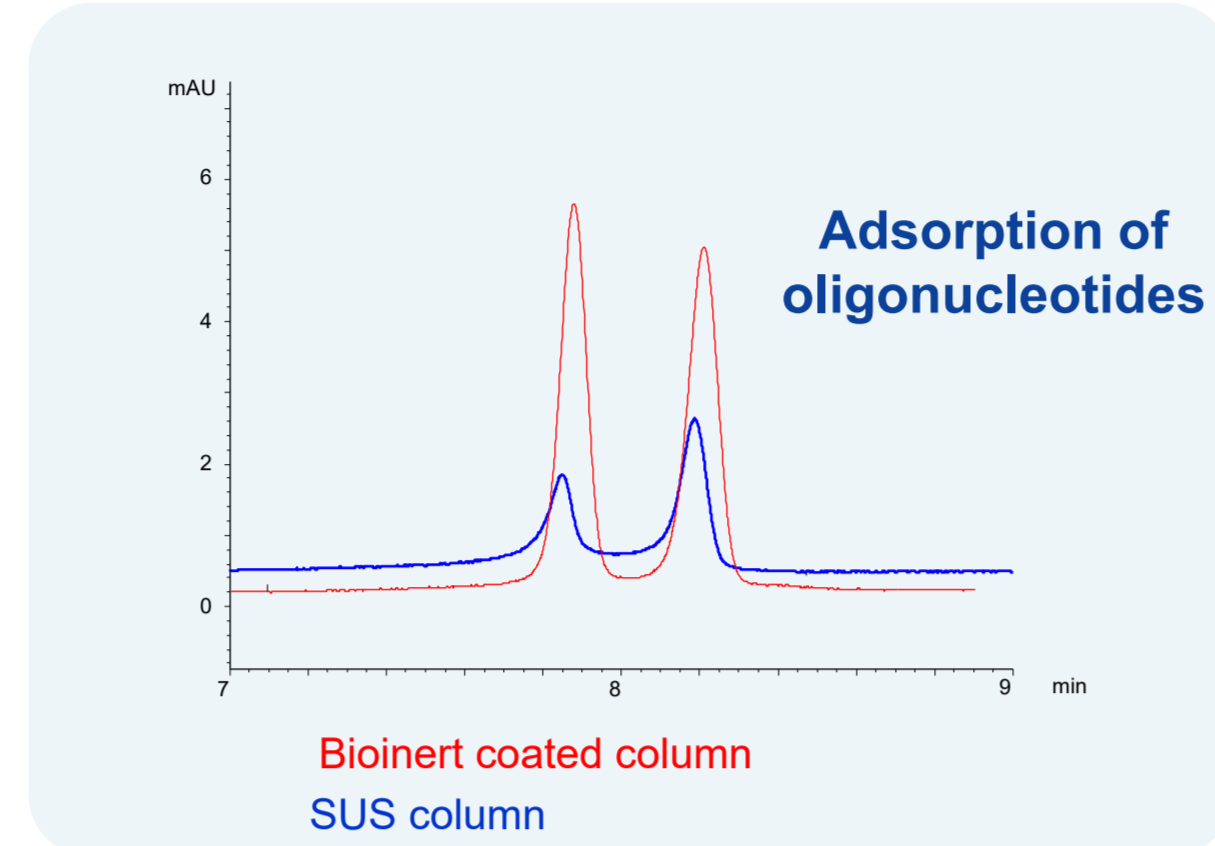
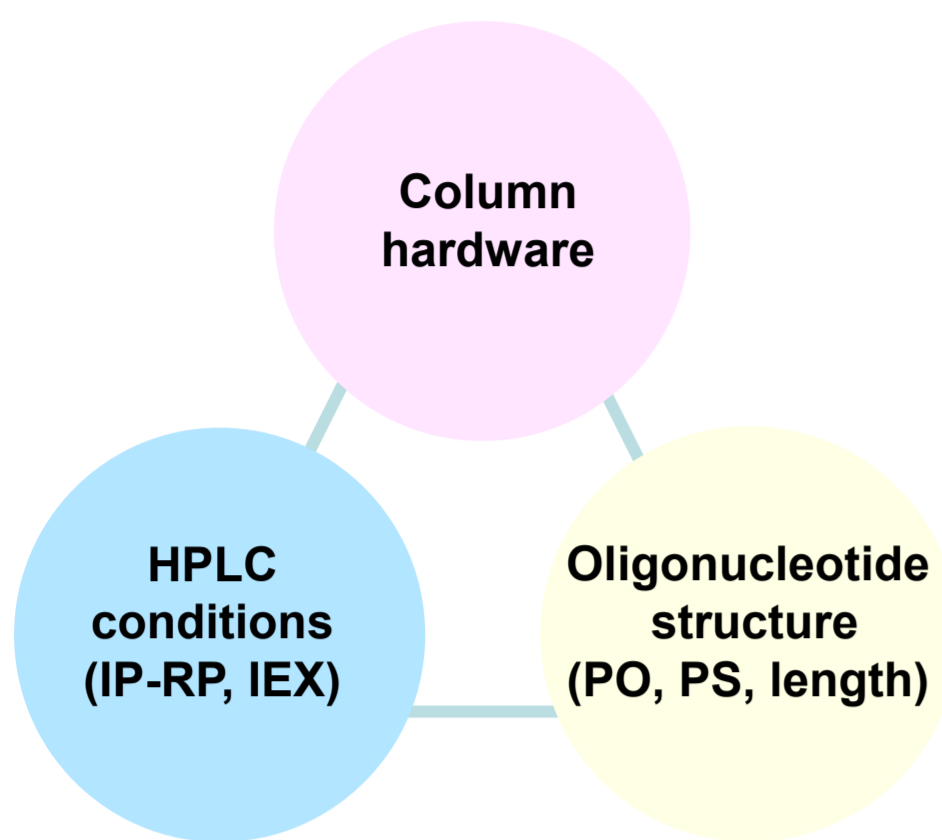


## Introduction

In the development of nucleic acid therapeutics, liquid chromatography is used for qualitative/quantitative analysis, impurity analysis and bioanalysis of oligonucleotides. An important issue in these analyses is the adsorption of oligonucleotides, which causes peak broadening and carry-over, resulting in reduced quantitative performance or LC-MS sensitivity. Therefore, understanding the influence of the combination of column hardware, sample and mobile phase on oligonucleotide adsorption in different separation modes is useful for the development of robust analytical methods.

### Adsorption influencing factors



## Materials and methods

### Column hardware

	Bioinert coating	PEEK	SUS
Column material	bioinert coated stainless steel	PEEK-lined stainless steel (used in IP-RPLC)	stainless steel

### Conditions

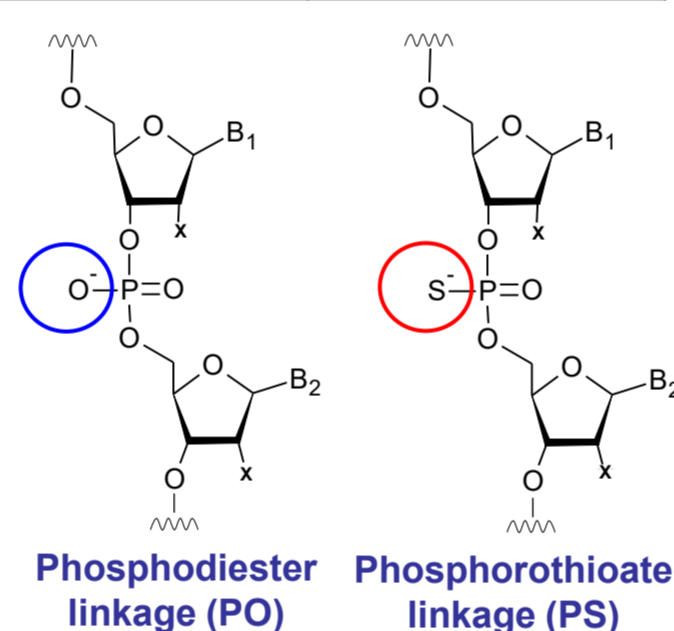
Mode / stationary phase	Eluent	Sample	Data
<b>IP-RPLC</b> (ion-pairing reversed phase liquid chromatography)  • YMC Accura Triart Bio C18 • YMC-Triart Bio C18 metal-free • YMC-Triart Bio C18 (SUS) (1.9 µm, 30 nm) 50 x 2.1 mm ID	A) 15 mM TEA <sup>+</sup> -400 mM HFIP <sup>**</sup> B) methanol	PS RNA	I
	A) 2 mM TEA-50 mM HFIP B) methanol	PS RNA	II
		PO RNA	III
<b>IEX</b> (ion exchange chromatography)  • YMC Accura BioPro IEX QF • BioPro IEX QF (PEEK) • BioPro IEX QF (SUS) (5 µm, non-porous) 50 x 4.6 mm ID	A) 20 mM Tris-HCl (pH 8.1) B) 20 mM Tris-HCl containing 1 M NaCl	PO RNA	IV
	A) 10 mM NaOH (pH 12) / methanol (70/30) B) 10 mM NaOH containing 1 M NaClO <sub>4</sub> / methanol (70/30)	PS RNA	V
	A) 20 mM Tris-HCl (pH 8.1) B) 20 mM Tris-HCl containing 1 M NaCl	dT, DNA ladders	VI

### Other conditions

<b>IP-RPLC</b> Flow rate: 0.42 mL/min Temperature: 65°C Injection: 1 µL	<b>IEX</b> Flow rate: 0.5 mL/min Temperature: 25°C for PO RNA, 65°C for PS RNA Injection: 4 µL (5 nmol/mL)
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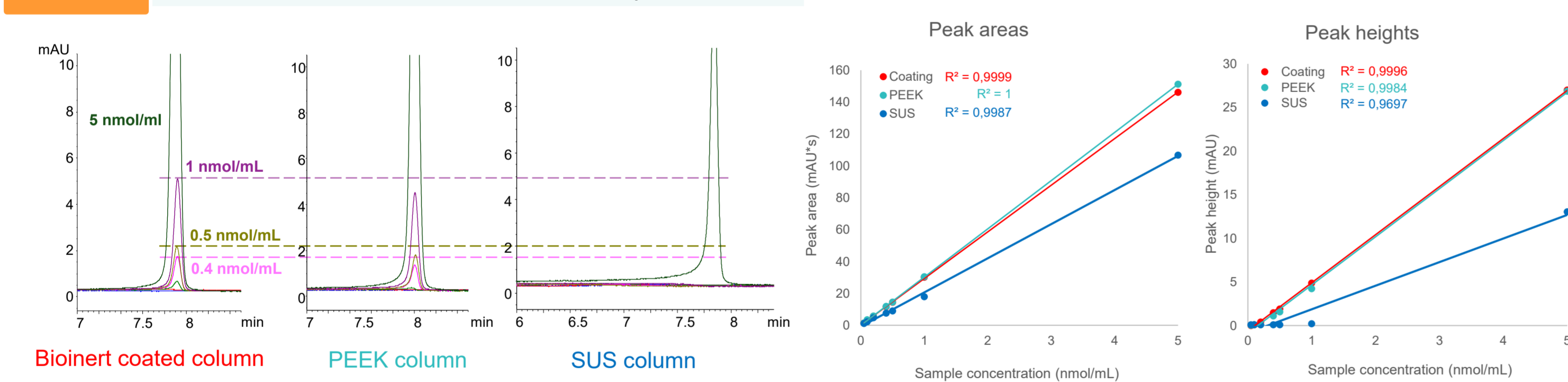
### Sample

Single strand RNA	5'-U C A U C A C A C U G A A U A C C A A U-3' (All PO RNA 20mer)
*=Phosphorothioated	5'-U <sup>*</sup> C <sup>*</sup> A <sup>*</sup> U <sup>*</sup> C <sup>*</sup> A <sup>*</sup> C <sup>*</sup> A <sup>*</sup> C <sup>*</sup> U <sup>*</sup> G <sup>*</sup> A <sup>*</sup> U <sup>*</sup> A <sup>*</sup> C <sup>*</sup> C <sup>*</sup> A <sup>*</sup> A <sup>*</sup> U-3' (All PS RNA 20mer)

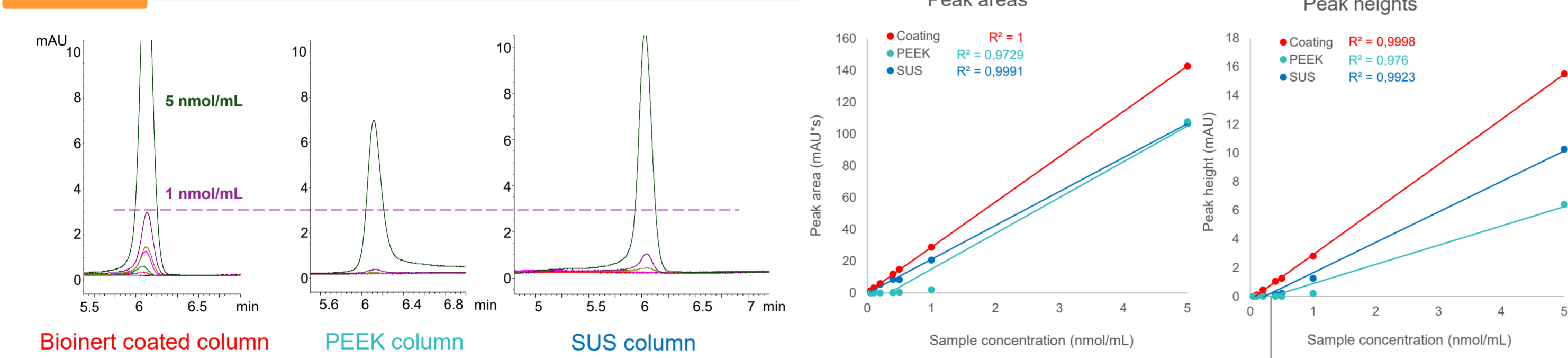


## IP-RPLC : Effect of TEA-HFIP concentration as mobile phase additive

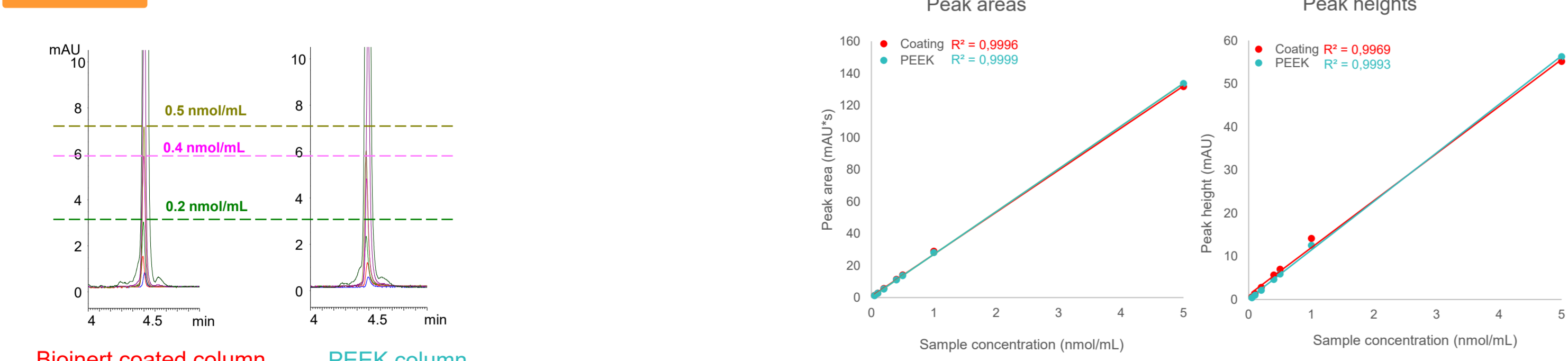
**Data I** Eluent: 15 mM TEA-400 mM HFIP / sample: PS RNA Sample concentration: 0.05, 0.1, 0.2, 0.4, 0.5, 1.0, 5.0 nmol/mL



**Data II** Eluent: 2 mM TEA-50 mM HFIP / sample: PS RNA



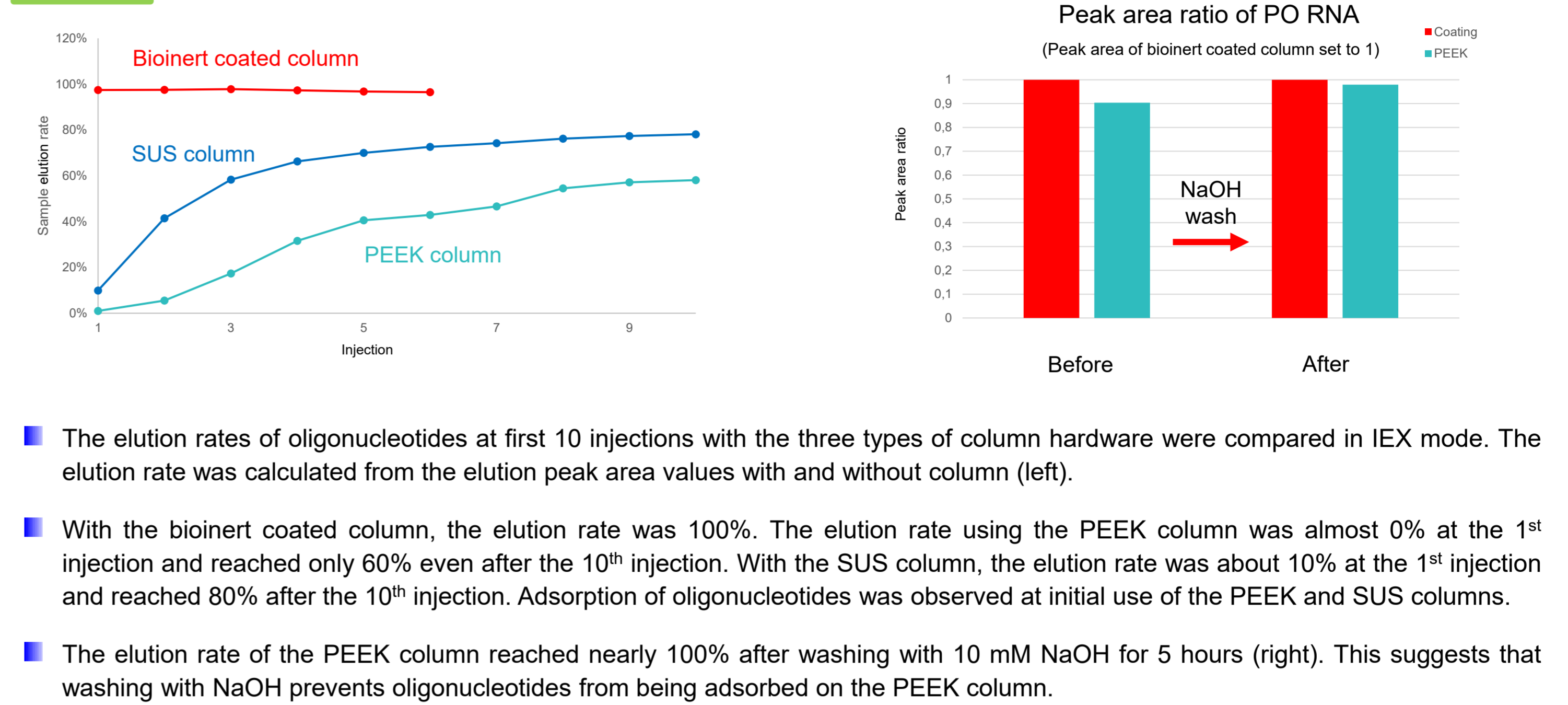
**Data III** Eluent: 2 mM TEA-50 mM HFIP / sample: PO RNA



- The peak area and height of All PS RNA were compared at two different concentrations of TEA-HFIP with three types of column hardware packed with YMC-Triart Bio C18 (Data I and II).
- Using 15 mM TEA-400 mM HFIP as eluent, there is no difference in the peak areas and peak heights between the bioinert coated and the PEEK column. But with the SUS column, peak area and height were lower than with the bioinert coated and the PEEK column. This is due to interactions between sample and metal surface. However, using 2 mM TEA-50 mM HFIP as eluent, the obtained peak areas and heights with not only the SUS but also with the PEEK column were lower than those with the bioinert coated column. It is presumably due to hydrophobic interactions between the PEEK hardware and the oligonucleotide.
- The peak areas and heights of All PS RNA and All PO RNA (the same nucleotide sequence) were compared using 2 mM TEA-50 mM HFIP eluent condition, and with the bioinert coated and the PEEK column packed with YMC-Triart Bio C18 (Data II and III).
- There is no difference in the peak areas and heights of PO RNA between the bioinert coated and the PEEK column. But those of PS RNA with the PEEK column were lower than those of the bioinert coated column. This might be a result of the higher hydrophobicity of PS RNA compared to PO RNA.

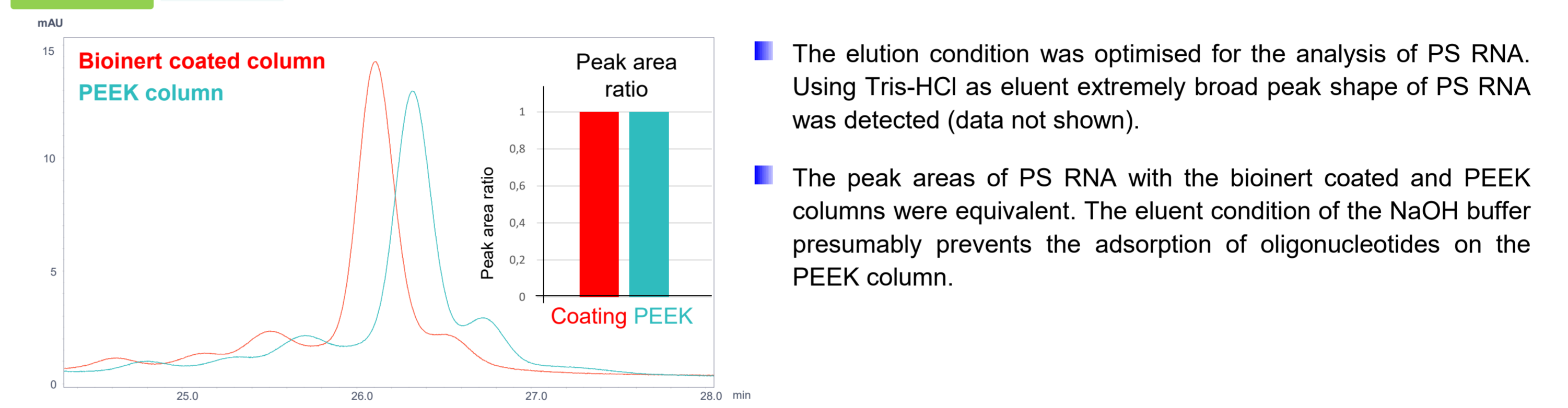
## IEX : Adsorption to IEX column hardware

**Data IV** Elution rates of PO RNA at first 10 injections



- The elution rates of oligonucleotides at first 10 injections with the three types of column hardware were compared in IEX mode. The elution rate was calculated from the elution peak area values with and without column (left).
- With the bioinert coated column, the elution rate was 100%. The elution rate using the PEEK column was almost 0% at the 1<sup>st</sup> injection and reached only 60% even after the 10<sup>th</sup> injection. With the SUS column, the elution rate was about 10% at the 1<sup>st</sup> injection and reached 80% after the 10<sup>th</sup> injection. Adsorption of oligonucleotides was observed at initial use of the PEEK and SUS columns.
- The elution rate of the PEEK column reached nearly 100% after washing with 10 mM NaOH for 5 hours (right). This suggests that washing with NaOH prevents oligonucleotides from being adsorbed on the PEEK column.

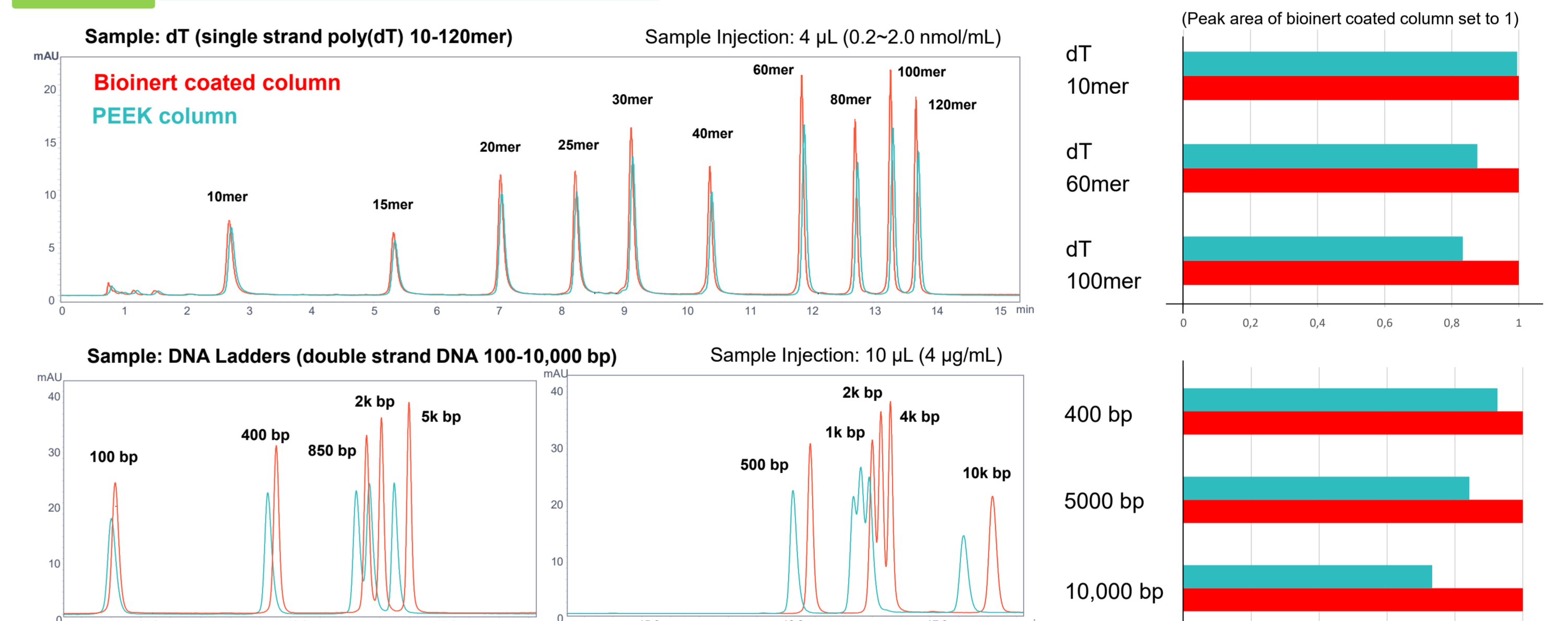
**Data V** PS RNA



- The elution condition was optimized for the analysis of PS RNA. Using Tris-HCl as eluent extremely broad peak shape of PS RNA was detected (data not shown).
- The peak areas of PS RNA with the bioinert coated and PEEK columns were equivalent. The eluent condition of the NaOH buffer presumably prevents the adsorption of oligonucleotides on the PEEK column.

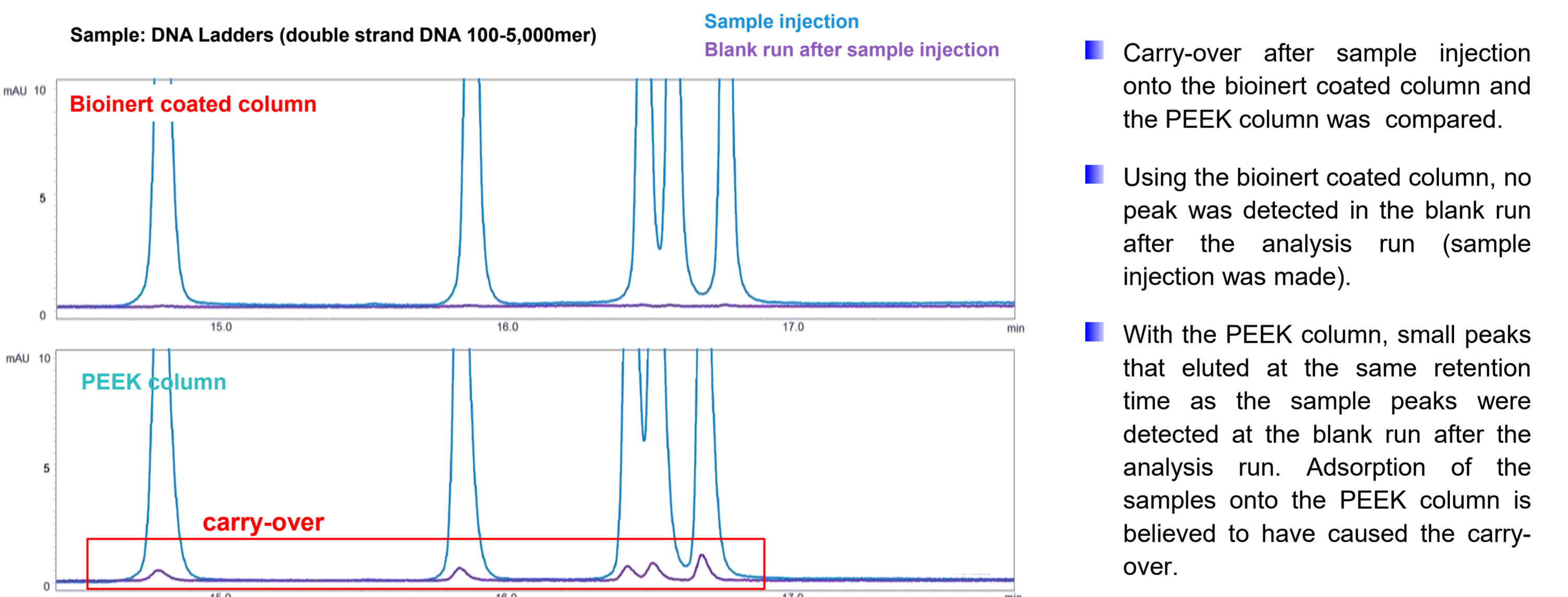
## IEX : Adsorption depending on the size of oligonucleotides

**Data VI** dT 10-120mer, DNA ladders 100-10,000 bp



- Using 20 mM Tris-HCl (pH 8.1) as eluent, single strand poly(dT) (10-120mer) and double strand DNA (100-10,000 bp) were analysed using the bioinert coated and the PEEK column.
- The resulting peak areas using the PEEK column were lower than with the bioinert coated column. Nucleotides with a longer strand length tend to have a greater difference in the peak areas between the bioinert coated column and the PEEK column. This suggests that the longer stranded nucleotides are more likely to get adsorbed onto the PEEK column.

## IEX : Carryover after sample injection



- Carry-over after sample injection onto the bioinert coated column and the PEEK column was compared.
- Using the bioinert coated column, no peak was detected in the blank run after the analysis run (sample injection was made).
- With the PEEK column, small peaks that eluted at the same retention time as the sample peaks were detected at the blank run after the analysis run. Adsorption of the samples onto the PEEK column is believed to have caused the carry-over.

## Conclusions

Mode	Data	Eluent	Sample	Adsorption evaluation results		
				Coating	PEEK	SUS
IP-RPLC	I	A) 15 mM TEA-400 mM HFIP B) methanol	PS RNA	○	○	▲
	II	A) 2 mM TEA-50 mM HFIP B) methanol	PS RNA	○	▲	▲
	III		PO RNA	○	○	—
IEX	IV	A) 20 mM Tris-HCl (pH 8.1) B) 20 mM Tris-HCl containing 1 M NaCl	PO RNA	○	▲	▲
	V	A) 10 mM NaOH (pH 12) / methanol (70/30) B) 10 mM NaOH containing 1 M NaClO <sub>4</sub> / methanol (70/30)	PS RNA	○	○	—
	VI	A) 20 mM Tris-HCl (pH 8.1) B) 20 mM Tris-HCl containing 1 M NaCl	dT, DNA ladders	○	▲	—

- The effect of the analytical conditions on the adsorption of oligonucleotides in IP-RPLC and IEX has been studied.
- It is assumed that oligonucleotides adsorb onto some types of hardware; onto the SUS hardware due to metallic interaction regardless of conditions, and onto the PEEK hardware due to hydrophobic interaction depending on eluent conditions.
- When developing analysis conditions for oligonucleotides, it is important to consider the effect of the hardware.