Novel Analytical Applications of DNA Biosensors

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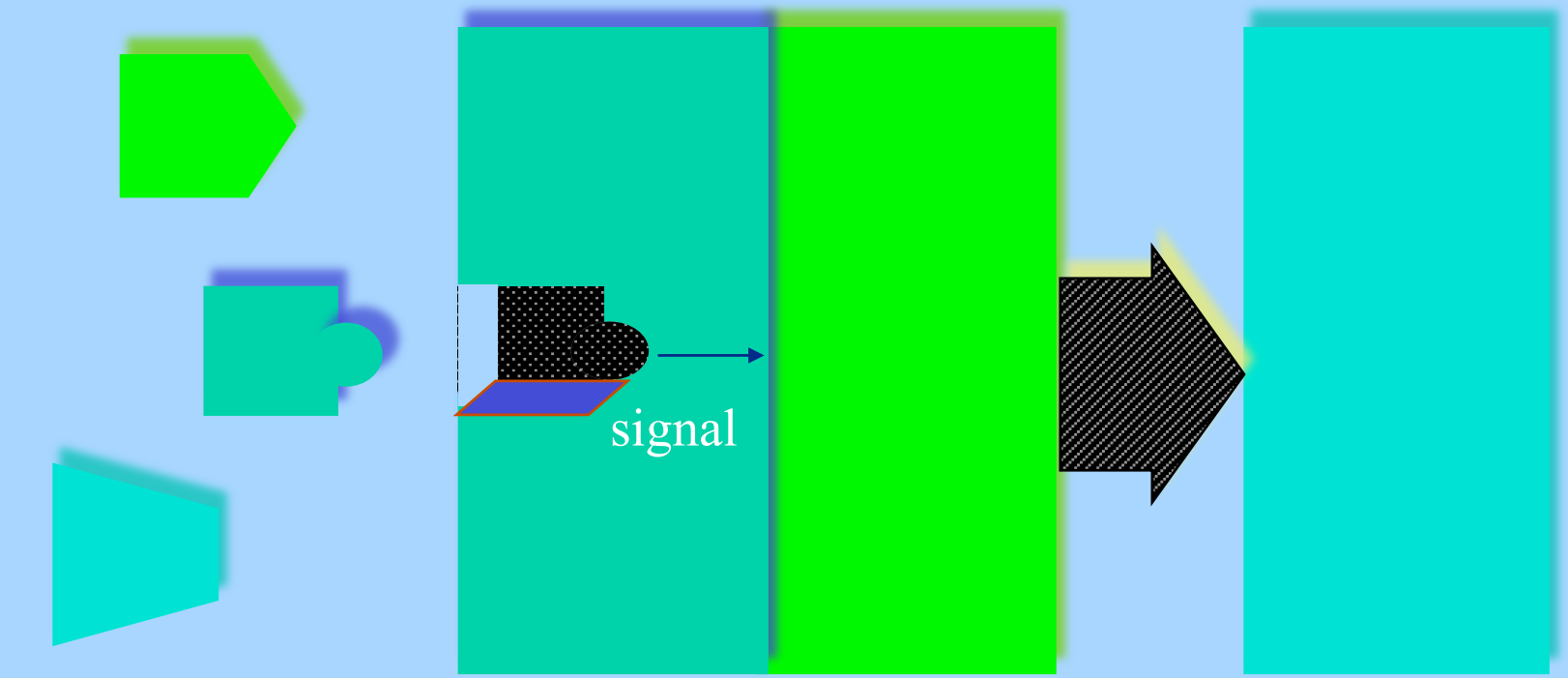
"*Biosensors* are analytical devices incorporating a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids etc.), a biologically derived material or biomimic intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, or magnetic."

***Biosensors* usually yield a digital electronic signal which is proportional to the concentration of a specific analyte or group of analytes. While the signal may in principle be continuous, devices can be configured to yield single measurements to meet specific market requirements." (One-shot biosensors)**

Biosensors & Bioelectronics (2000)

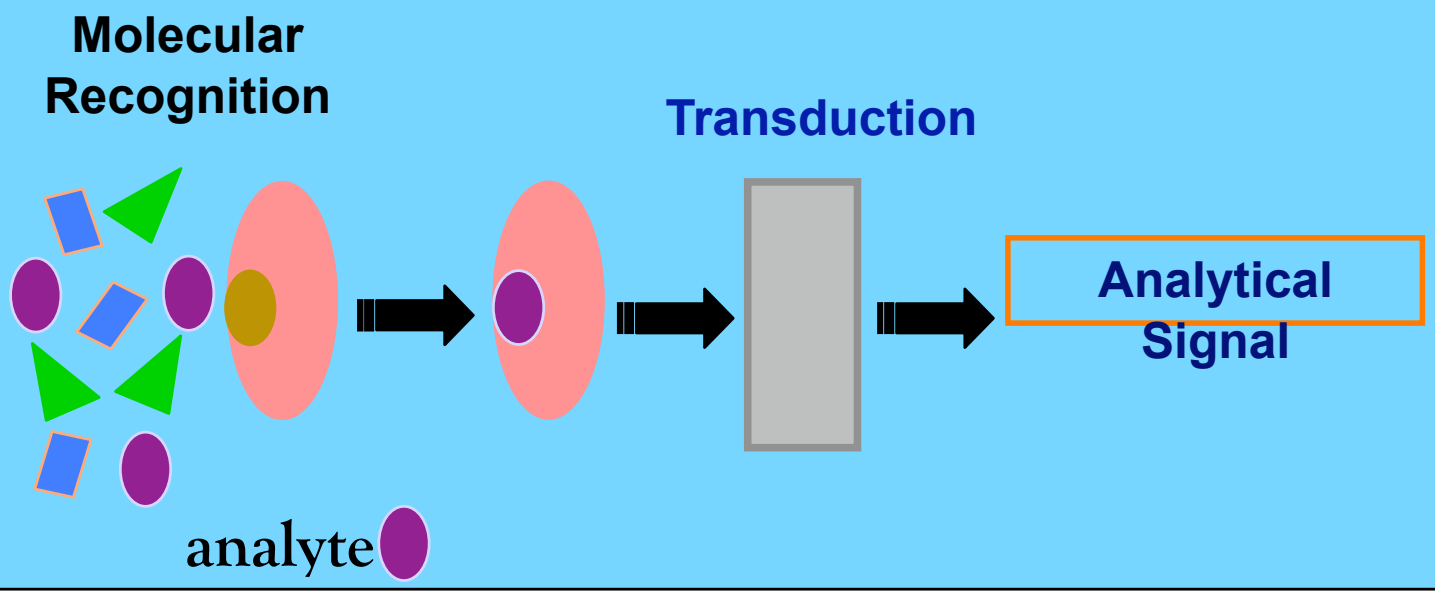
The Biosensor



DNA BIOSENSORS

- **Trace measurements of pollutans (intercalators, binders of DNA)**
- **Hybridization indicator (bacteria , virus , genetic inherited diseases)**
- **Biosensing of drugs**

AFFINITY BIOSENSOR



MOLECULAR RECOGNITION ELEMENTS

BIOLOGICAL

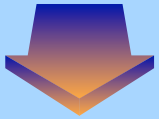
- Antibodies
- Receptors
- Nucleic Acids

BIOMIMETIC

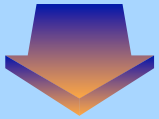
- Molecular Imprinted Polymers (MIP)
- Oligonucleotides
- Oligopeptides
- Aptamers

DNA Based Biosensor

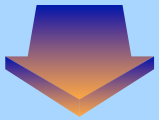
Immobilisation of a “probe” complementary to the target sequence onto the solid support of a sensor



Addition of the target DNA sequence (sample)



Formation of a complementary complex



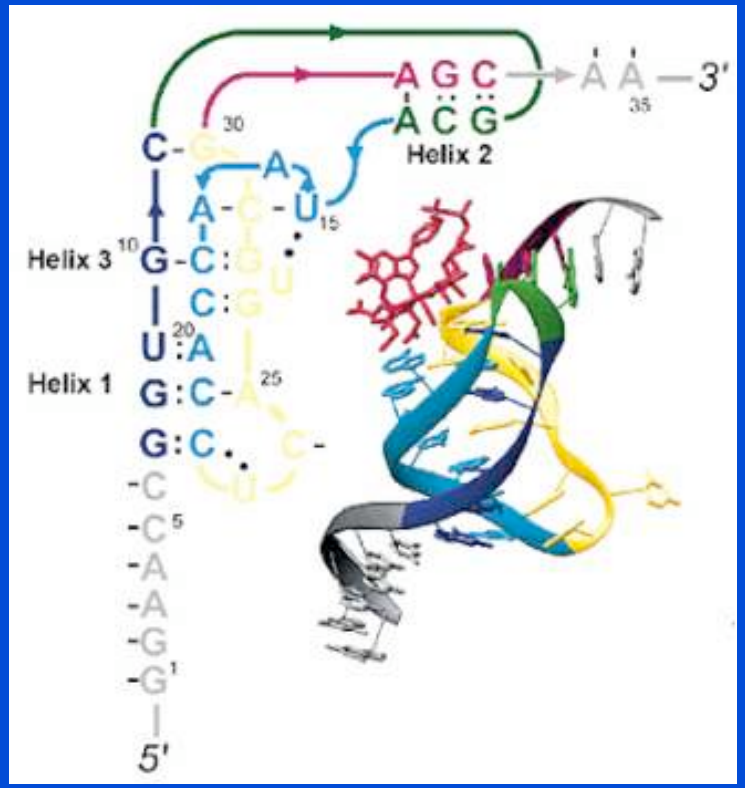
Changes in the physicochemical parameters of the layer formed on transducer (piezoelectric, electrochemical, optical, etc.)

Aptamers

Aptamers are oligonucleotides (DNA or RNA molecules) that can bind with high affinity and specificity to a wide range of target molecules (proteins, peptides, drugs, vitamins and other organic or inorganic compounds).

They were "discovered" in 1990 by the development of an *in vitro* selection and amplification technique, known as SELEX (Systematic Evolution of Ligands by Exponential enrichment).

Their name is derived from the Latin word "aptus" which means "to fit" .



APTAMERS

Aptamers are oligonucleotides that are identified through a combinatorial selection process for high affinity binding to target molecules. In the selection process, a combinatorial library of oligonucleotides is passed through a column containing the immobilized target.

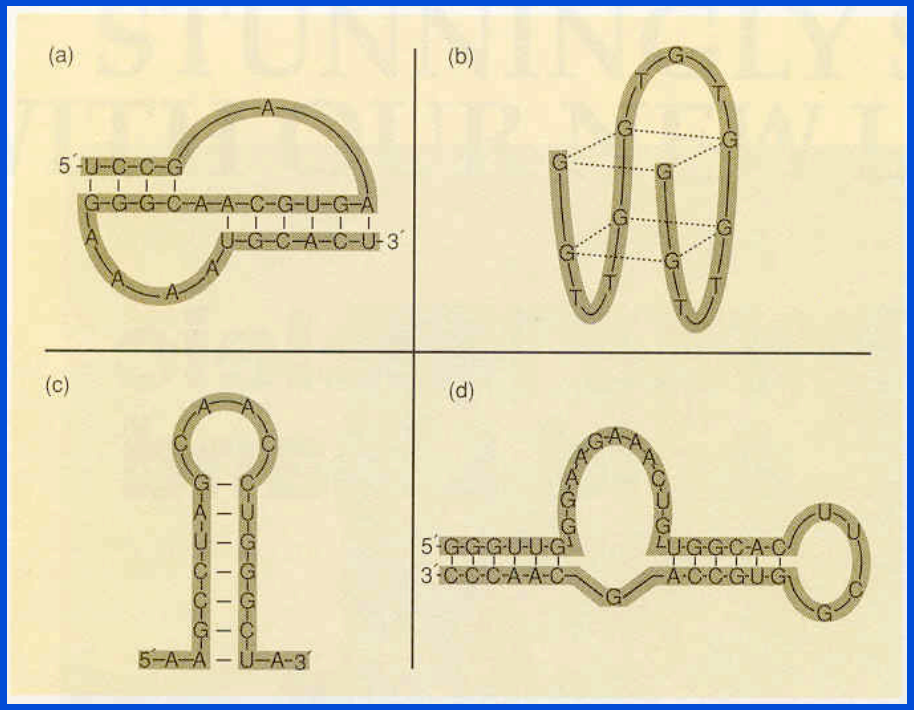
Those oligonucleotides that do not bind are discarded, while those that bind are collected and amplified.

This cycle is repeated several times until a small number of affinity binders, or aptamers, have been isolated from the combinatorial pool.

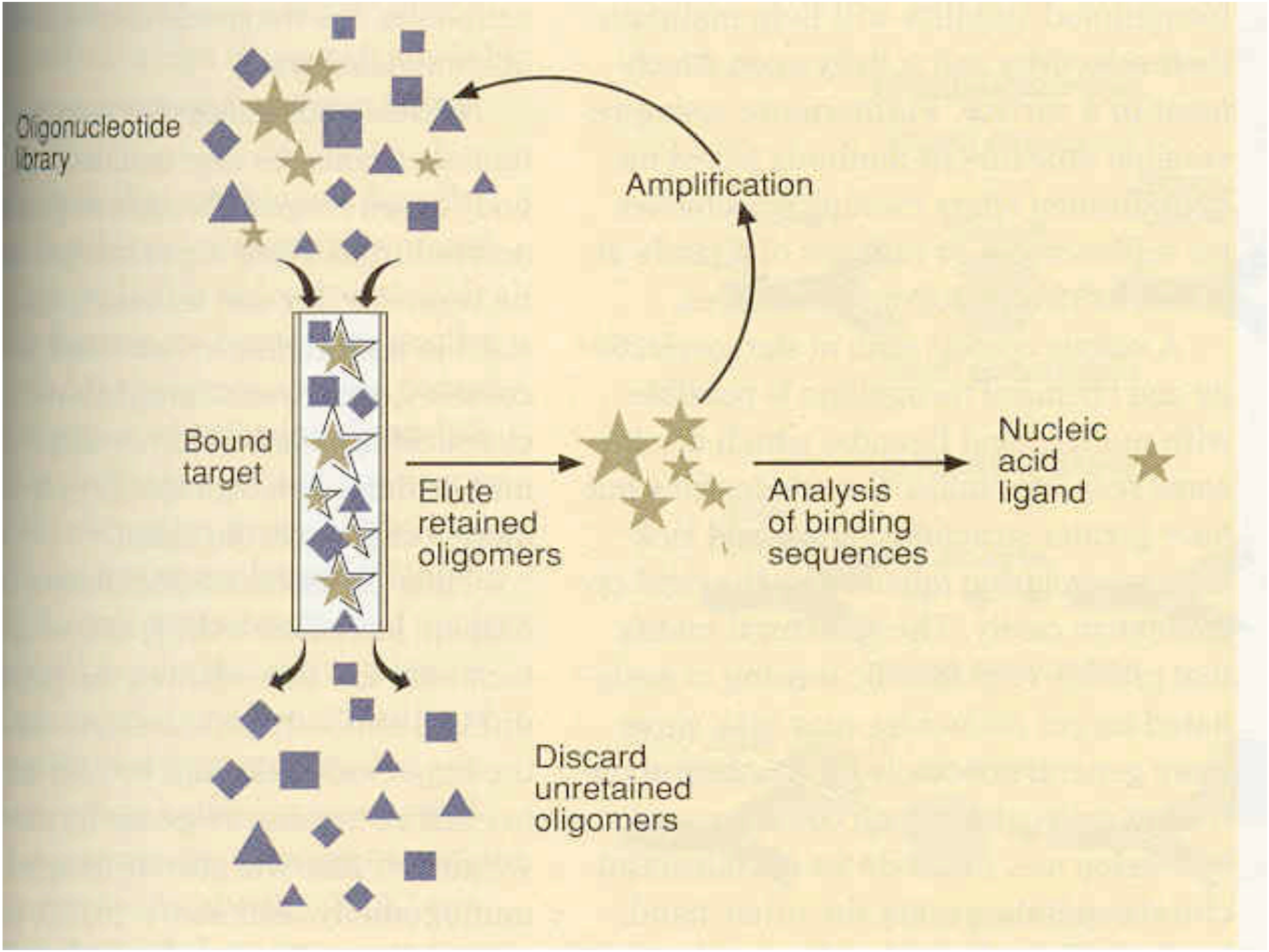
APTAMERs

- Synthetic sequence (30-40 mer) of nucleic acids, single strand DNA or RNA, obtained by an *in vitro* selection (SELEX)
- Molecolar recognition higly selective for the structures selected
- Possibility to obtain aptamers for a wide kind of structures
- Thermal stability and lifetime higher in comparison with protein receptors (antibodies)
- Any animal involved ;the procedure is suitable also for small molecole or any toxicants
- Time for obtaining : 2-3 months (v. monoclonal antibodies)

The majority of aptamer structures result from intramolecular base pairing to produce loops or bulges, forming structures such as the hairpin, the pseudo knot, and the stem-loop/bulge. A different type of structural motif is the G-quartet, also known as “quadruplex”, “tetraplex” or “G4” DNA.



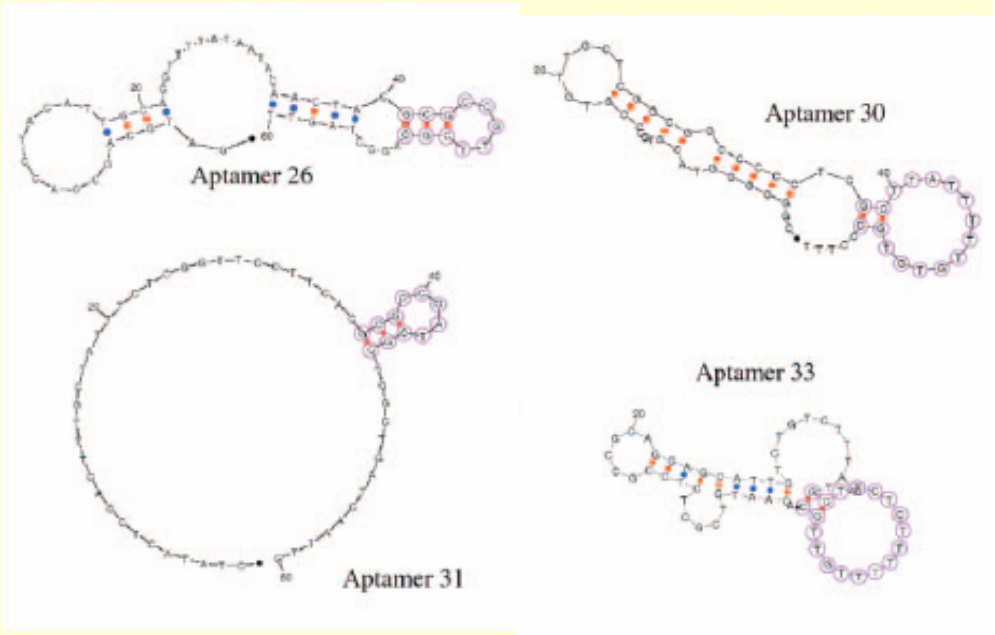
A) Pseudoknot , b) G-Quartet, c) hairpin, d) stem-loop/bulge
From Anal. Chem. 1995, 664A


Oligonucleotide library
Amplification
Bound target
Elute retained oligomers
Analysis of binding sequences
Nucleic acid ligand
Discard unretained oligomers

Aptamers are oligonucleotides (DNA or RNA molecules) that can bind with high affinity and specificity to a wide range of target molecules (proteins, peptides, drugs, vitamins and other organic or inorganic compounds).

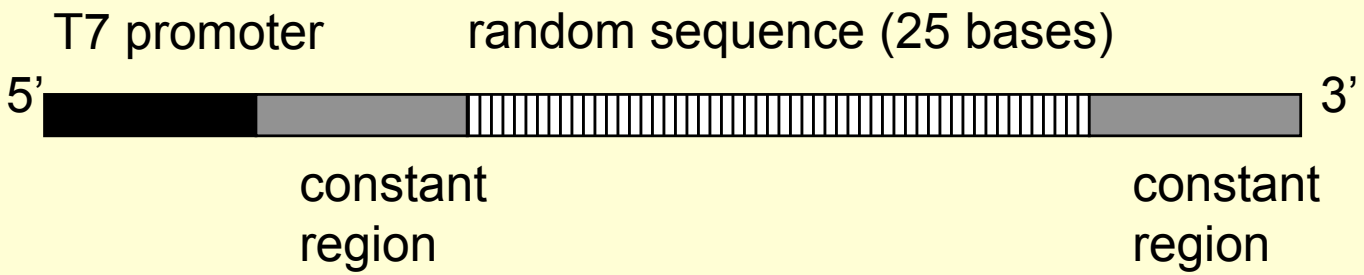
They were "discovered" in 1990 by the development of an in vitro selection and amplification technique, known as SELEX (Systematic Evolution of Ligands by Exponential enrichment).
(Ellington et al., **Nature** 346, 818; Tuerk and Gold, **Science** 249, 505)

Their name is derived from the Latin word "**aptus**" which means "to fit".



Similar to proteins short oligonucleotides can adopt complex three-dimensional structures

Combinatorial oligonucleotide library

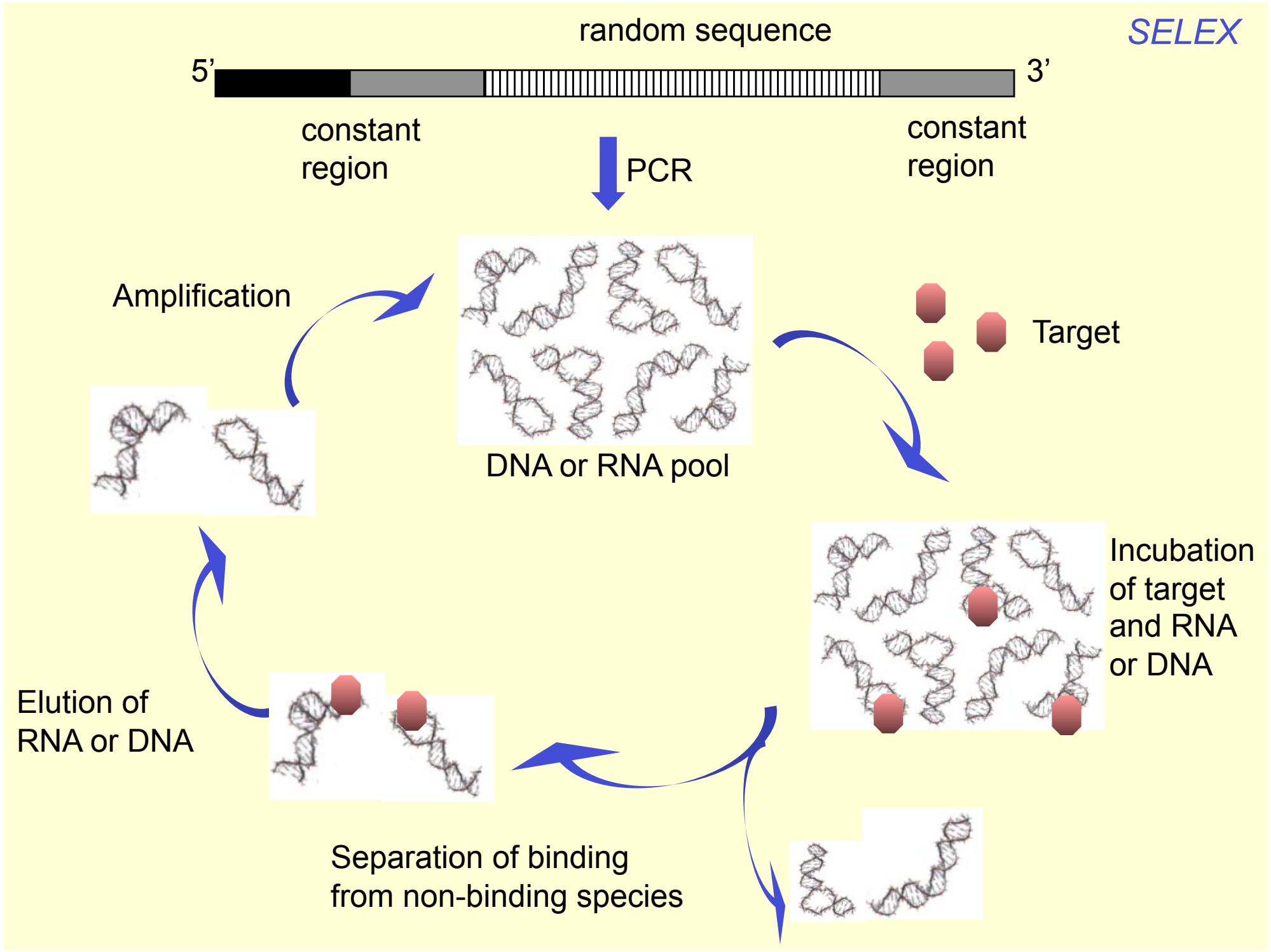


A, **G**, **C**, **U**(**T**)

$4^1 = 4$
$4^2 = 16$
$4^3 = 64$
$4^4 = 256$
$4^5 = 1024$
……
……
……
$4^{25} = 1125899906842624$

Pool of randomized DNA or RNA

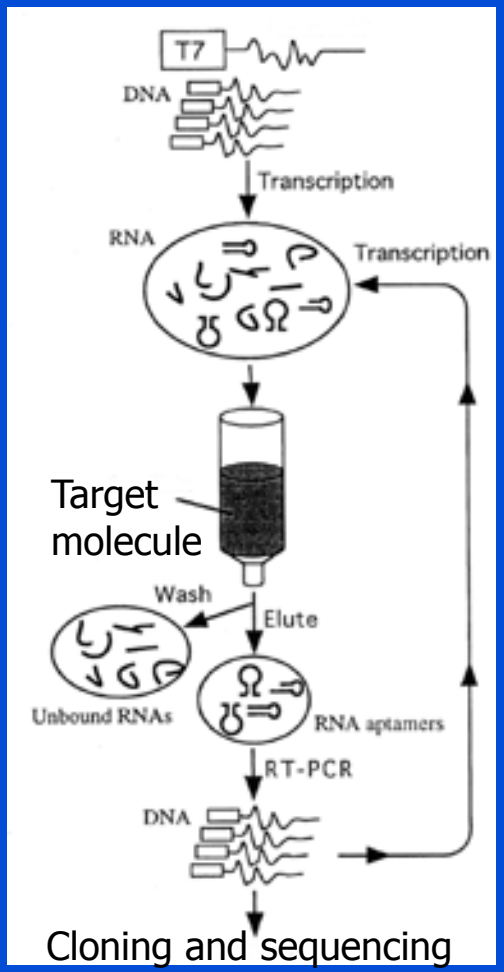
10^{15} different sequences!!!!

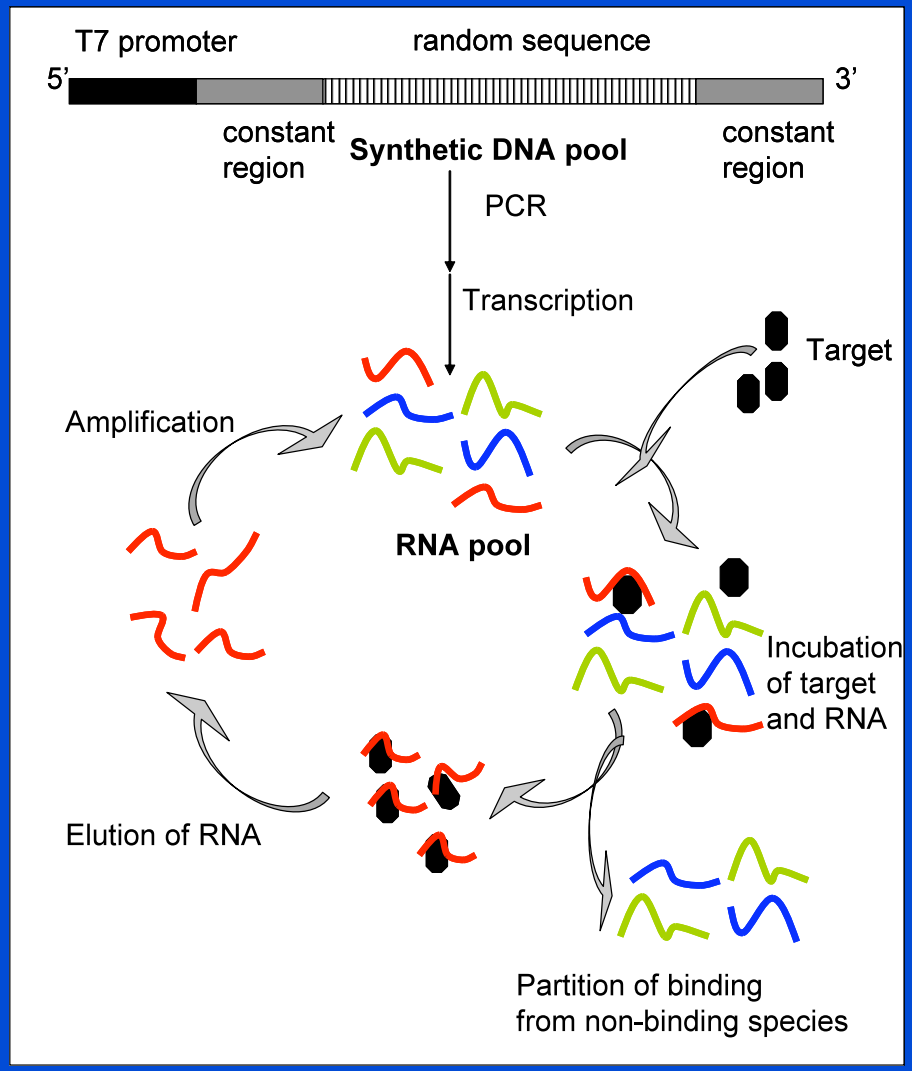

SELEX
random sequence
5'
3'
constant region
constant region
PCR
DNA or RNA pool
Target
Incubation of target and RNA or DNA
Separation of binding from non-binding species
Elution of RNA or DNA
Amplification

The SELEX process

Systematic Evolution of Ligands by Exponential enrichment

SELEX was first reported in 1990 (Ellington et al., **Nature** 346, 818; Tuerk and Gold, **Science** 249, 505)





A library containing a 40-nucleotide random region is represented by 4^{40} ($\sim 10^{24}$) individual sequences available for partitioning.

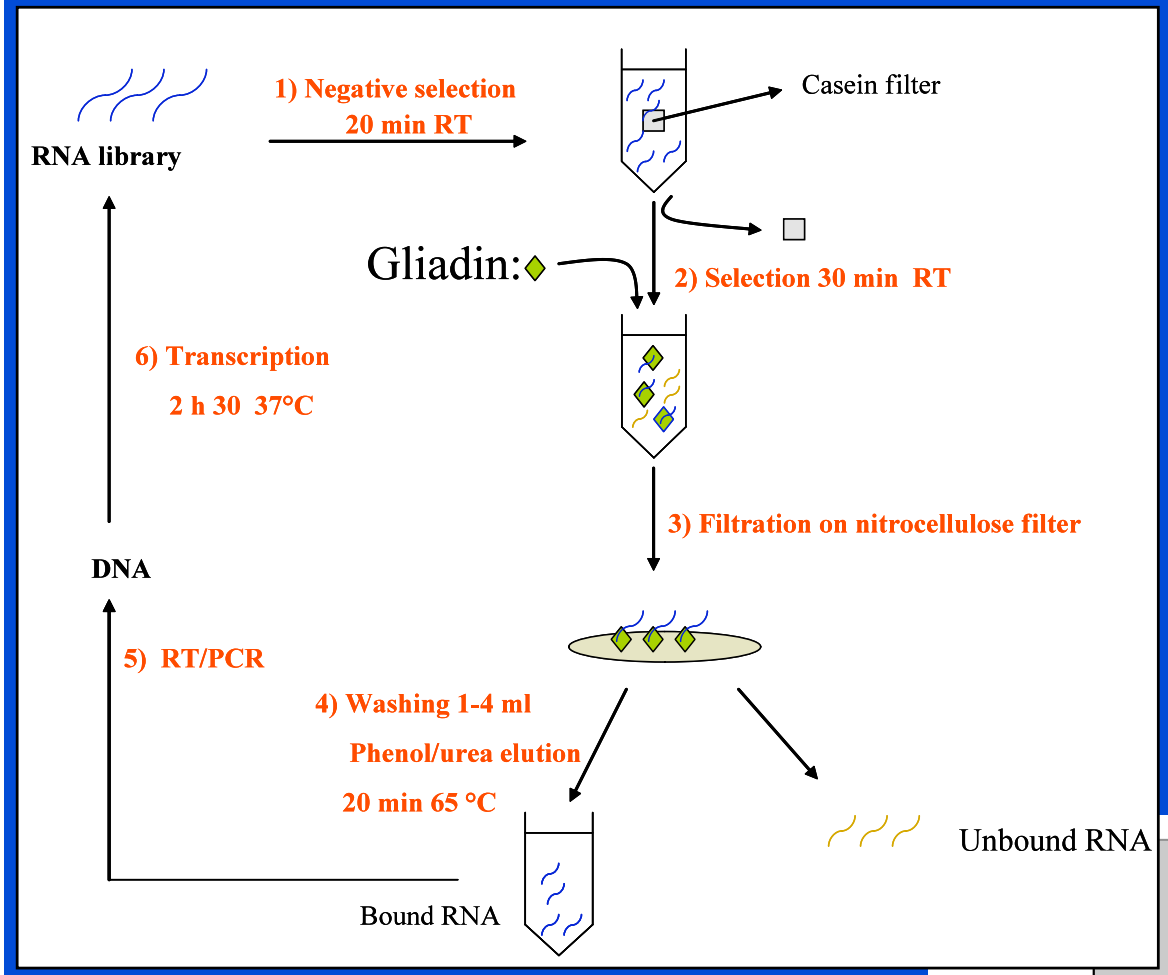
Normally, the starting round contains **10^{14}-10^{15} individual sequences**.

Tetranucleotides = 4 exp 4 = 264

Tetrapeptides = 20 exp 4 = 160.000

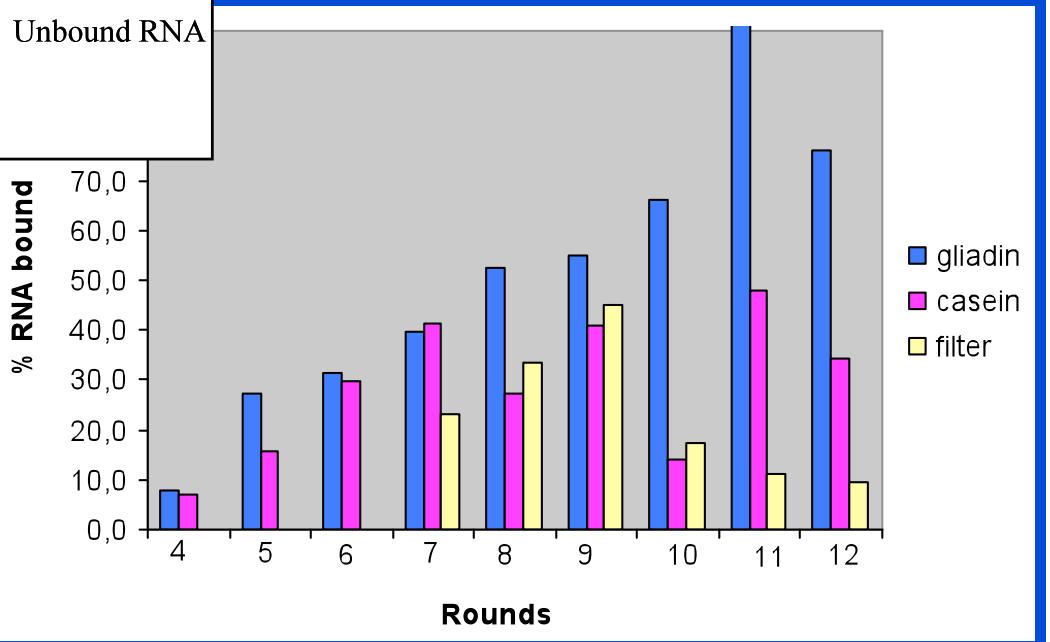
Tetrasaccharides = few millions of structures

SELEX: an example



Target: α-Gliadin (IRMM-480 from european wheat)

SELEX evolution after 12 rounds



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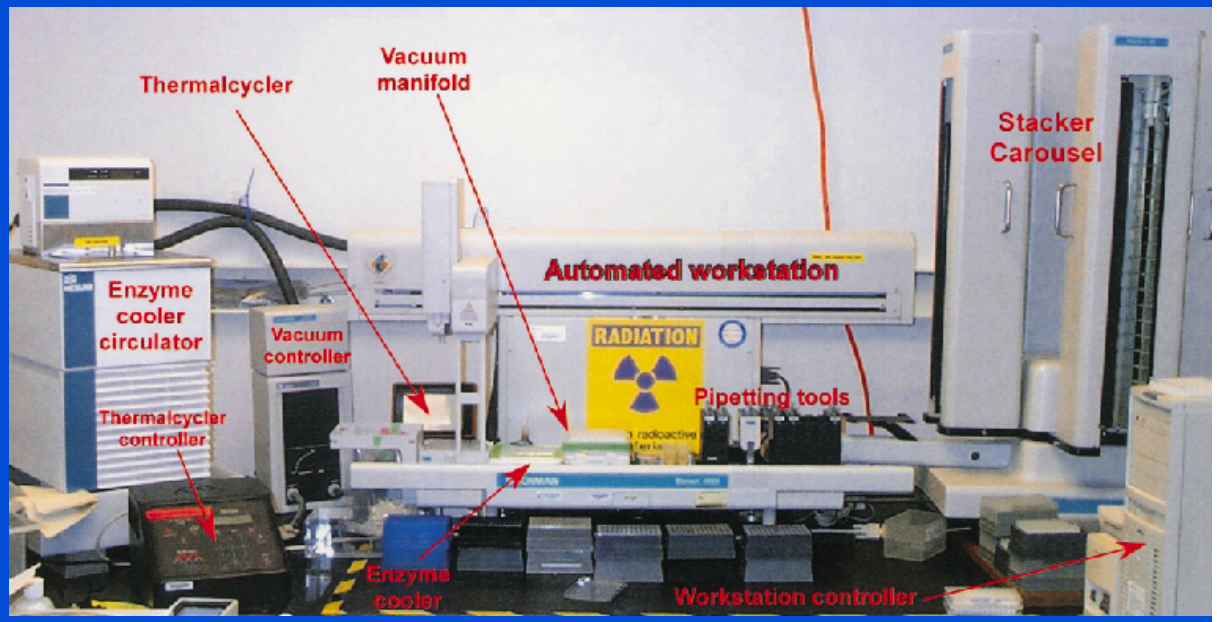
Prof. J.J. Toulmè

June-October 2004

Automation and modification of the SELEX process

- Automated selection of aptamers

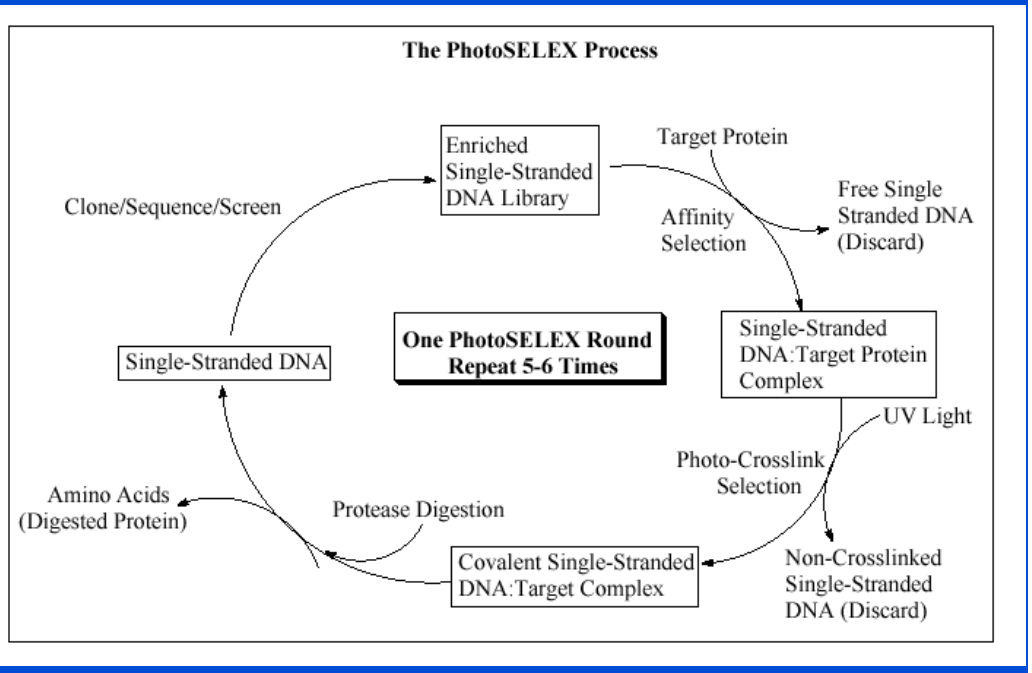
J.C. Cox, A.D. Ellington, Bioorg. Med. Chem. 9, 2525-2531, (2001)



- PhotoSELEX:

modified ssDNA aptamers capable of photocross-linking the target molecule.

M.C., Golden, B.D. Collins, M.C. Willis, T.H. Koch, J. E 81, 167-178, (2000)
C. Bock et al., Proteomics, 4, 609-618, 2004



Target molecules

PROTEINS

Syrian golden hamster prion
Escherichia coli SelB
L-selectin
Tyrosine phosphatase
Ff gene 5
Thrombin
HIV-1 Tat
HIV-1 Rev
Vascular endothelial growth factor
Prostate specific antigen
Human IgE
Taq DNA polymerase
Iron regulatory protein
Human oncostatin M
Human neutrophil elastase
Human CD4 antigen
Lysozyme
C-reactive protein
Tumor necrosis factor α
NF-kB
Acetylcholine receptor
Thyroid transcription factor

INORGANIC COMPOUNDS

Malachite green
Mg^{2+}

ORGANIC COMPOUNDS

ATP
FMN
Theophylline
Organic dyes
Cocaine

VITAMINS

Cyanonobalamin
Biotin

DRUGS

Neomycin B
Stretpomycin
Tobramycin
Tetracyclin
Kanamycine A
Dopamine

TOXINS

Cholera toxin
Staphylococcal enterotoxin B

POLLUTANTS AND CARCINOGENIC COMPOUNDS

4-chloroaniline
2,4,6-trichloroaniline
Pentachlorophenol
Methylenedianiline

OTHERS

Bacillus anthracis spores

Applications

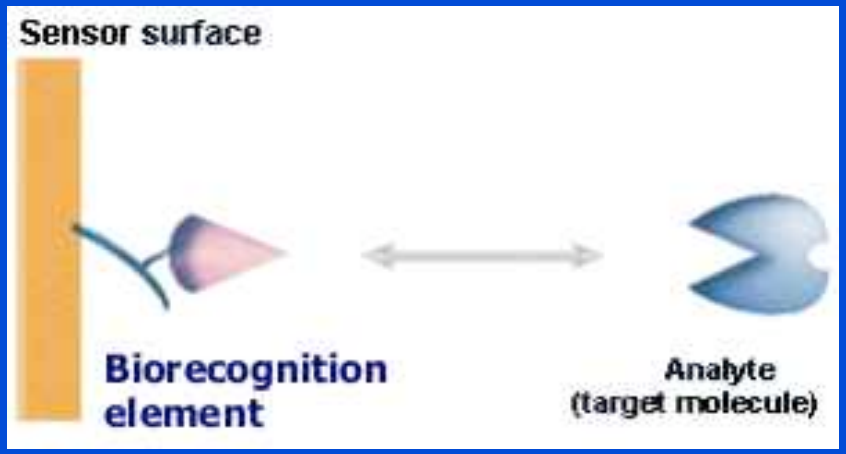
Applications based on molecular recognition:

Therapeutics: aptamers have been selected to disrupt the function of their targets and to inhibit or modify the metabolism associated with that target

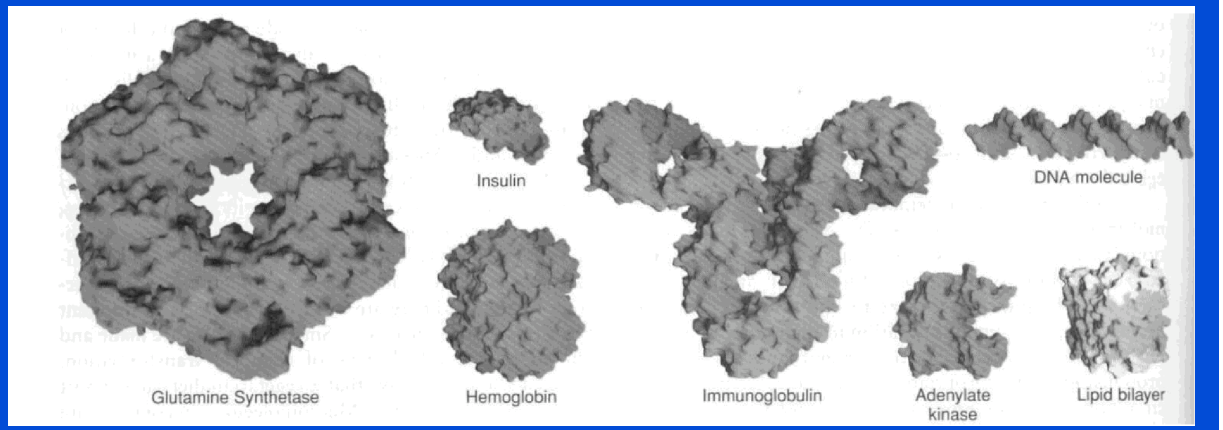
Diagnostics: the impressive discrimination between two molecules of very similar structure has suggested that aptamers can be potential diagnostic reagents

Analytical tools: flow cytometry
capillary electrophoresis and electrochromatography
affinity chromatography
biocomponents in biosensors

Affinity Biosensors



Biomolecules (natural)



Synthetic receptors:

Peptide Nucleic Acids (PNAs)
Molecularly Imprinted Polymers (MIPs)
Oligopeptides
Aptamers

Why aptamers can rival antibodies?

- Overcoming of the use of animals for their production

The immune response can fail when the target molecule, i.e. protein, has a structure similar to endogenous proteins and when the antigen consists of toxic or non-immunogenic compounds

- After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-to-batch variation found when using antibodies

- By chemical synthesis, modifications in the aptamer can be introduced enhancing the stability, affinity and specificity of the molecules

- Higher temperature stability

- Because of their small size, denser receptor layers can be generated

- Amplification by PCR

Advantages of Aptamers

- **-Malleability : the properties of aptamers can be changed on demand**
- **Targets that would not normally elicit a good immune response can be used to generate high-affinity aptamers**
- **No batch to batch variation in aptamer production since they are produced by chemical synthesis**
- **Reporter or functional molecules can be attached to aptamers at precise locations.**
- **Denaturation is a reversible process**
- **Do not cause much immunogenecity when administrated as drugs**
- **Can be created very rapidly**

Why aptamers can rival antibodies and other synthetic receptors?

- Overcoming of the use of **animals** for their production
- After selection, aptamers are **produced by chemical synthesis** and purified to a very high degree by eliminating the batch-to-batch variation found when using antibodies
- By chemical synthesis, **modifications** in the aptamer can be introduced enhancing the stability, affinity and specificity of the molecules
- Higher **temperature stability**
- Because of their **small size**, denser receptor layers can be generated

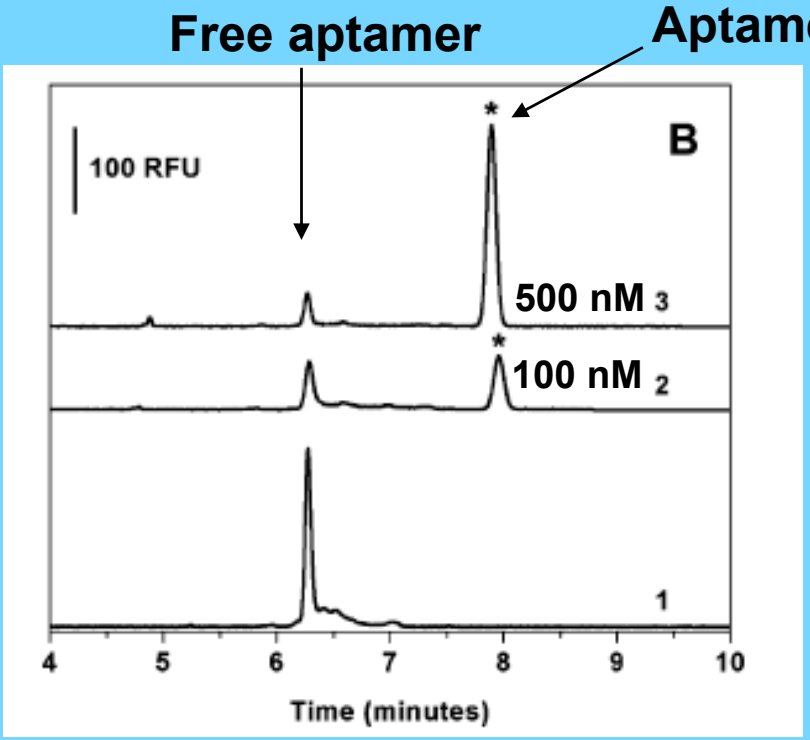
- **Amplification** by PCR during their selection

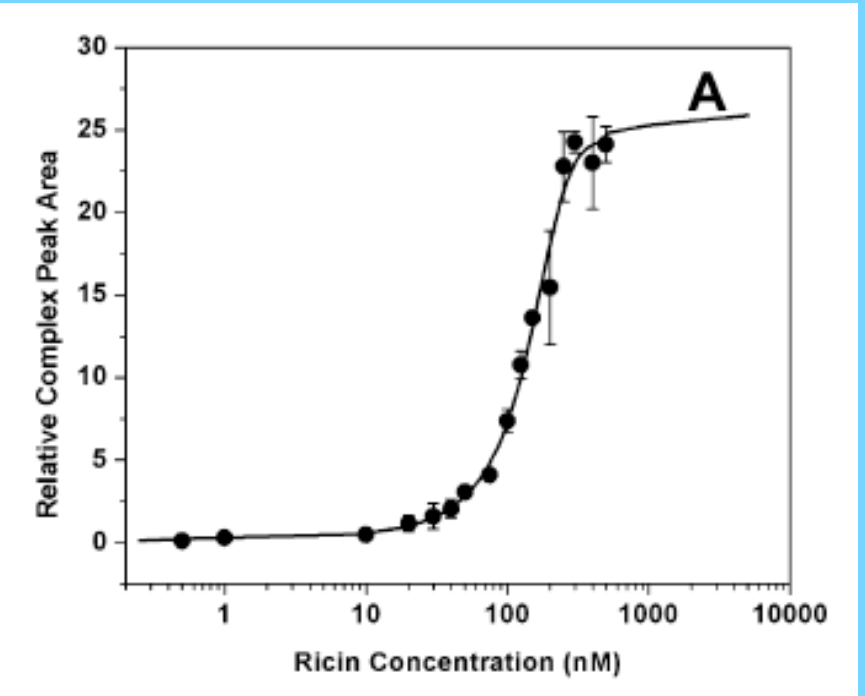
Advantage respect to other synthetic receptors such as **oligopeptides** which have a higher number of possible structures due to the higher number of “building blocks” (21 aminoacids), but they can not be amplified during the “production”

Capillary electrophoresis

The integration of laser-induced fluorescence-based capillary electrophoresis with fluorescently labelled aptamers provides a novel approach for the detection of ricin. This free solution assay offers an alternative technique for protein detection in comparison to standard immunoassay and ELISA methods.

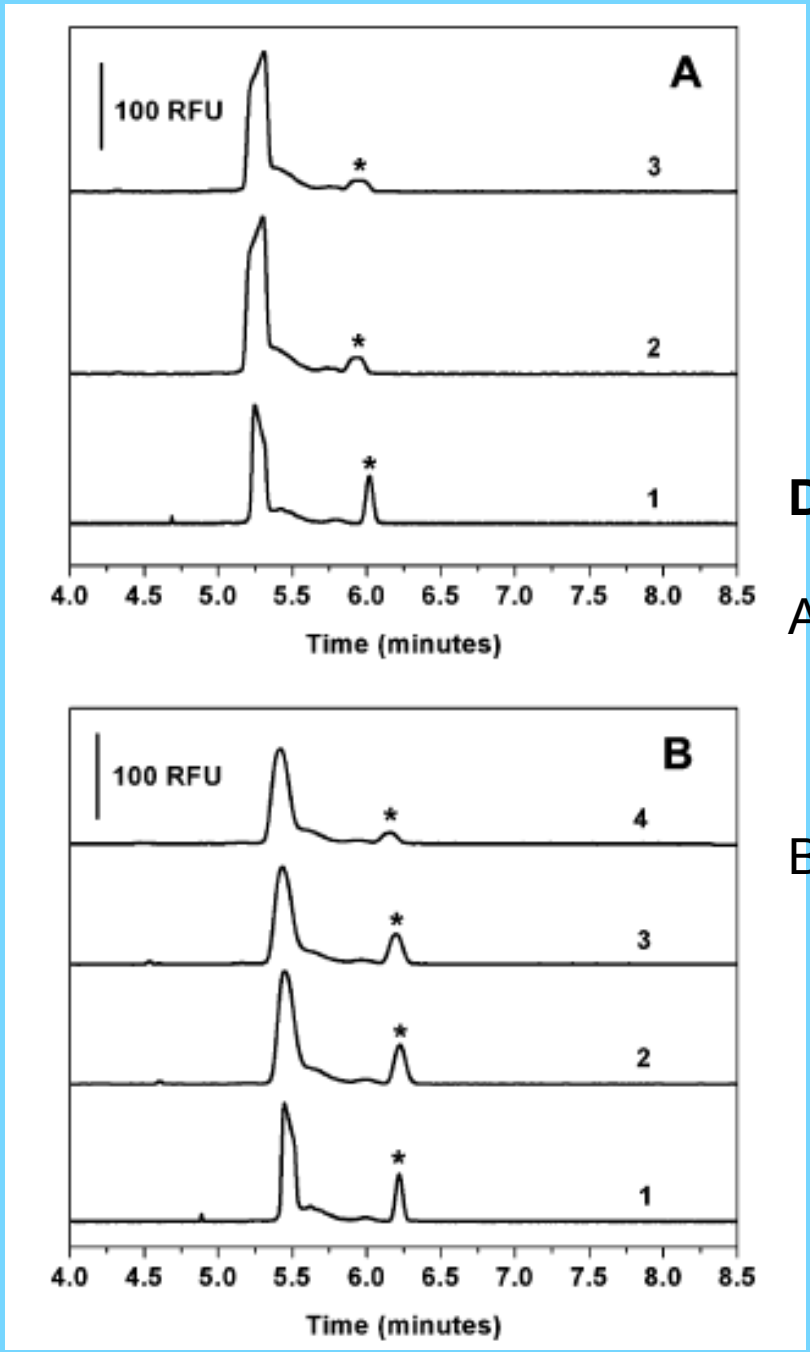
- Target molecule: Ricin
- RNA aptamer
- Transducer: affinity probe-based capillary electrophoresis





A.J. Haes et al., Anal. Chem., 78, 3758-3764, (2006)

Capillary electrophoresis



- Detection limit 500 pM
- Dynamic range low nM- low μM
- K_d K_d=134 nM

Detection of ricin in protein mixtures:

A) (1) 50 nM ricin
(2) 50 nM ricin and 50 μg/mL BSA
(3) 50 nM ricin and 100 μg/mL BSA

B) (1) 50 nM ricin
(2) 50 nM ricin and 50 μg/mL casein
(3) 50 nM ricin and 100 μg/mL casein
(4) 50 nM ricin and 150 μg/mL casein

Transducers

- Acoustic sensors
- Cantilever-based biosensors
- Optical sensors
- Electrochemical sensors

Critical aspects to be considered when developing an aptamer-based biosensor

APTAMER IMMOBILIZATION

The procedure to fix the aptamer to the biosensor/bioanalytical device surface is of paramount importance to obtain an ordered layer able to exploit as much as possible the flexibility of the bioreceptor without altering its structure and its affinity for the target molecule.

The immobilization of the aptamer on a solid support must avoid any steric hindrance or constraint which could prevent the folding of the aptamer in the correct conformation

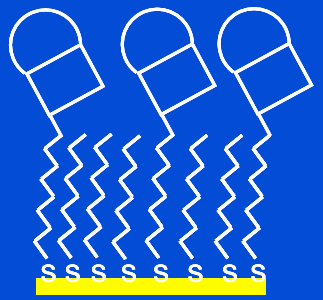
BINDING PROTOCOL

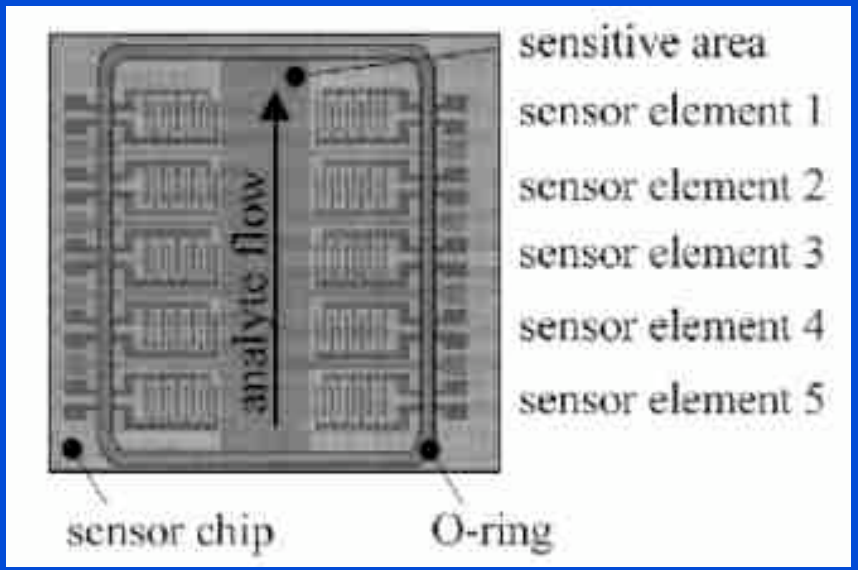
From the examination of the different protocols employed in aptamer-based assays, one important point must be emphasized and that is the nature, conformation and sequence of each aptamer should be carefully considered and also stress that optimal working conditions can remarkably vary from one aptamer to another

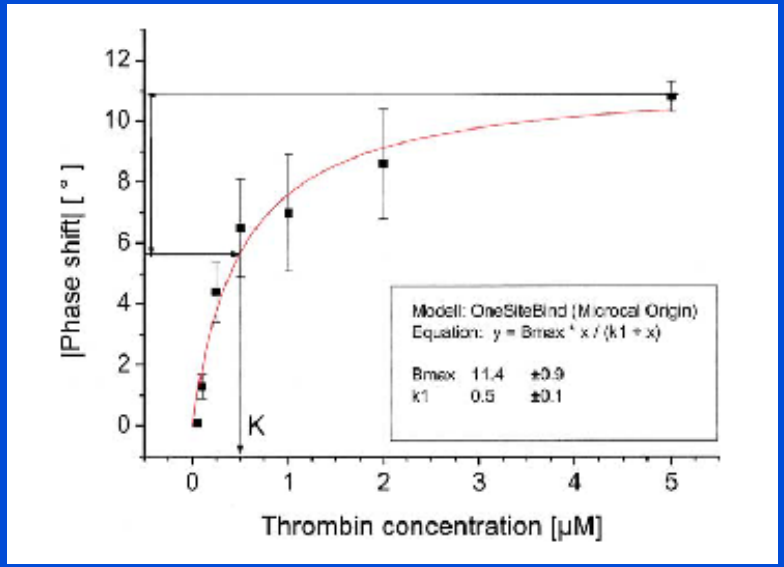
Love-wave biosensor

Love-wave sensors: highly sensitive analyte detection can be achieved in parallel fashion opening up the possibility of using the sensor-principle in an array format

- Target molecule: Thrombin and Rev peptide
- DNA aptamer
- Transducer: SAW Love-wave sensor
- Immobilisation of the aptamer on the sensor surface:







- Detection limit — 72±11 pg/cm² (thrombin); 77±36 pg/cm² (Rev peptide)
- Dynamic range — low nM- low µM
- Affinity-like constant — K=500 nM

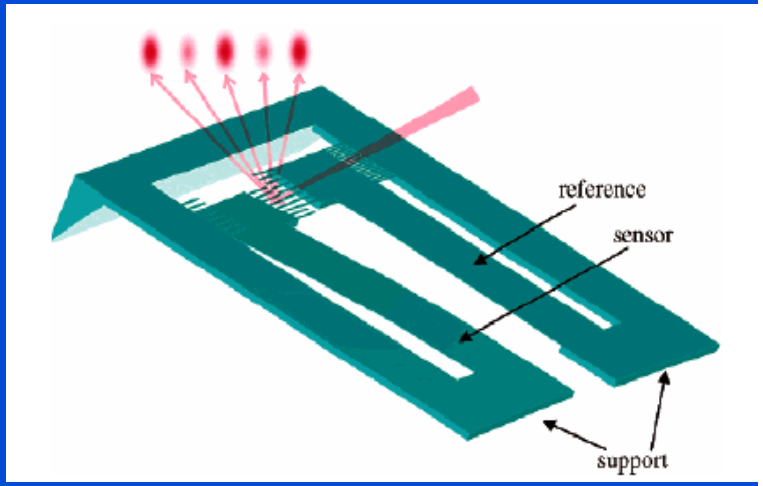
M.D. Schlensog, T.M.A Gronewold, M. Tewes, M. Famulok, E. Quandt, Sensors Act. B, 101, 308-315, (2004)

Cantilever-based biosensor

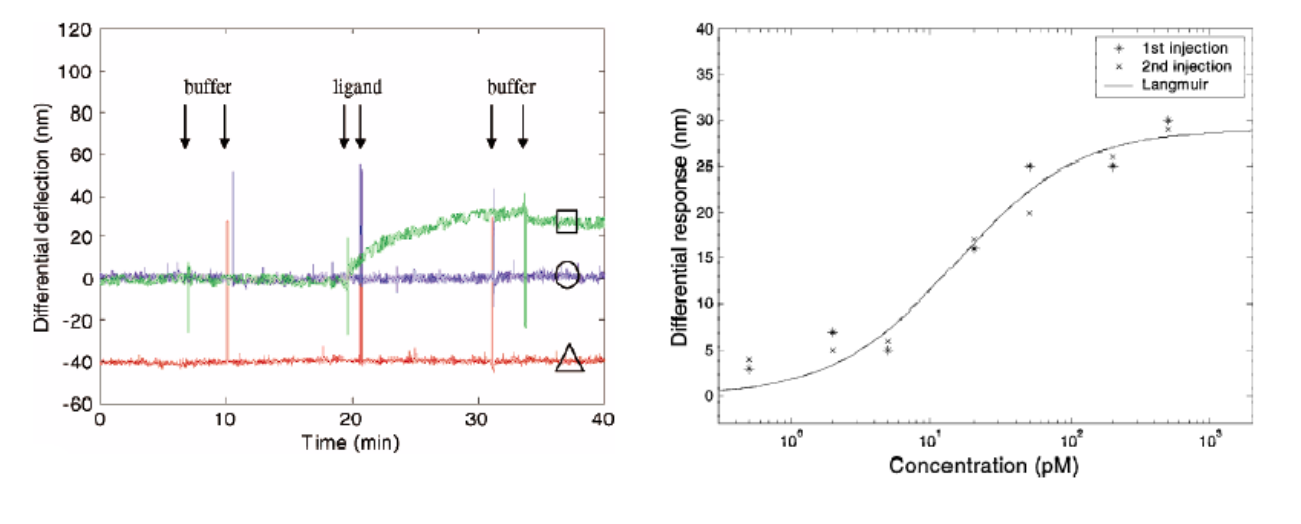
Cantilever-based biosensing:

Label-free detection
Batch-fabricated
Small scale

} Arrays can be used in parallel to detect various proteins simultaneously



- Target molecule: Taq DNA polymerase
- DNA aptamer
- Transducer: cantilever
- Immobilisation of the aptamer on the sensor:
  5' thiolated aptamer immobilised on gold

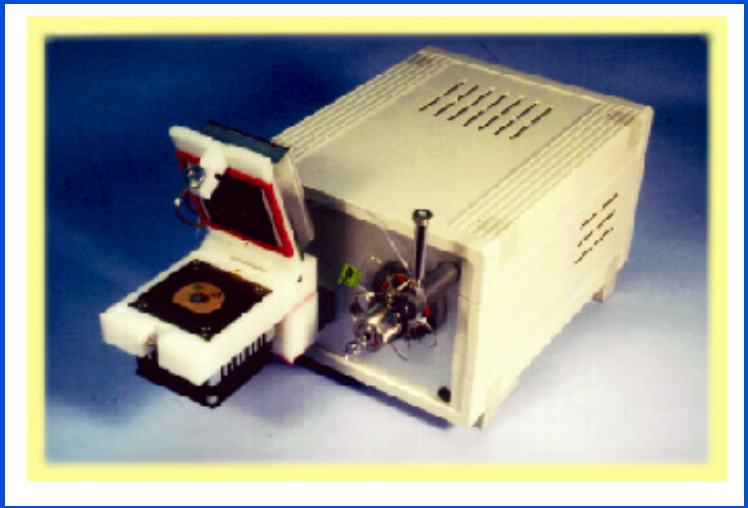


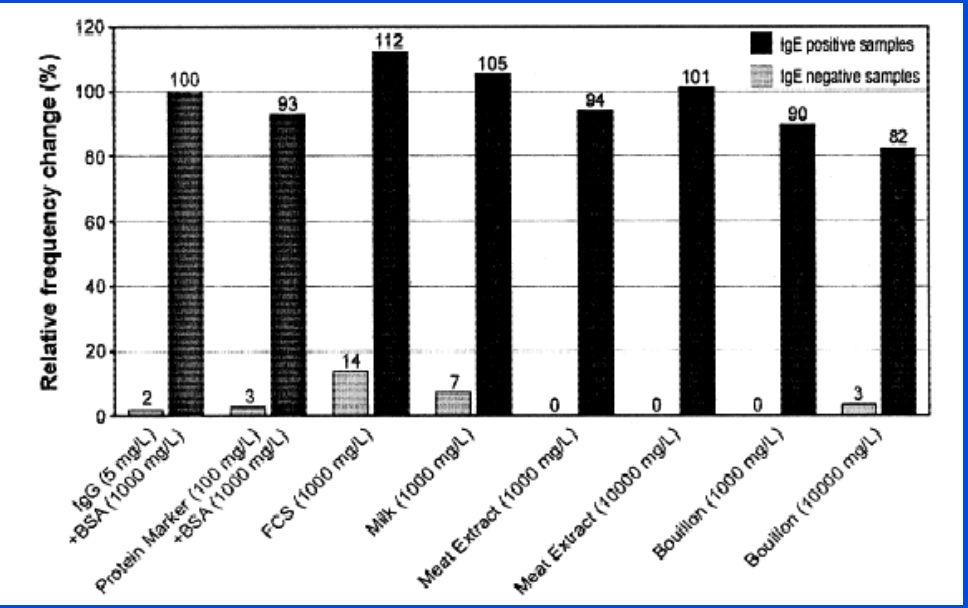
- Affinity: Kd=15 pM

C.A. Savran, S.M. Knudsen, A.D. Ellington, S.R. Manalis, Anal. Chem. 76, 3194-3198, (2004)

Quartz crystal biosensor 1

- Target molecule: human IgE
- DNA aptamer compared with anti-IgE antibody
- Transducer: quartz crystal microbalance
- Immobilisation of the aptamer on the sensor surface: 5' biotinylated aptamer immobilised on streptavidin fixed on the gold surface with DSP.





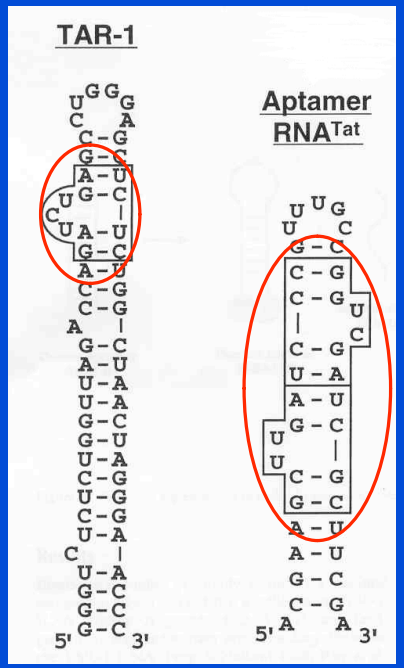
- Detection limit 100 μg/L (Ab and aptamer)
- Linear range 0.1-1 mg/L (Ab)
  0.1-10 mg/L (aptamer)
- Affinity Kd= 1.9 nM (Ab)
  Kd= 3.6 nM (aptamer)
- Stability crystals modified with aptamers could be stored for several weeks

M. Liss, B. Petersen, H. Wolf, E. Prohaska, Anal. Chem., 74, 4488-4495, (2002)

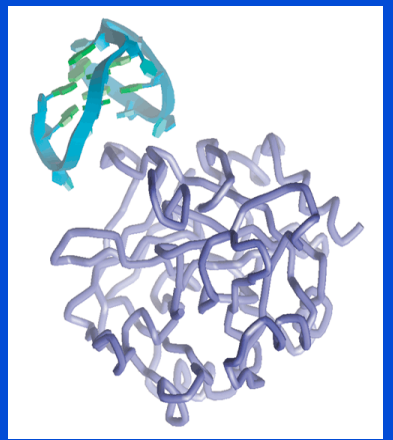
Quartz crystal biosensor and Surface Plasmon Resonance biosensor

- Target molecule: HIV-1 tat protein and thrombin
- RNA aptamer (tat) and DNA aptamer (thrombin)
- Transducer: quartz crystal microbalance and SPR

Tat is a small polypeptide of 86-102 amino acids comprising a few functional regions, controlling the HIV-1 replication cycle. The arginine-rich region (49-57) of Tat is involved in binding the RNA trans -activation response element (TAR).



Thrombin is a serine protease and its function is to cut specifically the large protein fibrinogen into fibrin monomers. The conversion of the plasma precursor prothrombin (factor II) to α-thrombin is one of the final steps in the blood cascade.

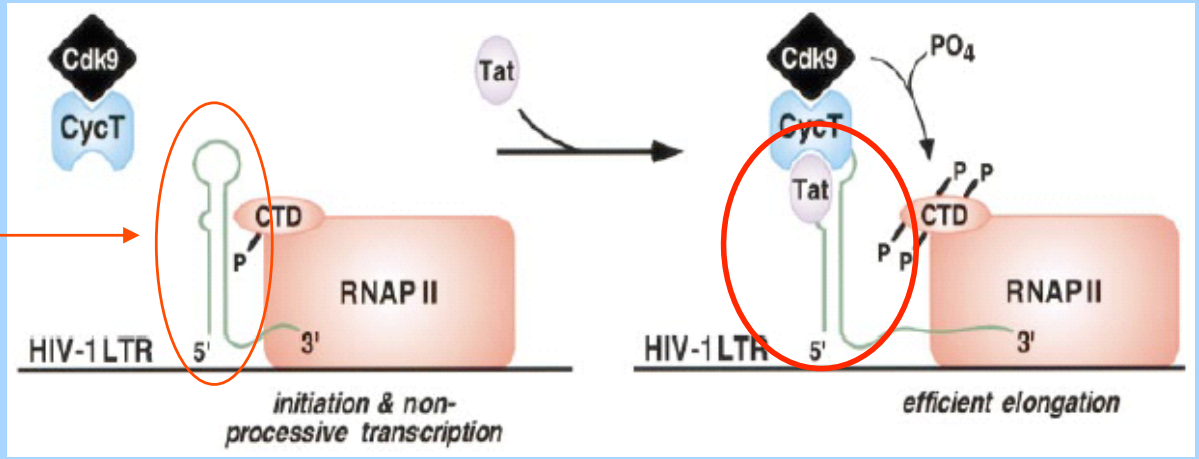


Tat Protein (trans-activating protein)

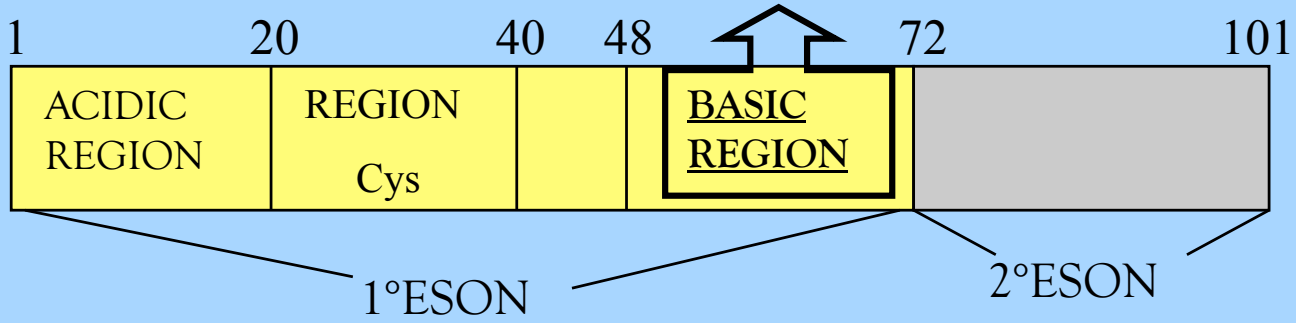
- Protein containing 86-101 aa
- Replication HIV virus and HIV pathologies related
- Vaccin Experimental work
- Diagnostic interest

TAR

(trans-activating region)



In the region 49-57 the domaine for binding TAR is present



Measurement optimisation (tat protein)

Binding buffer optimisation (buffer, pH, ionic strength, Mg content):

a) Tris 10 mM, NaCl 70 mM, EDTA 0.2 mM, pH 7.4
b) Sodium citrate 50 mM, NaCl 150 mM, pH 6.5
c) Buffer a) + BSA 0.1 %
d) Biacore running buffer (HEPES 10 mM, NaCl 150 mM, EDTA 3 mM, Tween 20 0.005%, pH 7.4) + BSA 0.1%

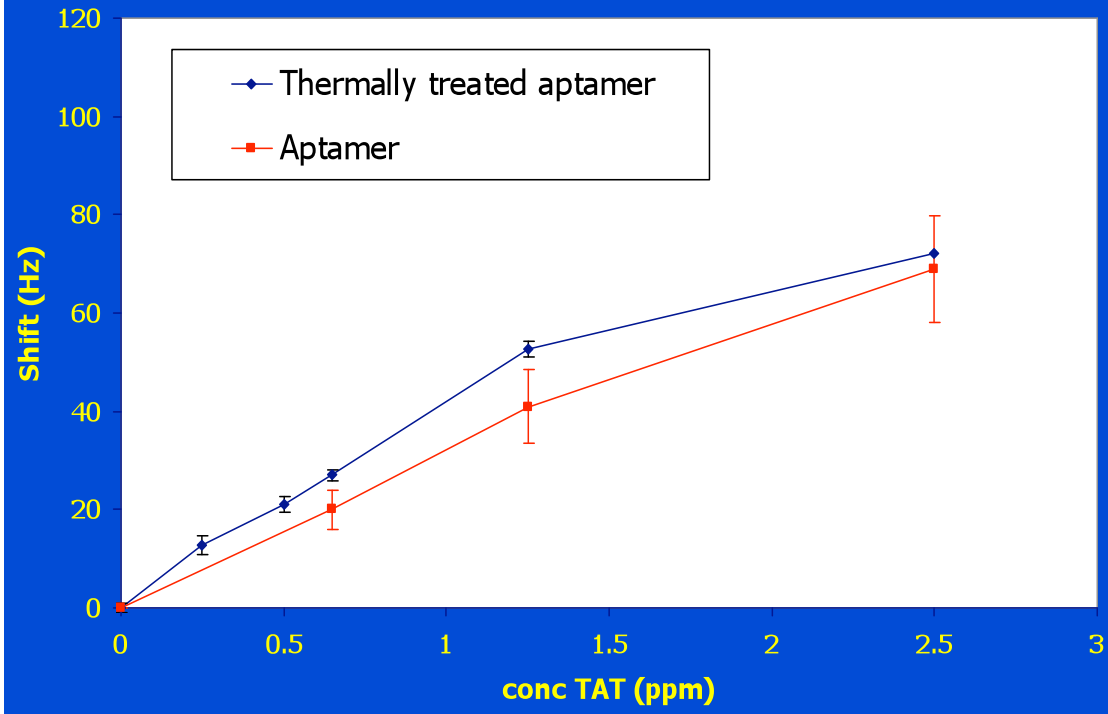
Interaction time: 15 minutes

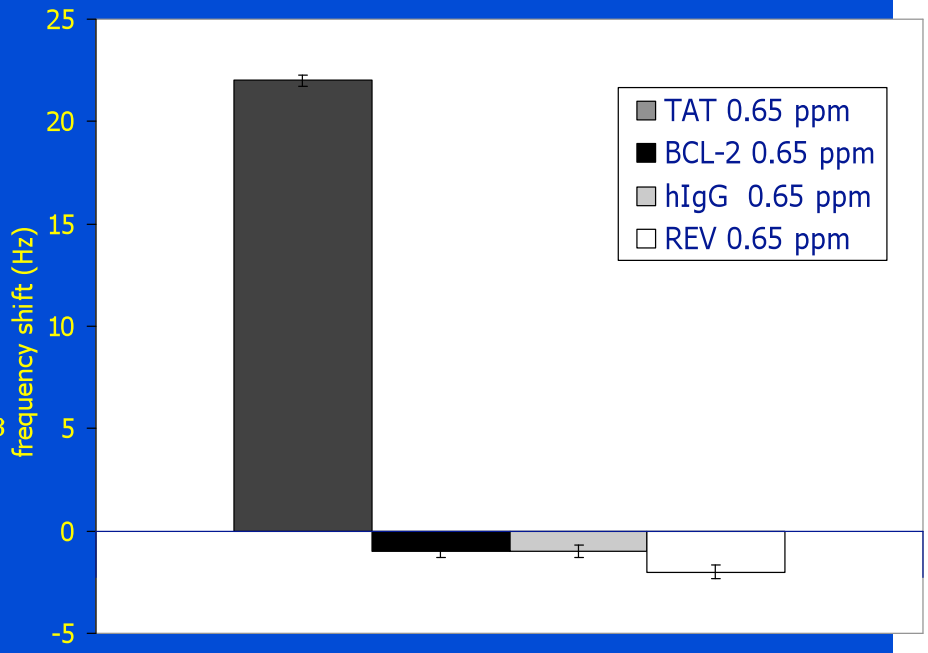
Regeneration

Regeneration solution 1: binding buffer + SDS (0.1%)
more than 5 steps of 30 sec

Regeneration solution 2: NaOH 12 mM + EtOH 1.2%
complete regeneration in 2 steps of 30 sec

Piezoelectric biosensor results





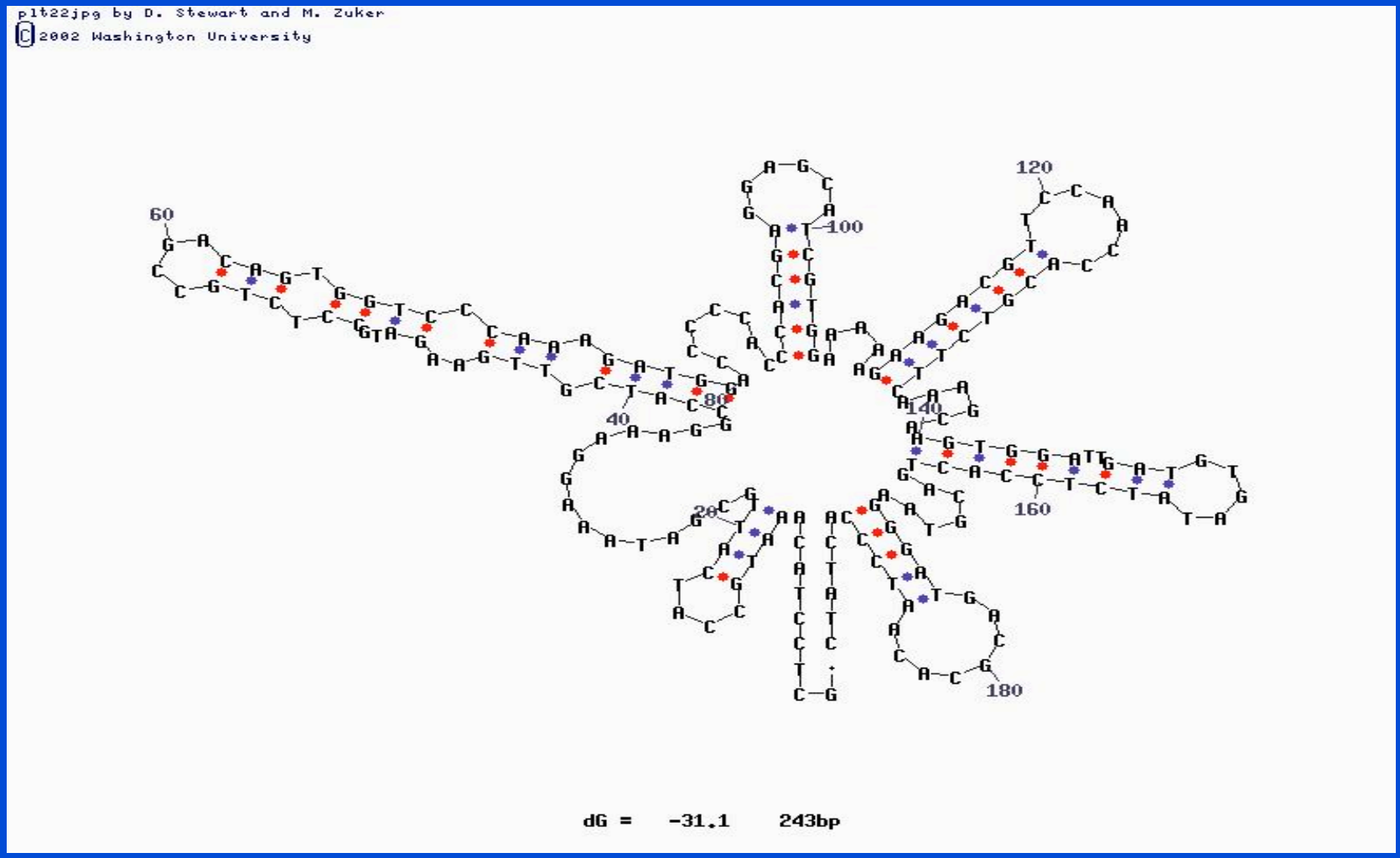
Improvements in reproducibility

Non-treated aptamer: CV%=16% (n=3 for each concentration) (1 crystal);
CV%=21% (8 crystals)

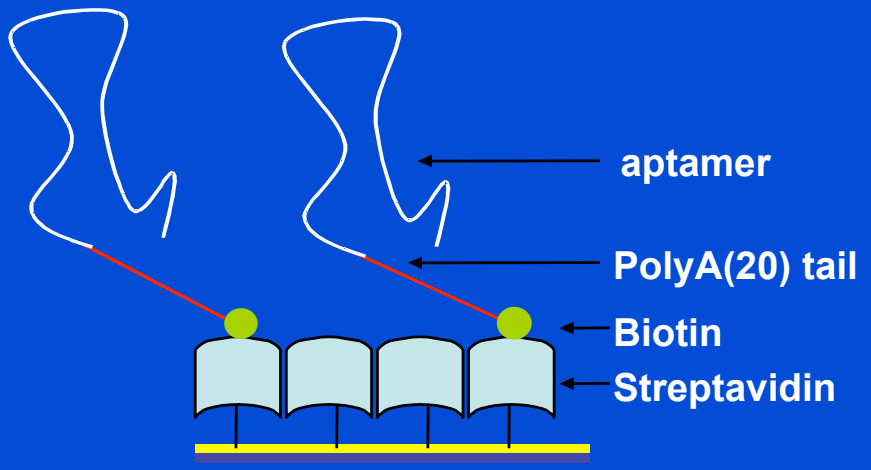
Thermally treated aptamer: CV%=6% (n=3 for each concentration) (1 crystal);
CV%=8% (8 crystals)

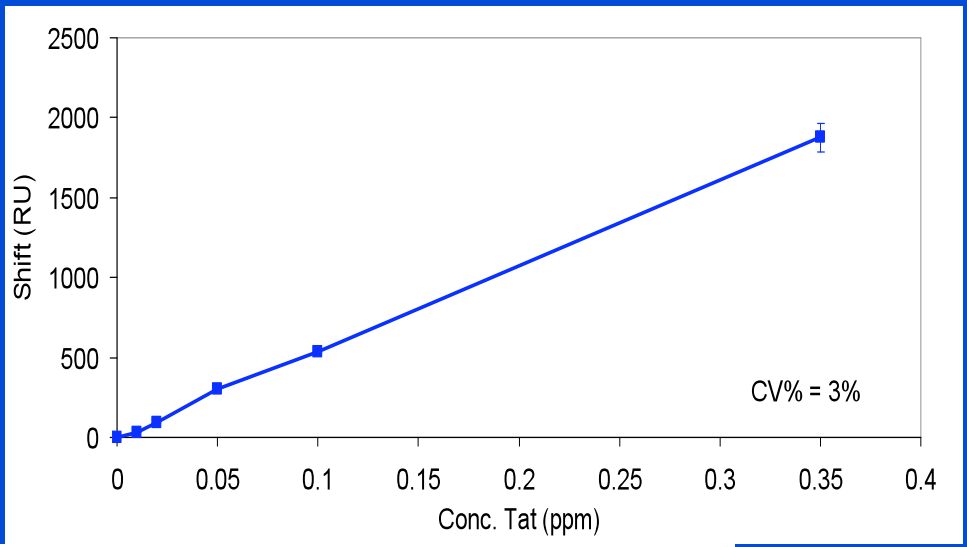
M. Minunni, S. Tombelli, A. Gullotto, E. Luzi, M. Mascini, Biosens. Bioelectron, 20 (2004) 1149-1156

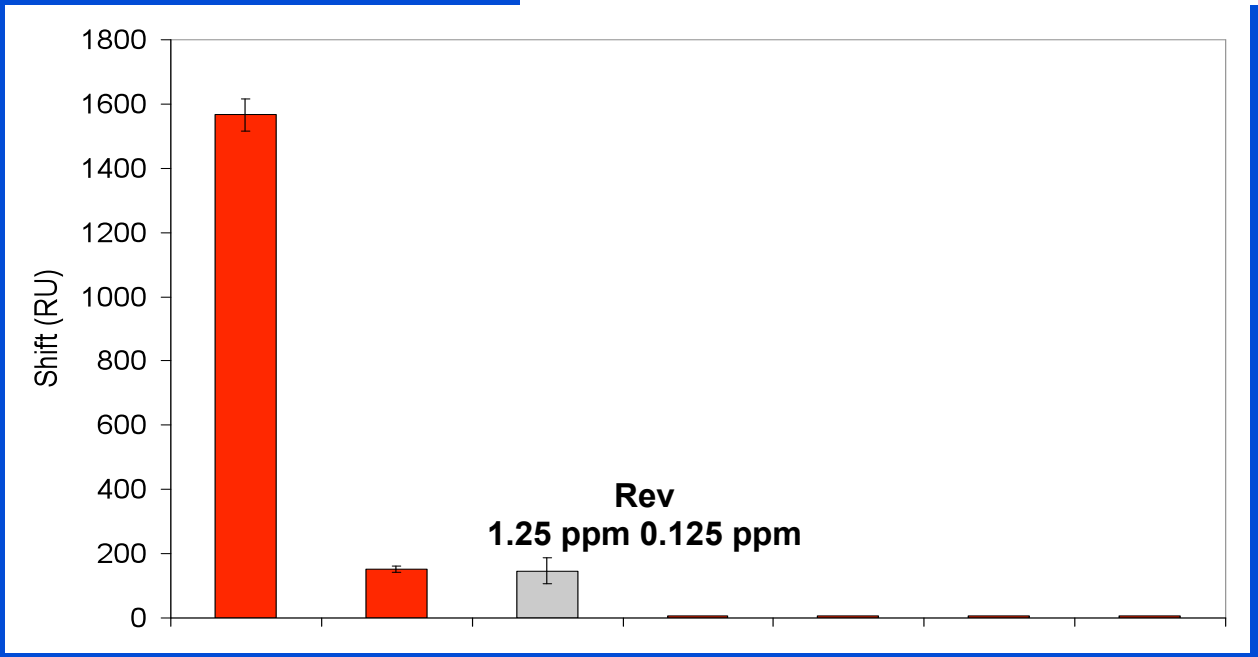
Secondary structures of ssDNA of P35S



SPR biosensor results (aptamer with polyA tail)







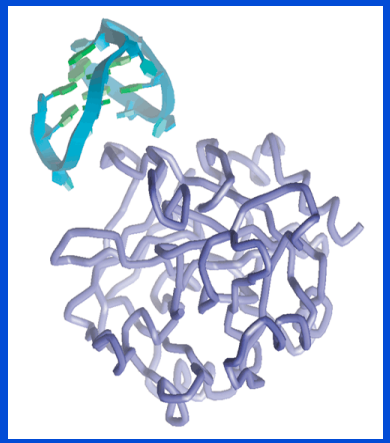
M. Minunni, S. Tombelli, E. Luzi, M. Mascini, Bioelectrochemstry, 67(2) (2005) 135-141.

Quartz crystal biosensor and Surface Plasmon Resonance biosensor

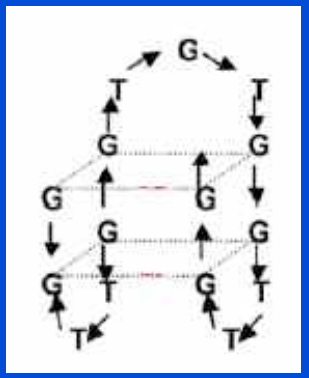
- Target molecule: Thrombin
- DNA aptamer
- Transducer: quartz crystal microbalance and SPR

Thrombin is a serine protease and its function is to cut specifically the large protein fibrinogen into fibrin monomers.

The conversion of the plasma precursor prothrombin (factor II) to α-thrombin is one of the final steps in the blood cascade.

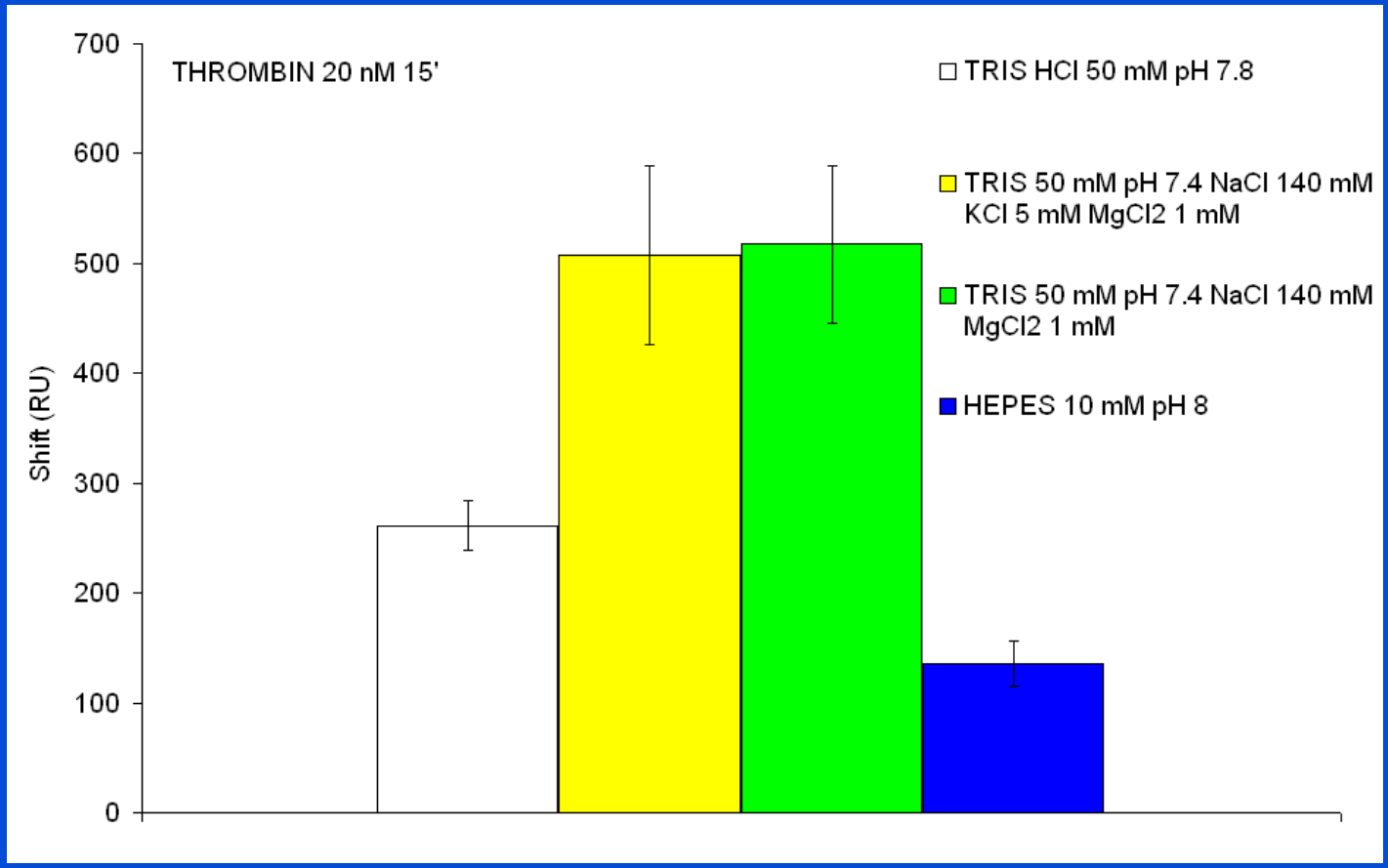


The DNA oligonucleotide d(GGTTGGTGTGGTTGG) (thrombin aptamer) binds to thrombin and inhibits its enzymatic activity in the chain of reactions that lead to blood clotting



Measurement optimisation (thrombin) SPR

Binding buffer optimisation (buffer, pH, ionic strength, Mg content):

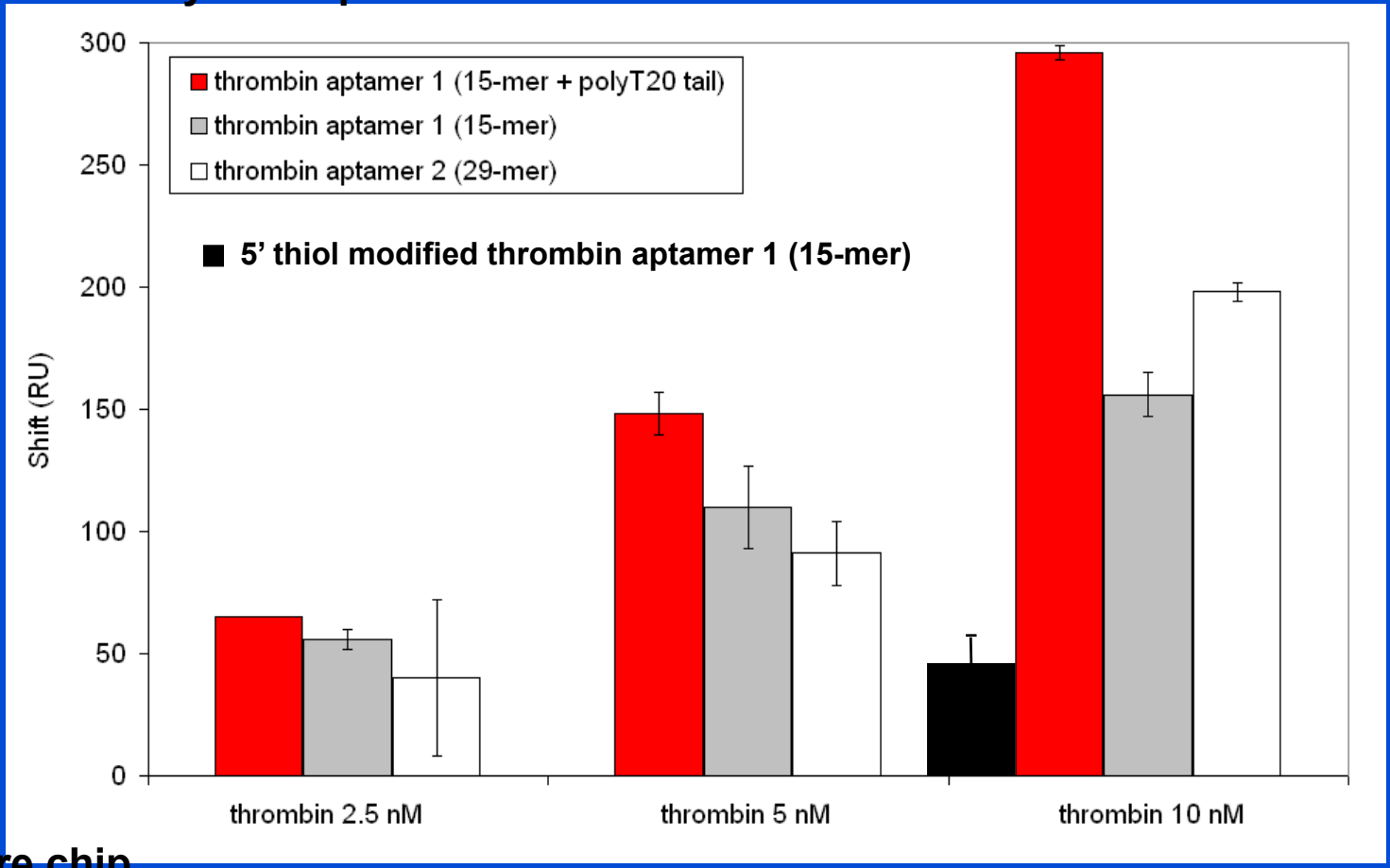


Regeneration

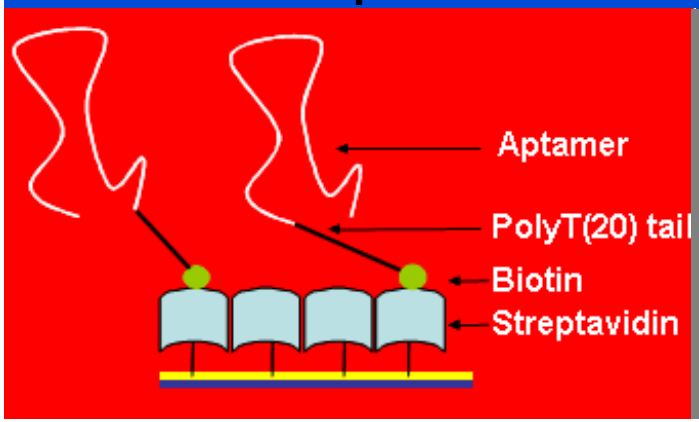
Regeneration solution: SDS (0.025%)
NaOH 12 mM + EtOH 1.2%
HCl 50 mM
NaCl 2 M

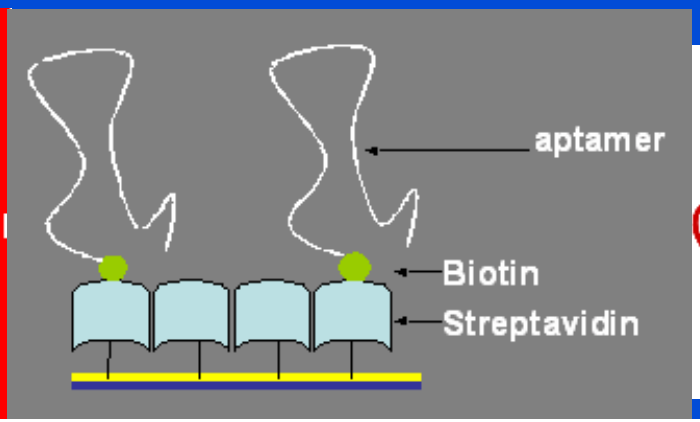
Immobilization optimization (SPR)

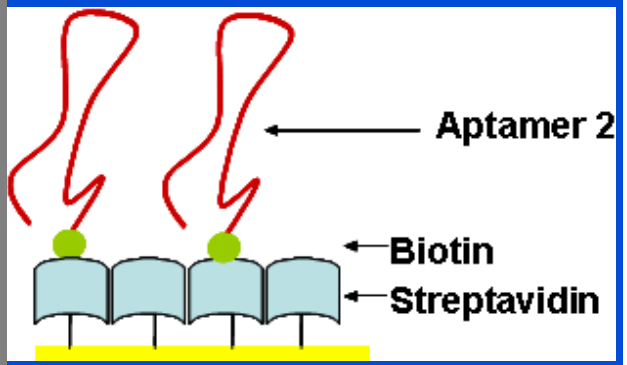
5' biotinylated aptamers



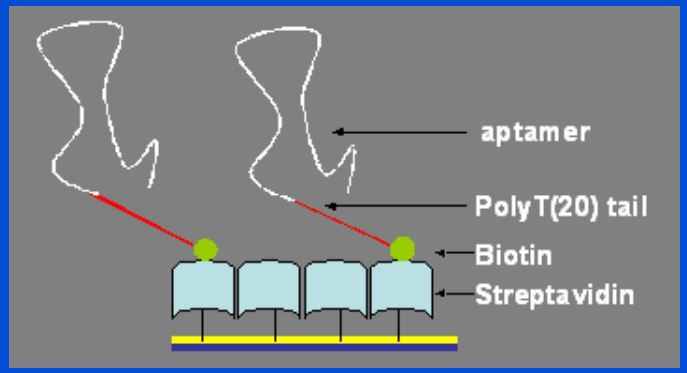
CM5 Biacore chip

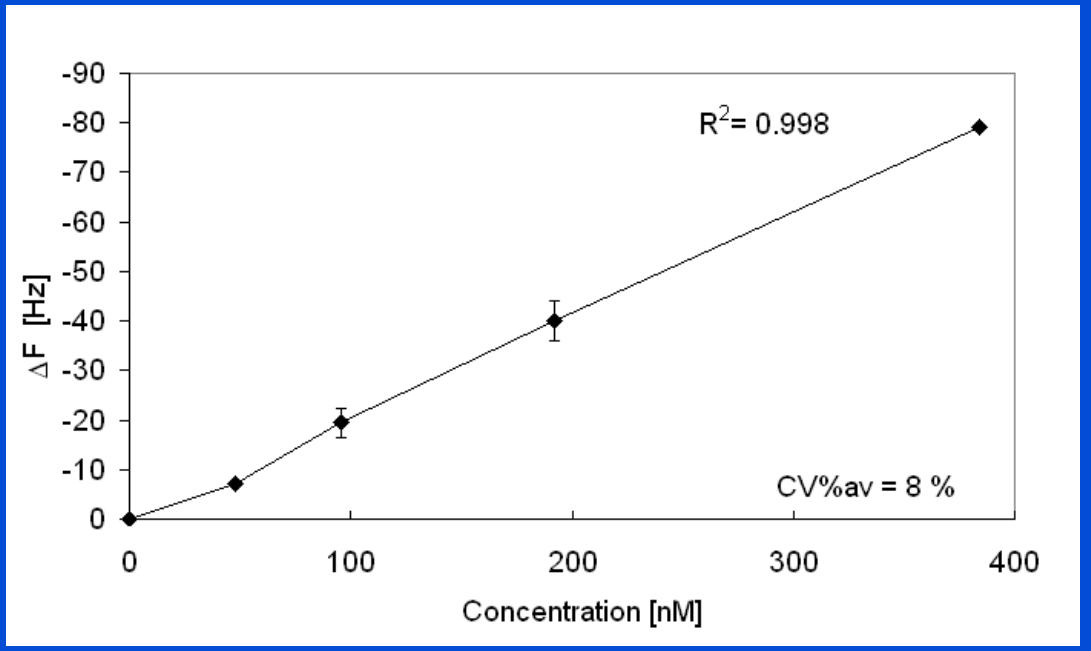


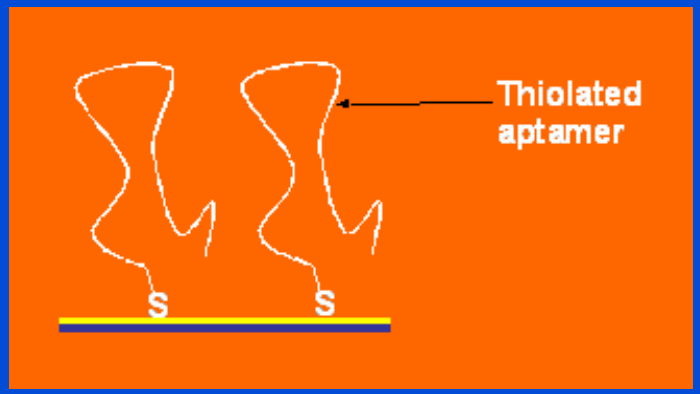


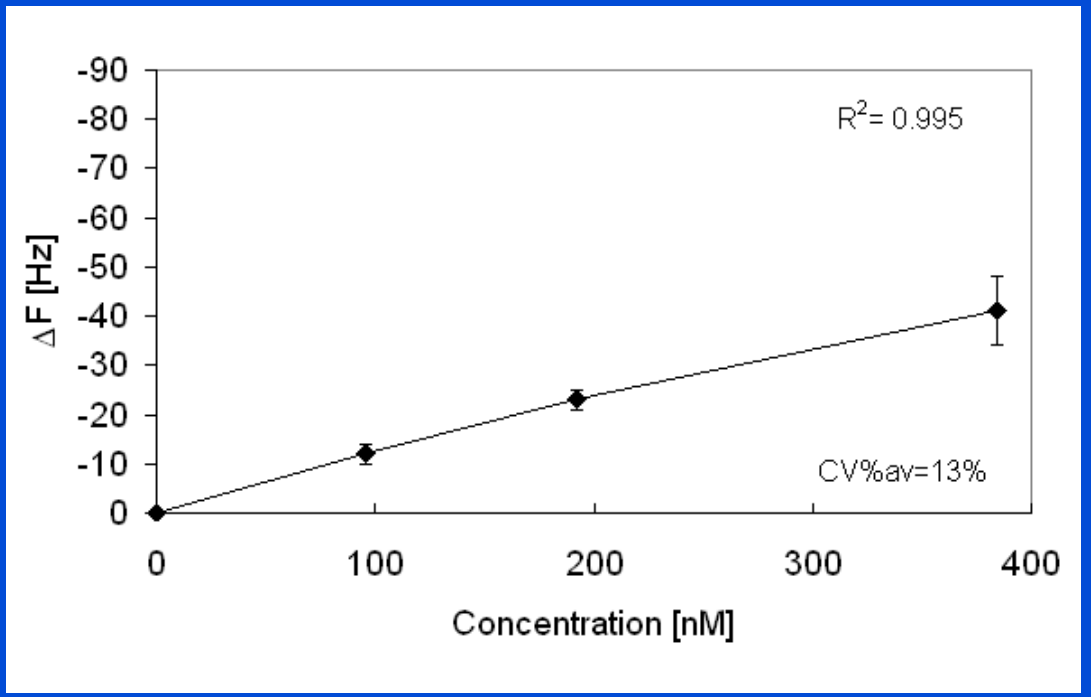


Immobilisation optimisation QCM

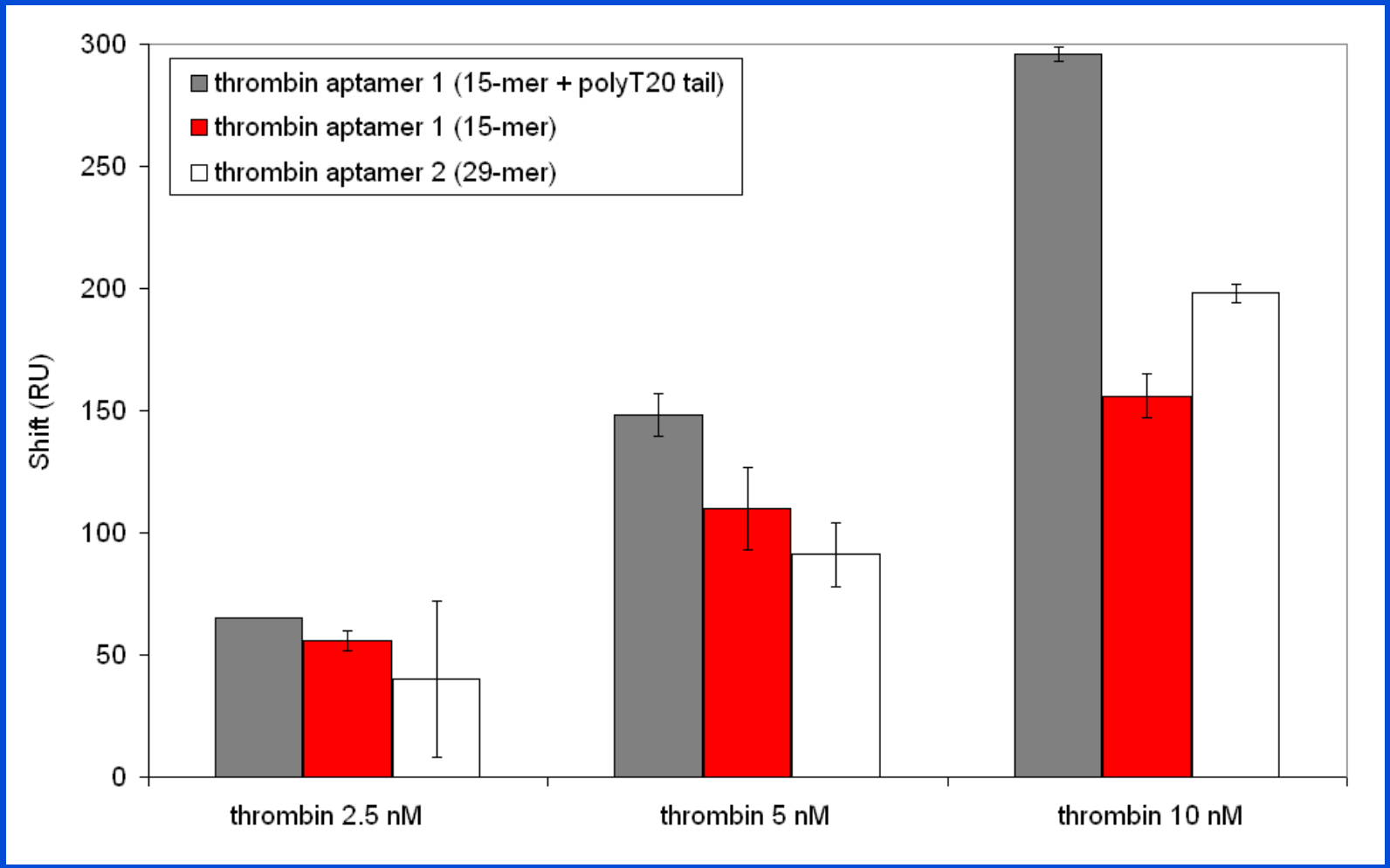


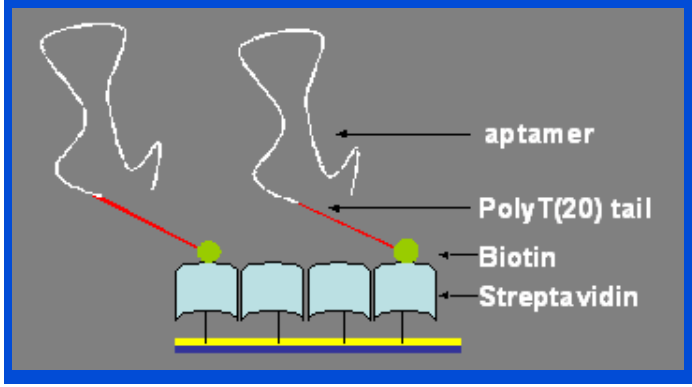


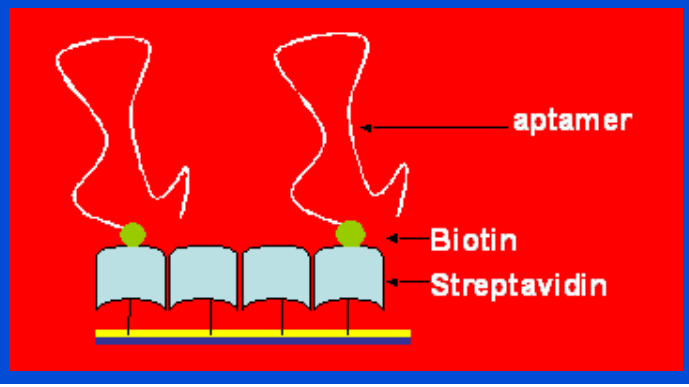


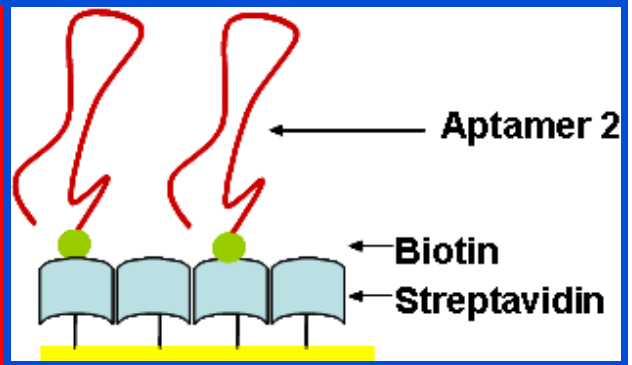


Immobilisation optimisation SPR

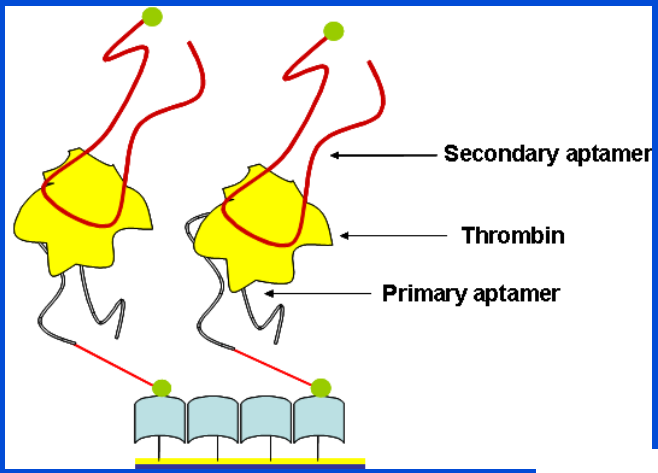


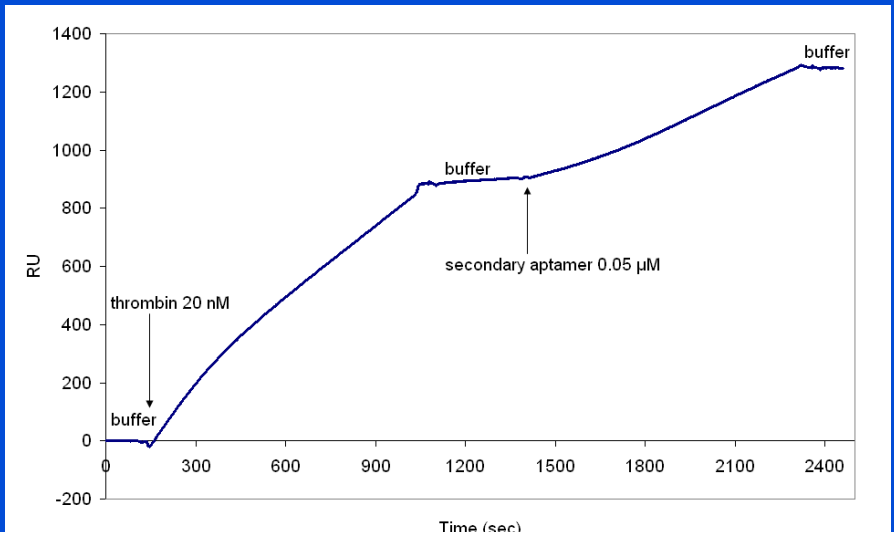






Sandwich assay

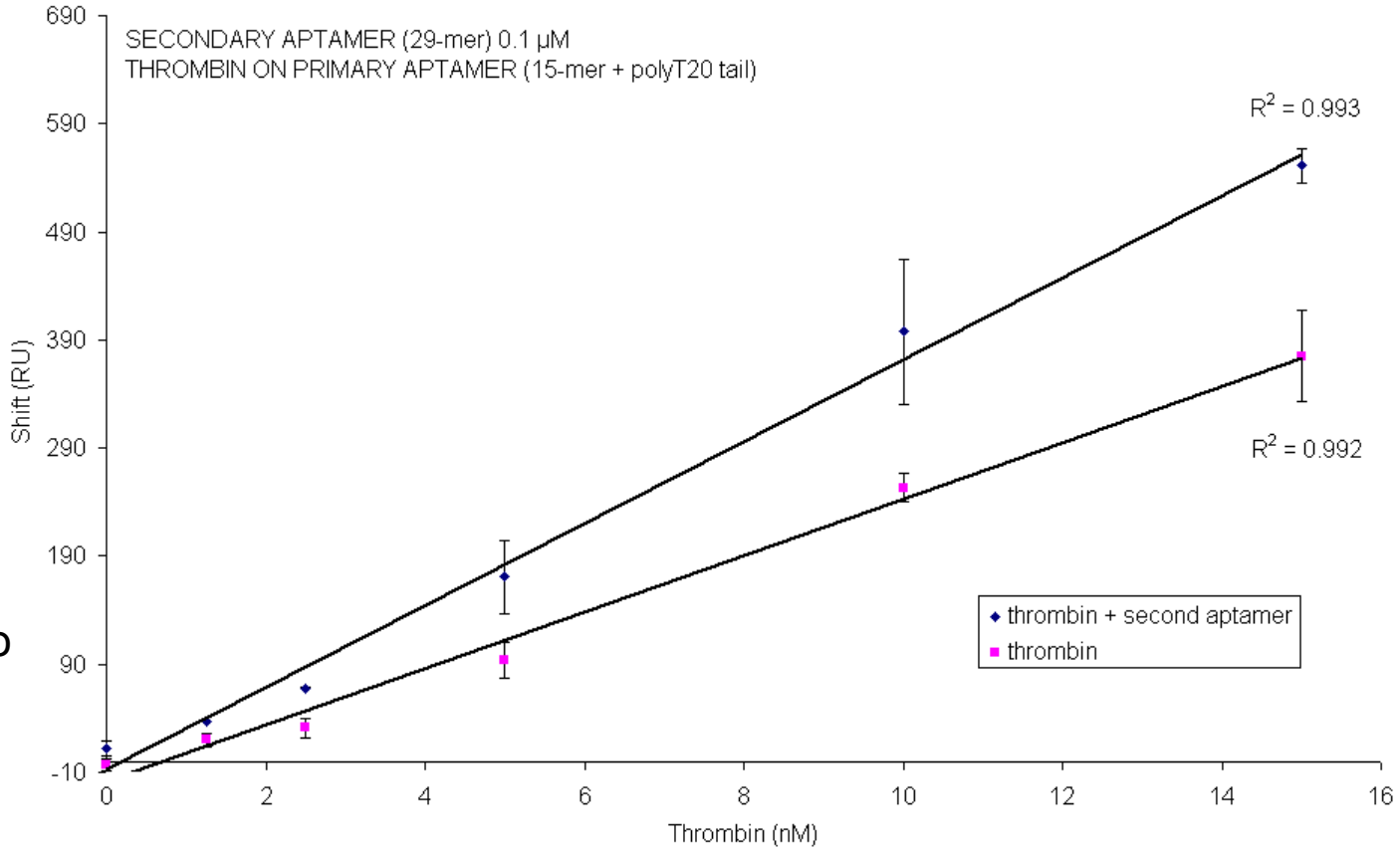




Detection limit

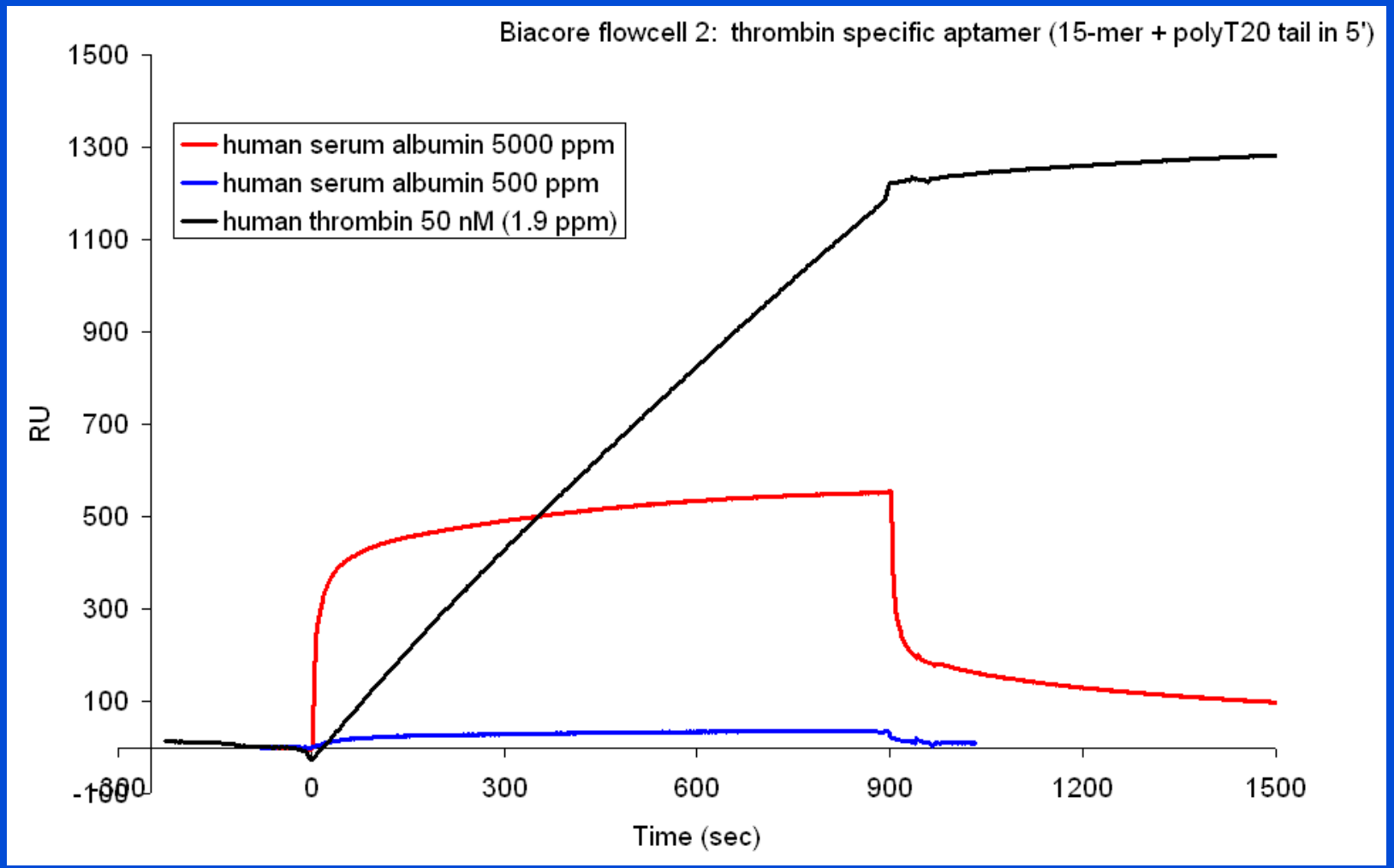
1.4 nM
0.7 nM (sandwich)
(thrombin physiological conc. range: low nM – low µM)

Protocol to be transferred onto an electrochemical biosensor coupled with magnetic beads



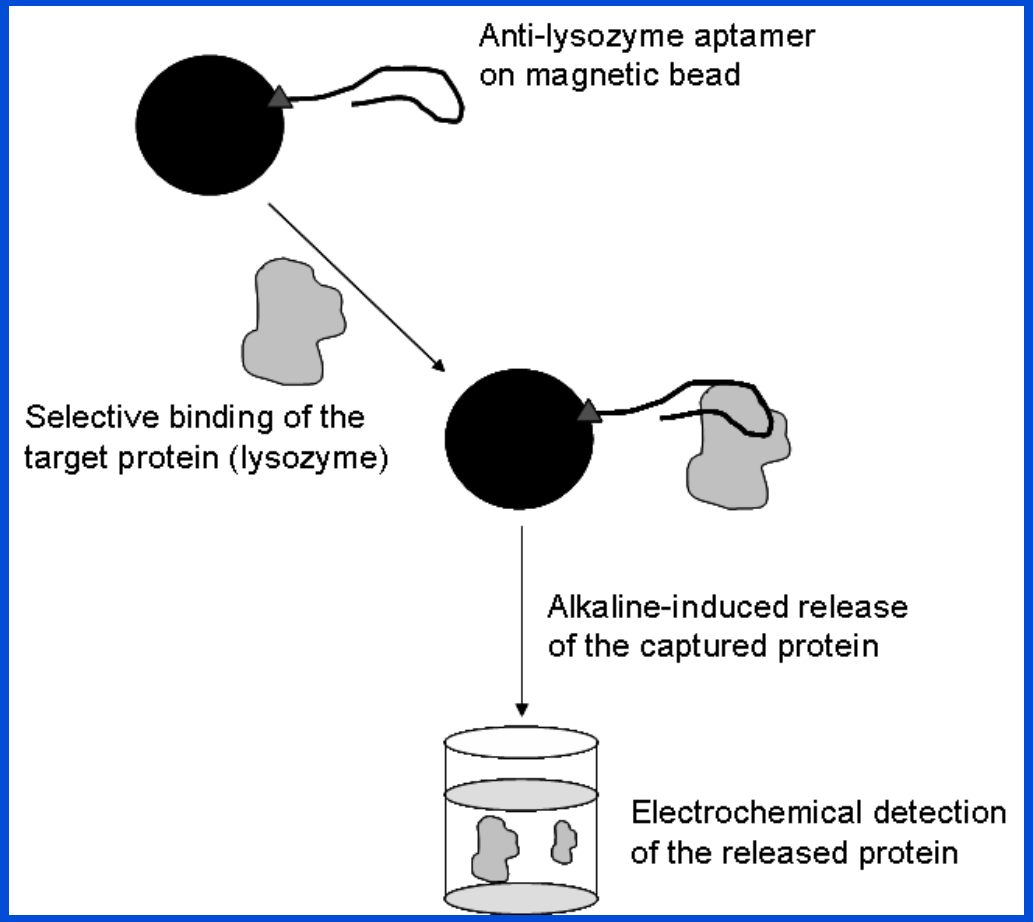
Specificity test SPR

Thrombin 50 nM (1.9 ppm)	1340 RU
Human serum albumin 500 ppm	9 RU
Human serum albumin 5000 ppm	57 RU



Electrochemical sensors

- Target molecule: Lysozyme
- DNA aptamer
- Transducer: Electrochemical detection
- Immobilisation of the aptamer on magnetic beads



This reagentless label-free detection cannot be accomplished with traditional immunoassays due to the presence of the electroactive residues both in the target protein and in the antibody.

method presented as an alternative technique for the development of protein biochips

Kawde, A., Rodriguez, M.C., Lee, T.M.H., and Wang, J., Label-free bioelectronic detection of aptamer-protein interactions, Electrochem. Comm. 7, 537, 2005

Electrochemical sandwich assay

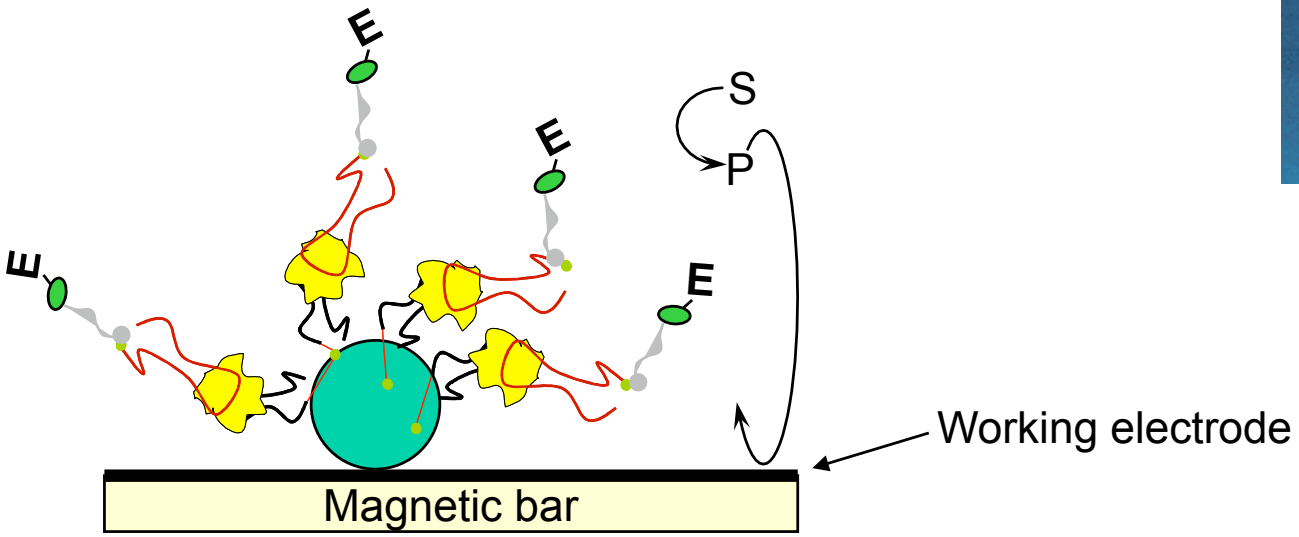
Streptavidin-coated magnetic bead

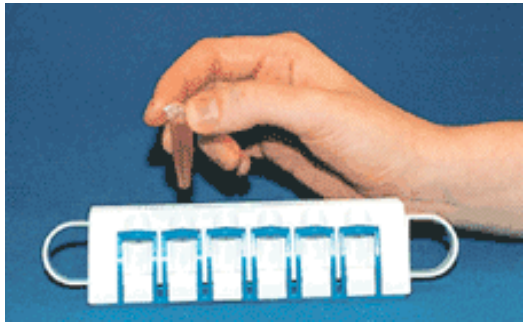
Thrombin

E Streptavidin-alkaline phosphatase conjugate

5’ biotinylated aptamer + polyT tail (20-mer)

B 5’ biotinylated secondary aptamer





Magnetic separator

Immunochemical reactions and magnetic separation

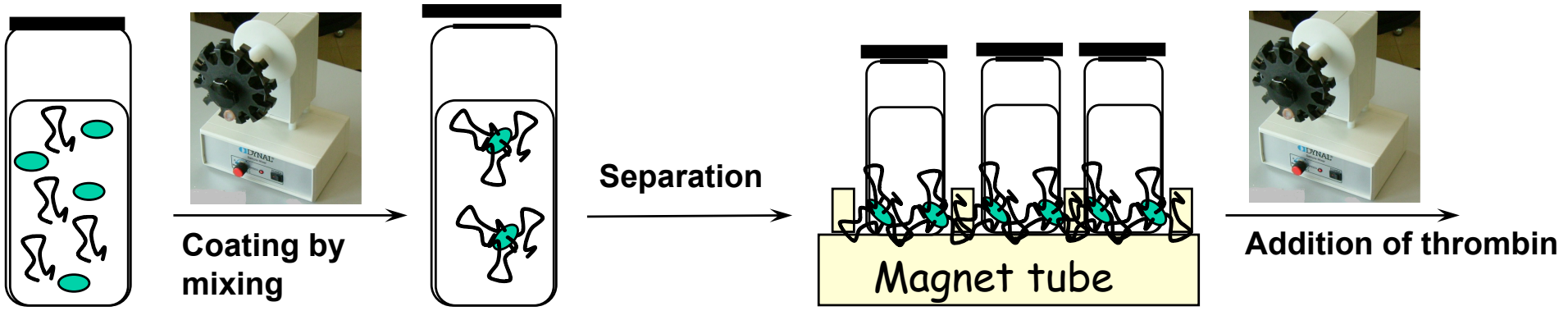
Streptavidin-coated magnetic bead

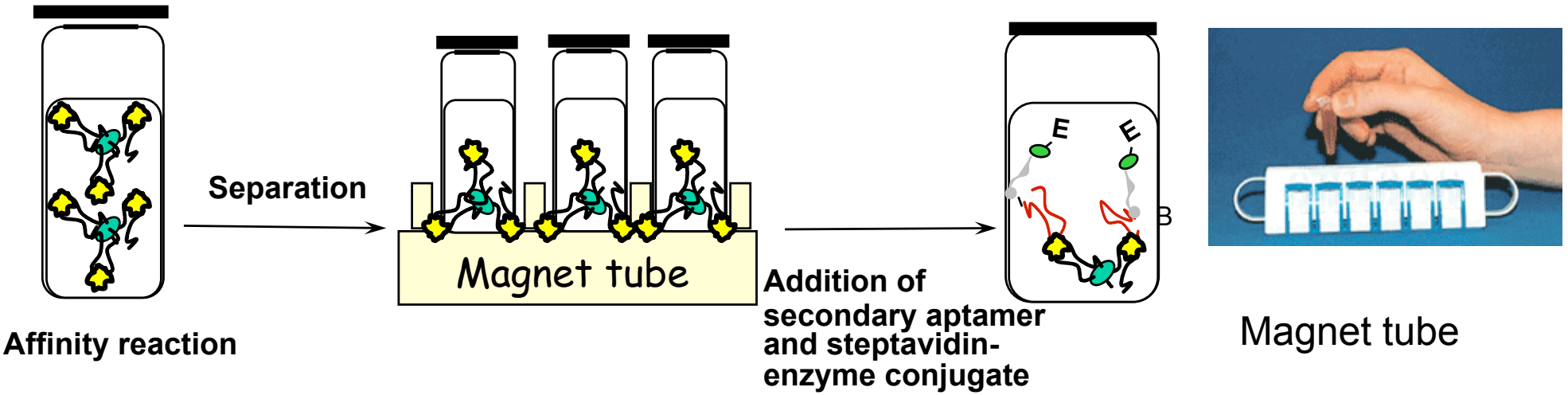
Thrombin

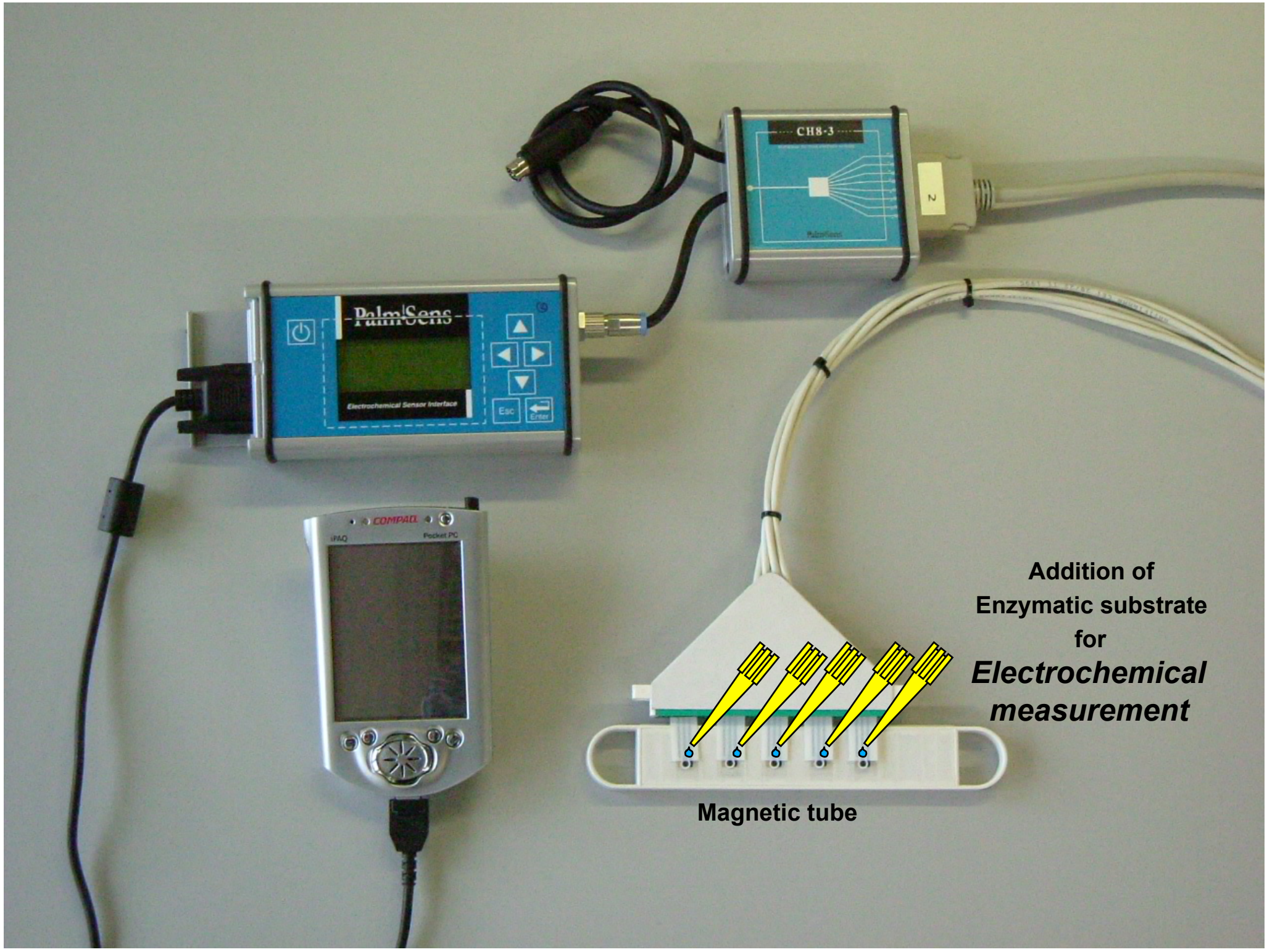
E Streptavidin-alkaline phosphatase conjugate

5’ biotinylated aptamer + polyT tail (20-mer)

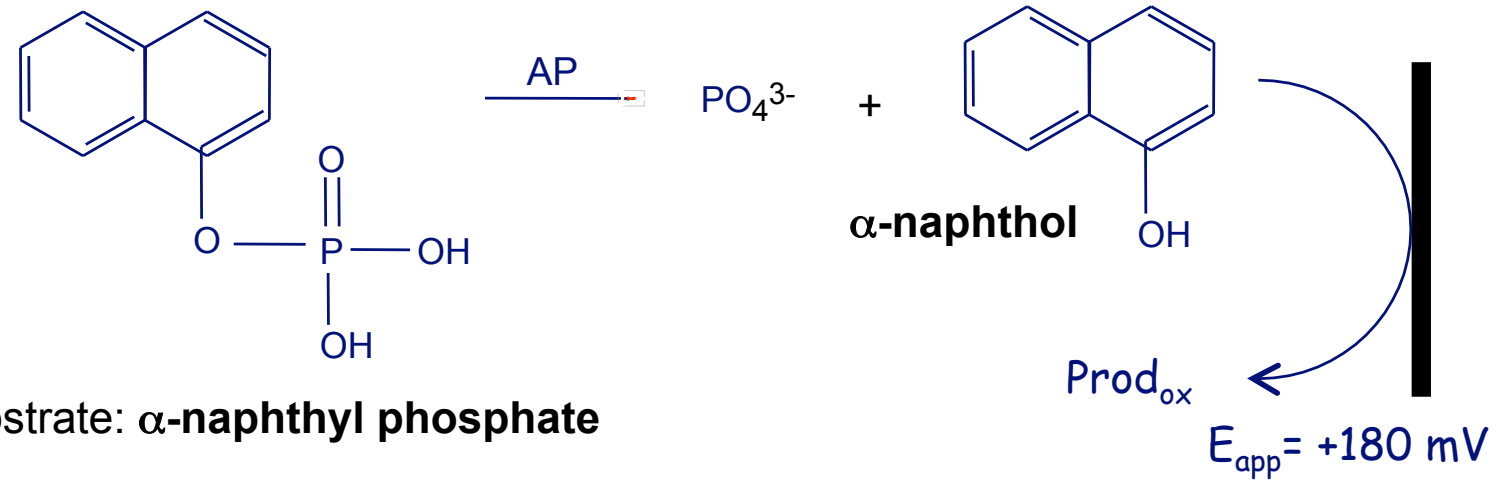
B 5’ biotinylated secondary aptamer






CH8-3
PalmSens
Electrochemical Sensor Interface
Esc
Enter
COMPAQ
iPAQ
Pocket PC
Addition of
Enzymatic substrate
for
Electrochemical
measurement
Magnetic tube

Alkaline Phosphatase (AP)

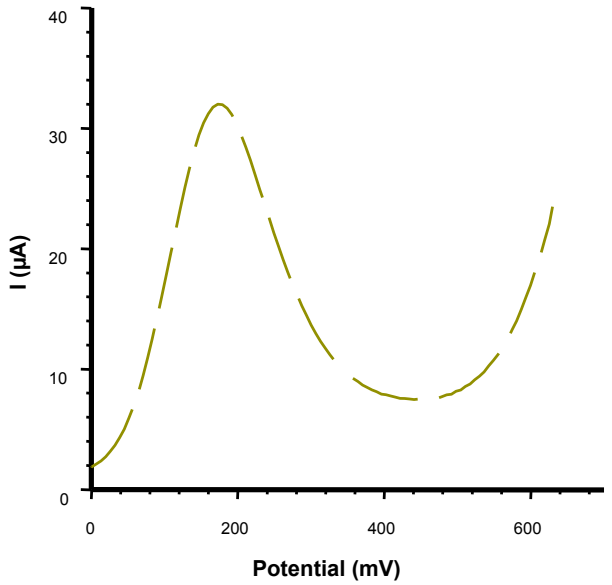


Substrate: α-**naphthyl phosphate**

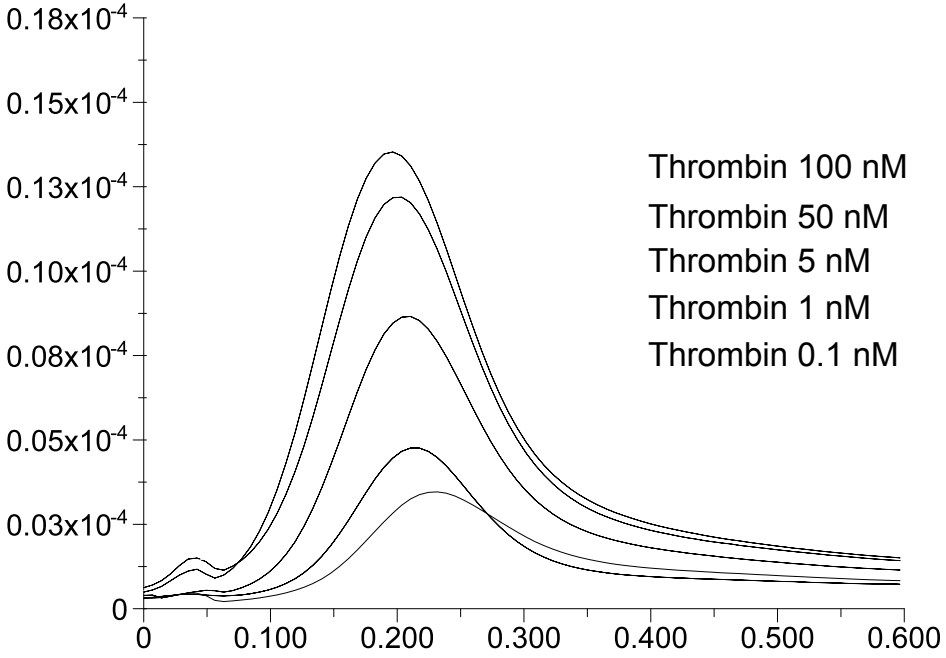
Differential pulse voltammetry (DPV) measurements:

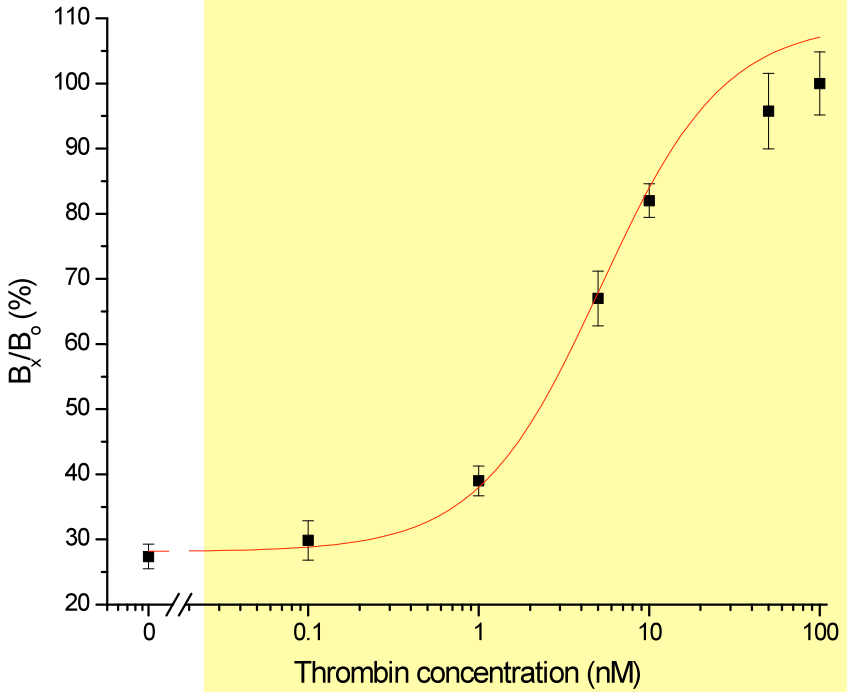
- Range potential: 0/+900 mV
- Scan rate: 70 mV/s
- Pulse amplitude: 70 mV

- Substrate: 1 mg/mL in DEA buffer

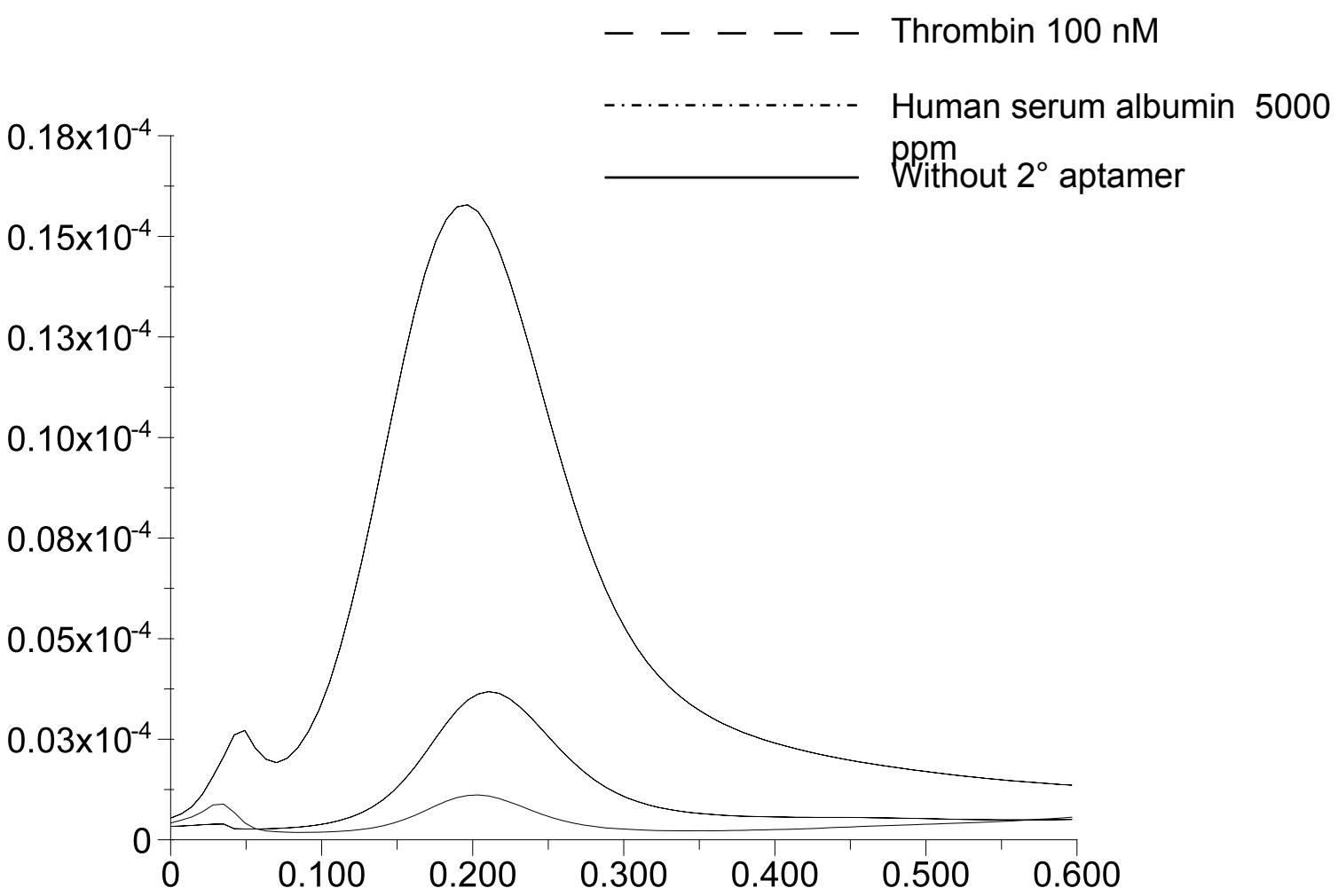


Dose-response curve

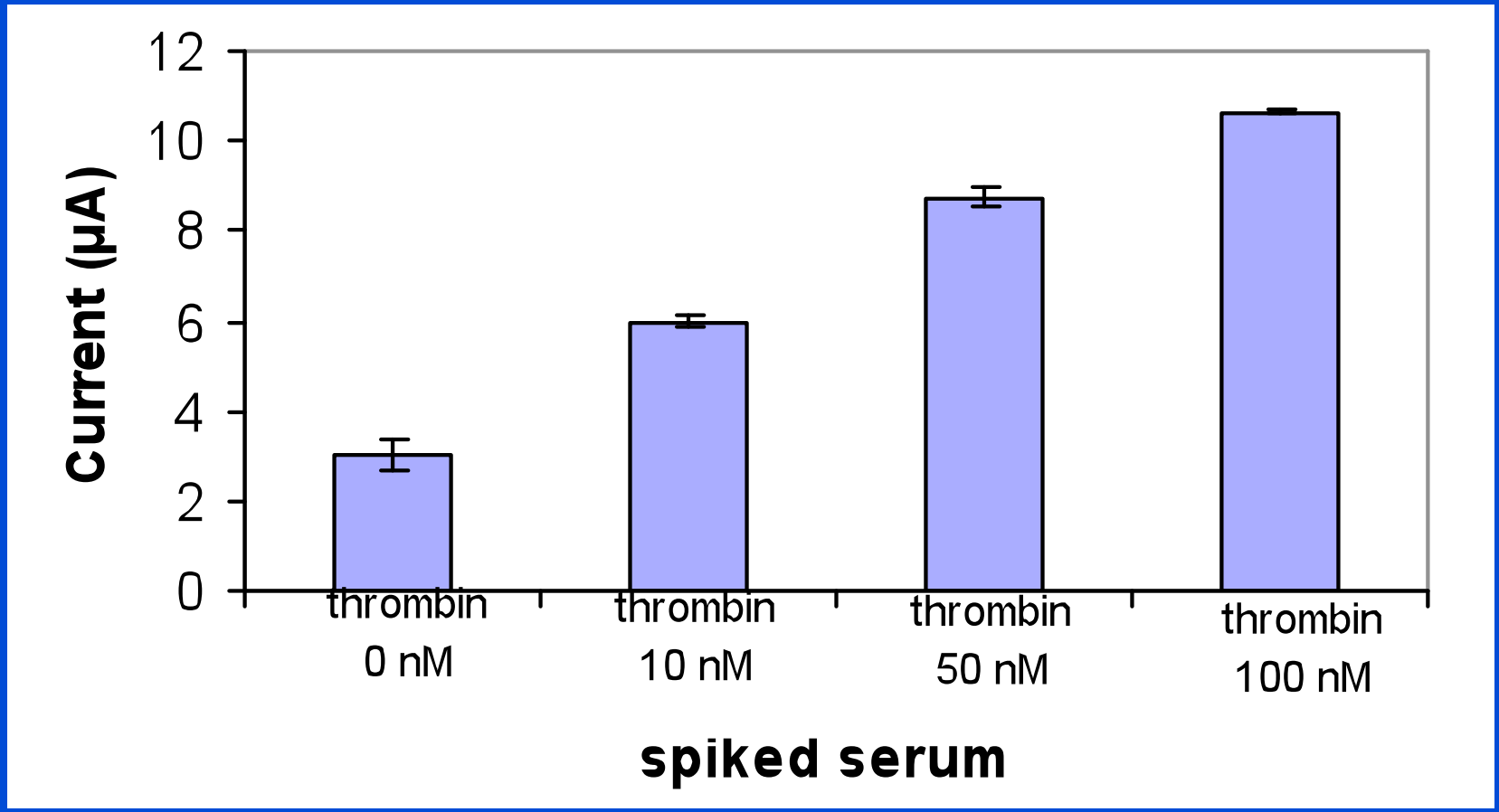




Specificity of the assay

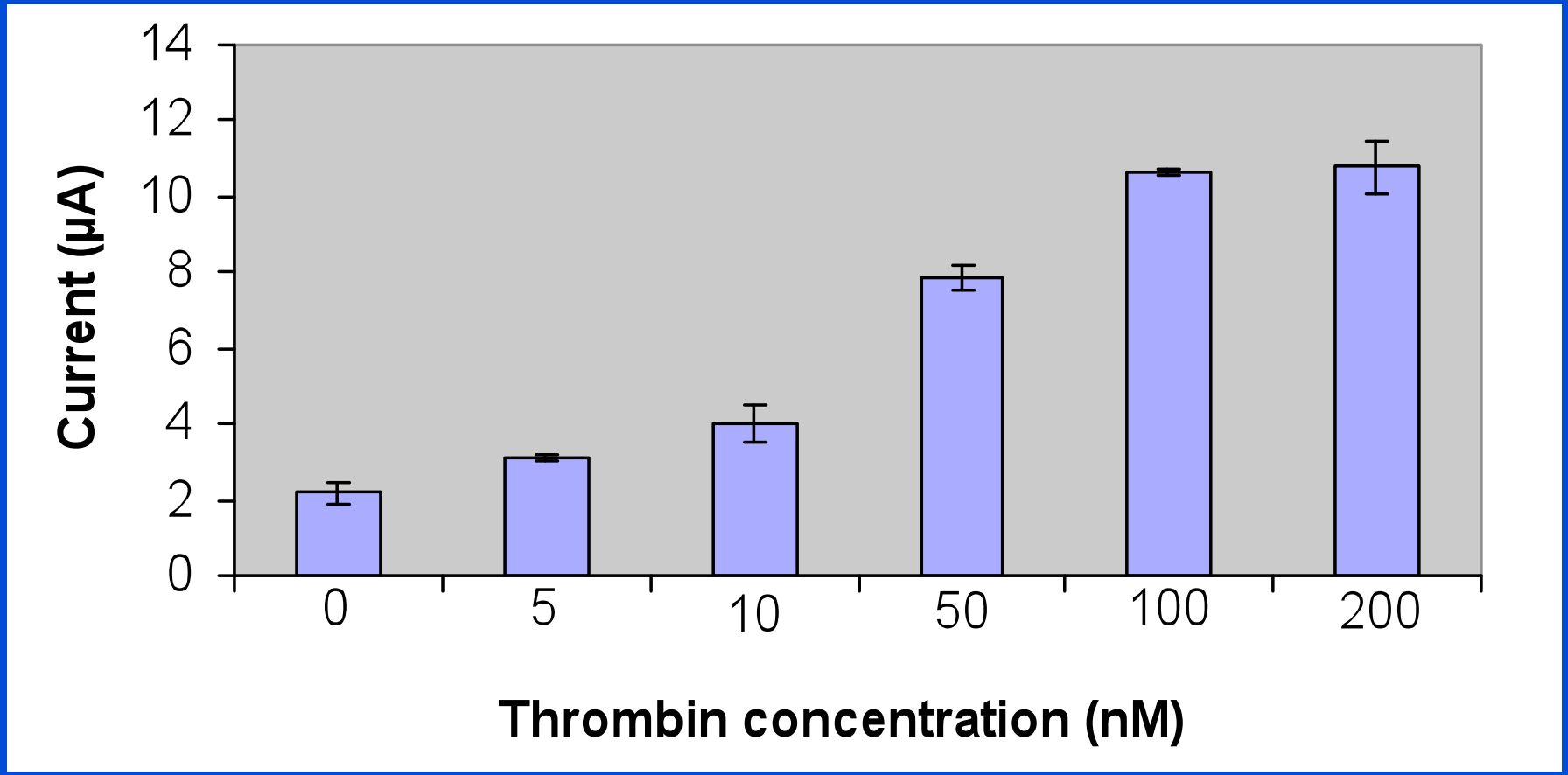


Measurements in serum



Measurements in plasma

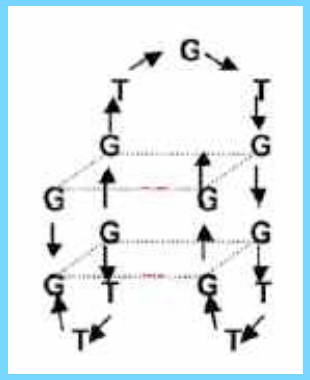
- *Precipitation of fibrinogen by $(NH_4)_2SO_4$*
- *Addition of thrombin standard solutions to plasma*

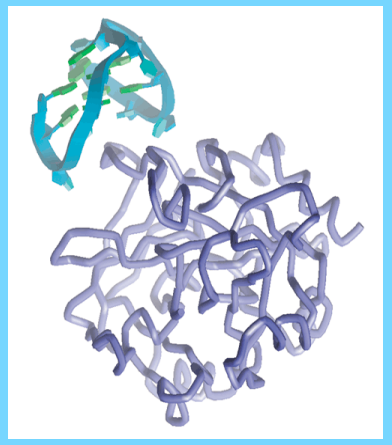


Thrombin binding aptamer-based QCM biosensor

- Target molecule: Thrombin
- DNA aptamer
- Transducer: quartz crystal microbalance

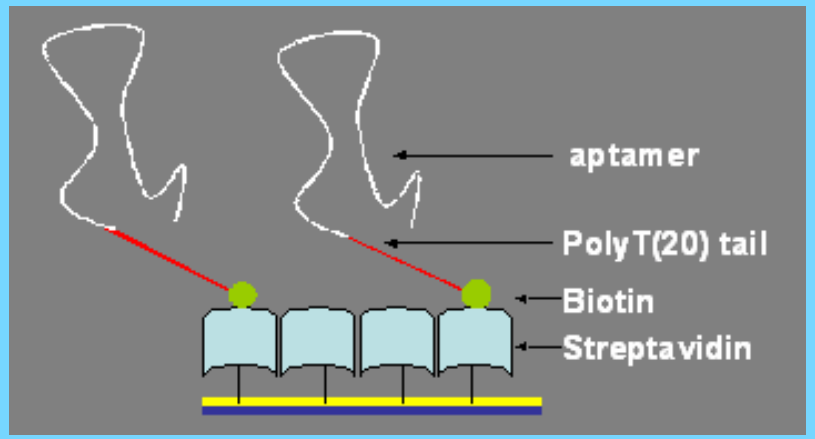
Quartz crystal microbalance
(QCMagic, Elbatech, Italy)





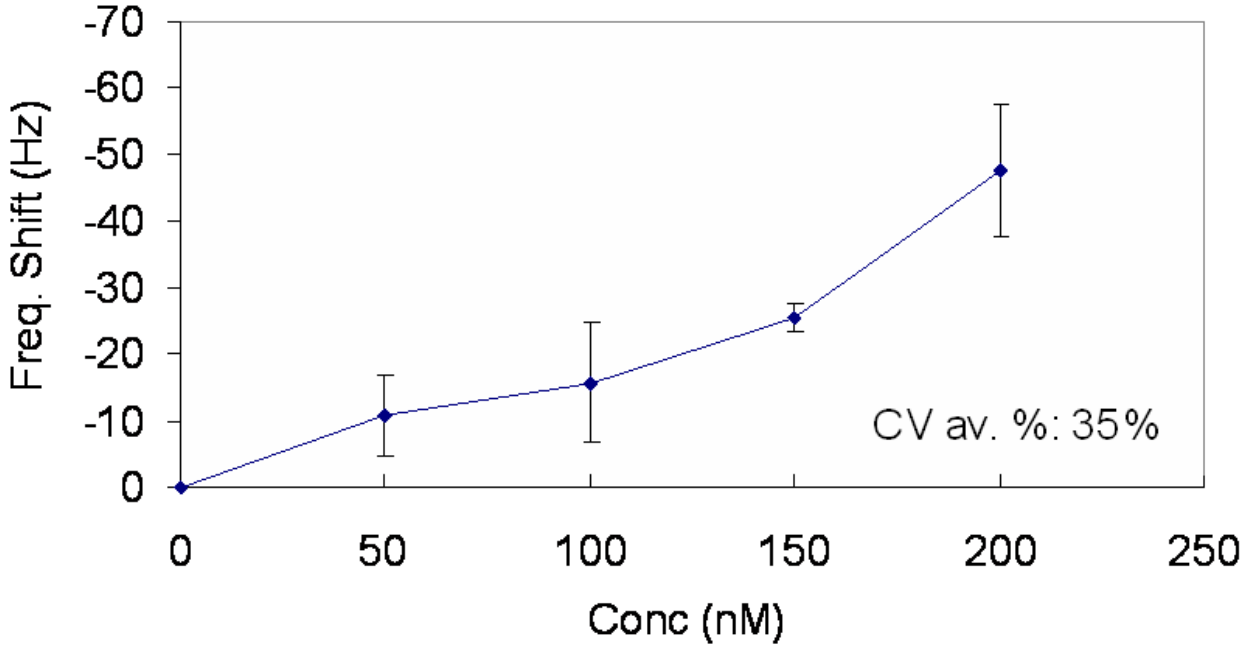
Thrombin-binding aptamer

Immobilization



Optimization

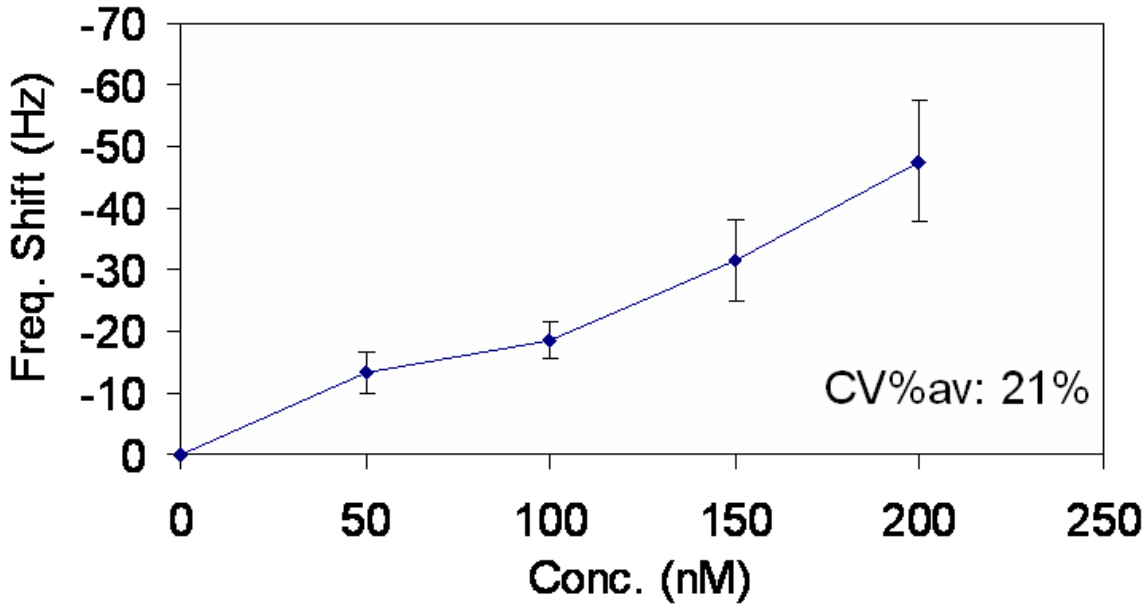
1) Immobilization: **1 µM** biotinylated aptamer (with polyT tail) Interaction time: **20'**



Number of cycles
for each crystal: 10-14

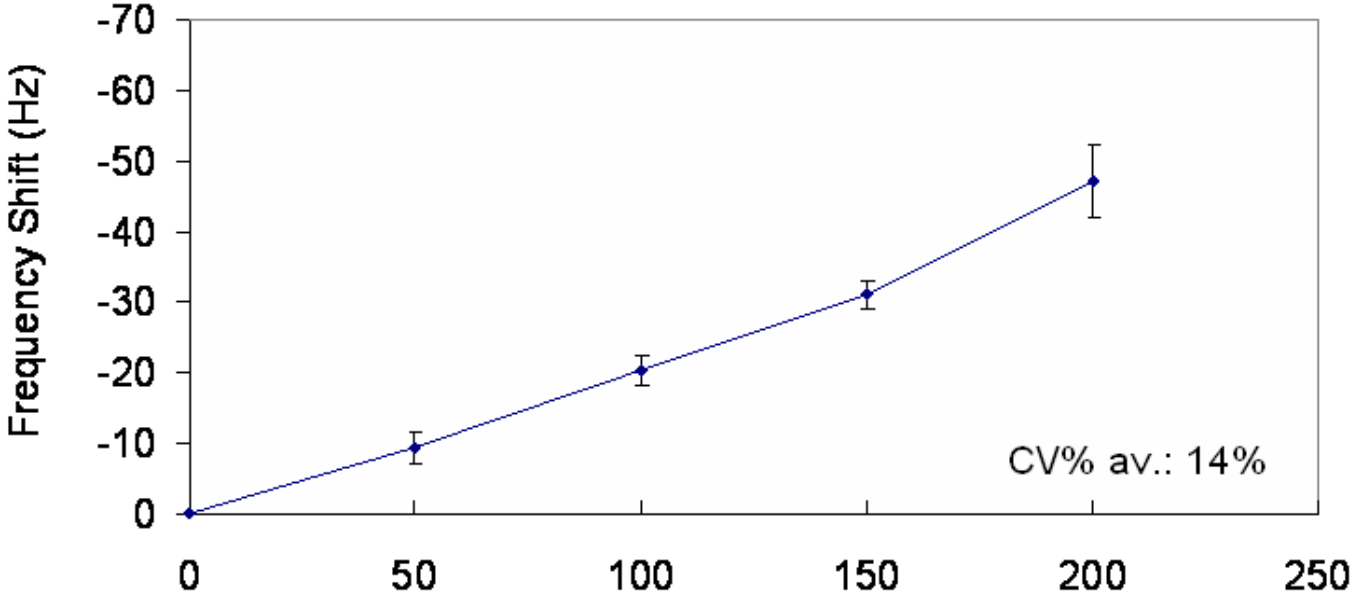
2) Immobilization: 1 µM biotinylated aptamer (with polyT tail) **after thermal treatment**

Interaction time: 20'



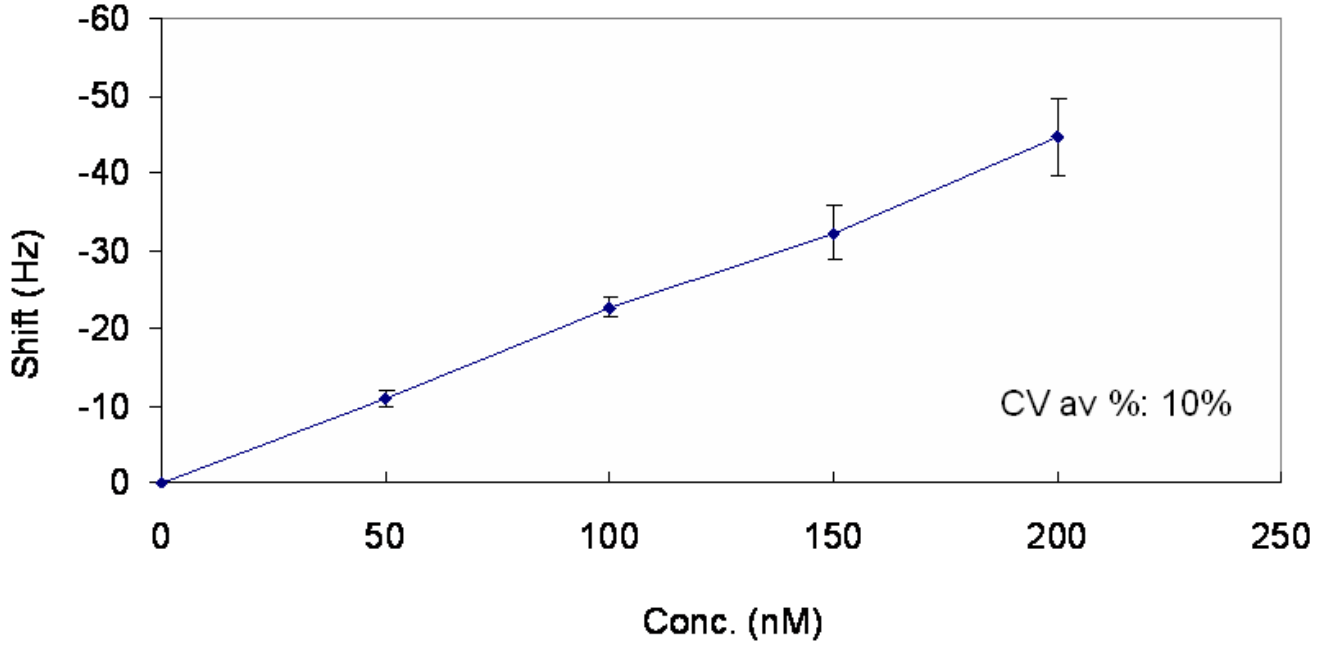
3) Immobilization: **0.5 µM** biotinylated aptamer (with polyT tail) after thermal treatment

Interaction time: 20’



4) Immobilization: 0.5 µM biotinylated aptamer (with polyT tail) after thermal treatment

Interaction time: **30’**



Conclusions

Several applications based on aptamers have been reported, focusing on the parameters that need to be optimized when developing such assays (i.e. immobilization protocols, etc.). Different bioanalytical methods based on aptamers have been considered both for the detection of proteins or small molecules.

From the examination of the different protocols employed in such assays, one important point must be emphasized and that is the nature, conformation and sequence of each aptamer should be carefully considered and also stress that optimal working conditions can remarkably vary from one aptamer to another.

These important characteristics, together with the shortening of the time required for the selection process, demonstrate that aptamers can actually represent the alternative for the development of bioanalytical methods with the possibility of producing new multi-analyte aptamer-based arrays.

Thank…..

…..you!