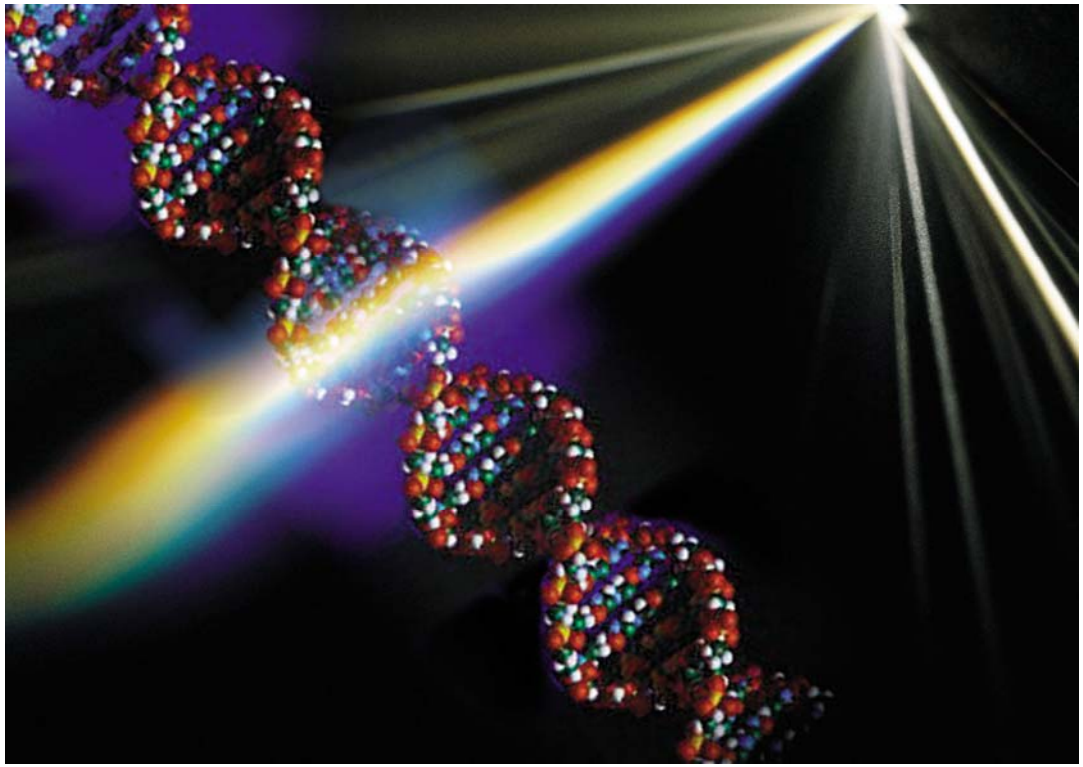
Aptamers in Bioanalysis



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Bioanalytical Chemistry
Lectures 2009

Affinity Probes

Affinity probes – biopolymers or small molecules which bind to a target molecule with *high affinity* and *specificity*. These probes are used for quantitative analysis of targets which cannot be otherwise detected

1. The probe should be labelled with a detectable tag (fluorescent molecule, radioactive isotope)
2. The known excess of probe is mixed with the unknown amount of target
3. Probe-target complex and excess of the probe are separated and quantified

Affinity Probes Contd.

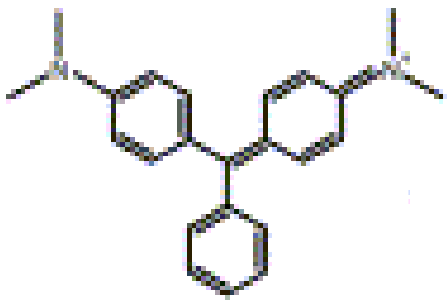
Types of affinity probes:

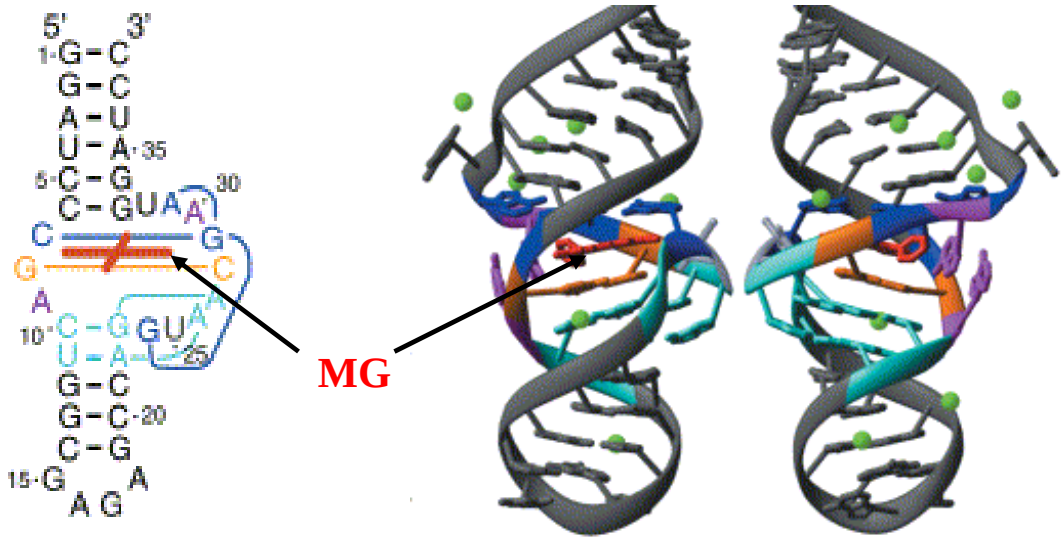
1. Antibodies – proteins used for the analysis of proteins. Produced *in vivo* by shuffling the parts of the genes as a response to antigene (target) introduction
2. Small molecules (for example, ethidium bromide for dsDNA analysis)
3. Oligonucleotides – DNA or RNA probes for quantitative hybridization analysis of DNA or RNA. Hybridization probes should be complimentary to their targets
4. ***Oligonucleotide aptamers*** – ssDNA or ssRNA used for the analysis of proteins and haptens. Selected *in vitro* from large libraries of random oligonucleotide sequences
5. ***Peptide aptamers*** – peptides used for the analysis of proteins and haptens. Selected *in vitro* from large libraries of random peptide sequences

Oligonucleotide Aptamers

The concept of aptamers (from *apt*: fitted, suited; Latin *aptus*: fastened) was introduced by Szostak's and Gold's groups in 1990.

RNA aptamer to Malachite Green





Malachite Green (MG)

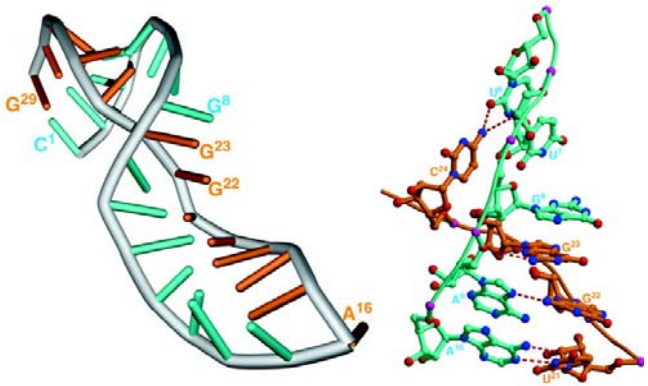
Secondary structure of the aptamer with the target MG, K_d = 800 nM

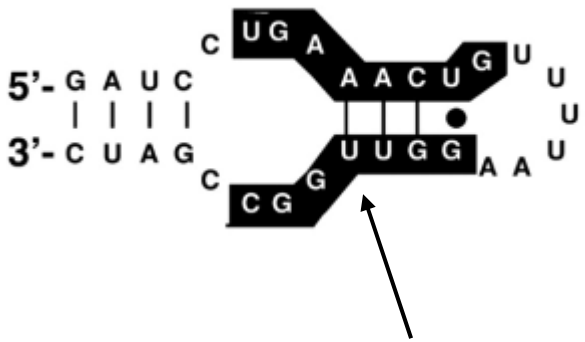
Tertiary structure of the aptamer with MG

J. Mol. Biol. 2000, 301, 117-28

Examples of Aptamers Contd.

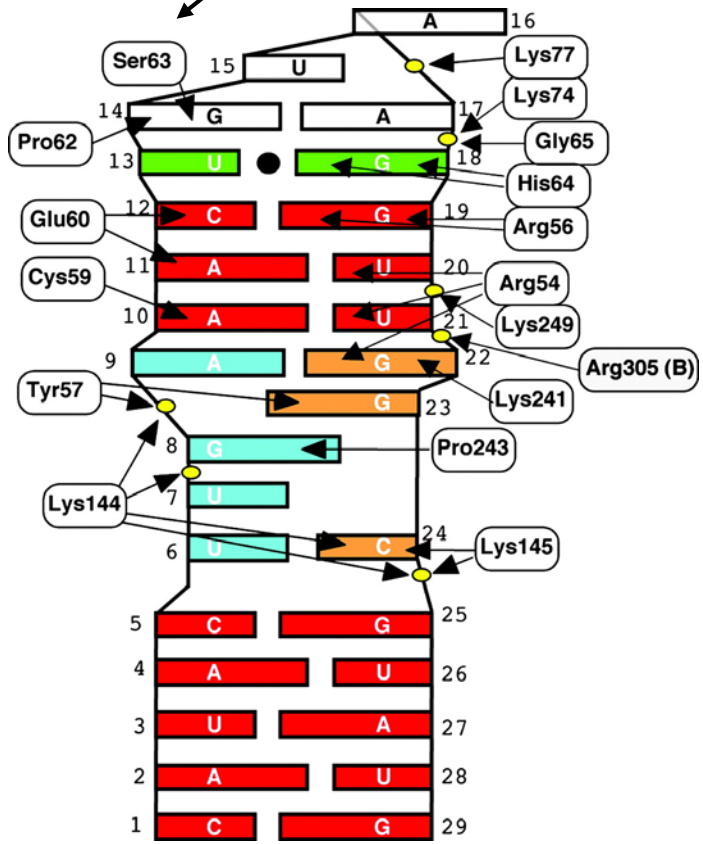
RNA aptamer to NP50 protein





Conserved sequence

Interactions with aminoacids

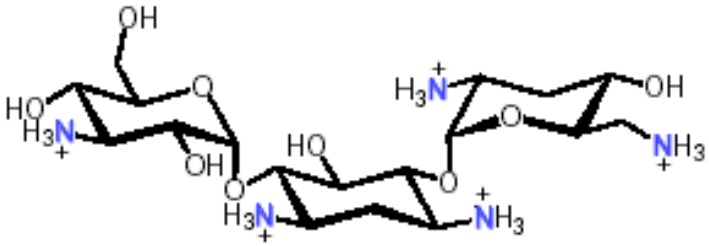


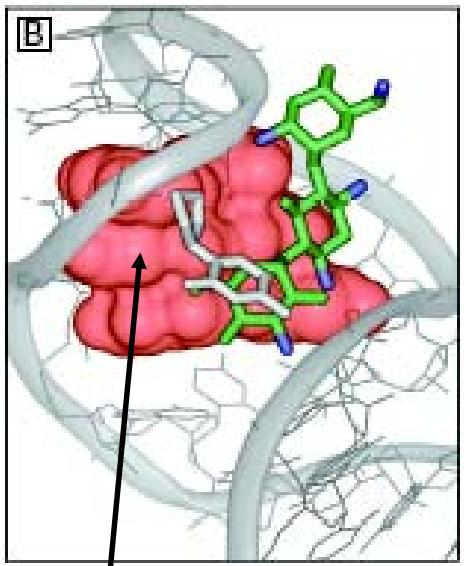
K_d = 5 nM

PNAS 2003, 100, 9268-73

Examples of Aptamers Contd.

Tobramycin (antibiotic) bound to its RNA aptamer



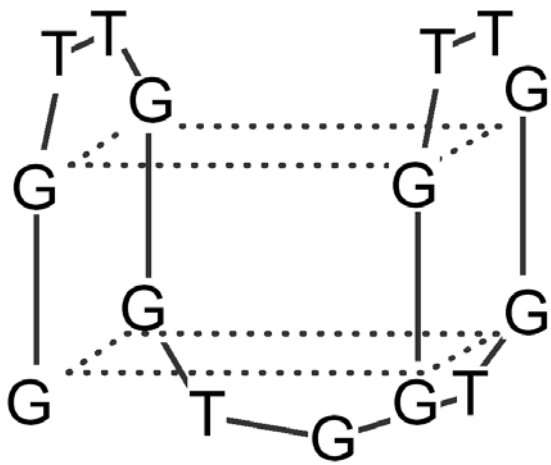


K_d = 1 nM

Negatively charged pocket in RNA structure displays the shape complementarity to the cationic ammonium groups in tobramycin.

Science 2000, 287, 820-25

Thrombin-binding DNA aptamer



G-quartets dominate the structure of DNA aptamers

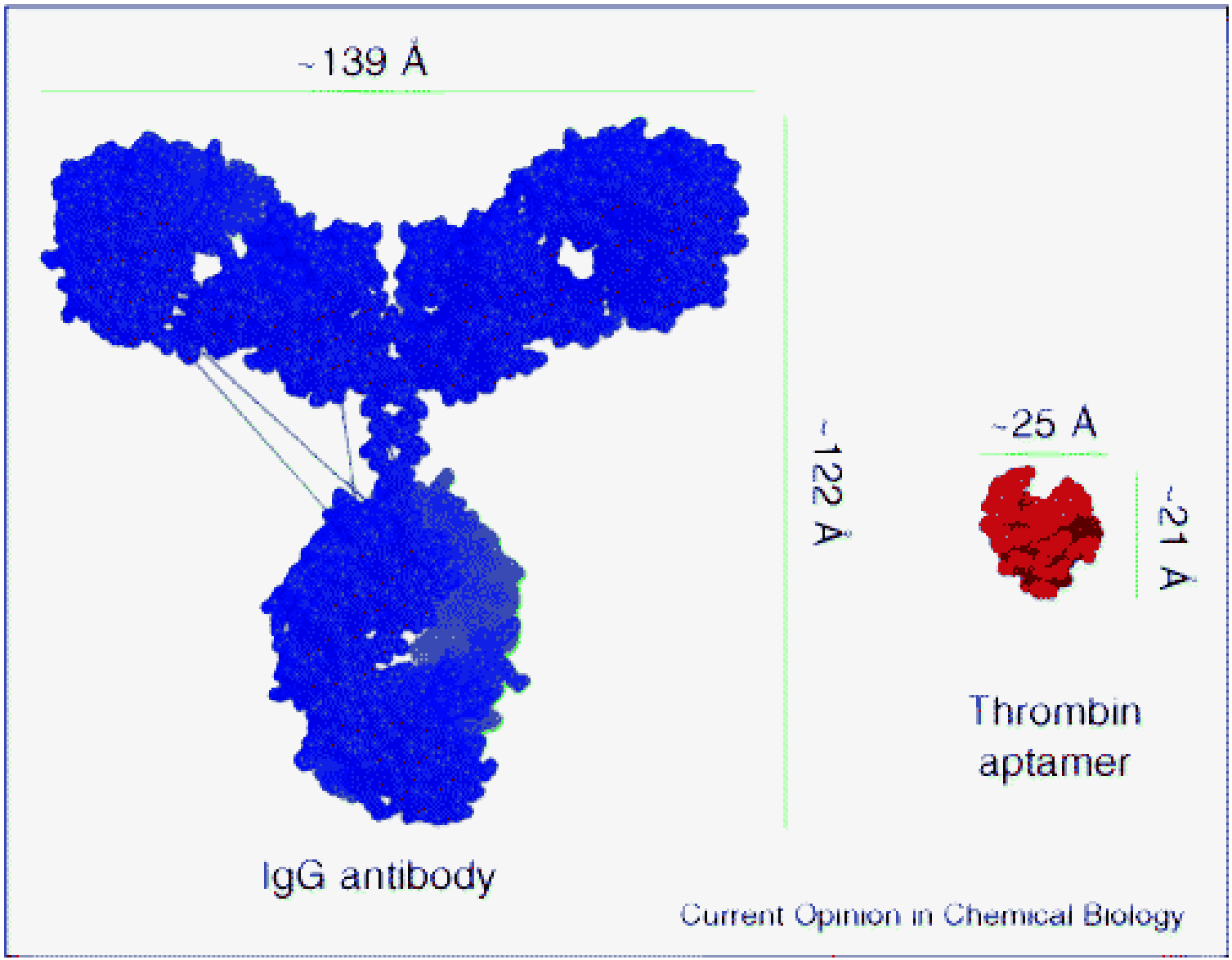
PNAS 1993, 90, 3745-49

Aptamers rival antibodies in affinity analyses

Aptamers (oligonucleotides)	Antibodies (proteins)
Binding affinity is in low nanomolar to picomolar range	Binding affinity is in low nanomolar to picomolar range
Selection is an *in vitro* process that can target any small molecule, biopolymer, or cell	Selection requires a biological organism and is inefficient with toxins and small non-immunogenic molecules
Selection of aptamers is inexpensive and takes few weeks	Screening of monoclonal antibodies is expensive and time consuming
Uniform activity regardless of the batch	Activity of antibodies varies from batch to batch
Affinity parameters can be controlled on demand ("*smart aptamers*")	Difficult to modify affinity parameters
Wide variety of chemical modifications are introduced to diversify properties and functions	Very limited modifications
Return to original conformation after temperature insult	Temperature causes irreversible denaturation
Unlimited shelf-life	Limited shelf-life
No evidence of immunogenicity	Significant immunogenicity
CE analysis: non-sticky to capillary walls, light ligands (5-15 kDa), easy to separate Apt•P from Apt	CE analysis: sticky to capillary walls, bulky (150 kDa), difficult to separate Ab•P from Ab

Annu. Rev. Med 2005, 56, 555-83.

Size: Antibody vs. Aptamer



Targets

Successful aptamer selection targets:

Inorganic ions (Zn^{2+})
Small molecules (biotin)
Organic dyes (malachite green)
Nucleotides (ATP)
Aminoacids (citrulline, arginine)
Neutral disaccharides
Aminoglycoside antibiotics
Oligopeptides
Proteins
Large glycoproteins (CD4)
Viruses
Anthrax spores
Cells

Affinity

- aptamers against small molecules have affinity in micromolar range (K_d ~ μM);
- affinity for proteins is usually higher (nanomolar to picomolar range).

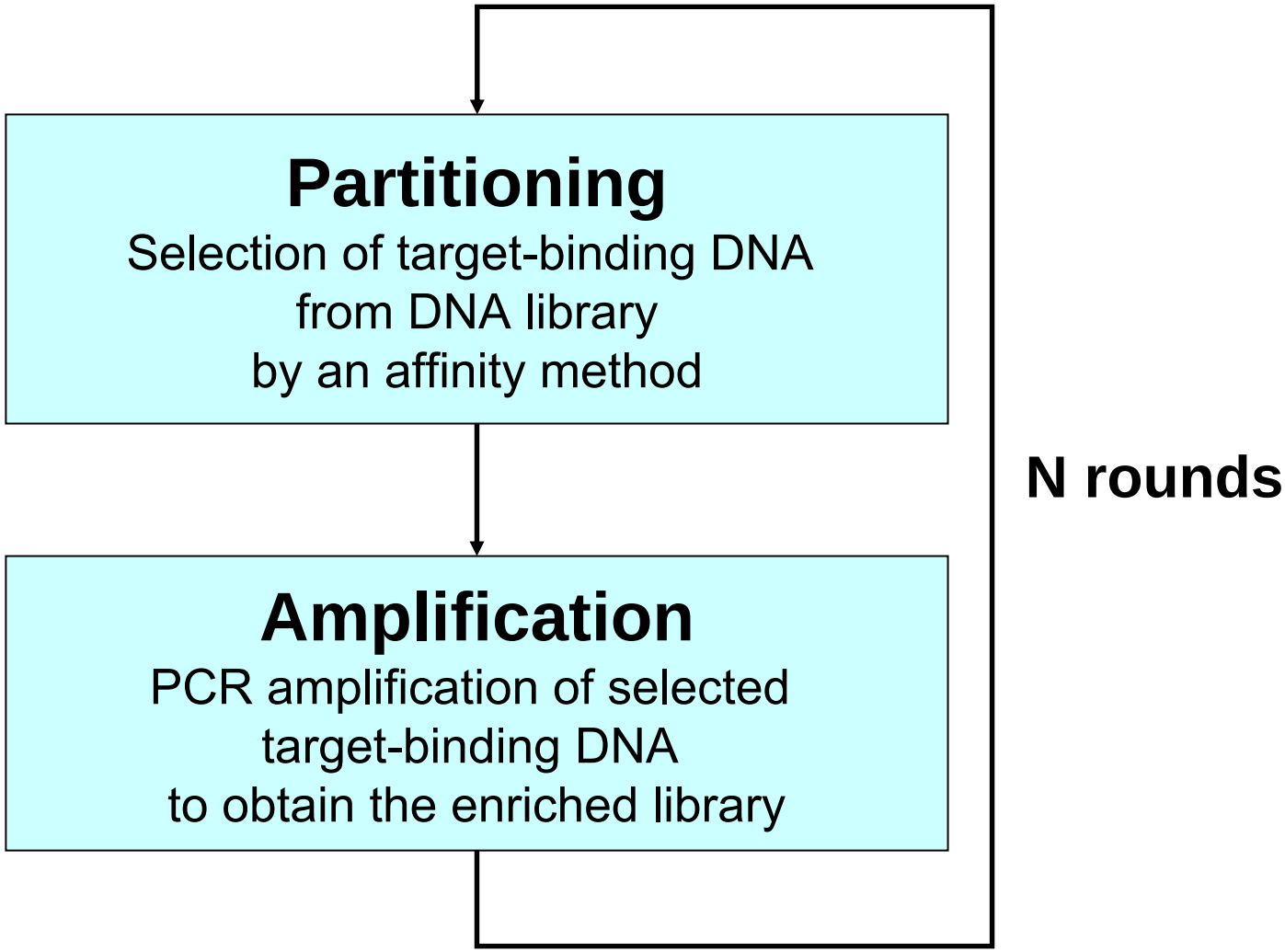
Specificity

- aptamers as well as antibodies are usually very specific to target molecules;
- aptamers can discriminate enantiomers and protein isoforms.

Science, 2000, 287, 820-825

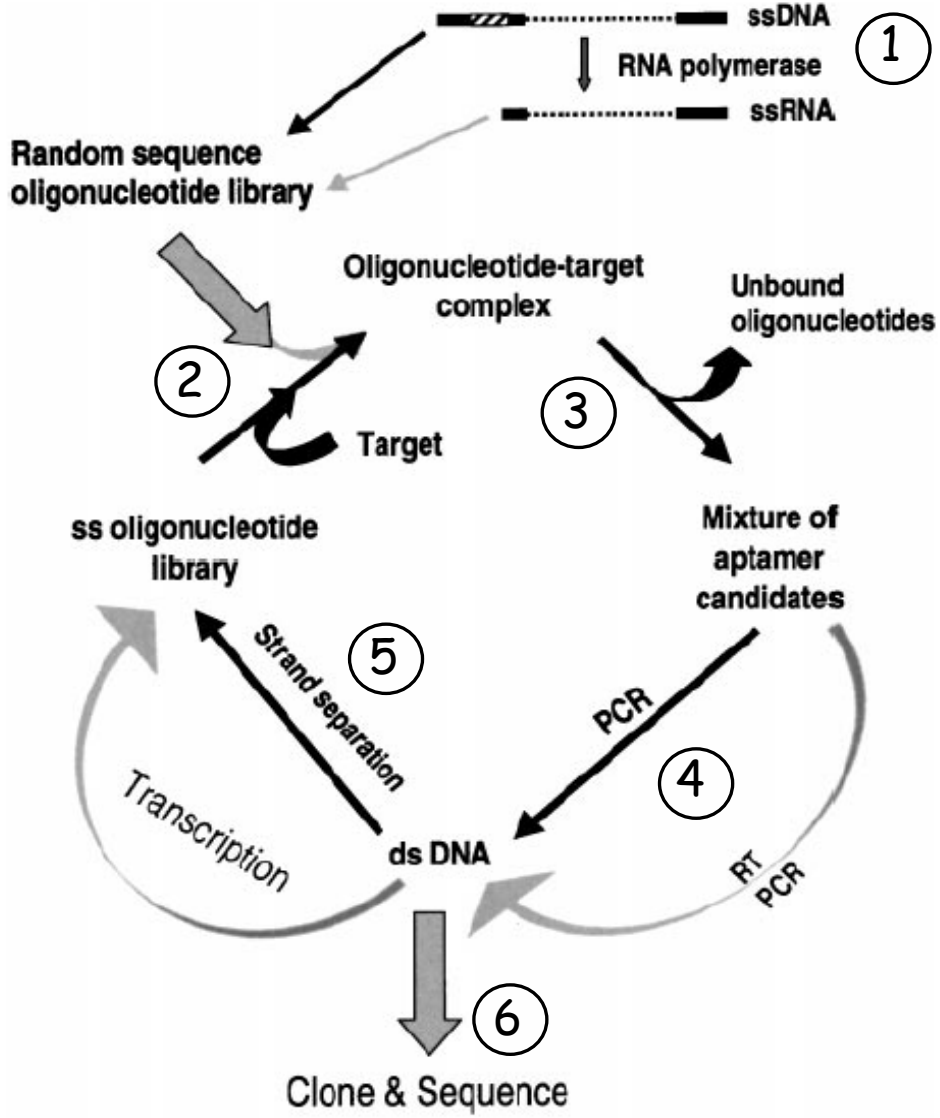
SELEX (Systematic Evolution of Ligands by EXponential Enrichment) is a general concept of aptamer selection

Tuerk, C. and Gold L. Science 1990, 249, 505-510



SELEX Contd.

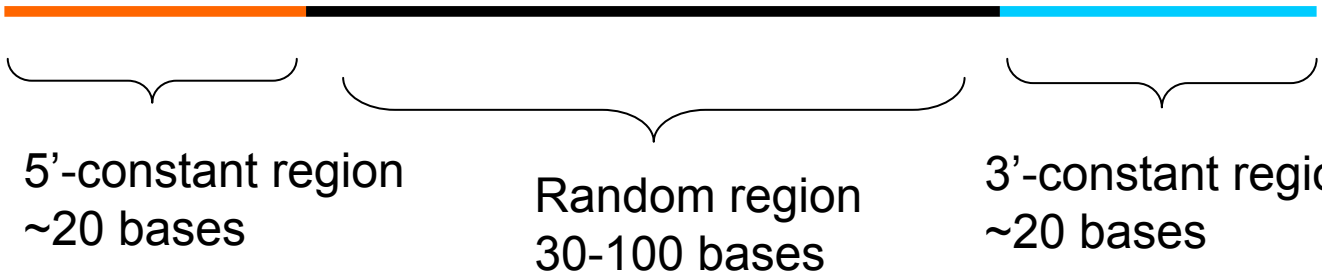
SELEX is a multi-step process in which strongly binding ligands are preferably selected by rounds of affinity assays and PCR amplification



Clin. Chem. 1999, 45, 1628-50

SELEX Contd.

1. The ssDNA library is synthesized with a random sequence in the middle and constant regions at the ends:



Selection of RNA aptamers also requires a T7-promotor at 5'-constant region of DNA. RNA polymerase uses it to transcribe DNA library to RNA library

2. Target T is mixed with the DNA (or RNA) library and allowed to reach equilibrium in the complex formation reaction. The number of equilibria is equal to the number of unique sequences in the library:

$$T + L_1 \overset{^1K_d}{\rightleftharpoons} T\bullet L_1$$

$$T + L_2 \overset{^2K_d}{\rightleftharpoons} T\bullet L_2$$

$$T + L_3 \overset{^3K_d}{\rightleftharpoons} T\bullet L_3$$

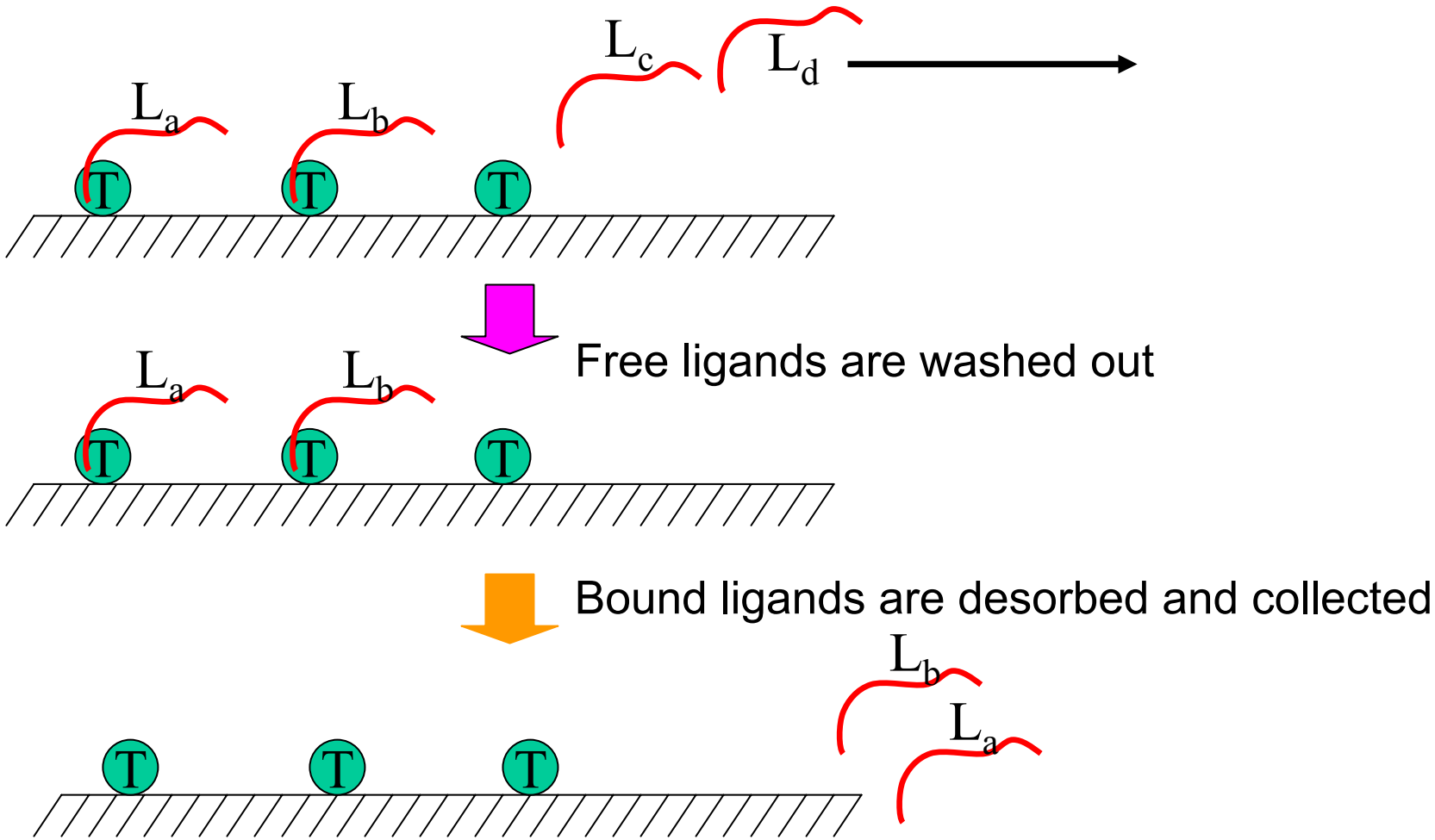
.....

.....

$$T + L_N \overset{^NK_d}{\rightleftharpoons} T\bullet L_N$$

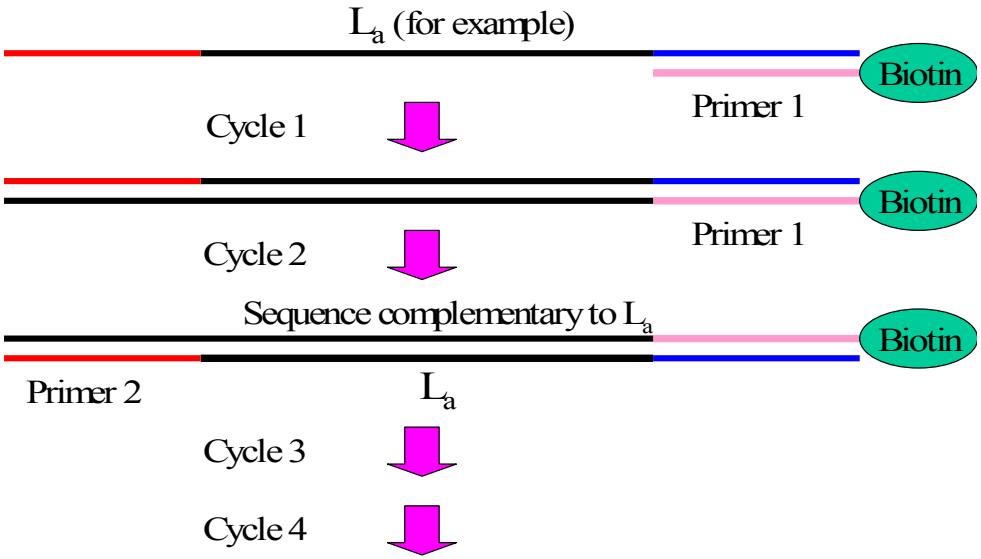
SELEX Contd.

3. Bound and unbound ligands are separated by a "partitioning" process, which is typically a heterogeneous binding assay. Target-ligand complexes are adsorbed on the surface that binds target (protein) but does not bind ligands (DNA):



SELEX Contd.

4. Bound ligands are amplified in PCR:



PCR products contain equal amounts of L_a and the complementary to L_a sequence labeled with biotin

Error-prone PCR: introduces random mutations during amplification, used for diversification of sequence space in the selected pool or individual sequence.

For a sequence of length *n* that is mutagenized with an error rate ε, the probability of introducing *k* mutations is given by the equation:

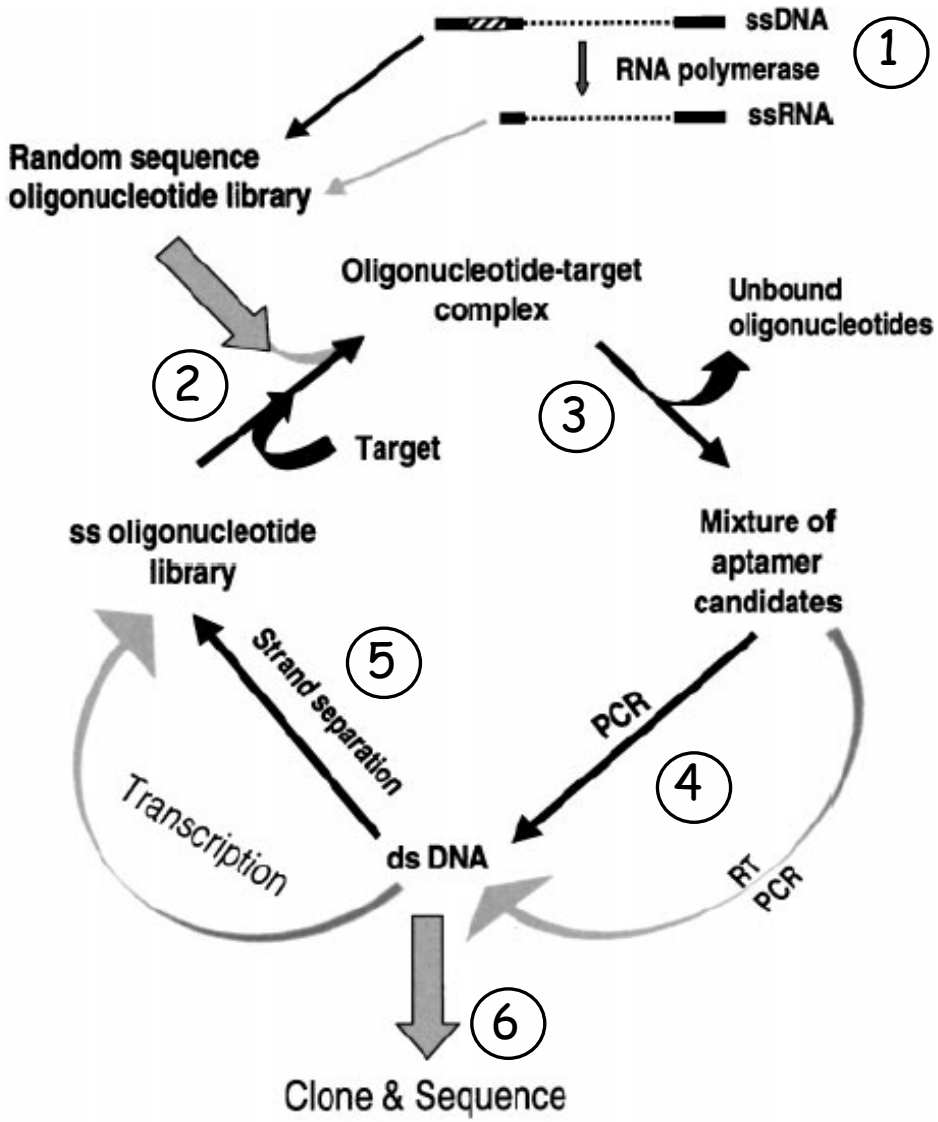
$$P(k,n,\varepsilon) = (n!/[(n-k)!\,k!])\,\varepsilon^{k}\,(1-\varepsilon)^{n-k}.$$

The number of different types of sequences in each error class is given by:

$$N_k = (n!/[(n-k)!\,k!])\,3^{k}.$$

For example, mutagenesis of 10^{15} molecules of the same sequence (n = 100, ε = 0.0066) will lead to about 1.1×10^{12} copies of each of the possible one-error mutants , 2.5×10^{9} copies of each of the two-error mutants and so on.

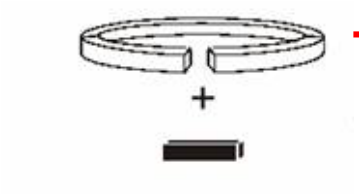
SELEX Contd.


ssDNA
RNA polymerase
ssRNA
1
Random sequence
oligonucleotide library
Oligonucleotide-target
complex
Unbound
oligonucleotides
2
Target
3
ss oligonucleotide
library
Mixture of
aptamer
candidates
Strand separation
5
PCR
4
Transcription
ds DNA
RT
PCR
6
Clone & Sequence

SELEX Contd.

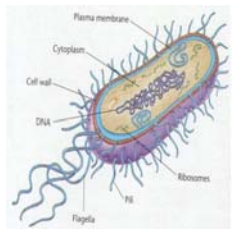
6. Cloning and Sequencing

Bacterial plasmids



dsDNA pool (thousands of sequences)

Modified plasmids



Delivery into bacteria

Growing bacteria

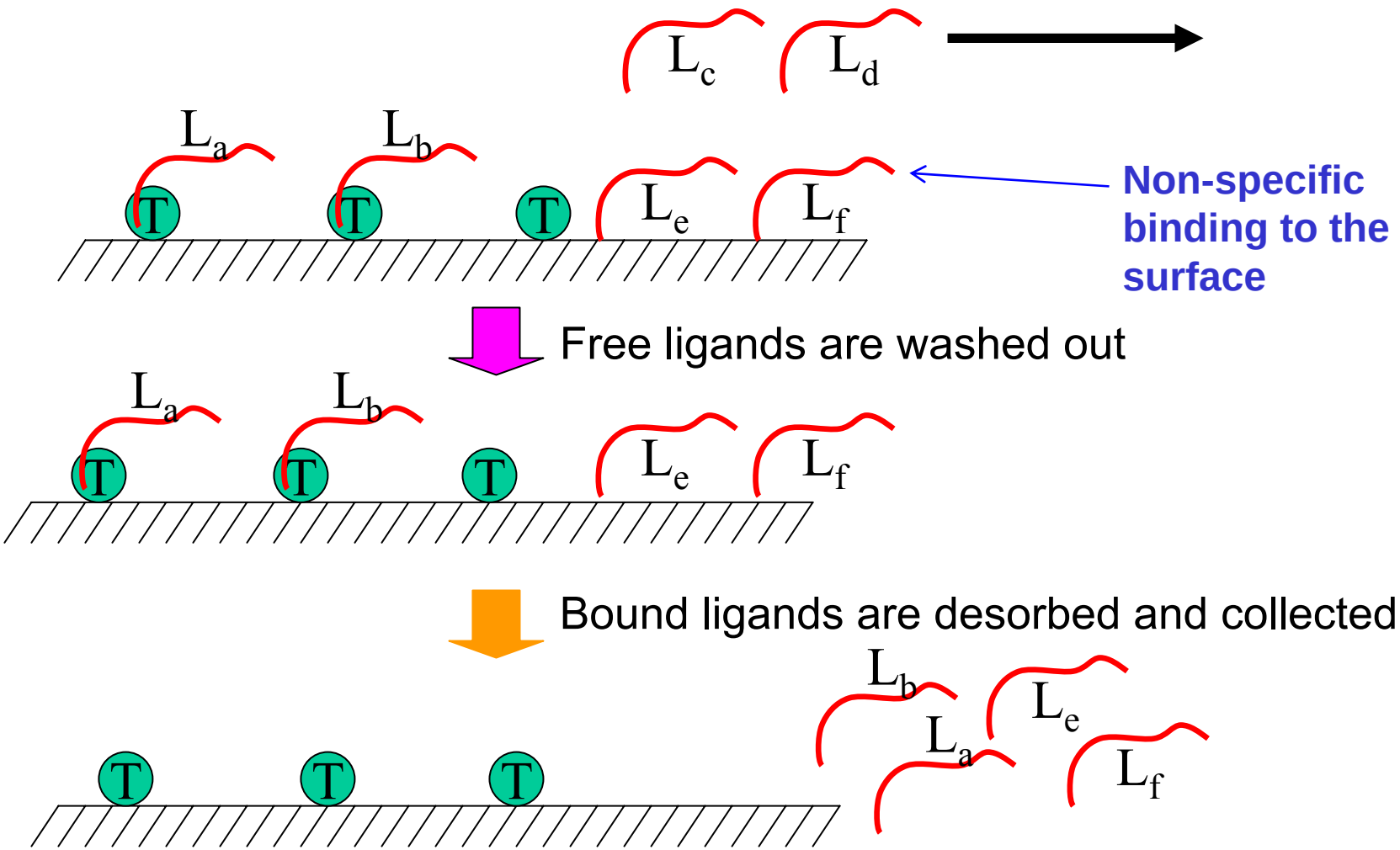
Only bacteria containing an aptamer insert grow. Each colony contains an individual aptamer sequence.

Plasmids from each colony are sequenced

Random site of aptamer sequence is unravelled

CTTCTGCCCGCCTCCTTCCTGGTAAAGTCATTAATAGGTGTGGGGTGCCGGGCATTTCGGAGACGAGATAGG

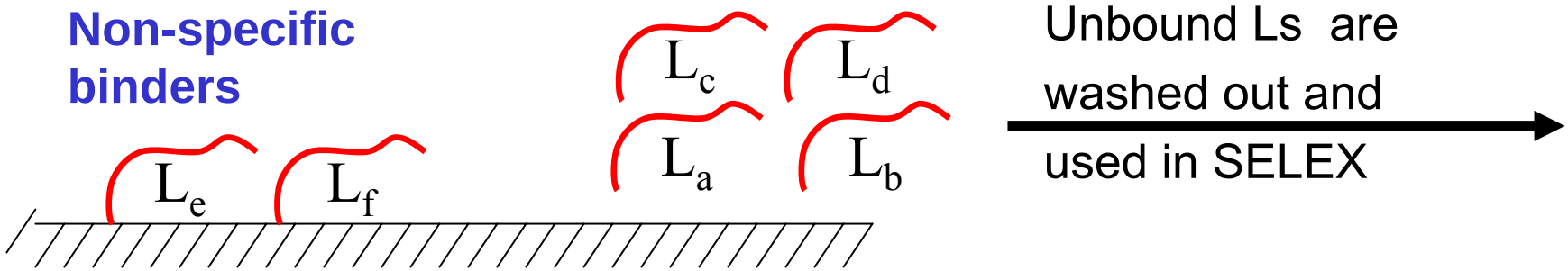
Non-Specific Binging of Ligands to the Surface



Non-specific binders will be amplified in PCR and thus contaminate true ligands

Negative selection to remove non-specific binders

The library is reacted with the surface (in the absence of the target) before the analysis. Non-specific binders bind to the surface. Non-binding ligands are washed out and used in SELEX



This method is not very efficient

Library Properties in SELEX

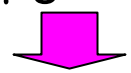
If the random sequence contains 35 nt then the complete library has $4^{35} = 10^{22}$ unique sequences

To have a complete library we have to synthesize 10^{22} molecules = 10^{-2} moles of oligonucleotide (1 mole ~ 10^{24} molecules)

Molar weight of a 75 nt long sequence (35 random bases + 2 constant regions of ~20 bases) is $75 \times 320 = 24000$ g/mole = 24 kg/mole

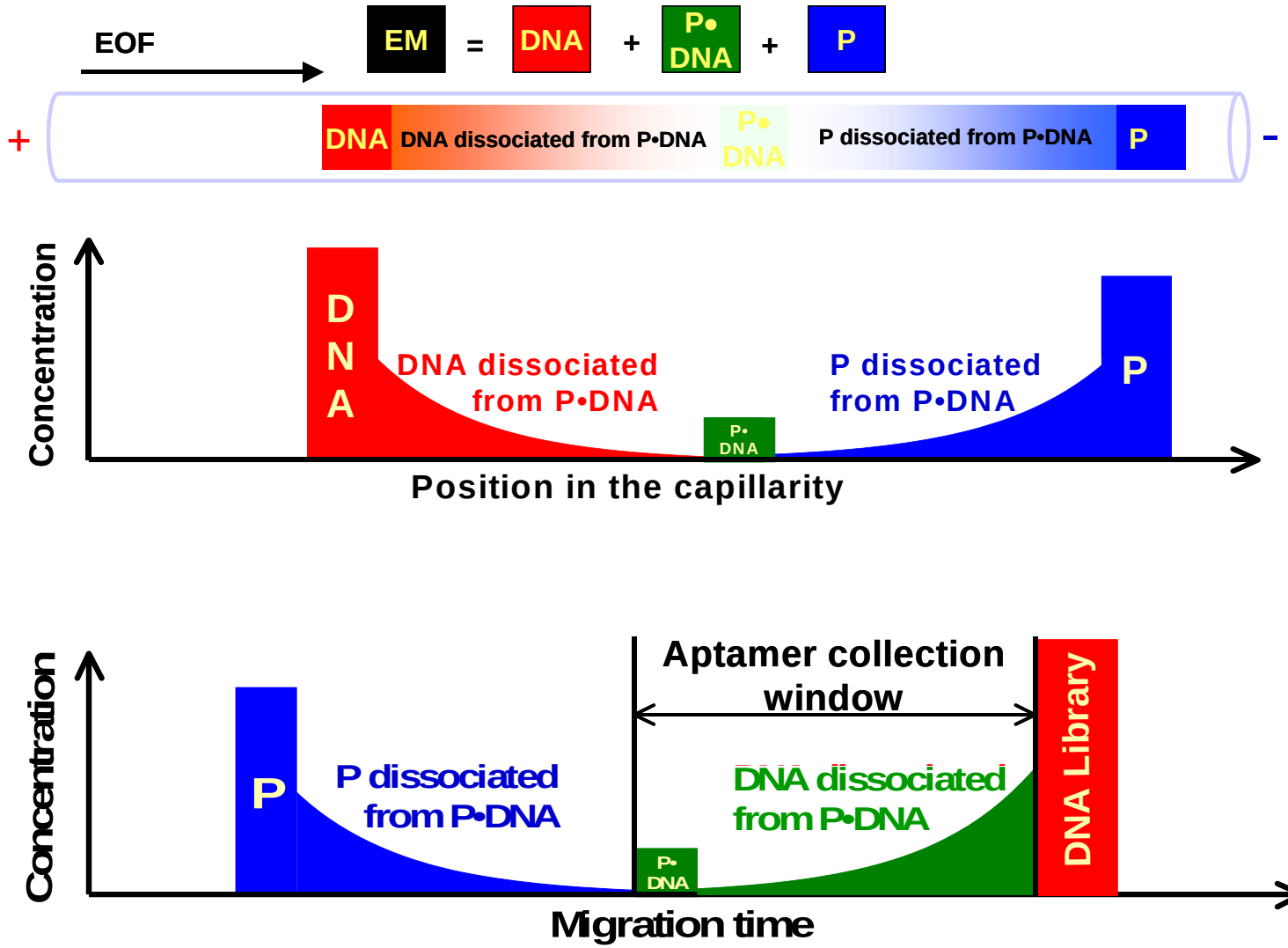
A complete library would weigh 10^{-2} moles $\times$ 24 kg/mole = **240 g (thousands $$$)**

Typical selection starts with ~ 10 μg of the library or ~ 10^{15} molecules



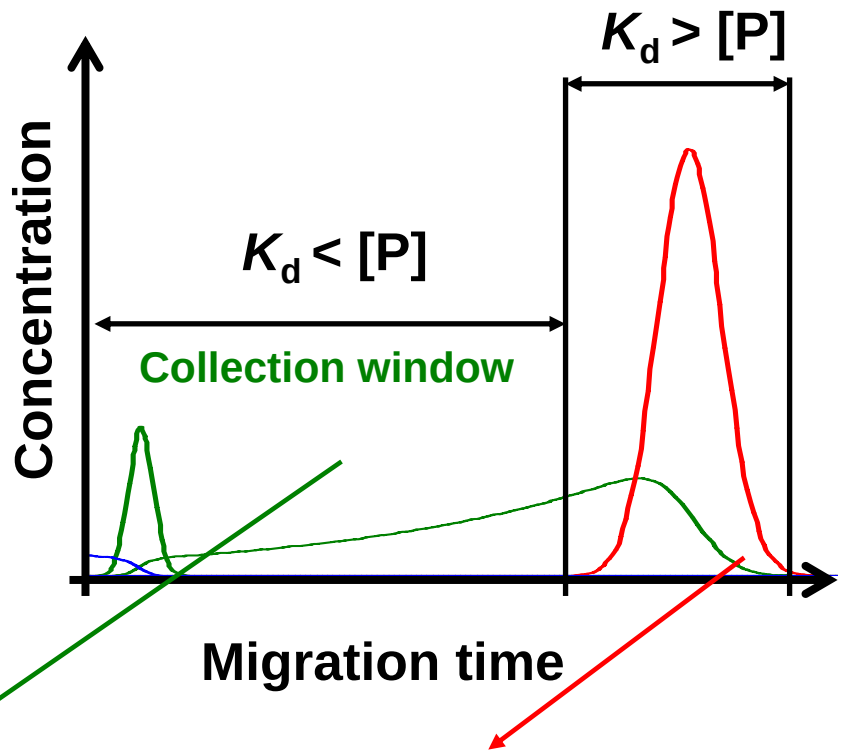
All ligands at the beginning of SELEX have statistically unique sequences

NECEEM-based selection of DNA aptamers for proteins



NECEEM facilitates selection of smart aptamers – aptamers with pre-defined binding parameters K_d, k_{off}, k_{on}

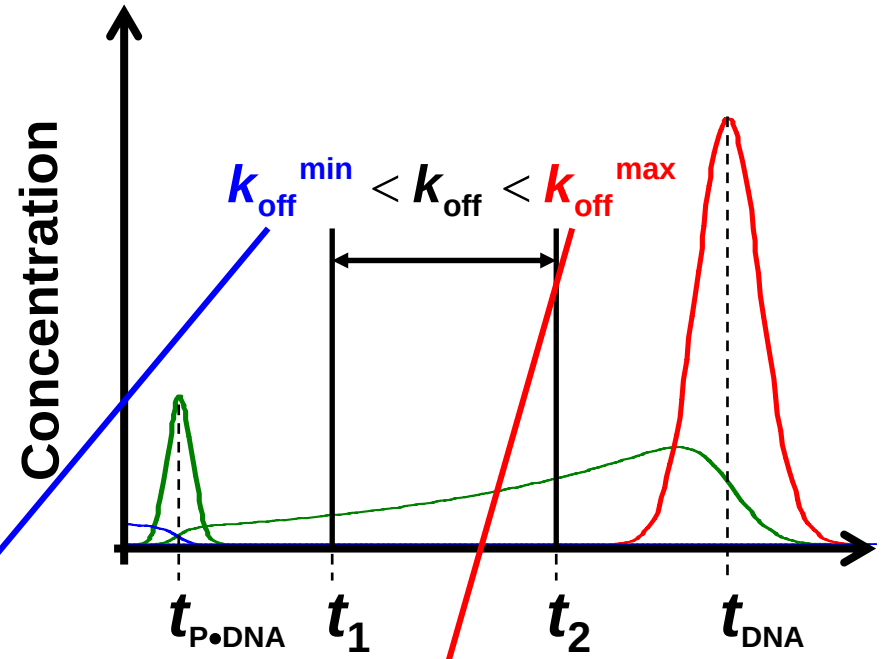
$$K_d = \frac{[P] \times [DNA]}{[P \bullet DNA]}$$



Aptamers with K_d < [P] are mainly bound to protein and collected

Aptamers with K_d > [P] are mainly non-bound to protein and discarded

NECEEM facilitates selection of smart aptamers – aptamers with pre-defined binding parameters K_d, k_{off}, k_{on}



$$k_{off}^{min} = \frac{1}{t_{P•DNA}} \frac{t_{DNA} - t_{P•DNA}}{t_{DNA} - t_1}$$

$$k_{off}^{max} = \frac{1}{t_{P•DNA}} \frac{t_{DNA} - t_{P•DNA}}{t_{DNA} - t_2}$$

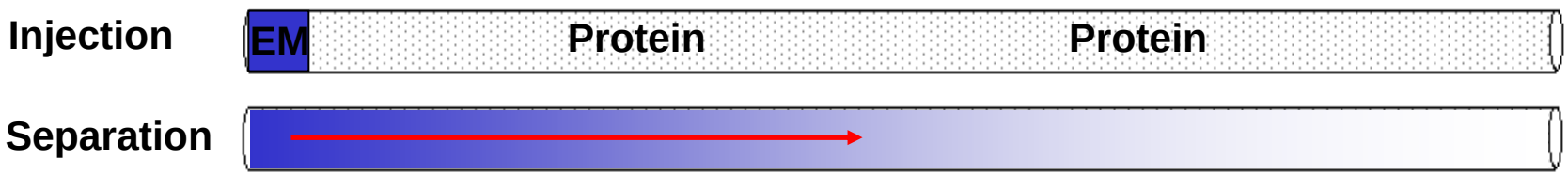
ECEEM

Equilibrium Capillary Electrophoresis of Equilibrium Mixtures

- Variation of Affinity Capillary Electrophoresis (ACE) with emphasis on maintained dynamic equilibrium during separation
- First method for the selection of smart aptamers with pre-defined equilibrium (K_d) parameters

Equilibrium mixture **EM** **= protein-DNA complex + free Protein + free DNA**

$[\text{Protein}]_{\text{EquilibriumMixture}} = [\text{Protein}]_{\text{Running Buffer}}$

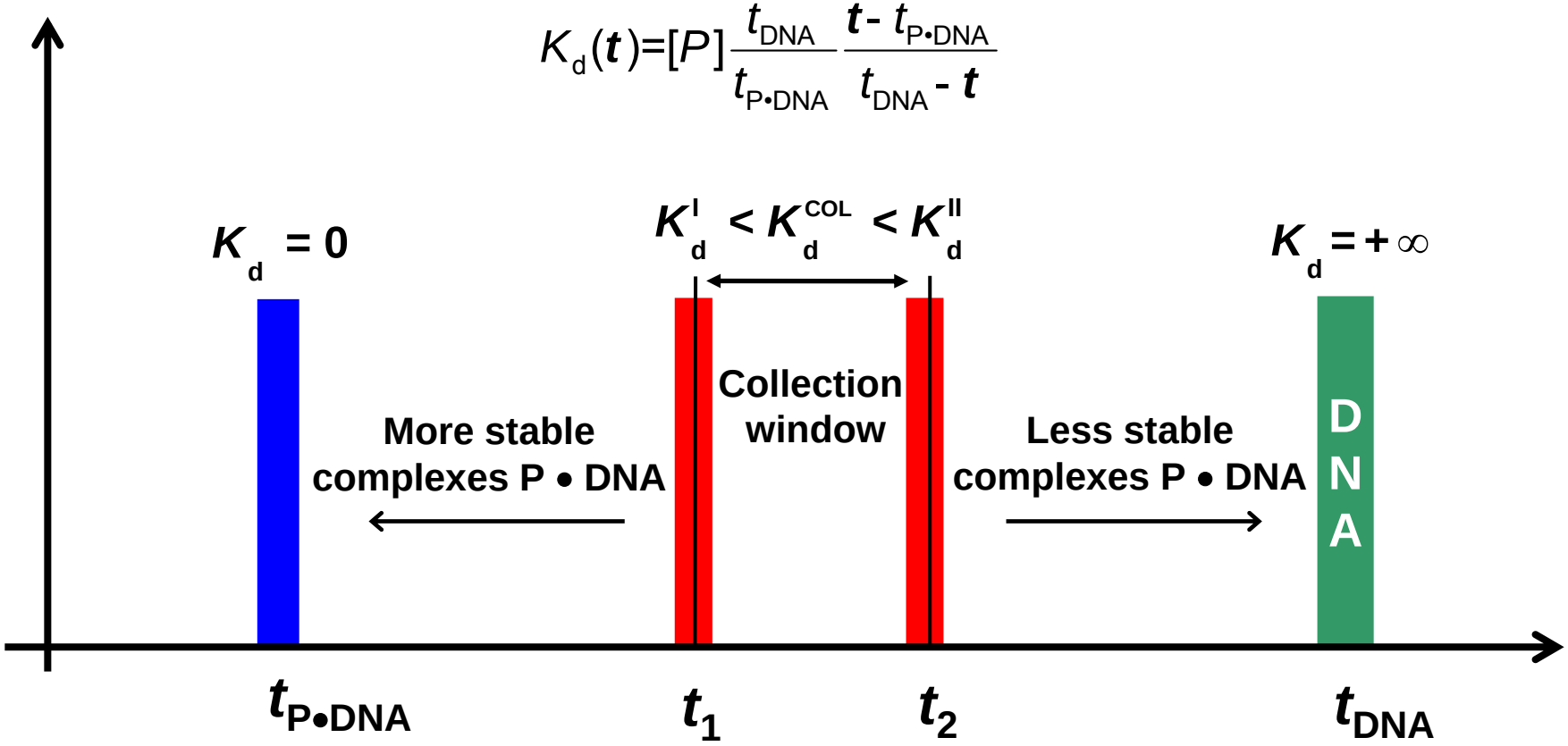


Constant flow of protein in the running buffer

$$\frac{1}{t_L^{app}} = \frac{1}{t_L^0}\frac{K_d}{[P]+K_d} + \frac{1}{t_{P\bullet L}^{\infty}}\frac{[P]}{[P]+K_d}$$

ECEEM Contd.

$$K_d(t)=[P]\frac{t_{DNA}}{t_{P\bullet DNA}}\frac{t-t_{P\bullet DNA}}{t_{DNA}-t}$$



Example: ECEEM was used to select a panel of "smart" aptamers for MutS protein with K_d range 5 - 1000 nM

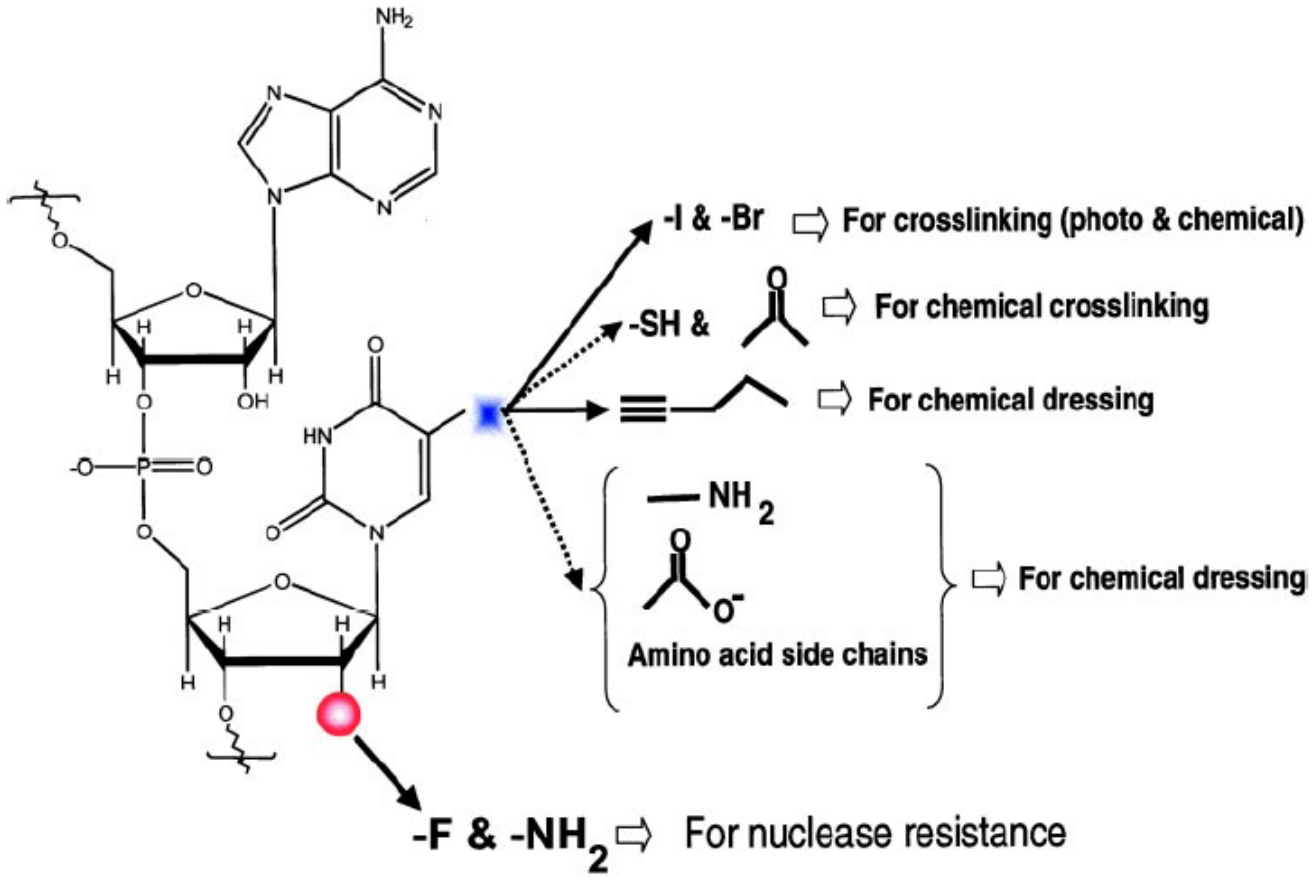
JACS, 2005, 127, 11224

Modification of structure of aptamers

- Pre-SELEX modifications: should undergo amplification by DNA or RNA polymerases!

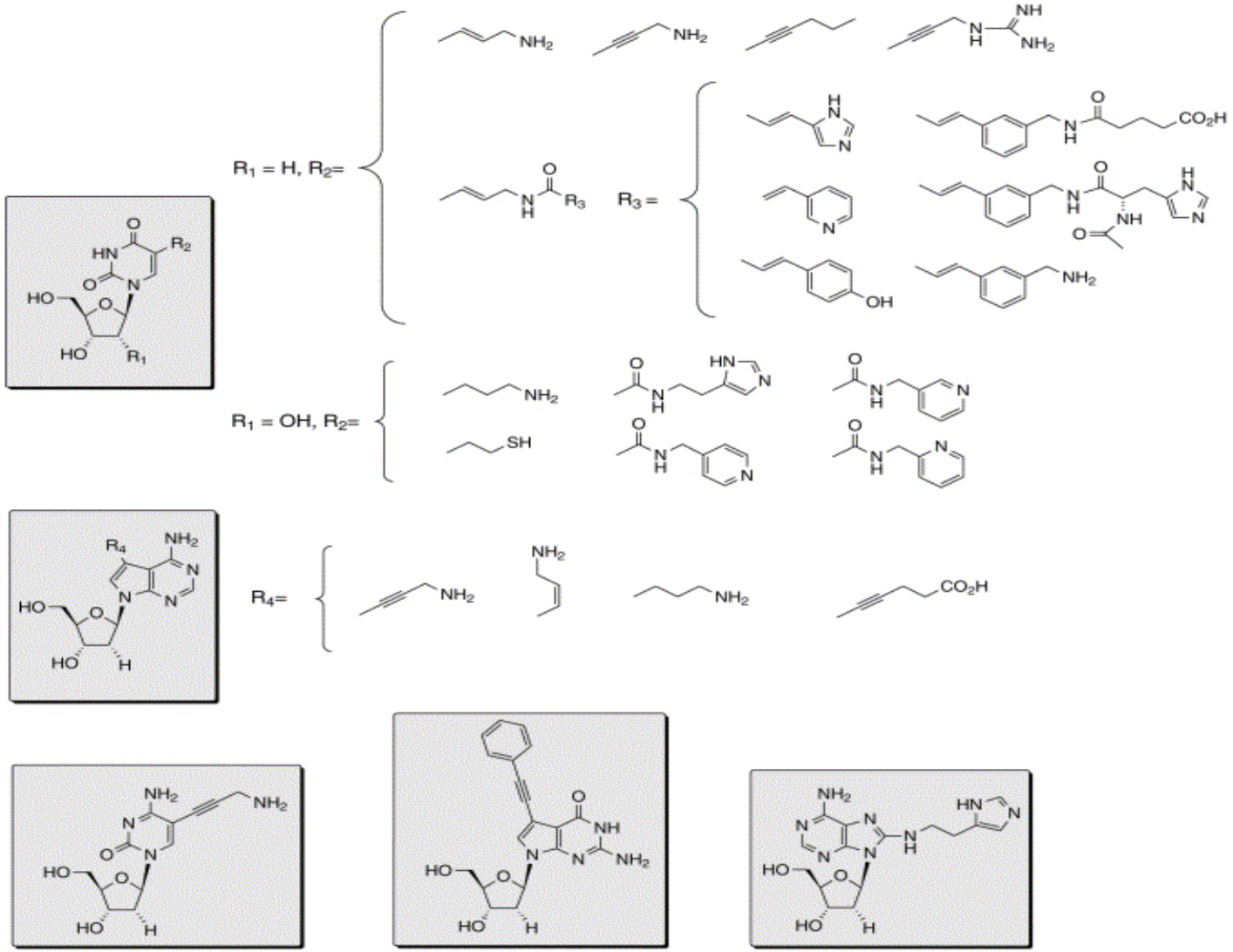
- Post-SELEX modifications: should not greatly affect the initial affinity and specificity.

Modification at the 2’ position of the sugar confers nuclease stability, whereas various modifications at the C-5 position of the pyrimidines could be used either to attract certain classes of targets or to generate covalent cross-links with targets



Clin. Chem. 1999, 45, 1628-50

Modification of structure of aptamers Contd.

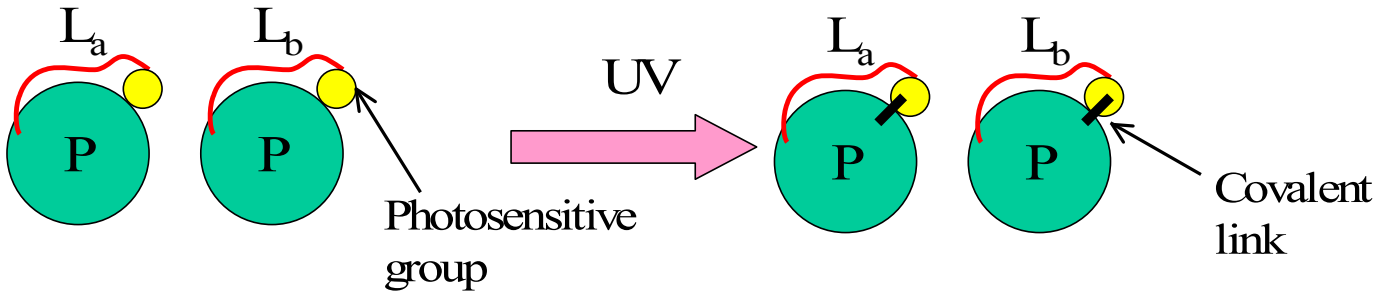


Modified nucleotides reported to serve as substrates from DNA or RNA polymerase enzymes

Curr. Opin. Chem. Biol. 2002, 6, 367-74

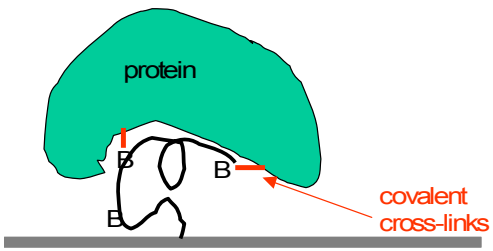
PhotoSELEX and Photoaptamers

Oligonucleotide library is made with photosensitive iodine and bromine-modified nucleotides which can form a covalent bond with protein upon UV irradiation. Reaction occurs only in aptamer-protein complex



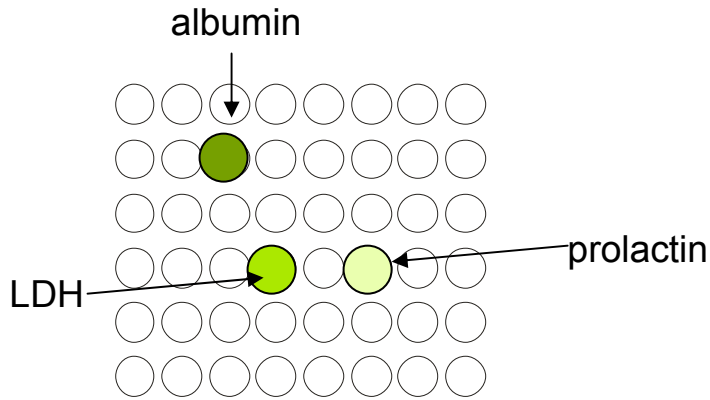
- Complexes are partitioned from free ligands in a typical way (interaction with the protein-binding surface).
- Protein in the complex is digested to release the ligand, which can be then amplified and send for the next step of SELEX.

Photoaptamers in analysis : microarrays with aptamers for simultaneous analysis of hundreds of proteins



Wash stringently to produce a low background.

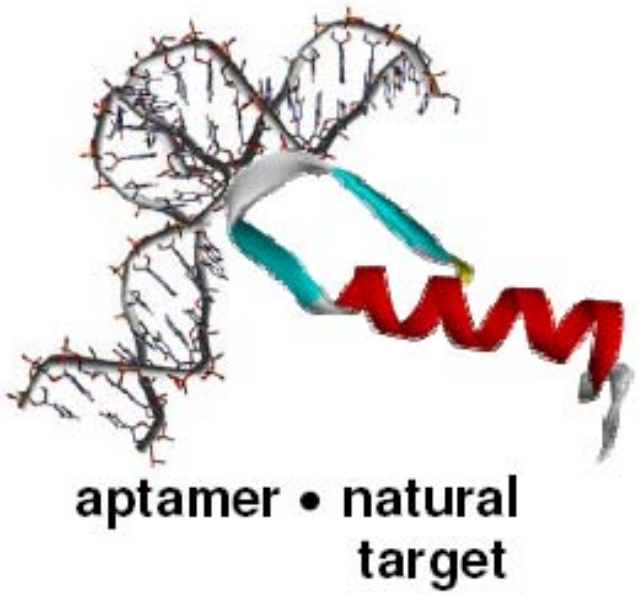
Stain with a protein-specific sensitive fluorescent stain



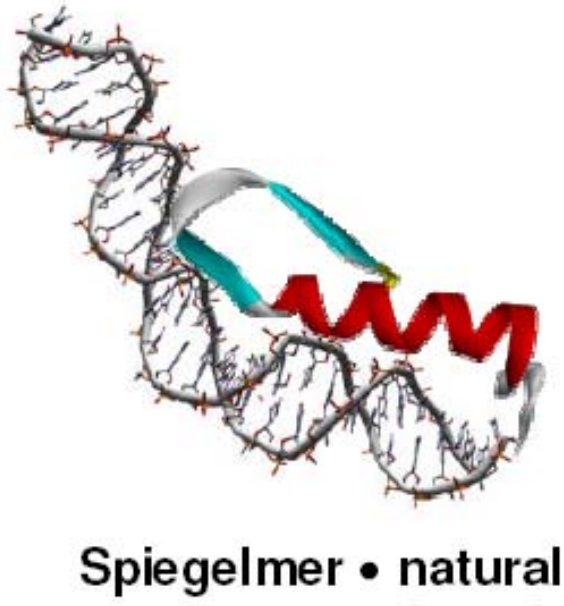
Spiegelmers (German *spiegel*: mirror)

Biostable aptamers

Natural enantiomers



D-oligonucleotide **L**-peptide



L-oligonucleotide **L**-peptide

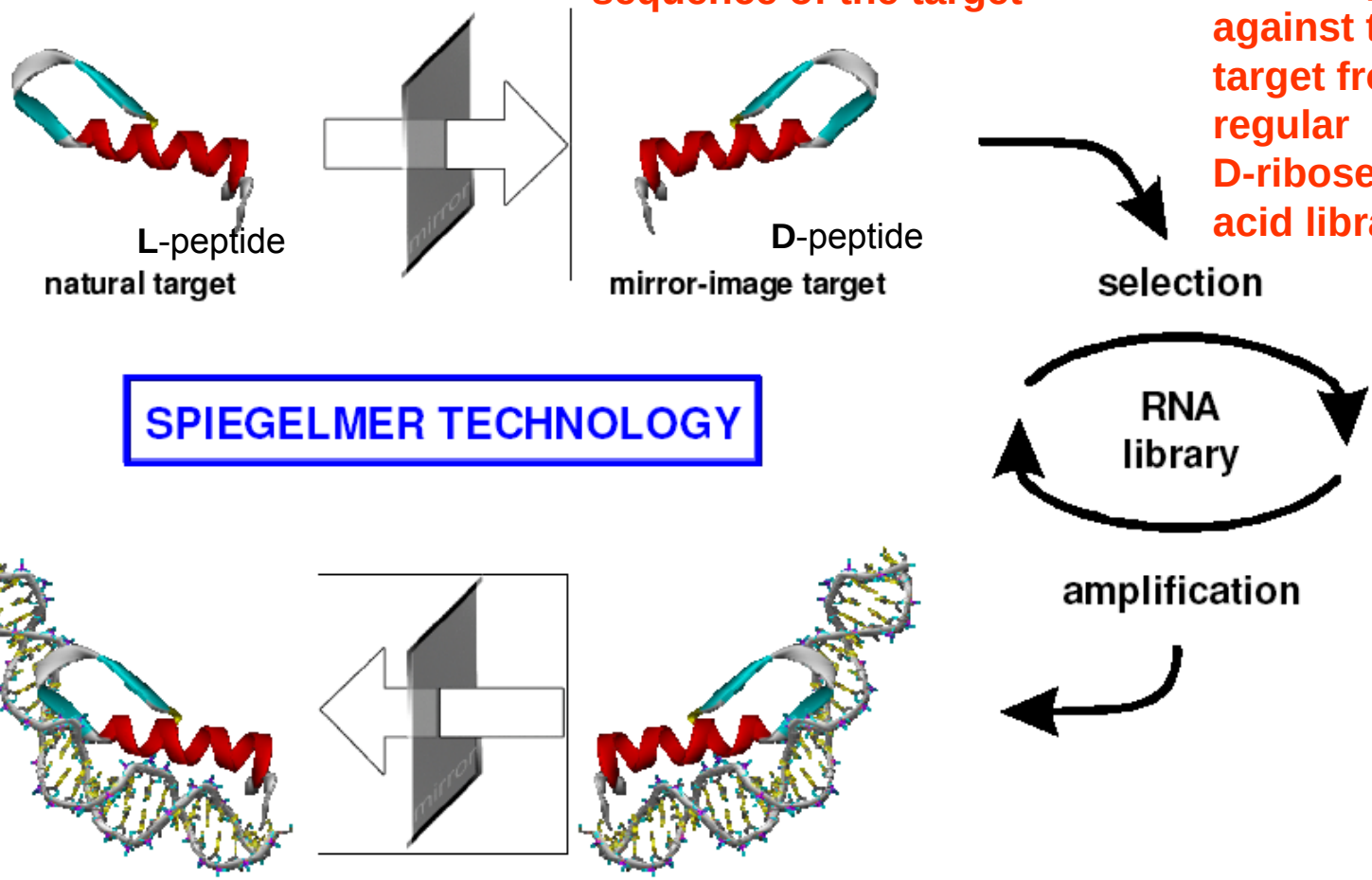
Unmodified aptamers are degraded by nucleases. Half-life in the human serum:

- unmodified aptamers – seconds
- 2'-modified aptamers – hours
- speigelmers - days

Spiegelmers Contd.

Synthesize D-amino acid sequence of the target

Select aptamers against the mirror target from the regular D-ribose nucleic acid library



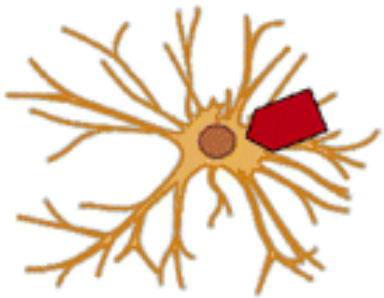
Obtain L-RNA (spiegelmer) resistant to nucleases

Sequence and synthesize the L-ribose version of the selected aptamer

Noxxon (Germany)

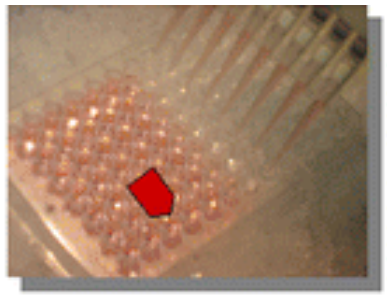
First products:
Anti-CGRP
Anti-Grehlin

Aptamers in Biomedical Sciences



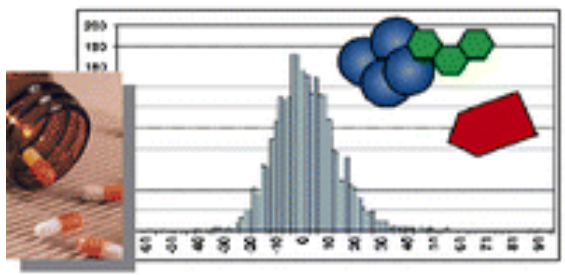
Target validation

(*e.g.* Marro et al., 2005)



Aptamers as diagnostics

(*e.g.* Fredrikson, 2002)



High-throughput screening

(*e.g.* Green et al., 2001)

Aptamers as therapeutics

(*e.g.* Rusconi et al., 2004)

Aptamer

Appl. Microbiol. Biotechnol., 2005, Nov 11, 1-8

Diagnostics

Aptamers can replace antibodies in a plenty of assays such as ELISAs and protein microarrays

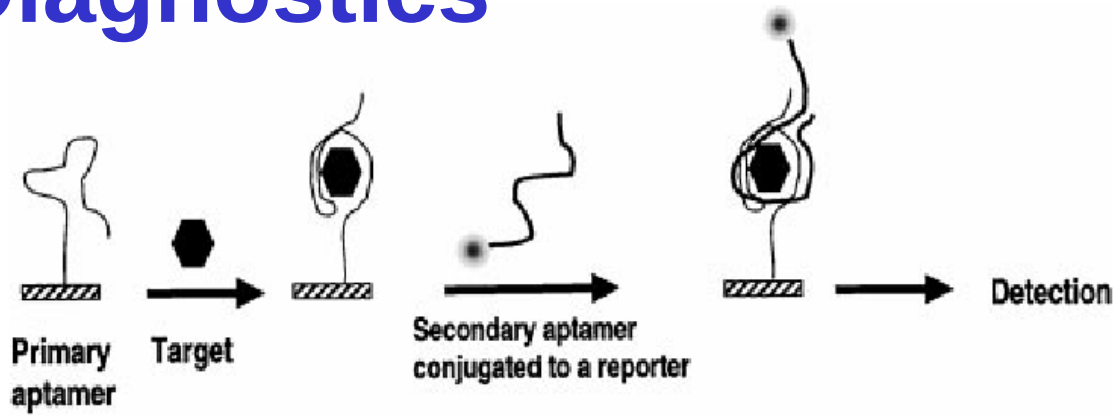


Fig. 5. Aptamer-based assay using a secondary aptamer that specifically recognizes primary aptamer-target complex.

Clin. Chem. 1999, 45, 1628-50

Ultra-Wide Dynamic Range Analysis of Proteins Using Smart Aptamers

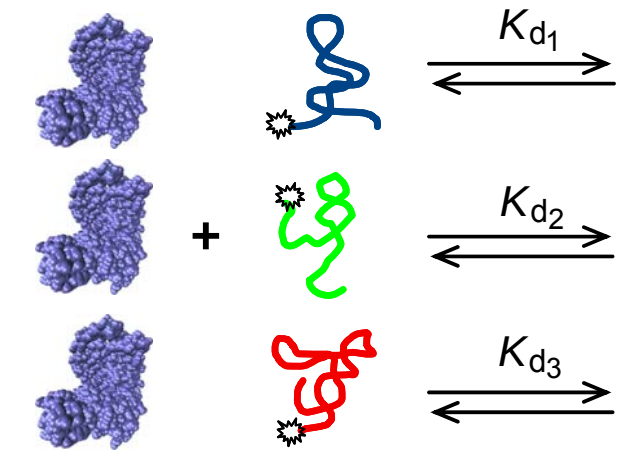
- Such an analysis requires multiple affinity probes with significantly different equilibrium constants (K_d)
- Each probe is detecting the target in the range of concentrations around its K_d value

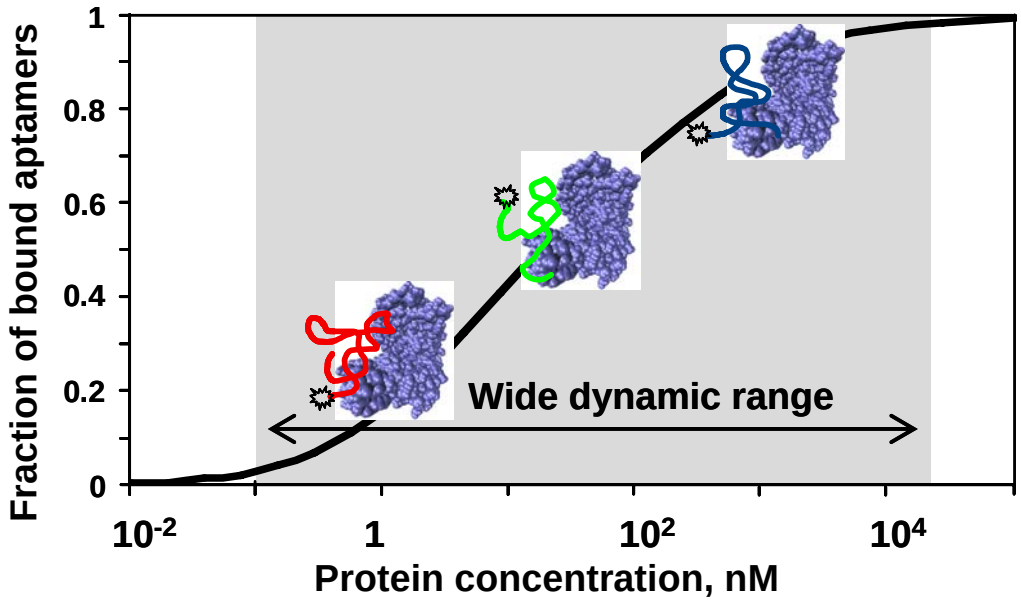
~~Antibodies~~ Smart aptamers

JACS 2007, *129*, 7260-7261

Diagnostics, Contd.

Ultra-Wide Dynamic Range Analysis of Proteins Using Smart Aptamers





Experimentally, fraction ***f*** is found (for example, with NECEEM):

$$f = \frac{[\mathrm{P{\bullet}Apt}_1] + [\mathrm{P{\bullet}Apt}_2] + \ldots + [\mathrm{P{\bullet}Apt}_n]}{[\mathrm{Apt}_1]_0 + [\mathrm{Apt}_2]_0 + \ldots + [\mathrm{Apt}_n]_0}$$

To find the total concentration of the target protein $\mathbf{[P]_0}$, the following general equation is used for ***n*** probes (smart aptamers):

$$\sum_{i=1}^{n} \frac{[\mathrm{Apt}_i]_0}{K_{d_i} + [\mathrm{P}]_0 - f \cdot \sum_{j=1}^{n} [\mathrm{Apt}_j]_0} = \frac{f \cdot \sum_{i=1}^{n} [\mathrm{Apt}_i]_0}{[\mathrm{P}]_0 - f \cdot \sum_{i=1}^{n} [\mathrm{Apt}_i]_0}$$

$\mathbf{[Apt}_i\mathbf{]_0}$ and $\boldsymbol{K_{di}}$ are the total concentration and affinity of aptamer ***i***, respectively

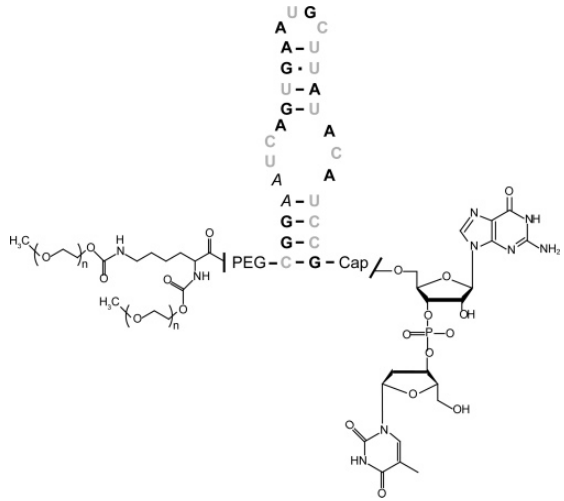
JACS 2007, *129*, 7260-7261

Therapy

Therapeutic aptamers in clinical development to treat :

- all forms of Age-related Macular Degeneration
- lung cancer, melanoma, cutaneous T-cell lymphoma
- hepatitis C and HIV
- asthma and allergy
- blood coagulation in surgery (short half-life anticoagulants/antithrombotics)

MACUGEN® or PEGANTANIB - modified RNA aptamer that targets vascular endothelial growth factor VEGF (Kd = 200 pM) and prevents development of Age-related Macular Degeneration



MACUGEN® NAMED INNOVATIVE PHARMACEUTICAL PRODUCT OF THE YEAR AT THE 2005 PHARMACEUTICAL ACHIEVEMENT AWARDS

Nat. Rev. Microbiol. 2006, 4, 588-596

Drug Discovery

Competitive drug screening assay: active small molecule displaces an aptamer from active center of a target

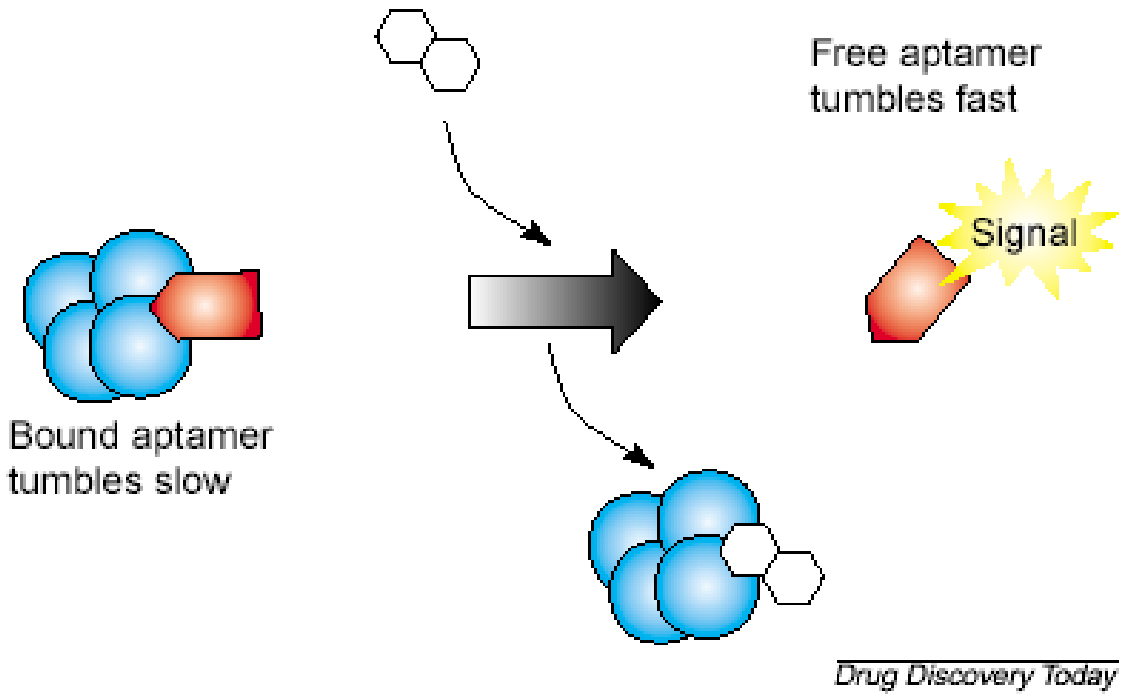


Fig. 2 Schematic representation of an aptamer-based fluorescence polarization assay. Competition of the bound aptamer from its cognate protein target by a small-molecule competitor results in a change in fluorescence polarization.

Drug Discov. Today, 2002, 7, 1221-8

Target validation

Determination that a target (protein) is involved in disease pathology. Aptamers are used to block functions of intracellular and extracellular drug targets at protein level.

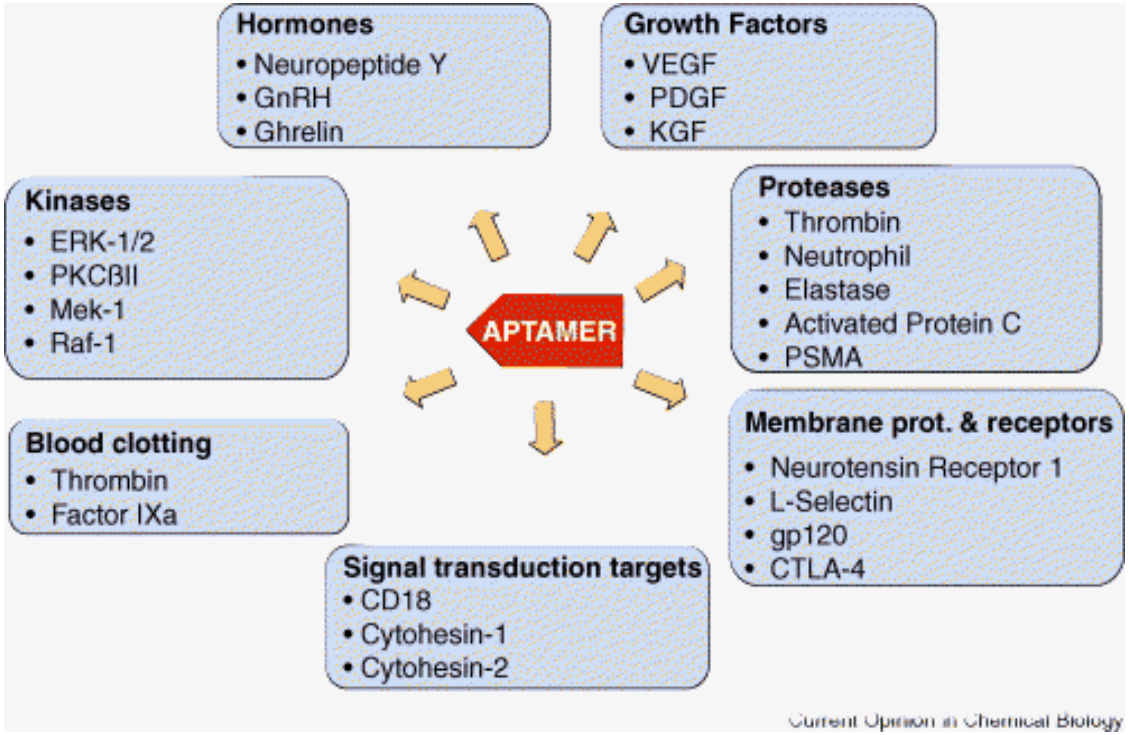
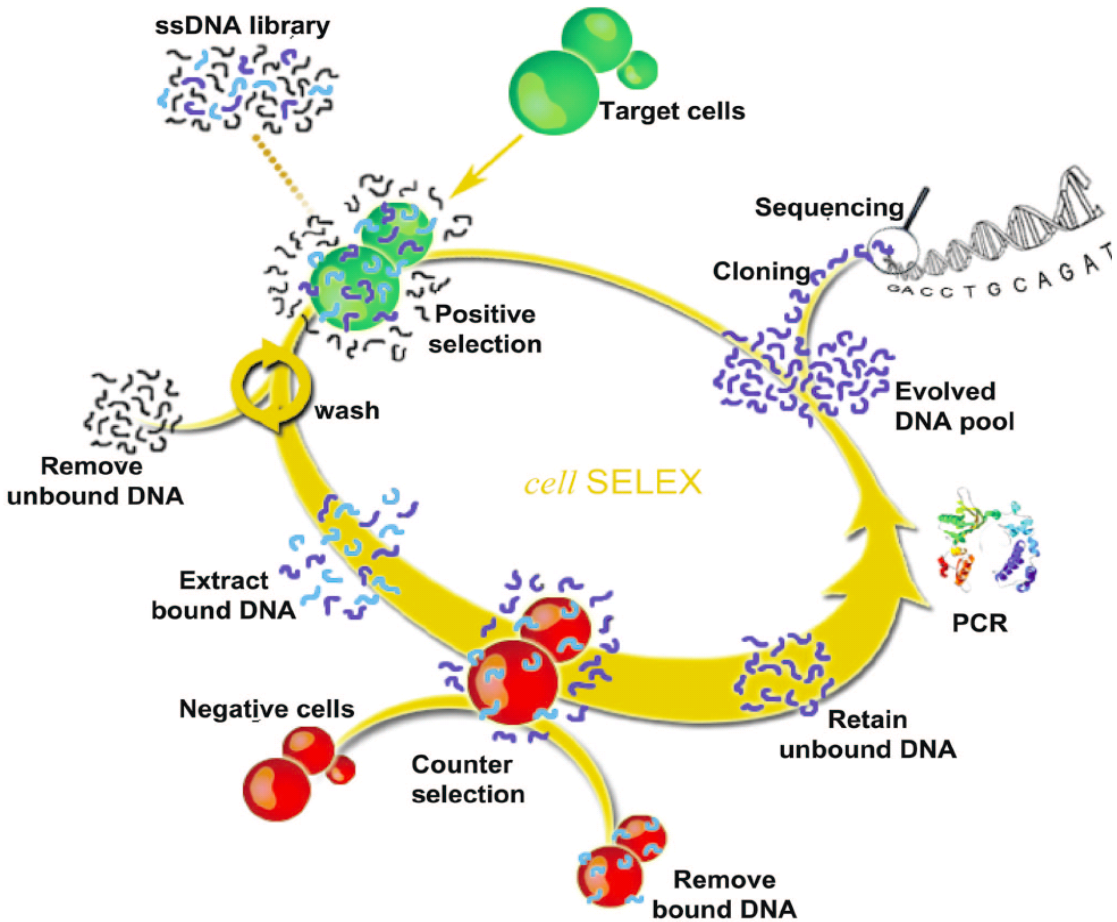


Fig.4 Aptamers are effective inhibitors of various target classes. Selected examples of published studies, in which aptamers have been used as specific inhibitors of diverse target protein families *in vitro* and *in vivo*.

Curr. Opin. Chem. Biol. 2005, 9, 336-42

Cell SELEX

- Cell SELEX – selection of aptamers to the whole cells
- Multiple targets on the surface of the cell



- *Cell-surface biomarker discovery*: targets are purified with a pool of aptamers and identified with mass-spectrometry
- *Cell imaging*: staining of the cell surface with fluorescent aptamers
- *Cell sorting*: aptamers are conjugated to nanoparticles and used to purify specific cells (cancer cells, stem cells)