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Towards Direct Enzyme Wiring: a Theoretical Investigation of Charge Carriers Transfer Mechanisms between Glucose Oxidase and Organic Semiconductors

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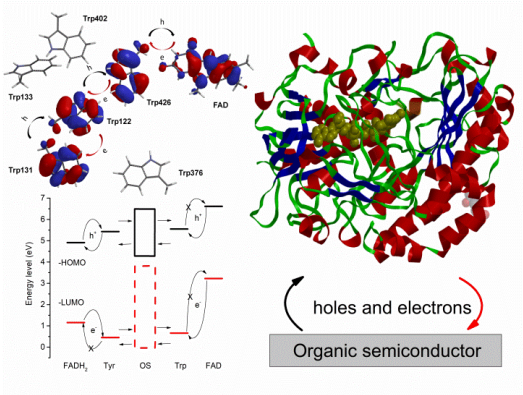
TOC GRAPHICS



KEYWORDS: Marcus-Hush theory, organic semiconductors, glucose oxidase, glucose biosensor, carriers hopping.

ABSTACT: In this work, a general theoretical and numerical approach based on semiconductor theory, which could be applied to a study of direct enzyme wiring, has been discussed. Marcus-Hush theory was applied to evaluate the potential transfer of charge carriers (holes and electrons) between glucose oxidase (GOx) and organic semiconductor. Two mechanisms of multistep hopping of charge to/from oxidised/reduced flavin-based moiety through residues of aromatic amino acids located in GOx and a long range charge direct tunnelling from cofactor to the organic semiconductor surface have been proposed and evaluated. It was determined that the hole-hopping mechanism is possible and proceeds at a low ionization potential of organic semiconductor. The calculations reveal that a hopping of electrons is blocked, but a direct electron tunnelling between cofactor and organic semiconductor is still probable. The most optimal conditions and options characteristics of GOx-based biosensors such as ionization potential, electron affinity of organic semiconductors and a distance between the enzyme and surface were estimated for the first time.

Introduction

Charge carriers (hole – h^+ and electron – e^-) transfer (CT) reactions are vital in natural and artificial biological systems including metabolic pathways, photosynthesis systems, biosensors and biofuel cells.[1–4] Both above mentioned types of charge carriers can be involved into CT processes within and between various phases and interphases, which are found in structures of various bioelectronics devices. It should be also taken into account that in a nature, some CT processes, especially those, which involve redox-active proteins such as flavoenzymes and cytochromes, are based on a transfer of charge carriers by hopping and/or long rage tunnelling.[1,5] Both these charge transfer mechanisms can be observed in the intra-enzyme media and between the enzyme and charge acceptor.[6] It should be noted that in complex bioelectronics devices, CT mechanism depends on potentials of electrodes and materials, which are used for the modification

of electrodes. Therefore, in addition to previously mentioned electron hopping and/or long rage tunnelling, hole-based charge transfer ways are possible in enzyme-based bioelectrochemical devices, because when energy levels (potentials) of oxidation or reduction processes are suitable, then both types of charge carriers can be transferred to/from the enzyme, respectively. However, recent developments of theoretical calculations and practical investigations still are not very far and are considering mostly only electron transfer based reactions.[3] Therefore, more advanced point of view, which can consider both the hole- and electron-based CT options, is recently required for bioelectronics. Moreover, electrodes of various bioelectrochemical devices are very often modified by semiconducting materials, which supports either hole- either electron-based conductivity, therefore, CT between such organic semiconducting materials and enzymes should be also evaluated in such way that many different types of charge transfer are possible between organic semiconductors and enzymes. While, recently in bioelectronics related researches, only electron transfer based mechanisms are mostly taken into account for such CT calculations if any are applied.

According to analytical signal registration principle, amperometric biosensors are divided into several generations.[7] In a first-generation glucose biosensors, charge carriers are transferred to one of substrates of enzyme (e.g., to molecular oxygen in the case of oxidases) and then the concentration of reduced product (e.g., hydrogen peroxide) is determined electrochemically. Second-generation of biosensors utilizes the artificial redox mediators, which freely diffuse in solution and in such way transfer the charge from enzyme to the electrode. In third-generation biosensors, charge is transferred directly from the enzyme to the electrode, which in some particular cases can be modified by additional charge transfer able material. Therefore, in all generations of amperometric biosensors as well as in biofuel cells high and balanced rate of CT

between the active site of enzyme and the electrode surface is a crucial and in many cases it is the most challenging factor in the design of electrochemical biosensors.[8] Therefore, the detailed understanding of charge carrier dynamics inside redox enzymes is essential to an efficient interpretation of catalytic action and charge transfer mechanisms, which both are critical in advanced understanding of an action of electrochemical biosensors. Thus, aromatic amino acids such as tyrosine (Tyr) and tryptophan (Trp) are involved in both intrinsic and extrinsic CT pathways of various biological systems,[9–11] taking into account this fact Winkler and Gray[12] have proposed that hole-hopping based charge transfer can protect enzymes from an oxidative damage, which is induced by the harmful molecular oxidants. Therefore, some well-known a p-type semiconductors (e.g., poly(3,4-ethylenedioxythiophene) (PEDOT)), which due to a low ionisation potential (below 5.0 eV) have a good hole-injection ability[13] and these materials are widely applied in organic electronics and even showed good applicability in stable hole transfer-based glucose amperometric biosensors, which are operating at a positive potential and has advanced stability due to exclusion of oxidative damage based inactivation.[14–16] Such developments opens a promising direction in bioelectronics where a low solubility of organic semiconductors in water can be exploited as an advantage suitable to a formation of organic semiconductor-based layers on the electrode surface.[17,18]

Inspired by this idea, we have recently developed a stable bio-sensing system, which is based on a hole-transporting (p-type) semiconductor derivative and glucose oxidase (GOx) covalently attached to this organic semiconductor.[19] Therefore, in recent work, we are presenting advanced and versatile theoretical and computational research based on our previously published experimental achievements.[19] In this research, we have applied the advanced theoretical and numerical approaches to a prediction of organic semiconductors' adaptability for GOx-based

biosensors and to a modelling of CT mechanism inside of GOx and between the enzyme and organic semiconductor. Subsequently, values of the simulated rate constants were compared with that determined experimentally in other researches.

Models and methods

General equation. The seminal Marcus-Hush theory (MHT) can be applied for a calculation of charge transfer (CT) rate between the charged and neutral sites in the biomolecular systems.[20] However, according to MHT, molecular vibrations are not considered because the most intramolecular vibrational frequencies of sites are above the thermal energy at a room temperature (i.e., around 25-70 meV).[2] Therefore, this theory is not suitable at high-temperatures when the thermal energy exceeds the vibrational energy. In order to determine the rate of CT between redox-able biomolecules and redox-able organic semiconducting compounds simplified MHT based equation (Eq. 1)[21] has been applied:

$$k_i = \frac{2\pi}{\hbar} |\Delta_i|^2 \frac{1}{\sqrt{4\pi\lambda_i k_b T}} exp\left(-\frac{\left(\lambda_i+\Delta G_i^0\right)^2}{4\lambda_i k_b T}\right) \quad (1)$$

where k_i is an charge carrier transfer rate constant for pathway i, $\hbar$ is reduced Planck constant, Δ_i is electronic coupling (electronic tunnel splitting) between the initial and final states, λ_i is total reorganization energy of i pathway and ΔG^0_i is total reaction Gibbs free energy of CT reaction for pathway i, respectively, k_b is the Boltzmann constant and T is an absolute temperature (298 K). In this model, an influence of electric field has not been considered.

Gibbs free energies. The driving force ΔG^0_i as the energy difference between product and reactant states was approximated as the difference of the ionization (oxidation) (*IP*) and electron affinity (reduction) (*EA*) energies between a charge acceptor (A) and a donor (D) *versus* electron. The classical MHT represents the non-adiabatic case, i.e., calculated coupling integral must be weaker than the reorganization energy ($\Delta_i << \lambda_i$) because the parabolic approximation of molecule potential

energy map in the other case does not make any sense. Moreover, non-adiabatic behaviour is expected to occur in materials with large charge-hopping distances. Therefore, by using the Koopman's theorem at the non-adiabatic mode, the *IP* and *EA* energies can be directly calculated from energies of highest occupied molecular orbital (HOMO) and lowest unoccupied molecular frontier orbital (LUMO) as IP = -HOMO and EA = -LUMO, respectively. Moreover, Thompson et al. have determined that theoretical HOMO and LUMO levels in organic semiconductors linearly correlate with the IP and EA , which were determined by electrochemical measurements, respectively.[22,23] Entropic (or temperature) terms were neglected in this work at a room temperature conditions. Thus, the Gibbs free energy for hole-hopping can be estimated by Eq. 2:

$$\Delta G_i^{0h} = E_{HOMO}^{D} - E_{HOMO}^{A} \qquad (2)$$

and energy for electron-hopping can be estimated, respectively:

$$\Delta G_i^{0e} = E_{LUMO}^{D} - E_{LUMO}^{A} \qquad (3)$$

To calculate the corresponding revisable processes, the opposite $E_{HOMO}^{A} - E_{HOMO}^{D}$ and $E_{LUMO}^{A} - E_{LUMO}^{D}$ parts of the equations 1 and 2 were used. All the neutral sites HOMO/LUMO were estimated by using a semi-local hybrid functional B3LYP and 6-31+G(d) basis set with a diffuse function (+). When larger basis was used, the calculations did not show any significant difference in the computed values for the smaller systems studied here, and hence, therefore, less demanding basis set-up of parameters was applied. To approximate the effects of solvent or enzymatic media to the energy levels, polarizable continuum model (PCM) of the enzymatic media (ε_{st}=4), which is consisting of aromatic amino acids and cofactor, and PCM of water media (ε_{st}=78) for organic semiconductor, which describes the frontier energy levels on semiconductor-electrolyte interface, was used. Moreover, we proposed that the distance between the acceptor and

donor has more significant effect on the variation of coupling integrals than on change of the Gibbs free energy.

Reorganization energies. This energy is defined as the energy, which is required for the distortion of neutral nuclear configuration into configuration of charged state. This energy is usually split into domains of inner- and external-contributions, which are stemming from the rearrangement of bonds within the donor and acceptor and/or a reorientation of surrounding molecules/fragments. According to the reference[26], the computed and experimentally estimated values of external reorganization energy, λ_{ext}, for the charge transfer reactions in organic semiconductors (OS) are very small compared to the component of internal reorganization energy (i.e., λ_{ext} is typically lower than 0.1 eV) due to very low dielectric permittivity of OS.[27] However, the values of λ_{ext} in enzymes can exceed 1 eV due to high CT distance in comparison with that in organic semiconductors.[28] For this reason, the external reorganization energies for all the pathways of charge transfer in the GOx in the best way can be estimated by the Marcus equation:[29]

$$\lambda_{ext}^{enz} = \frac{(\Delta q\, e)^2}{4\pi\varepsilon_0}\left(\frac{1}{\varepsilon_{op}} - \frac{1}{\varepsilon_{st}}\right)\left(\frac{1}{2R_A} + \frac{1}{2R_D} - \frac{1}{d_i}\right) \qquad (4)$$

where Δq is transferred the part of charge (for details, please see below), e is the elementary charge of electron, ε_0 is the electric permittivity of vacuum, ε_{st} is the static dielectric permittivity of material (i.e., for proteins/polypeptides, ε_{st} = 4 was used)[30] and d_i is a distance of CT pathway i. ε_{op} and ε_{st} are the optical and static dielectric permittivities, which corresponds to dielectric permittivities of the relaxed and non-relaxed dipoles of the medium, respectively, R_A, R_D and d_{AD} are radii of the donor and acceptor molecule (see in Table 1) and the distance of d_i (see above) in glucose oxidase. For R calculations, 3-methyl-1*H*-indole as tryptophan (Trp), *p*-cresol as tyrosine (Tyr) and isoallozazine as flavin adenine dinucleotide (FAD) cofactor models were approximated because an electrical charge density is the mostly delocalized on these aromatic moieties as it is

evidential from the density function theory (DFT) calculations. Unfortunately, this continuum model does not reveal the whole picture of charge transfer between all media because the approximate descriptors of organic semiconductor and water media are applied in this model. Moreover, by using the dielectric continuum model,[31] λ_{ext} should be considered to be

$$\lambda_{ext}^{i\to j} = \frac{(\Delta q\, e)^2}{4\pi\varepsilon_0}\left[\left(\frac{1}{\varepsilon_{op}^{i}}-\frac{1}{\varepsilon_{st}^{i}}\right)\frac{1}{2R_D}-\left(\frac{M_{op}^{i+1\to i}}{\varepsilon_{op}^{i}}-\frac{M_{st}^{i+1\to i}}{\varepsilon_{st}^{i}}\right)\frac{1}{4d_i}+\left(\frac{\varepsilon_{op}^{i+1}M_{op}^{i+1\to j}}{\left(\varepsilon_{op}^{i+1}+\varepsilon_{op}^{i}\right)^2}-\frac{\varepsilon_{st}^{i+1}M_{st}^{i+1\to j}}{\left(\varepsilon_{st}^{i+1}+\varepsilon_{st}^{i}\right)^2}\right)\frac{1}{d_i+d_{i+1}}\right] \quad (5)$$

where

$$M_{op,st}^{i\to j} = \frac{\varepsilon_i-\varepsilon_j}{\varepsilon_i+\varepsilon_j} \qquad (5')$$

In these equations, the ε_i values (i= I (enzyme), II (water), III (organic semiconductor)) are the dielectric optical and static permittivities of all three media (Table 1).

Table 1. The used dielectric optical and static permittivities of I-III media, radii and distances of corresponding redox moieties and CT pathways.

Media	ε_{st}/ ε_{op}	$R_{A,D}$ (Å)[a)]	d_{AD} (Å)
I (enzyme)	4/ 2.5	4.3 (Trp) 4.1 (Tyr) 4.9 (FAD)	20
II (water)	78/ 1.8	-	10
III (OS)	3.5/ 2.5	6.0 (OS)	4

a) The $R_{A,D}$ values were calculated by optimizing structures of corresponding residues and equation of $R = \sqrt[3]{\frac{3V}{4\pi}}$ plus 1 Å where V is molecule volume calculated by the B3LYP/6-31+G(d) level of theory.

The values of internal reorganization energy, λ_{int}, for the modelled structures were calculated by using the four points of adiabatic potential energy surface,[27] and at the B3LYP/6-31+G(d) level of theory applied for calculations according to Equation 6:

$$\lambda_{int} = [E^{\pm}(g^0) - E^{\pm}(g^{\pm})] + [E^0(g^{\pm}) - E^0(g^0)] \qquad (6)$$

where *E* corresponds to the energy of neutral molecule (g^o) in the geometry of cationic/anionic species ($g^{\pm}$), respectively. The λ-values for a cross-reaction between the different fragments and additional external λ_{ext} of corresponding pathway *i* can be estimated according the self-exchange reorganization energies for each *m* and *n* fragment:

$$\lambda_{tot} = \frac{1}{2}(\lambda_{mm} + \lambda_{nn}) + \lambda_{ext} \qquad (7)$$

Reorganisation energies of organic semiconductors for electron and hole transfer are 0.2 eV because the values in this range are very common as it was confirmed in our previous works.[24,25,27,32] All the modelling data are summarised in Table 2 and Table 3.

Table 2. Energy levels estimated by B3LYP/6-31+G(d) method and the internal reorganization energies of OS, amino acid residues and reduced and oxidised Flavin cofactor.

Compound	-HOMO / eV	-LUMO / eV	λ(h) / eV	λ(e) / eV
OS	4.5-6.5	0-4	0.20	0.20
Trp	5.55	0.45	0.772	0.362
Tyr	5.37	0.66	0.589	0.278
$FADH_2$	4.91	1.15	0.625	1.153
FAD	6.61	3.22	0.224	0.428

Electronic coupling integrals. For biomolecules, the electronic couplings can be estimated from the semiempirical rules.[33,34]. In this work, we offer a simpler model for the estimation of Δ_i based on density function theory (DFT) prediction as well as the electron tunnelling theory and the orientation function. On an atomistic level, the coupling integrals at large distance between the sites, which exceeds double of Van der Waals radius (about 3Å), can be overestimated by DFT.[35,36] Therefore, the values of Δ_i(3Å) integrals without the tunnelling effects were estimated when the distances between the redox sites are 3Å, and when the sites are interacting in parallel way. The values of Δ_i(3Å) for the pathways between the fragments *m* and *n* were obtained by using the fragment charge difference scheme (Eq. 8):[37]

$$\Delta(3\text{Å})_i = \frac{(E_m - E_n)|\Delta q_{mn}|}{\sqrt{(\Delta q_m - \Delta q_m)^2 + 4\Delta q_{mn}{}^2}} \qquad (8)$$

where E_m and E_n are the energies of corresponding frontier orbitals (i.e., HOMO/HOMO-1 and LUMO/LUMO+1) of *m* and *n* fragments in the dimer, which can be derived from a one-electron theory. One-electron energy is based on Koopmans' theorem with the Hartree-Fock wavefunctions.[38] The energy for a charged state is derived from the energy of a corresponding molecular orbital of the neutral dimer. Δq_{mn} is a charge of ±1 of the dimer. Values Δq_{m} and Δq_{n} are a charge on the *m* and *n* fragment of the dimer, which were calculated by summing the natural charges on each atom. All these values were computed by using the long-range corrected hybrid density functional ωB97X-D and the Pople 6-311+G(d,p) basis set. This theoretical approach showed good matches to the predictions of charge carrier mobility in organic semiconductors as it is reported in the most recent works.[27,32,39] Then, starting coupling integral Δ_i(3Å) was multiplied by an orientation coefficient and a tunnelling effect of charge-hopping by using Equation (9):

$$\Delta_i(d) = \Delta_i(3\text{Å})\ (cos\,\alpha \cdot sin\,\theta)\ exp[-\tfrac{1}{2}\beta(d_i - 3)] \qquad (9)$$

where β is a distance-decay constant of 1.1 and 1.6 $Å^{-1}$ for the tunnelling through peptide matrix and though water media[40] (i.e., though water hydration shell of enzyme), respectively. Additionally, the orientation of active sites is important for the determination of coupling integrals.[41] Therefore, additional descriptors α and θ were added to Eq. 9, where α is the angle between the two molecular planes and θ is the shear angle of the sites (Table S1). From this equation, the highest coupling integrals are observed when the sites are parallel to each other (α=0º) and the shear angle is of 90º. These descriptors were generated from the oscillator (symmetry) vectors of HOMO and LUMO of the redox-able moieties of enzyme involved into hopping of holes and electrons (Fig. S1). Therefore, the orientation coefficient of FAD redox-able cofactor and the organic semiconductor (electrode) surface (or surfaces whose orientation is unknown) for direct charge tunnelling process was used as 2/3.[42]

An X-ray structure analysis of GOx from *Aspergillus niger* (a code in Protein Data Bank is 1cf3), the pathways of hole and electron hopping, with the shortest distances from FAD cofactor and surface of GOx, were generated (see Fig. 2b).[43] In order to prove this way of modelling, $FADH_2$ and FAD as the reduced and oxidized forms of cofactor were changed, respectively, and the charge (hole and/or electron) transfer rate constants were calculated separately by using particular calculation methods for each type of charge carriers. Table 3 summarises all calculated rate constants.

Results and discussion

Theoretical modelling of interaction between organic semiconductors and biological redox systems (e.g., redox enzymes, parts of photosystems, respiratory complexes etc.) and the

prediction of adaptability aspects are the central tasks, which can be solved by describing the evaluation of charge carriers' mobility by using well-established empirical and quantum mechanical techniques.[44] Recently, molecular dynamics simulations are the most frequently used for an investigation of charge transfer reactions in biological processes.[45] Contrary from molecular dynamics simulations, we have applied the static picture of CT between residues of aromatic amino acids and organic semiconductor, which has been empirically determined in this work.

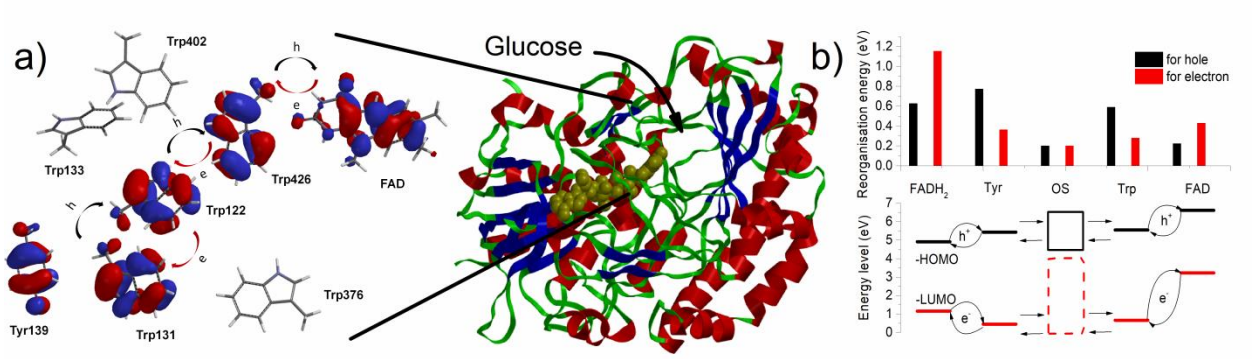


Figure 1. a) Structural model of charge carriers hopping pathways within GOx with the representation of corresponding HOMO-N orbitals; b) theoretically estimated energy levels of HOMO and LUMO and internal reorganization energies of the redox-able sites.

For theoretical modelling of the CT pathways and a determination of rate constants, the Marcus-Hush expression (Eq. 1) has been employed for the calculation of major characteristics of each predicted CT pathway. The shortest charge transfer distances from FAD cofactor towards a surface of GOx were predicted and are outlined in Figure 1a. In order to prove this model, CT efficiency from the reduced and oxidized forms of flavin cofactor ($FADH_2$ and FAD, respectively) by (h^+ and/or e^-) hopping and tunnelling was estimated. CT pathway from organic semiconductor (OS) → [Tyr(139) or Trp(131)] → Trp(122) → Trp(426) → $FADH_2$ (FAD) with the CT distances

of 9, 11, 14 and 12 Å, respectively, has been deducted and evaluated (Fig. 1a). It should be mentioned that the Tyr(139) and Trp(131) residues are close to the surface of GOx and, therefore, CT to OS from these residues of amino acids is possible. By the evaluation of X-ray data,[43] it was estimated that the direct charge tunnelling (d_{CT}) distance from cofactor to Tyr(139) or Trp(131) through the enzyme is ~20 Å. Moreover, a water hydration shell of the enzyme is in a range of approximately 10 Å, which is formed around the side chains on the enzyme surface, is taken into account in this model.[46]

The values of internal (λ_{int}), external (λ_{ext}) and total reorganization energies (λ_{tot}) were estimated, which was applied in the calculation of energies for each fragment of CT pathway. The λ_{tot} values of pathways for h^+ and e^- hopping are summarized in Table 3. The dielectric continuum model[31] has been applied for the estimation of λ_{ext} in different media such as the enzyme and water. To support here calculated λ_{tot} values, a difference between maxima of the absorption and emission spectra (Stokes shifts) of GOx can be applied for the determination of λ_{tot} within the enzyme (in the enzyme media).[47] For the coenzyme-free[48], oxidized and reduced GOx[49], these values were calculated to be 0.81, 0.56 and 1.2 eV, respectively. Thus, the ranges of λ_{tot} well correspond to averaged values of the charge transfer of h^+ and e^- (Table 3) and literature data of charge transfer in some other biological systems.[28]

Table 3. Estimated CT lengths, coupling integrals, total reorganization energies for holes and electrons calculated for various pathways in GOx and between GOx and of organic semiconductor.

Entry	Pathway	d_{CT} (Å)	$\lvert\Delta\rvert$ for h^+ (eV)	$\lvert\Delta\rvert$ for e^- (eV)	λ_{tot} for h^+ (eV)	λ_{tot} for e^- (eV)
1	$FADH_2 \rightarrow$ Trp(426)	12	5.4×10^{-3}	2.0×10^{-3}	0.96	1.02

2	FAD → Trp(426)	12	8.3×10^{-4}	3.7×10^{-4}	0.76	0.66
3	Trp(426) → Trp(122)	14	2.9×10^{-4}	3.7×10^{-4}	1.06	0.65
4	Trp(122) → Trp(131)	11	2.5×10^{-3}	3.1×10^{-3}	1.02	0.61
5	Trp(131) → Tyr(139)	9	2.8×10^{-3}	3.4×10^{-3}	0.88	0.52
6	Trp(131) → OS[a)]	10	1.2×10^{-3}	1.2×10^{-3}	0.77	0.54
7	Tyr(139) →OS[a)]	10	1.2×10^{-3}	1.2×10^{-3}	0.68	0.50
8[b)]	$FADH_2$ → OS[a)]	30	1.2×10^{-7}	1.2×10^{-7}	0.70	0.94
9[b)]	FAD → OS[a)]	30	1.2×10^{-7}	1.2×10^{-7}	0.50	0.57

a) Long range tunnelling pathways;

b) For these calculations, coupling integral between oxidized/reduced cofactor ($FAD/FADH_2$) and OS of 0.50 eV and internal reorganization energy (λ_{int}) of OS of 0.20 eV have been applied.

Coupling integrals, Δ, are among the key parameters, which determine the charge carrier mobility.[50] Therefore, the charge tunnelling effect through proteins is an important factor for CT in enzymes.[51] In this work, we offer a simplified model for the estimation of Δ_i based on DFT prediction, the electron tunnelling theory and the orientation function (for details see electronic supplementary material). All the calculated range of Δ values for h^+ and e^- in the GOx are in a range of 10^{-3}-10^{-4} eV. Therefore, we have predicted that the lowest Δ values of about 10^{-7} eV are for a direct CT between flavin cofactor and OS due to the long distance of about 30 Å between these two redox-able parts of CT pathway. Moreover, ranges of magnitude of the predicted Δ values are similar to those reported in other references.[5]

Gibbs free energies for the pathways were approximated to provide the difference between the ionization (oxidation) and electron affinity (reduction) energies of charge acceptor and donor

(or *vice versa* for the opposite CT process) (see Eq. 2-3). Figure 1b shows theoretically estimated energy levels of redox-able residues. According to the semiconductor theory, the holes and electrons are transported through the empty and occupied molecular orbitals, in other words, the highest occupied (HOMO) and lowest unoccupied molecular orbitals (LUMO), respectively. Furthermore, the energy difference between these levels is of great importance for the probability of CT in redox and semiconducting systems. During the modelling of the third generation glucose biosensors, which are based on a direct CT, these energy levels of the redox-active moieties were estimated by DFT (Fig. 1b and Table 2). The HOMO energies of the Trp and Tyr amino acids residues were found from experimental data[52] to be -5.55 and -5.37 eV (a potential of reference Ag electrode of -4.66 eV *versus* electron in vacuum was applied), respectively. The LUMO estimated values are not available to compare with experimental results. However, the LUMO energies of organic semiconductor well correlate with the DFT obtained energies, which are estimated to be -0.45 and -0.66 eV for Trp and Tyr in this work, respectively (Fig. 1b).[22]

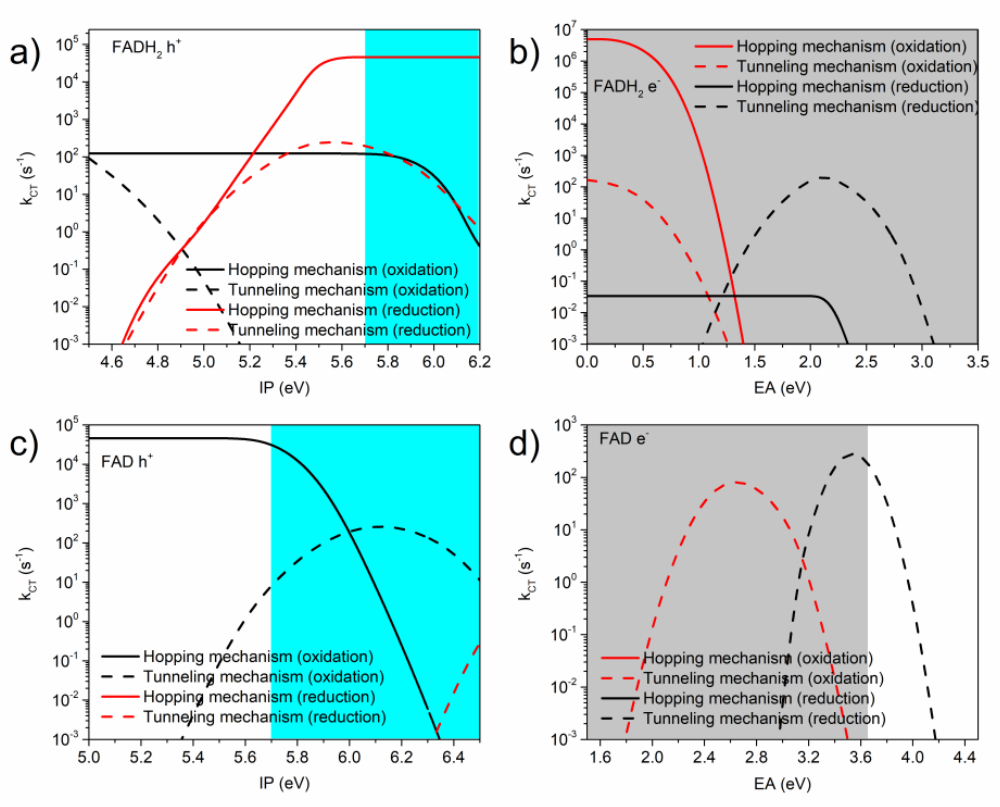


Figure 2. Dependences of oxidation and reduction rate constant on ionization potential and electron affinity of the organic semiconductor surface: a) the dependence of limiting step rate (for oxidation and reverse process – reduction of $FADH_2$ by h^+ on IP of OS; b) the dependence of oxidation and reduction rates of $FADH_2$ by e^- on EA of OS; c) rates of oxidation/reduction of FAD by h^+ dependence on IP; d) the dependence of oxidation and reduction rate of FAD by e^- on EA.

Direct CT from organic semiconductor to Trp/Tyr residues, which are located close to the surface of GOx, and the enzyme's cofactor $FAD/FADH_2$ has exhaustively been analyzed in this work. Figures 2 show the dependences of oxidation and reduction rate-limiting step constants (k_{ct}) on the frontier energy levels of organic semiconductors. In order to find an optimal condition of carriers hopping and tunnelling, the dependences on ionization energy of OS and distance between

the covalently immobilized enzyme and the surface of OS, which is equal to the length of applied tag or water hydration shell of the enzyme, for the oxidation and reduction charge transfer constants of rate-limiting step of carriers hopping were estimated by the model applied for the calculations. Therefore, the ranges of ionization energy (or -HOMO level) of 4.5-6.5 eV and affinity for electron (-LUMO) of 0-4 eV have been used in this model, respectively. These frontier energy levels are typical for the organic semiconductors.[7] In the theory of chemical kinetics, the overall rate of a reaction is often determined approximately, by the slowest step, known as the rate-limiting step. Moreover, we proposed that a one electron process between the redox residues is reversible. First of all, for a catalytic action of GOx, a reduction of oxidized cofactor FAD to $FADH_2$ is performed by a reaction of FAD + glucose → $FADH_2$ + gluconolactone.[53] From our model, the direct reduction of FAD is possible by only electrons in the cases when the electron affinity (*EA*) of OS is in the interval of 3.0-4.2 eV (Fig. 2d). According to the published data, the second order rate constant of this reaction is 3×10^4 M^{-1} s^{-1}.[54]. Therefore, the pseudo-first order rate constant is calculated in the range of 120-240 s^{-1} for this process at normal physiological concentration of 4-8 mM of glucose. Based on this pseudo-first order rate constant of the reduction of FAD with glucose and the model results in Figure 2d, the direct reduction of FAD by electron from OS-modified electrode is only possible by electron tunnelling mechanism when *EA* of OS is around 3.6 eV. In this case, the maximal rate constant of this CT step is equal to 400 s^{-1}. In almost all cases, glucose much faster reduces FAD cofactor than e^- is transferred to the electrode. Moreover, Figure 2c shows that the FAD reduction (reversible process) by h^+ transfer is blocked in all pathways. Therefore, a diffusion of glucose to the active centre of GOx and the proton tunnelling from neighbouring histidine moiety are the limiting steps in FAD reduction. Secondly, an injection of h^+ from Trp(426) to $FADH_2$ located within the enzyme *via* hopping mechanism is

a limited step when *IP* of OS is lower than 5.2 eV because, at higher potential, the reverse (reduction) process is faster (Fig. 2a). The rate constant of h^+ in Trp(426) to $FADH_2$, which reaches 120 s^{-1}, does not depend on *IP* of OS and on a distance between the enzyme and the OS surface. It should be noted that the hole-tunnelling mechanism (OS → $FADH_2$) is possible when the values of IP of OS are lower than 4.9 eV (i.e., oxidation potential of $FADH_2$), but such semiconductors are rare and unstable.

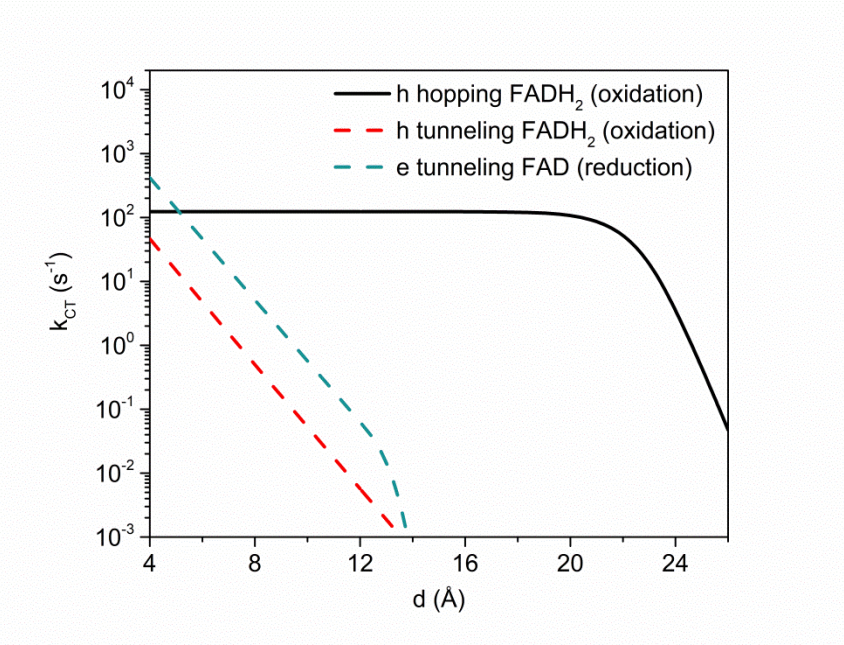


Figure 3. The dependence of rate constants on the distance between the enzyme and OS when optimal values of IP= 5 eV and EA = 4 eV are applied.

Oxygen and water reduction and oxidation should be taken into account when her proposed model was applied. Water is oxidized at the around 5.7 eV potential *vs* e^- in vacuum (cyan area in Fig. 2a and 2c). On the other hand, an appropriate *EA* level of n-type OSs should be below 3.7 eV in that air instability usually is not due to the degradation of intrinsically chemically unstable OSs,

but arises from the charge carrier trapping under an ambient condition by H_2O or O_2.[55] These conditions are shown in Figures 2b and 2d as the grey areas. If an oxidation of $FADH_2$ by carriers from electrode is blocked than a H_2O_2 formation occurs by a reaction of $FADH_2 + O_2 \rightarrow FAD + H_2O_2$, because flavins display an intrinsic reactivity with O_2 leading to the accumulation of reactive species, in particular flavin radicals and $O_2^{-\bullet}$ or $HO^{\bullet}$.[56] These active radicals are the most harmful molecular oxidants and due to oxidative damage of enzymes they significantly reduce the durability of the biosensor. Hence, from our model, the oxidation of $FADH_2$ by e^- is blocked and it is possible only by h^+ (Figs. 2a and 2b). This principle of first-generation glucose biosensors relies on the tuning of operating potential to the optimal region from 0 to -0.2 V *vs* Ag/AgCl where contributions from easily oxidizable interfering substances are eliminated.[7] However, for third generation glucose biosensors, the detection relies on the tuning of operating potential to interval from -1.0 to -0.5 V *vs* Ag/AgCl (i.e., 3.7 – 4.2 eV of *EA vs* e^- in vacuum), which was deducted from the non-blocked rate constants of e^- tunnelling (Fig. 2d) and well correlated with the experimental data.[7] Moreover, Figure 3 shows the limited k_{ct} dependences on the distance range of 4-25 Å between GOx and surface, which is determined by the length of applied tag, when optimal values of *IP* = 5 eV and *EA* = 4 eV were applied to OS. A rate *via* hole-hopping mechanism, which practically (up to 20 Å) does not depend on the distance, is dominant when the distance is higher than 8 Å in all the cases. Only reduction of FAD by electron tunnelling mechanism is possible at short tag lengths (i.e. < 6 Å) and the rate constants of this process decrease rapidly.

To support our mechanistic proposal, we have compared the experimental rate constants, which we found in the literature, with our modelled results. First of all, we have modelled our recently prepared biosensing system based on a hole-transporting (p-type) carbazole

semiconductor and covalently immobilized GOx monolayer.[19] The charge transfer rate constants between this OS and GOx have experimentally been determined to be around 130-160 s^{-1}. By using this theoretical model and the estimated internal reorganisation energy for e^-/h^+ transfer is 0.28/0.23 eV for 9,9'-diethyl-9*H*,9'*H*-3,3'-bicarbazole, which was used as a model compound, and *IP/EA* of 5.2/1.0 eV as well as the distance between GOx and surface, which is determined by the length of applied tag, which is of 15 Å, respectively. The h^+ transfer k_{ct} between the $FADH_2$ - Trp(426) residues as limited step was estimated to be 120 s^{-1}. This result well correlates with experimental one. Furthermore, in this publication, we have proposed that hole from electrode at 0.2 V *vs* Ag/AgCl can be reversibly oxidised the reduced flavine ($FADH_2$) to $FADH^{\bullet}$ and the next step at 0.8 V *vs* Ag/AgCl the second hole is injected into a $FADH^{\bullet}$-glucose complex. On the other hand, Willner et al.[57] have reported a method used to assemble a GOx monolayer on the Au electrode *via* reconstitution of the apo-protein with the 4,5-dioxo-4,5-dihydro-1*H*-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid (PQQ)/FAD monolayer, which yields a functionalized electrode for electrooxidation of glucose at a high rate constant of around 600 s^{-1}. For this model, the oxidized PQQ_{ox} and reduced 4,5-dihydroxy-1*H*-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid forms were applied. The internal reorganization energies for e^- of the oxidized and for h^+ of reduced PQQ forms were estimated to be 0.53 and 0.48 eV, respectively. Moreover, the frontier HOMO= -6.1 eV and LUMO= -3.9 eV (or *–IP* and *–EA* from the Koopmans' approximation) energies of appropriate forms of PQQ_{ox} and PQQ_{red} were determined. From the structure, which was optimized by DFT, the covalently connected PQQ and flavin adenine dinucleotide adduct (Fig. S3), the distance of 25 Å between PQQ and FAD was determined. For such system by our model, the calculated h^+ hopping mechanism seems not possible due to a higher oxidation potential of PQQ_{red} than that of water. However, due to a suitable electron affinity of PQQ_{ox}, FAD is reduced

by electron tunnelling from PQQ and rate constant of 640 s^{-1} of this process was estimated in that the research.[57] However, intra-enzyme e^- hopping is blocked because the *EA* levels of FAD and Trp are very different for the injection of e^-.

Conclusions

In this research, we have evaluated the two mechanisms of a multistep hopping of charge carriers through the oxidised and reduced flavin cofactor and aromatic redox-able amino acids in the glucose oxidase and direct tunnelling between the cofactor of enzyme and the organic semiconducting modified electrode surface. From our here presented theoretic model, the hole-hopping to reduced flavin is possible when the ionization potential of organic semiconductor is lower than 5.2 eV. However, electron from oxidized cofactor is transferred by only the long range direct tunnelling mechanism in a narrow potential range and strongly depends on the distance between enzyme and surface. These theoretical results well correlate with the experimental data and provide a promising opportunity for the design and construction of stabile and effective third generation biosensors based on glucose oxidase and p-type organic semiconductors.

Supporting Information. Orientation descriptors and coordinates of calculated systems are presented in ESI. The Supporting Information is available free of charge.

Conflicts of interest

There are no conflicts to declare.

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