ELECTROCHEMICAL DETECTION OF ABL1 TYROSINE KINASE ACTIVITY AND INHIBITION

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Protein kinases

- enzymes that catalyse the chemical **addition** of a **phosphate** group from an **ATP** molecule to a **substrate** protein (phosphorylation).

Phosphorylation

- is an important mechanism in **transduction of extracellular signals** to the cell interior;
- responsible for the **regulation of cell** proliferation, differentiation and transformation;
- uncontrolled signalling frequently leads to **diseases** such as cancer.

Abl1 - tyrosine kinase (Abl1-TK)

- **Bcr-Abl tyrosine kinase** – the biomarker of chronic myeloid leukaemia (CML);
- maintain Abl1-TK activity but is responsible for **uncontrolled signaling**;
- target for drug development.

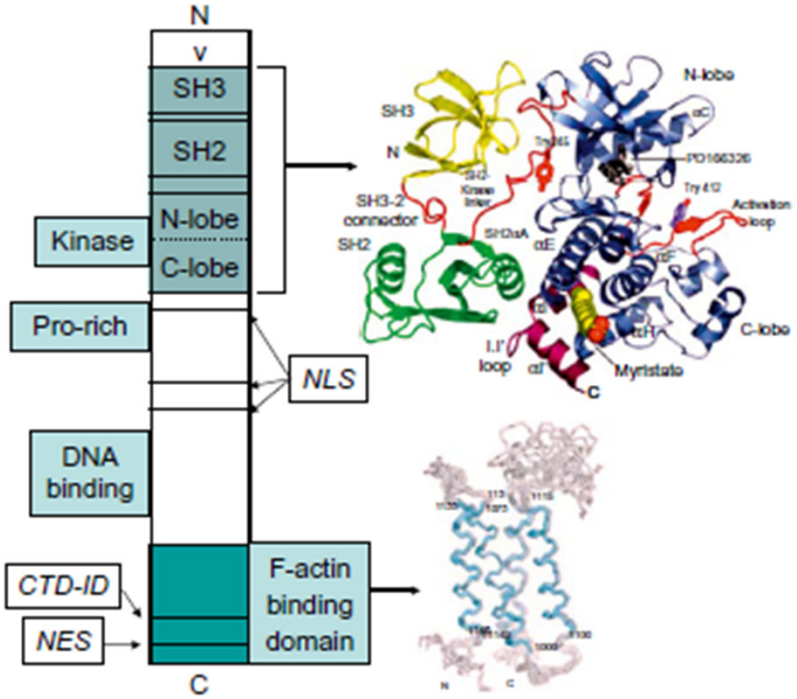
Inhibition of Abl1-TK

- natural and synthetic inhibitors;
- block substrates or ATP binding;
- prevents signalling;
- through degenerated libraries.

OBJECTIVES

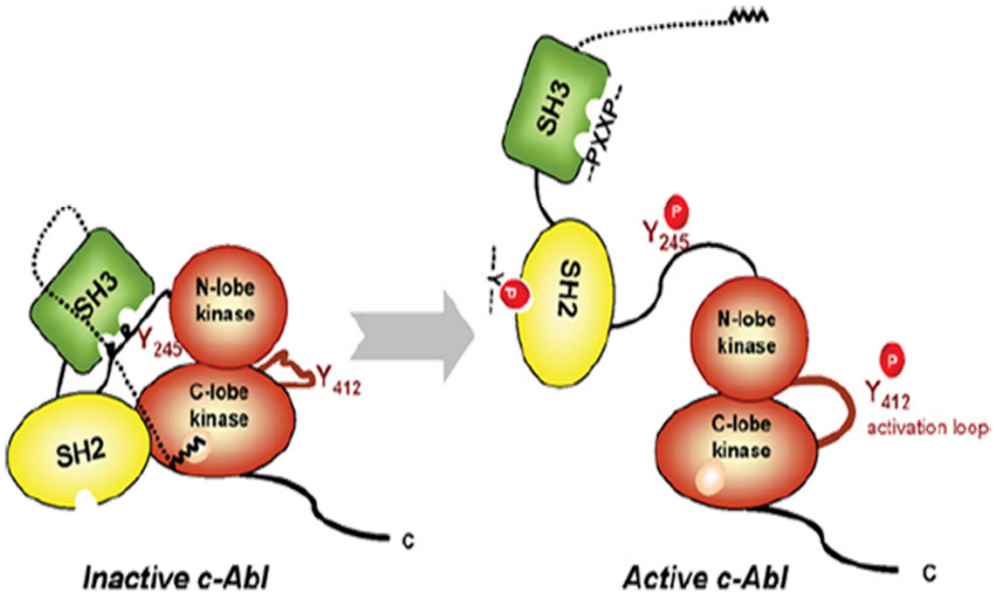
- electrochemically asses Abl1-TK activity;
- factors that lead to kinase-inhibitor interaction;
- development of methodology for detection of inhibitors.

Abl1-TK structure



The kinase domain is highly conserved among kinase family and species;
The N and C lobes contain the catalytic core, where ATP and substrates are binding.

Abl1-TK is activated by (auto)phosphorylation of several serine/threonine and tyrosine residues



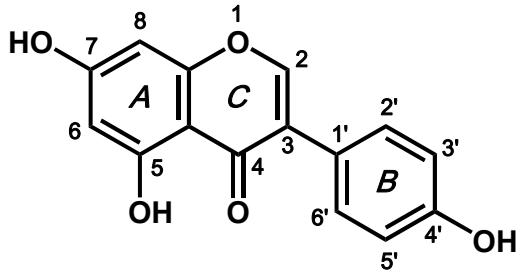
Electrochemical oxidation of Abl1-TK inhibitors

- Natural inhibitors: genistein and apigenin
- Synthetic inhibitors: imatinib mesylate, danusertin and nilotinib

Natural inhibitors

Genistein

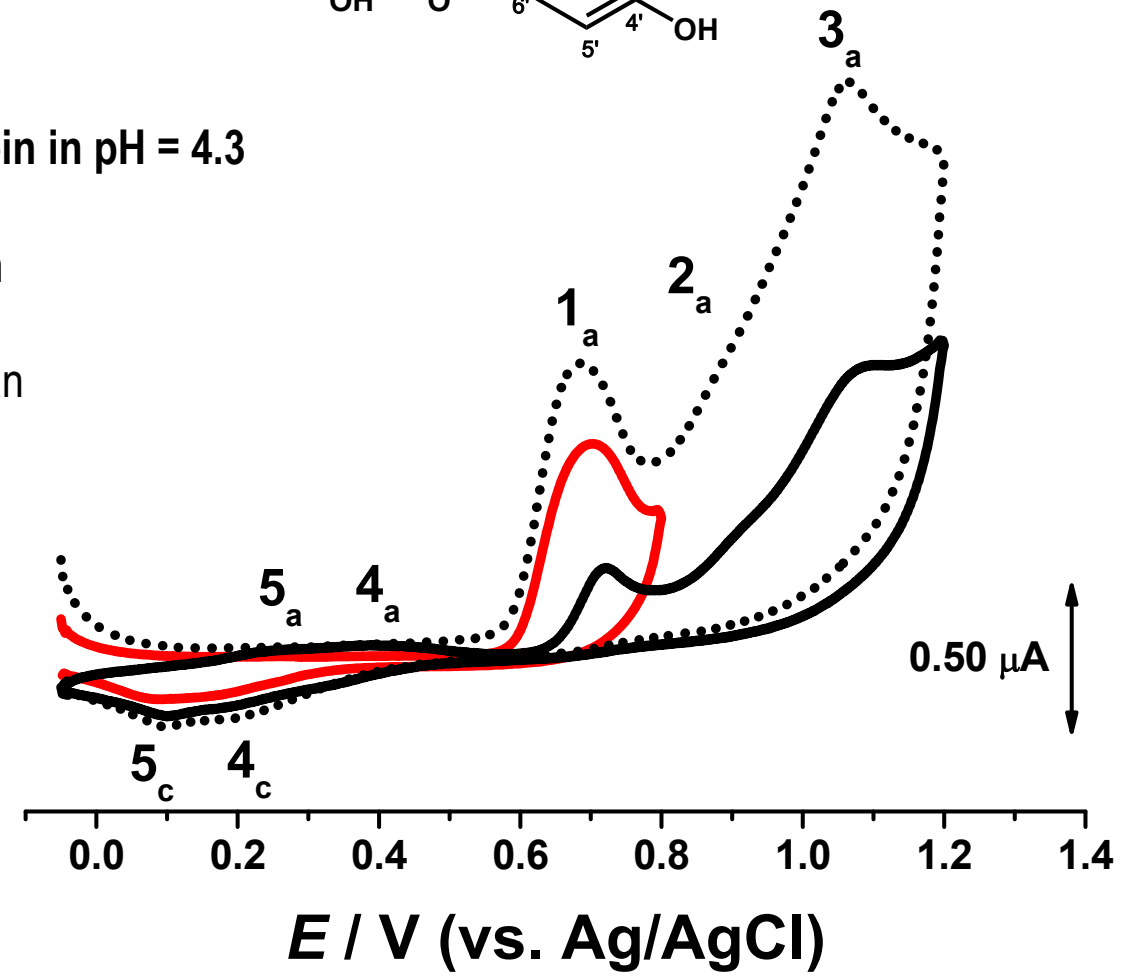
Cyclic voltammetry



GCE; 50 µM genistein in pH = 4.3

(•••) first scan

(—) third scan



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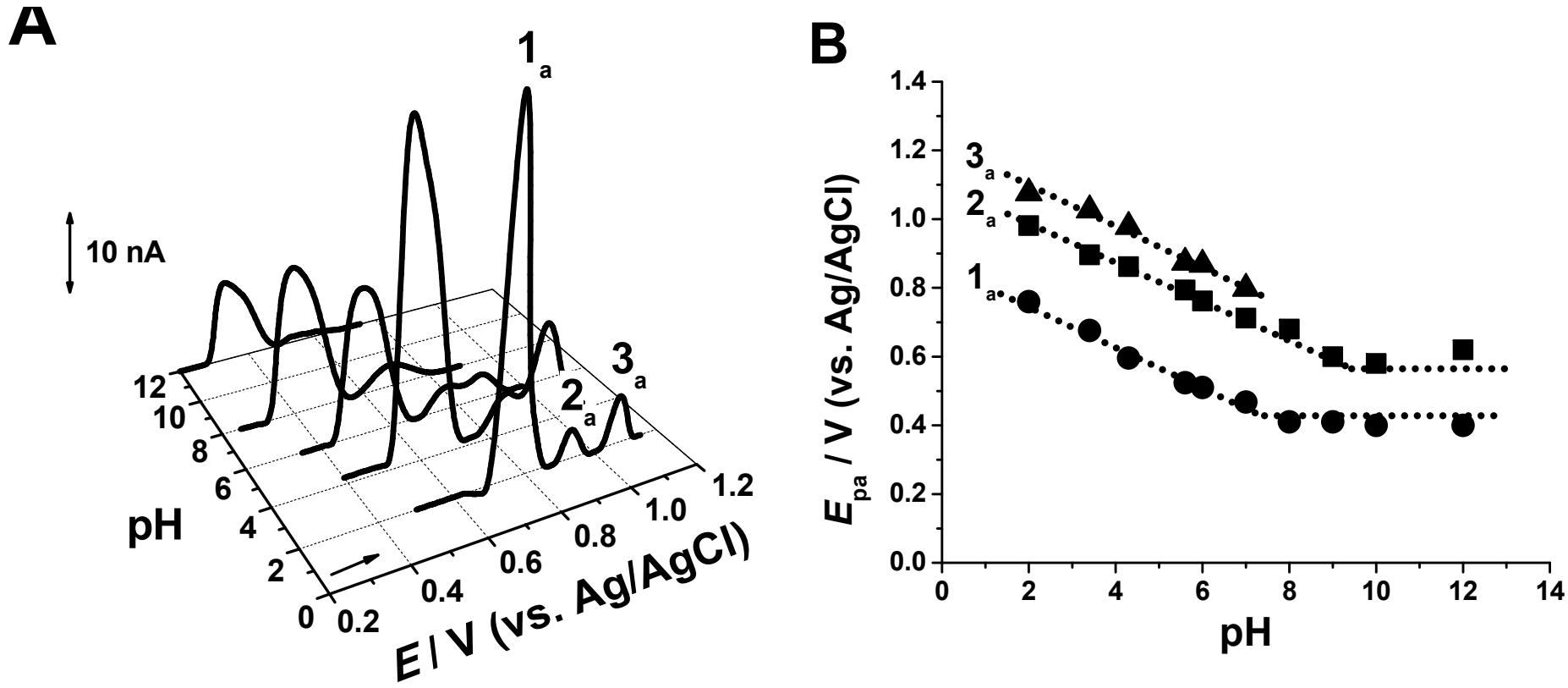
Differential pulse voltammetry

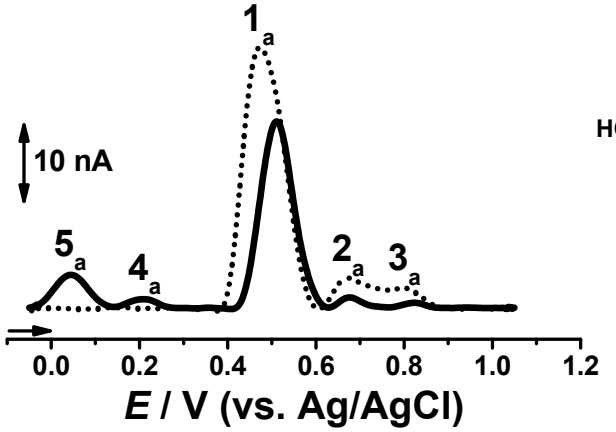
5 µM genistein



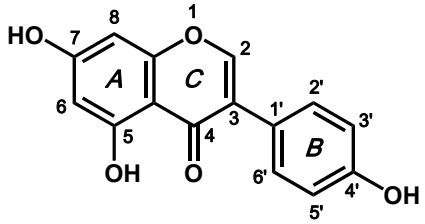
The oxidation of genistein occurs in three consecutive steps, each involving the transfer of one electron and one proton.

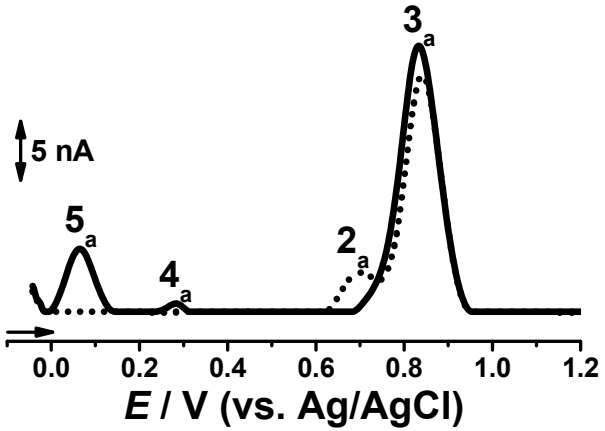
pKa1 ~ 8.0 and pKa2 ~ 10.0

Oxidation mechanism

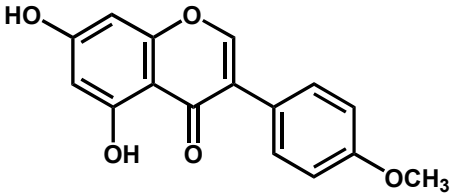


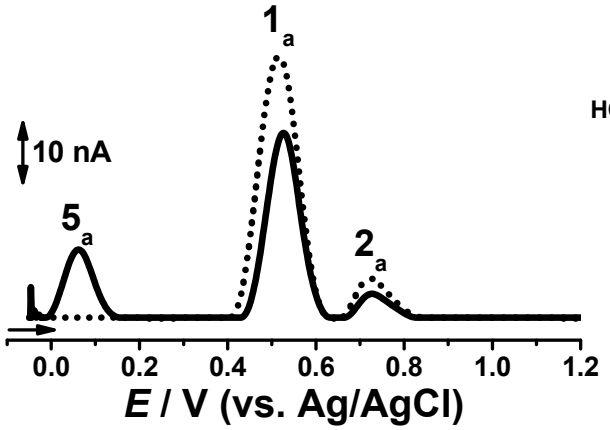
genistein



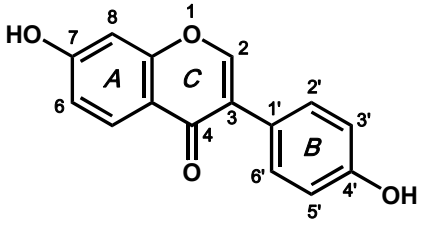


biochanin A





daidzein



The oxidation of genistein involves transfer of electrons and protons from each hydroxyl group in its structure with the formation of quinone-like compounds

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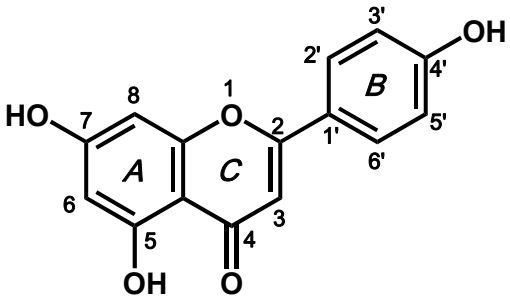
Apigenin

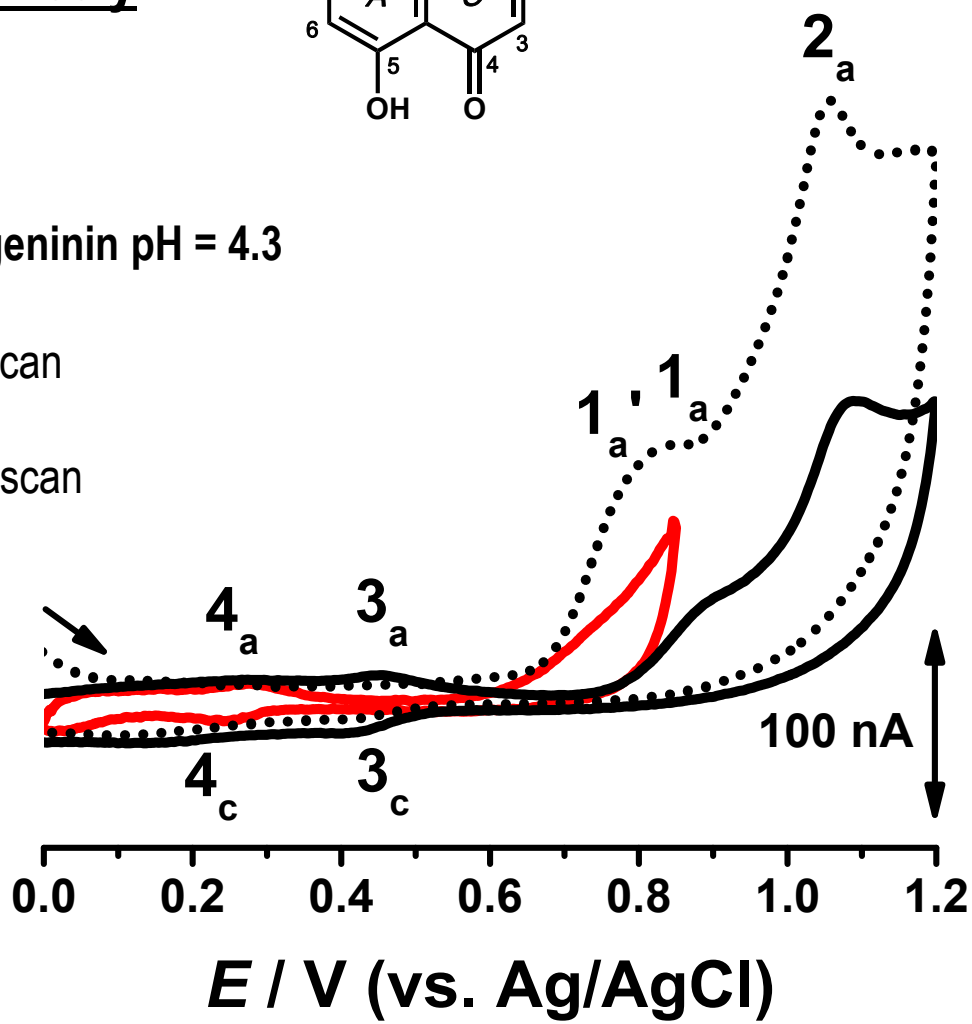
Cyclic voltammetry



GCE; 50 µM apigeninin pH = 4.3

(•••) first scan

(—) third scan



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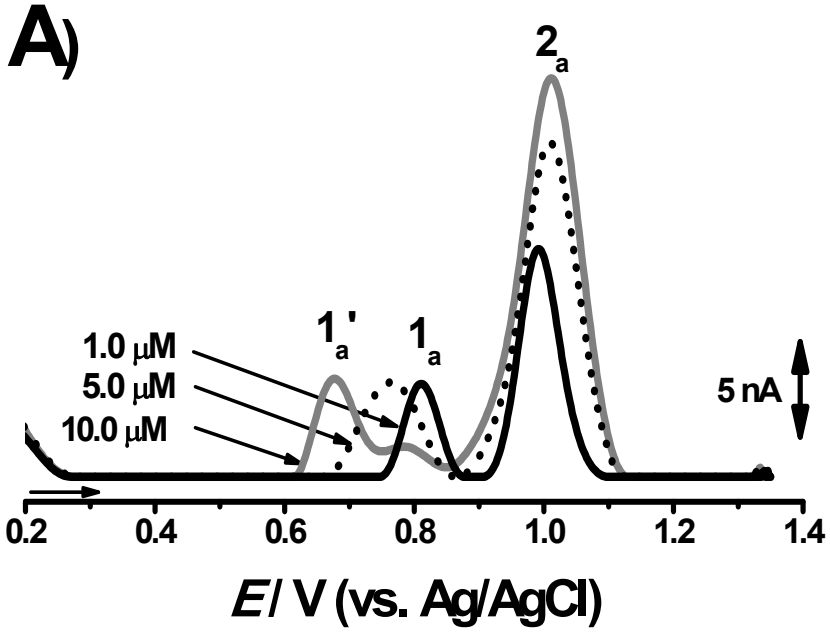
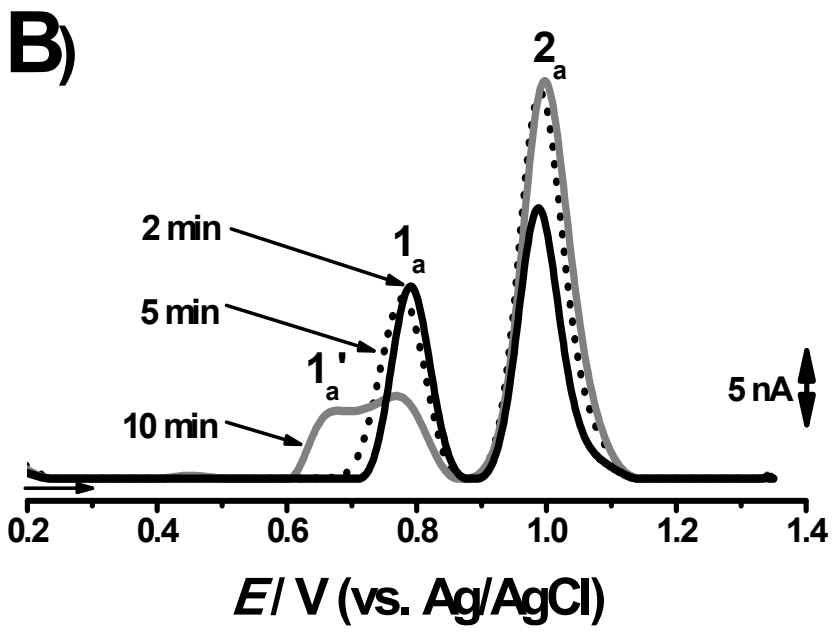
Differential pulse voltammetry

concentration effect
at fixed adsorption time

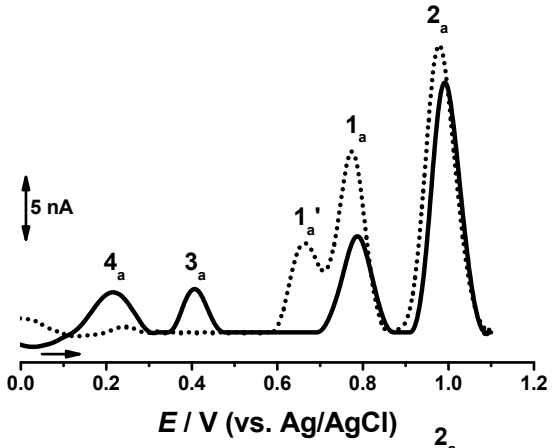


adsorption time effect
at fixed concentration

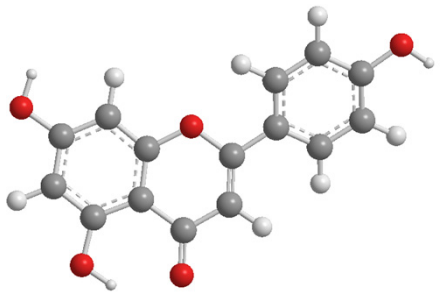


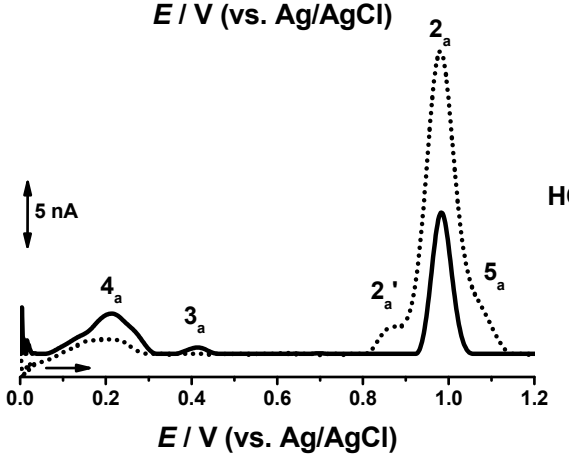
Peaks 1_a' and 1_a are due to different orientations of apigenin molecules at the GCE surface

Comparison between genistein and apigenin

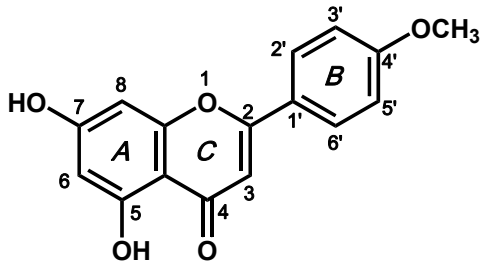


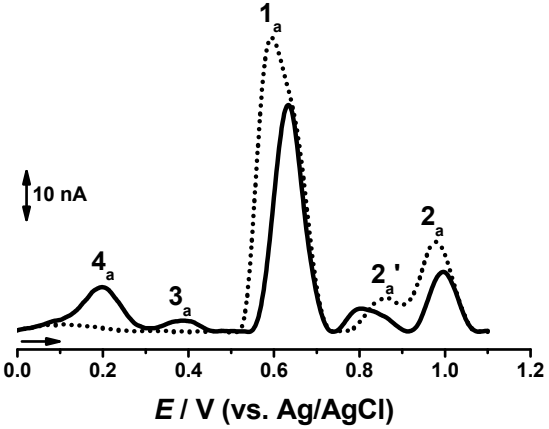
apigenin



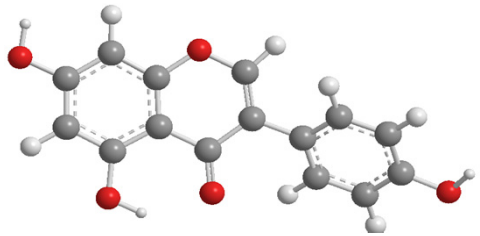


acacetin





genistein

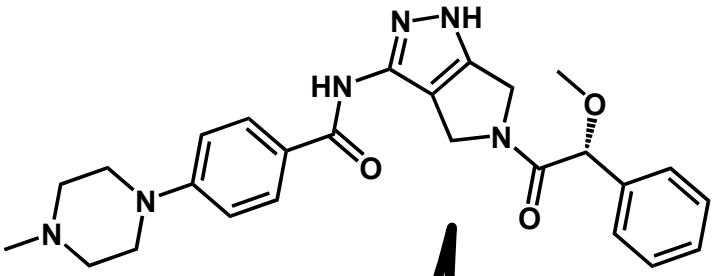


the lower oxidation potential of genistein relative to apigenin is due to the influence of the oxygen atom at position 4 in ring C on the electroactive centre

O.M. Popa, V.C. Diculescu, Electroanalysis, 25 (2013) 1201–1208.
O.M. Popa, V.C. Diculescu, J. Electroanal. Chem., 708 (2013) 108-115.0

Synthetic inhibitors
Danusertib

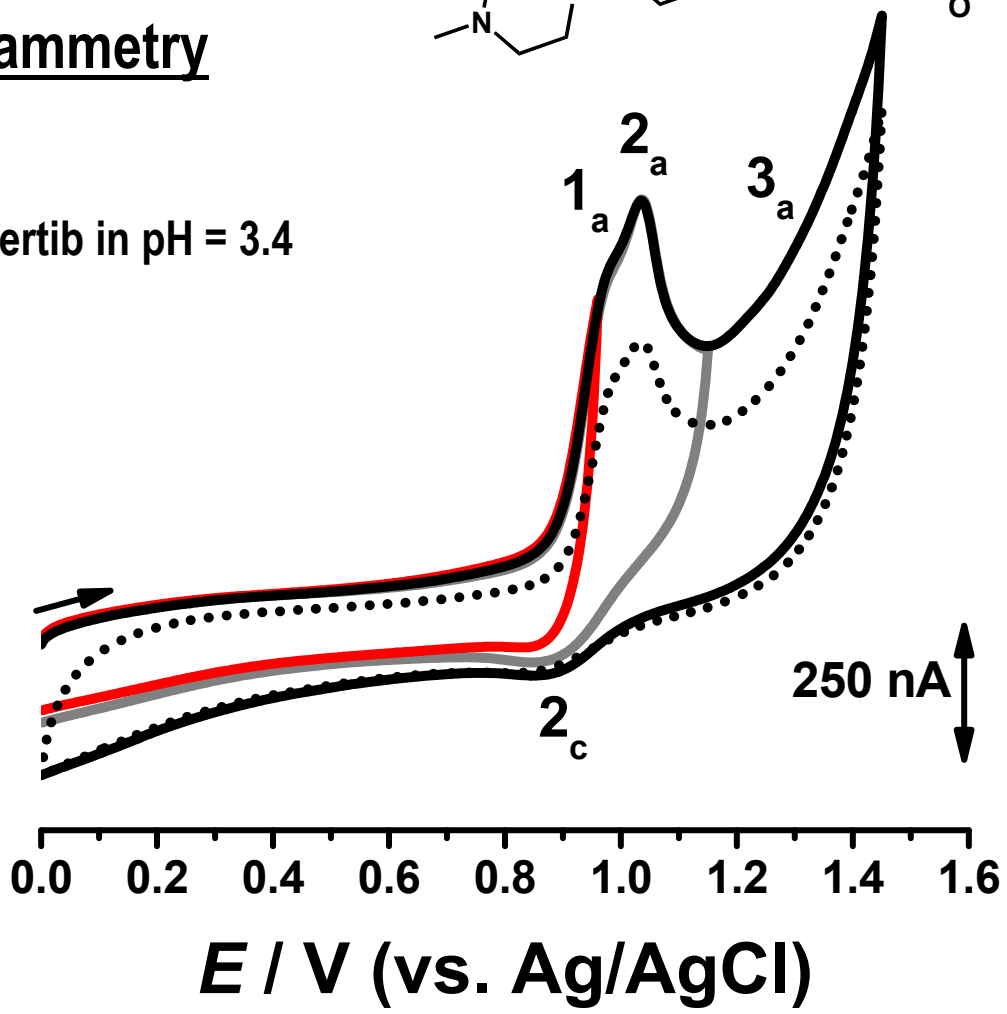
Cyclic voltammetry



GCE; 50 µM danusertib in pH = 3.4

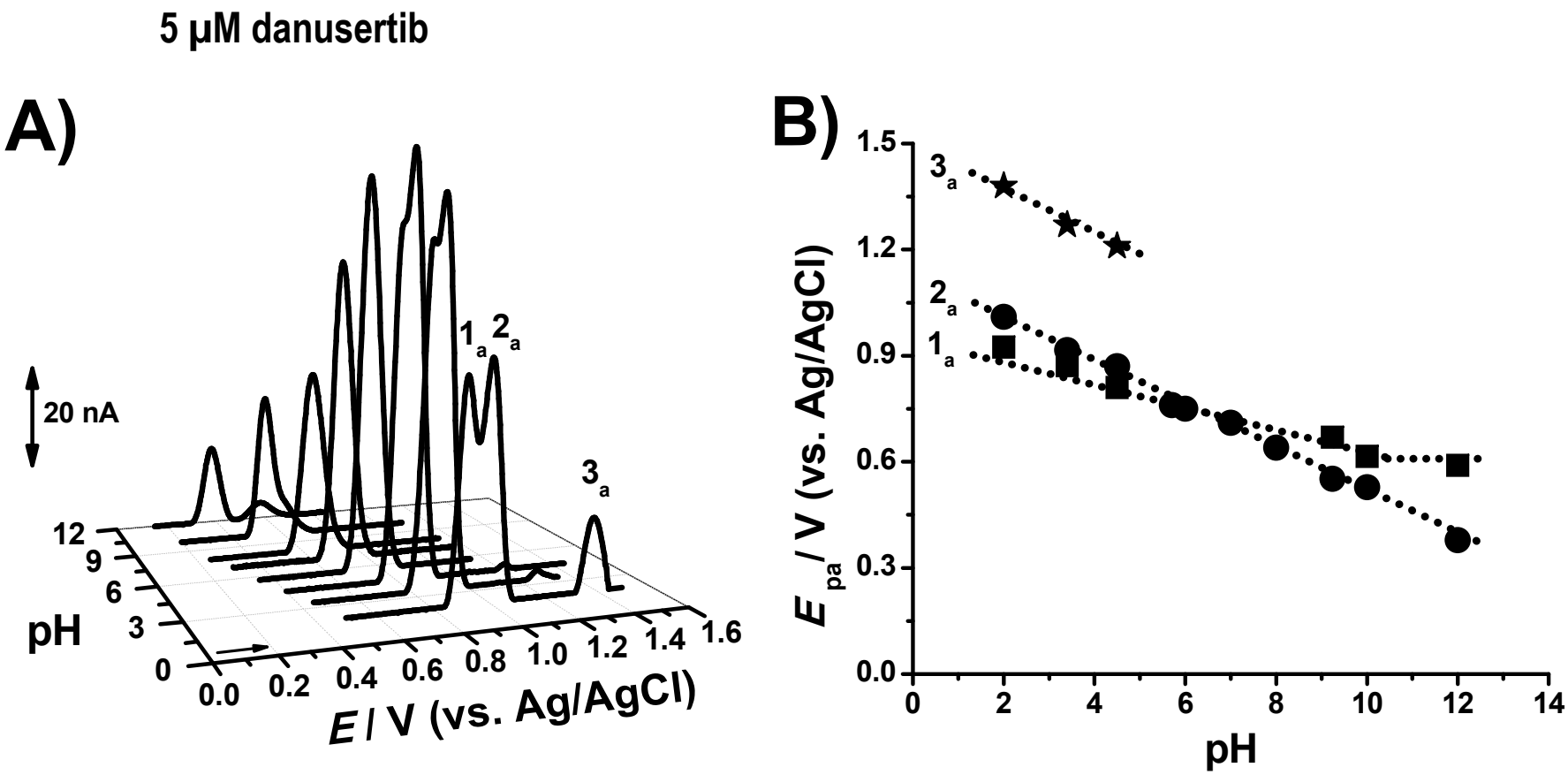
(—) first scan

(•••) third scan



O.M. Popa, V.C. Diculescu, Electrochim. Acta., 112 (2013) 486-492.

Differential pulse voltammetry

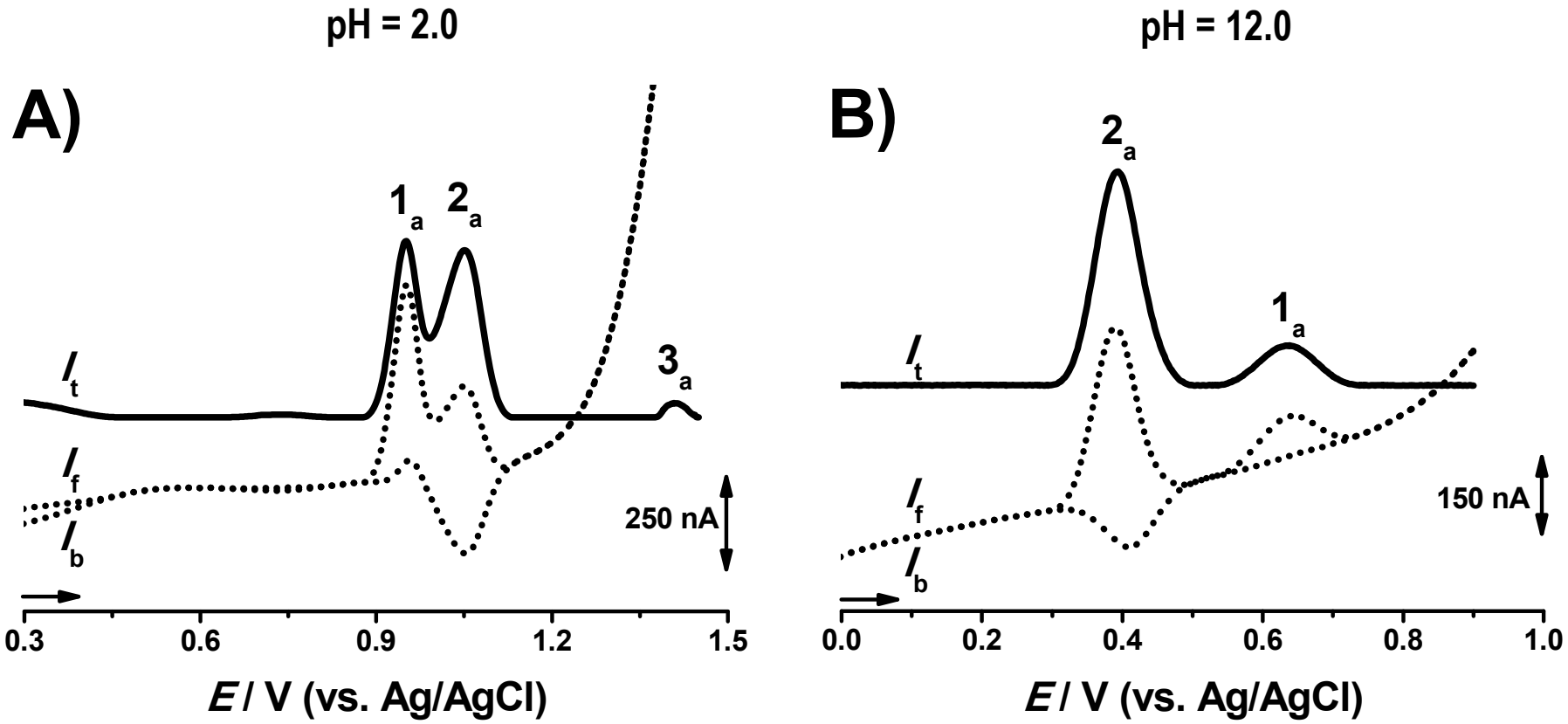


Oxidation at: - peak 1_a with the transfer of 2 electrons and 1 proton

- peaks 2_a and 3_a with 2 electrons and 2 protons

Square wave voltammetry

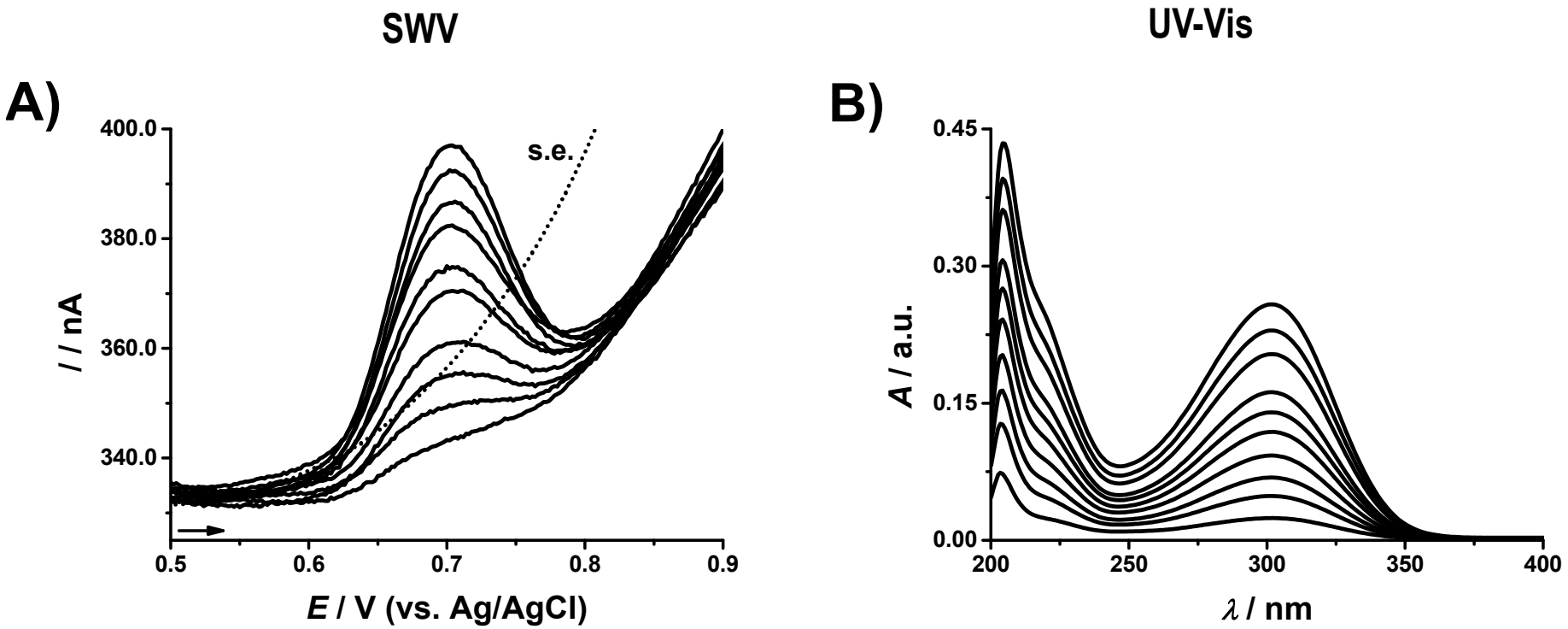
5 µM danusertib



The oxidation at: - peak 1a is quasi-reversible due to a chemical reaction of the oxidation product

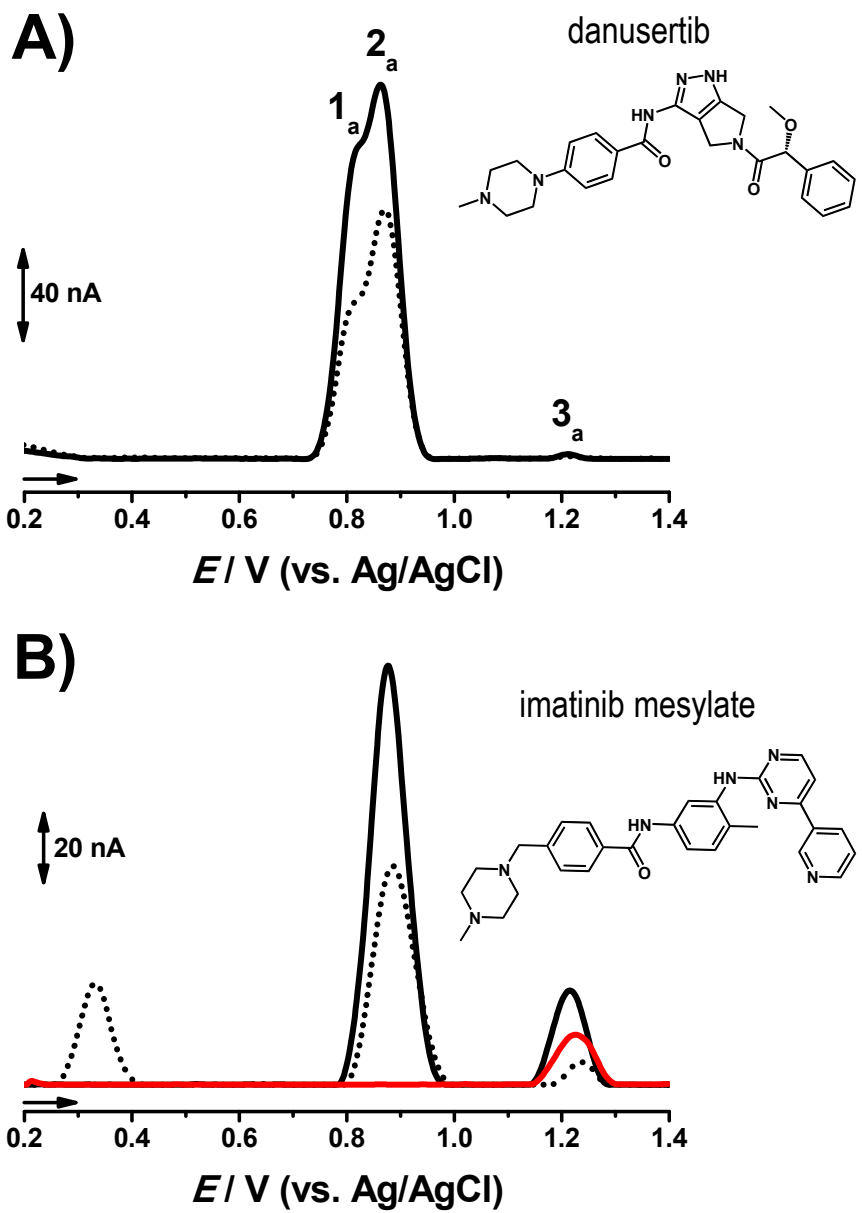
- peak 2a is a reversible process

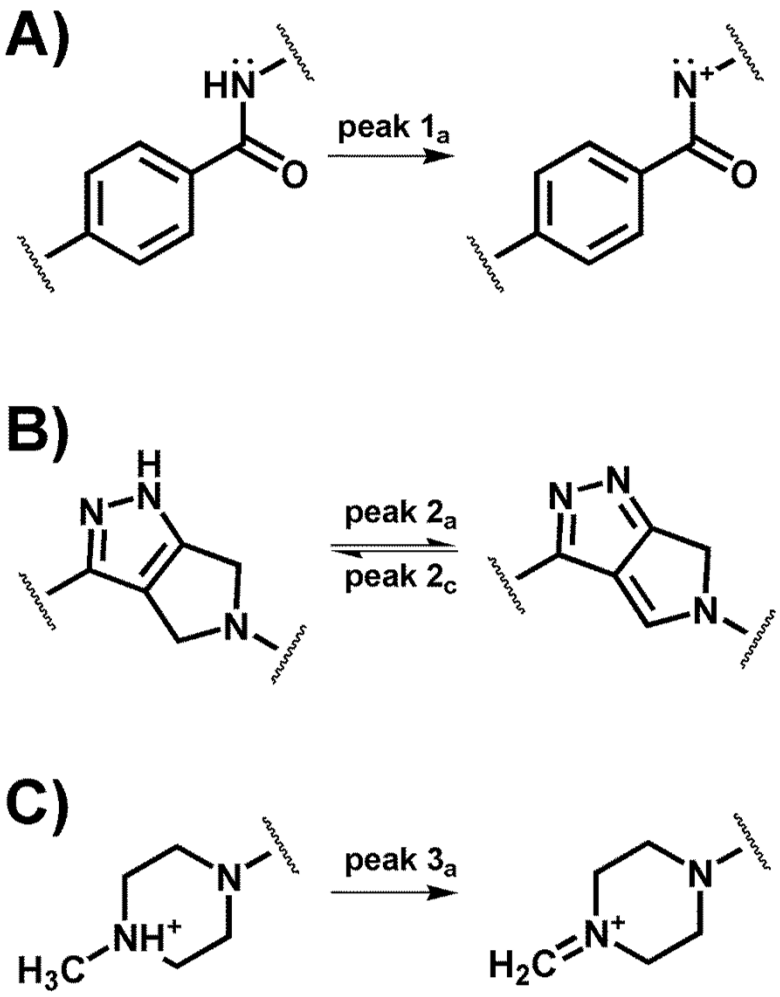
Analytical determination



method	sensitivity	intercept	LOD	LOQ	R^2	S.D.	R.S.D.
SWV	0.099 ± 0.002 nA/nM	-3.789 ± 0.646 nA	27.4 nM	91.2 nM	0.997	0.903 nA	5.4 %
UV-VIS	0.024 ± 0.001 a.u./µM	0.001 ± 0.002 a.u.	0.5 µM	1.6 µM	0.996	0.004 a.u.	0.5 %

Oxidation mechanism



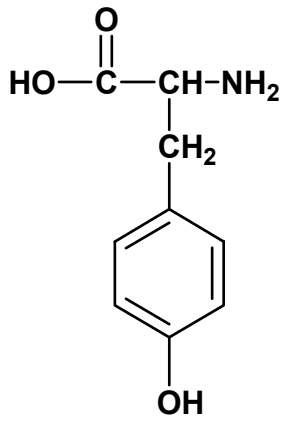


O.M. Popa, V.C. Diculescu, Electrochim. Acta., 112 (2013) 486-492.

Electrochemical oxidation of phosphotyrosine

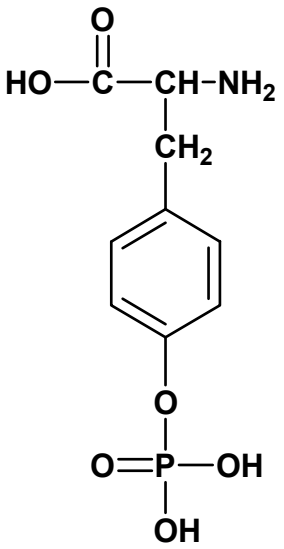
tyrosine – Tyr

A



phosphotyrosine - pTyr

B

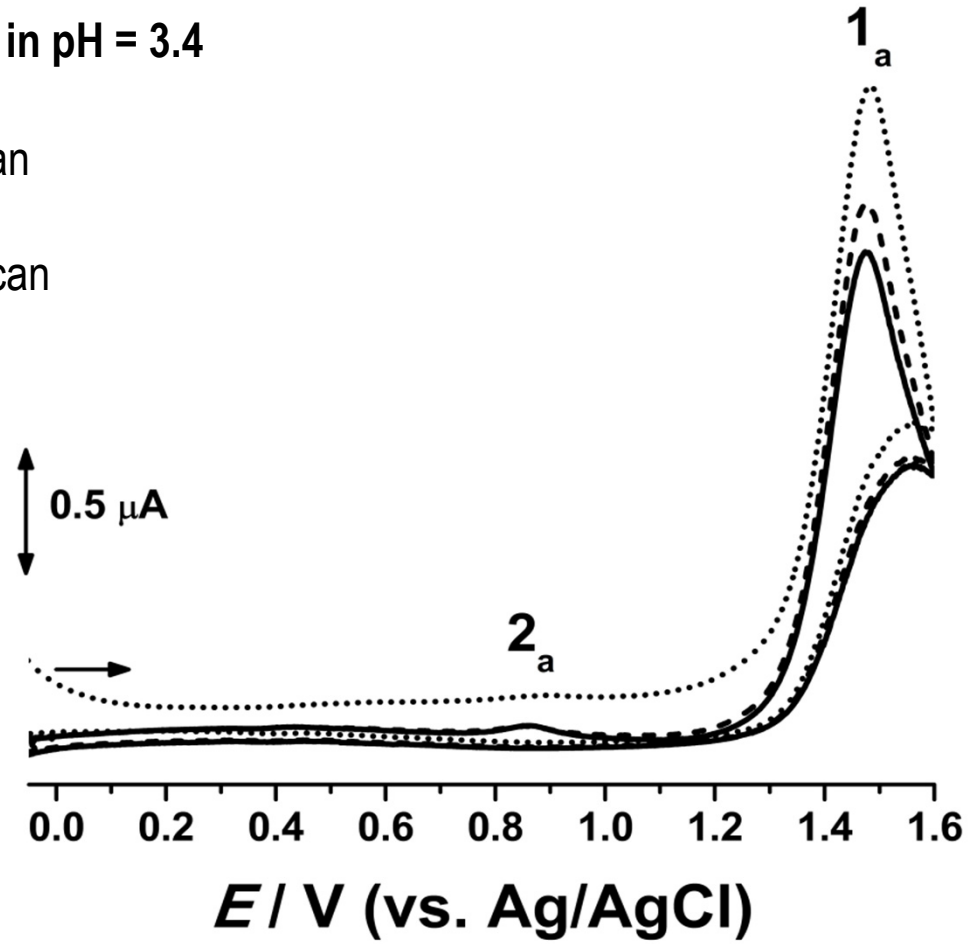


Cyclic voltammetry

GCE; 100 µM pTyr in pH = 3.4

(•••) first scan

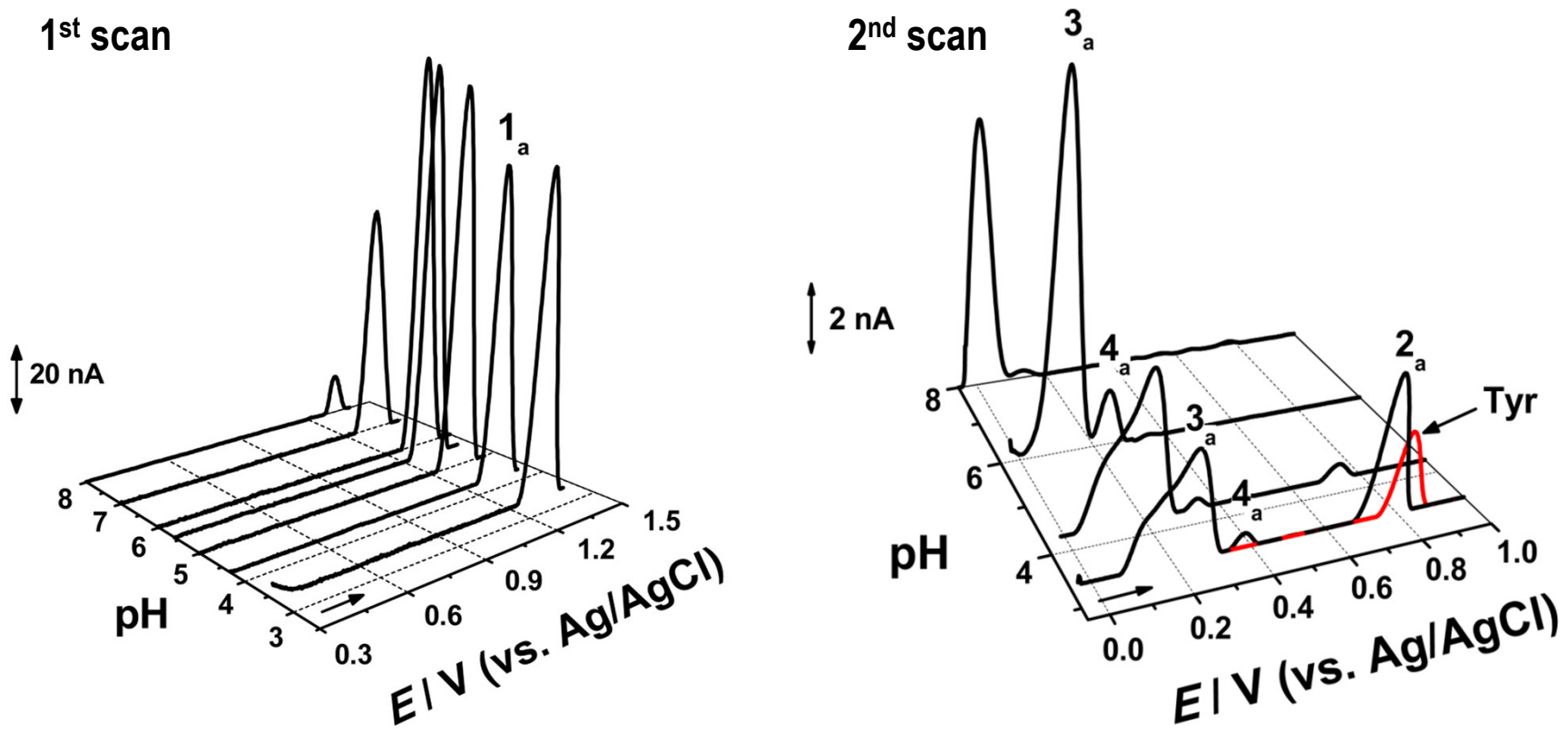
(—) third scan



O.M. Popa, V.C. Diculescu, J. Electroanal. Chem., 689 (2013) 216-222.

Differential pulse voltammetry

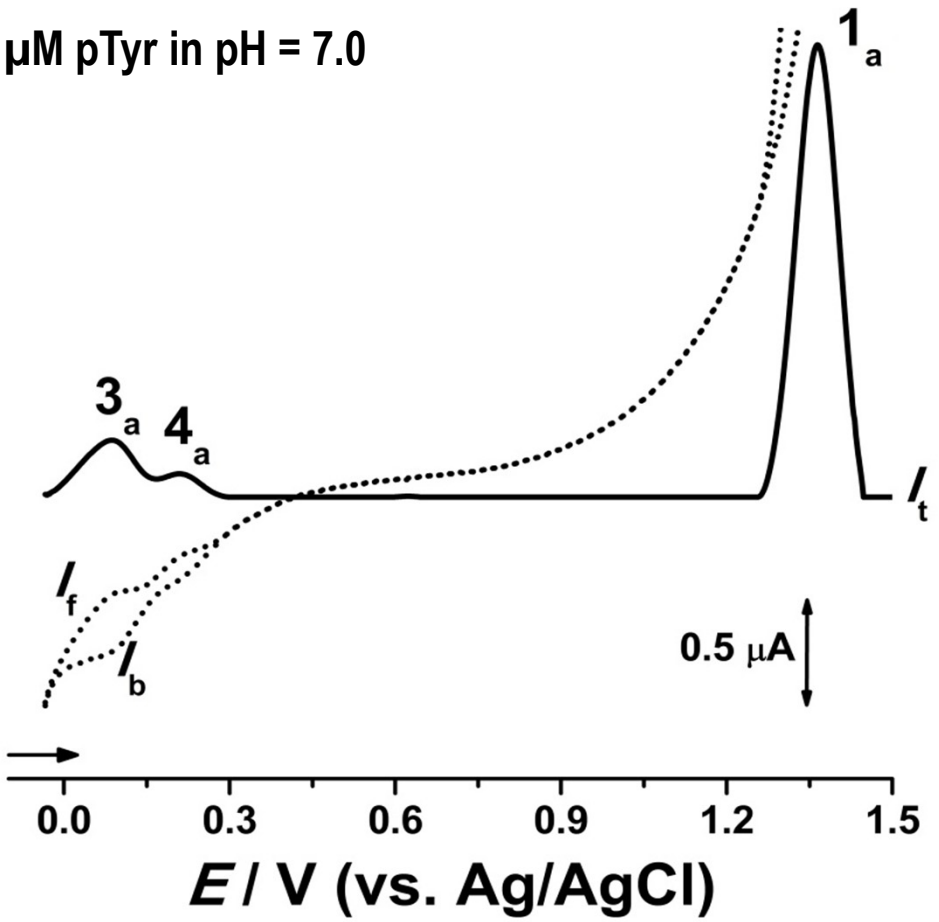
100 µM pTyr



The oxidation of pTyr is pH-independent and involves the transfer of one electron. It leads to the formation of three oxidation products that undergo pH-dependent redox reactions with the transfer of two electrons and two protons.

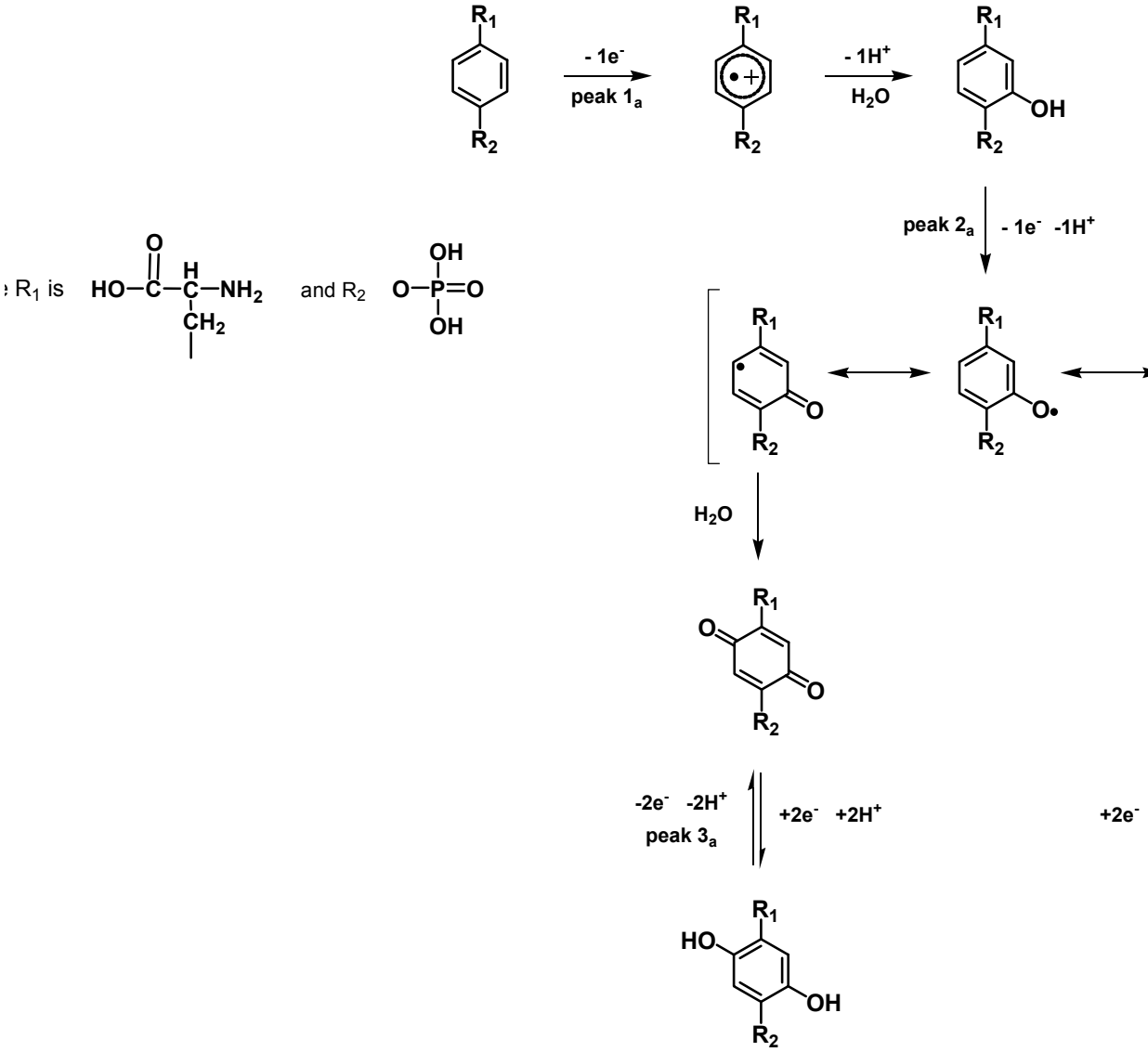
Square wave voltammetry

Second scan in 200 µM pTyr in pH = 7.0



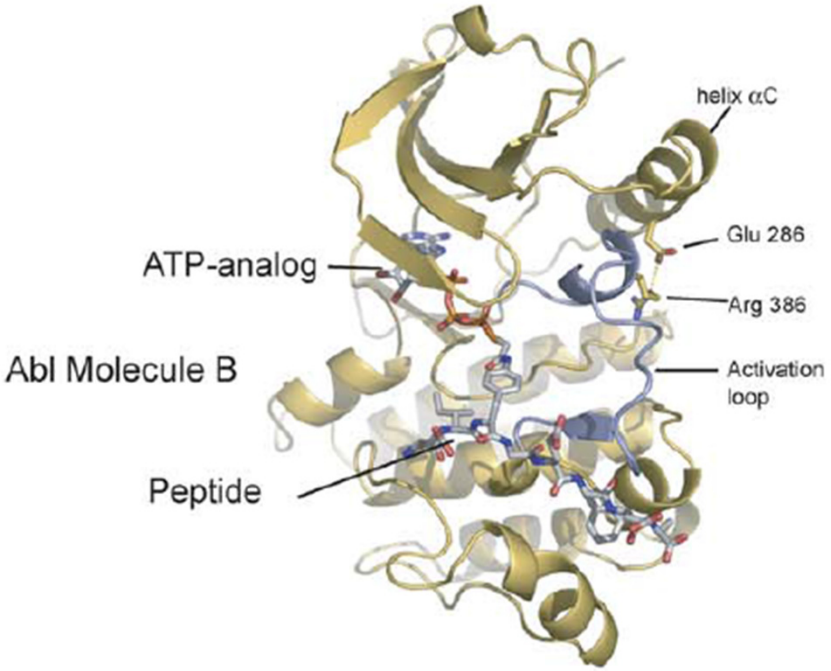
The oxidation products of pTyr undergo reversible redox reactions

Oxidation mechanism



O.M. Popa, V.C. Diculescu, J. Electroanal. Chem., 689 (2013) 216-222.

Electrochemical characterisation of Abl1-TK and interaction with substrate, ATP and inhibitors



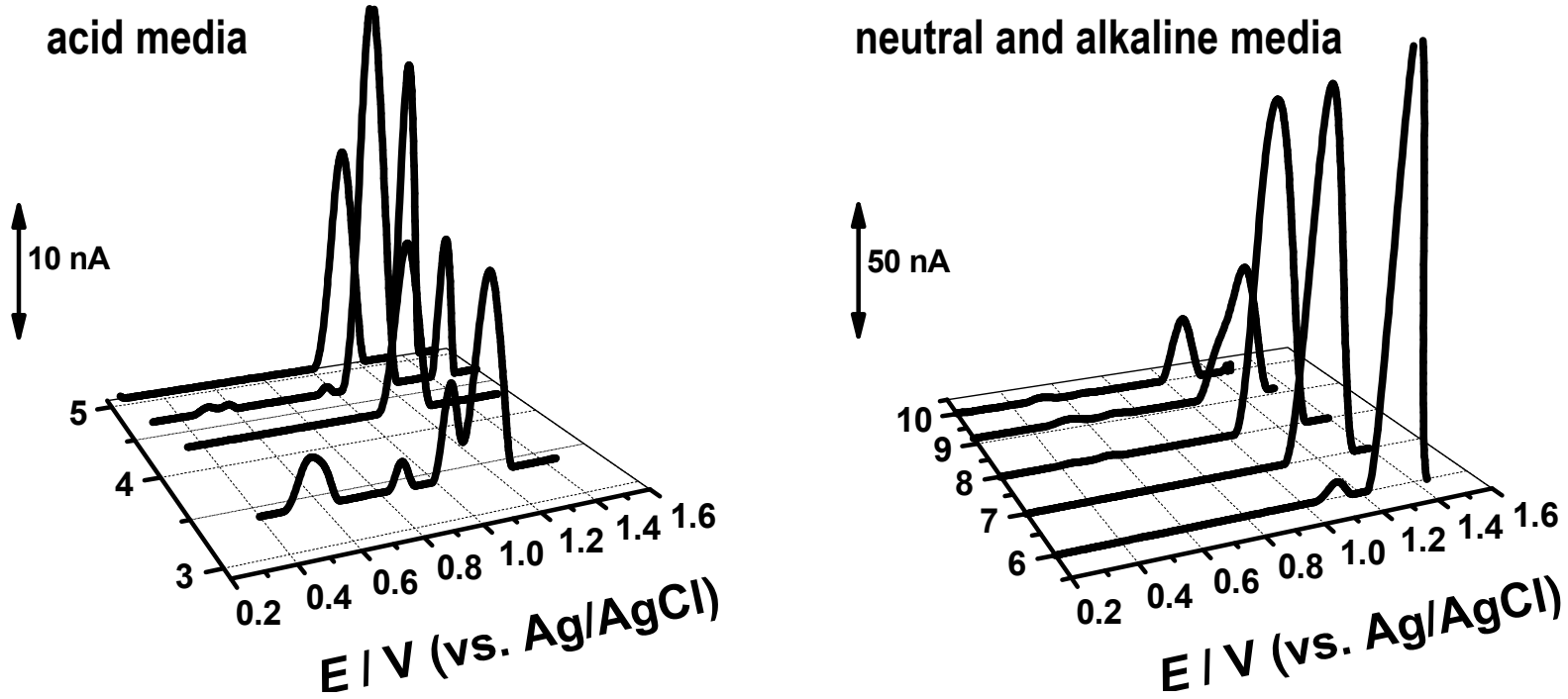
SUBSTRATE-competitive inhibitors (genistein, apigenin)

ATP-competitive inhibitors (imatinib, danusertib, nilotinib)

Electrochemical oxidation of Abl1-TK

Differential pulse voltammetry

1 µg mL^{-1} abl1-TK



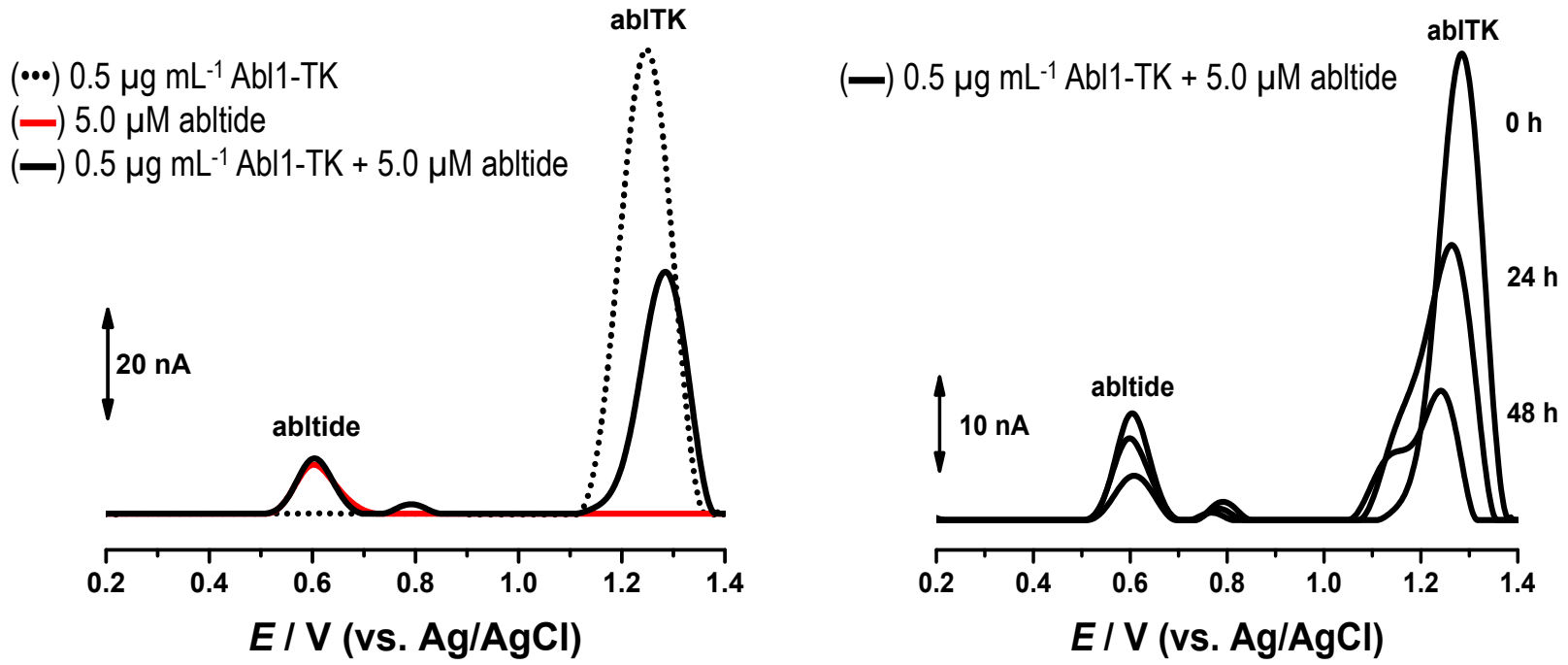
aminoacid residue	number of residues	pH = 4.5			pH = 7.0		
		E_p1_a (V)	E_p2_a (V)	E_p3_a (V)	E_p1_a (V)	E_p2_a (V)	E_p3_a (V)
tyrosine (Y)	31	0.79	-	-	0.63	-	-
phosphotyrosine	> 3	1.37			1.37		
tryptophan (W)	13	0.76	1.11	-	0.63	1.08	-
histidine (H)	24	1.35	-	-	1.15	-	-
cysteine (C)	14	0.70	0.88	1.35	0.52	0.88	1.27
methionine (M)	18	1.05	1.25	-	-	1.25	-

Interaction with substrate, ATP and inhibitors

Abltide and substrate-competitive inhibitors

ABLTIDE Glu-Ala-Ile-**Tyr**-Ala-Ala-Pro-Phe-Ala-Lys-Lys-Lys (EAI**Y**AAPFAKKK)

pH = 7.0 0.1 M phosphate buffer

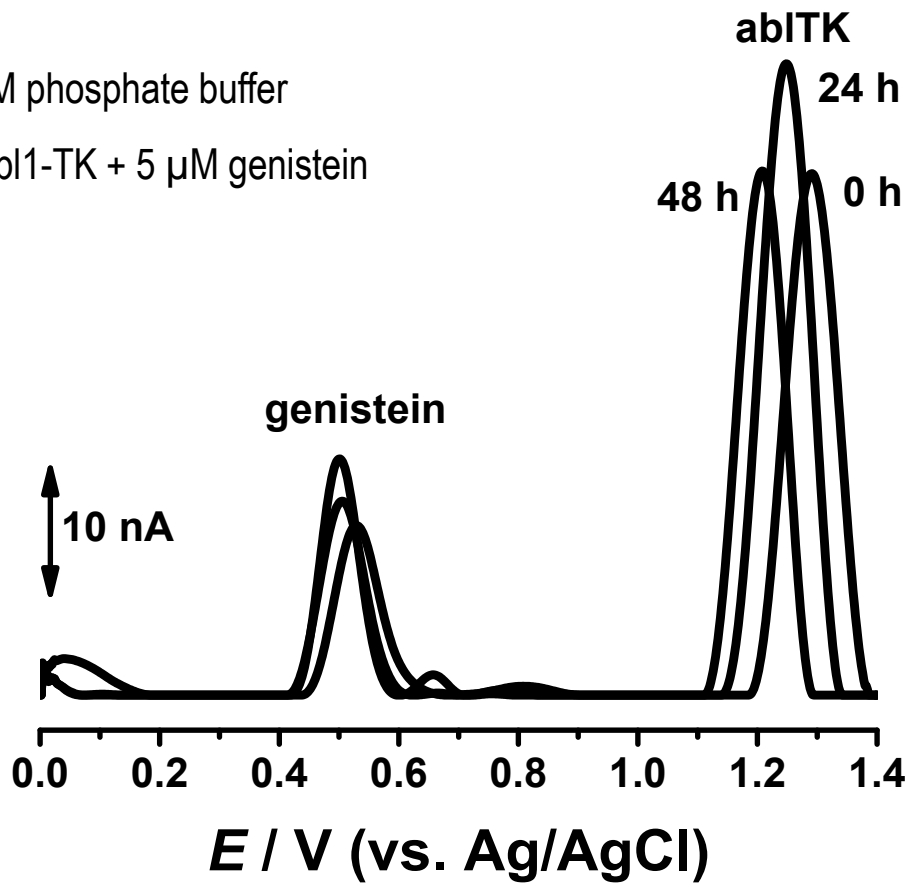


Formation of stable complex and conformational modification of Abl1-TK

Genistein

pH = 7.0 0.1 M phosphate buffer

(—) 1.0 µg mL^{-1} Abl1-TK + 5 µM genistein

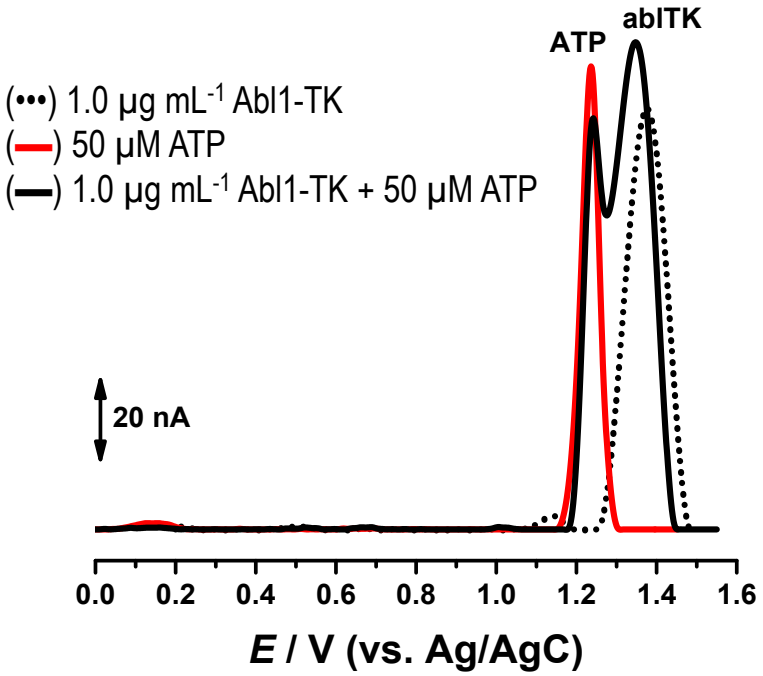


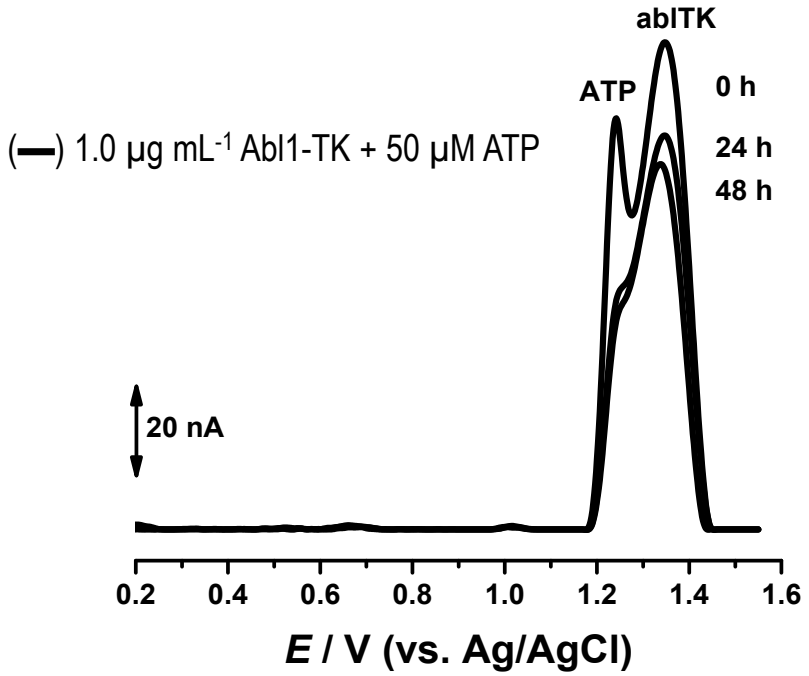
The interaction between genistein and Abl1-TK resembles that with the substrate abltide, where structural modifications of enzyme led to occurrence of new oxidation peaks

ATP and ATP-competitive inhibitors

ATP

pH = 5.8 0.1 M acetate buffer

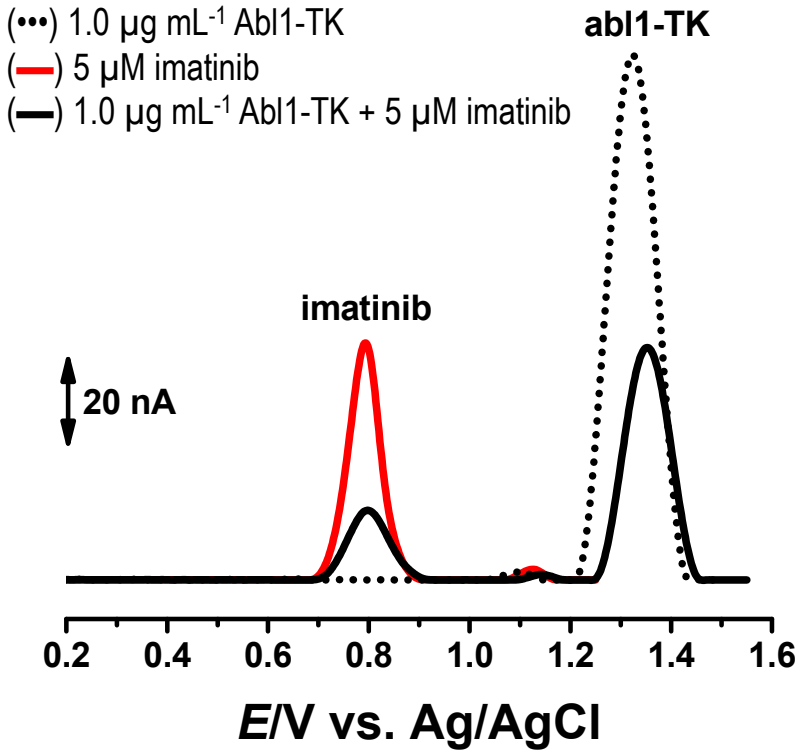


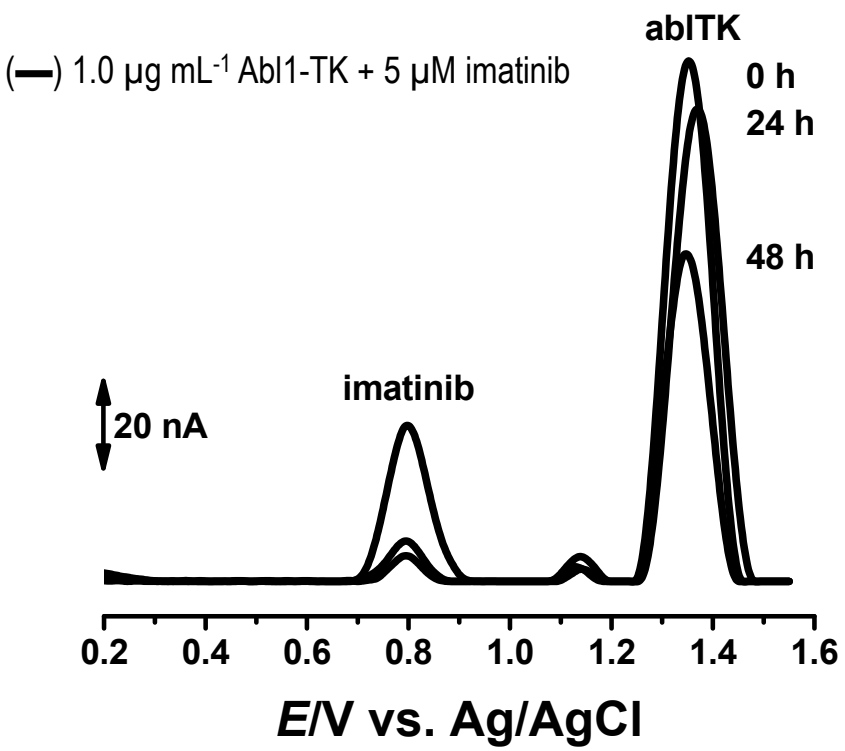


Formation of stable complex and no relevant conformational modification of Abl1-TK

Imatinib

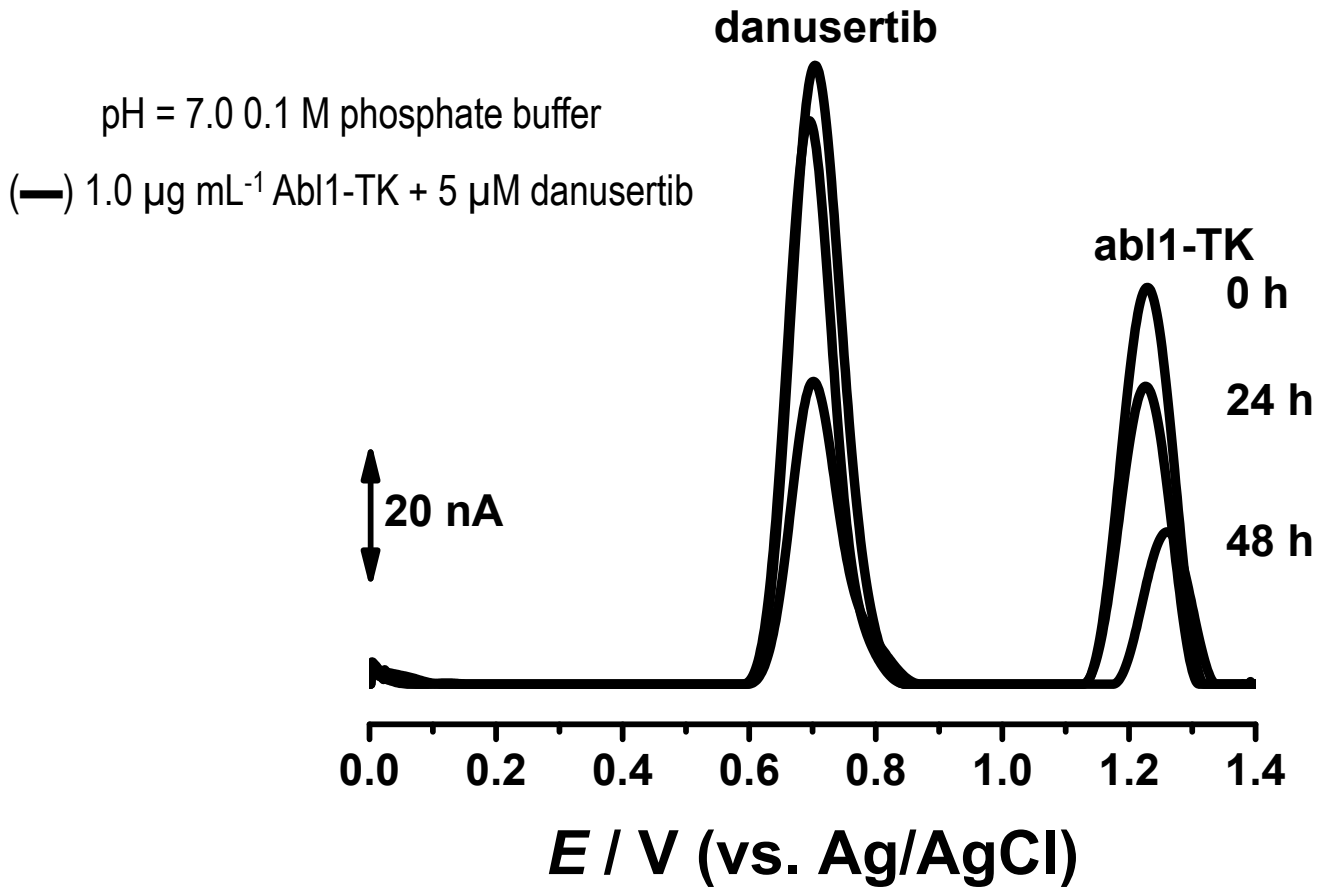
pH = 5.8 0.1 M acetate buffer





Formation of stable complex with conformational modification of Abl1-TK

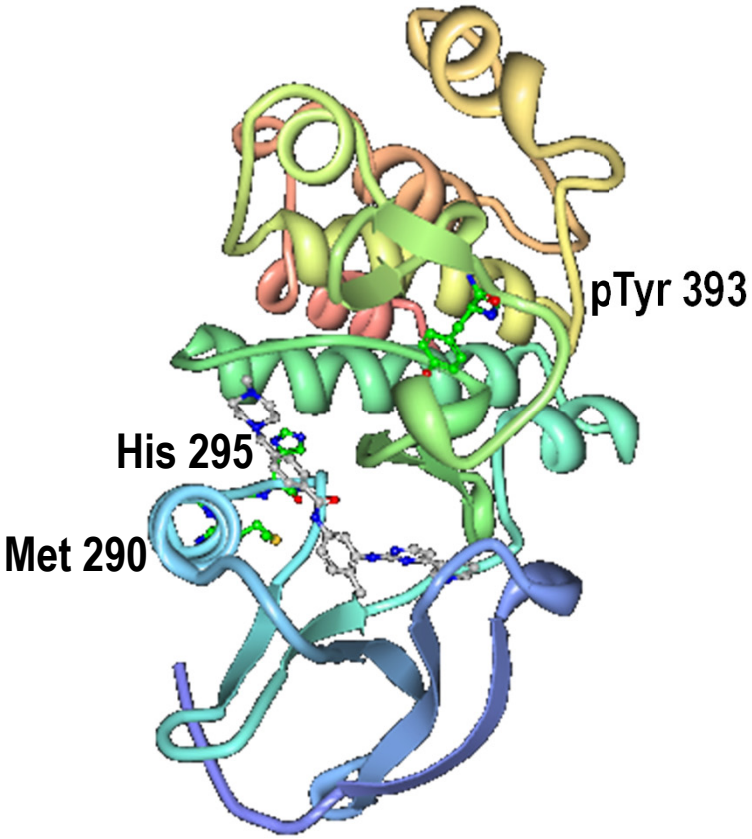
Danusertib



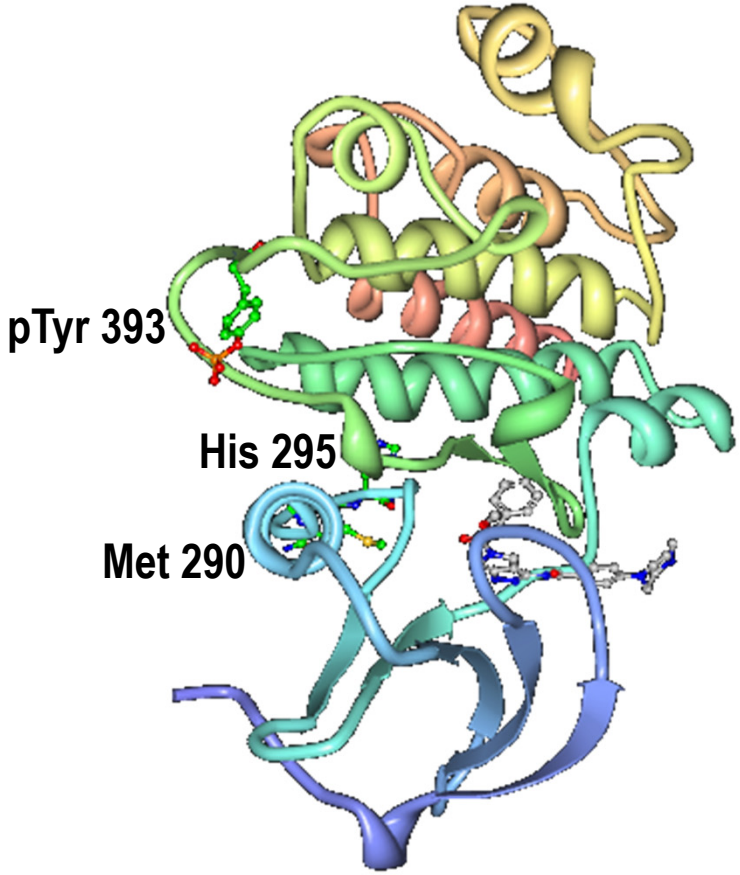
Formation of stable complex with relevant conformational modification of Abl1-TK

Comparison of structural modifications upon interaction with inhibitors

Abl1-TK complex with imatinib



Abl1-TK complex with danusertib



Electrochemical characterisation of Abl1-TK catalysed-phosphorylation and inhibition

- Incubated solutions
- Electrochemical biosensor

Incubated solutions

Procedure and detection of phosphorylation

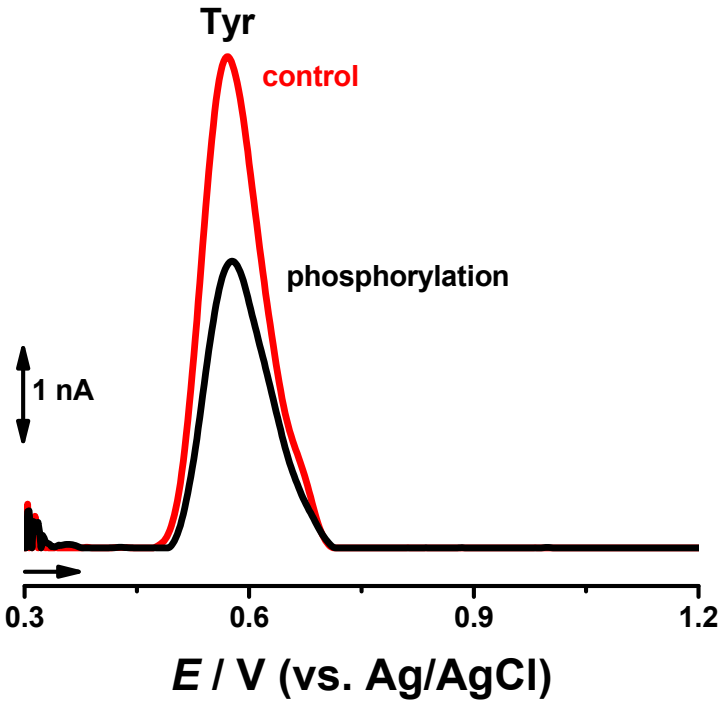
ABLTIDE EAIYAAPFAKKK

Incubation Procedure:
-5 µL H_20 (4 °C)
-5 µL abltide 25 µM
-5 µL ATP 500 µM
-10 µL Abl1-TK
For control ATP replaced by ADP

5 µL of incubated solution was dropped on the GCE surface and allowed to adsorb during 3 min

The GCE was washed and transferred to pH = 7.4 0.1 M phosphate buffer and DPV performed

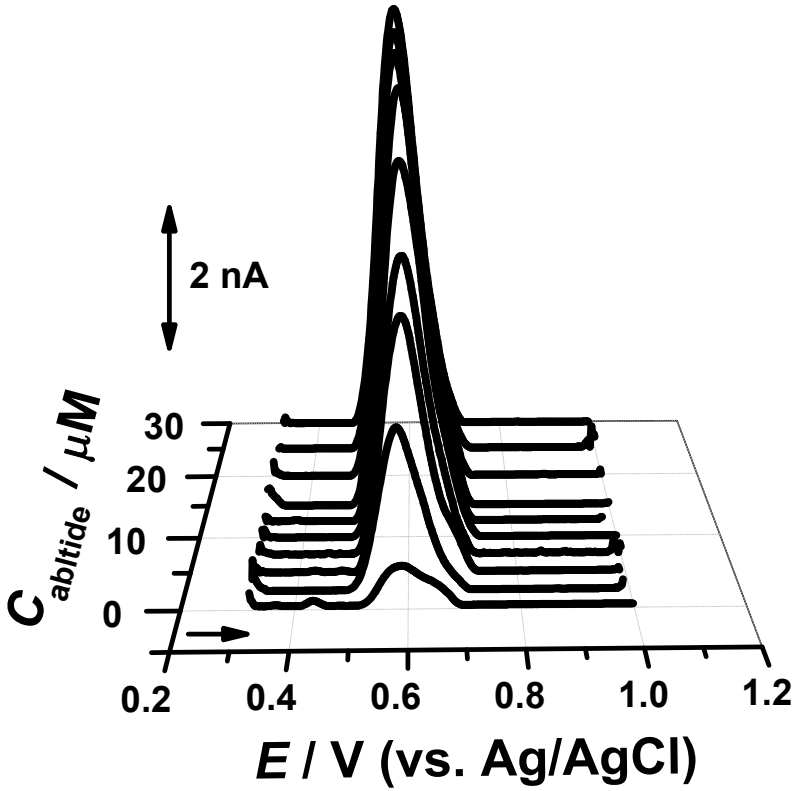
5 µM abltide phosphorylated during 15 min in the presence of 0.20 ug mL^{-1} Abl1-TK and 100 mM ATP

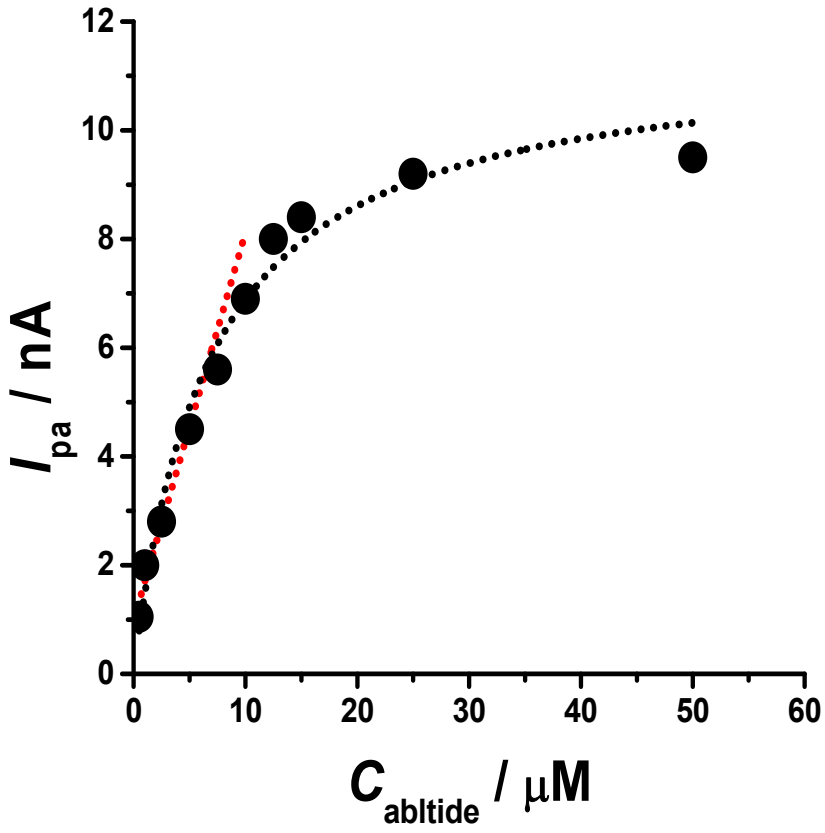


The decrease of the concentration of Tyr residues available for oxidation after the phosphorylation reaction. The occurrence of a smaller Tyr oxidation peak is attributed to abltide molecules that were not phosphorylated.

Abltide calibration curve

DPV in pH = 7.4 0.1 M phosphate buffer after adsorption during 3 min in abltide

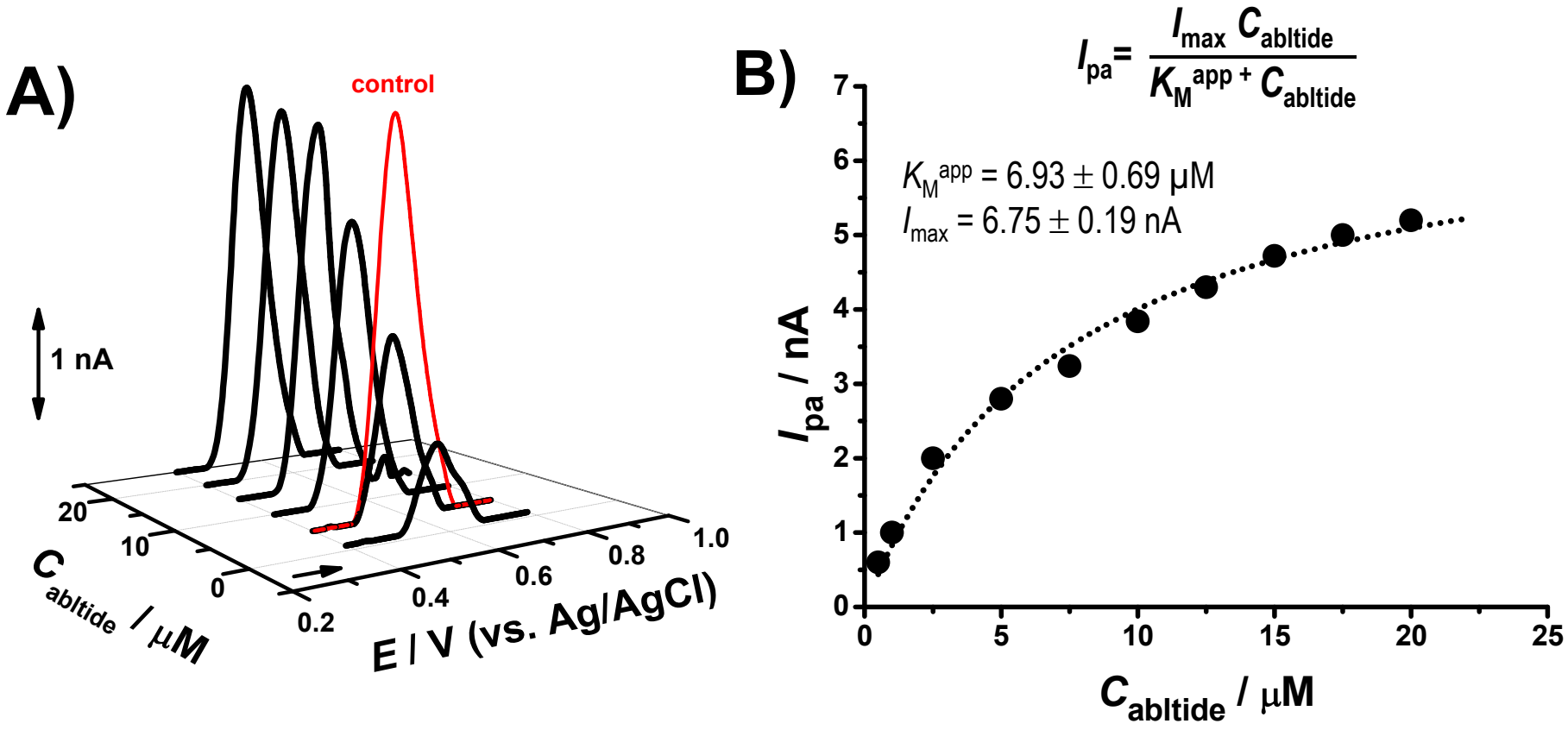




I_{pa} (nA) = 0.98 + 0.73 $C_{abltide}$ (μM) (R^2 = 0.989)

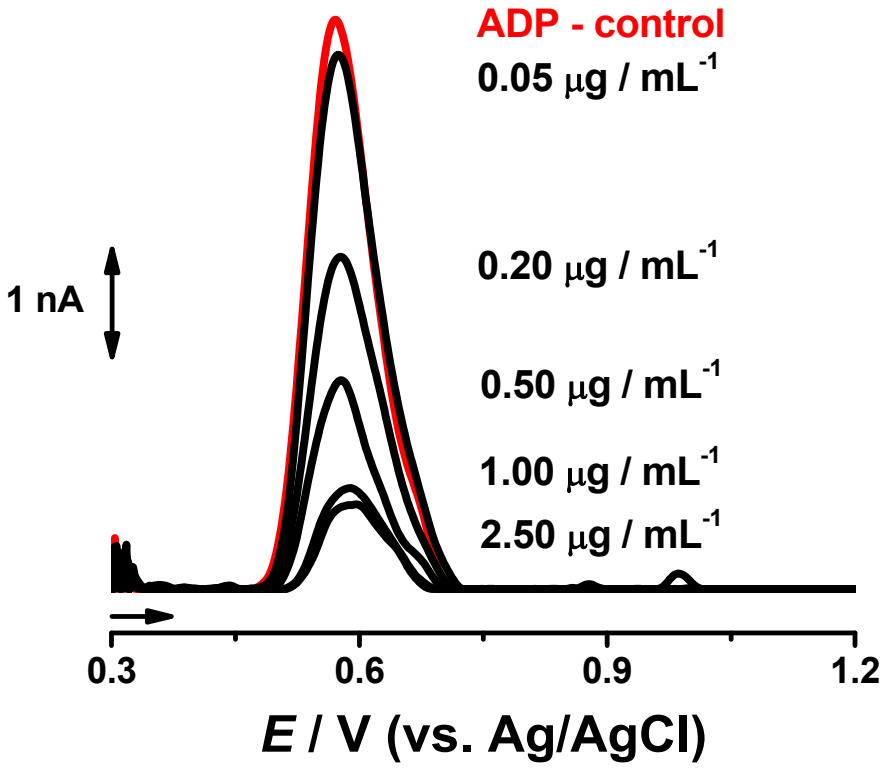
Abltide concentration effect

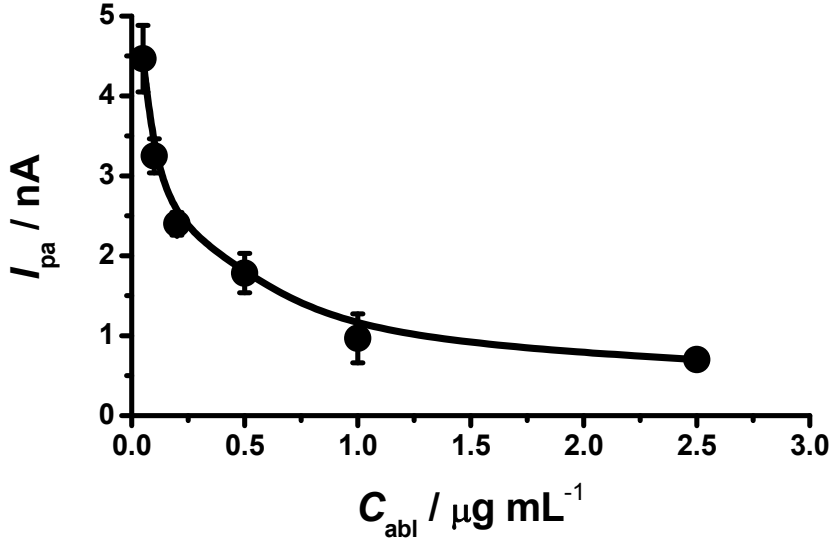
phosphorylation during 15 min by 0.10 µg mL^{-1} Abl1-TK in the presence of 100 µM ATP

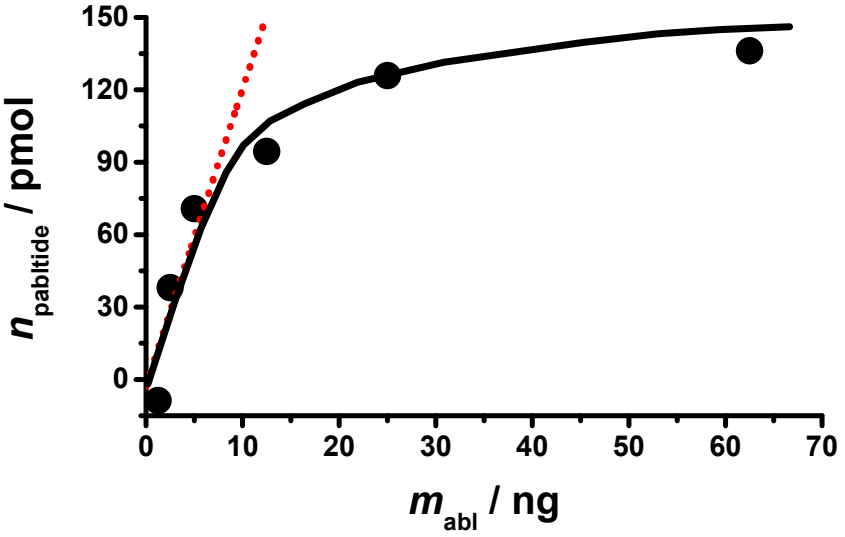


The co-adsorption of not-phosphorylated abltide may influence the result

Abl1-TK concentration effect



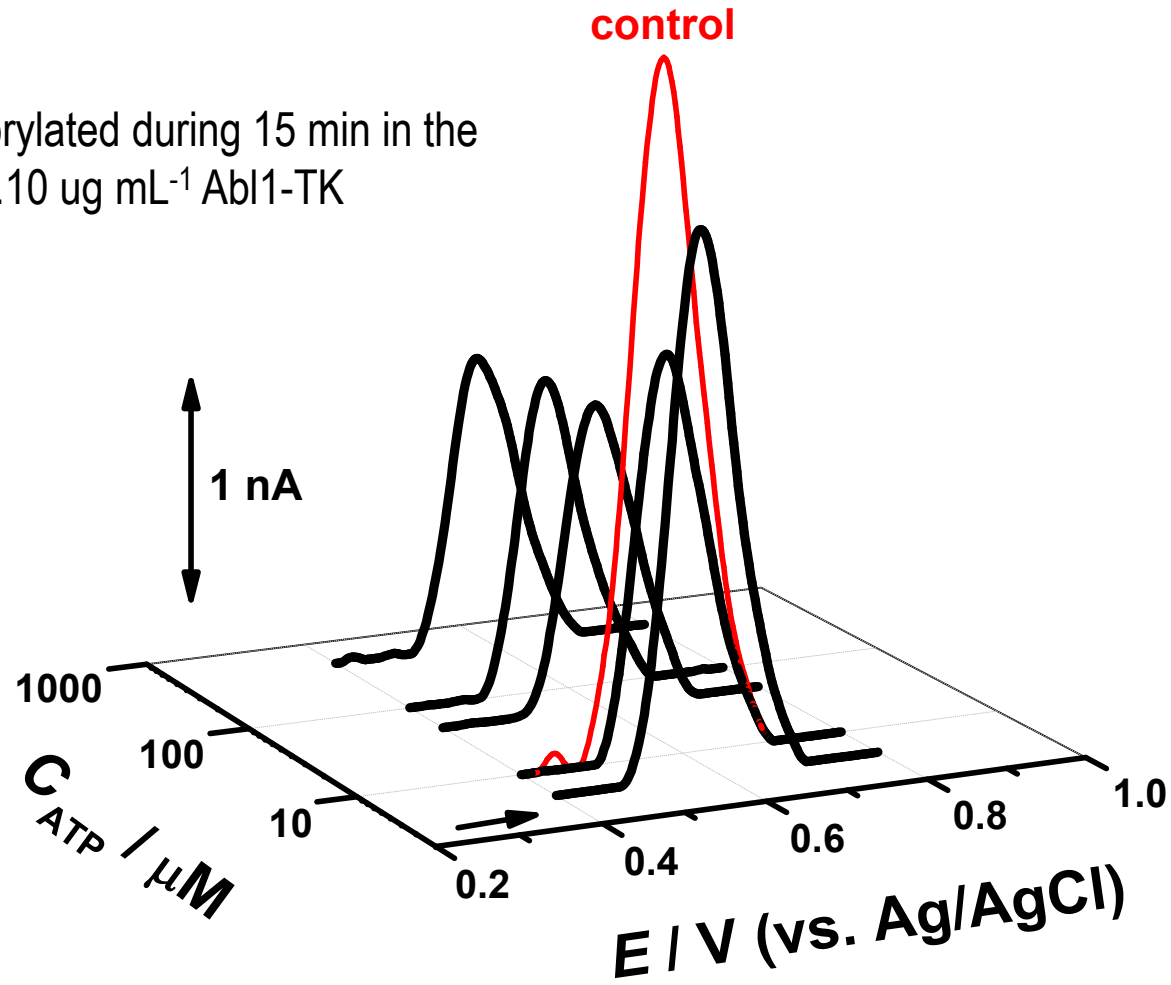




Abl1-TK specific activity
821 ±. 159 nmol min^{-1} mg^{-1}

ATP concentration effect

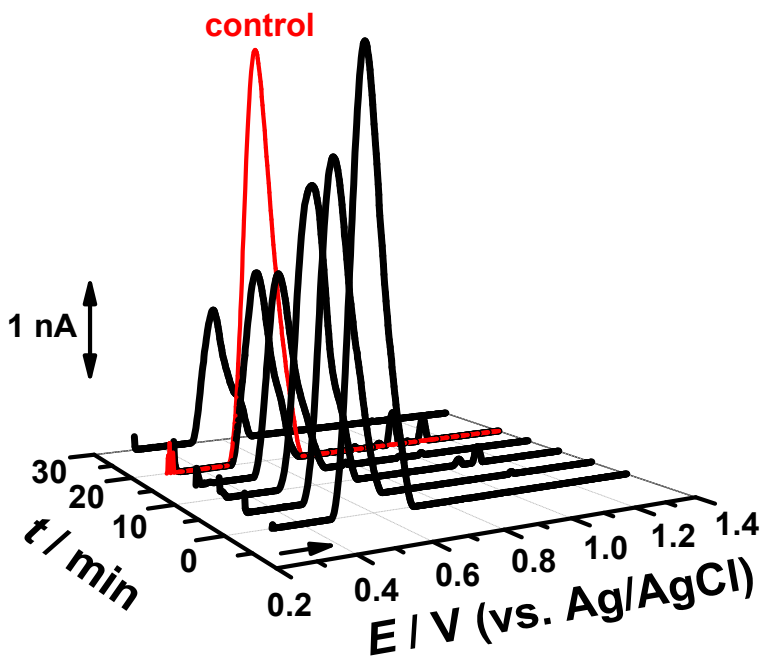
5 µM abltide phosphorylated during 15 min in the presence of 0.10 ug mL^{-1} Abl1-TK

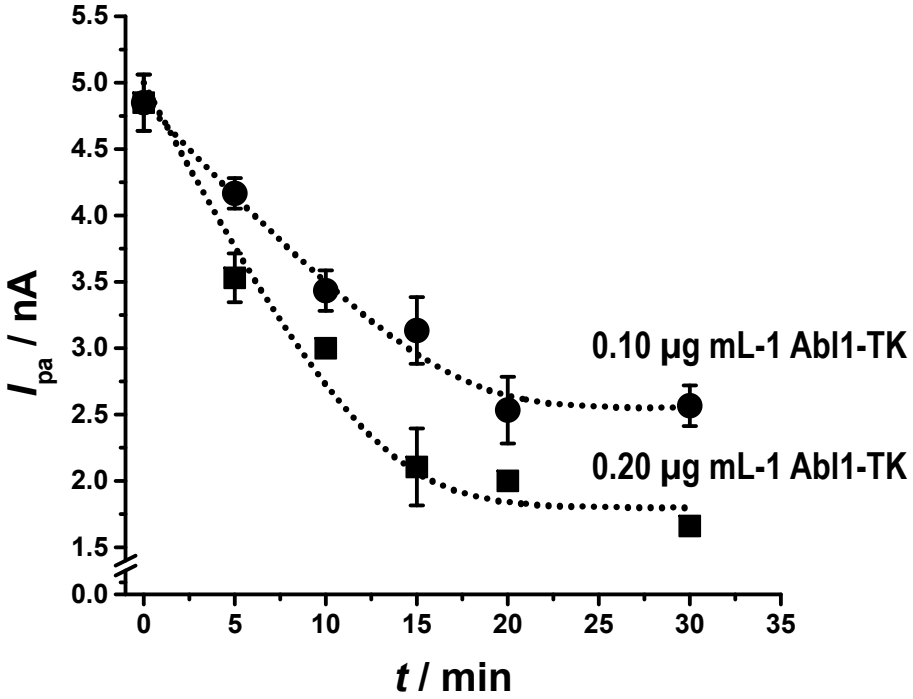


C_{ATP} > 100 µM ATP constant currents were recorded

Time dependence of the phosphorylation reaction

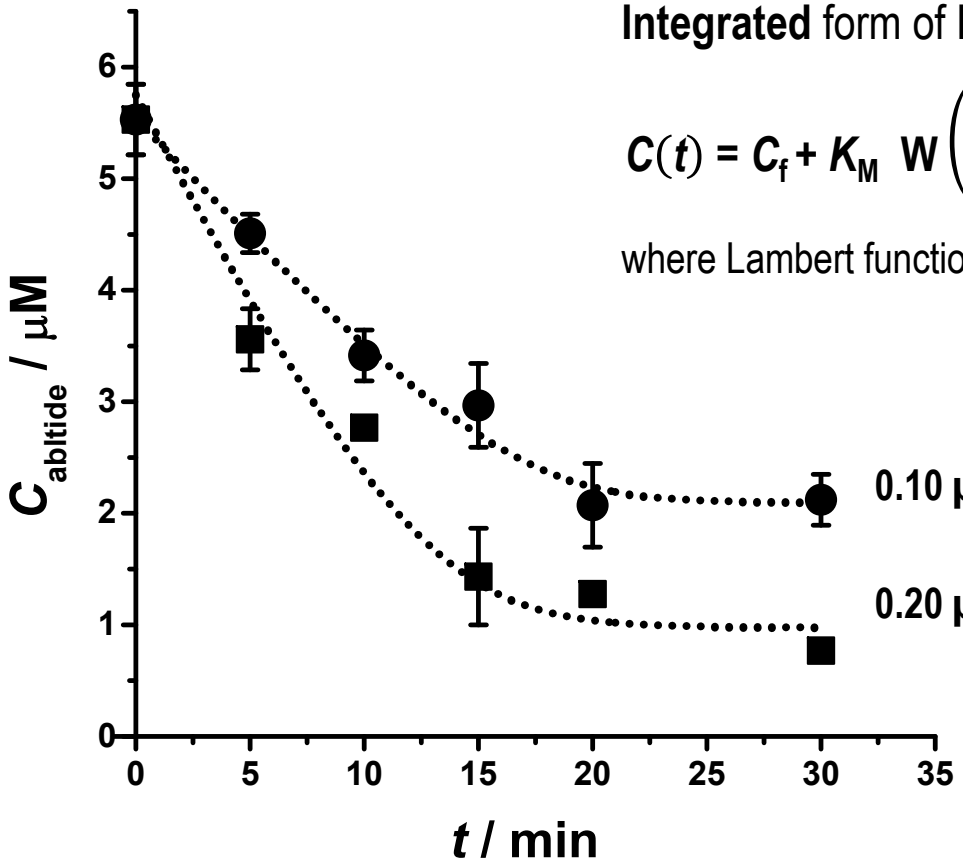
5 µM abltide phosphorylated in the presence of 0.10 ug mL^{-1} Abl1-TK





abltide oxidation peak decreased in time-dependent manner but reached constant values for t > 20 min

Abl1-TK progress curves



Integrated form of **Michaelis-Menten** equation

$$C(t) = C_f + K_M \, W\left(\frac{C_0 - C_f}{K_M} \, e^{\frac{C_0 - C_f - V_{max}t}{K_M}}\right)$$

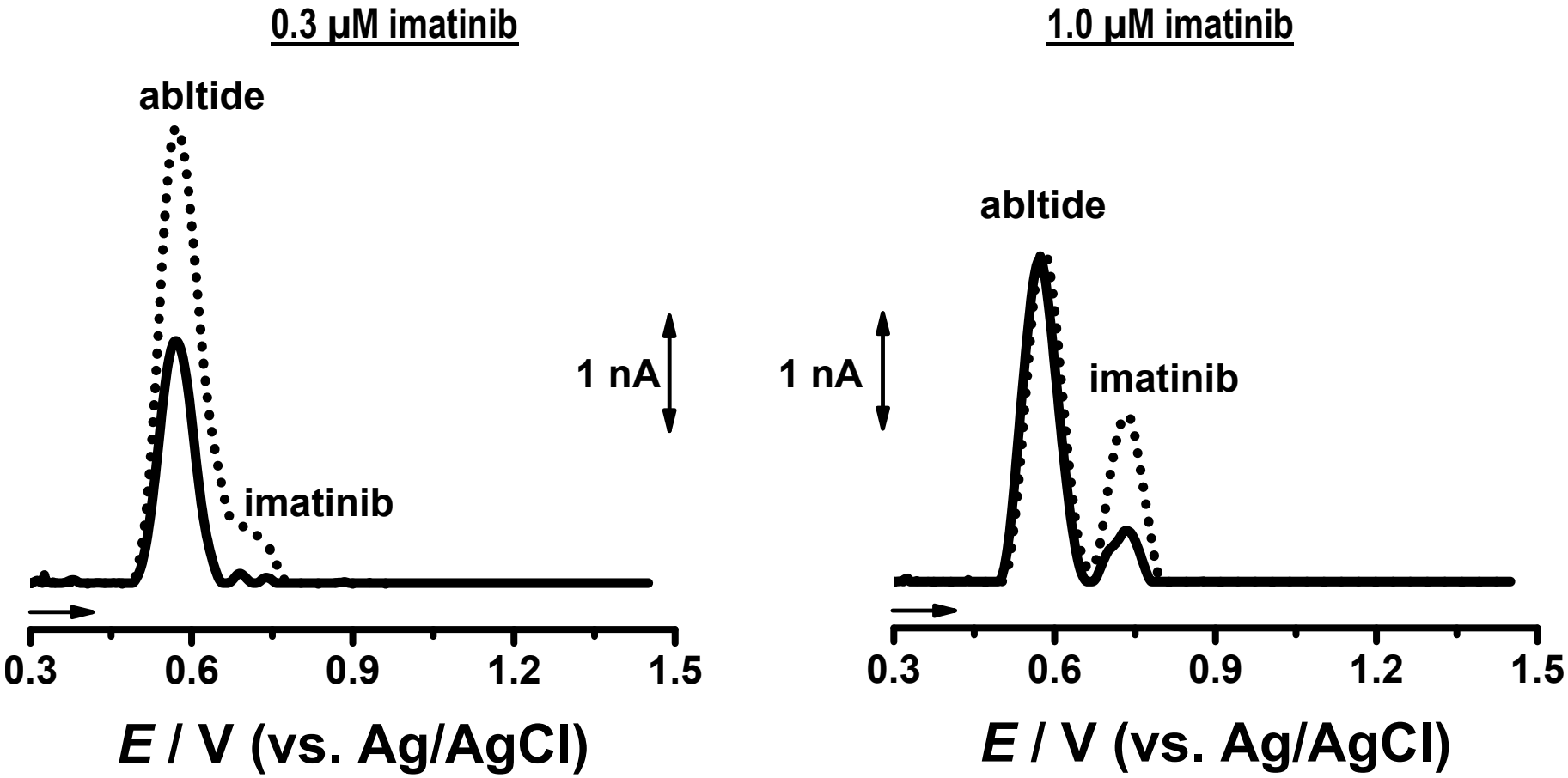
where Lambert function W(x) is the solution of $x = W(x)e^{W(x)}$

$C_{Abl1\text{-}TK}$ (nM)	K_M (µM)	V_{max} (µM min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M^{app} (s^{-1} µM^{-1})
0.74	4.04 ± 0.86	0.44 ± 0.06	9.90	2.45
1.48	4.06 ± 0.62	0.83 ± 0.07	9.33	2.29

Evaluation of Abl1 – TK inhibition

Imatinib

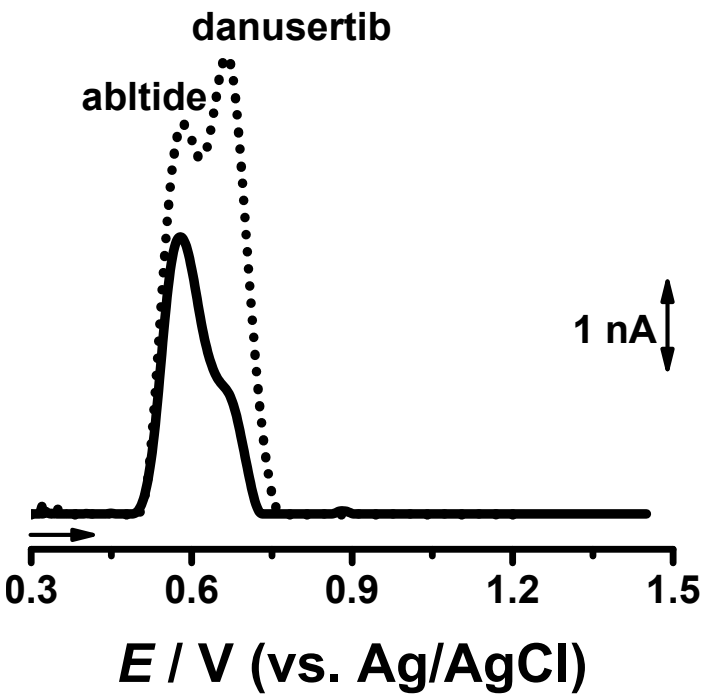
Explain the conditions of control – made with no



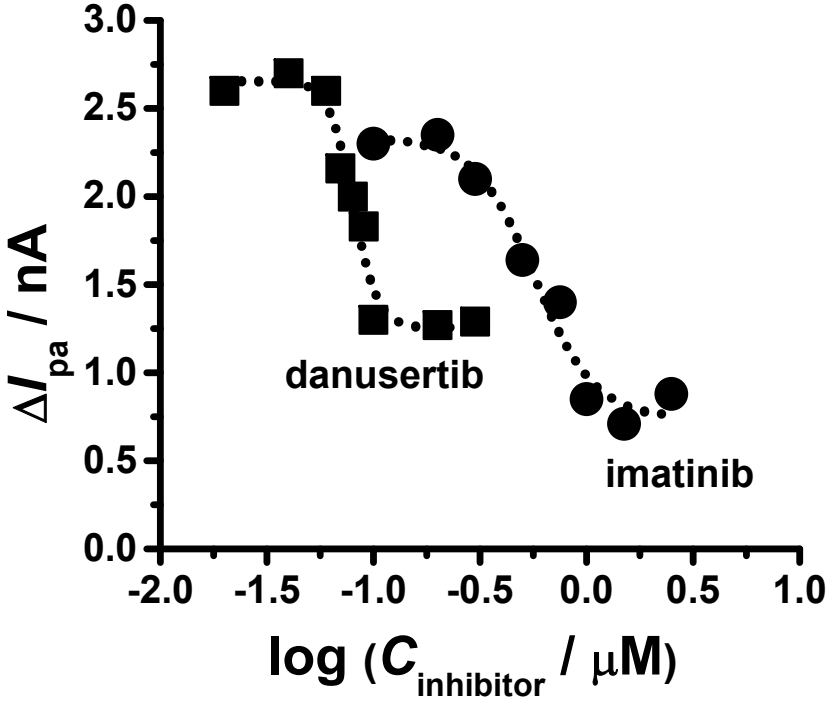
Co-adsorption of inhibitor influences abltide oxidation peak

Danusertib

0.2 µM danusertib



Dose-response analysis



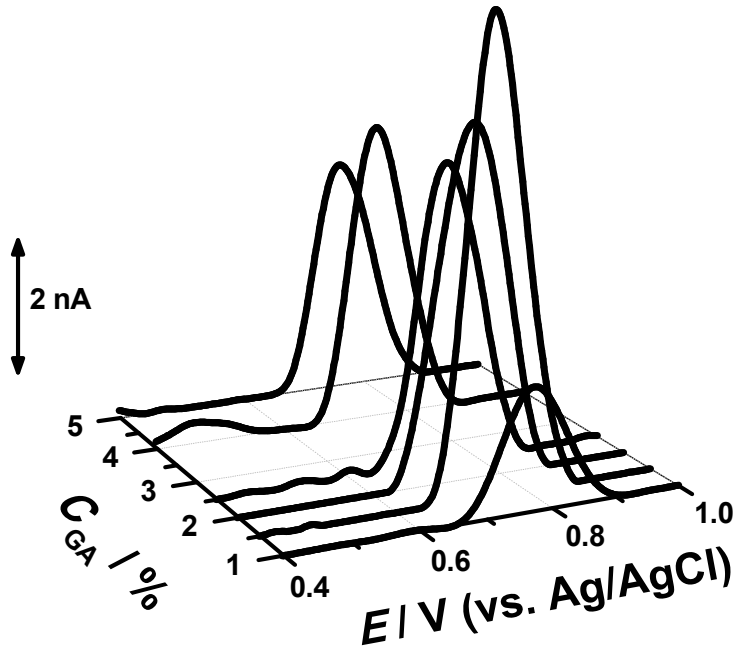
$$\triangle I_{pa} = \triangle I_{min} + (\triangle I_{max} - \triangle I_{min})\,(1 + 10^{(\log IC50 - C)p})$$

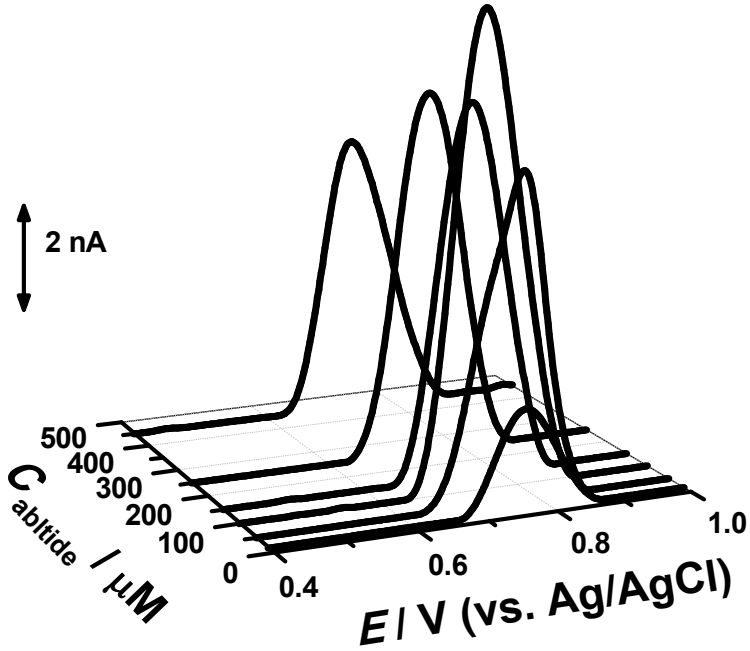
$IC50_{imatinib}$ = 0.53 µM

$IC50_{danusertib}$ = 80 nM

Electrochemical biosensor

Immobilisation of abltide at liposome and glutaraldehyde modified GCE

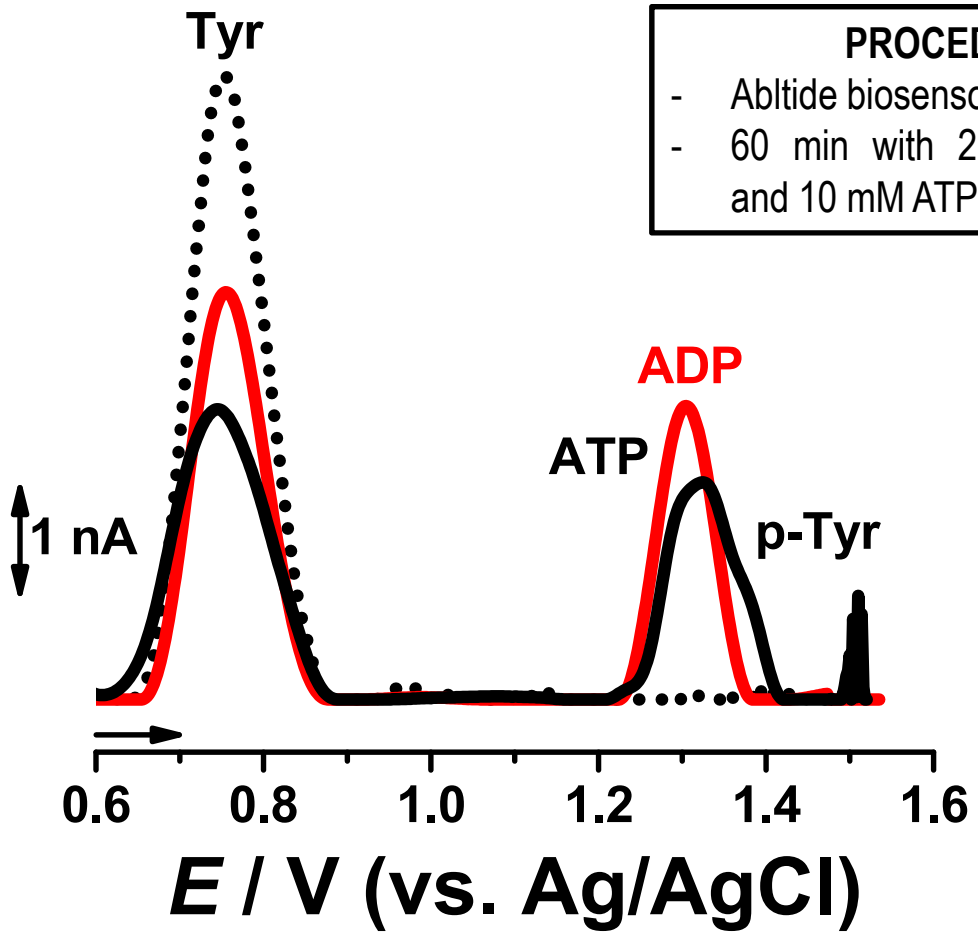




GCE surface modification

1.5% GA followed by adsorption during 10 min in 50 μM abltide solution.

Detection of phosphorylation reaction

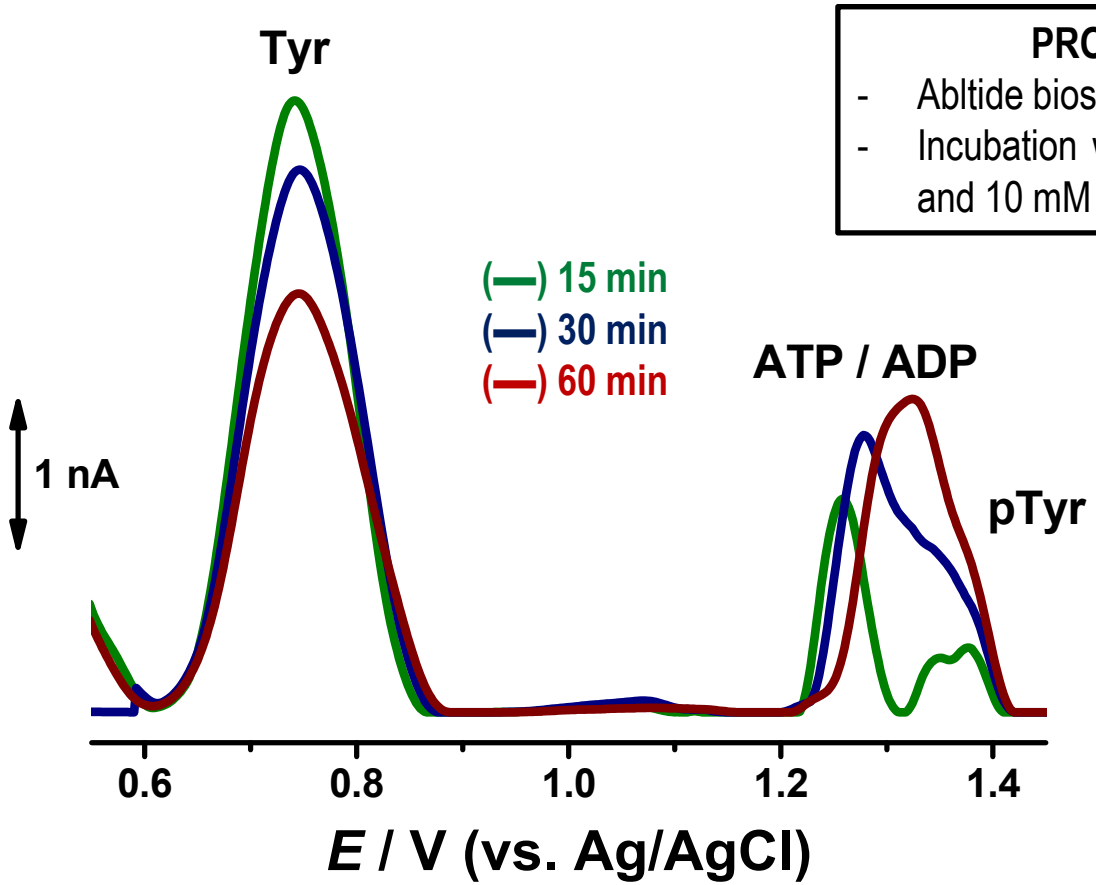


PROCEDURE

- Abltide biosensor
- 60 min with 2 µg mL^{-1} abl1-TK and 10 mM ATP / ADP

ATP and ADP non-specifically adsorbed influencing the Tyr peak. Occurrence of pTyr is an advantage and revealed the phosphorylation

Time dependence of the phosphorylation reaction



PROCEDURE

- Abltide biosensor
- Incubation with 2 µg mL^{-1} abl1-TK and 10 mM ATP / ADP

pTyr oxidation peak increase whereas Tyr decreases with incubation time. Both ATP and ADP non- specifically adsorption influences the results.

CONCLUSION

The electrochemical behaviour of natural and synthetic inhibitors of Abl1-TK:

- important for understanding structure-activity relationships of Abl1-TK complex with inhibitor.

The electrochemical behaviour of phosphotyrosine:

- phosphorylation suppresses Tyr oxidation but pTyr is still electroactive;
- phosphotyrosine as a electroanalytical signal for studying kinases-catalysed phosphorylation.

The electrochemical behaviour of Abl1-TK and interaction with inhibitors:

- resembles that between Abl1-TK and its substrates and involves the formation of stable complexes with conformational modification of enzyme structure.

The electrochemical detection of Abl1-TK catalysed phosphorylation was studied:

- in incubated solution. The analysis of progress curves in optimised conditions allowed K_M, K_{cat} and enzyme efficiency. The inhibitory power of imatinib and danusertib was evaluated.

- with an electrochemical biosensor in which the abltide substrate was immobilised in a GA matrix. The phosphorylation reaction can be observed through both the decrease of substrate Tyr residues and occurrence of pTyr oxidation peaks.