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Molecularly "Wired" Cholesterol Oxidase for Biosensing

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Abstract The influence of several factors on the activity of cholesterol oxidase (ChOx) transiently exposed to a room temperature ionic liquid (RTIL) was studied. Presence of flavin adenine dinucleotide (FAD, prosthetic group of ChOx) during exposure to RTIL makes the procedure enzyme-friendly, while the use of RTIL (green reagent) makes it environmentally-friendly. Following exposure to RTIL and its subsequent removal, FAD becomes part of the molecular structure of the refolded protein (a molecular "wire"). This makes the procedure used here a molecular one. The factors studied were: FAD presence in RTIL during modification, water presence during exposure to RTIL, and ratio FAD:RTIL during "wiring". Performance parameters monitored were: enzyme activity before and after "wiring" (expressed as (dA/dt)/mg enzyme, and measured spectrophotometrically), peak current in an amperometric biosensor for cholesterol detection, and linearity of the biosensor response depending on cholesterol concentration. After RTIL removal, the modified enzyme (ME) retained a high percentage of the added FAD, which supplemented that of the native enzyme (functioning as a "wire" and enhancing electron transfer kinetics), and a fraction of the initial activity. Used in an amperometric biosensor, ME showed catalytic activity, linear behavior as a function of cholesterol concentration, and stability.

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Abbreviations

ChOx	Cholesterol oxidase
HPLC	High performance liquid chromatography
RTIL	Room temperature ionic liquids
FAD	Flavin adenine dinucleotide
$[EMIM]BF_4$	1-Ethyl-3-methylimidazolium tetrafluoroborate
LDH	Lactate dehydrogenase
ME	Modified enzyme
GC	Glassy carbon
SCE	Standard calomel electrode
EtOH	Ethanol

1 Introduction

Cholesterol is an important analyte for health sciences, as well as for the food industry and biotechnology. Measurement of blood cholesterol concentration is a routine practice in medical screening and diagnosis. Thus, it is desirable to develop techniques which allow convenient and rapid determination of cholesterol levels in the blood. Prevalent techniques are: (1) the Lieberman–Burchard reaction involving the colorimetric determination of cholesterol in chloroform [1], (2) colorimetry of dyes produced during the enzymatic reaction, catalyzed by horseradish peroxidase, of 4-aminoantipyrine and phenol with the hydrogen peroxide produced by oxidation of cholesterol with cholesterol oxidase (ChOx), [2], and (3) separation methods, mostly high

performance liquid chromatography (HPLC) [3]. These methods, however, need either pre-separation of serum and plasma to avoid optical interferences (colorimetry), pre-treatment of the samples (HPLC), and/or require much time and expensive reagents (Lieberman–Burchard reaction). Thus an in-place reliable, fast alternative to these methods is highly desirable. An amperometric biosensor for cholesterol is one of the most promising candidates.

Ionic liquids are ionic compounds which melt below 100 °C. Room temperature ionic liquids (RTIL) have melting points below 30 °C. It is known that exposure of proteins to RTIL can result in reversible or partially reversible denaturation of proteins [4]. The effect of the RTIL exposure on the stability of protein structure in solution [5] together with that on aggregation of proteins [6] was used mainly in green syntheses where RTIL replaced the traditional organic solvents [7]. As a principle, the alkyl groups of the RTIL's tetraalkylammonium interact with the hydrophobic portions of proteins preventing intermolecular associations while the charged part of the RTIL stabilizes electrostatic interactions. In enzymatic syntheses using ionic liquids as solvents, lipases were reported to maintain the highest activity [8].

We pioneered the use of RTILs in conjunction with enhancing oxidoreductases (enzyme "wiring") [9]. In this study, we present the influence of several factors on the activity of cholesterol oxidase (ChOx) transiently exposed to a RTIL. The ChOx molecularly "wired" in an RTIL environment was subsequently used as sensing element in an amperometric biosensor. Flavin adenine dinucleotide (FAD) was used as molecular "wire". The factors considered were: the FAD presence in RTIL during modification, water presence during exposure to RTIL, and the ratio FAD:RTIL during the "wiring". The performance parameters monitored were: the enzyme activity before and after "wiring" (expressed as (dA/dt)/mg enzyme, and measured spectrophotometrically; A is the absorbance, dimensionless, and t is time, in seconds), the peak current in an amperometric biosensor, and the linearity of the biosensor response as a function of cholesterol concentration.

"Wiring" of enzymes was introduced to prevent mediators from diffusing out of films deposited on electrodes [10]. It results in an enhancement in electron transfer kinetics as well. Instead of the toxic mediators typically used as "wires" and reported in the literature [11] we used FAD which is non-toxic, is intrinsic to ChOx (a flavoenzyme), and was instrumental in enhancing enzyme kinetics and stability in synthesis [12] and in biosensors for lactate analysis [13]. Following the exposure to the RTIL and its subsequent removal, FAD used as a "wire" becomes part of the molecular structure of the refolded protein. This makes the procedure recommended here a molecular one while the biosensors recommended in the literature for cholesterol analysis only use immobilization in conducting polymers as a method to enhance electron transfer kinetics [14].

2 Materials and Methods

2.1 Materials

The following reagents were used in the experiments: 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM]BF_4, used as RTIL), cholesterol oxidase from *Brevibacterium* sp. (E.C.1.1.3.6, 500U), cholesterol, monobasic and dibasic sodium phosphate, glutaraldehyde 25 %, and Tween 80. All reagents were purchased from Sigma Aldrich. The water used was deionized water.

2.2 Instrumentation

The spectrophotometer used was the Perkin-Elmer® Lambda-20 UV–Vis Spectrometer, controlled by the Perkin-Elmer's UV Kinlab® software. The electrochemical experiments were conducted with a Hokto potentiostat/galvanostat in a 3-electrode configuration for cyclic voltammetry.

2.3 Experimental Protocol

All operations were performed at room temperature 20 ± 0.5 °C.

2.3.1 "Wired"/Modified Enzyme (ME) Samples Preparation

The common features of all sample preparations are: exposure to RTIL for 24 h, under magnetic stirring, followed by 24 h dialysis, and subsequent lyophilization. Dialysis was performed at 20 ± 0.5 °C, under gentle stirring for 18 h, against 50 mM phosphate buffer pH 7 using a dialysis membrane with a cutoff molecular weight of 3 kDa. The volume of the dialysis buffer was 500 fold larger than that of the sample. The lyophilization cycle consisted of four steps. First step is a freezing step down to −55 °C (to provide homogenous freezing of the samples), followed by an annealing step at −6 °C to further homogenize the physical characteristics. The third step was drying at 0.05 mbar chamber vacuum and −5 °C, until all ice has sublimated followed by secondary drying of the powder at 0.05 mbar and 25 °C.

The composition of the solutions used for sample preparation is shown in Table 1, where the identity of the samples is assigned as 1.1, 1.2, etc., as detailed in the table.

Table 1 Composition of the mixtures used in enzyme modification

Sample	ChOx (mg)	[EMIM]BF_4 (mL)	Water (ml)	FAD (mg)
1.1	3.8	0.25	0	0
1.2	3.8	0.25	0	0.89
1.3	3.8	0.5	0	0
1.4	3.8	0.5	0	1.65
2.1	3.8	0.25	1.7	0
2.2	3.8	0.25	1.7	0.89
2.3	3.8	0.5	1.7	0
2.4	3.8	0.5	1.7	1.65

Each sample had an initial activity of 62.5 U

Samples 1.x had no intentionally added water, while the 2.x samples were added 1.7 mL of deionized water.

Table 2 illustrates that, following the enzyme modification in FAD-containing mixtures/RTIL, the uptake of FAD was in excess of 97 %. The uptake was estimated by measuring FAD concentration spectrophotometrically ($\lambda = 450$ nm) in the dialysis solution.

2.3.2 *The Spectrophotometric Assay of ChOx is Based on the Oxidation of Cholesterol to Cholest-4-en-3-one*

The product absorbs at 240 nm, with a molar absorptivity of 1,220 L cm/mol. The solutions used for spectrophotometric assays were prepared as follows: (1) 0.05 M sodium phosphate buffer (pH 7.0) with 0.5 % Tween 80 (added for the solubilization of cholesterol). The phosphate buffer solution (pH 7.0) was prepared by mixing 57.7 mL solution 1 M Na_2HPO_4, with 423 mL solution 1 M NaH_2PO_4 and bringing the volume to 2.00 L with deionized water; (2) cholesterol oxidase and modified enzyme samples were prepared by dissolving 50 mg in 1 mL of 0.05 M sodium phosphate buffer (pH 7.0) with 0.5 % Tween 80; (3) 6 mM cholesterol solution in ethanol. The spectrophotometer cuvette contained 3 mL buffer and 0.05 mL of cholesterol solution. When starting the measurement, 50 µL enzyme solution were added, and the results are expressed as rate of change in absorbance at 240 nm, dA/dt [15].

2.3.3 *Electrochemical Assay Preparation and Protocol*

Cyclic voltammetry was performed using working electrodes prepared by placing 100 µL of the ME solution on a 3-mm diameter glassy carbon electrode and allowing it to air-dry. Then 100 µL of 0.75 % glutaraldehyde (used as a cross-linker) was placed on top of the film and allowed to air-dry as well.

The electrochemical cell contained 40 mL 0.05 M phosphate buffer (pH 7), 0.5 % Tween, and 10 mL 6.0 mM cholesterol solution in ethanol. The electrochemical cell contained also FAD as mediator (at a concentration of 0.1 mM), for the samples whose preparation involved no added FAD. Cyclic voltammograms (CV) were recorded with SCE as reference electrode and a platinum wire as counterelectrode. The voltammograms were run at 100 mV/s between −0.8 and +0.6 V versus SCE. The peak current values listed in Table 3, Δi_{max}, are the difference between the values of the peak current in the presence and in the absence of cholesterol.

3 Results

Table 3 summarizes the results of the assays (spectrophotometric and electrochemical, respectively) for all ME samples. The increase in activity is calculated with respect to the preceding sample in the table (e.g. for sample 1.2, the values are compared with those for sample 1.1). In all cases, the activity of the enzyme was decreased to 20–80 % of the initial activity of the sample as seen in columns three and four of Table 3, but in all cases the enzyme was still active after RTIL exposure.

The presence of FAD in the modifying medium proved to be beneficial to the activity of the ME in all cases. It resulted in an increase in specific activity by a factor of 1.81 compared to 1.28 in the case of denaturation/renaturation without the FAD present. In the case of the modification in the presence of water, increases in activities of

Table 2 FAD uptake during ME preparation

ME	FAD (mg)	Water (mL)	FAD in the wash (mg)	FAD incorporated in ME (mg)	FAD incorporated in ME (%)
1.2	0.89	0	0.023	0.867	97.42
1.4	1.65	0	0.031	1.619	98.12
2.2	0.89	1.7	0.021	0.869	97.64
2.4	1.65	1.7	0.032	1.618	98.06

Table 3 Summary of assays (spectrophotometric and electrochemical)

Sample	Specific activity dA/dt (s^{-1} mg^{-1})	Enzymatic activity (U)	Percent fraction of the initial activity (%)	Peak current (μA)	Increase in activity/mg (%)	Increase in peak current (%)
1.1	0.0277	15.8	25.3	371.643	–	–
1.2	0.0503	28.7	45.8	649.261	81.53	74.70
1.3	0.0247	14.1	22.5	422.211	–	–
1.4	0.0317	18.1	28.9	563.016	28.04	33.35
2.1	0.0393	22.4	35.8	562.024	–	–
2.2	0.0507	28.9	46.2	756.195	29.11	34.55
2.3	0.0477	27.2	43.5	257.019	–	–
2.4	0.0855	48.7	78.0	629.327	79.35	144.86

renatured enzymes were by a factor of 1.29 and by 1.79, respectively. From the point of view of specific enzymatic activity, one could say that the presence of water limited the extent of the denaturation while successfully assisting the refolding process.

Regarding the electrochemical activity, there is a distinct peak due to cholesterol oxidation in a typical cyclic voltammogram. The height of the oxidation peak is also affected by the presence of FAD in the treatment mixture, with the corresponding enhancement factors of 1.75 and 1.33 for the dry treatment, and 1.08 and 2.45, respectively, for the case in which the RTIL mixture contained added water. The trend parallels the one in specific activity, in which the increase in the amount of "wire" enhanced activity by a lower factor in "dry" RTIL, but by a higher factor in the "wet" RTIL.

A higher ratio RTIL: enzyme during the modification procedure does not seem beneficial to ME although it affords a modest increase in the FAD entrapped within its 3-D structure.

Thus, we believe that the transient exposure of the enzyme to an RTIL containing a mediator and subsequent removal of RTIL afford an enzyme (ME) with enhanced kinetics for electron transfer. We tested the analytical potential of the electrode that exhibited the most enhanced electrochemical peak (ME 2.4), hence the highest catalytic effect (Table 3), prepared from a water-containing mixture (Table 1). The peak current exhibited linearity with respect to cholesterol concentration between 0.55 and 1 mM cholesterol (Fig. 1). The strong correlation between the peak current and the cholesterol concentration encourages further optimization efforts towards the development of an amperometric biosensor for cholesterol.

The ME 2.4-based electrode was also tested for stability. The electrode retained 88 % of the oxidation peak current after storage for 12 days in 0.05 M pH 7 phosphate buffer. The peak current at the electrode was not affected by the presence of 0.1 mM ascorbate in solution. The lack of interference by ascorbate is important for the analysis of clinical samples, since ascorbic acid causes a false decrease in the measured values for total cholesterol in serum [16].

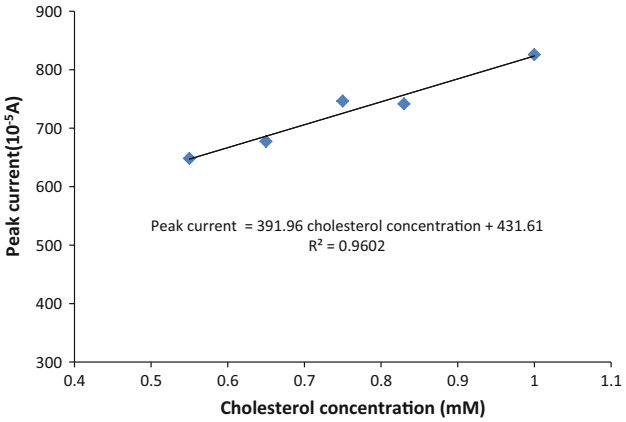

Fig. 1 Dependence of oxidation peak on cholesterol concentration for biosensor prepared with sample ME 2.4. Working electrode: GC/ME 2.4/glutaraldehyde 0.75 %, Pt counterelectrode, standard calomel electrode (SCE)—reference electrode. Cell: 0.05 M phosphate buffer, pH 7, 1 % EtOH, 0.5 % Tween 80

4 Discussion

In the present work, we investigated the behavior of ChOx subjected to transient exposure to a RTIL. After RTIL removal the ME preserved a fraction of the activity of the initial enzyme. This behavior was attributed to partially reversible denaturation of the enzyme [17].

In cases when FAD, the prosthetic group of ChOx, was present during modification it ended entrapped (almost totally) in the 3-D structure of the renatured enzyme, becoming a part of the molecular structure of ChOx, and supplementing the intrinsic FAD of the native enzyme. As

a molecular "wire" it became part of the 3-D structure of the renatured enzyme, and it resulted in enhanced electron transfer kinetics at the electrode [18]. "Wiring" does not make the bulk of the enzyme conductive but establishes preferential paths for electron transfer. Even when immobilized on the electrode only a small fraction of the enzyme is properly oriented for electron transfer [19]. The mediator molecules important for electron mediation between enzyme and electrode are only those accessible to both the prosthetic group of the enzyme and to the electrode, and they are mostly located on or close to the protein surface. From this fact a discrepancy between the enzymatic activity of the ME and the amperometric response may occur. This discrepancy suggests different impact of exposure to RTIL on the two steps involved in the catalytic action of ChOx (oxidation and isomerization).

Higher content of RTIL in the "wiring" mixture, while negatively impacting renaturation, resulted in increase in FAD entrapped in ME. This effect was compensated for by the presence of water during the modification. The role of water may be twofold: limit denaturation and assist renaturation.

The procedure presented herein has the advantage of being an enzyme-friendly one (since ChOx is a flavoenzyme) as well as an environmentally-friendly one since the "wire" (FAD) is not toxic; the compounds typically used in oxidoreductase "wiring" are toxic (Os or Ru complexes).

The refolded structure, although non-native is a stable one. The biosensor having "wired" ChOx as sensing element displayed, after repeated measurements and 12 days in storage, 88 % of the initial oxidation peak. Use of FAD as "wire" for lactate dehydrogenase (LDH) afforded higher increases in enzyme activity [20]. This different behavior was correlated with the FAD present in the native enzyme: while LDH contains only non-covalent FAD, ChOx from *Brevibacterium* sp. contains both non-covalent and covalent FAD (less stable) [21].

A further direction of research is to determine the optimal conditions for biosensor preparation by transient exposure to RTIL. A complete design of experiments will include temperature, length of exposure to RTIL, and other experimental factors in addition to the factors mentioned in this article: amount of enzyme, water content, and ratio of "wiring" agent to enzyme. In this study, a partial factor matrix was used and the results should be considered as guidance for further study.

Usually, 70–80 % of total serum cholesterol is esterified with fatty acids with a variety of degrees of saturation and molar masses [22]. As future direction for the research, to fit clinical analysis needs, we will focus on a biosensor including cholesterol esterase, to transform the two thirds of serum cholesterol that appears in the ester form, into free cholesterol.

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