Fabrication and principles of amperometric biosensors

Clark Oxygen Electrode: Principle

Importance of oxygen detection:

- Photosynthesis and respiration rates are dependent on oxygen levels, and they play significant roles in crop yield and staple food production.
- Detection of oxygen in blood.
- The quality of water in the ocean, seas, coastal, underground, and treatment plants uses oxygen level as an indicator.

In 1954 Leland Clark conceived and constructed the first membrane-covered oxygen electrode having both the anode and cathode behind a nonconductive polyethylene membrane.

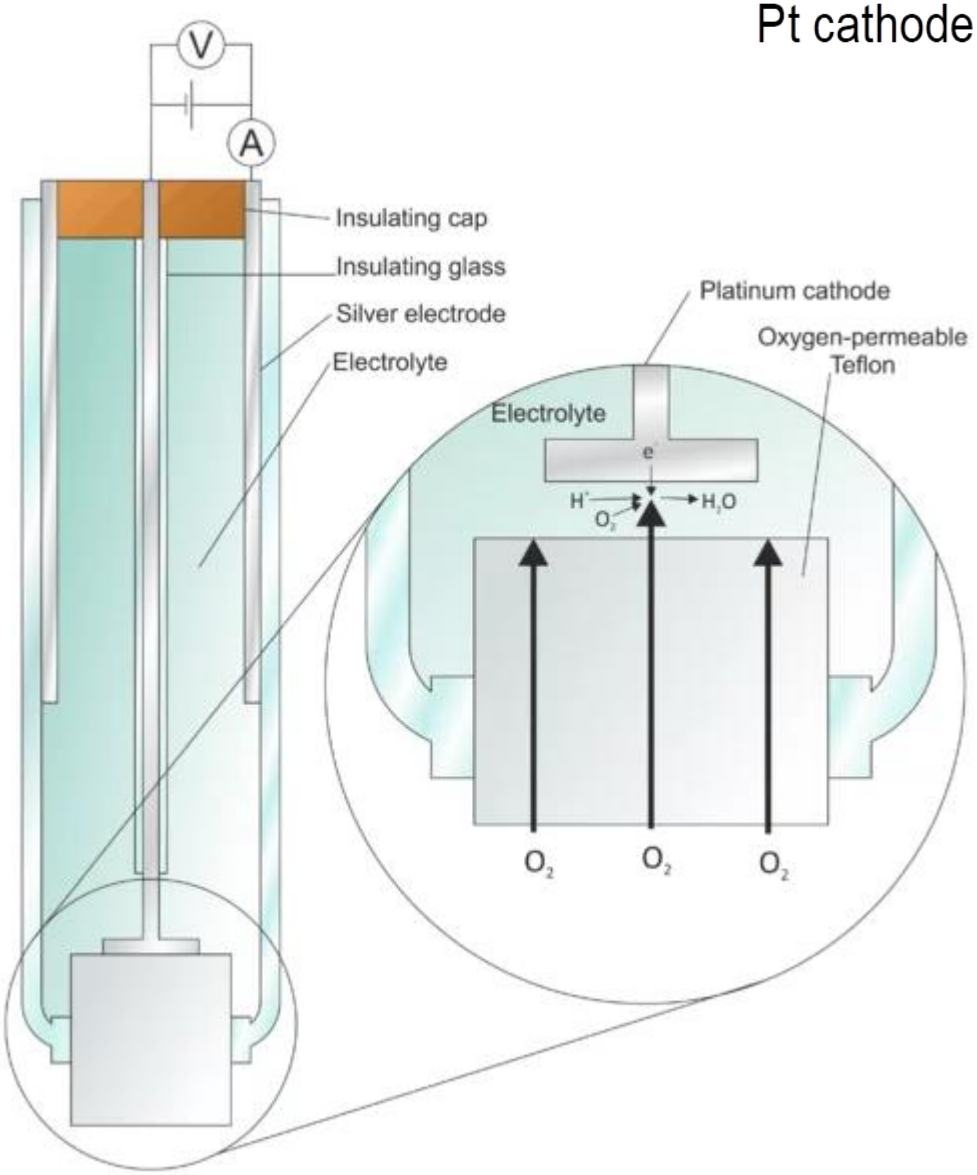
Originally conceived as a medical device, the Clark electrodes have been adapted for non-medical uses. It is the basis of modern blood gas analyzers, which quantify the blood pH, oxygen, and carbon dioxide level in vitro and in vivo.

It is also the foundation of modern-day gas measuring devices and biosensors, which not only measures oxygen but also other biologically relevant molecules.

The Clark Oxygen Electrode

Ag anode $4Ag^0 + 4Cl^- \longrightarrow 4AgCl + 4e^-$

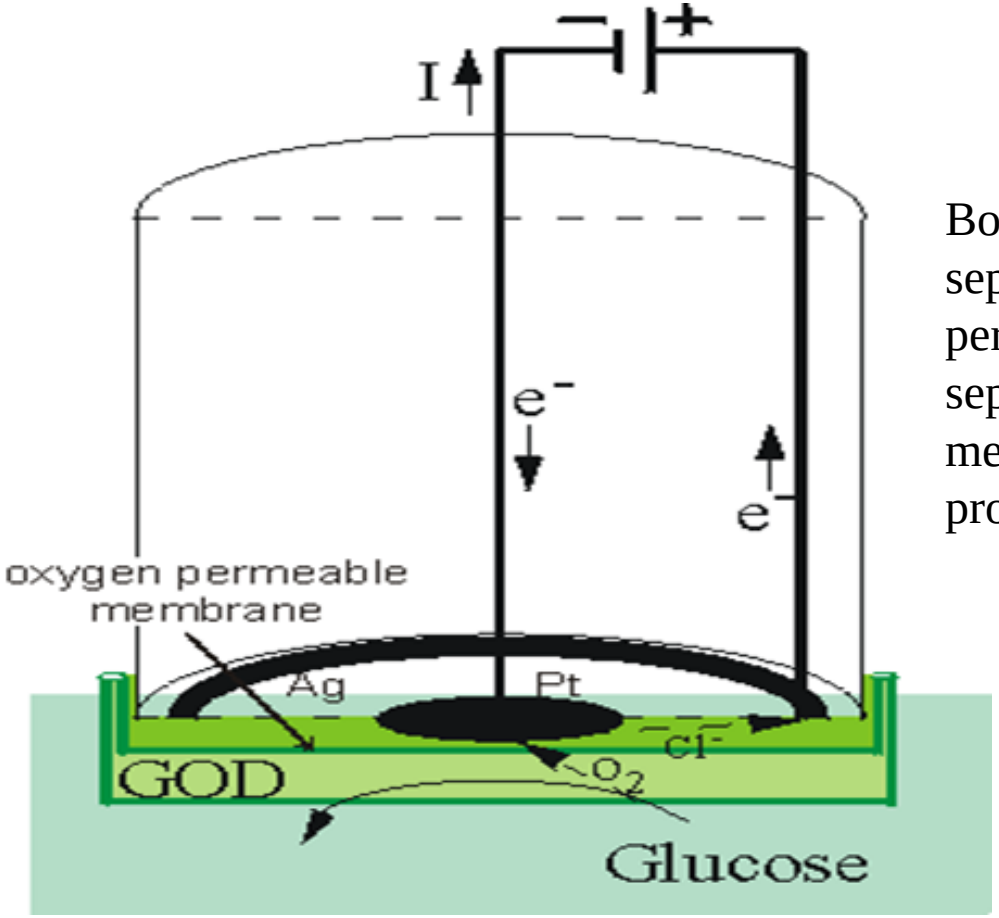
Pt cathode $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$



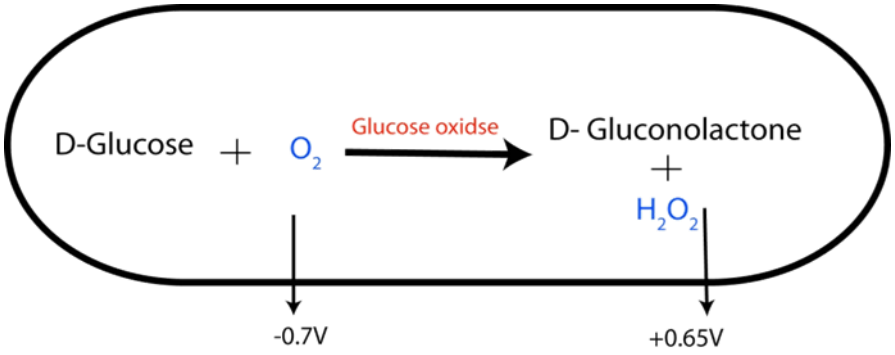
The magnitude of current is proportion to the partial pressure of oxygen (pO2) dissolved in the sample. In medicine, this is referred to as the oxygen tension, which reflects the level of oxygen in the arterial blood.

https://conductscience.com/clark-oxygen-electrode/

Glucose biosensor is created from a modification of the Clark oxygen electrode. The enzyme electrode is the modification of the Clark electrode, which resulted in the first commercially available glucose biosensor.

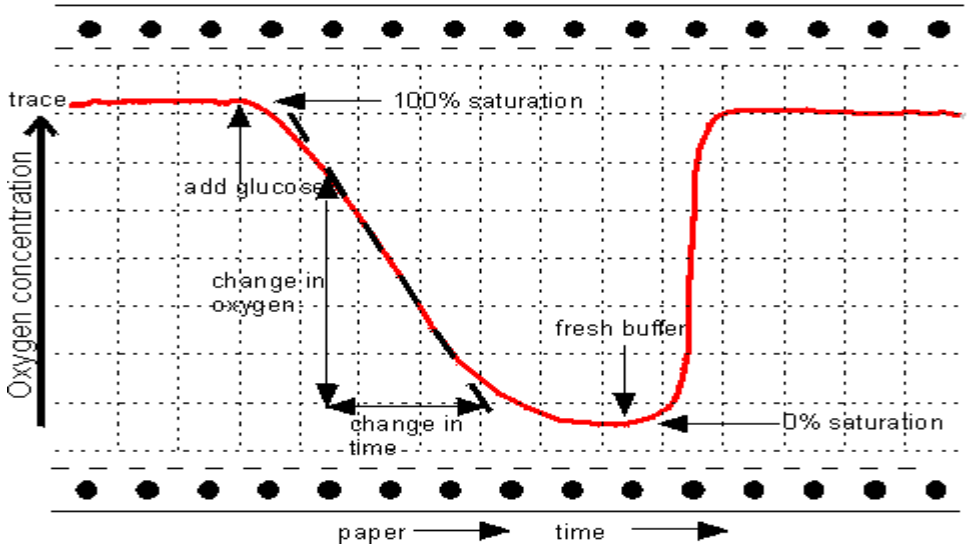


Both electrodes are bathed in saturated KCl, separated from the bulk solution by an oxygen-permeable membrane, analyte solution is separated from the biocatalyst by another membrane permeable to the substrate(s) and product(s).



When a potential of -0.7 V, relative to the Ag/AgCl electrode is applied to the platinum cathode, a current proportional to the oxygen concentration is produced.

The rate of this electrochemical reduction therefore depends on the rate of diffusion of the oxygen from the bulk solution, which is dependent on the concentration gradient and hence the bulk oxygen concentration.



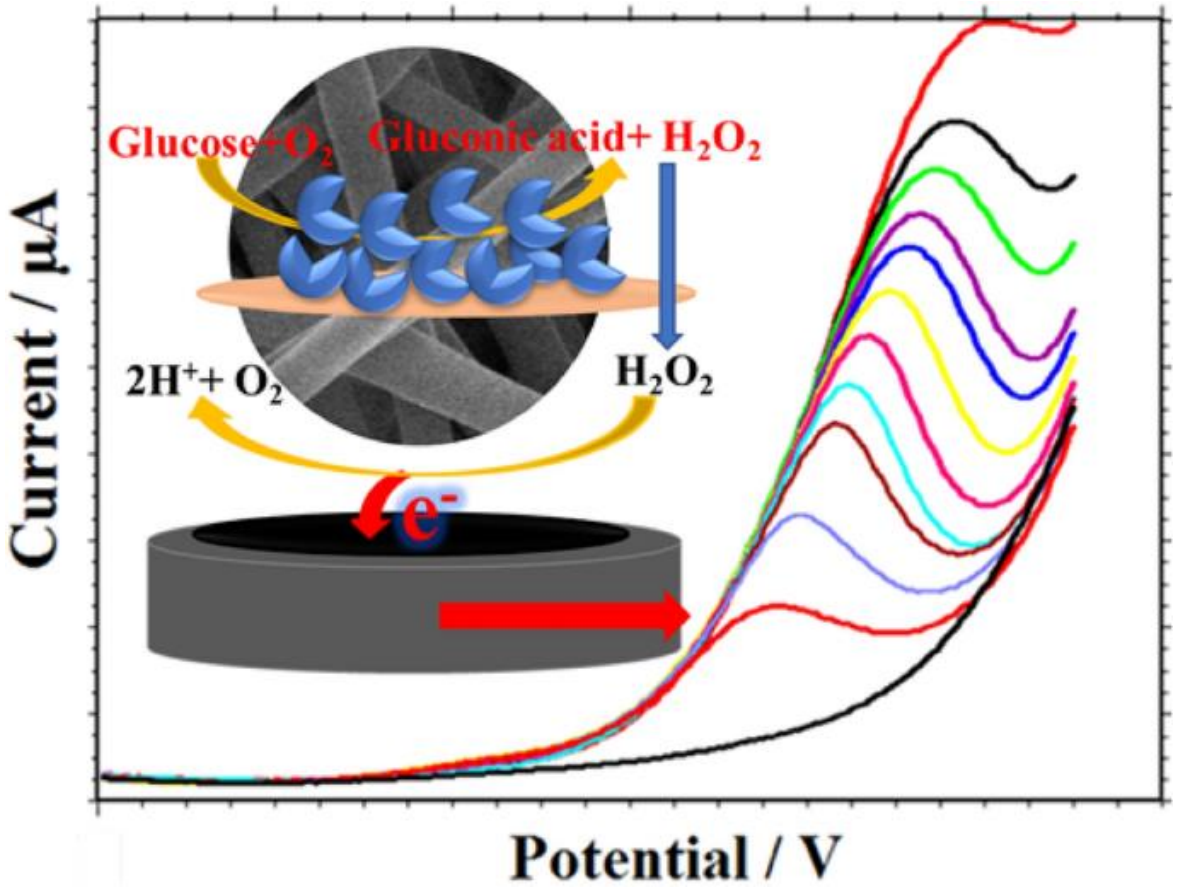
The steady rates of oxygen depletion may be used to generate standard response curve to determine glucose in the samples.

This biosensor is normally about 1 cm in diameter but has been scaled down to 0.25 mm dia using a Pt wire cathode within a silver plated steel needle anode and utilizing dip-coated membranes.

An alternative method for determining the rate of this reaction is to measure the production of H_2O_2 directly by applying **+0.68 V** to the Pt electrode, relative to the Ag/AgCl electrode, and causing the reactions:

Pt anode H_2O_2 -----→ O_2 + 2H+ + 2e-

Ag cathode 2AgCl + 2e- ------→$2Ag^0$ + 2Cl-



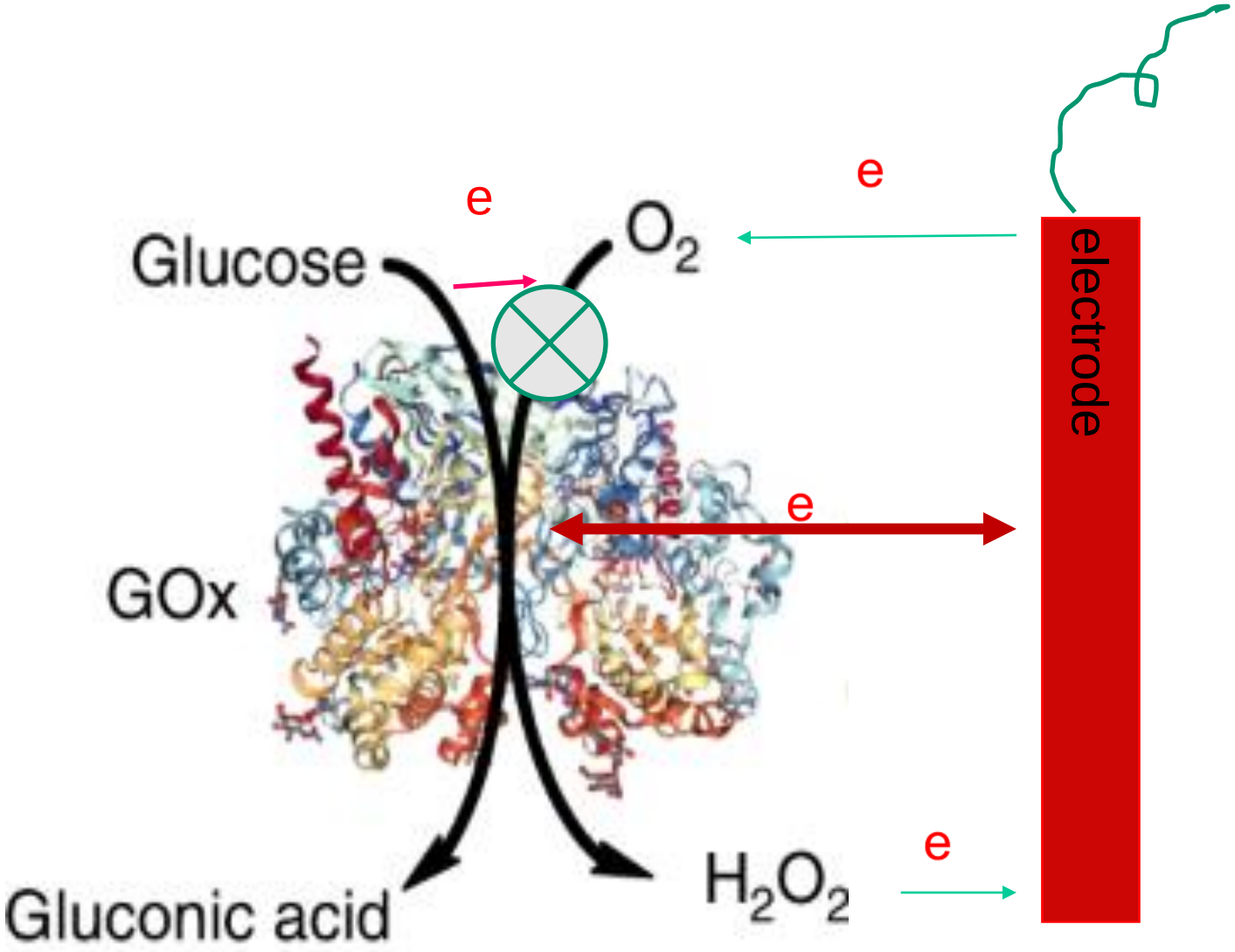
J.Appl Electrochemistry **51**,283–293 (2021)

A large number of enzymes use oxygen as electron acceptor and produce hydrogen peroxide as byproduct (e.g. glucose oxidase, cholesterol oxidase, alcohol oxidase, L-aminoacid oxidase and many more). Among the redox enzymes, oxidase can catalyze the oxidation of substances by oxygen, and dehydrogenase can catalyze the removal of hydrogen from material molecules.

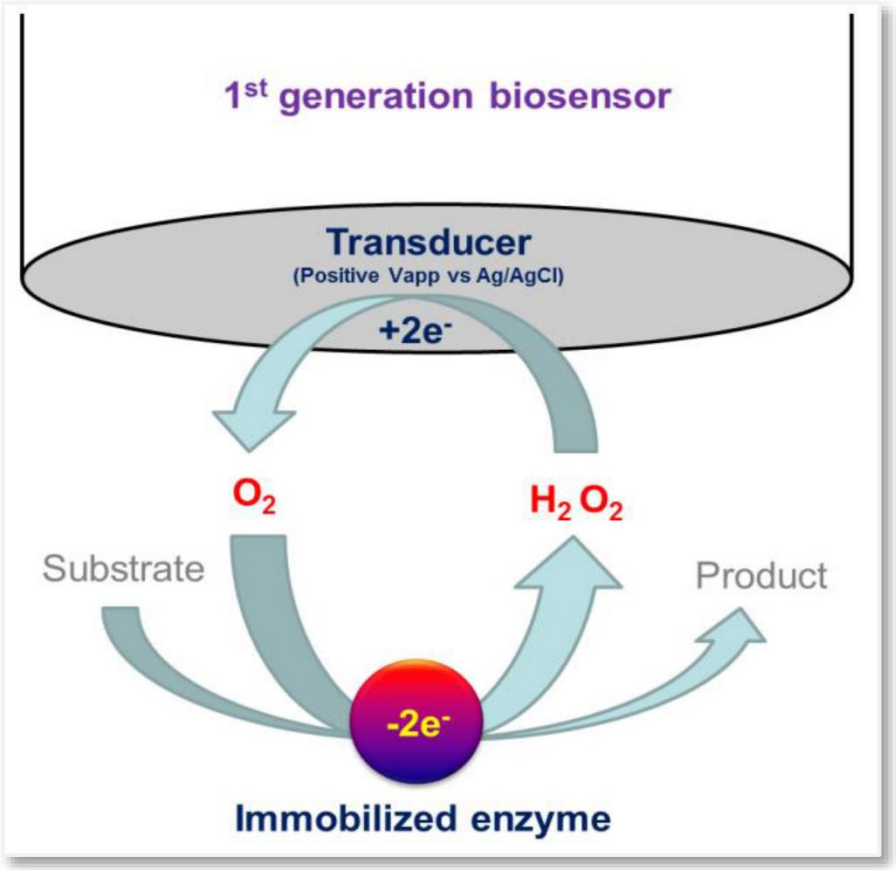
However, the requirement of high over-potential to oxidize hydrogen peroxides (+0.65V) sometimes affects the specificity of the constructed biosensors. This is because, at this high over-potential many molecules present in the biological samples become susceptible for oxidation that impairs the specificity of the sensor.

The major problem with these biosensors is their dependence on the dissolved oxygen concentration or high over potential to oxidize hydrogen peroxides.

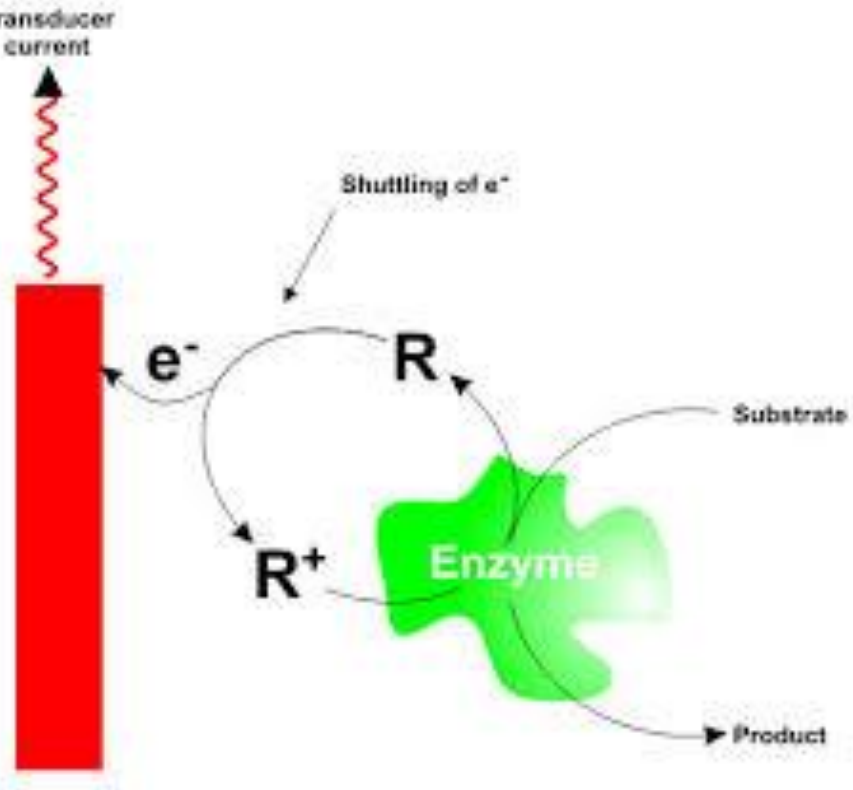
WHAT IS THE SOLUTION ??



Oxidation reaction



2nd generation biosensor



In the process of shuttling charge between the redox-center and the electrode, the mediator is cycled between its oxidized and reduced states. The process is called Mediating Electron Transport (MET).

MARCUS ELECTRON-TRANSFER THEORY

The feasibility of electron exchange between the redox centers of proteins and the electrodes may be assessed from the electron transfer rate constant (/s):

$$k_{et} \propto e^{[-\beta(d-d_0)]} e^{\left[\frac{-(\Delta G^0+\lambda)^2}{4RT\lambda}\right]}$$

$(\Delta G^o + \lambda)^2 / 4\lambda = \Delta G^*$

$\Delta G^o = \sim -n\ F\ \eta$

Over potential $\eta = E - E^o$
E and E^o are the applied and standard potential, respectively.

d = distance of electron transfer.

d_o = distance of closest contact.

λ = re-organization energy for electron transfer (energy it would take to force the reactants to have the same nuclear configuration as the products without letting the electron transfer).

ΔG^o = Gibbs free energy

ΔG^*: activation energy

β = coupling constant (typically in 8.5-11 nm^{-1}

Distance and Driving force

Marcus and Sutin 1985, Biochim. Biophys.Acta – Rev. Bioenerg. 811, 265–322

2nd generation amperometric biosensors

From the above Marcus theory, we see that the ways to facilitate the electron transfer between an enzyme and an electrode surface include:

- Decreasing “d” by using small **electron mediator** to relay the electron between the electrode and enzyme.,
- Decreasing λ by employing a mediator having a fast self-exchange rate.

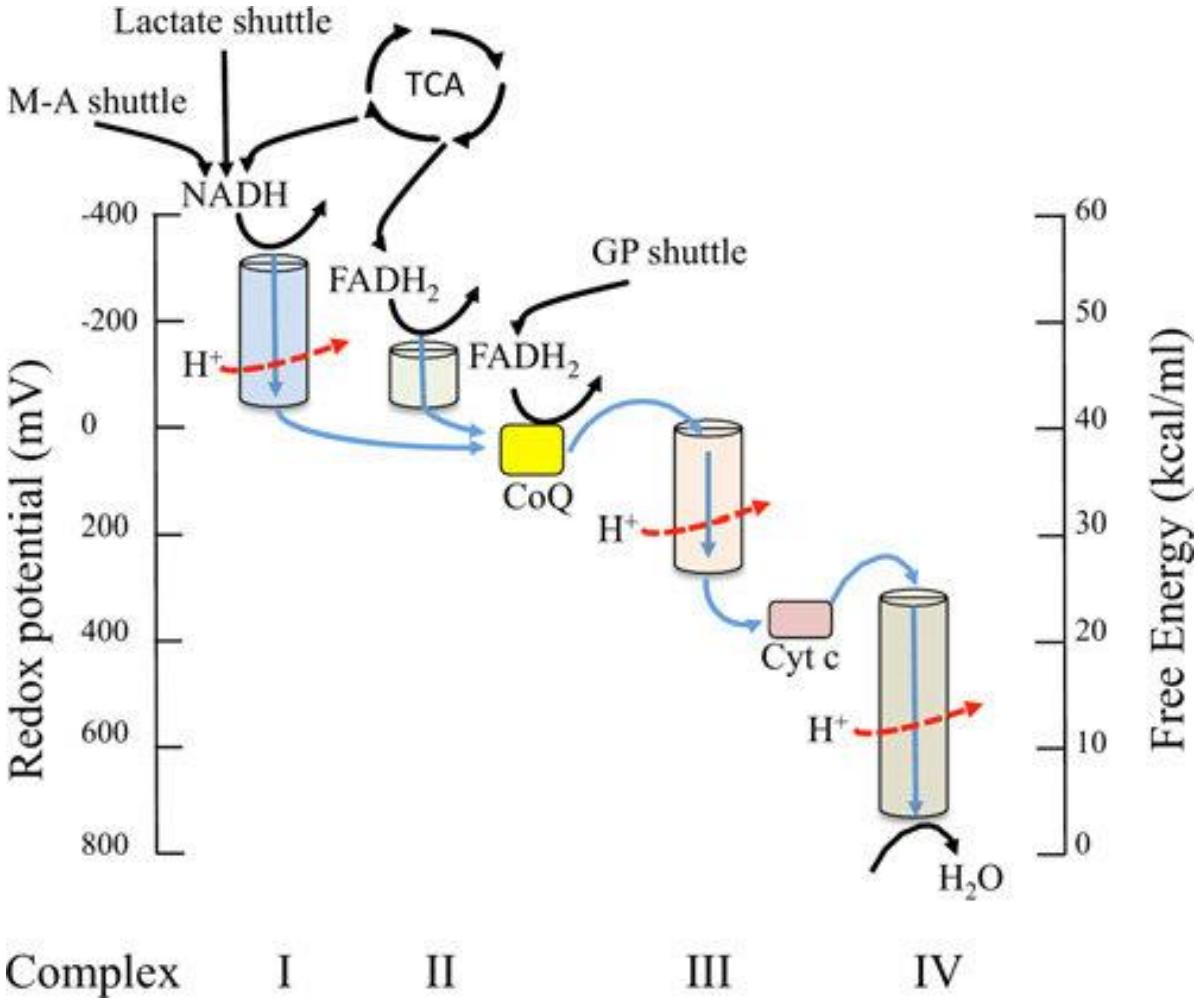
Small chemical compounds (e.g. ferrocene derivatives, ruthenium or osmium complexes and viologens) are frequently used for this purpose

- ‘Mediators' transfer electrons directly to the electrode bypasses the reduction of the oxygen co-substrate.
- Synthetic or biologically-active charge-carriers as intermediates between the redox-center and the electrode.
- These artificial electron donor or acceptor molecules (in case of reductive or oxidative enzymes, respectively), usually referred to as **electron-transfer mediators (ETM),** can be accepted by many redox-enzymes in place of their natural oxidants or reductants.
- They have a wide range of structures, and hence properties, including a range of redox potentials.
- The redox potential of a suitable mediator should provide an appropriate potential gradient for electron-transfer between an enzyme active site and an electrode.

Mediators should fulfill the following requirements:

- The redox potential of the mediator, E^0_M, should be more positive or more negative than the redox potential of the enzyme active site. E^0_E, in the case of oxidative $E^0_M > E^0_E$ and reductive $E^0_M < E^0_E$ bioelectrocatalysis.
- They must react rapidly with the reduced form of the enzyme.
- They must be sufficiently soluble, in both the oxidised and reduced forms, to be able to rapidly diffuse between the active site of the enzyme and the electrode surface.
- This solubility should, however, not be so great as to cause significant loss of the mediator from the biosensor's microenvironment to the bulk of the solution.
- The overpotential for the regeneration of the oxidised mediator, at the electrode, should be low and independent of pH.

Mediator mimics what happen in biology. Here and oxidative process, the respiratory chain



The redox potential increases continuously along the respiratory chain to reach its highest value at oxygen, which therefore has the highest affinity for the electrons and gets to keep them. Reduced oxygen, which recombines with protons to yield water, is the end product of respiration.

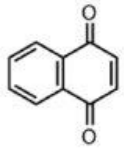
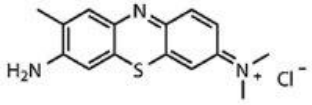
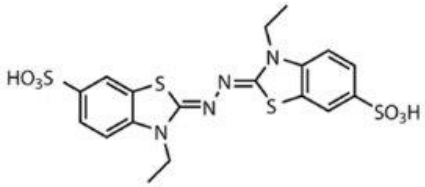
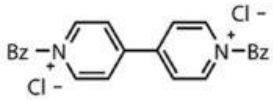
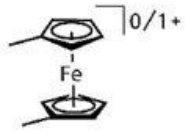
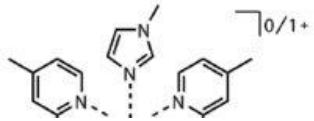
- The reduced form of the mediator should not readily react with oxygen

- The hydrophobic/hydrophilic properties of the mediator and the enzyme.

- The size and shape of the mediator and

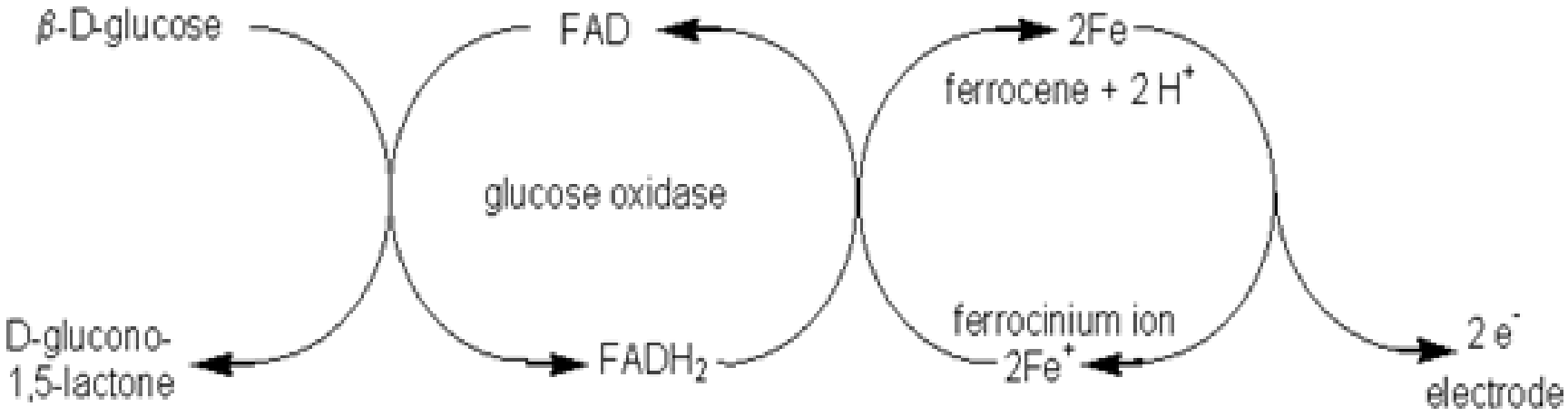
- The electrostatic charge interactions between the mediator and the enzyme.

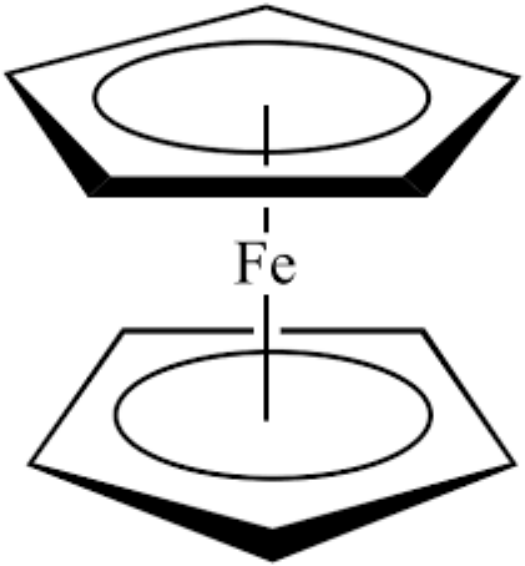
Some mediators commonly used in biosensor area

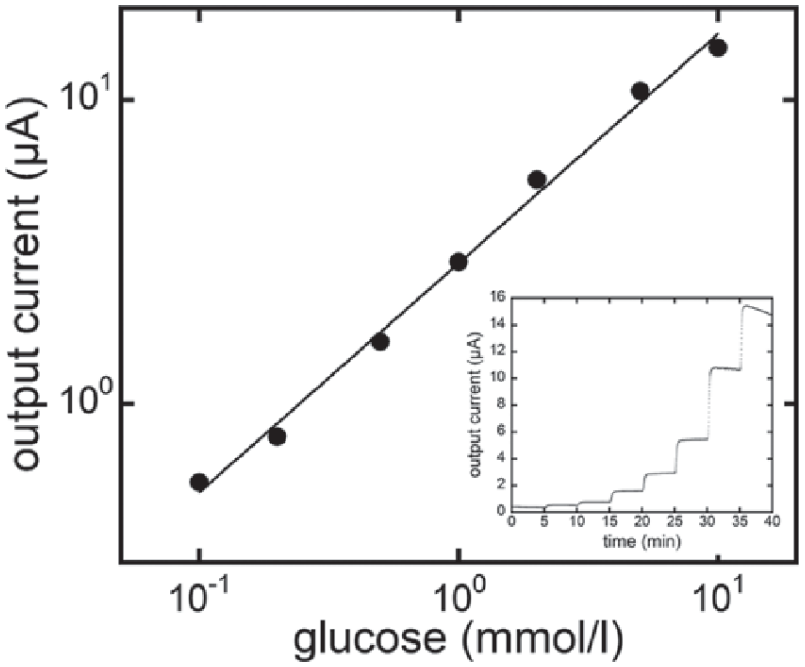
Mediator name	E°/V *vs.* SHE	Structure	Ref.
Benzoquinone	0.280		35
Naphthoquinone	− 0.145		36
Toluidine blue-O	0.027		19
2,2′-azino-bis(3-ethylbenzo-thazoline-6-sulphonc acid) (ABTS)	0.670		37
1,1′-Dibenzyl-4,4′-bipyridyl (benzyl viologen)	−0.480		38
1,1′-Dimethyl-ferrocene	0.515		28
Os(im)(dm-bpy)$_2$Cl	−0.026		26

The Os is coordinated by bpy rings (not shown)

Example : Glucose sensor based on Ferrocene mediated oxidation of glucose by GOx:







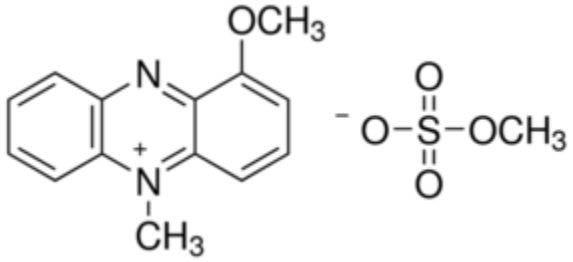
Example 2: Amperometric DNA sensor using the pyrroquinoline quinone glucose dehydrogenase-avidin* conjugate.

Ikebukuro et al. *Biosensors and Bioelectronics* 17,1075-1080(2002).

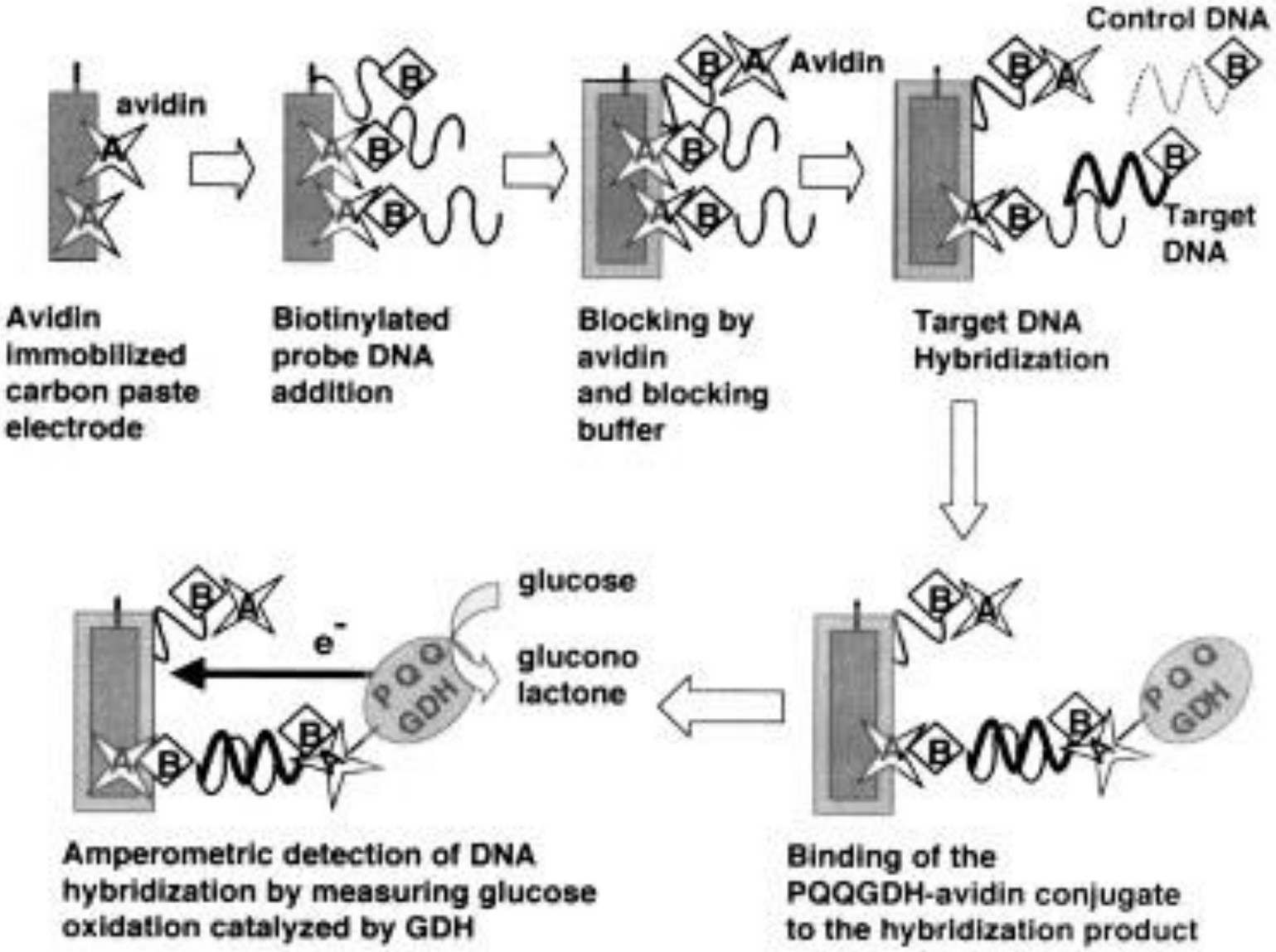
Aim: to detect the DNA sequence of the *invA* virulence gene from the pathogenic bacterium *Salmonella* (that cause typhoid/diarrhea).

- *Target and probe DNA sequence:*
  - *Target DNA: 5'-bio-TCGGCATCAATACTCATC-3'.*
  - *Probe DNA: 5'-bio-GATGAGTATTGATGCCGA-3'*

Electron transfer mediator:
1-methoxyphenazine methosulphate (m-PMS).



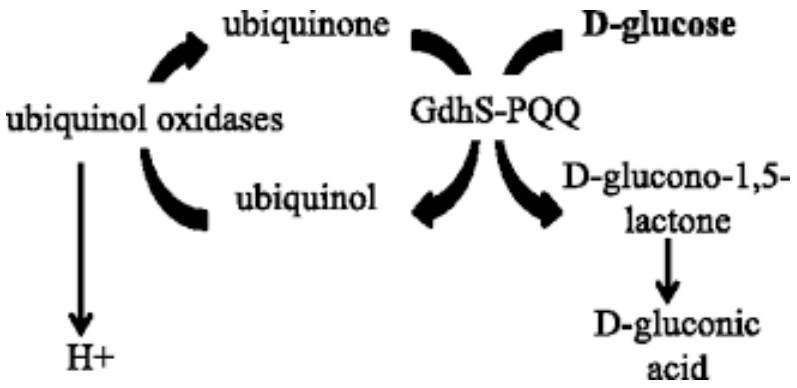
*Avidin is a protein produced in the eggs of birds, reptiles and amphibians. This tetrameric protein contains four identical subunits, each of which can bind to biotin (Vitamin B_7, vitamin H) with a high degree of affinity and specificity. The dissociation constant of avidin is measured to be $K_D \approx 10^{-15}$ M, making it one of the strongest known non-covalent bonds.

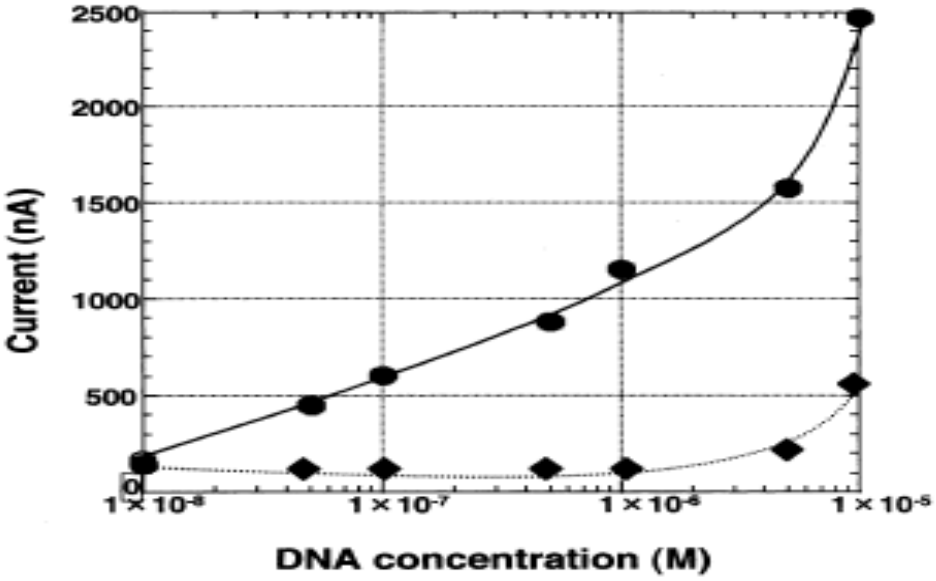


This (PQQ)GDH/avidin conjugate based DNA biosensor is highly sensitive and selective to the target *Salmonella inv*A virulence gene.

Circles, the target DNA (from *Salmonella).*

Diamonds, the control DNA, a random sequence.





The sensor response increased with the addition of glucose and in the presence of 6.3 mM glucose the response increased with increasing DNA in the range 5.0×10^{-8}-1.0×10^{-5} M.

This DNA biosensor would be applicable for single nucleotide polymorphism detection