Biosensors Based on Electrogenerated Chemiluminescence (ECL) Detection

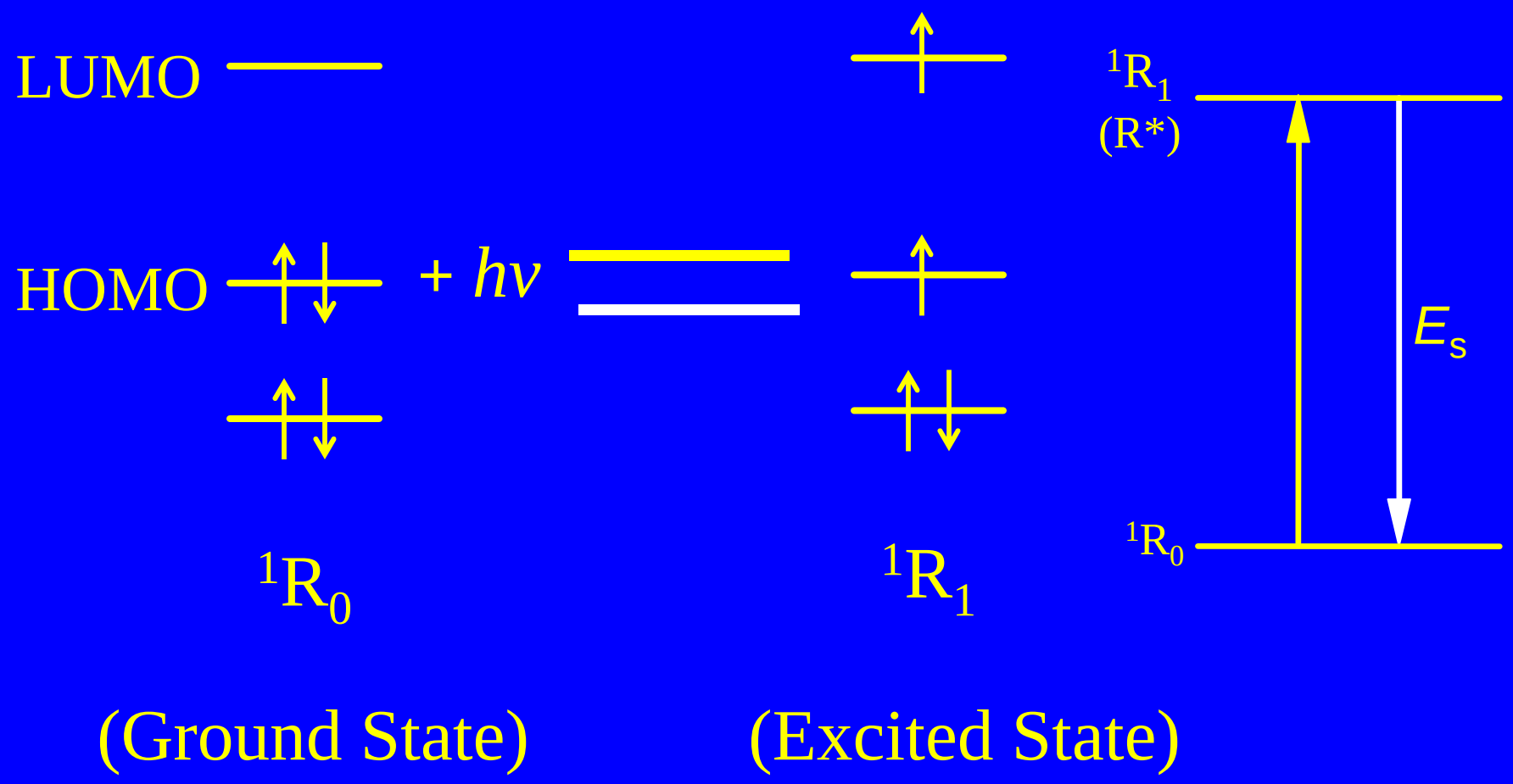
ECL Determination of Immobilized DNA and CRP Protein on Au (111) Electrodes Using $Ru(bpy)_3^{2+}$ Labels

Wujian Miao

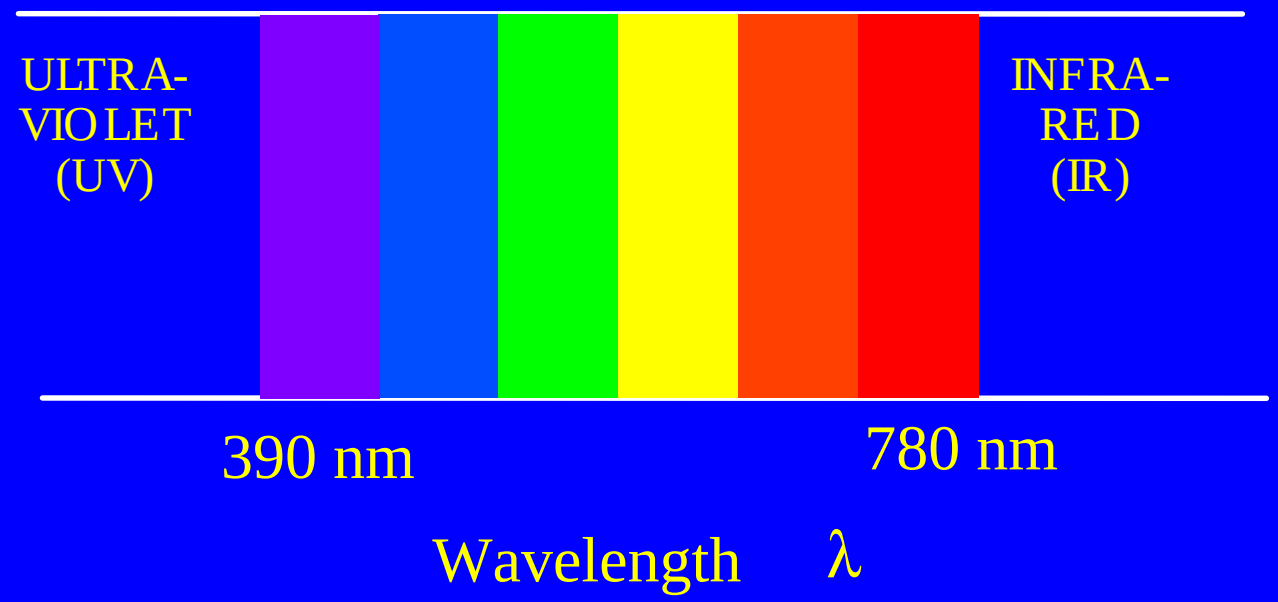
Outline of Talk

- Introduction and General Principles
- Annihilation and Coreactant ECL
- DNA Hybridization and C-Reactive Protein (CRP)
- $Ru(bpy)_3^{2+}$ Entrapped Microspheres
- Conclusions

Photoluminescence

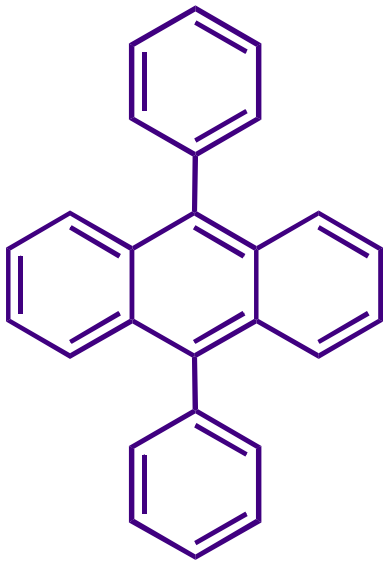


VISIBLE REGION

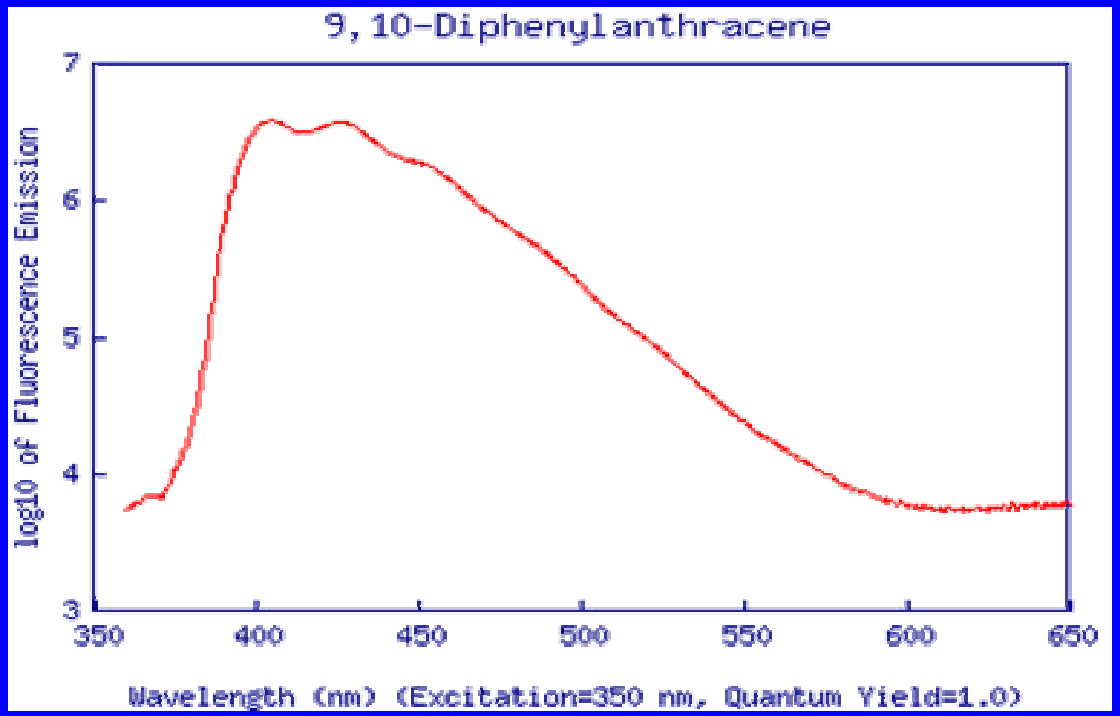


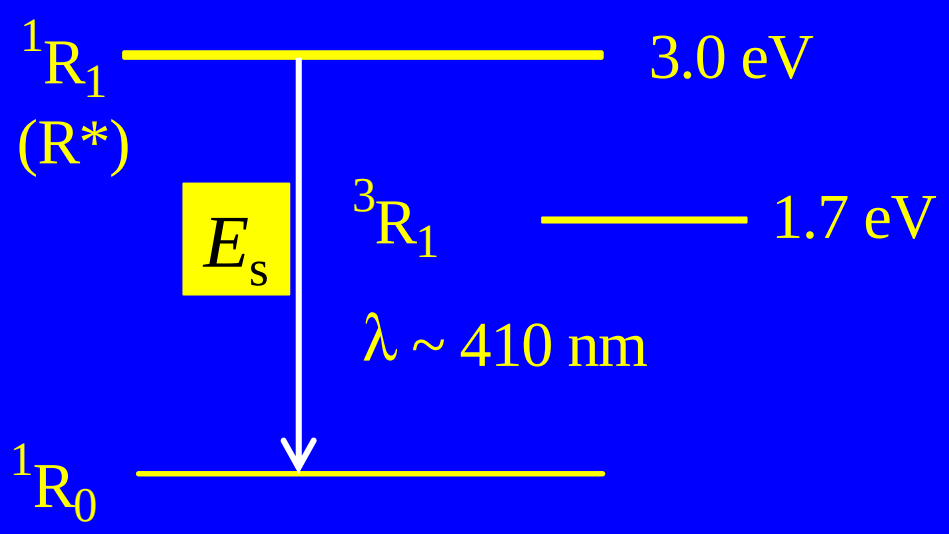
$$3.2\ \text{eV} > E_s > 1.6\ \text{eV}$$

$$E = h\nu = hc/\lambda = 4.13\times10^{-15}\ (\text{eV s})\times3.00\times10^{8}\ (\text{m/s})/\lambda\ (\text{m})$$



9, 10-Diphenylanthracene
(DPA)





Fluorescence Emission Spectrum of DPA In Cyclohexane

Photoluminescence

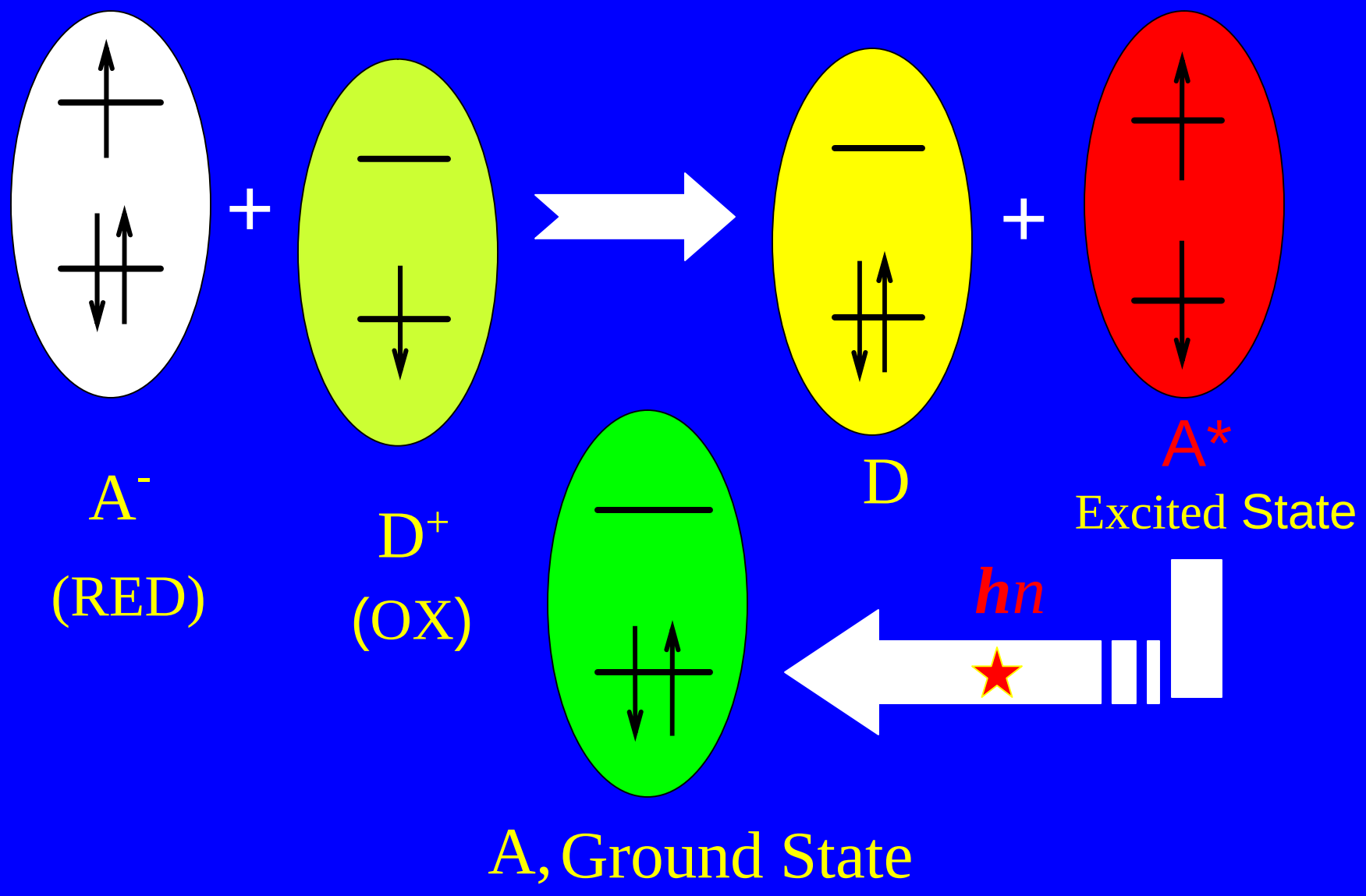
is a process

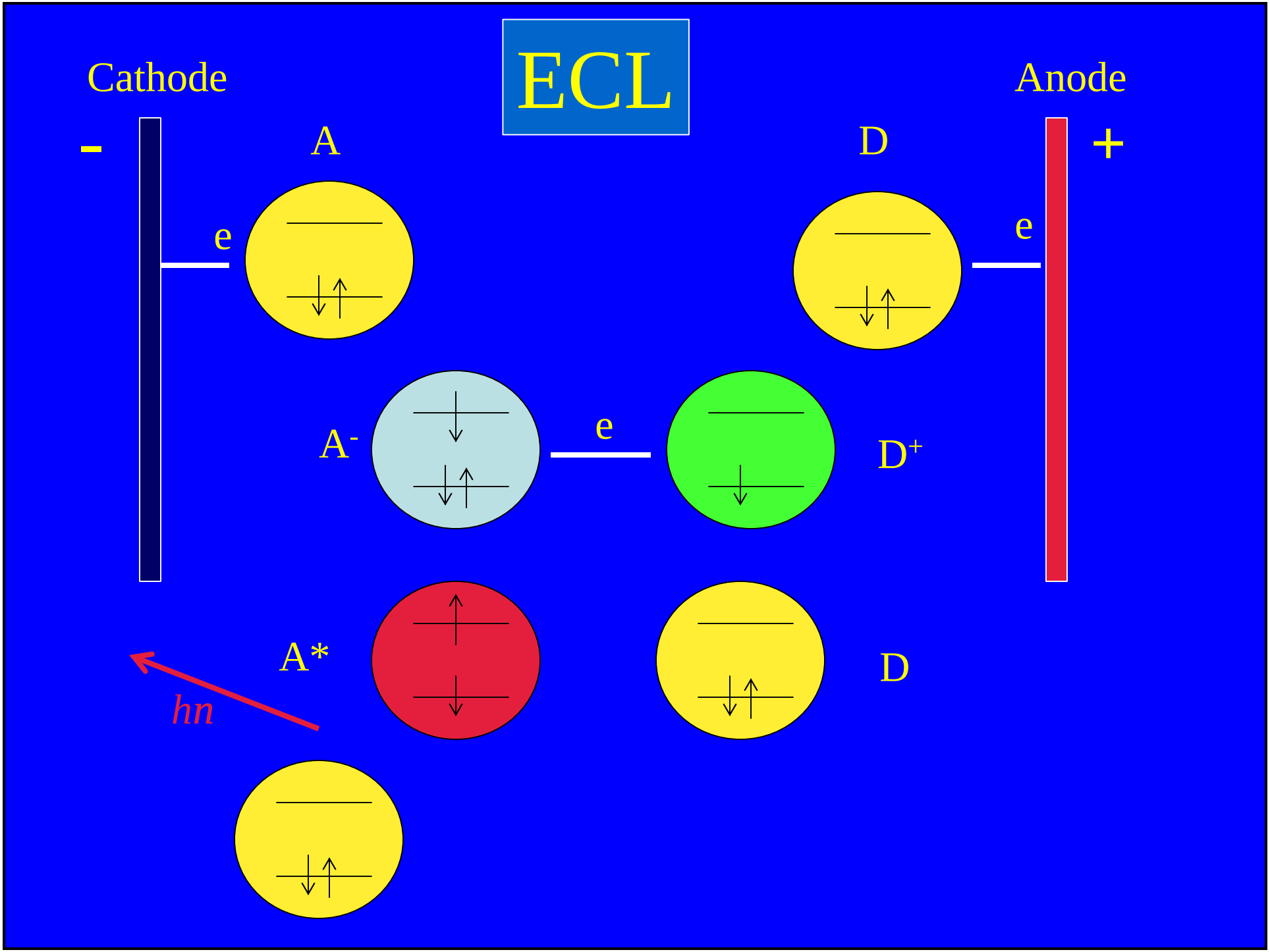
that using light to generate light

Chemiluminescence (CL)—Cool light

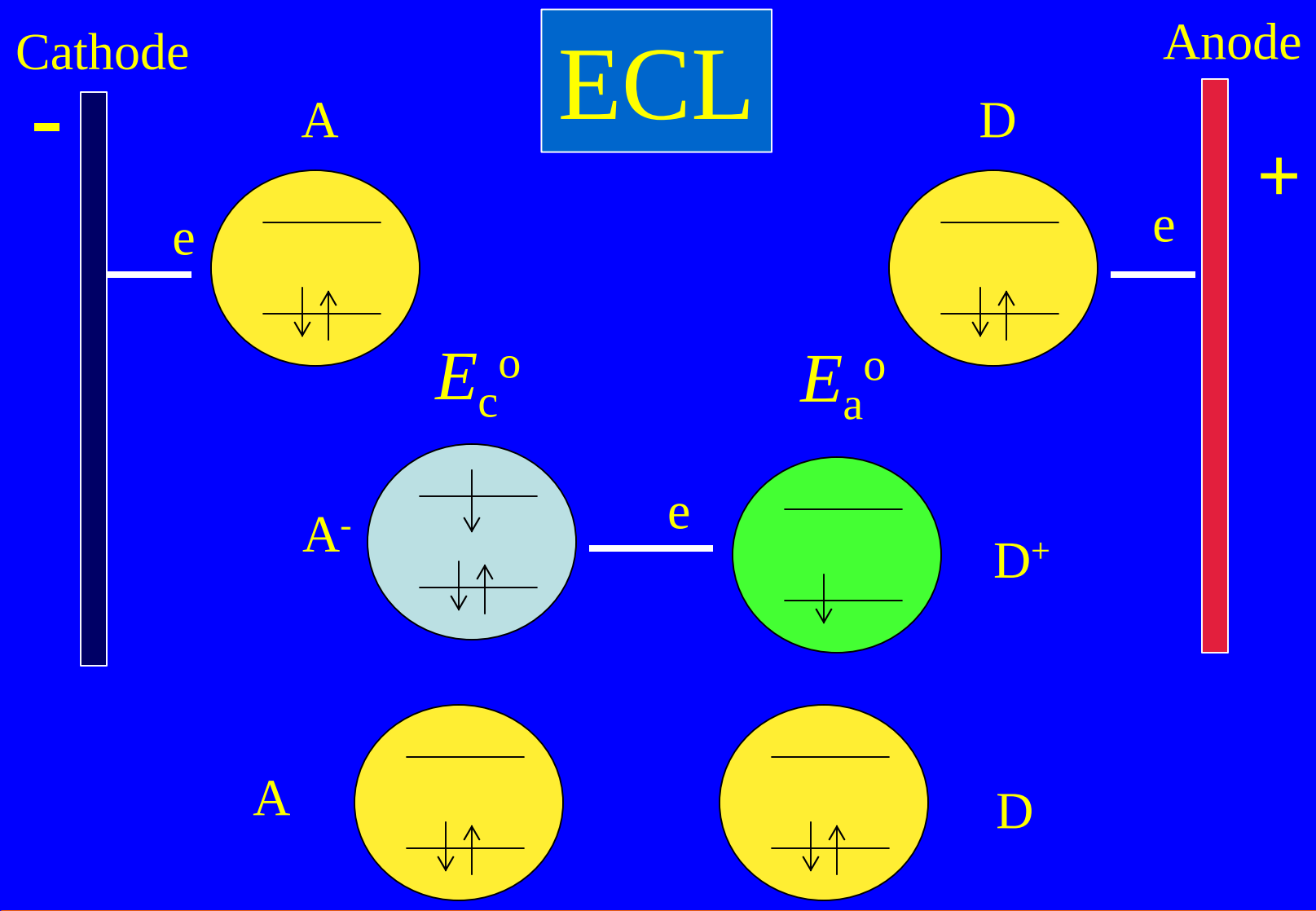
Reactions
that produce light
without heat
are called
Chemiluminescent
(CL) Reactions

Chemiluminescence--Mechanism

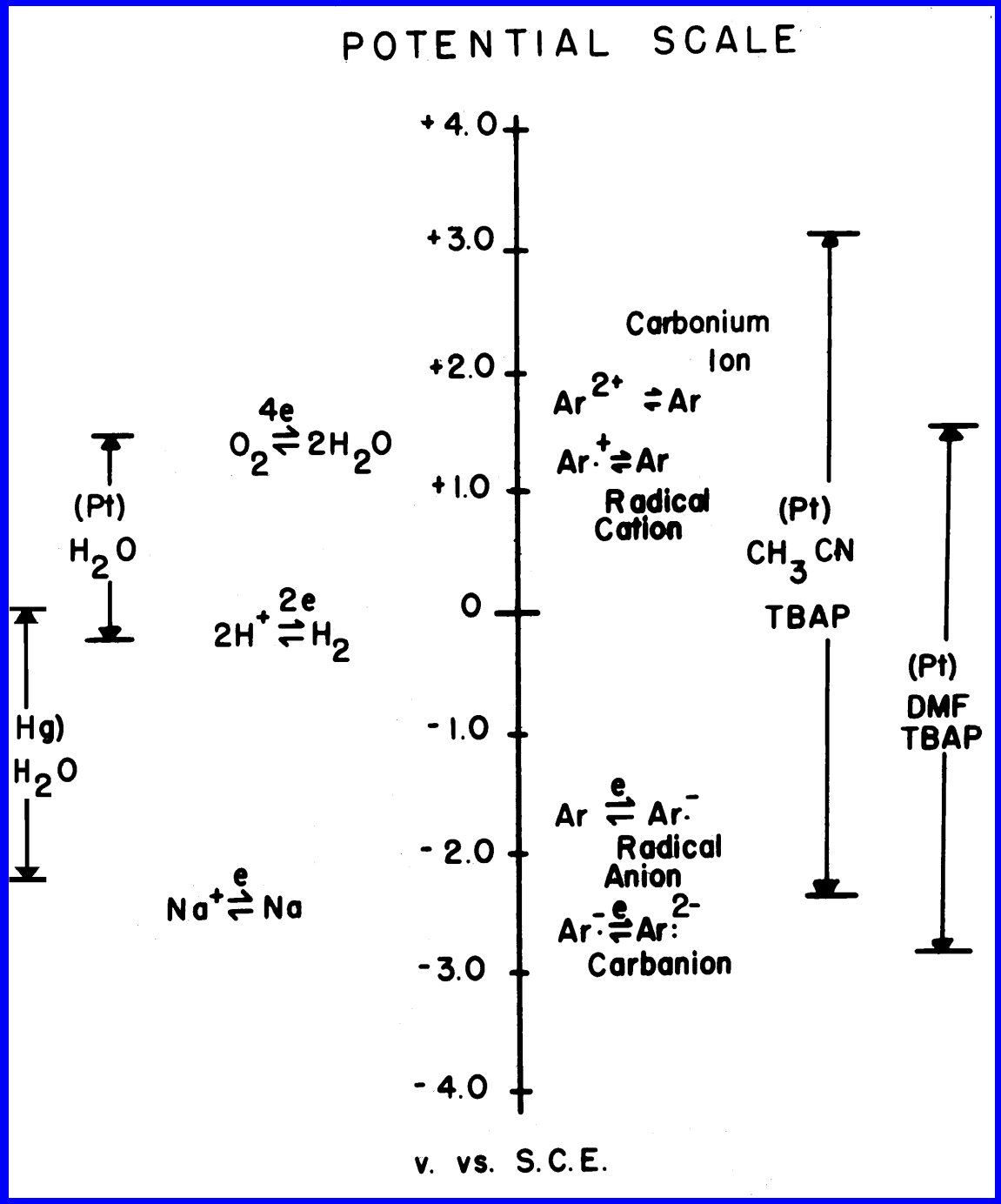


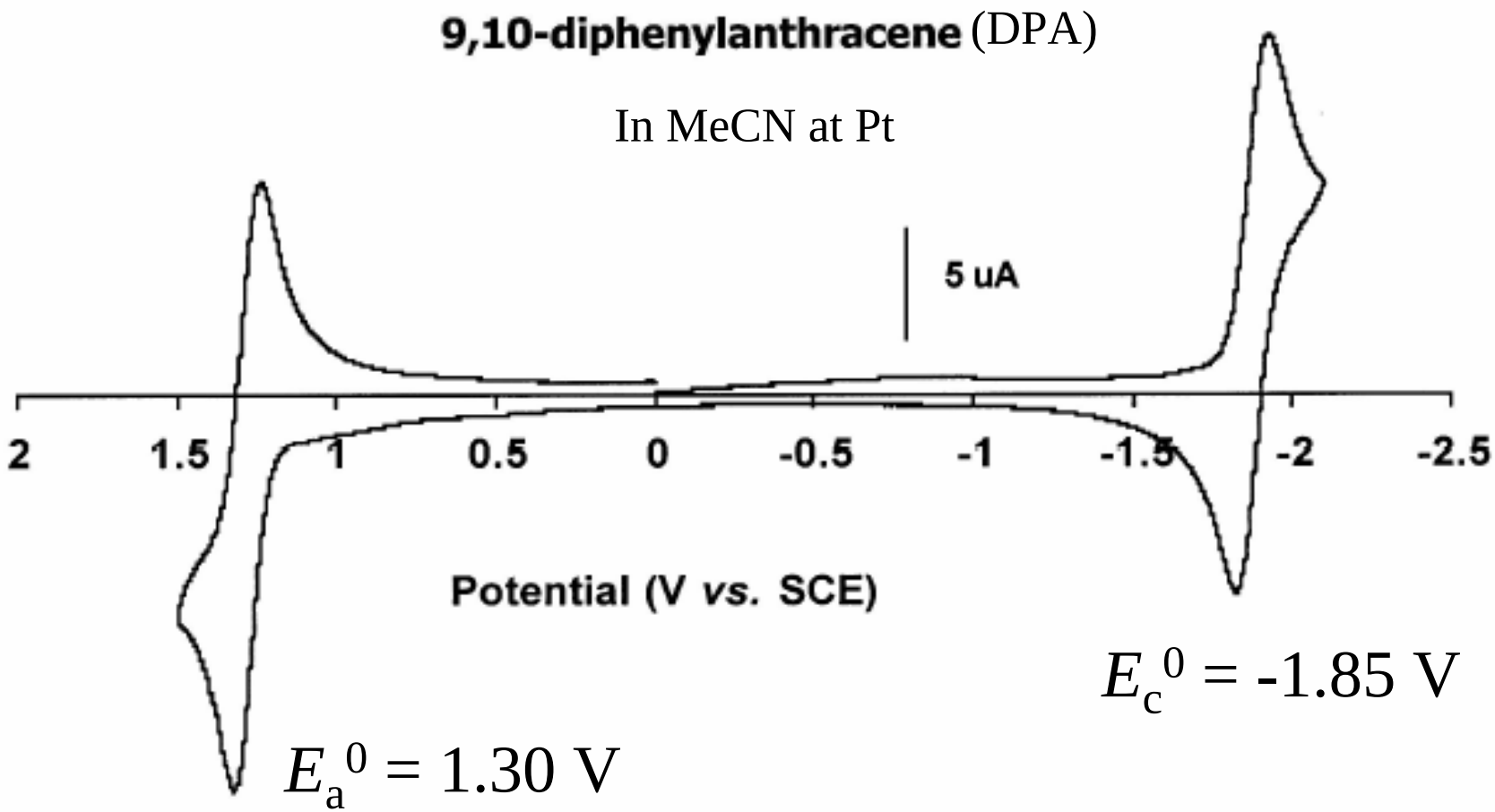

ECL
Cathode
Anode
-
+
A
D
e
e
A-
e
D+
A*
D
hn

ECL



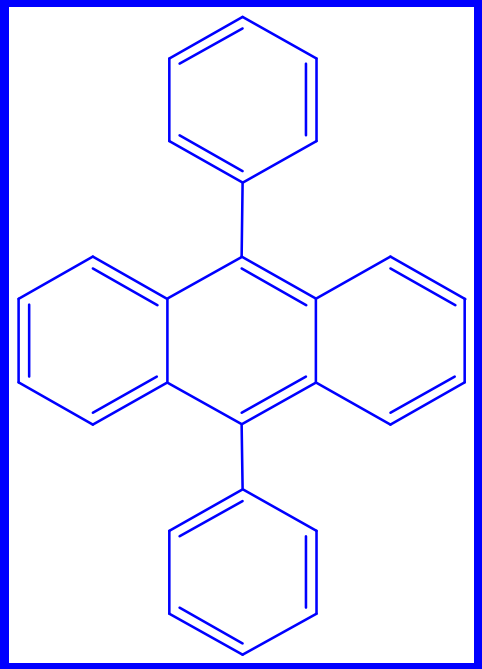
Criterion for excited state formation → $E_a^o - E_c^o - 0.1 > E_S$ ($E_s \sim 2$ to 3 eV)


POTENTIAL SCALE
+4.0
+3.0
+2.0
+1.0
0
-1.0
-2.0
-3.0
-4.0
v. vs. S.C.E.
Carbonium Ion
$Ar^{2+} \rightleftharpoons Ar$
$Ar\cdot^{+} \rightleftharpoons Ar$
Radical Cation
$Ar \overset{e}{\rightleftharpoons} Ar\cdot^{-}$
Radical Anion
$Ar\cdot^{-} \overset{e}{\rightleftharpoons} Ar:^{2-}$
Carbanion
$O_2 \overset{4e}{\rightleftharpoons} 2H_2O$
$2H^{+} \overset{2e}{\rightleftharpoons} H_2$
$Na^{+} \overset{e}{\rightleftharpoons} Na$
(Pt)
H_2O
Hg)
H_2O
(Pt)
CH_3CN
TBAP
(Pt)
DMF
TBAP



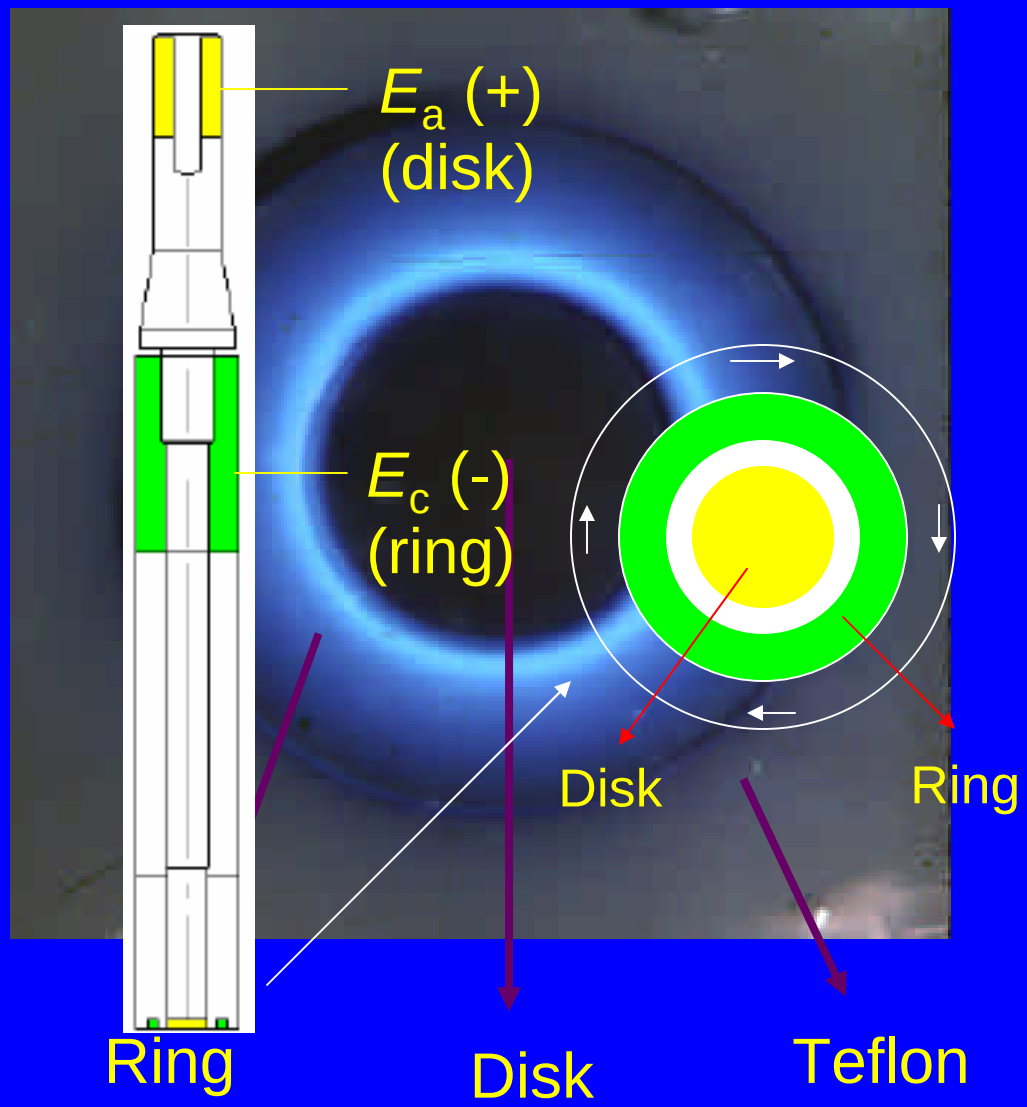
$E_a^0 - E_c^0$ - 0.1 = 1.30 – (-1.85) – 0.1 = 3.05 eV > E_s (3.0 eV)

9,10-diphenylanthracence in MeCN at Rotating Ring Disk Electrode



(DPA)

E_s = 3.0 eV
l ~ 410 nm

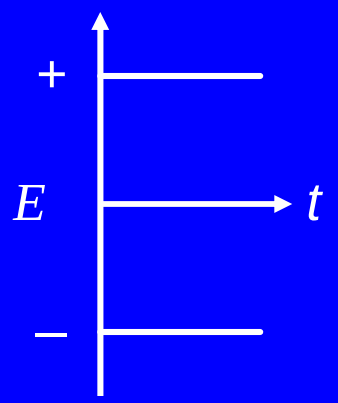


Two Types of ECL

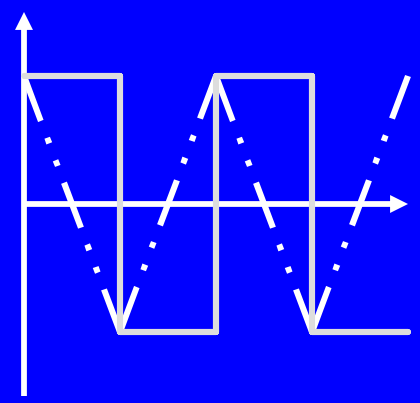
1. Annihilation ECL (A = D = DPA)

$$D^+ + A^- \longrightarrow A + D^*$$

$$D^* \rightarrow D + h\nu$$

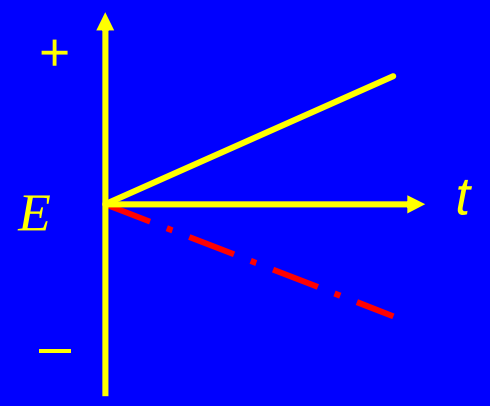


(Two Electrodes)



(One Electrode with an AC Potential)

Two Types of ECL



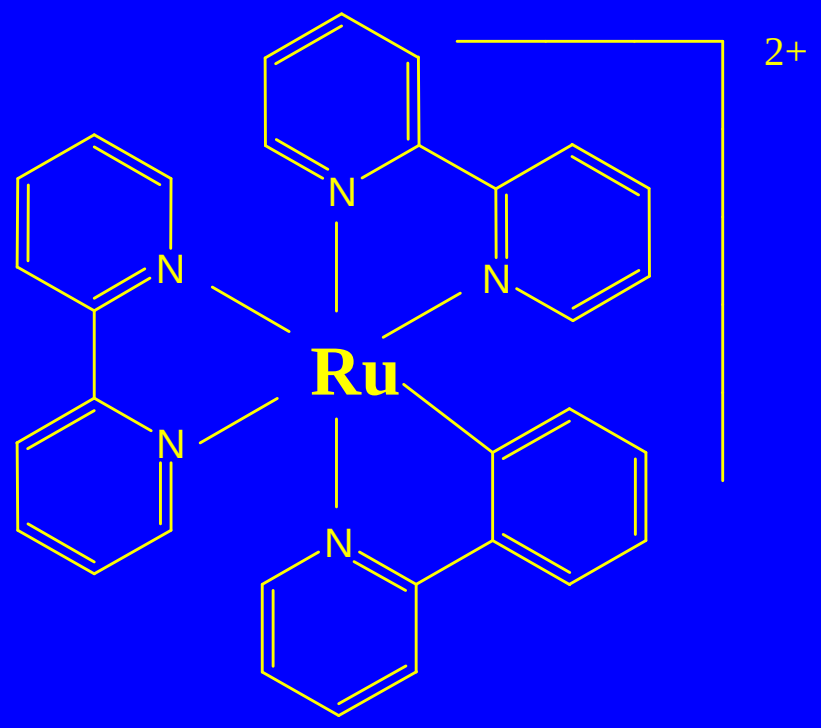
2. Coreactant ECL
(D + Coreactant)

$$D^{+} + Int \longrightarrow D^{*} + Product$$

$$D^{*} \longrightarrow D + h\nu$$

(Where Int is formed on Oxidation)

Coreactant ECL



$Ru(bpy)_3^{2+}$

$(CH_3CH_2CH_2)_3N$:

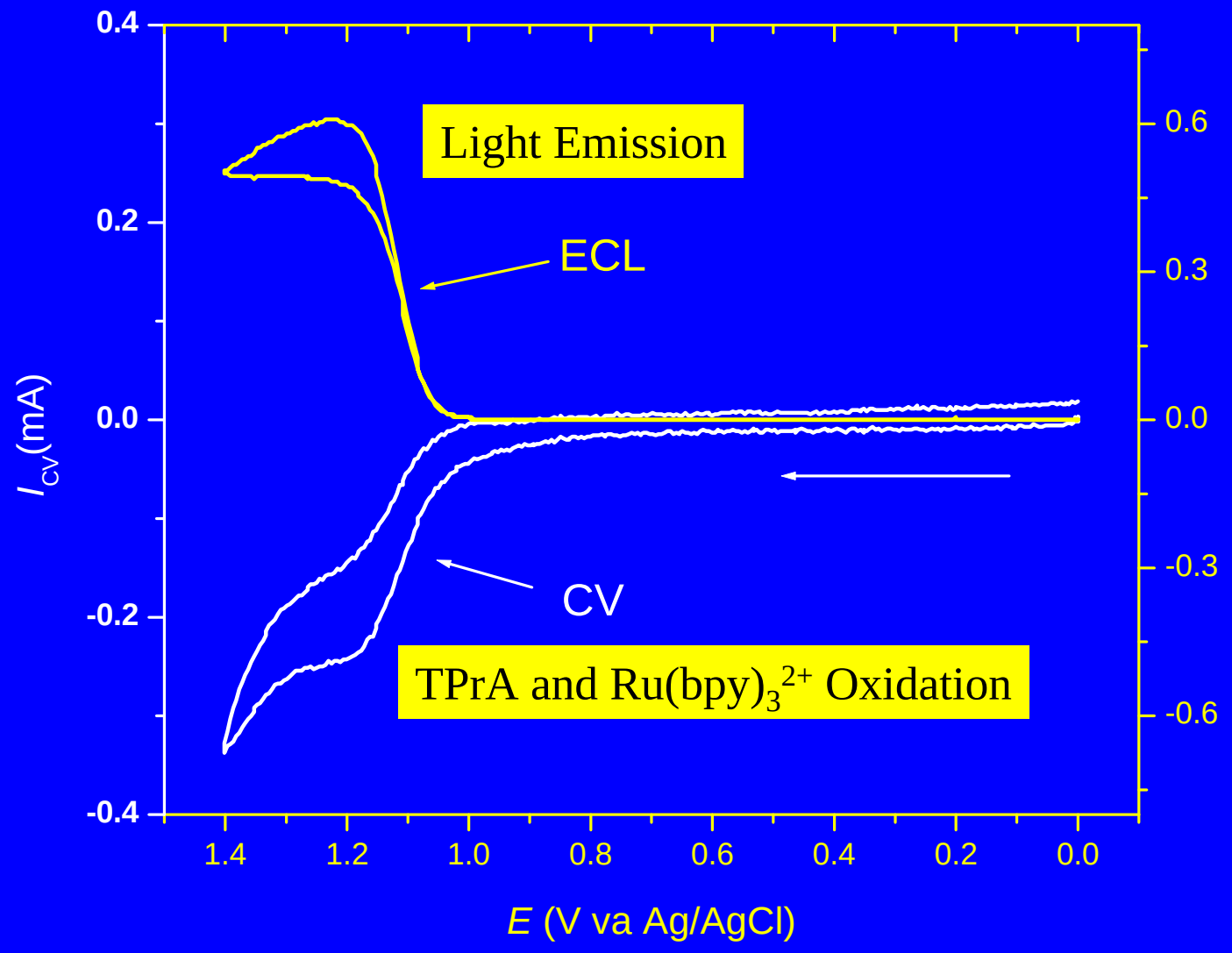
(***T***ri***Pr***opyl ***A***mine)

TPrA

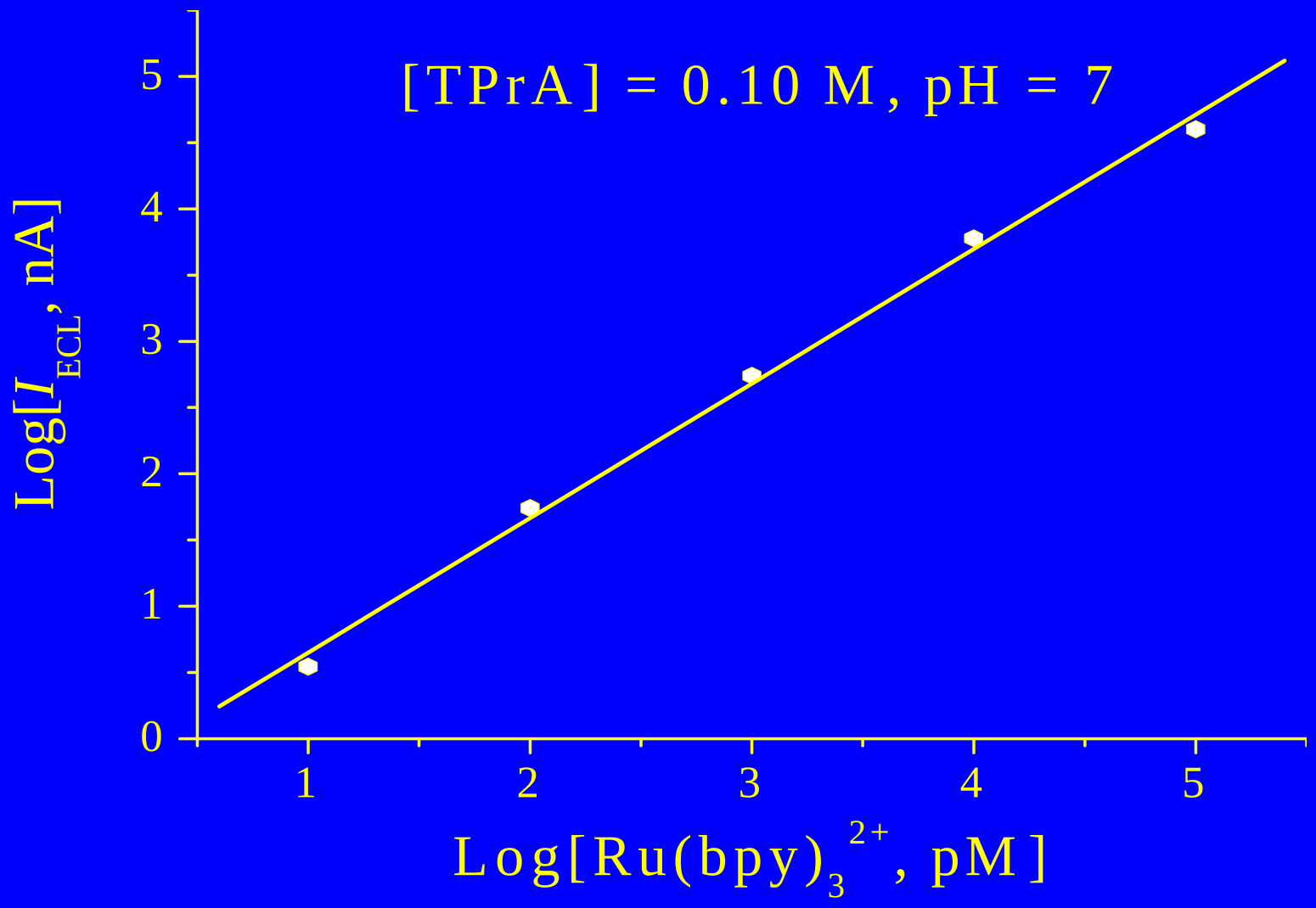
(As coreactant)

This System Gives the Most Efficient ECL Known So Far.

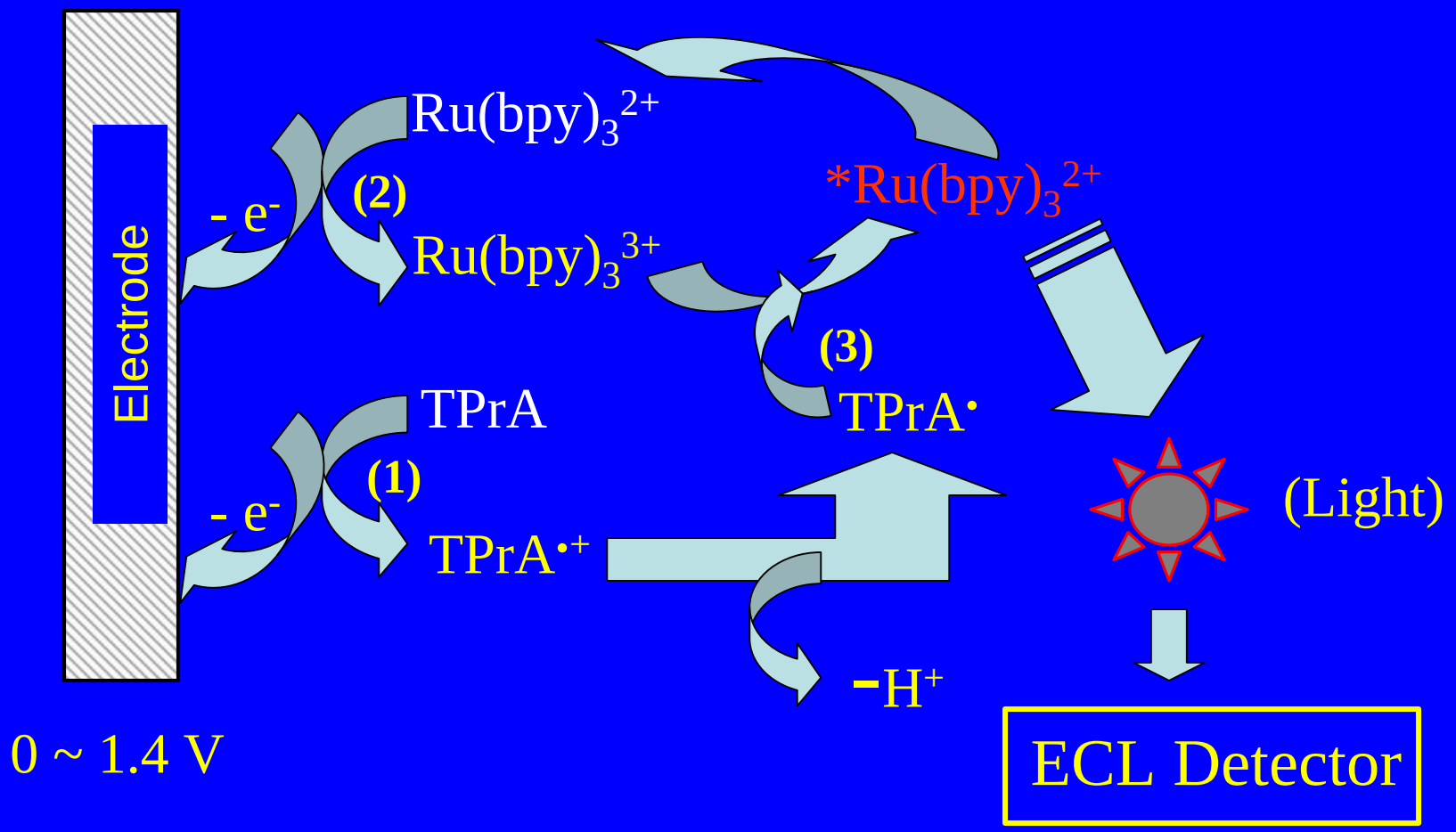
I_{ECL} & I_{CV} ~ E Profiles for $Ru(bpy)_3^{2+}$(1 mM) /TPrA (10 mM) System



$[Ru(bpy)_3^{2+}]$ 8 I_{ECL}



ECL Mechanism



0 ~ 1.4 V

TPrA = Tripropylamine

*$Ru(bpy)_3^{2+}$ = Excited $Ru(bpy)_3^{2+}$

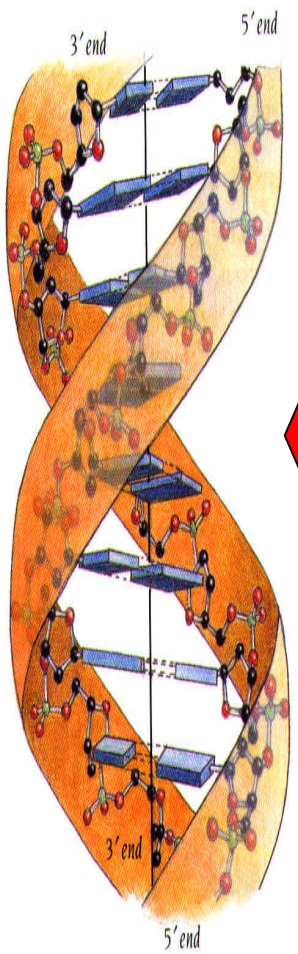
(λ_{max} ~ 620 nm)

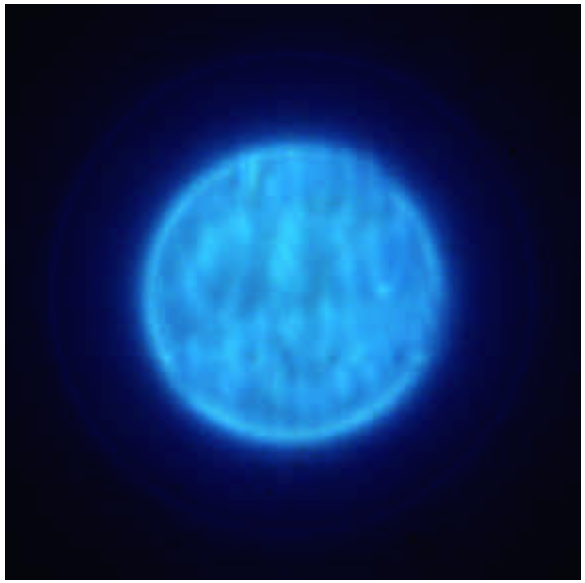
W. Miao, J-P, Choi, A.J. Bard, *J. Am. Chem. Soc.*, **124** (48), 14478 -14485, 2002.

ECL Analysis Advantages

- No Light Source Required
- No Scattered Light
- No Interference From Luminescent Impurities
- High Sensitivity, Selectivity and Stability
- Reactions Are Localized and Controllable
  --Good Spatial and Temporal Resolution

ECL Applications



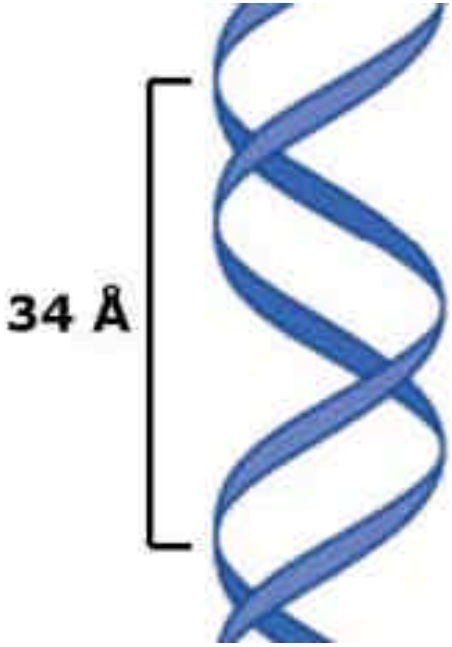


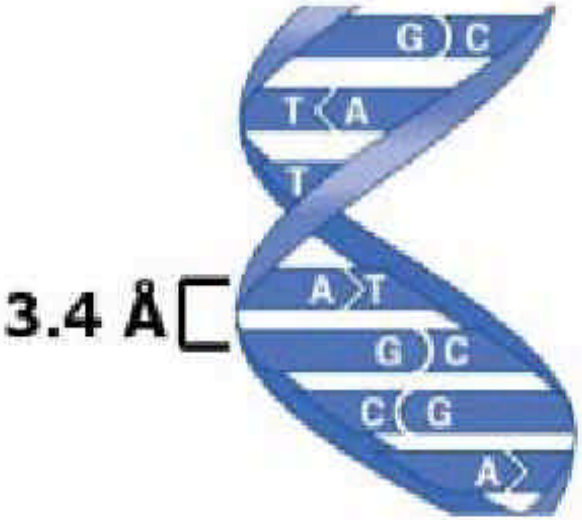
DNA

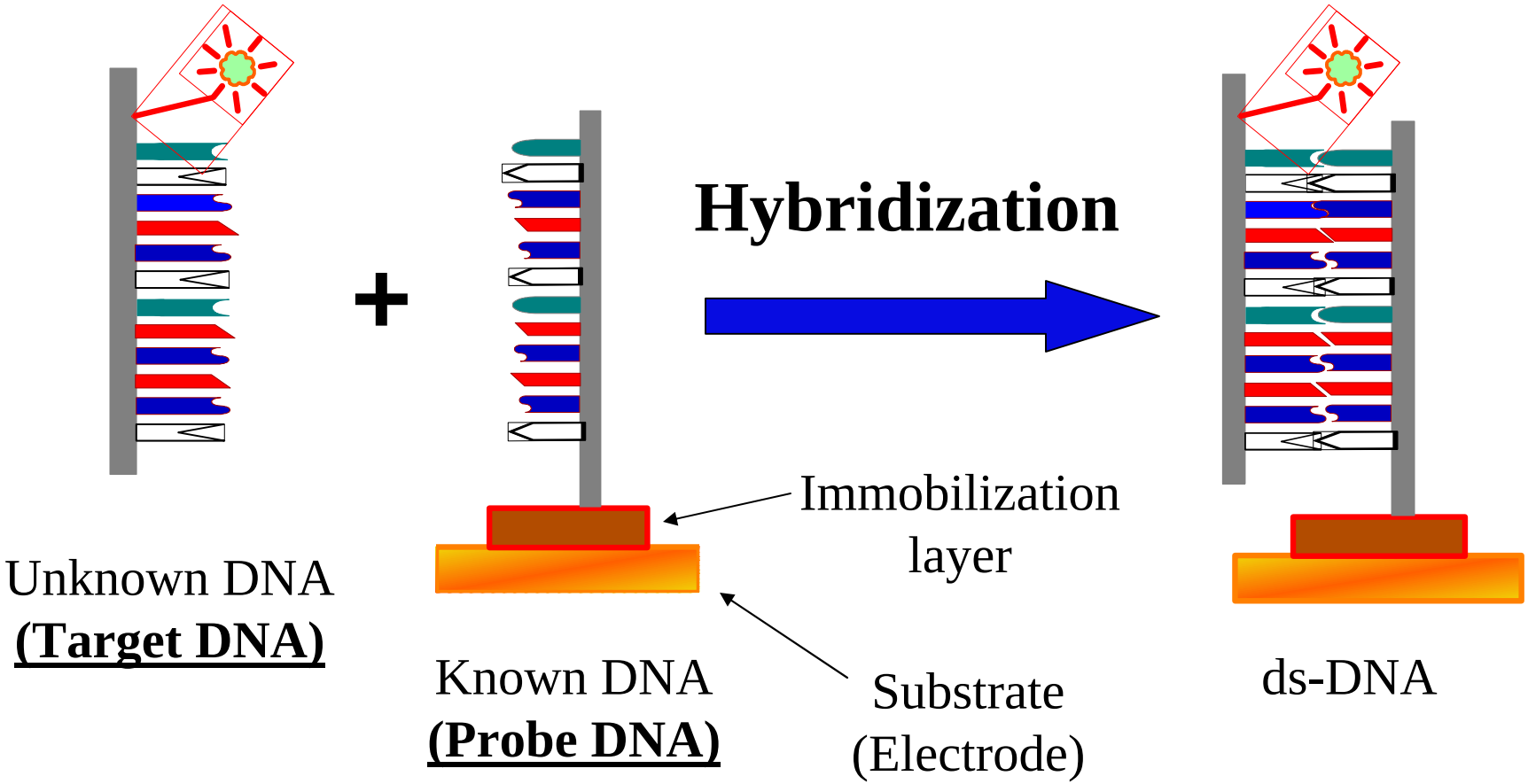
ECL

Antibody-Antigen

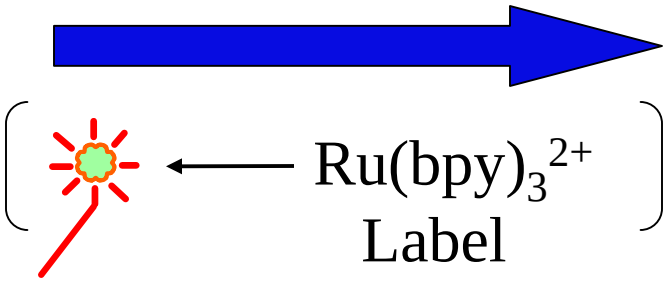
DNA
Double Helical Structure





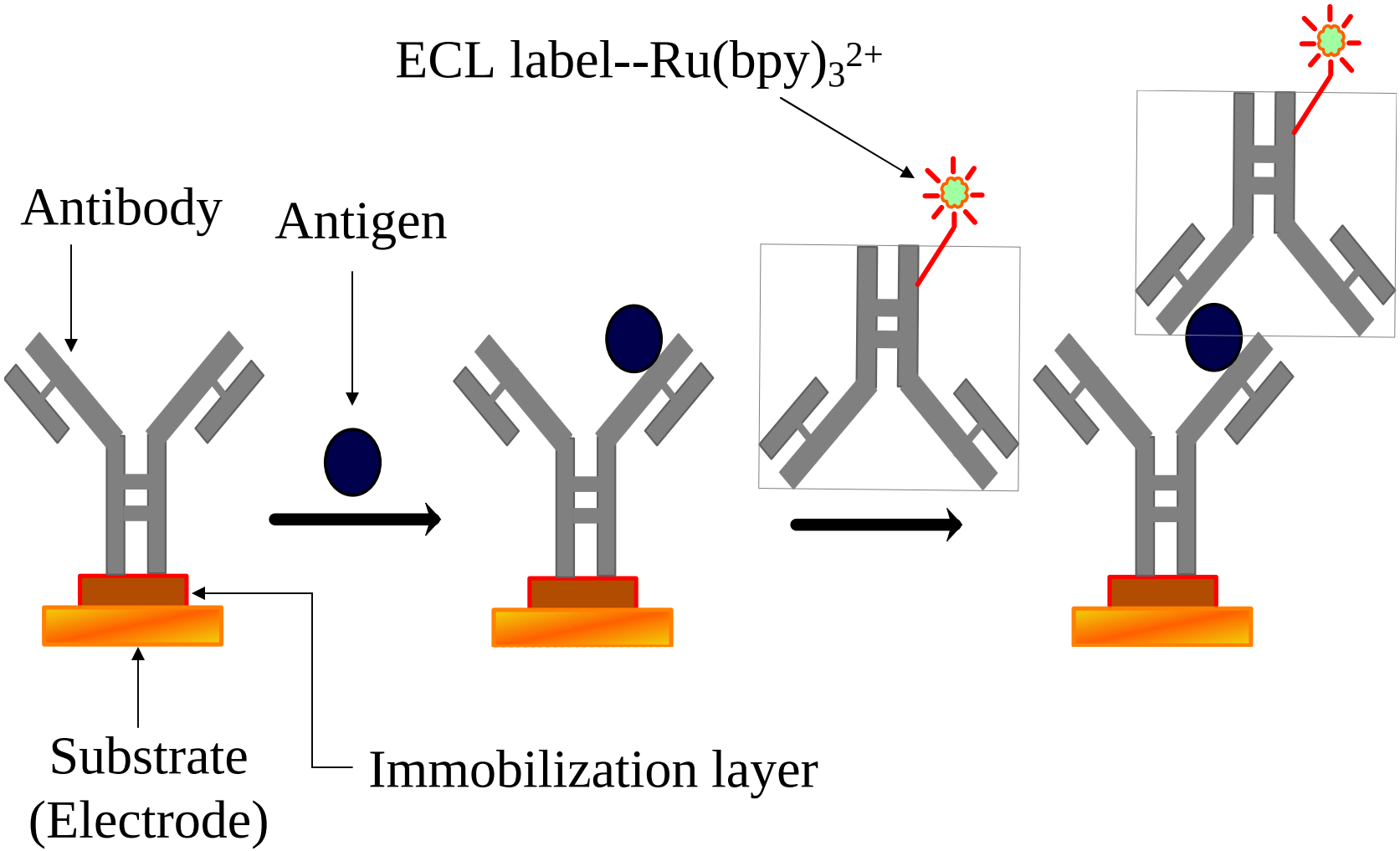


ECL Detection

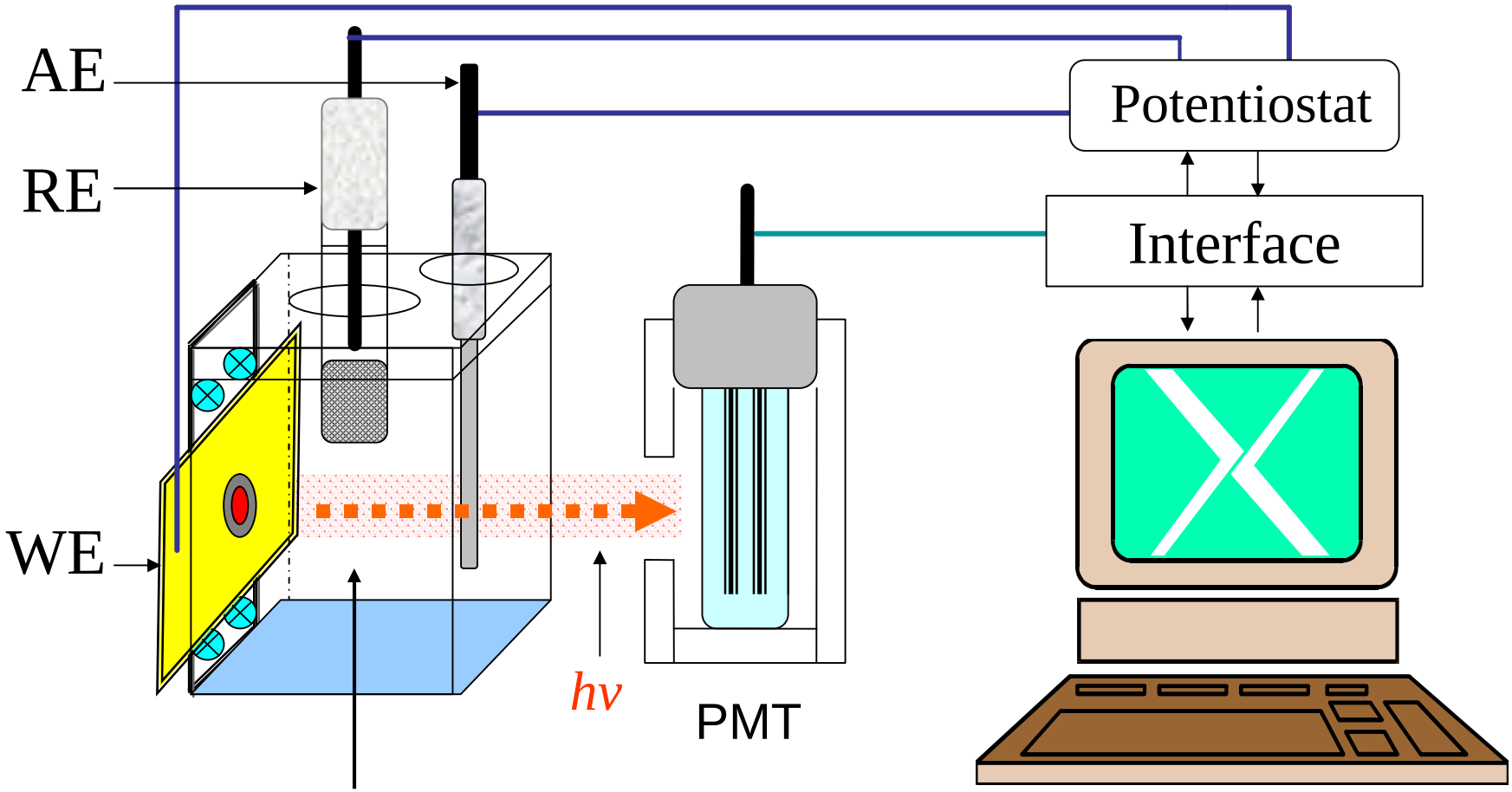


- Large I_{ECL} → Complementary
- Small I_{ECL} → Base-pair mismatched
- $I_{ECL} \sim 0$ → Non-complementary

ECL Detection with Sandwich Type Immunoassay



ECL Experimental Set-up

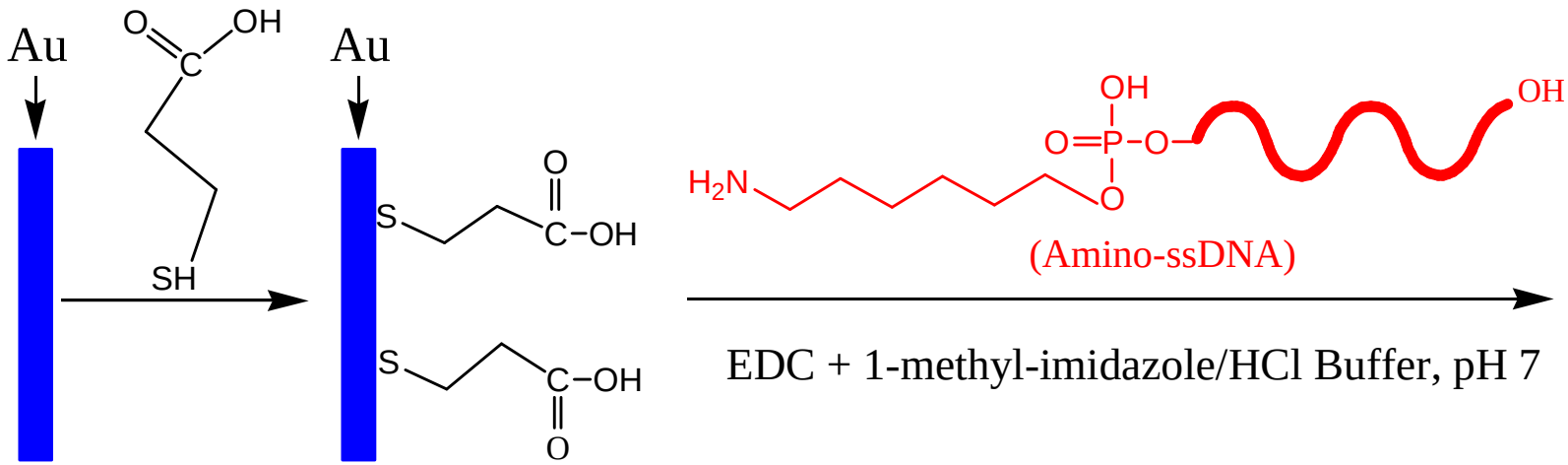


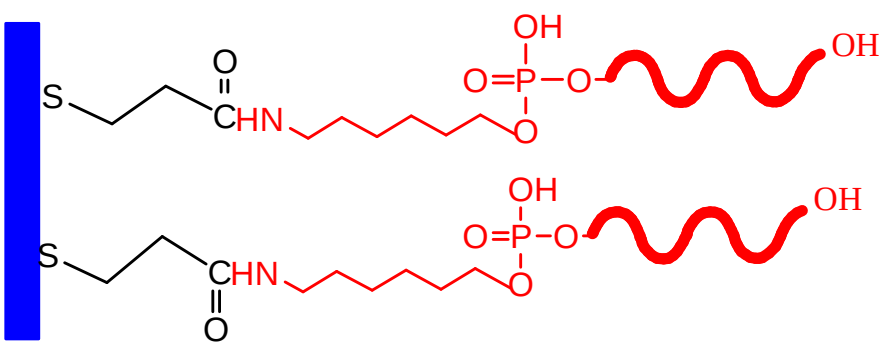
Electrolyte Solution

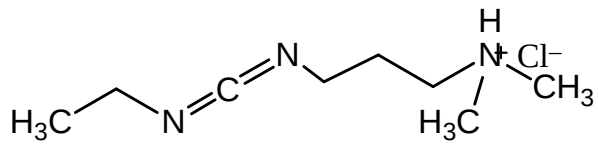
0.10 M TPrA/$LiClO_4$/Tris, pH 8

→ Coreactant--Tripropylamine

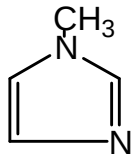
Immobilization of ssDNA onto Electrode





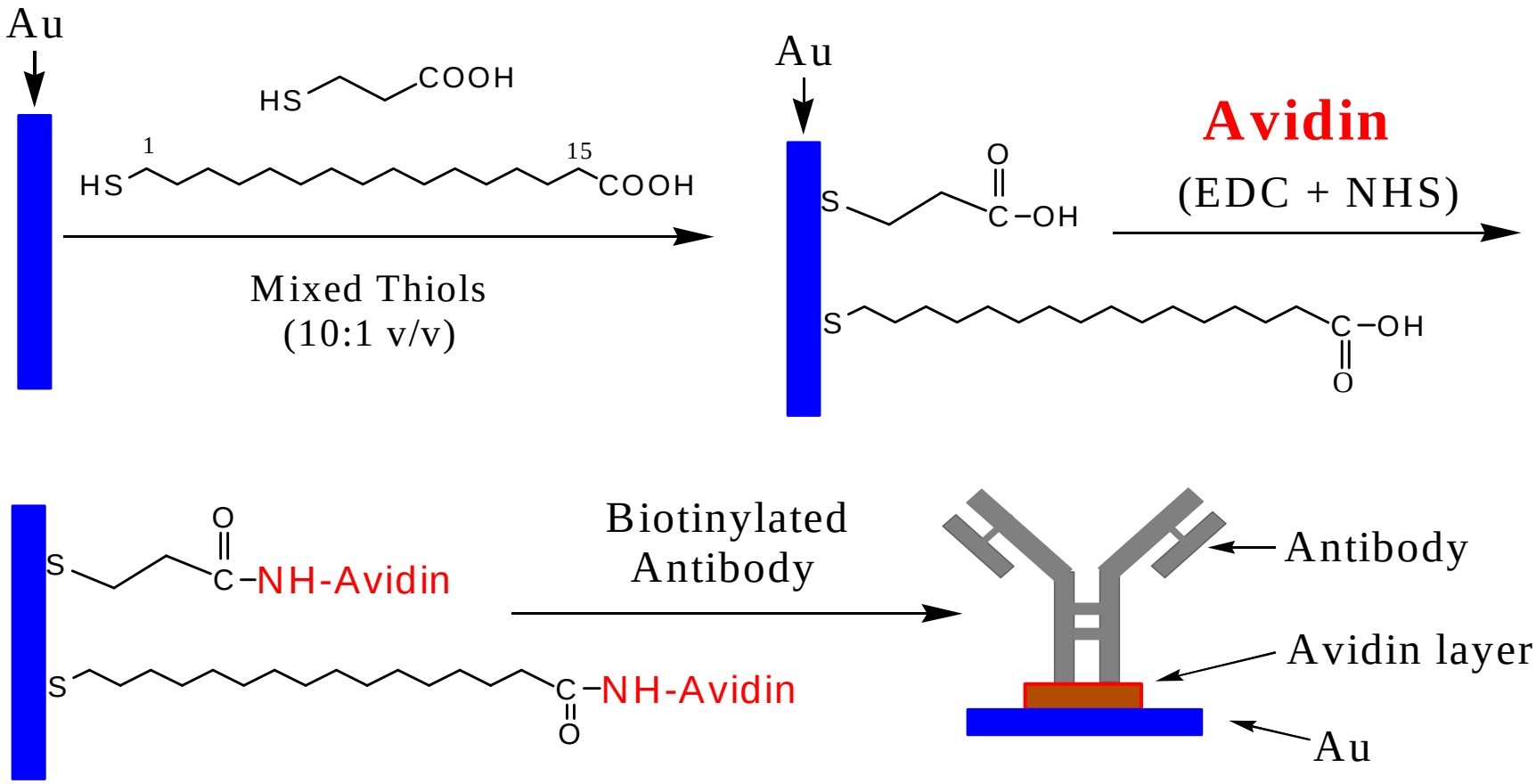


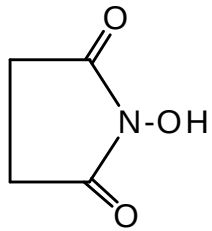
EDC/EDAC
1-Ethyl-3-(3-dimethylaminopropyl)
Carbodimide Hydrochloride



1-methyl-imidazole

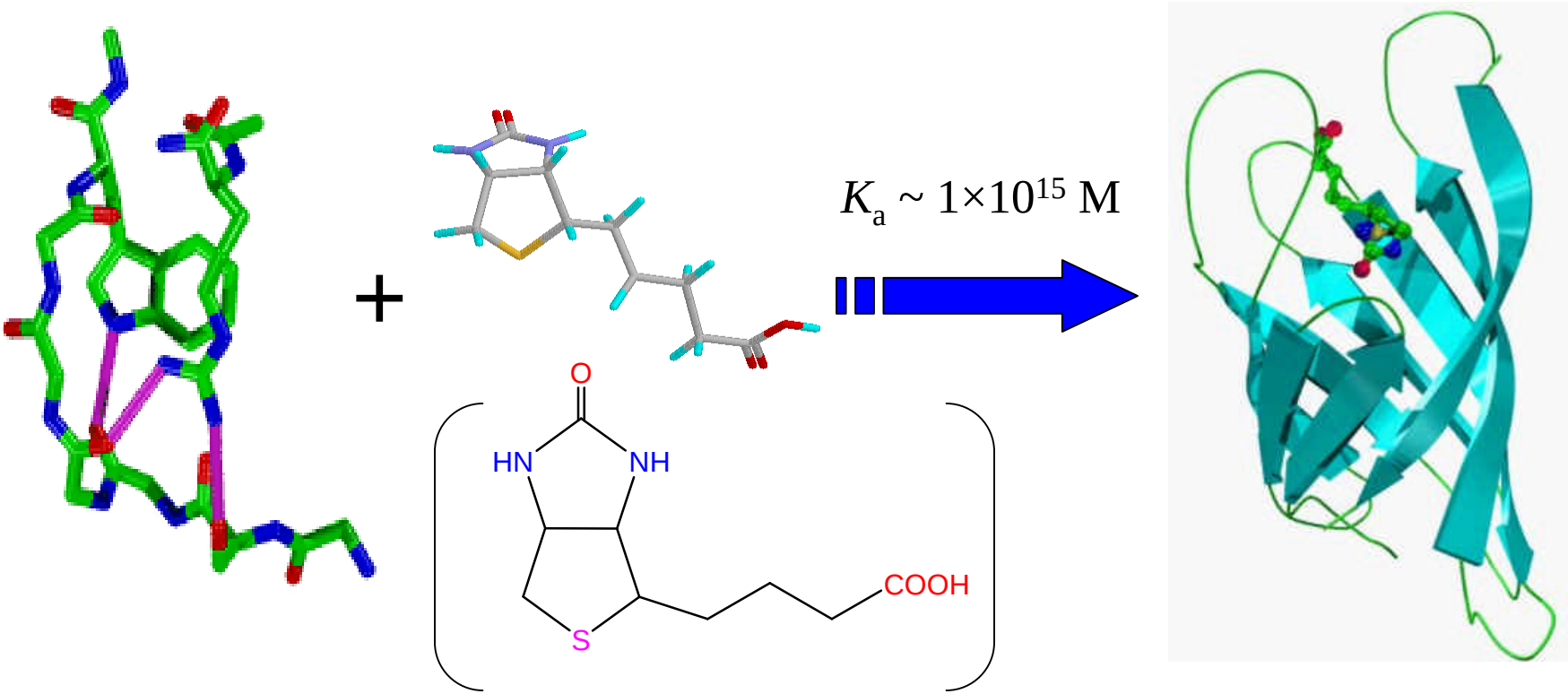
Immobilization of Antibody onto Electrode





NHS (N-Hydroxysuccinimide)

Avidin—Biotin Interaction (Non-Covalent Binding)



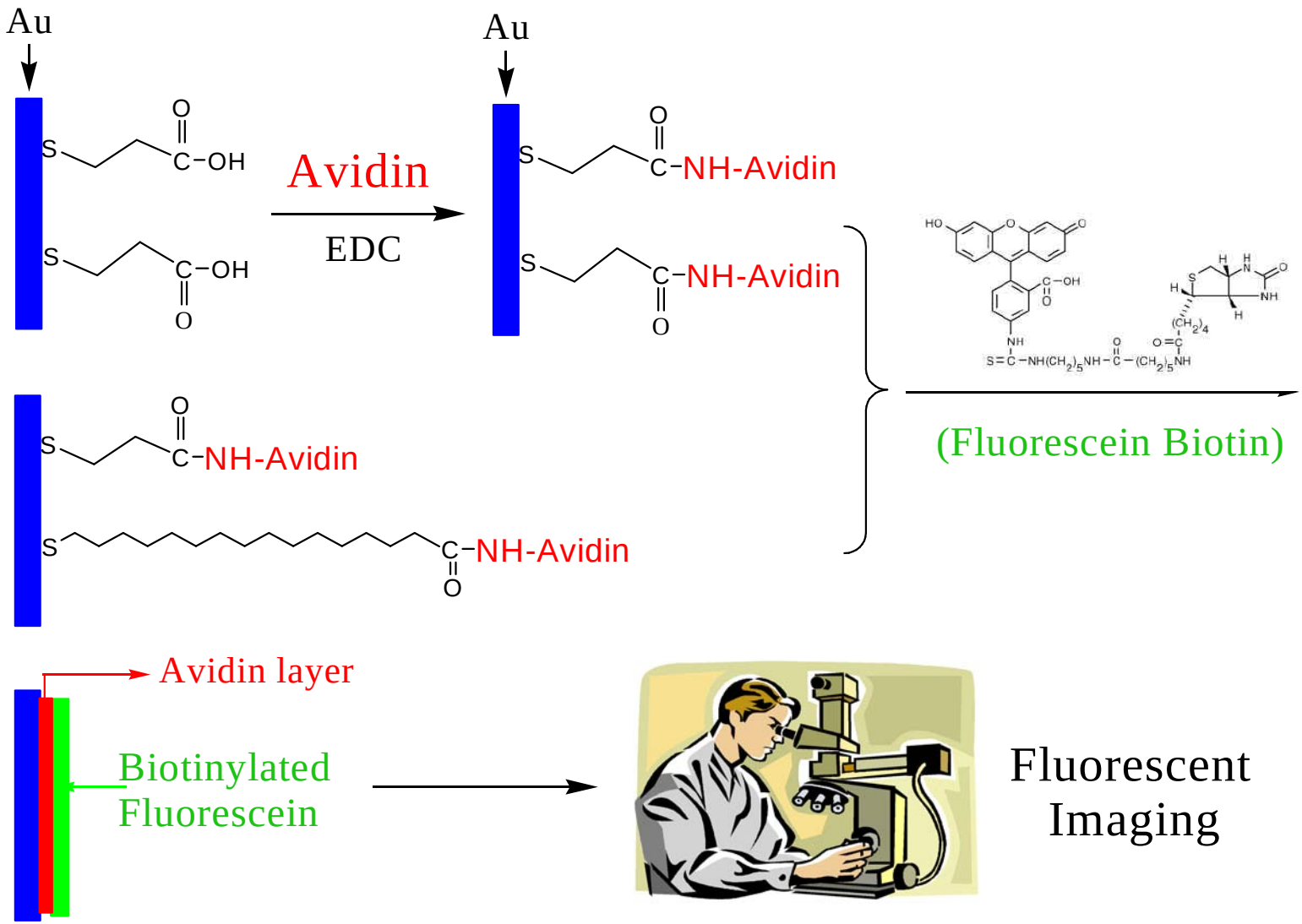
Avidin

Biotin

Avidin—Biotin Conjugate

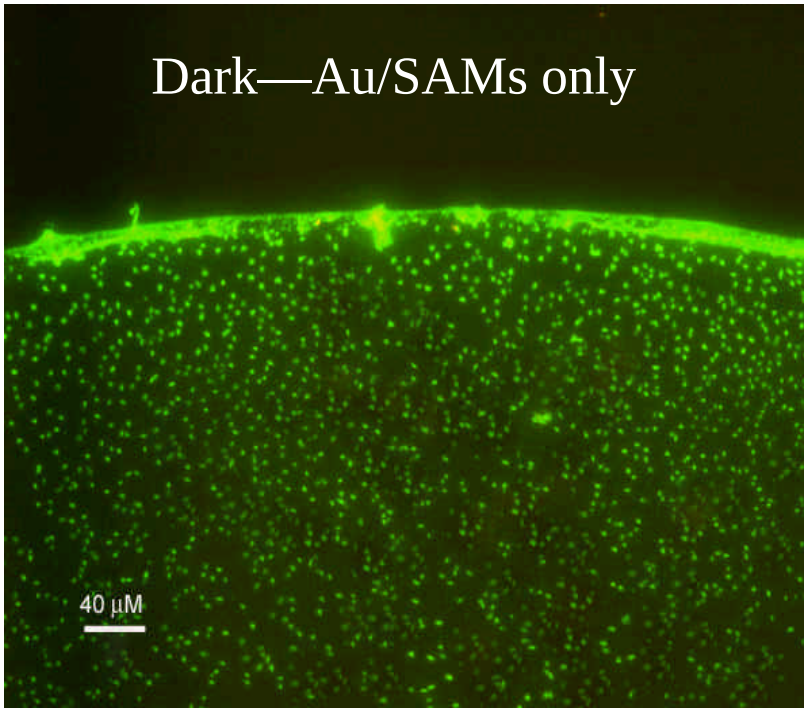
K_a-- Affinity Constant (Biological Reaction)

Surface Coverage Verification of SAMs and/or Avidin Layer on Electrode

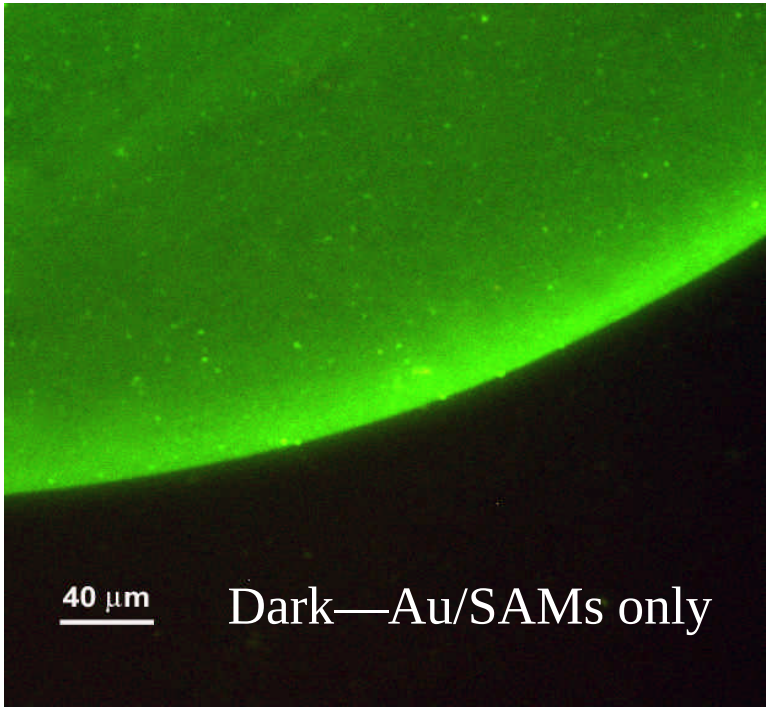


Fluorescent Images

(Excited at ~490 nm, Monitored at ~520 nm)

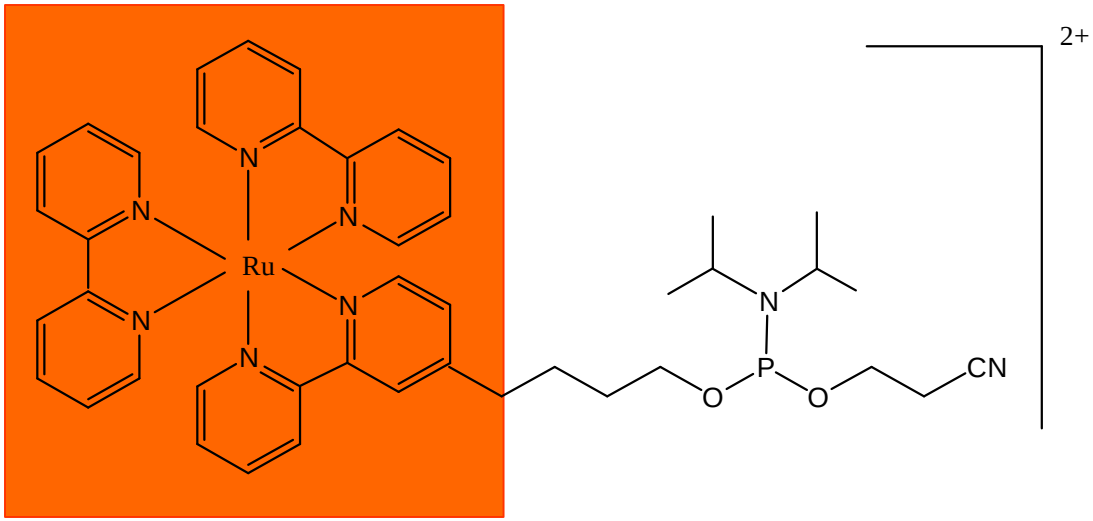


Au/S$(CH_2)_2$CO-NH-Avidin
←Fluorescein Biotin
(Exposure time: 3s)

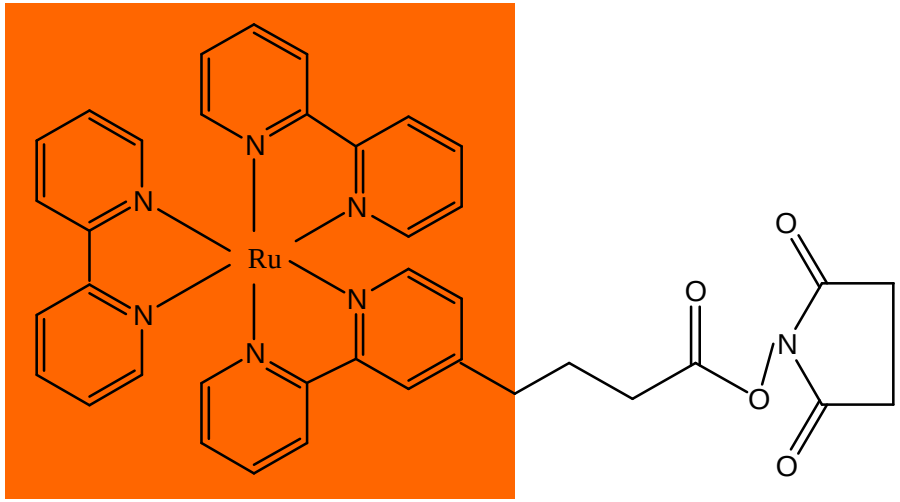


Au/S$(CH_2)_n$CO-NH-Avidin
←Fluorescein Biotin (n=2 & 15,
Exposure time: 30s)

ECL Tags Used for DNA and Antibody Labeling



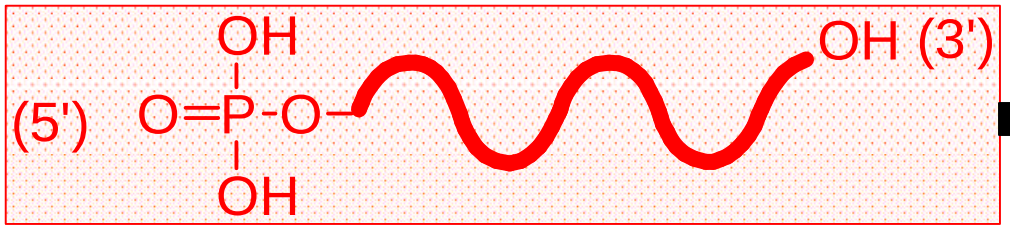
$Ru(bpy)_3^{2+}$
Phosphoramidite
for DNA Labeling



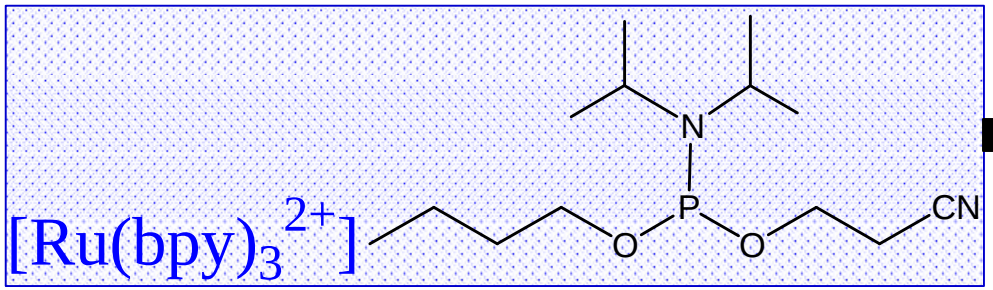
$Ru(bpy)_3^{2+}$ NHS Ester
for Antibody Labeling

Available from IGEN, Inc.

Modification of ssDNA with Ru-Tag



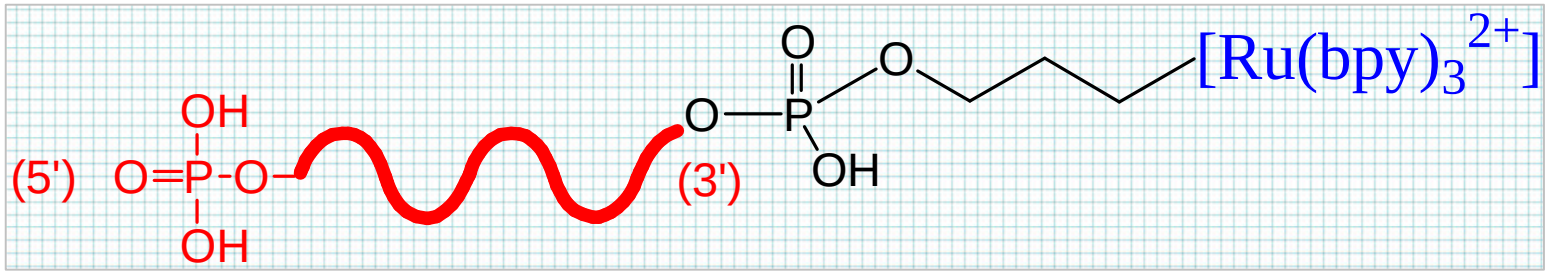
ssDNA



Ru-Phosphoramidite

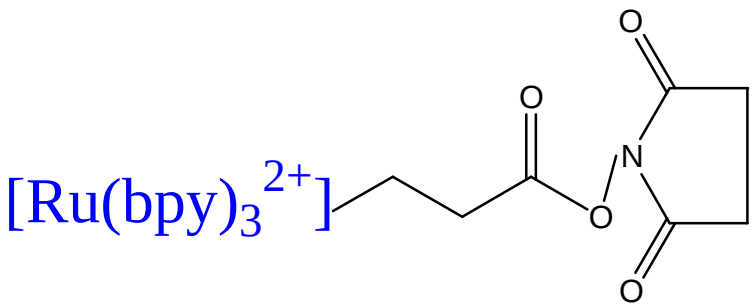
Exp. Conditions:

(1) Pure ssDNA
(2) Excess of Ru-Tag
(~100 times to DNA)
(3) In CH_3CN
(+ ~H_2O for a long DNA sequence)
(4) R.T., 30 ~ 60 min
with shaking in the dark
(5) Gel filtration/separation

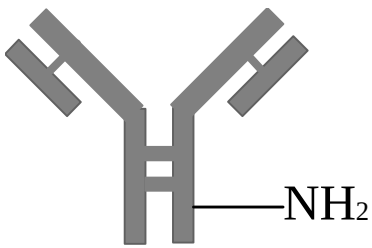


ssDNA tagged with $Ru(bpy)_3^{2+}$

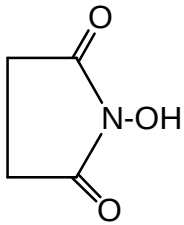
Modification of Antibody with Ru-Tag



Ru-NHS Ester

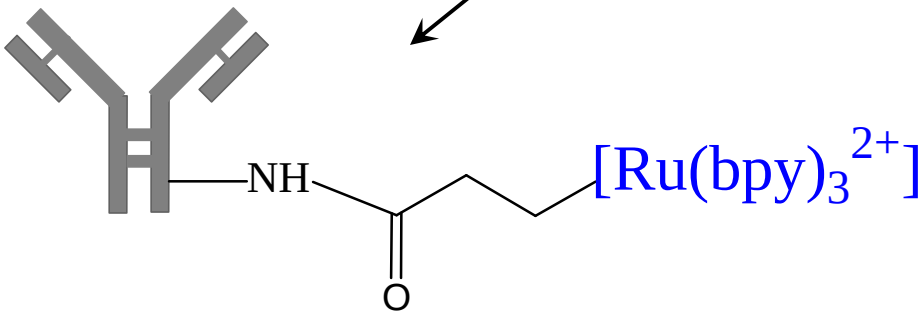


Antibody



NHS

(1) Ru-NHS pre-dissolved in DMSO
(2) In PBS buffer, pH ~7.2
(3) R.T. for ~ 1hr with shaking in the dark
(4) Gel filtration/separation

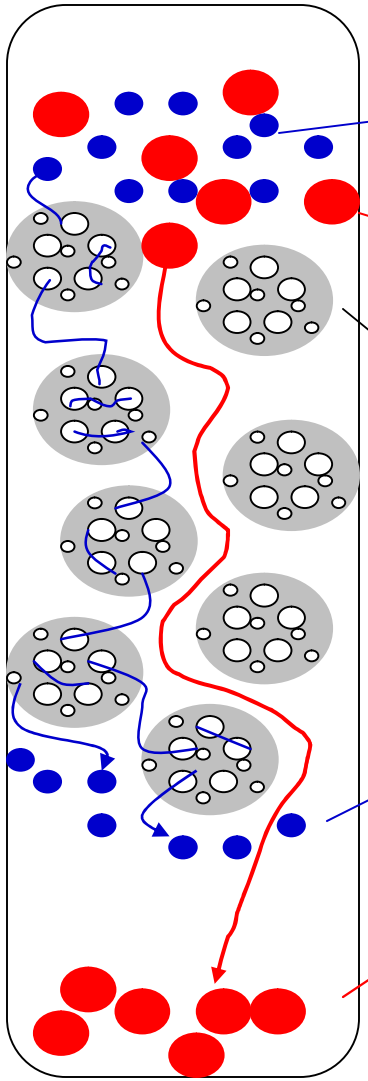


Antibody Tagged with $Ru(bpy)_3^{2+}$

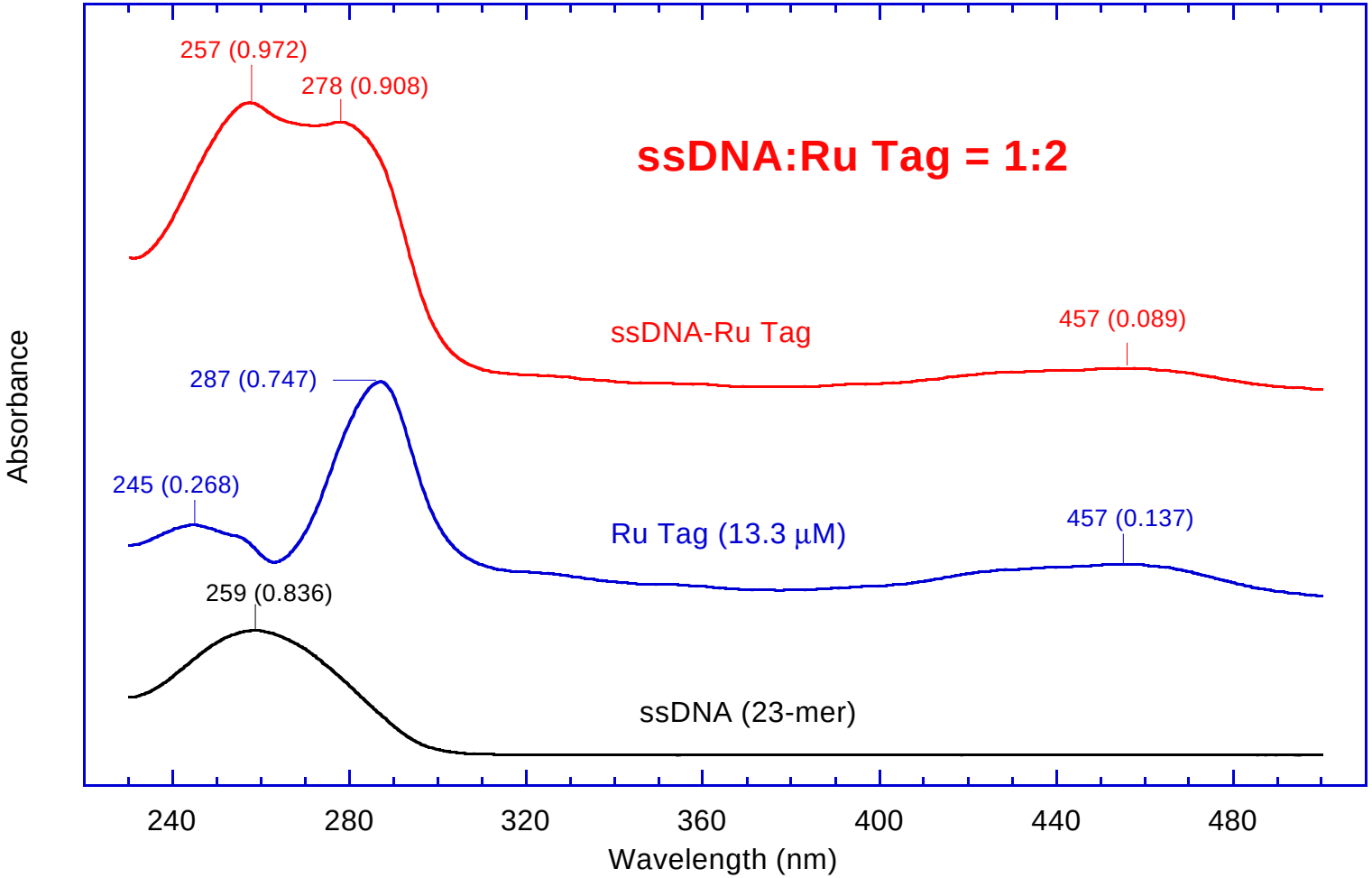
1. How to separate the newly formed complexes from the starting material $Ru(bpy)_3^{2+}$ tag?

2. What is the composition of the complexes?

Principle of Gel Filtration

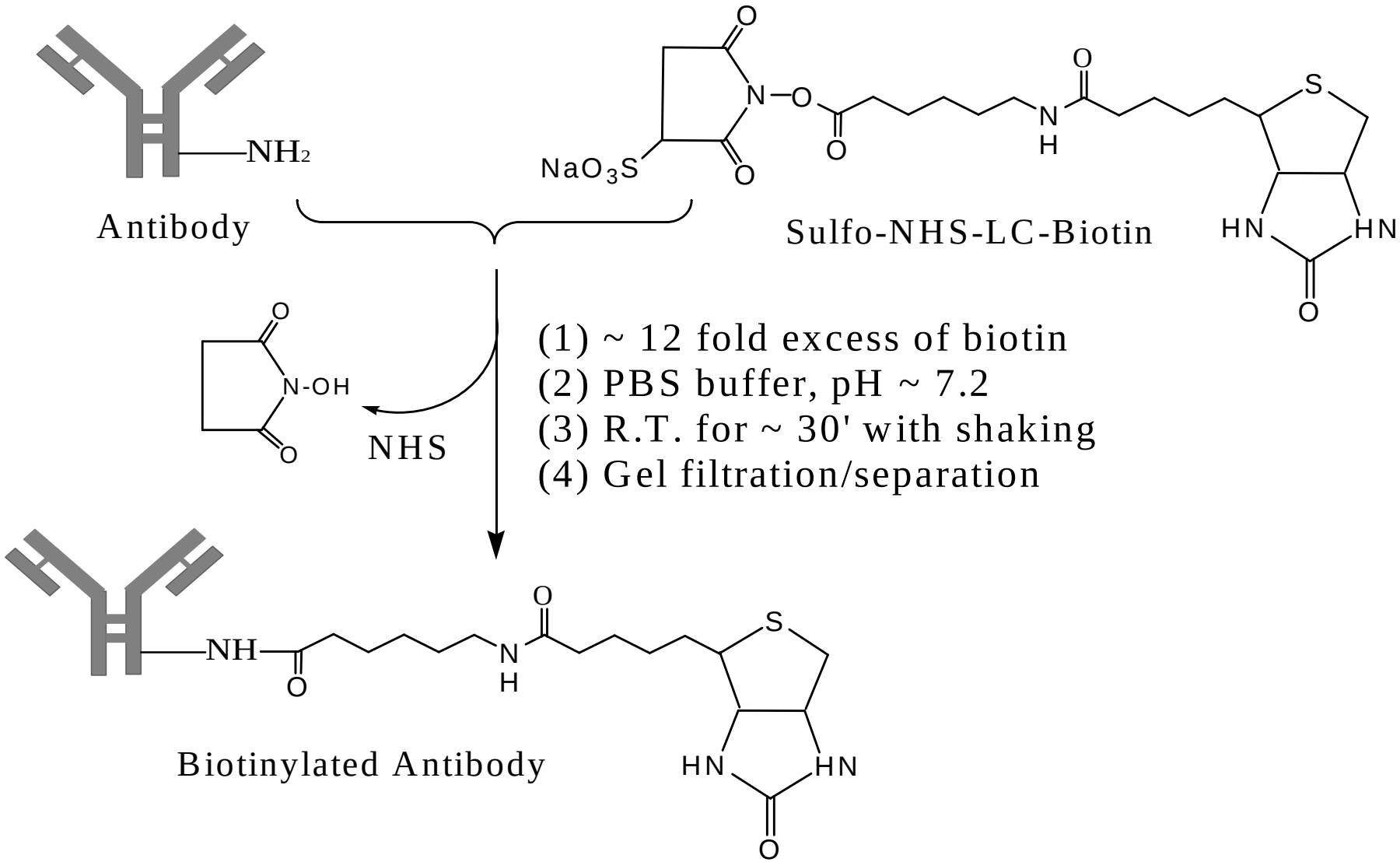


Verification of DNA-Ru Tag Formation

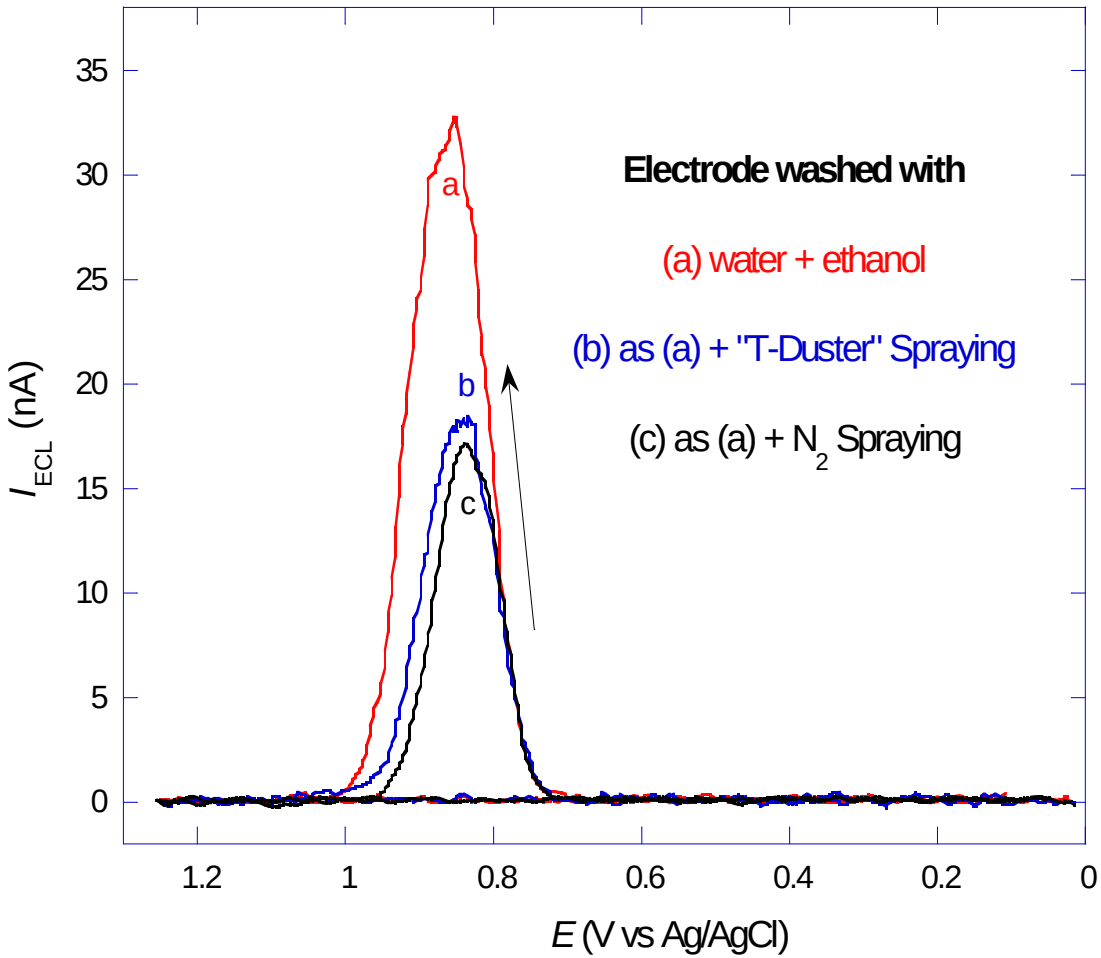


ssDNA: 5'-CTCCA AATGT AGGAG CTATC GTT-3' (23-mer)

Biotinylation of Antibody With Sulfo-NHS-LC-Biotin



Inert Gas Spraying Reduces Non-specific Adsorption of $Ru(bpy)_3^{2+}$



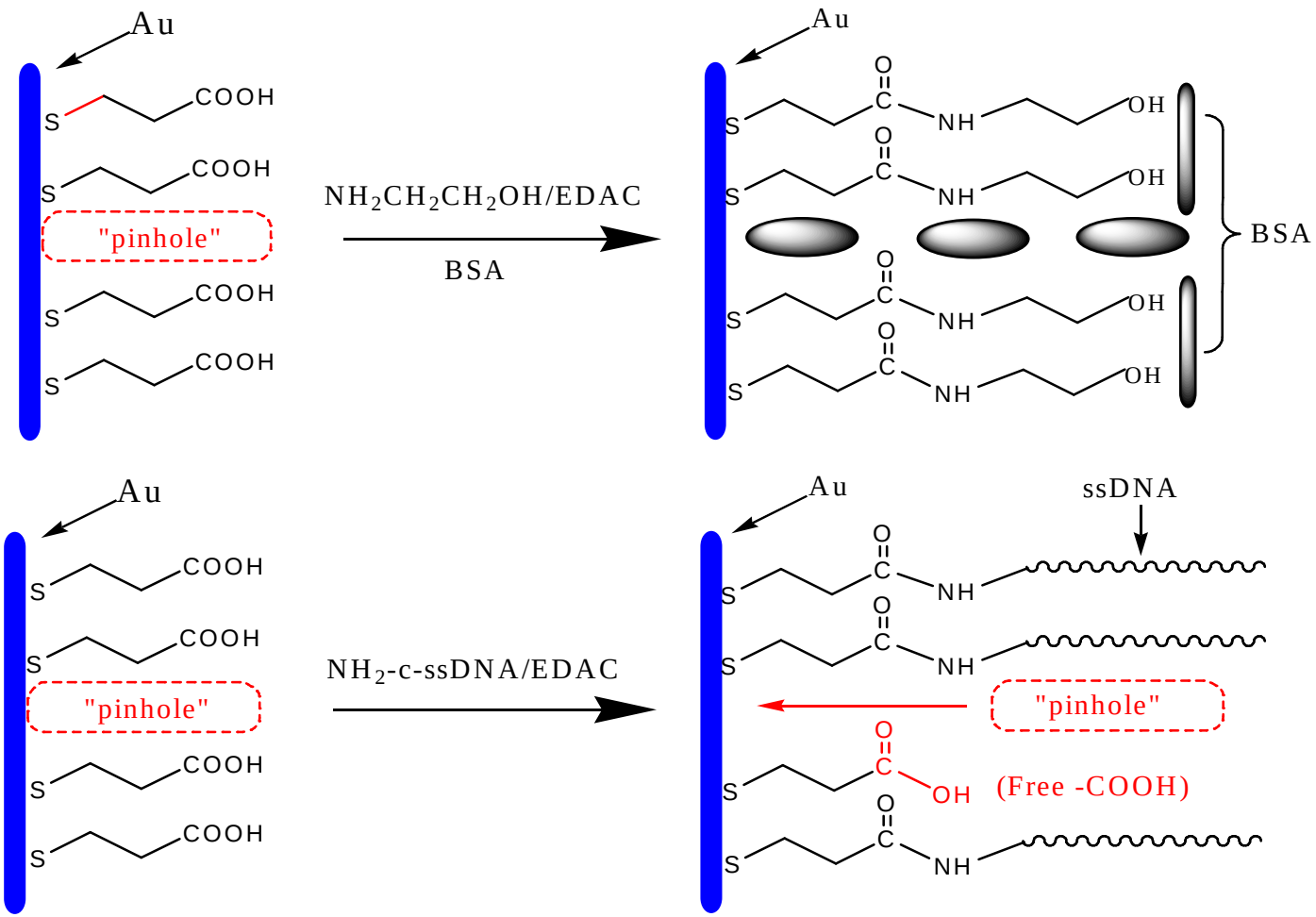
Au/Si pre-cleaned with UV (10')—MeOH (20')

Au/Si Immersed in 1 mM $Ru(bpy)_3^{2+}$ (60')

ECL carried out in 0.10 M TPrA/$LiClO_4$ /Tris buffer, pH 8

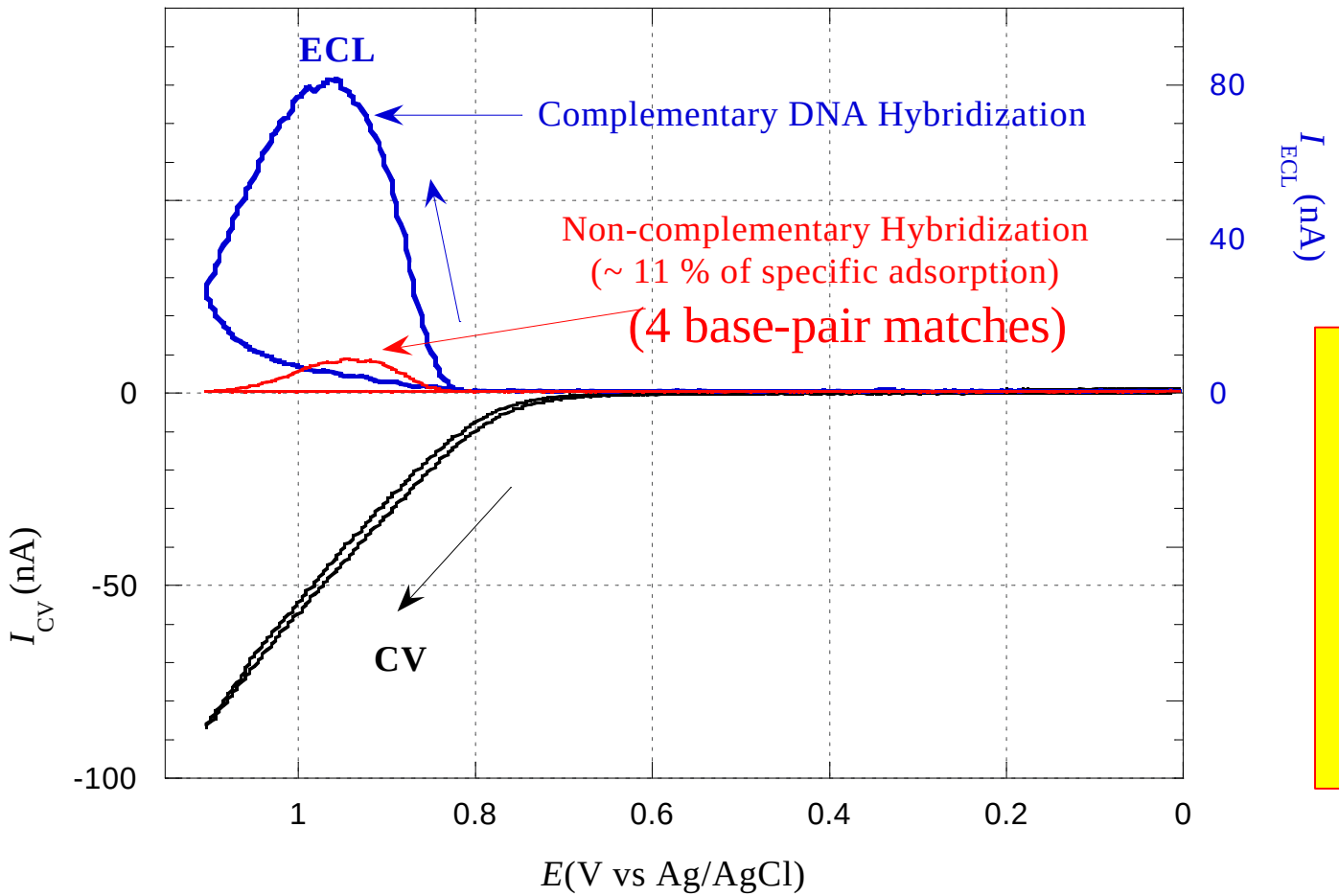
Scan rate: 50 mV/s

Strategies for the Reduction of ssDNA-Ru Tag Non-specific Adsorption



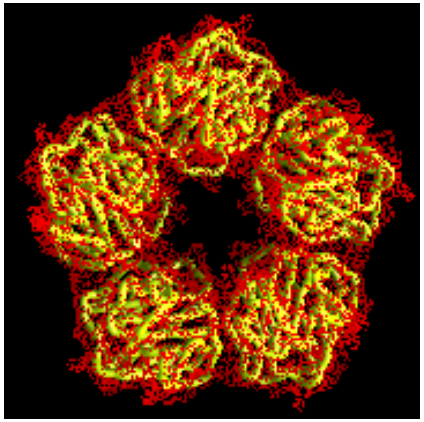
BSA: Bovine Serum Albumin

ECL Detection of DNA Hybridization



ssDNA derived from the *Bacillus anthracis*

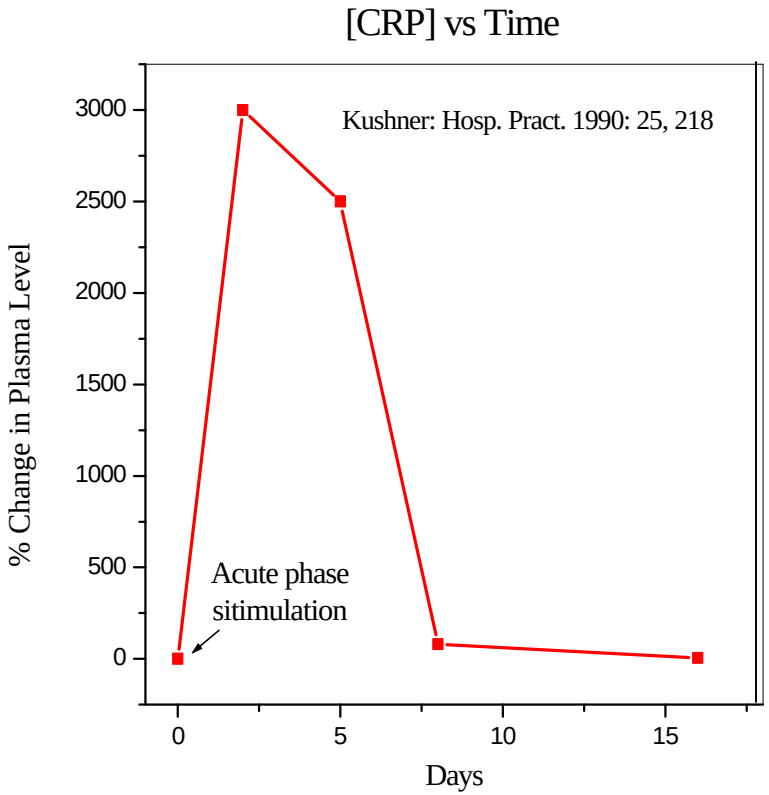
ssDNA-Ru Tag: Ru-5'-CTCCA AATGT AGGAG CTATC GTT-3'-Ru (23-mer)
c-ssDNA: 5'-Amino-AACGA TAGCT CCTAC ATTTG GAG-3' (23-mer)
Non-c-ss-DNA: 5'-Amino-TTAACACCTTAGCGACGGCTAGT-3' (23-mer)



*

C Reactive Protein (CRP)

- An acute phase reactant.
- Released by the body in response to acute injury, infection, inflammatory stimuli.
- Silent Predictor of Heart Disease.



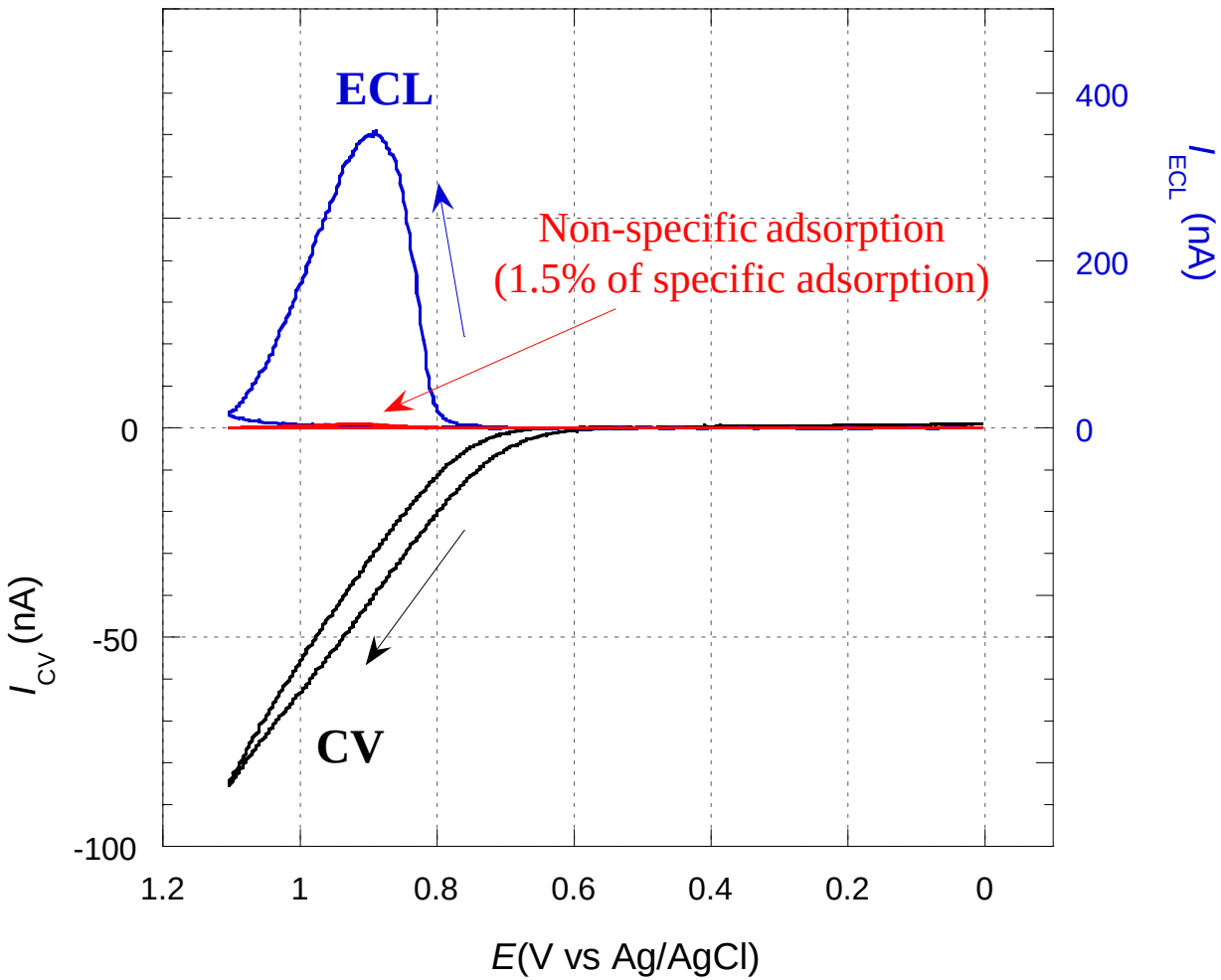
*CRP Structure, http://www.keele.ac.uk/depts/ph/research/px/home.html

Correlation between [CRP] and the risk of coronary heart disease

Quintile	CRP (mg/mL)	Risk of Coronary Heart Disease
1	< 0.7	Lowest Risk
2	0.7 - 1.1	Low risk
3	1.2 - 1.9	Moderate Risk
4	2.0 - 3.8	High Risk
5	3.9 - 15.0	Highest Risk

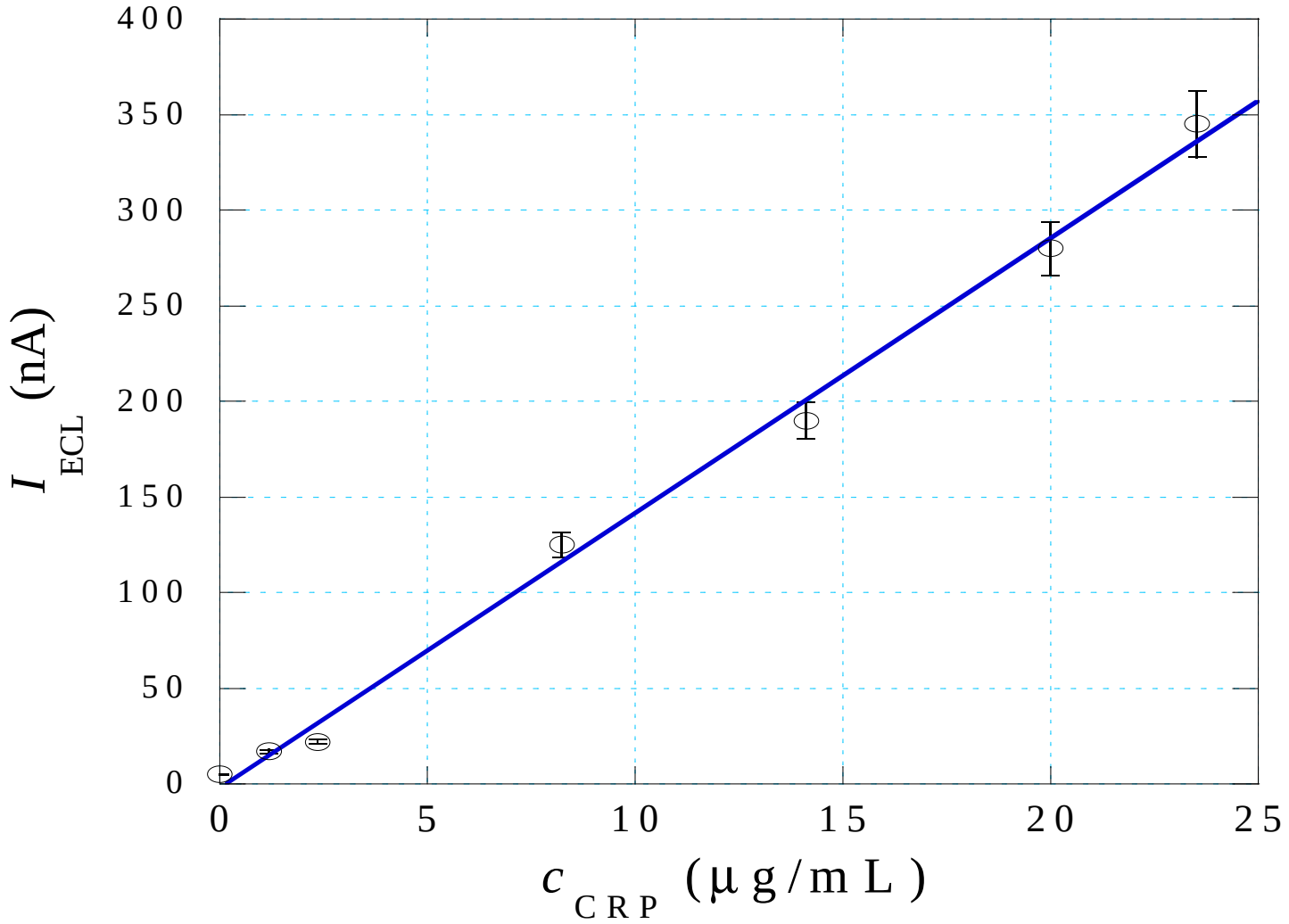
For apparently healthy people: Mean value of CRP: ~1.9 μg/mL.

ECL Detection of C Reactive Protein

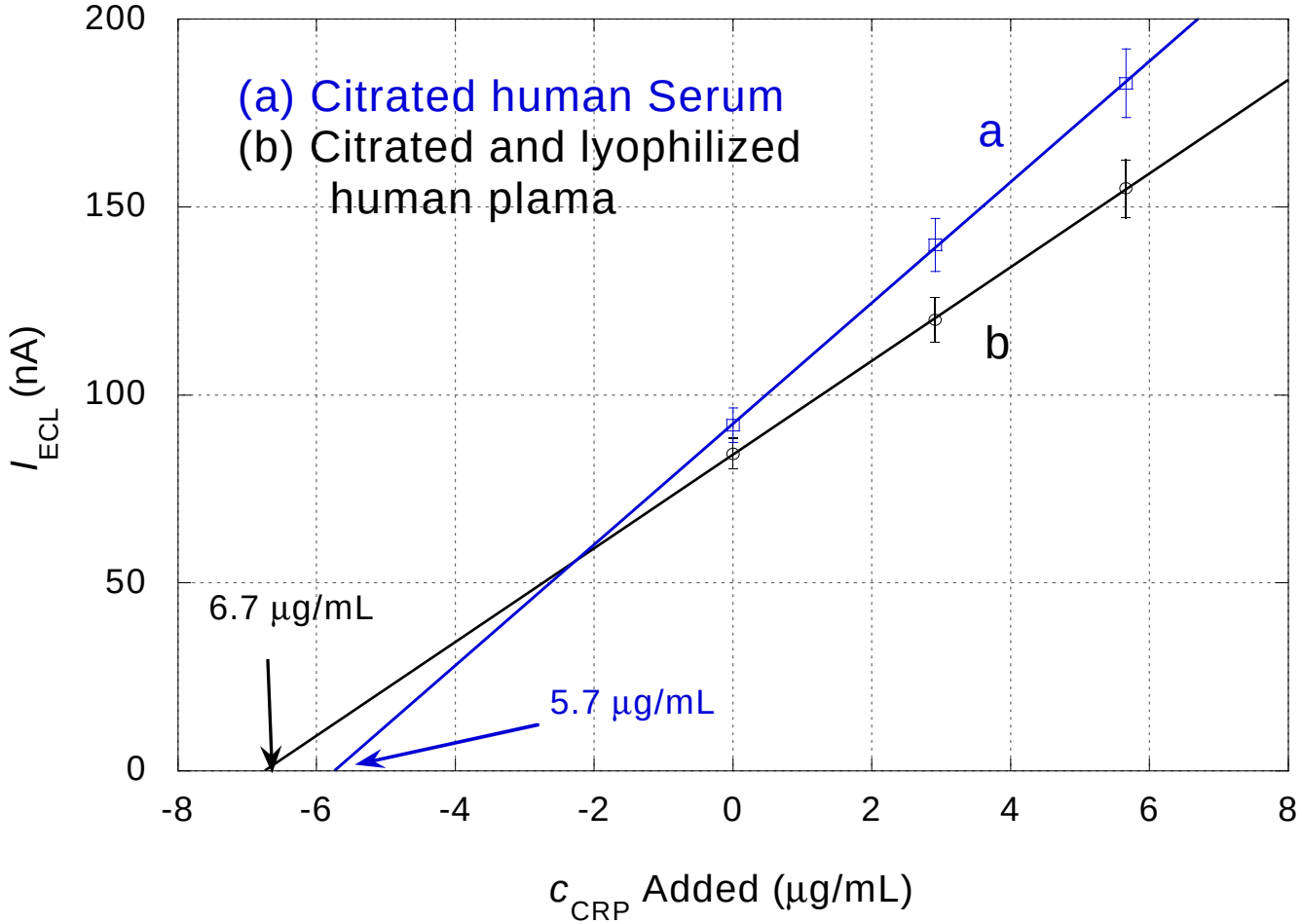


When c_{CRP} = 23.5 μg/mL

Correlation Between c_{CRP} and I_{ECL}



Determination of [CRP] in Human Serum and Plasma



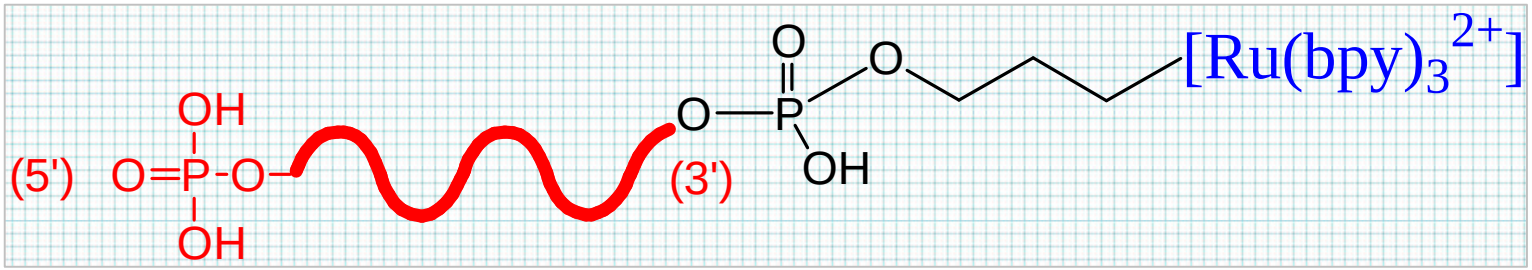
(Standard Addition Method)

So far,.....

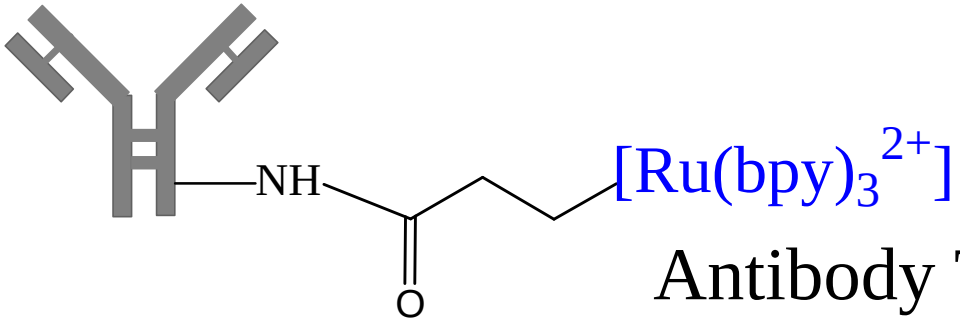
- General principles of ECL
- Attachment of receptor to electrode
- Characterization
- Labeling and binding of analyte
- DNA and CRP Detection

ECL Based Biosensors –Promising

Limitations of Currently Used Method



ssDNA tagged with $Ru(bpy)_3^{2+}$

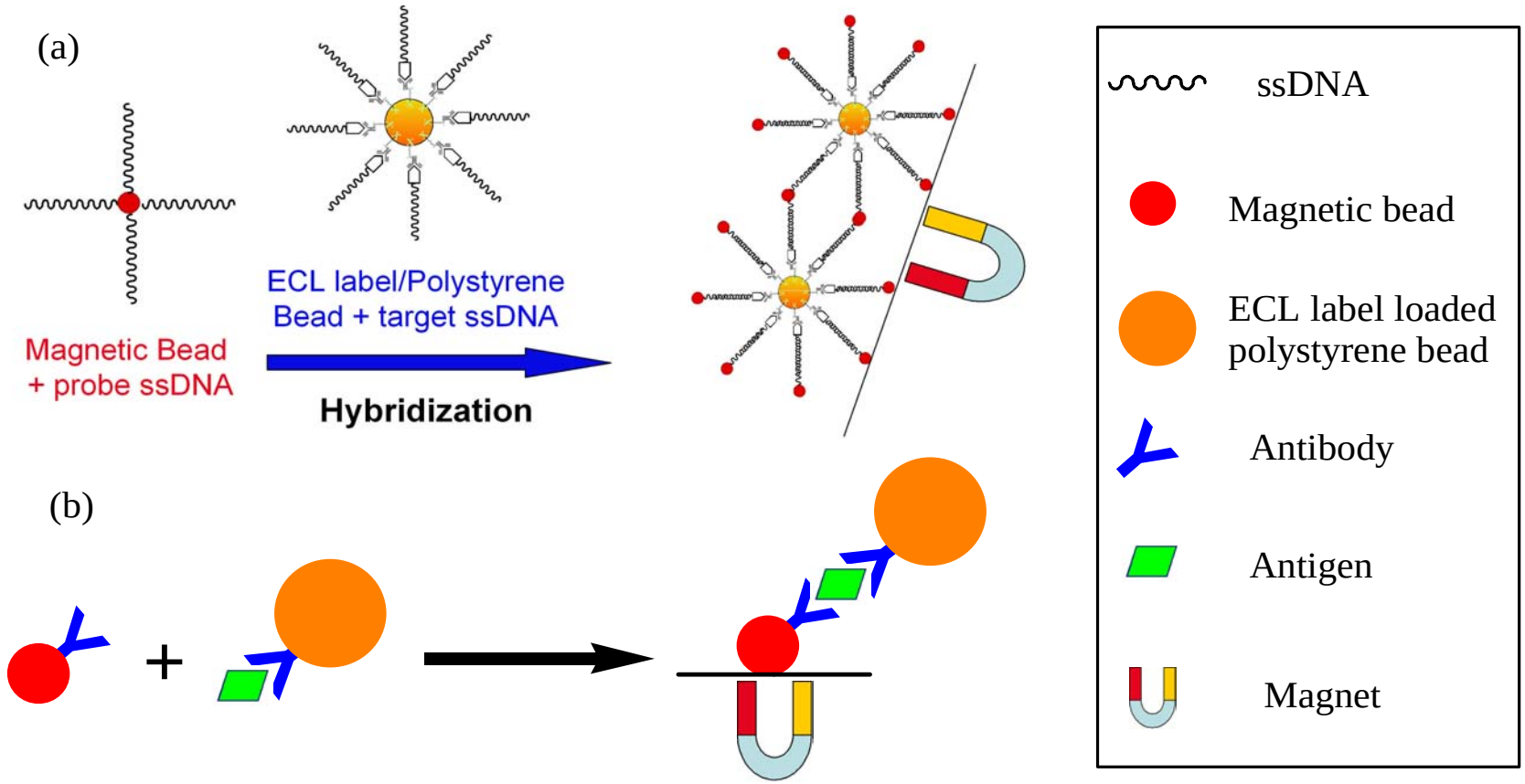


Antibody Tagged with $Ru(bpy)_3^{2+}$

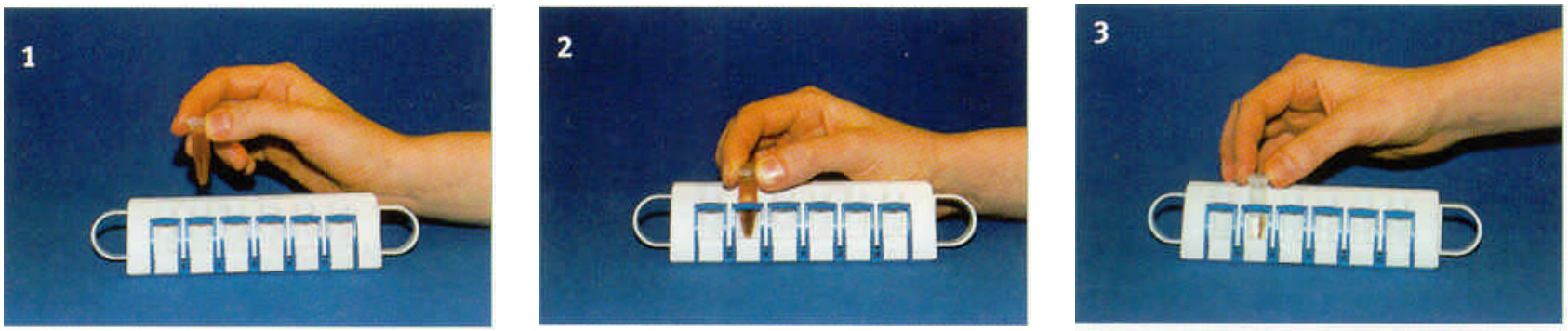
1.Only one or few ECL Labels can be attached to t-DNA or antibodies → Low detection limit

2. Two different ECL labels for DNA and antibody labeling

New Strategies…



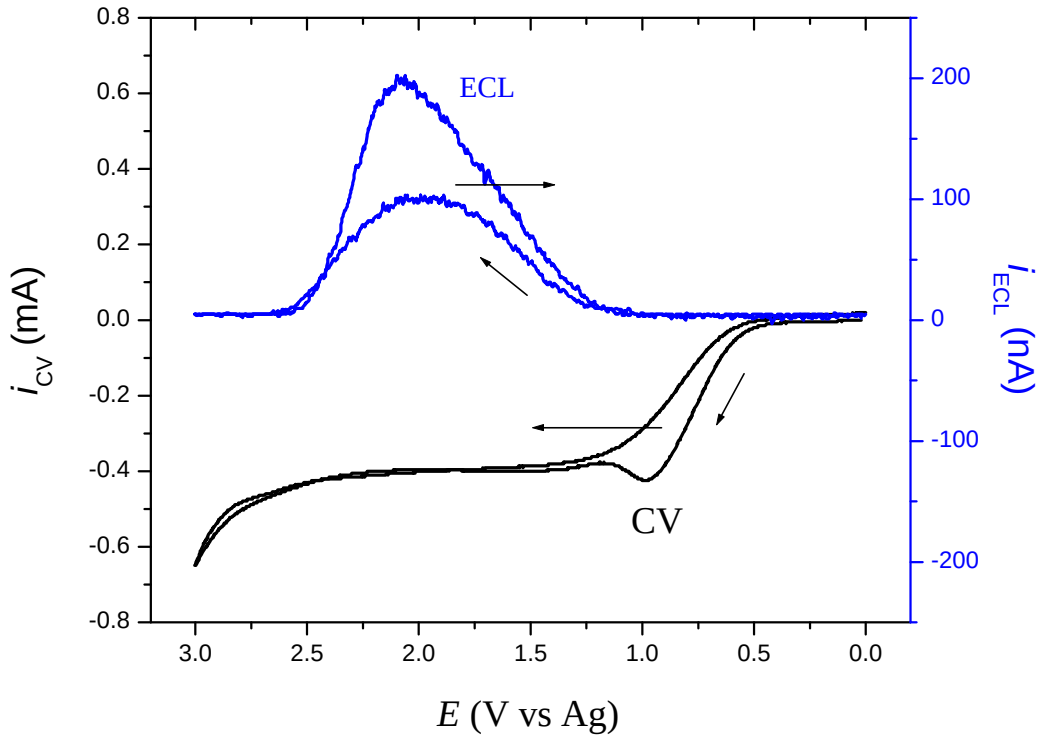
Schematic diagram of (a) DNA hybridization, and (b) sandwich type immunoassay



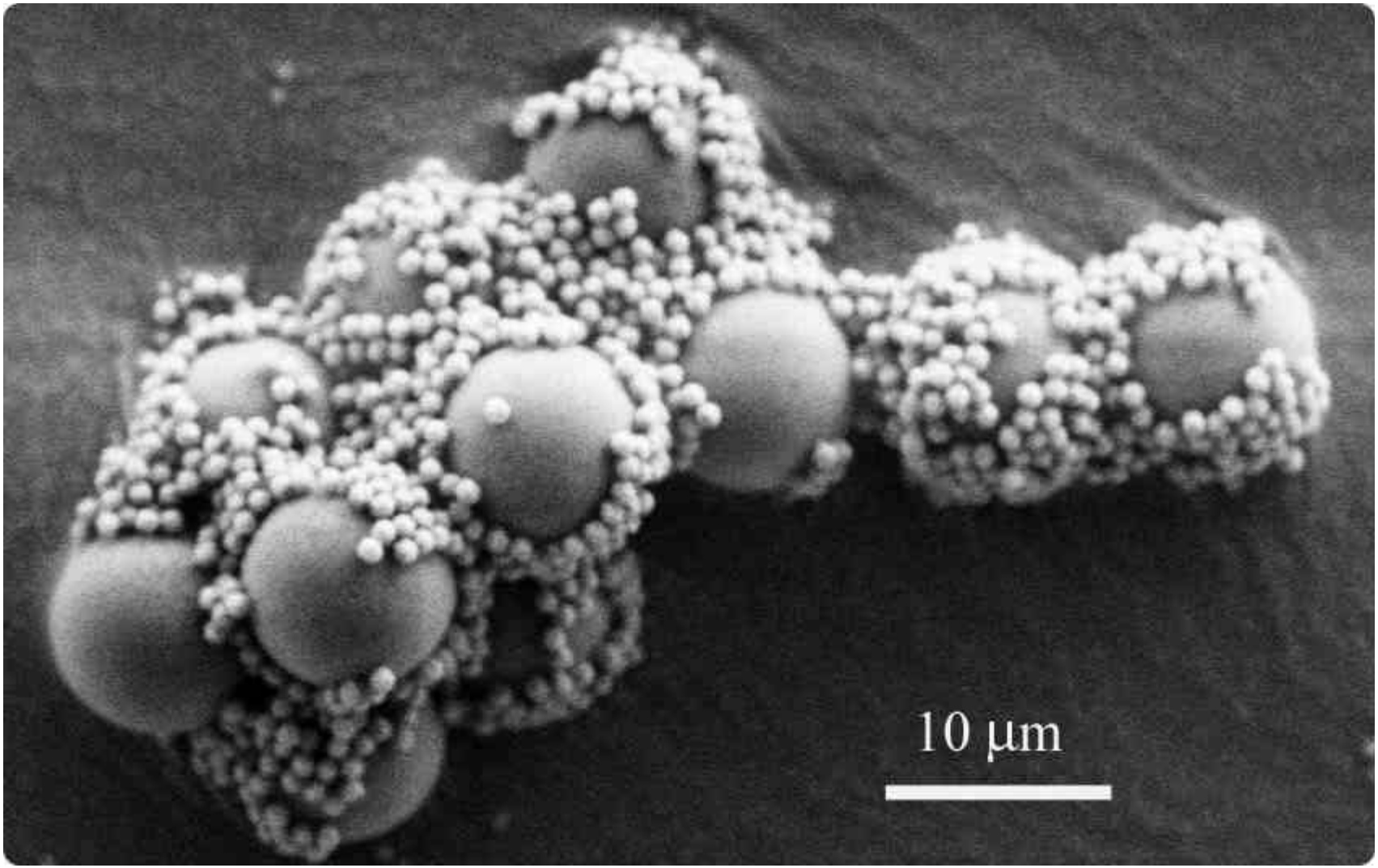
Beads Dissolution
In MeCN

Anodic Coreactant
(TPrA) ECL Detection

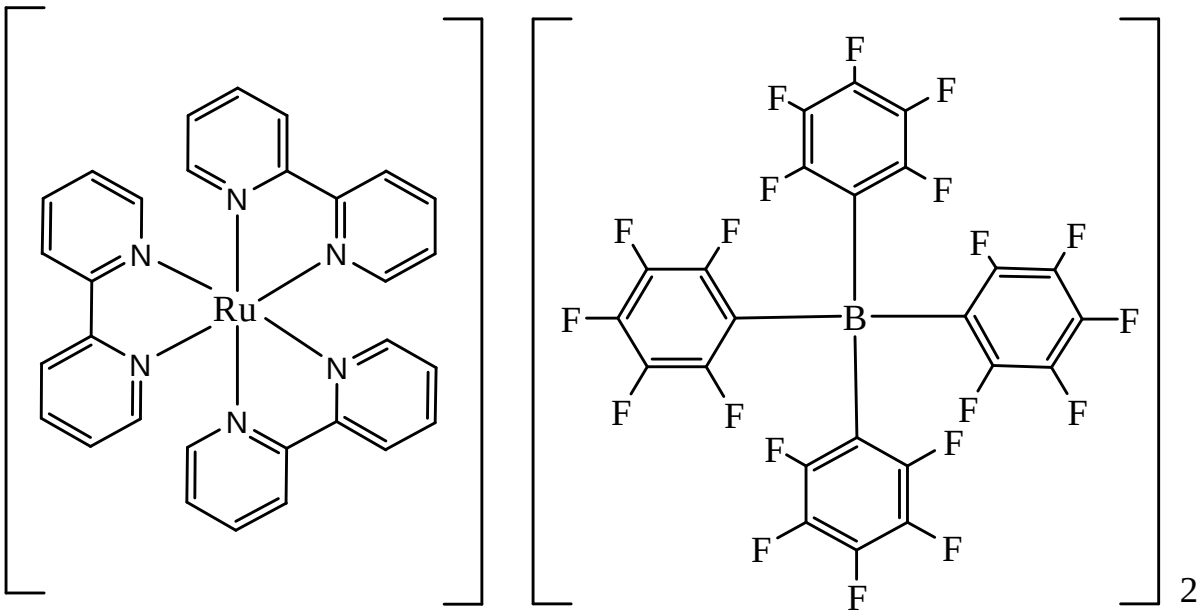
ECL Detection of DNA hybridization



SEM Images of c-DNA Hybridization



ECL Labels

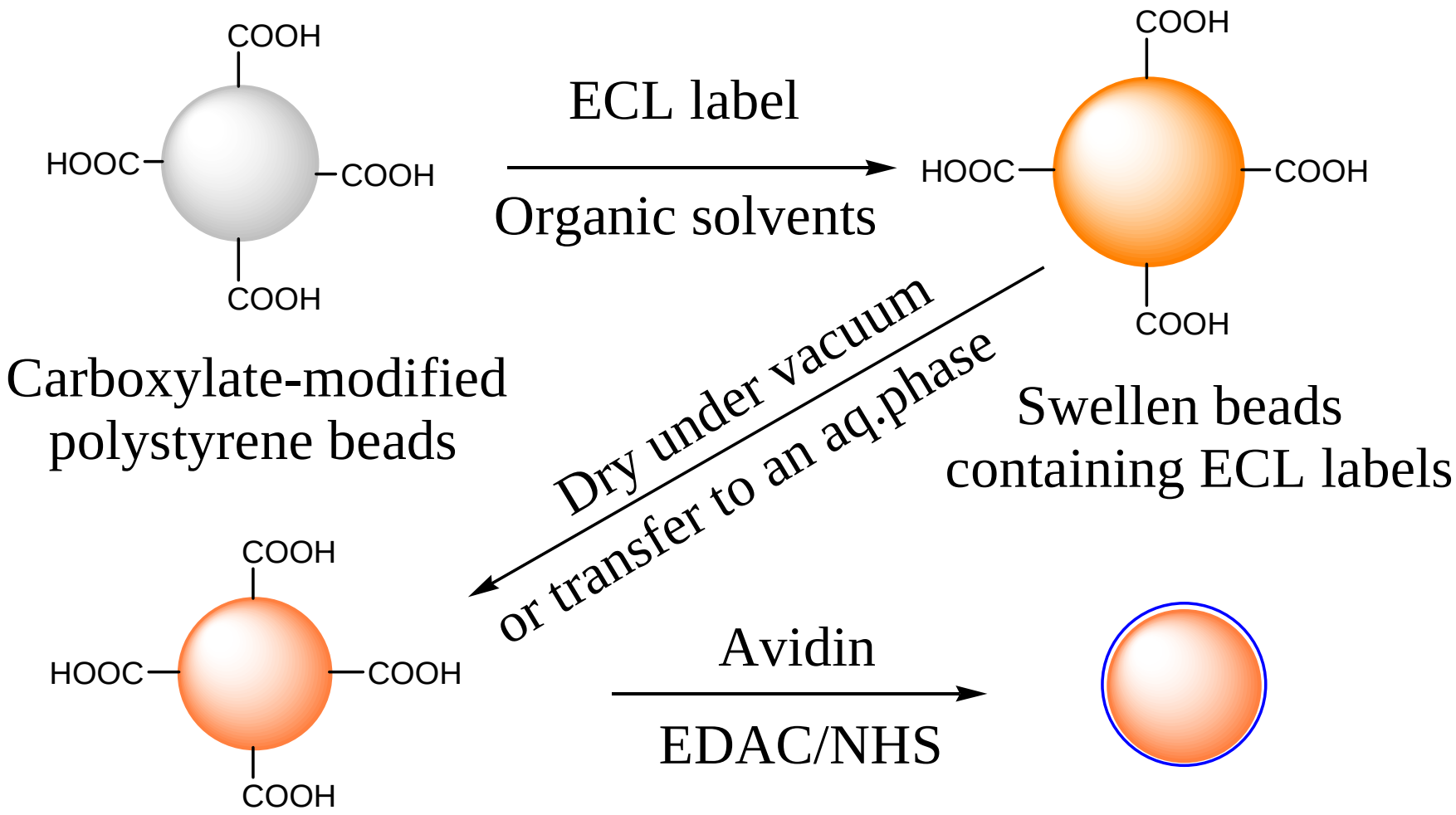


Ruthenium(II) tris(2,2'-bipyridine) tetrakis(pentafluorophenyl) borate

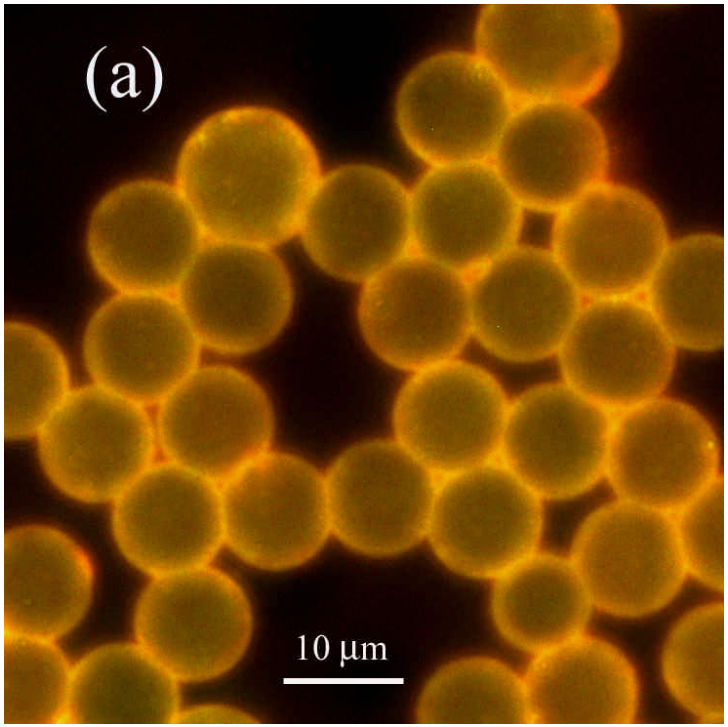
$Ru(bpy)_3[B(C_6F_5)_4]_2$—$Ru(bpy)_3(Brookhart)_2$

- High ECL efficiency
- Sufficiently soluble in organic solvents, but completely insoluble in aqueous solutions

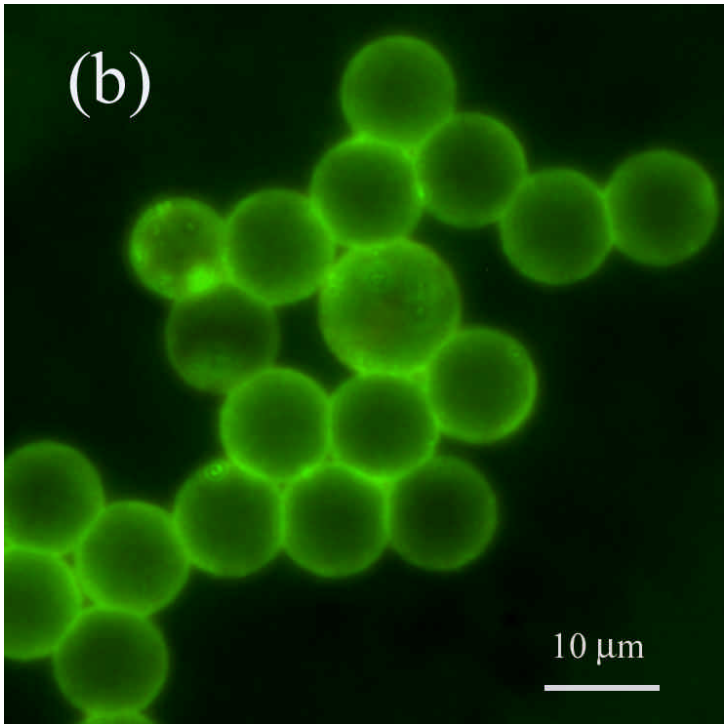
How to Effectively Load ECL Labels into Polystyrene Beads?



Fluorescent Images



After $Ru(bpy)_3^{2+}$ Loading

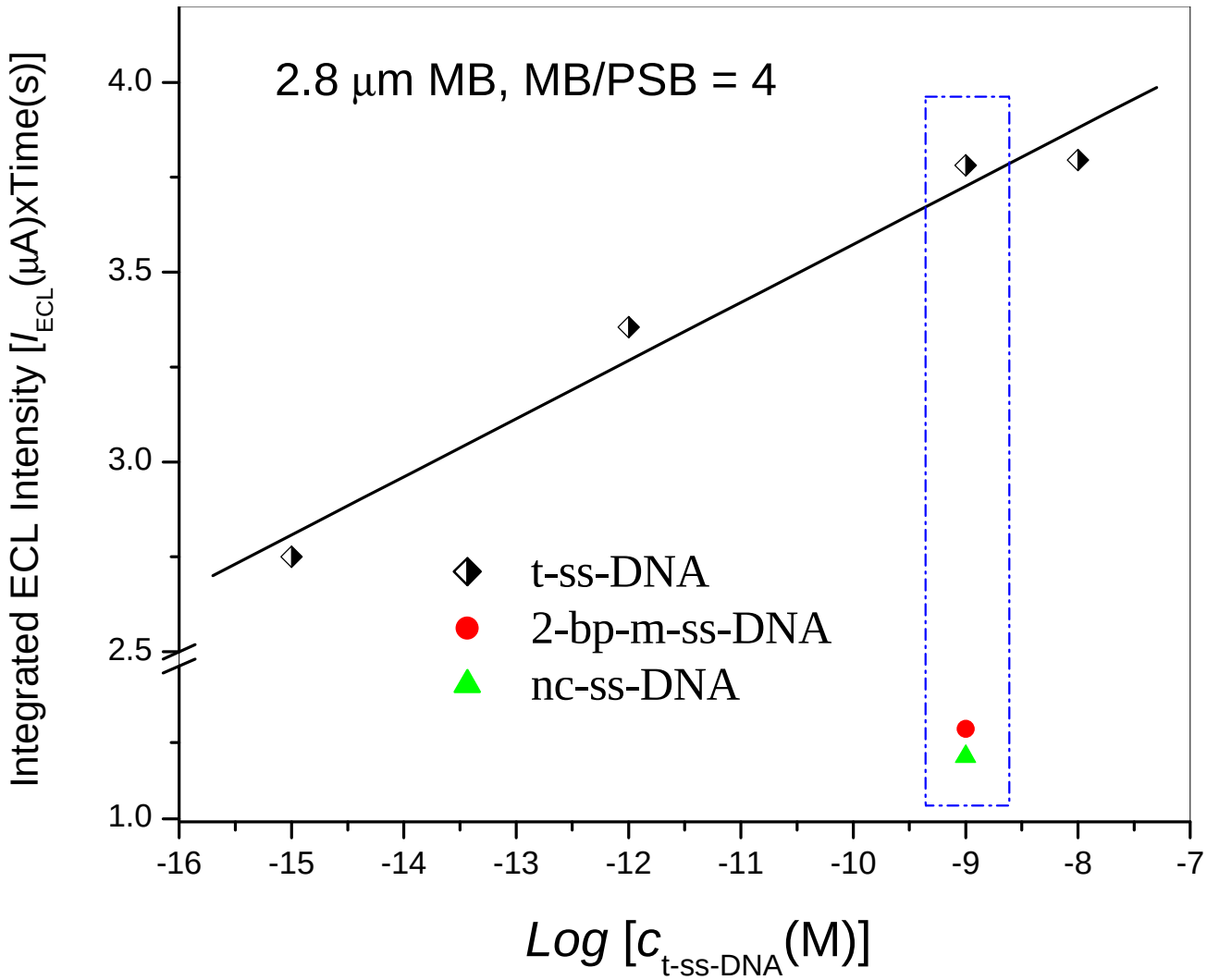


(a) + Avidin ← Fluorescein Biotin

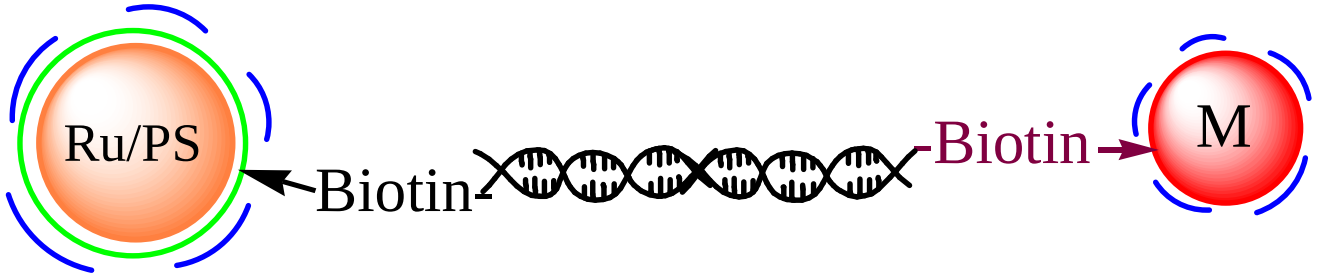
Loading capacity:
7×10^9 molecules/bead

Amplification factor:
7×10^9 $Ru(bpy)_3^{2+}$/1×10^5 ssDNA
70,000 $Ru(bpy)_3^{2+}$/ssDNA

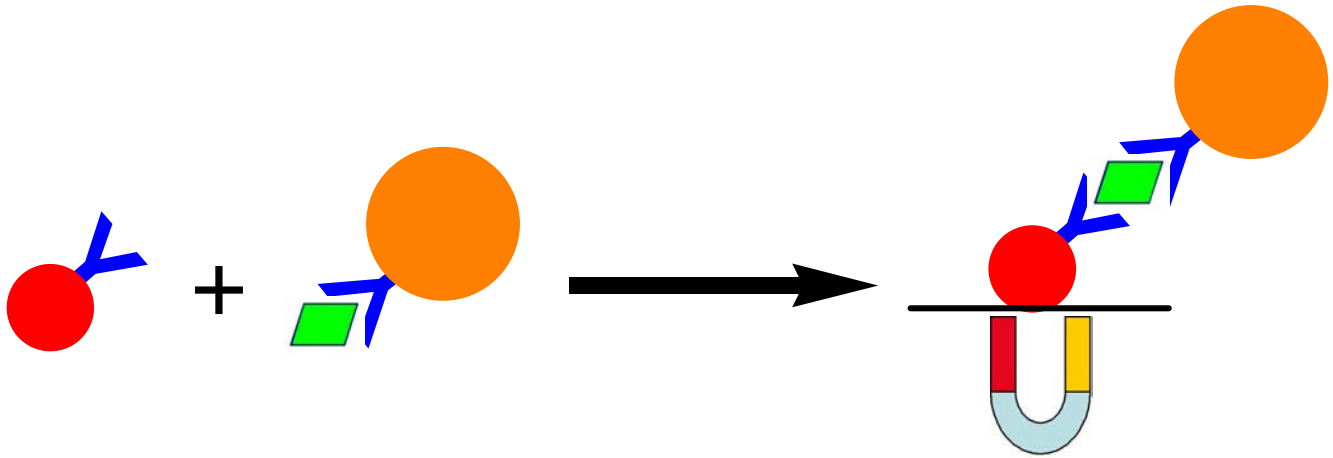
ECL Detection of DNA Hybridization Using $Ru(bpy)_3^{2+}$ Entrapped Microspheres



Towards a Single Biomolecule Detection



DNA Hybridization



Immunoassays

Conclusions

- Biosensors based on ECL are promising.
- The new strategy could have the ECL signal amplified by 4 to 5 orders of magnitude.
- $Ru(bpy)_3^{2+}$ entrapped microspheres can be used to label both DNA and Proteins.
- Single biomolecule ECL detection could be achievable.

Acknowledgements

Prof. Allen J Bard

Support

MURI - DAAD 19-99-1-0207 (DOD, UT)
IGEN, Inc.

Questions & Answers

Why Biosensors?
(DNA Probe Assays and Immunoassays)

A wide range of applications in the areas of, e.g.,

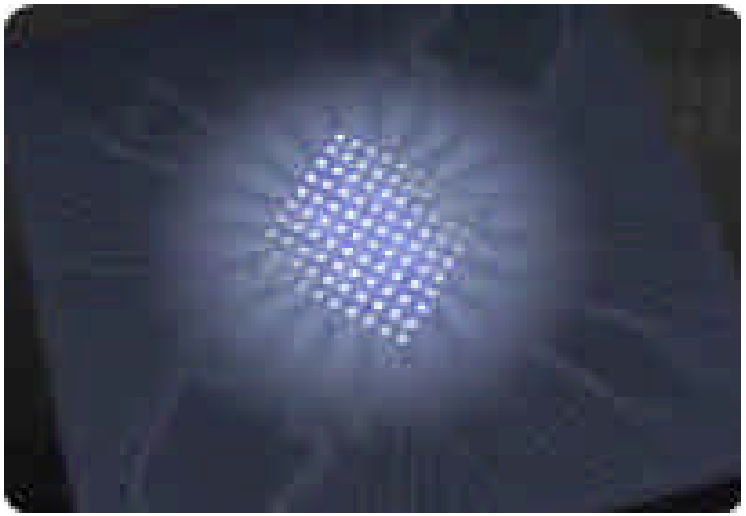
- Clinical Diagnostics
- Forensic Chemistry
- Environmental Investigations
- Pharmaceutical Studies
- Biological Warfare Agent Detections

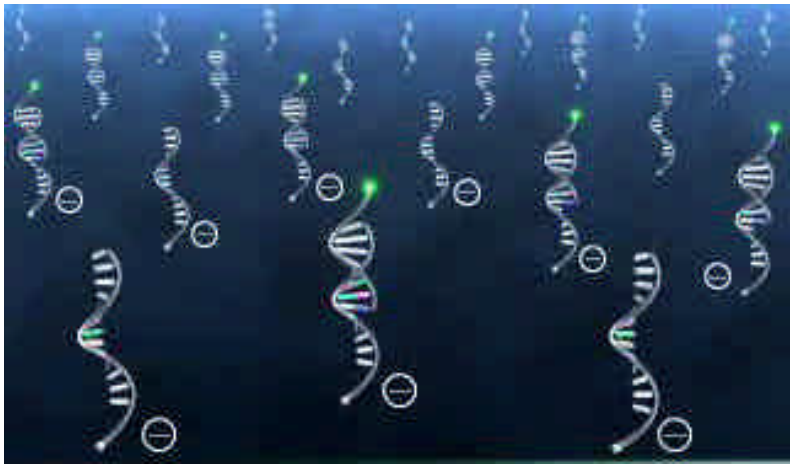
Why ECL Based Biosensors?

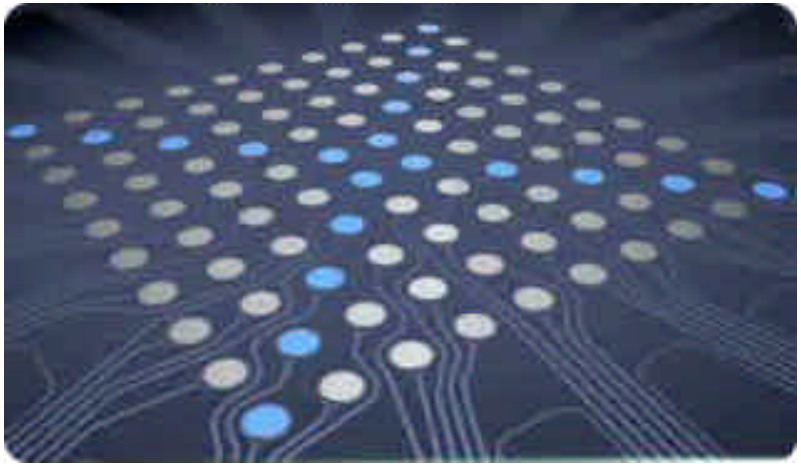
ECL→ Electrochemical + Spectroscopic Methods

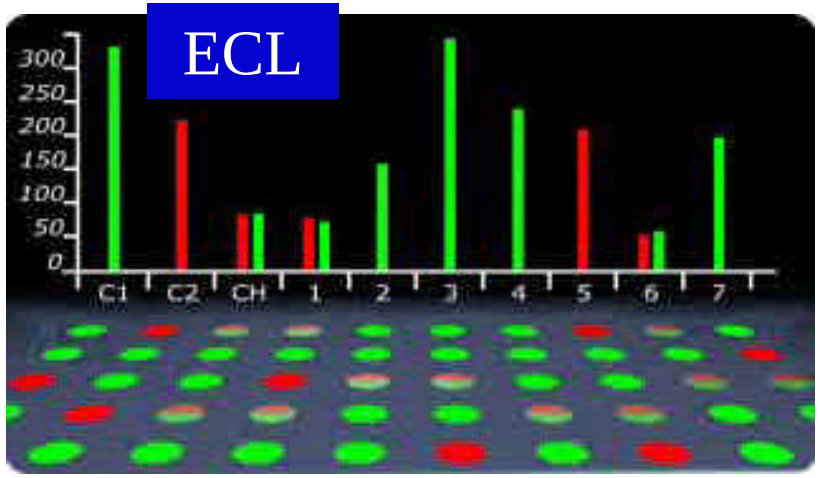
- Very sensitive (~10 pM $Ru(bpy)_3^{2+}$ in TPrA)
- No background ECL
- No light source required
- Reactions are localized and controllable
- Relatively simple instrumentation

Technology Transfer: Biochips



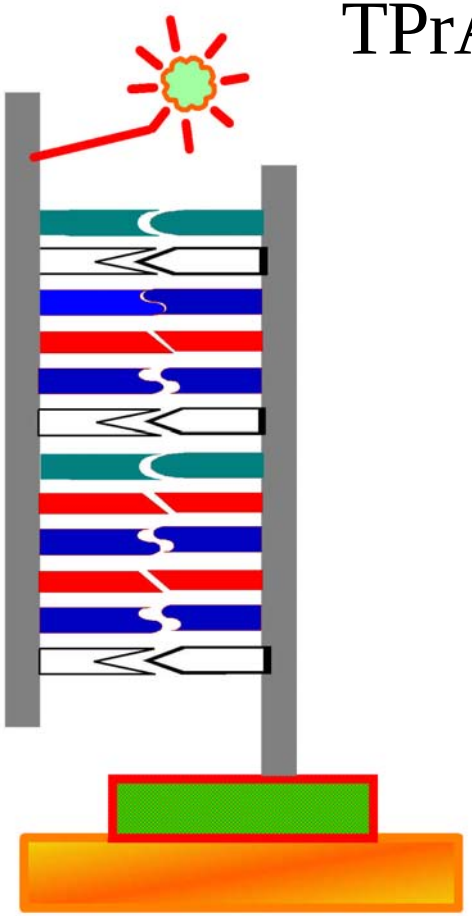


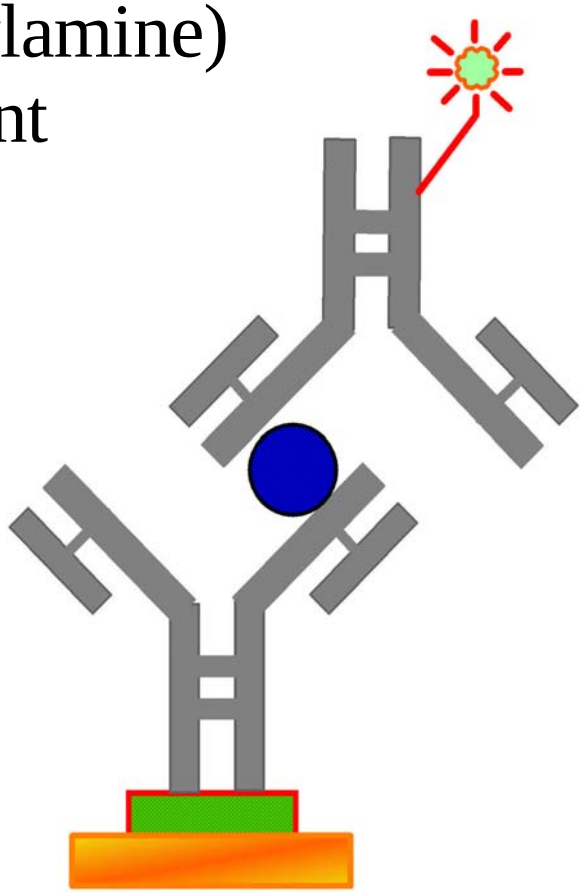




Dose ECL Emission Need Direct Oxidation of $Ru(bpy)_3^{2+}$?

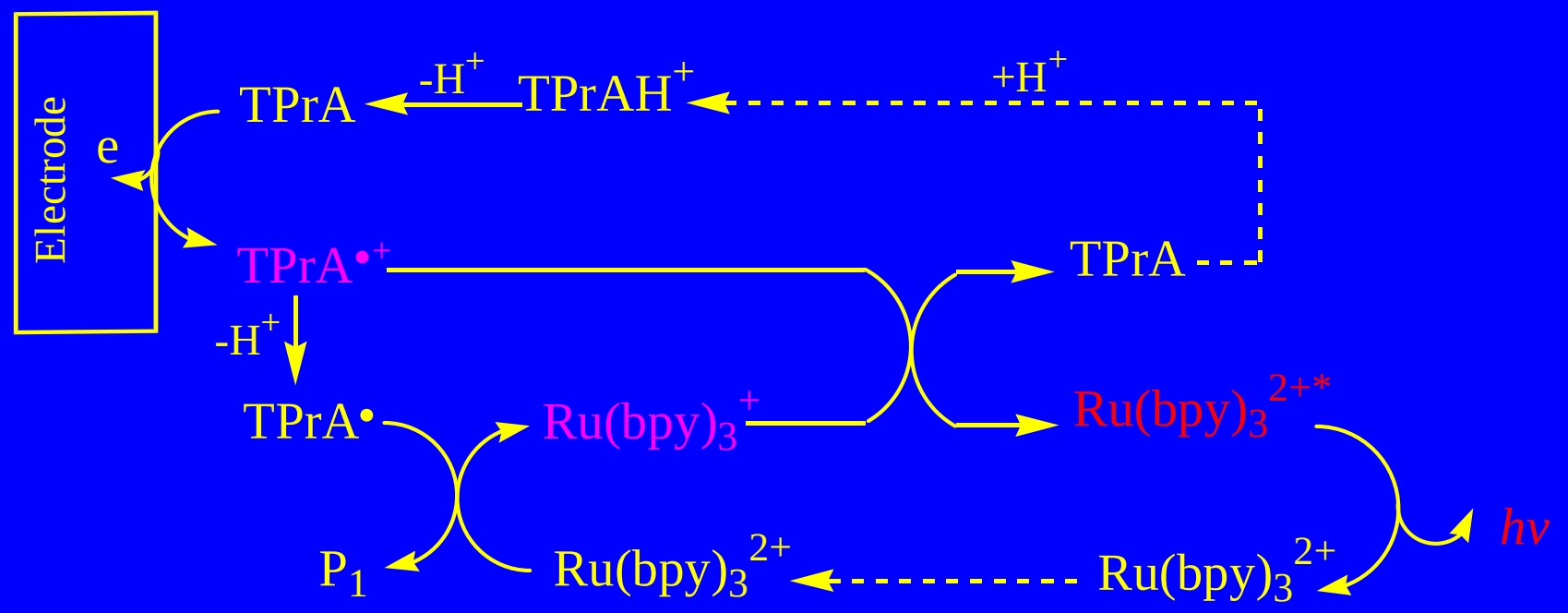
TPrA(Tripropylamine)
Coreactant





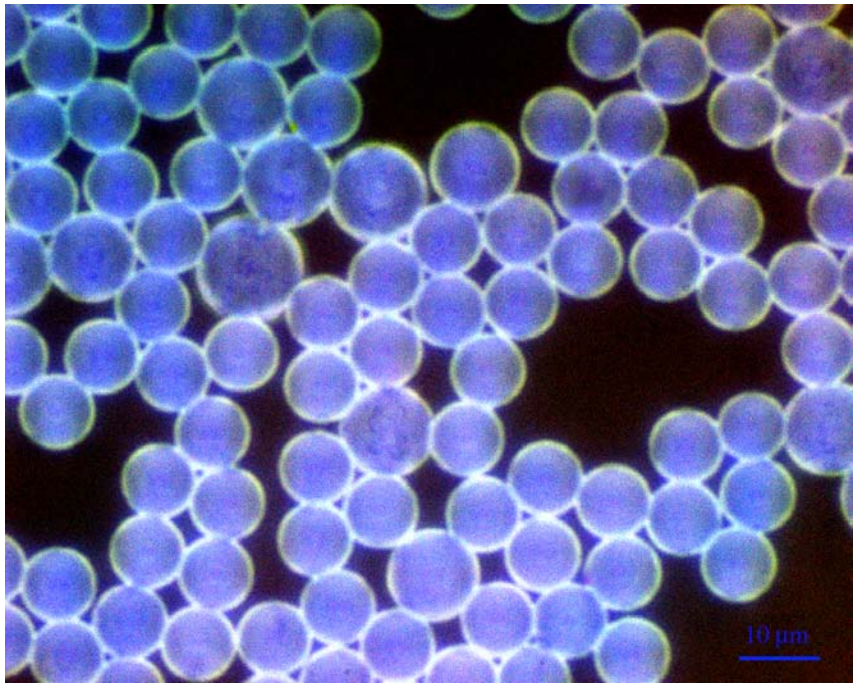
Potential Scanning from 0 to 1.4 V vs Ag/AgCl

A Route for the Generation of $Ru(bpy)_3^{2+*}$ in DNA/Protein-Ru(II)/TPrA System

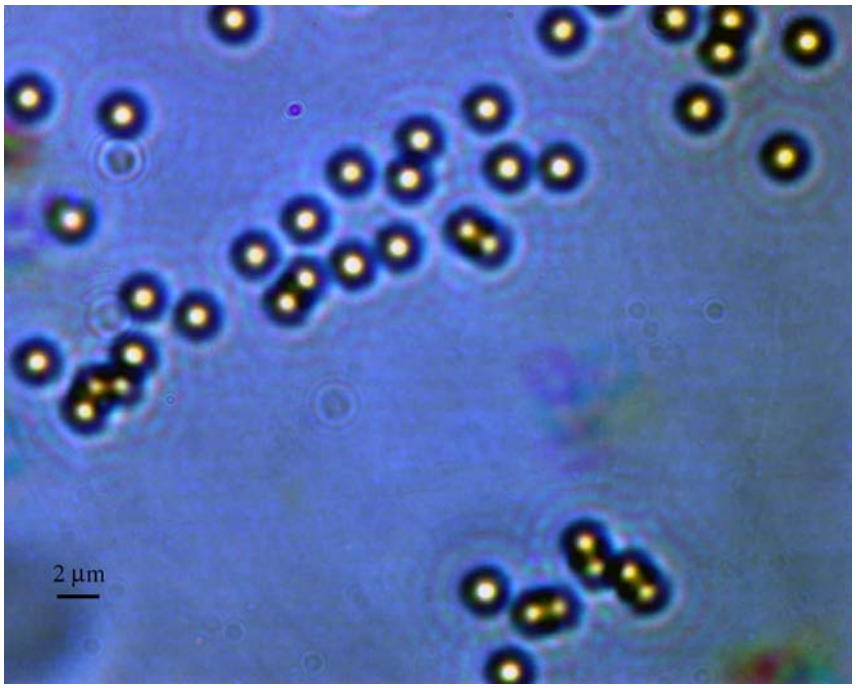


1. W. Miao, J-P, Choi, A. J. Bard, *J. Am. Chem. Soc.*, **124** (48), 14478 -14485, 2002
2. W. Miao, A. J. Bard, *Anal. Chem.*, **75**, 5825-5834, 2003

Carboxylated Polystyrene Beads (PS, d ~10 μm)



Magnetic Beads (MB, d ~1 μm)



Synthesis of the ECL Labels

$Ru(bpy)_3Cl_2$ (aq) + $2Li[B(C_6H_5)_4]\cdot nEt_2O$ (n = 2-3)

"Lithium Brookhart Salt"

$\Downarrow$

$Ru(bpy)_3[B(C_6F_5)_4]_2$ ($\downarrow$) + 2LiCl + $2nEt_2O$

(a) Washed with water

(b) Recrystallized from MeCN/water soln

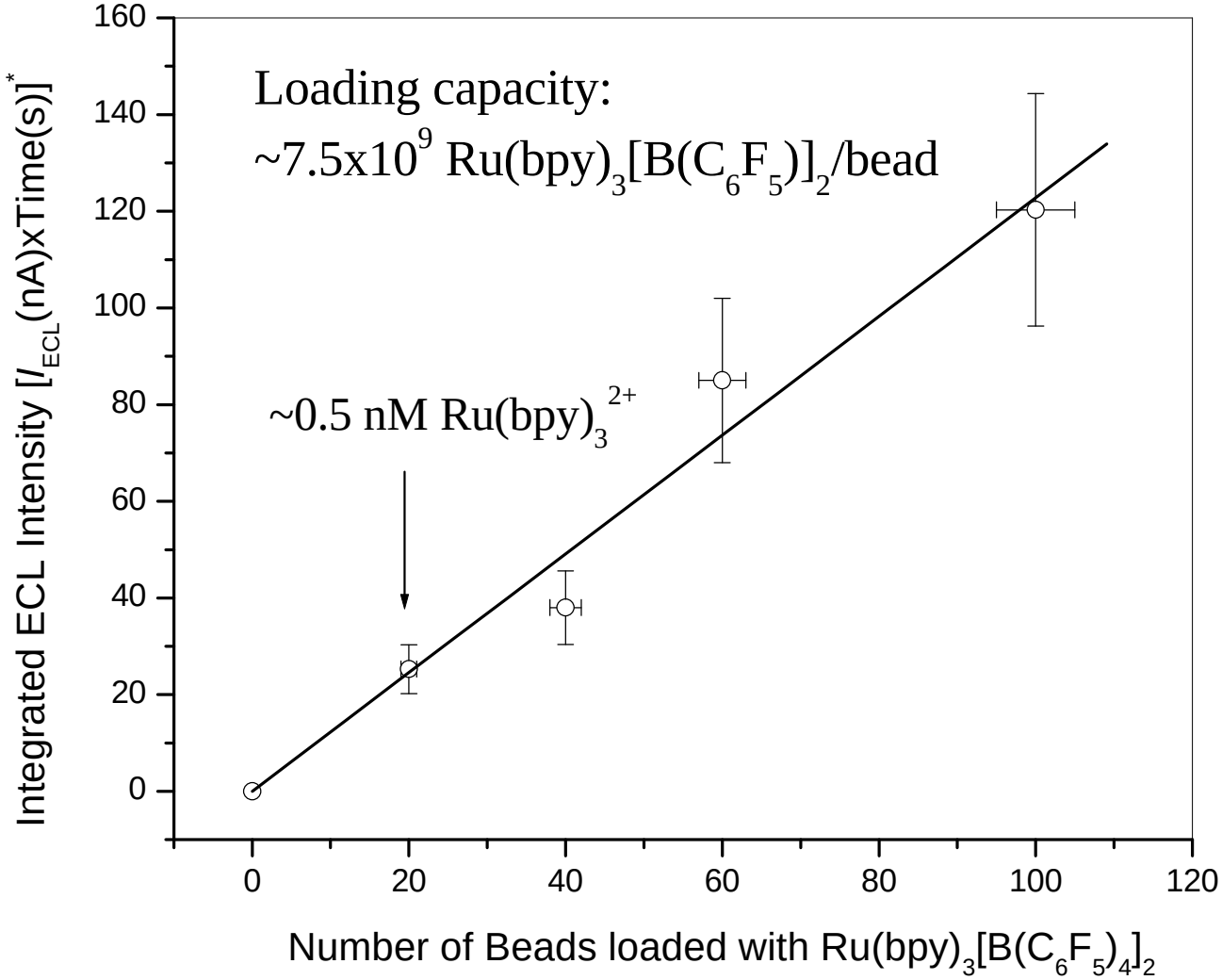
$\downarrow$

$Ru(bpy)_3[B(C_6F_5)_4]_2$

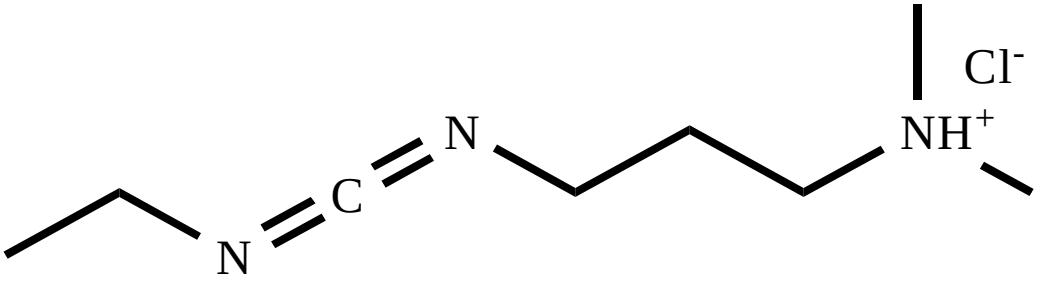
(Dried under vacuum)

How Many Ru(II)/PSB Can Be Detected?

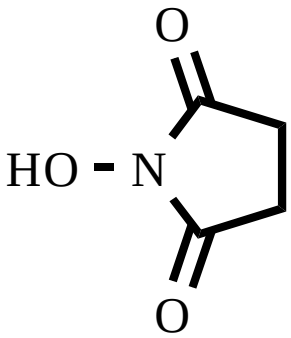
(In 0.50 mL 0.10 M TPrA-0.055 M TFAA-0.10 M (TBA)BF_4-1% H_2O, 10 μm PSB, Pt)



** With background ECL subtraction*

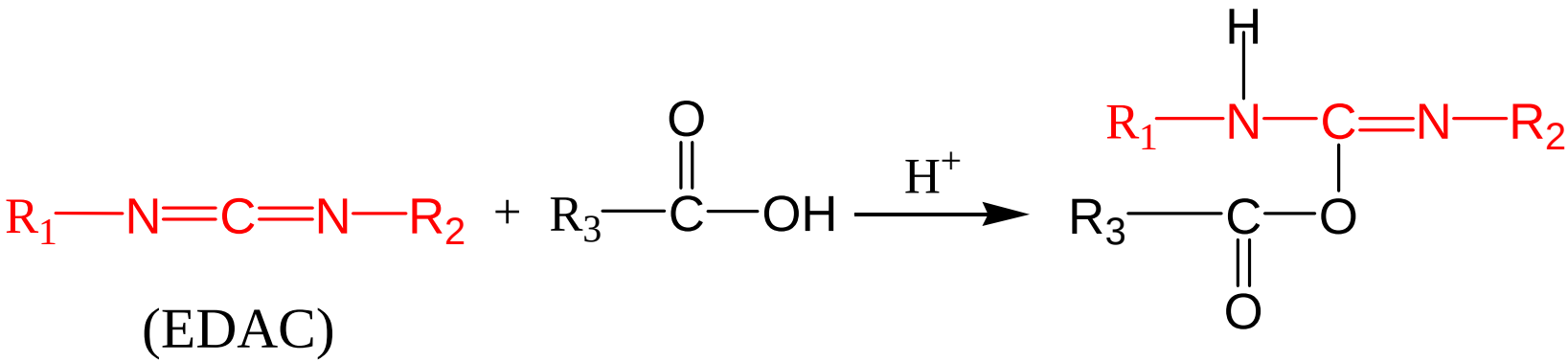


1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC)

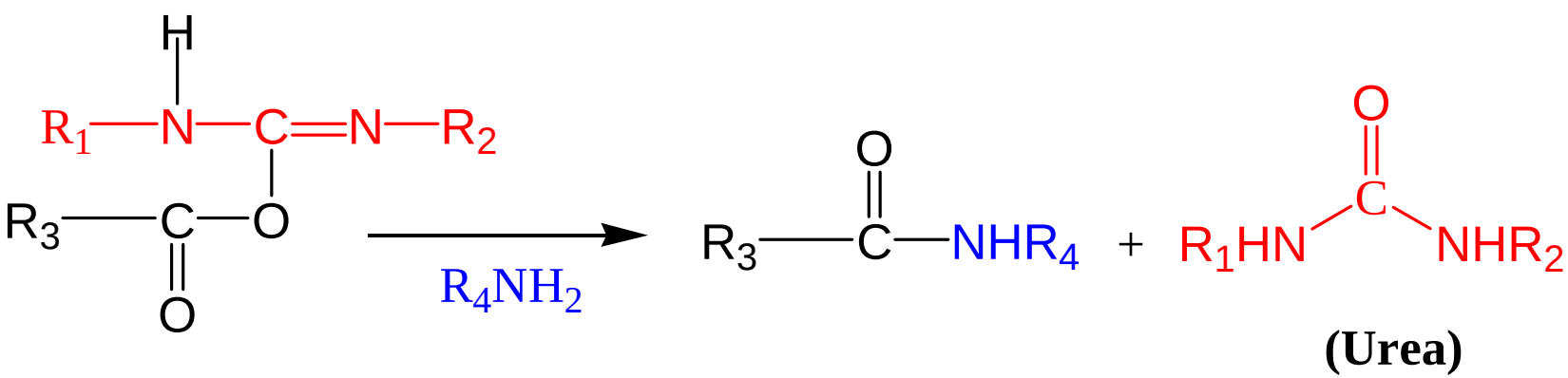


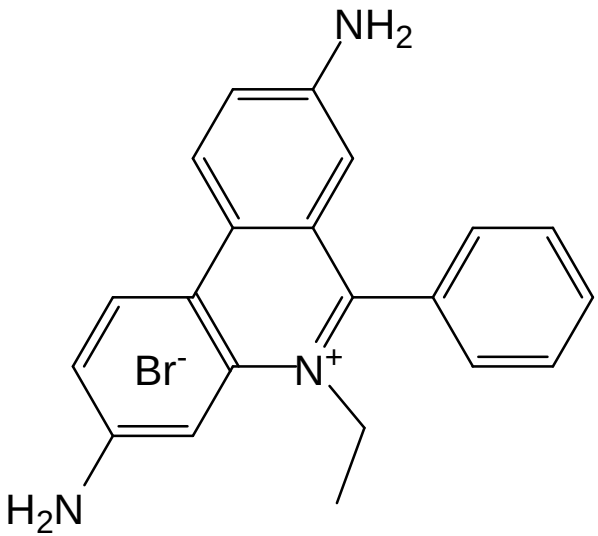
N-hydroxysuccinimide (NHS)

Carbodiimide Reaction



EDAC reacts with carboxylic acid group and activates the carboxyl group, allowing it to be coupled to the amino group (R_4HN_2) in the reaction mixture.





Ethidium bromide

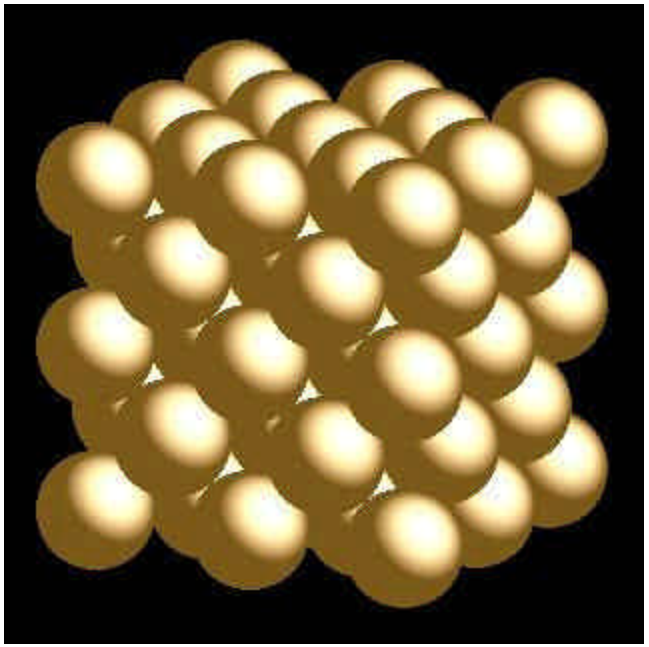
The detection limit of Ethidium Bromide stained DNA using an UV transiluminator is generally accepted to be approximately 1 ng. ~0.1 μM dsDNA (with 25-mer)

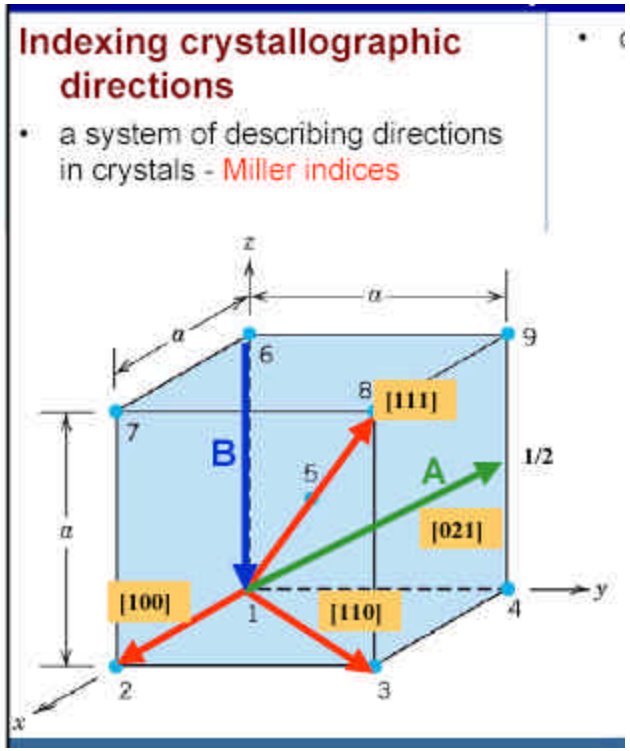
Gold Crystal Structure

Space group: Fm-3m (Space group number: 225)

Structure: ccp (cubic close-packed)

Cell parameters:	***a* /pm**	***b* /pm**	***c* /pm**	**a / °**	**ß / °**	**? / °**
	407.82	407.82	407.82	90.000	90.000	90.000

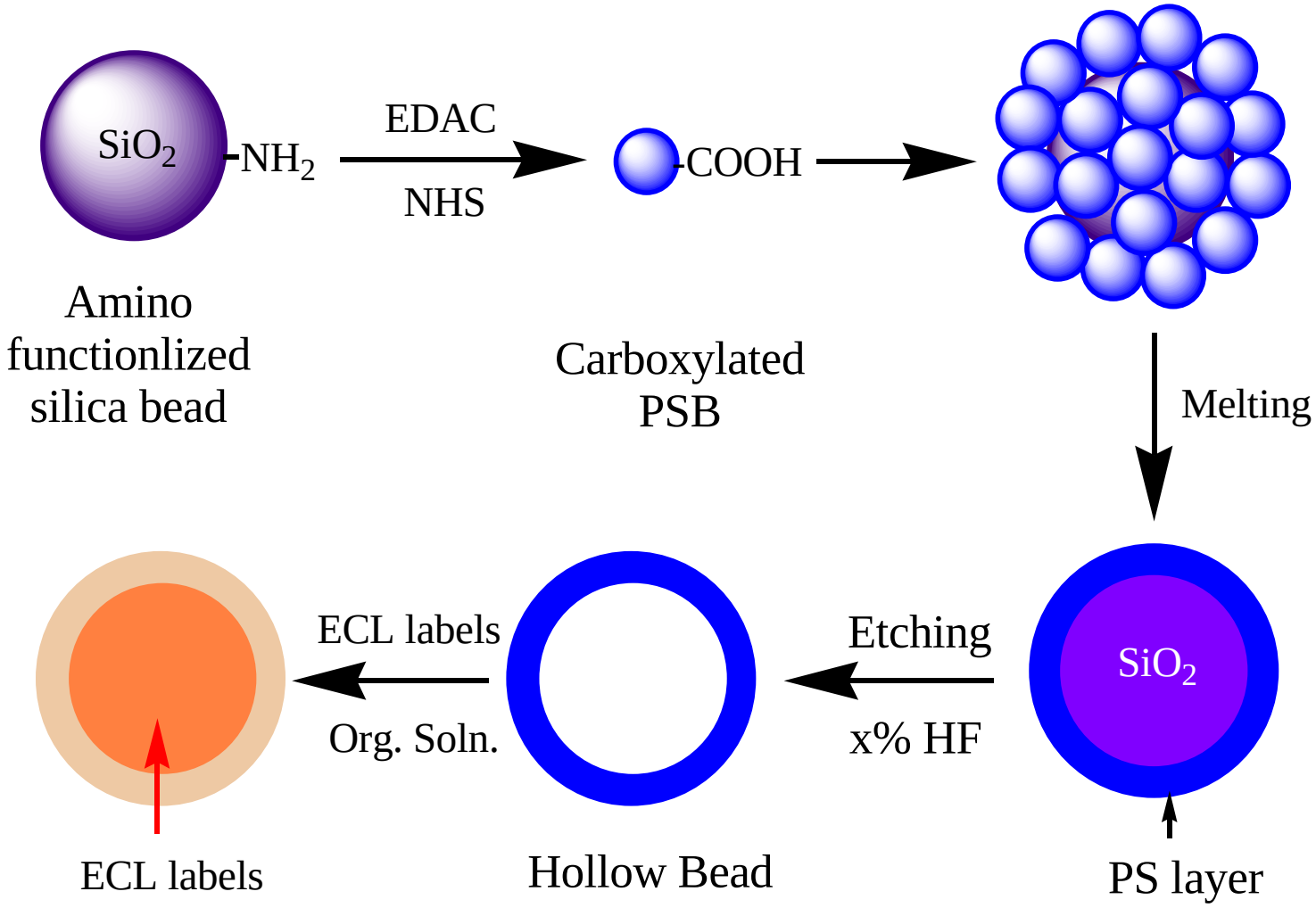




Increasing $Ru(bpy)_3^{2+}$ Loading Capacities

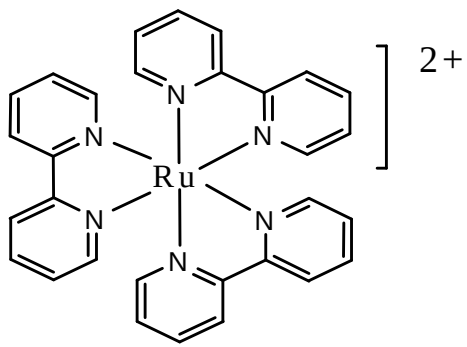
1. Increasing the size of PSB
2. Producing “hollow polymeric beads” capable of encapsulating of solution-phase or solid-state $Ru(bpy)_3^{2+}$
3. Loading ECL labels during the polymerization process of bead formation

Hollow Polymeric Beads



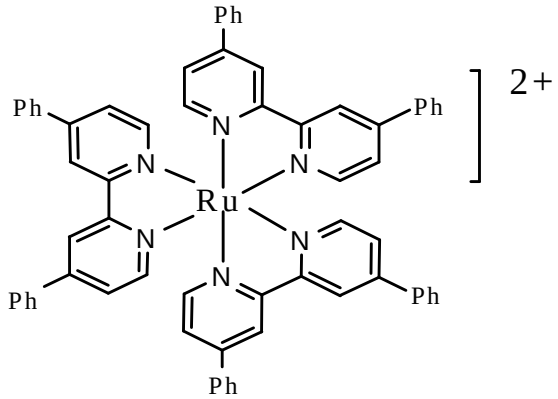
Using Different ECL Labels

(A)



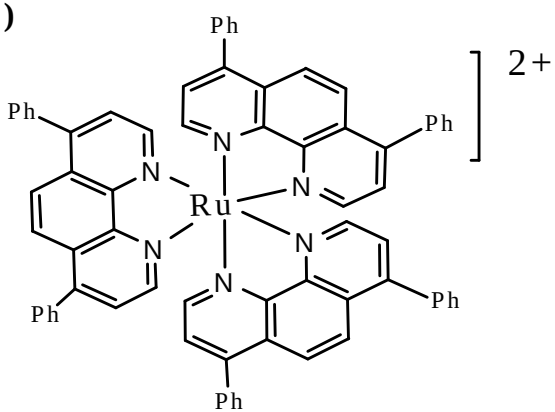
Ruthenium(II) tris(2,2'-bipyridine)

(B)



Ruthenium (II) 4,4'-diphenyl-2,2'-bipyridine

(C)



Ruthenium(II)
4,7-diphenyl-1,10-phenanthroline

ECL Label	ECL Efficiency
$Ru(bpy)_3^{2+}$	5%
$Ru(dp\text{-}bpy)_3^{2+}$	14%
$Ru(dp\text{-}phen\text{-})_3^{2+}$	24%

From P McCord, AJ Bard, JEAC, 318(1991) 91-99

ECL Detection of DNA and Antibody/Antigen

- DNA: Synthesized DNA Sequences
- Proteins: Human serum albumin (HAS)/Anti-HAS; *E* Coli/Anti-*E* Coli; CRP/Anti-CRP; $p16^{INK4A}$ gene/Anti-$p16^{INK4A}$ gene (a predictive factor in many cancers)…
- Real samples: Human Serum/Blood…