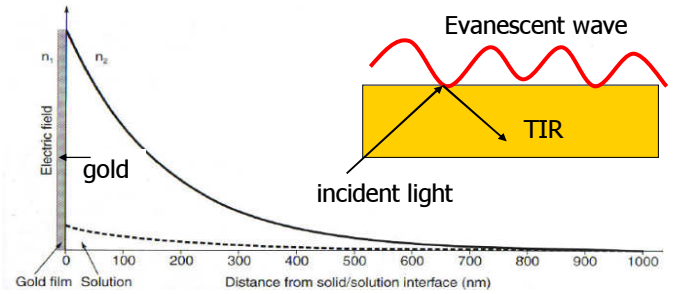
Welcome to Lecture 11

Evanescent wave biosensors

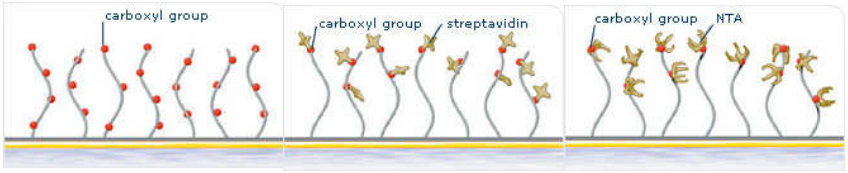


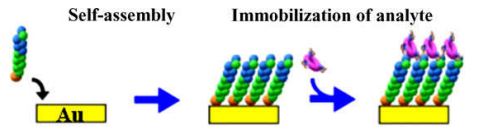
Evanescent electric field amplitude vs distance from the surface

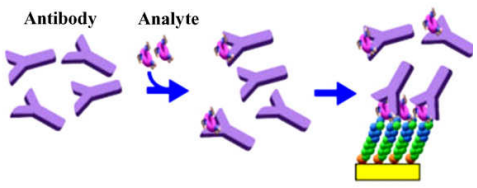
This is a great tool to focus specifically on the binding events occurring at the interface

Sensor chips

Biacore chips: plenty of receptors in the 400nm layer







Self-assembly of mono-layers right on the transducer surface: easy and great control

However, in many SPR sensors amplification with a secondary Ab or assays in the competitive format were used

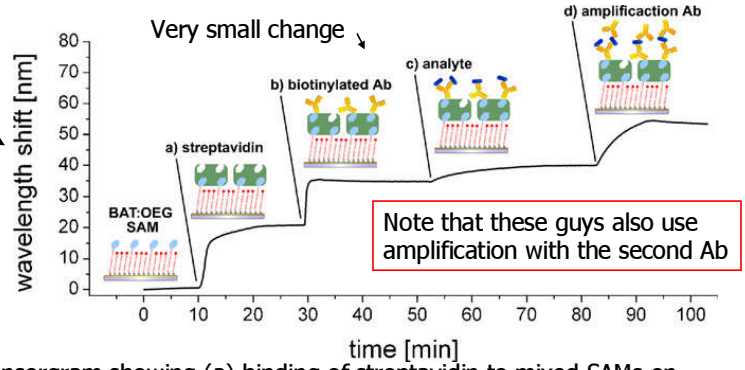
WHY?

SPR signal

Quantitative and simultaneous detection of four foodborne bacterial pathogens with a an 8-channel SPR biosensor

Or more often the resonant angle shift

Either way, its RI at the surface

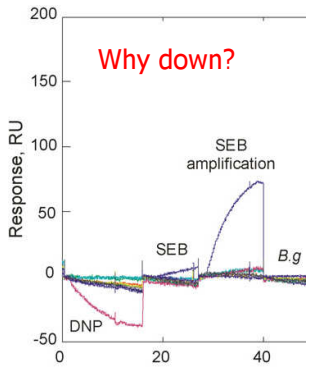


Sensorgram showing (a) binding of streptavidin to mixed SAMs on Au, (b) binding of biotinylated PAb to streptavidin, (c) direct detection of an analyte, and (d) secondary amplification using PAb

Biosensors and Bioelectronics 22 (2006) 752–758

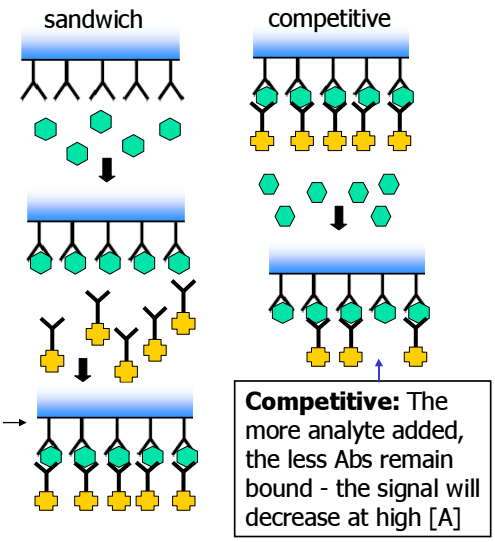
Change in both directions

SPR signal



Sandwich: The more analyte bound to the 1st Ab, the higher the binding of the 2nd Ab hence **a typical response (up)**

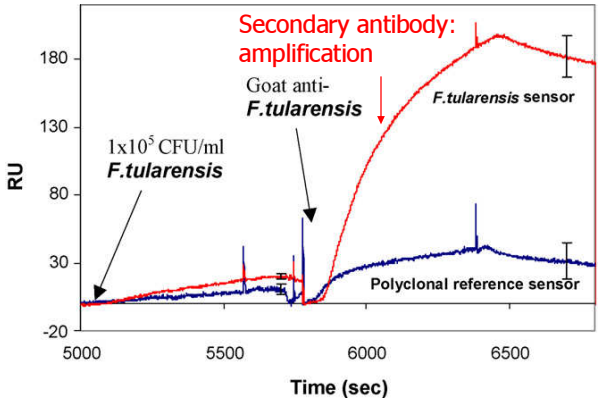
We have seen it with Abs too:



Competitive: The more analyte added, the less Abs remain bound - the signal will decrease at high [A]

Amplification

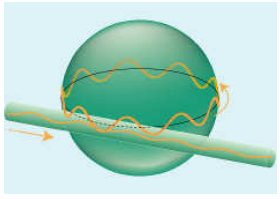
Detection of 1×10^5 CFU/ml F. tularensis



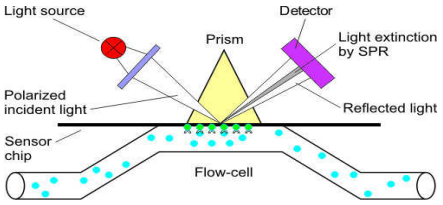
The average response of three sensor amplified goat anti-F. tularensis vs normal goat serum surfaces

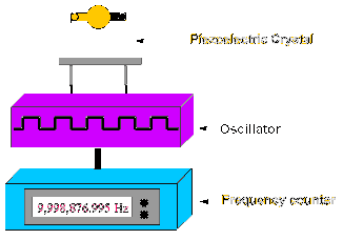
Sensitivity

This is a great solution to the problem of sensitivity, but there is no perfection in this world - immobilization is a little more involving…



Label free biosensors





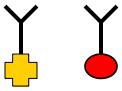
Advantage: no labeling required; just add your sample and go

Is this the future of biosensors?

May be or may be not

Why is labeling a problem?

ELISA – the end user doesn't label anything but buys an appropriately labeled Abs



The extra cost of labeling per se is marginal…

Hence, the real problem is requirement for the secondary Ab and additional operations it brings – add, wash, etc

It would be great to have a signal generating label right on the actual bioreceptor i.e. "reagentless" sensor

How?

Förster resonance energy transfer (FRET) is an energy transfer mechanism between two chromophores e.g. **Fluorescent resonance energy transfer** ☺

FRET is a REALLY cool bioanalytical tool

What is FRET?

A donor fluorophore in its excited state can transfer energy by a non-radiative, long-range dipole-dipole coupling mechanism to an acceptor chromophore in close proximity, typically <10nm

The FRET efficiency (E) is the fraction of energy transfer that occurred per donor excitation event

The physics of dipole-dipole coupling mechanism is such that E depends on the donor-to-acceptor separation distance r with an inverse power of 6:

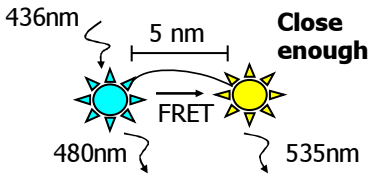
$$E = \frac{1}{1 + (r/R_0)^6}$$

Thus, FRET can only be observed when the two participants, the donor and acceptor, are VERY (i.e. "molecularly") close

For example

Let us take CFP (cyan fluorescent protein)

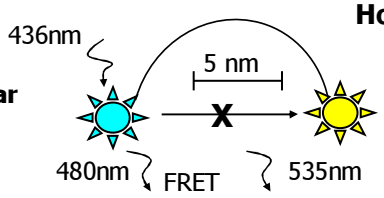
Suppose we make a fusion protein between CFP and YFP (yellow fluorescent protein)



If the two proteins are adjacent to each other, the energy transfer is significant and a large proportion of the energy from CFP will be transferred to YFP

However,

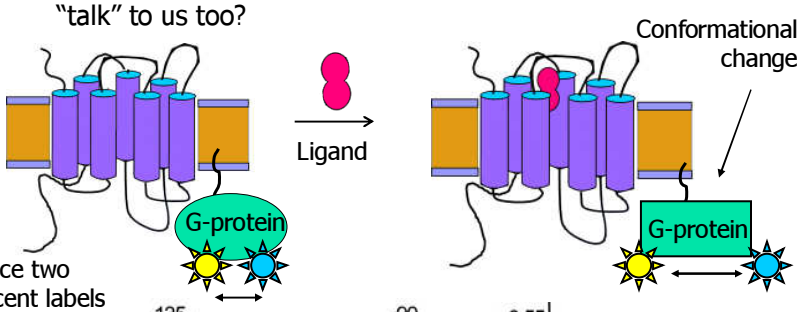
Too far



It does not have to be proteins – any suitable pair of labels will work

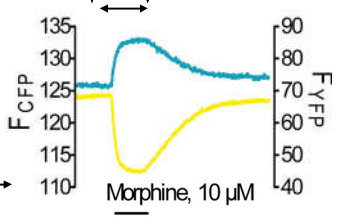
We have seen it in action

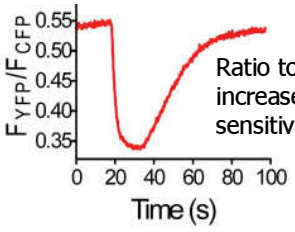
Hijacking cells' signaling: Can we get G-proteins to "talk" to us too?



Introduce two fluorescent labels into the G-protein

When morphine binds the two proteins move apart

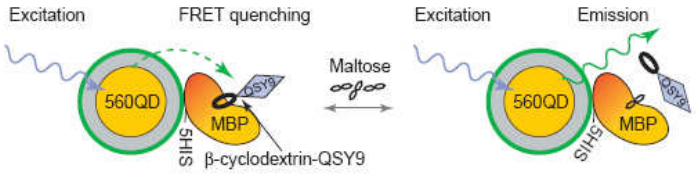




Ratio to increase sensitivity

Quenching works too

Receptor proteins and QD: Competitive donor–acceptor separation in a receptor/binding protein

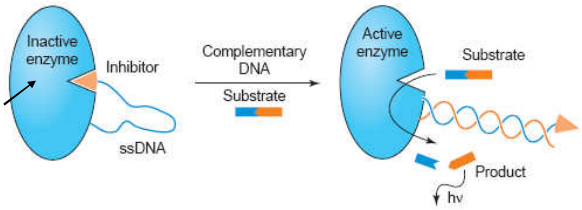


- Maltose-binding protein (MBP; just a model to how the principle) is labeled with a QD which emits light at 560 nm
- QD emission is quenched by a quencher (QSY9) conjugated to a substrate analogue (β-cyclodextrin) bound in the MBP binding site
- In the presence of substrate maltose, β-cyclodextrin–QSY9 is displaced and this results in increased QD emission

Enzyme amplification

Enzyme-amplified DNA detection

An inhibitor-DNA–enzyme (IDE) complex



In IDE an enzyme is conjugated with a ssDNA which is tagged with an enzyme inhibitor

Initially, the enzyme is inactivated because conformational mobility of ssDNA allows it to get into the active site (intramolecular inhibitor)

Binding of target DNA (complementary to the ssDNA) leads to the formation of a rigid duplex and the inhibitor is pulled from the active site

Thus activated enzyme cleaves its substrate e.g. separates a fluorophore and a quencher labels at either end of the substrate molecule leading to the appearance (and amplification) of fluorescent signal

Medintz, I.L. et al. (2003) Nat. Mater. 2, 630–638

Biosensing on the surface

A hair-pin RNA/DNA as a biosensor

Recognition loop complementary to the target

Short complementary sequences to bring the two ends together

Donor/acceptor Fret interaction (excitation or quenching)

What will happen?

Target sequence

If the recognition loop sequence is longer than the end sequence, hybridization with the target will lead to a conformational change and generate a signal

Change in fluorescence

And you can easily get it on the surface of a traducer too

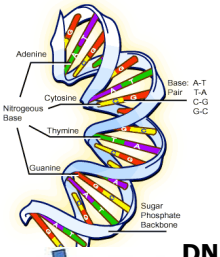
Will it be up or down signal?

Nucleic acids

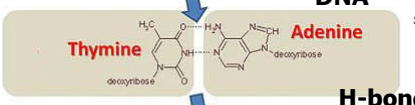
3-D DNA structure

Pairing of complimentary DNA strands leads to the Double Helix

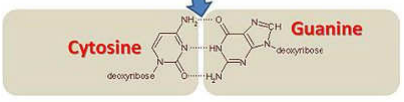
Because the energy is the same either can be used; in practice DNA is often preferred



DNA

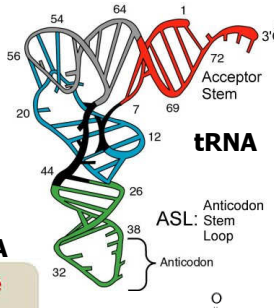


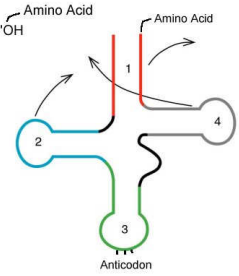
H-bonding



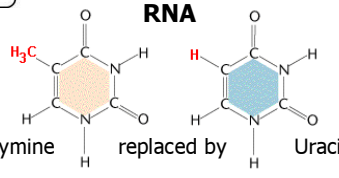
3-D RNA Structure

Pairing of partially complimentary sequences leads to RNA "hair-pins"





RNA

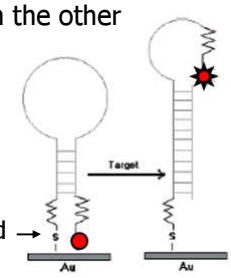


Thymine replaced by Uracil

Molecular beacons

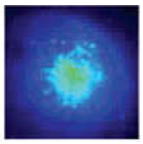
Molecular beacons consist of a DNA hairpin functionalized at one end with a fluorophore and a FRET active group e.g. a quenching agent on the other

DNA hairpin incorporating portions of *Staphlococcus aureus* genes, bearing a 5/-end-linked disulfide and a 3/-end-linked rhodamine (fluorescent label)



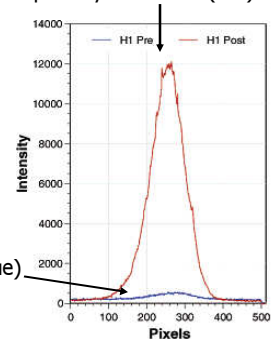
Signal: CCD image from the sensor

Prehybridization



After hybridization with complementary sequence

post-hybridization (red)



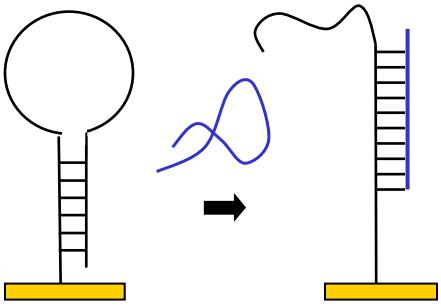
pre-hybridization (blue)

Excitation at 514 nm; emission 585 nm strong

J. AM. CHEM. SOC. 2003, 125, 4012

Electron transfer

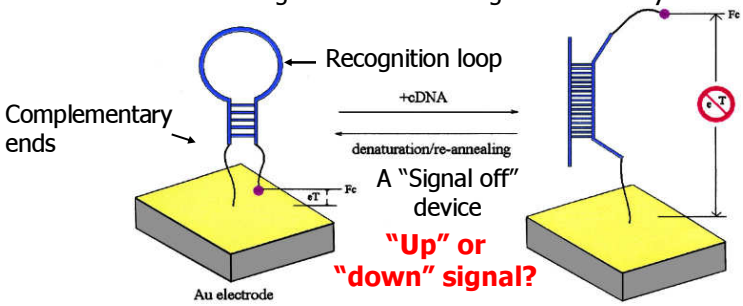
Bioreceptors can be brought very close to the surface and they can change conformation on binding



Hence, if there was a mechanism to transfer energy DIRECTLY to the electrode we would get a very cool **bioelectronic device**

E-DNA sensor

A stem-loop oligonucleotide with terminal thiol and a ferrocene group is immobilized at a gold electrode through self-assembly



- In the absence of target, the stem–loop structure holds the ferrocene tag in close proximity to the electrode surface, thus ensuring rapid electron transfer and efficient redox cycling of the ferrocene
- On hybridization with the target sequence, a large change in redox currents is observed

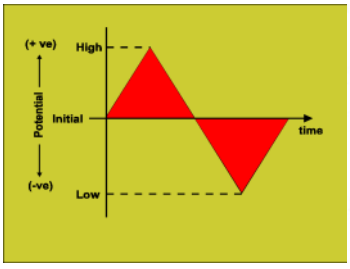
Fan et al. (2003) PNAS 100, 9134-9137

How can we measure more than just a single e⁻ jump?

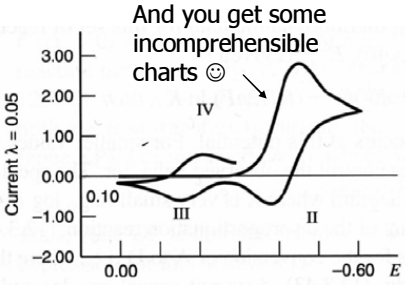
Cyclic voltammetry

Cyclic voltammetry is the measure of the current flowing between a reference and working electrode as the potential difference is linearly cycled between a maximum and minimum value

The analyte solution is not stirred, so the current is due to electrochemical changes at/near the working electrode surface



And you get some incomprehensible charts ☺

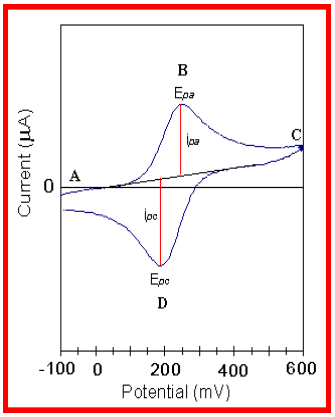


Cyclic voltammetry

Starting with reduced sample (A) little current is detected unless higher potential is applied, with a max near the oxidation potential. Approaching this value the current rises as the sample at the surface is oxidized (B), and then decays until only species diffusing through the solution can be oxidized. At C the max potential applied

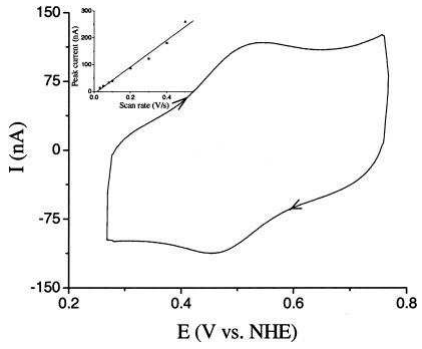
The reverse now occurs as we reversed the voltage - the current increases in the opposite direction reaching max near the reduction potential (D) and then drops to a residual value due to reduction of any oxidized sample diffusing back to the electrode

The CV for a single, reversible oxidation-reduction reaction at the electrode



E-DNA sensor

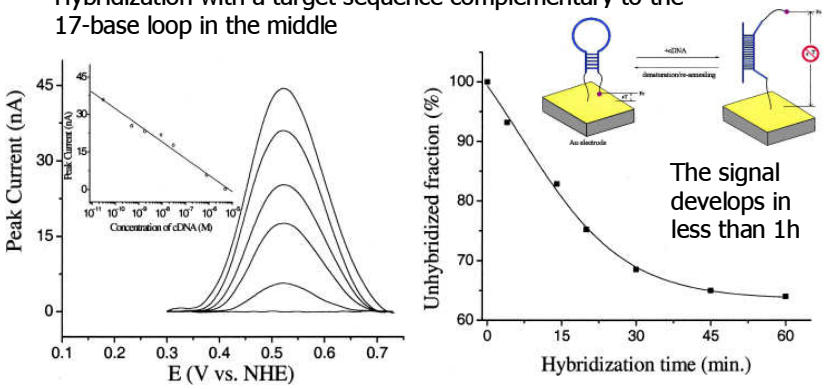
Signal detection – cyclic voltammetry



A cyclic voltammogram for a gold electrode modified with the ferrocene-tagged, stem–loop oligonucleotide in the absence of target DNA. The linear relationship between peak currents and scan rates confirms that the redox species is confined to the electrode surface

E-DNA sensor: signal detection

Hybridization with a target sequence complementary to the 17-base loop in the middle

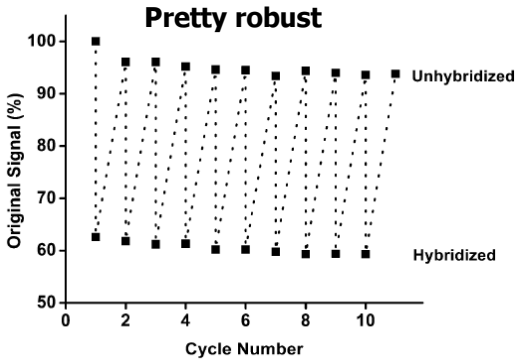


The signal develops in less than 1h

Left: Background-subtracted voltammograms for the E-DNA sensor in the presence of complementary DNA at 0 M, 30 pM, 500 pM, 30 nM, 800 nM, and 5 μM (from top to bottom) – **note the sensitivity**

Fan et al. (2003) PNAS 100, 9134-9137

Stability and reproducibility



- Highly reproducible response after successive regeneration cycles, although there is sole signal loss after the first use/wash cycle
- E-DNA sensors built using micro-fabricated gold electrodes are similarly reusable and reproducible: 100.9 $\pm$ 1.4% signal recovery per wash and 21.4 $\pm$ 0.7% signal drop per use over 13 iterations

Anal. Chem. **2006,** 78, 5671

Works in real samples

DNA analysis – medical and environmental samples

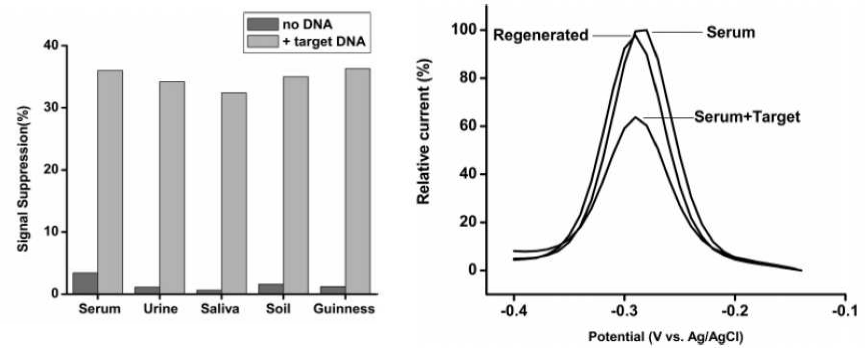


Table 1. Detection of Target DNA by E-DNA in Complex, Contaminant-Ridden Samples

	sample tested[a]				
	buffer	blood serum	urine	saliva	soil
sample	0[b]	3.4 ± 1.6	1.1 ± 0.7	0.6 ± 0.5	1.6 ± 0.8
sample + target	36.5 ± 0.6	36.0 ± 14.2	34.2 ± 3.8	32.4 ± 1.2	35.0 ± 8.1

Why SNPs?

A single nucleotide polymorphism (SNP) is a single "letter" difference in DNA sequence in the genome of the same species

In humans common SNPs constitute ~90% of the variation in the population (the remaining 10% is a vast array of rare relatively variants), and the majority have only two alleles

By early 2007 more than 3 million of common SNP variations (~30%) in people from geographically diverse populations were catalogued (HapMap project*)

Why are SNPs so important?

Because gene variants affect how/whether a disease would develop and patients' response to drugs and other treatments

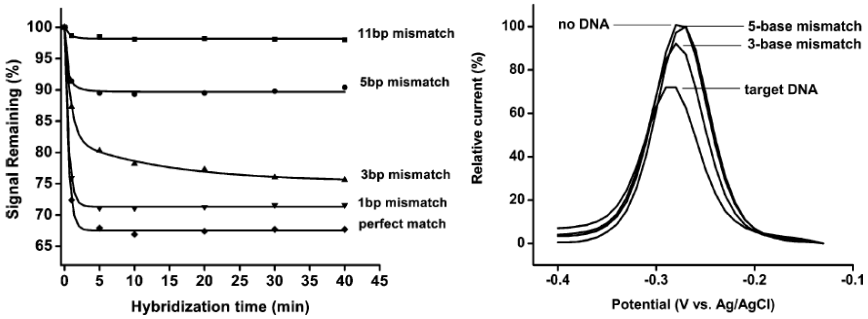
*Haplotype is a specific set of alleles on a [part of] chromosome ⟶

ApoE2: xxTxxxxTxx
ApoE3: xxTxxxxCxx
ApoE4: xxCxxxxCxx

- **Diagnostics**
- **Risk assessment**

SNPs – the ultimate selectivity

E-DNA's can identify SNPs under ideal laboratory conditions, but the detection of SNPs in samples like serum is still problematic

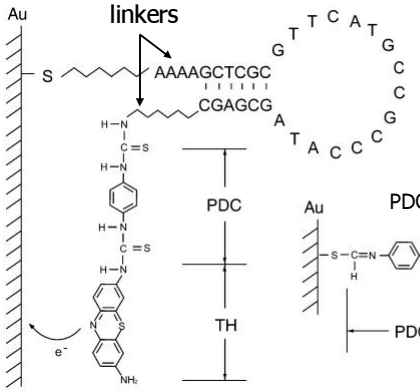


On the right: The sensor readily distinguishes 200 nM 3-, and 5-bp mismatches from a perfectly complementary target in a sample of fetal calf serum (**in situ!**), but the sensor's response to a single-base mismatch is within error of that observed with fully complementary targets

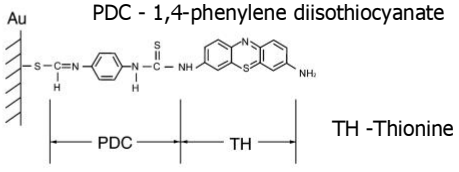
Anal. Chem. **2006,** 78, 5671

Another way to skin the cat

The hairpin DNA immobilized on the gold surface with redox-active moiety (TH) attached to the 3′-end through a linker; the 5′-end is also extended with four adenosines



As before, the loop consists of a probe sequence, which is complementary to a target sequence; the stem is short unrelated sequence

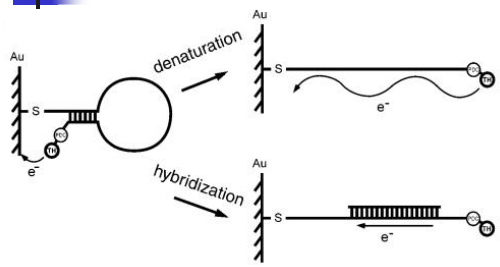


PDC - 1,4-phenylene diisothiocyanate

TH -Thionine

The electroactive group offers free electrons that are able to produce micro-current pulse, if a suitable electric potential is applied

Operating principle



- When the probes are not hybridized electron transfer produces a tunneling current
- Opening the hairpin by heating, greatly reduces the tunneling current, but when the probes is coupled with a target, the e⁻ can move along the ds-DNA as it was a conducting piece of wire ☺

TH+ + 2e⁻ + H⁺ ↔ leucothionine (LTH)

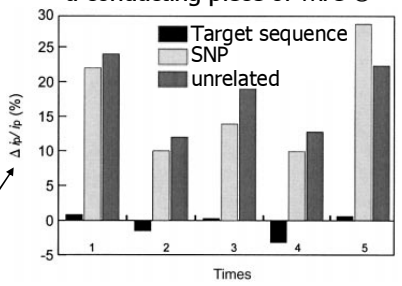
How good is this little sensor?

It can detect a single nucleotide mismatch (SNP) with the target

Very impressive but…

i_p is the peak current in the voltammogram; measurement are done at two temperatures

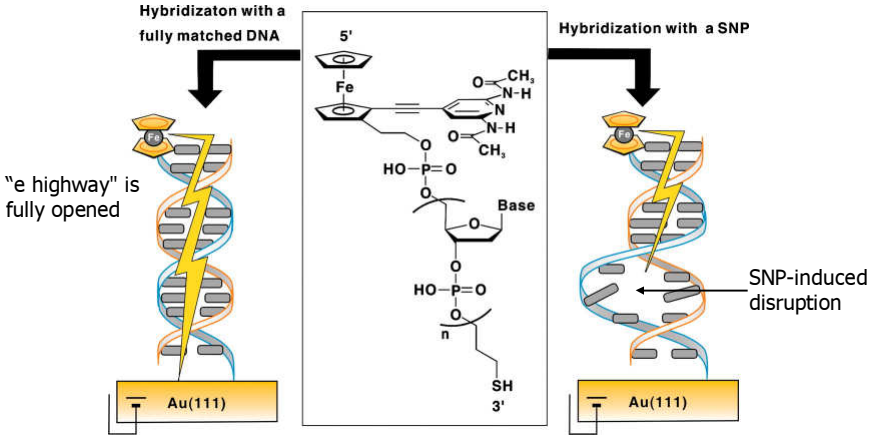
$$\Delta i_p/i_p = [i_p(20°C) - i_p(40°C)]/i_p(40°C).$$



Mao et al (2003) Nucleic Acids Res. 31, e108

A DNA-wire sensor

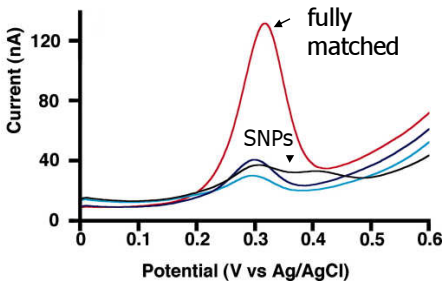
The presence of a single-base mismatch in the DNA duplexes results in a dramatic decrease in the electrochemical response



Inouye et al. (2005) Proc. Natl. Acad. Sci. USA 102, 11606-11610

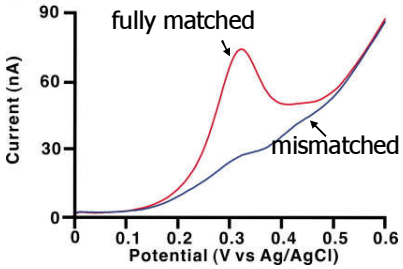
SNP detection

Detection of p53 mutations (cancer)



Uncorrected square wave voltammetry profiles at the gold working electrodes modified with fully matched DNA (red line), 248A SNP (light blue), 249 SNP (black), and 248T SNP (dark blue)

Inouye et al. (2005) Proc. Natl. Acad. Sci. USA 102, 11606-11610

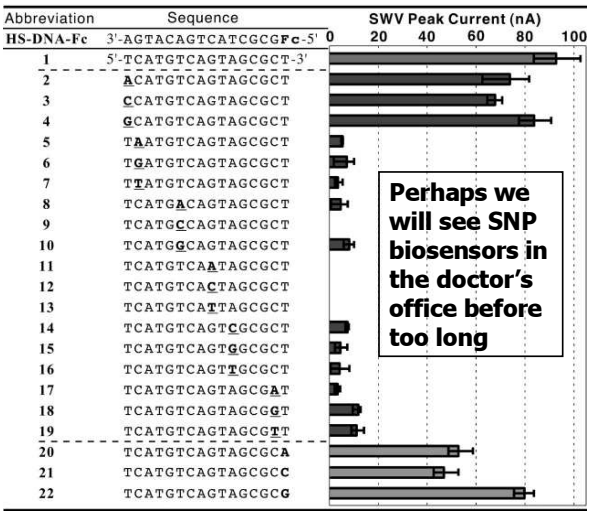


SNP discrimination for longer target strands (43-mer)

Let's take a good look at selectivity

As good as it gets

SNPs in positions **5**–**19** show only small or no electrochemical responses

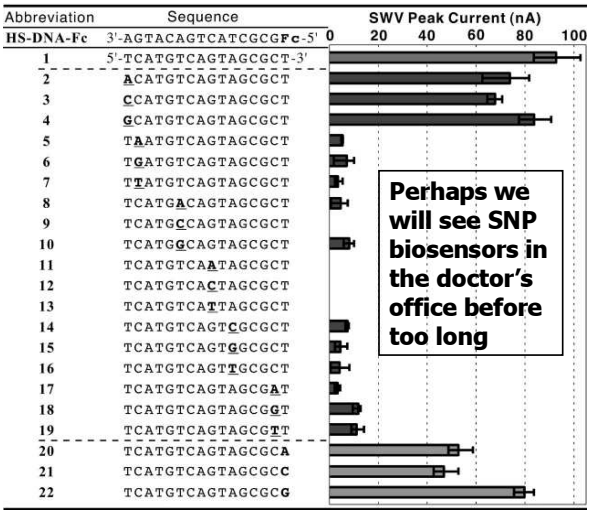


Perhaps we will see SNP biosensors in the doctor's office before too long

Can we do the same type of tricks with proteins or this surface technology only works with NAs?

As good as it gets

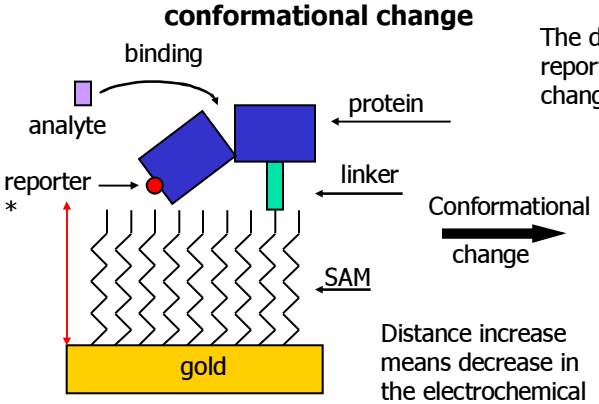
SNPs in positions **5–19** show only small or no electrochemical responses



Can we do the same type of tricks with proteins or this surface technology only works with NAs?

Bending hinge motion in proteins

Design of bioelectronic interfaces based on conformational change

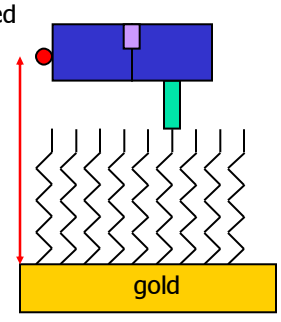


Reporter PF6: Ru(II)(NH3)4(1,10-henanthroline-5-maleimide)]

Conformational change

Distance increase means decrease in the electrochemical response in a [C]-dependent manner

The distance between the reporter and transducer has changed

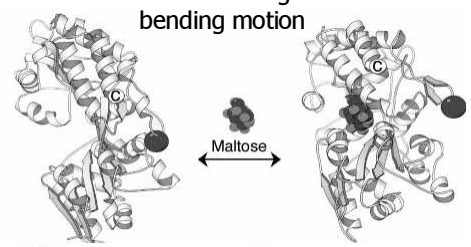


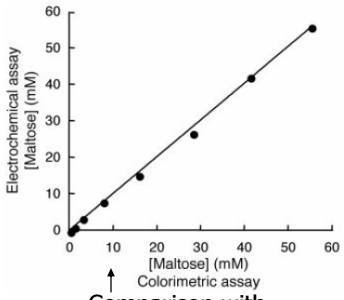
Receptor e.g. Maltose-binding protein

Science 293, 1641–1644

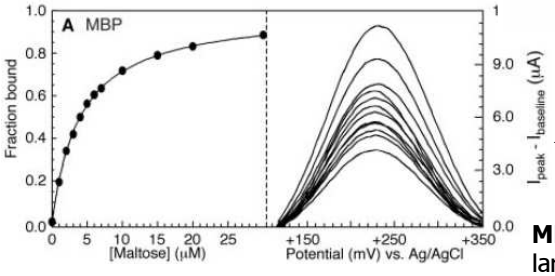
Bioelectronic interface

Based on hinge bending motion





Comparison with conventional colorimetric assay



← Ligand-mediated responses of MBP biosensor (left))

MBP again: good model – large conformational change

SCIENCE (2001) 293 31

Aptamers

Single-stranded polynucleotide binding molecules with high affinity and selectivity for the target – great capturing agents in biosensors

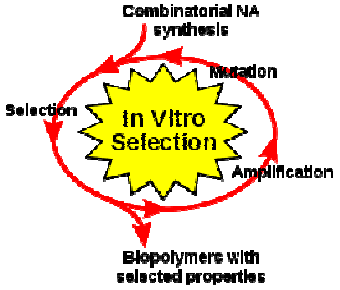
How to make them?

Solid phase synthesis: fast, not very expensive and scalable

How does one know which one to make?

For detecting NAs – mainly rational design but for protein and small molecule targets???

Directed "molecular" evolution ☺



Making aptamers

For any application aptamers are created *de novo* by selecting them from a library - a large random pool of sequences

The numbers game:

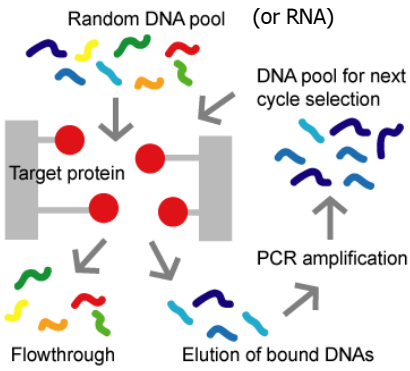
- Length: ~40 base pairs
  - Random incorporation of nucleotides in a 40-mer: $4^{40} = 1.2 \times 10^{24}$
    - Libraries of between 10^{14} and 10^{16} generally used

All sequences comprising the library are kept of the same length - random in the middle" and flanked by fixed 5' and 3' ends that serve as primers for amplification

The rest is just selection and amplification – **SELEX** molecular genotype-phenotype link

SELEX

Systematic Evolution of Ligands by Exponential Enrichment



Captured, partitioned, & purified sequences are amplified by PCR

New library is enriched for (high affinity) binders

Early rounds select against non-binding sequences by elimination

Later rounds select for high affinity-binding RNAs by competition

- Proteins or any other target can be used
- Some form of affinity chromatography

~15 rounds required but SELEX can be fully automated

Characterization & Optimization

Characterization

- At the end a small number of aptamers that bind tightly to the target are isolated
- Nucleotide sequences are determined
- Target binding affinity and specificity measured and compared
- Nucleotides not contributing to target binding or aptamer structure are optionally eliminated

Optimization

- Affinity enhancement by derivatization (e.g. 2'-deoxy -> 2'-O-methoxy nucleotides)
- Small size allows systematic synthesis & assay for improved functional activity
- A few such changes can result in increased affinity of up 100- to 1,000-fold
  - increased contacts with target
  - reduction of deleterious contacts
  - improved fold and stability

The final product

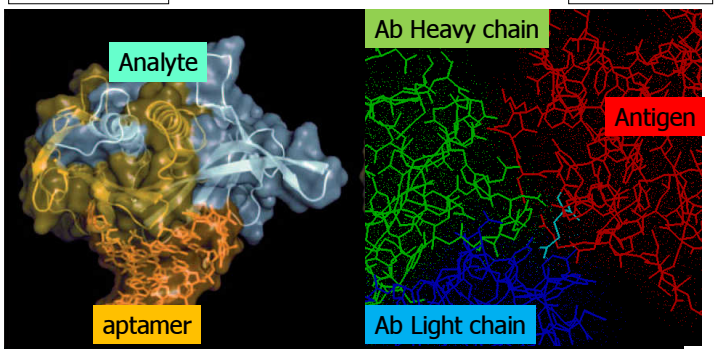
Diversity and affinity of selected aptamers

Ligand	Nucleic acid*	Affinity K_d [μM]	3D structure†
Theophylline	RNA (*4*)	~0.3	NMR, 1EHT (*5*)
FMN	RNA (*6*)	~0.5	NMR, 1FMN (*7*)
AMP	DNA (*9*)	~6	NMR, 1AW4 (*12*)
	RNA (*8*)	~10	NMR, 1AM0, 1RAW (*10*, *11*)
Arginine	2 DNA (*15*)	~125	NMR, 1OLD, 2ARG (*18*, *20*)
	RNA (*16*)	~60	NMR, 1KOC (*19*)
Citrulline	RNA (*16*)	~65	NMR, 1KOD (*19*)
Tobramycin	2 RNA (*25*)	~ 0.009	NMR, 1TOB (*32*)
		~ 0.012	NMR, 2TOB (*33*)
Neomycin B	RNA (*26*)	~ 0.115	NMR, 1NEM (*34*)
HIV-1 Rev peptide	2 RNA (*40*)	~ 0.004	NMR, 1ULL, 484D (*41*, *42*)
HTLV-1 Rex peptide	RNA (*43*)	~ 0.025	NMR, 1C4J (*44*)
MS2 coat protein	3 RNA (*45*)	ND	X-ray, 5-7MSF (*45*, *46*)
Thrombin	DNA (*47*)	~ 0.025	NMR, 148D (*38*); x-ray, 1HAO (*39*)

- SELEX has been used to evolve aptamers of extremely high binding affinity to a variety of targets
- BTW, this technology is being applied clinically too – the first aptamer drug (Macugen) has been approved for treating macular degeneration

Interactions with proteins

~10 kDa Comparison to antibodies ~150 kDa



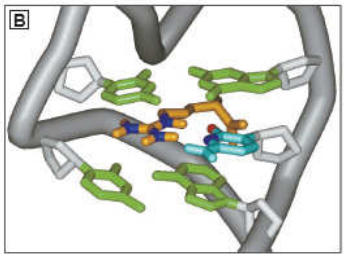
Dramatic conformational change Almost no conformational change

Typically aptamers require larger surface area of interaction with target protein to display affinity similar to Abs, **but**

Molecular interactions

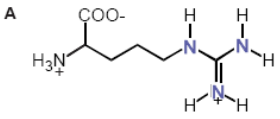
Antibodies:

- Hydrophobic Interactions
- Ionic
- Hydrogen bonding
- Van der Waals



Aptamers:

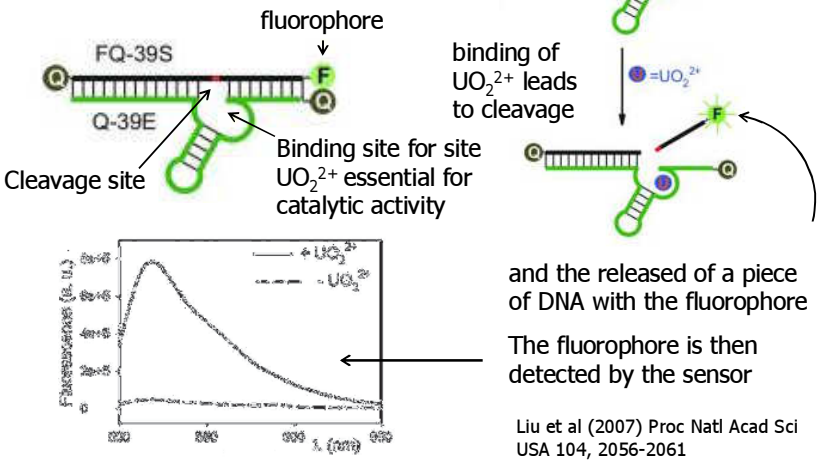
- Predominantly hydrogen bonding
- Within pockets:
  - –Stacking: p-orbital interactions between aromatic rings
  - –shape complementarity
  - –electrostatic interactions



Arginine binding to aptamer

Can be catalytic too

A small catalytic DNA that can cleave itself but only in the presence of UO_2^{2+}



and the released of a piece of DNA with the fluorophore

The fluorophore is then detected by the sensor

Liu et al (2007) Proc Natl Acad Sci USA 104, 2056-2061

Aptamers: summary

- Aptamers' binding affinity is comparable to that of antibodies - nM (10^{-9}) range, but sometimes even pM (10^{-12})
- SELEX is fast (weeks), relatively inexpensive, typically high success rate
- Very small molecular weight: truncated to core typically 20-40 nucleotides
- Chemically stable: can be boiled and frozen without loss of function
- Refold easily upon denaturation, thus reusable
- Amenable to a variety of chemical modifications - optimization, tailoring selectivity for specific applications, immobilization
- Synthesis – solid state

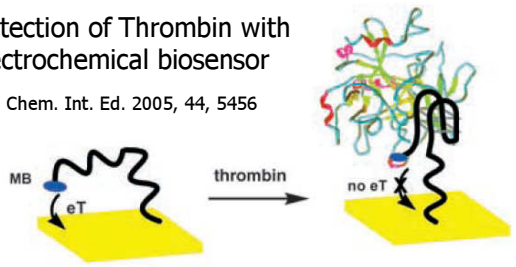
Commercialization?

Aptamer-based protein sensor

Electrochemical detection of Thrombin with Aptamer-based electrochemical biosensor

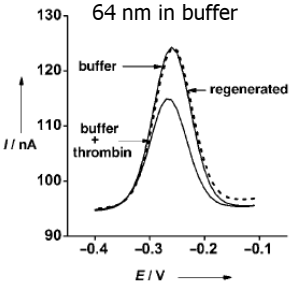
Sensor can be regenerated by a RT wash with 6M guanidine HCl. 93% of the signal is retained (dashed line) after two such cycles

Angew. Chem. Int. Ed. 2005, 44, 5456

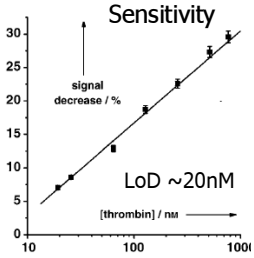


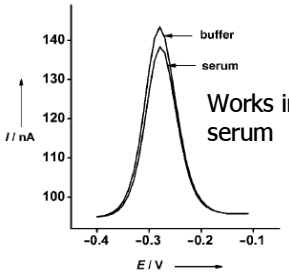
Once again, signal-off sensor

Signal: Thrombin 64 nm in buffer



Sensitivity



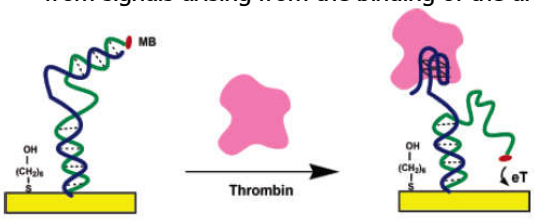


Works in serum

Signal-on architecture

Generally signal-off sensors have two drawbacks:

- Limited signal gain since - only 100% of the signal (max) can be suppressed
- Contaminants that degrade the bioreceptos or its redox label can give rise to "false positives" that are difficult to distinguish from signals arising from the binding of the analyte



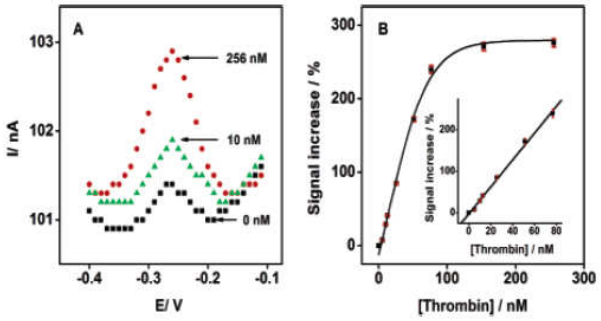
Thrombin binding kicks out the labeled piece of DNA – **SIGNAL!**

A short, methylene blue (MB)-tagged oligonucleotide that hybridizes with both the thrombin-binding portion of the same aptamer16 and the DNA sequence linking the aptamer to the electrode

J. AM. CHEM. SOC, 2006

Much improved sensitivity

At 19 nM thrombin produces a 7% signal drop in the off sensor and 60% increase in the on sensor; at 256 nM thrombin 270% signal gain



(A) Alternating current voltammetric (ACV) curves of the ds-DNA-aptamer functionalized surfaces

(B) A dose-response curve for the signal-on E-AB thrombin bisensor

And it can sniff out cocaine

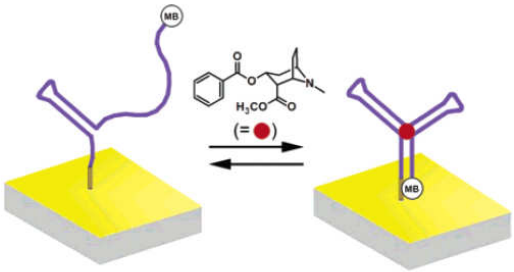


Figure 1. The electronic aptamer-based (E-AB) cocaine biosensor.

Table 1. Modified Aptamers[a] 30-mers

	aptamer sequence (5′ → 3′)
A1	HSC6-GACAAGGAAAATCCTTCAATGAAGTGGGTC-MB
A2	MB-GACAAGGAAAATCCTTCAATGAAGTGGGTC-C3SH
A3	HSC6-GACAAGGAAAATCCTTCAATGAAGT(MB)GGGTC
A4	HSC6-AGACAAGGAAAATCCTTCAATGAAGTGGGTCG-MB

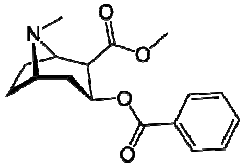
[a] Methylene blue (MB) redox tags are covalently attached via a seven-carbon linker to the termini, or an internal thymidine, as indicated.

J. AM. CHEM. SOC. 2006, 128, 3138

Cocaine

Cocaine is a powerful stimulant acting on the CNS; give users a quick and intense feeling of power and energy

Sold mostly in packets or small vials



Alkaloid obtained from the leaves of coca plant, native to South Am

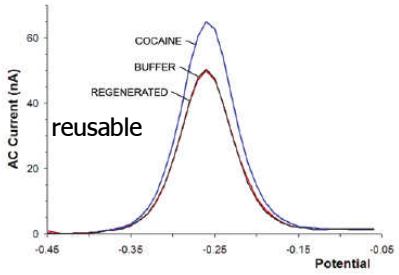
Normally detected by Scott Test

A three step procedure – cocaine is detected by appearance of blue precipitate →

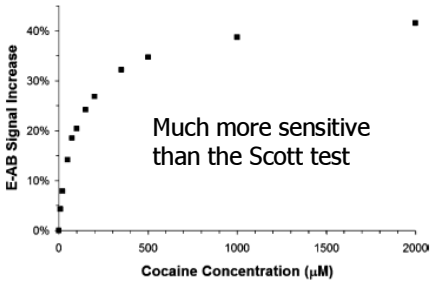
Major problem: Selectivity! The guys who cut it know well how the test works

Biosensor's performance

Response to 500 µM cocaine



Dose-response curve

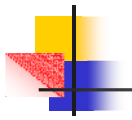


Much more sensitive than the Scott test

Table 2. Cocaine Detection in Cutting and Masking Adulterants[a]

adulterant	Scott Test	E-AB signal
none	positive	+31.4%
equal mass flour	positive	+31.4%
equal mass sugar	positive	+31.5%
equal mass baking soda	positive	+31.2%
equal mass coffee	positive	+29.5%
equal mass mustard powder	positive	+30.7%
10X mass cobaltous thiocyanate	negative	+24.2%

Works in the present of agents used to cut or mask cocaine



In conclusion

We have looked at a few VERY COOL sensors today

The bottom line:

There are numerous terrific platforms out there – the problem is not technology but the business model and competition

Have fun and

see you next week