. Middle panel: main peak in the buffer system containing 25 mM HA/100 mM HFIP. Lower panel: late eluting peaks in the buffer system containing 25 mM HA/100 mM HFIP. The signals at m/z = 1686.7, m/z = 1693.2, m/z = 1710.5 and m/z = 1773.1, represent fourfold charged GS-Luc (M_r = 6776.5), PS-Luc (M_r = 6751.5), PS + iBu (M_r = 6846.3) and GS + guanine (M_r = 7096.3) respectively.

bent surface may have become saturated, or micelles may have formed in solution, leading to a reduced availability of adsorption sites and hence decreased retention [47]. Based on these results, an optimized buffer system was developed, containing 15 mM HA and 50 mM HFIP in both mobile phases. The buffer composition represents the optimal concentration of ion-pairing reagents as established in the scouting runs, where mobile phase B did not include HA/HFIP. Peak resolution for optimal duplex and variant V1 was best at the lower temperatures (R_s = 1.42 at 15 °C and R_s = 1.35 at 20 °C). At column temperatures of 30 °C and above, partial dissociation of the main duplex was observed, resulting in peak broadening and decrease in resolution (data not shown).

3.3. Single-strand separation in IP-RP HPLC

In the HAA as well as the HA/HFIP buffer system, non-hybridized single strands eluted several minutes earlier than the duplex. Non-hybridized GS-Luc and its N – 1 shortmer (Short-G1) were separated to a resolution of R_s = 2.37 in the HAA system and to R_s = 1.22 using the HA/HFIP buffer. Separation between non-hybridized PS-Luc and its N – 1 shortmer (Short-P1) was R_s = 1.14 in the HAA system and R_s = 1.82 in the HA/HFIP buffer (data not shown). Hence, peak resolution in the HA/HFIP system was higher for PS-Luc and its shortmer, but lower for single stranded GS-Luc and its shortmer peak when compared to the HAA system. This apparent discrepancy was attributed to the physico-chemical properties of the individual single strands. In addition to their different base composition, full-length GS-Luc and PS-Luc differ significantly in the number of 2′-*O*-methyl-modifications. The passenger strand contains a total of eleven modifications, whereas the guide strand contains only three. As a result, PS-Luc is expected to be significantly more hydrophobic than GS-Luc. Short-P1 lacks one 2′-*O*-methyl-modified cytidine at the 5′-end, whereas the Short-G1 lacks an unmodified uridine. This results in divergent changes in the overall hydrophobicity of full-length strands and their shortmers. It is well established that retention times of single strands are sequence dependent [17,33,42,48]. HFIP has been described to reduce the impact of oligonucleotide hydrophobicity upon retention [43]. Consequently, in the HA/HFIP buffer system, the hydrophobicity of the single strands is expected to have a lower influence on peak separation than in systems containing acetate, where elution order of oligonucleotides have been reported to show dramatic changes with base sequence and hydrophobicity [43]. This is in accordance with the observations for the single strand separations in the HA/HFIP compared to the HAA system. The unique characteristic of HFIP to render the chromatography less dependent on base composition may also have benefited the analysis of the double stranded fraction of siRNA-Luc. Duplex separation has been reported to be sequence independent [42]. However, siRNA-Luc, like most siRNA, contains single stranded overhangs at the 3′-end. It is reasonable to speculate that the unpaired nucleotides of the overhangs contribute to the separation efficiency of siRNA in the HA/HFIP buffer system, possibly showing less sequence specificity than in acetate buffer systems.

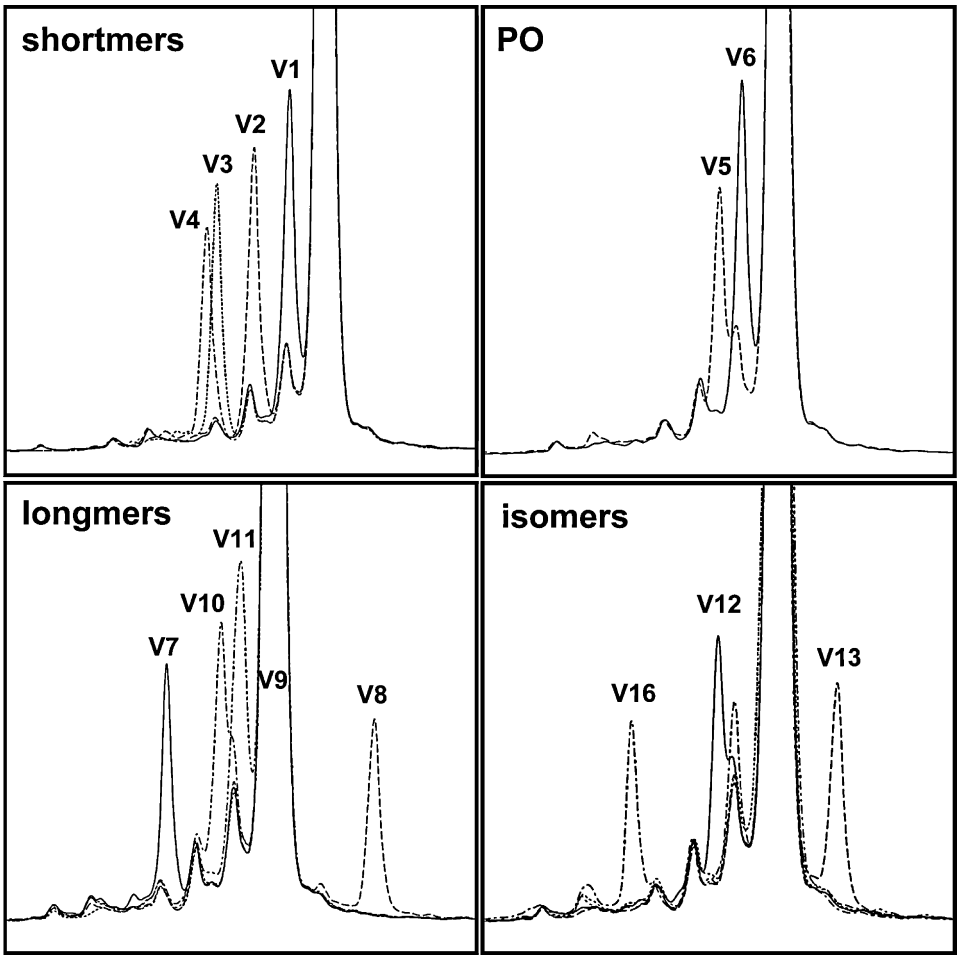

Fig. 5. Chromatographic separation of duplex variants from optimal duplex. UV-traces obtained with an optimized buffer system containing 15 mM HA and 50 mM HFIP in both mobile phases are shown. Solid lines indicate unspiked samples of siRNA-Luc; dashed and dotted lines indicate siRNA-Luc samples spiked with duplex impurity markers. Identity of all variants were confirmed by MS. Upper left: siRNA-Luc spiked with shortmers. Upper right: siRNA-Luc spiked with PO. Lower left: siRNA-Luc spiked with longmers. Passenger strand variant Long-P3 (M_r = 7123) was identified under the main peak by ESI-MS (V9). Lower right: siRNA-Luc spiked with isomers.

3.4. ESI-MS detection

In both buffer systems, HAA and HA/HFIP, full-length passenger and guide strand (PS-Luc and GS-Luc respectively) were identified under the main peak, as well as the duplex variants V1–V3 in the pre-peaks (Fig. 4, upper and middle spectrum). However, overall mass sensitivity in the HAA system was low compared to HA/HFIP (intensities of the duplex base peaks were 3.15×10^7 and 6.72×10^8 respectively). The low mass sensitivity in the HAA system was attributed to the quenching effect of acetic acid during mass spectrometric detection [38,49]. In the system containing acetate, no single strands other than PS-Luc and GS-Luc were detected eluting after the main peak. Similar to the results reported by McCarthy et al. [42], non-hybridized single strands, although not visible in the TIC, could only be detected by selective ion monitoring (data not shown).

In the HA/HFIP system, several mass signals were recorded after the main duplex peak. The identified masses of the late eluters included (a) GS-Luc and PS-Luc (molecular weight (M_r) = 6776.5 and M_r = 6751.5 respectively), (b) guide strand longmers containing one additional guanine-nucleotide (M_r = 7096.3) and (c) passenger strand with one additional isobutyryl protection group (M_r = 6846.3 spectrum) (Fig. 4, lower spectrum). The higher mass sensitivity in the system containing HFIP improved detection of low abundant duplexes and significantly facilitated identification of non-hybridized single strands.

3.5. Separation of non-optimal duplex variants

Four sets of siRNA-Luc samples containing typical duplex variants were analyzed using the optimized HA/HFIP buffer system (V1–V16, Table 2). Most siRNA duplex variants were separated from optimal duplex to baseline. All duplex variants containing shortmers (V1–V4) were separated from optimal duplex (Fig. 5, upper left panel). A similar level of peak separation was observed for the two duplex variants containing one phosphodiester linkage (PO) in either passenger or guide strand (V5 and V6; Fig. 5, upper right panel). Of the five duplex variants containing longmers (V7–V11), four were separated from the optimal duplex. One duplex variant (V9) eluted with the optimal duplex (Fig. 5 lower left panel), but could be identified using MS detection. Three of five duplex variants containing 2′,5′-isomers in either passenger or guide strand were separated from the optimal duplex (V12, V13 and V16; Fig. 5 lower right panel).

3.6. Impact of annealing conditions on siRNA composition

The annealing step is an integral part of the manufacturing of siRNA. During annealing, heating of a siRNA solution above its melting temperature leads to strand dissociation, resolving possible mismatched structures. Subsequent cooling of the mixture results in re-association of the single strands. A slow cooling process is expected to promote the formation of the thermodynamically

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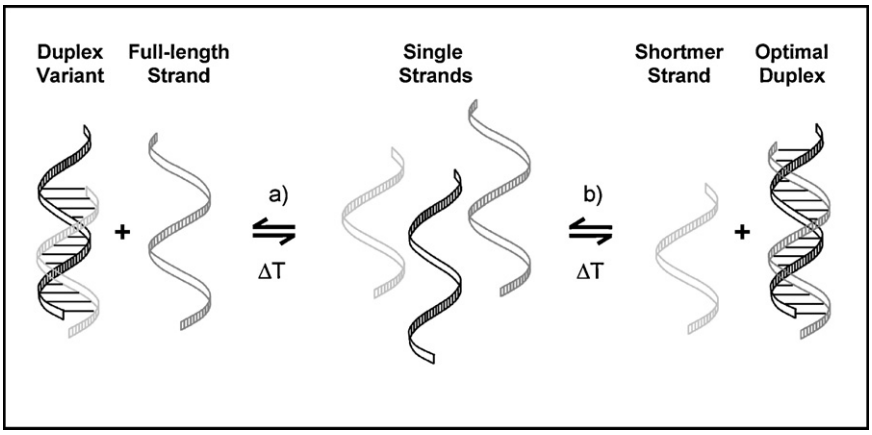

Fig. 6. Thermodynamically controlled annealing leads to formation of optimal duplex. (a) Heating of a siRNA leads to strand dissociation. Mismatched structures are resolved. (b) Cooling results in re-association of the single strands. A slow cooling process is expected to promote the formation of the thermodynamically preferred, optimal duplex.

preferred, optimal duplex. An annealing process that is under thermodynamic control should therefore increase the overall amount of optimal duplex in the annealing mixture (Fig. 6). However, the expected subtle changes in duplex composition of a typical siRNA sample during annealing have not yet been described in detail. This, in part, is due to the lack of high resolution non-denaturing analytical methods. The HA/HFIP method reported herein provides significantly improved peak resolution over previous methods and was therefore employed to characterize duplex samples before and after annealing.

Solutions of unheated 1000 μM siRNA-Luc were subjected to three different annealing procedures and analyzed for duplex con-

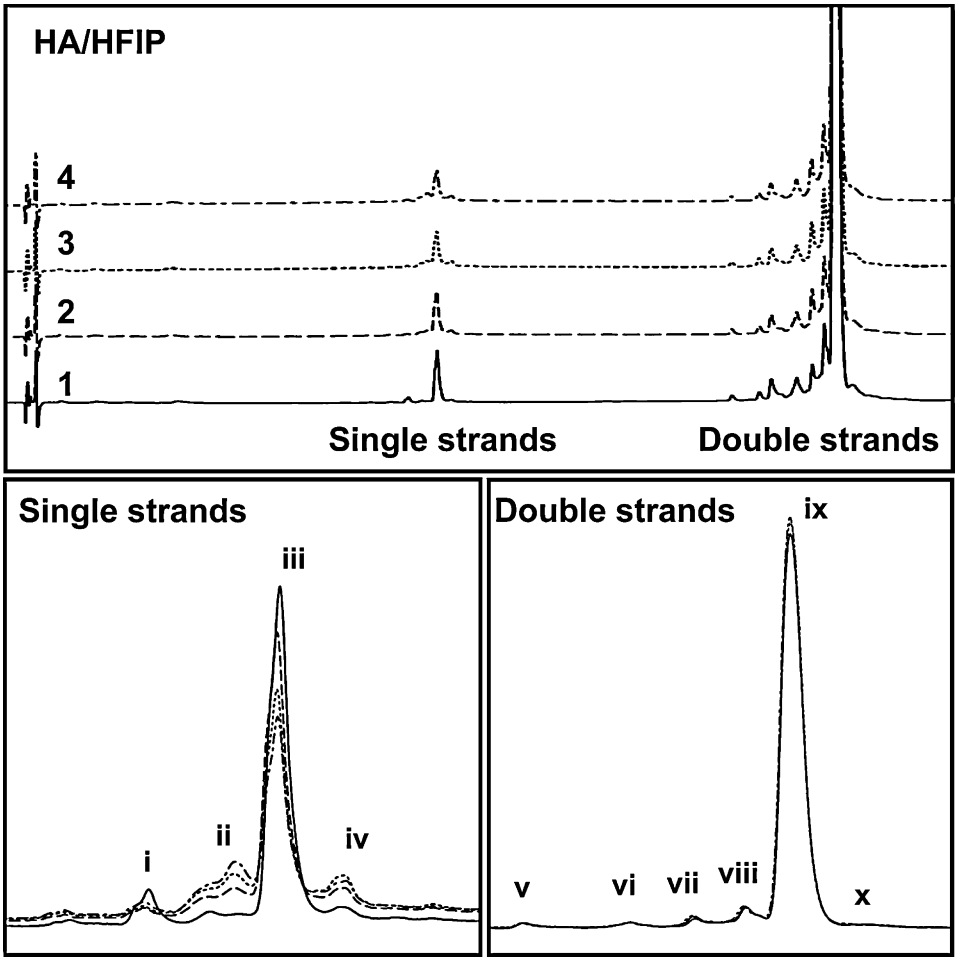

Fig. 7. Effect of annealing conditions on the composition of a siRNA-Luc sample. Upper panel depict UV-traces of the optimized non-denaturing method containing 15 mM HA and 50 mM HFIP in both mobile phases. Trace 1 = mixing at RT; trace 2 = snap cooling; trace 3 = fast cooling; trace 4 = slow cooling. Lower left panel depicts the enlarged single strand peak region. i = Short-G2; ii = Short-G1; iii = GS-Luc; iv = guide strand longmer with one additional adenosine (M_r = 7083.4). Lower right panel depicts the enlarged duplex peak region. v = GS-Luc/shortmer P4 5′N-4 (M_r = 5490.0); vi = GS-Luc/Short-P1; vii = PS-Luc/Short-G2; viii = PS-Luc/Short-G1; ix = optimal duplex; x = PS-Luc/guide strand longmer with one additional guanine (M_r = 7096.3); and GS-Luc/passenger strand longmer with one additional isobutyryl group (M_r = 6846.3).

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Table 4
Effect of annealing conditions on duplex composition. siRNA-Luc was annealed by heating to 85 °C for 5 min and either (a) snap cooling on ice (snap cooling), (b) cooling to RT in 30 min (fast cooling) or (c) cooling to RT in 3 h (slow cooling). Controls were kept at RT without heating (RT). Samples were analyzed by IP-RP HPLC. Duplex (opt.) describes the relative peak area of optimal duplex. GS-Luc (single) describes the relative peak area of non-hybridized full-length guide strand. Duplex (total) describes the sum of the relative peak areas of optimal duplex and all detected duplex variants.

[%]	Duplex (opt.) IP-RP	GS-Luc (single) IP-RP	Duplex (total) IP-RP
RT	82.3	2.6	96.1
Snap cooling	82.4	2.3	96.1
Fast cooling	83.3	1.9	96.1
Slow cooling	83.5	1.6	96.2

tent and composition using the optimized HA/HFIP method. Due to the high chromatographic resolution that was achieved for duplexes as well as for non-hybridized strands, gradual changes in the composition of the siRNA samples could be detected. The percentage of optimal duplex in the sample increased from 82.3% before annealing to 83.5% after thermodynamically controlled annealing (Fig. 7 and Table 4). In turn, the percentage of non-hybridized full-length single strand decreased one percent from 2.6 to 1.6% (peak iii in the lower left panel of Fig. 7 and Table 4). At the same time, non-hybridized shortmer and longmer peaks increased (peaks i, ii and iv in the lower left panel of Fig. 7). Strand degradation as the source of the detected changes in the annealing samples was considered unlikely, due to results described in Seiffert et al. Here, exposure of siRNA-Luc in water to elevated temperatures predominantly lead to the formation of 2′,5′-isomers and levels increased linearly with time [45]. According to the reported data, annealing at 85 °C for 5 min is expected to result in less than 0.2% isomerisation. These levels were considered too low to account for the observed changes in the samples. Hence, the changes in duplex composition are believed to be caused by the different annealing procedures.

Sensitive ESI-MS detection after chromatographic separation allowed for the identification of duplex and single strand impurities. The identified duplex variants contained the 5′N-4 passenger strand shortmer (peak v in the lower right panel of Fig. 7; M_r = 5490.0), Short-P1 (M_r = 6460.0; peak vi), Short-G2 (M_r = 6138.9; peak vii) and Short-G1 (M_r = 6446.9; peak viii). All identified variants containing longmers (peak x) eluted after the main peak. The detected molecular mass M_r = 7096.3 corresponded to guide strand longmers with one additional guanine nucleotide. The detected molecular mass M_r = 6846.3 corresponded to passenger strand longmers with one additional isobutyryl residue. In the non-hybridized single strand fraction, the detected impurities included Short-G1 and Short-G2 (M_r = 6446.9 and 6138.8) and N + A longmers of the guide strand (M_r = 7081.4) (Fig. 7, lower left panel). The total amount of duplex was approximately 96.1% in all four samples and appeared to be independent of annealing conditions. Since no change in total duplex content was observed before and after heating, improved quality of the siRNA mixture (higher percentage of optimal duplex) rather than an increase in the duplex content appears to be the consequence of annealing.

4. Conclusion

Taken together, our data confirm the beneficial effect of a thermodynamically controlled annealing step on siRNA quality and demonstrate the utility of the HA/HFIP buffer system for non-denaturing IP-RP chromatography in combination with ESI-MS. Our results highlight the need for a chromatographic method capable to document small changes in duplex composition for full characterization of oligonucleotide duplexes. The reported non-denaturing HA/HFIP IP-RP method showed separation efficiencies that have not been achieved with other buffer systems and/or chromatographic methods. The major related duplex variants were separated from optimal duplex and could be identified by MS detection. Mass sensitivity was more than 20-fold higher than in HAA buffer. Content of optimal duplex in a typical siRNA sample was determined to a fraction of a percent. Similar results were obtained for other siRNA (data not shown) and a general applicability of the method on other double stranded and/or structured RNA oligonucleotides appears likely. HA/HFIP chromatography is useful as an *in-process* control and release method for therapeutic double stranded nucleic acids such as siRNA.

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