



On the Mediated Electron Transfer of Immobilized Galactose Oxidase for Biotechnological Applications

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Abstract: The use of enzymes as catalysts in chemical synthesis offers advantages in terms of clean and highly selective transformations. Galactose oxidase (GalOx) is a remarkable enzyme with several applications in industrial conversions as it catalyzes the oxidation of primary alcohols. We have investigated the wiring of GalOx with a redox polymer; this enables mediated electron transfer with the electrode surface for its potential application in biotechnological conversions. As a result of electrochemical regener-

ation of the catalytic center, the formation of harmful H₂O₂ is minimized during enzymatic catalysis. The introduced bio-electrode was applied to the conversion of bio-renewable platform materials, with glycerol as model substrate. The biocatalytic transformations of glycerol and 5-hydroxymethylfurfural (HMF) were investigated in a circular flow-through setup to assess the possibility of substrate over-oxidation, which is observed for glycerol oxidation but not during HMF conversion.

Introduction

Several oxidation reactions in synthetic chemistry, for example, the conversion of alcohols to carbonyl compounds, are performed by using chemical oxidants that are required in stoichiometric amounts, meaning that for industrial applications large quantities of toxic and occasionally expensive reagents as well as subsequent separation processes are needed.^[1] Therefore, the possibility to implement catalytic conversions to perform these reactions has proven advantageous by decreasing waste generation, boosting selectivity, and improving conversion efficiencies.^[2,3] In particular, the use of enzymes for performing catalytic conversions offers several advantages, enabling environmentally friendly and highly selective transformations under mild and aqueous conditions. As a result, the

implementation of biocatalytic transformations is currently of high relevance in the fine chemical and pharmaceutical industries.^[4]

Galactose oxidase (GalOx) is a mononuclear copper enzyme that catalyzes the oxidation of primary alcohols to aldehydes using molecular oxygen as the natural electron acceptor. The active site comprises a tyrosine radical and a Cu²⁺ metal center as distinct one-electron acceptors. Thus, GalOx can exhibit three different oxidation states associated with two one-electron reduction steps: i) the fully oxidized form, consisting of Cu²⁺ and the free radical (Tyr[•]), the redox-active form for the oxidation of alcohols as substrates; ii) the semi-reduced form (Cu²⁺, Tyr), catalytically inactive; and iii) the fully reduced O₂-reactive form (Cu⁺, Tyr), which can participate in the catalytic cycle (Figure 1).^[5–7] The metalloradical complex acts as a two-electron redox unit during catalytic turnover. In an initial step, the transfer of two electrons and two protons from the alcohol substrate to the enzyme forms the aldehyde product and concomitantly the two-electron reduced enzyme, with one electron at each redox center. The enzyme is subsequently oxidized by the transfer of two electrons and two protons to O₂ to form H₂O₂, closing the catalytic cycle.^[5,6,8–10]

Due to the effective conversion of galactose, GalOx has been mainly applied in electrochemical devices for the development of biosensors for galactose detection,^[11] where the most common strategy used has been the detection of H₂O₂ generated during conversion of the analyte.^[12] Nevertheless, the enzyme GalOx presents an unusually broad substrate tolerance,^[9] being able to oxidize other primary alcohols as well, including simple three-carbon alcohols such as glycerol (Figure 1)^[13] at reasonable conversion rates.^[14] Although the lack of substrate selectivity is debated between a tradeoff for catalytic speed at the expense of selectivity^[8,9] or as a result of H₂O₂ being the biologically relevant product,^[5,6,8] this peculiarity makes GalOx a remarkable biocatalyst for the oxidation of

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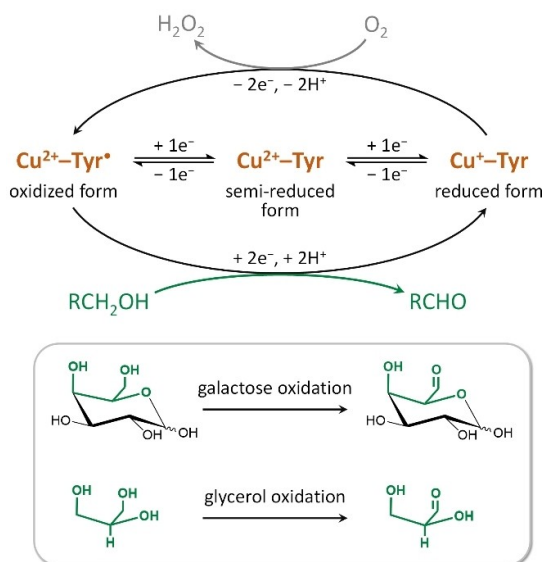


Figure 1. Schematic representation of redox processes with GalOx. The catalytic center comprises a copper ion and a tyrosine radical. For a detailed description of the redox interconversions, see text. The box shows examples of oxidation reactions catalyzed by the enzyme: conversion of galactose (natural substrate) and glycerol. The structural similarities of the molecules are highlighted in green. The enzyme performs the oxidation of the hydroxy group at the C6 position in galactose and the corresponding hydroxy group at glycerol.

primary alcohols. Consequently, GalOx has become an industrially relevant enzyme applied in biocatalytic transformations for the synthesis of diverse products of interest^[1,2] and has been widely explored in synthetic manufacturing, including the pharmaceutical industry.^[4,15]

Furthermore, GalOx is an attractive enzyme for biotechnological applications, as it also catalyzes the oxidation of bio-renewable platform products such as glycerol^[3] and 5-hydroxymethylfurfural (HMF).^[16] In contrast with other strategies for the catalytic conversion of these products,^[17] biologically driven catalytic transformations are inherently more selective and constitute a renewable alternative with the possibility to operate at lower applied potentials, under mild pH values, and ambient temperature conditions.

Despite the attractive potential applications of GalOx in synthetic and bio-renewable processes, several factors should be considered for ensuring efficient conversions. The regeneration of the catalytic center by O₂ implies the associated co-production of H₂O₂ (Figure 1). As it has been reported, the enzyme is irreversibly inactivated in the presence of H₂O₂ during catalytic turnover.^[2,14] Therefore, the removal of H₂O₂ is necessary for ensuring enzymatic function. Thus, technological applications of GalOx conventionally involve the addition of large amounts of catalase for catalyzing the dismutation of H₂O₂ into H₂O and O₂.^[1,3,4,15,16,18] Moreover, the Michaelis constant (*K_M*) for O₂ as substrate required for the regeneration of the enzyme active site has been estimated to be above 3 mM.^[2,8] As this value is higher than the solubility of O₂ in aqueous solutions under ambient conditions, the reaction rate

of conversion is highly affected by small variations in O₂ concentrations.^[2] Furthermore, the presence of single-electron oxidants in the reaction medium has proven essential in order to achieve sustained catalytic activity.^[2,4] The intermediate, semi-reduced form of the enzyme (see Figure 1) is periodically formed during conversion.^[4] Because the enzyme in this state is unable to oxidize an alcohol substrate or be reactivated by O₂, the semi-reduced form falls off the catalytic cycle and accumulates, causing an overall decrease in conversion over time.

The regeneration of the enzyme into its fully active form has been effectively shown using mild oxidants such as [Fe(CN)₆]³⁻,^[9] tetracyanoquinodimethane^[19] and ferrocene derivatives,^[20] as well as osmium^[21] and manganese complexes.^[4] Although most of the time these species are required in relatively large amounts, their effective use has suggested the possibility to replace O₂ and support turnover under anaerobic conditions,^[5] which could overcome the previously discussed limitations by performing an electrochemical regeneration of the enzyme.^[4]

The use of redox polymers for immobilization and wiring of enzymes is an attractive strategy for the implementation of heterogeneous catalytic systems, as it allows a fast regeneration of the enzymatic active site and a simple separation of the modified electrode from the reaction medium, which would be beneficial for continuous processing in industrial applications.^[22] However, limited reports have shown until now the possibility for integration of GalOx with redox polymers,^[23] encouraging further detailed investigations.

The aim of this work was to construct a modified electrode enabling mediated electron transfer of immobilized GalOx to minimize the influence of O₂ during catalytic conversions. The fabricated bioelectrode makes use of native GalOx and is investigated for the conversion of glycerol and HMF in a flow-through system, with emphasis on the qualitative identification of reaction products.

Results and Discussion

The redox polymer poly(1-vinylimidazole-co-allylamine)-[Os-(bpy)₂Cl]⁺ (P-Os, bpy = 2,2'-bipyridine, Figure S1 in the Supporting Information)^[24] was used to immobilize and wire the enzyme. The redox polymer matrix is solvated in aqueous solutions, leading to a hydrogel structure with entrapped enzymes that are wired to the electrode surface through collisional electron transfer by the polymer-bound redox centers. An effective mediated electron transfer was observed for GalOx embedded into the redox polymer, as evidenced by the catalytic wave in the presence of galactose with a midpoint potential matching that of the Os complex tethered to the redox polymer (Figure 2A). To attain an optimal performance, the amount of redox polymer, immobilized enzyme, and the overall film thickness were used as optimization parameters for achieving the maximum catalytic current under saturating galactose concentrations and to ensure minimal interference from ambient O₂ on the performance of the bioelectrode. The

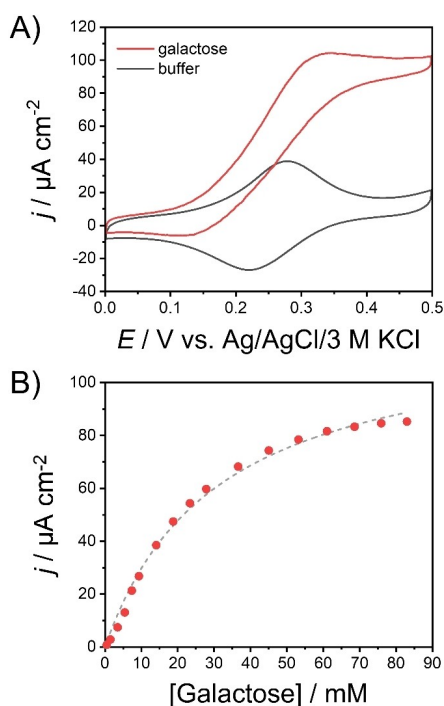


Figure 2. A) Cyclic voltammograms in the absence and presence of 45 mM galactose for a graphite electrode modified with GalOx embedded in P–Os. Tris buffer, pH 9.0. Scan rate: 5 mV s^{-1} . B) Calibration curve obtained after the addition of different galactose concentrations to the electrochemical cell, as presented in Figure S2. $E_{\text{app}} = 0.5 \text{ V vs. Ag/AgCl/3 M KCl}$. 10 mM HEPES buffer, pH 7.4. Dashed line: fitting to a Michaelis–Menten model.

investigation of the catalytic current response as a function of galactose concentration (Figure S2) followed a Michaelis–Menten model, with a K_M value of about 30 mM (Figure 2B). The obtained value was lower than previously reported for the free native enzyme ($K_M = 175 \text{ mM}$ at O_2 saturated conditions^[8]). However, it was comparable with other K_M values reported for recombinant forms of GalOx (47 mM, GalOx from *Fusarium oxysporum*^[7] and 35 mM, GalOx from *Fusarium graminearum*,^[25] both expressed in *Escherichia coli*). The fast diffusion of galactose through the redox polymer hydrogel reaching the immobilized enzyme was confirmed by a sharp increase in anodic current after the addition of substrate into the electrochemical cell (Figure S3).

As has been highlighted before, the presence of negative charges near the enzyme active site promotes efficient electronic communication when positively charged mediators are used.^[20] Moreover, the use of a redox polymer matrix was expected to provide a fast electron transfer between the enzyme and the electrode surface, supported by the characteristic electron hopping mechanism between redox centers tethered to the polymer backbone.^[26] Consequently, the electrochemical regeneration of the enzyme active site was expected to enable O_2 -independent catalysis. A comparison of catalytic currents obtained for different galactose concentrations under ambient conditions and an Ar atmosphere revealed that the GalOx-based bioelectrode was able to perform catalytic conversions with a minor influence from the availability of O_2 on

the conversion rate (Figure 3A); hence, minimizing the formation of harmful H_2O_2 alongside the chemical conversion of the substrate. In addition, the use of a $1e^-$ mediator as redox center in P–Os ensured the full regeneration of the enzyme after catalytic conversion, preventing the formation and accumulation of enzyme in the inactive semi-reduced form.

As a proof of concept for the biotechnological application of immobilized GalOx in a mediated electron-transfer configuration, the developed bioelectrode was used for the oxidation of glycerol. The catalytic current for increasing glycerol concentrations was investigated at different pH values of the electrolyte solution. The obtained results indicated the catalytic oxidation of glycerol with the bioelectrode, exhibiting an optimal conversion performance at a pH value of 8.0 (Figure 4A). In agreement with previous results shown when other redox mediators were used for the anaerobic reactivation of GalOx in solution,^[6,20,27] the optimum for catalytic conversion was attained at more alkaline pH values in comparison with the rather neutral pH values conventionally used for GalOx-catalyzed conversions. This could be explained as the requirement of a more alkaline pH value to promote the abstraction of 2H^+ from the catalytic center after oxidation of the substrate, in contrast with the regeneration of the enzyme by O_2 reduction leading to the formation of H_2O_2 after a $2e^-/2\text{H}^+$ transfer (Figure 1).

The possibility for bio-electrochemical conversion with a minimal interference from ambient oxygen was also proved using glycerol as substrate (Figure 3B). To confirm that the

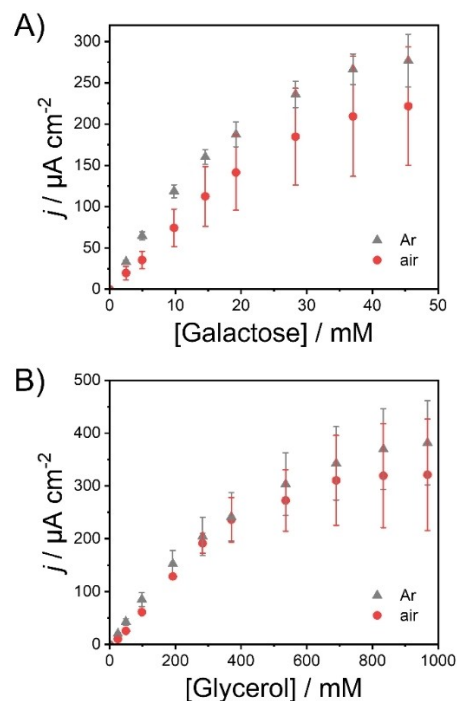


Figure 3. Investigation of the current response obtained for different substrates in the presence of solutions equilibrated in ambient air and under argon. A) Galactose conversion, $N = 4$ different electrodes. B) Glycerol conversion, $N = 3$ different electrodes. $E_{\text{app}} = 0.5 \text{ V vs. Ag/AgCl/3 M KCl}$. Phosphate-citrate buffer, pH 8.0. Error bars represent the standard deviation.

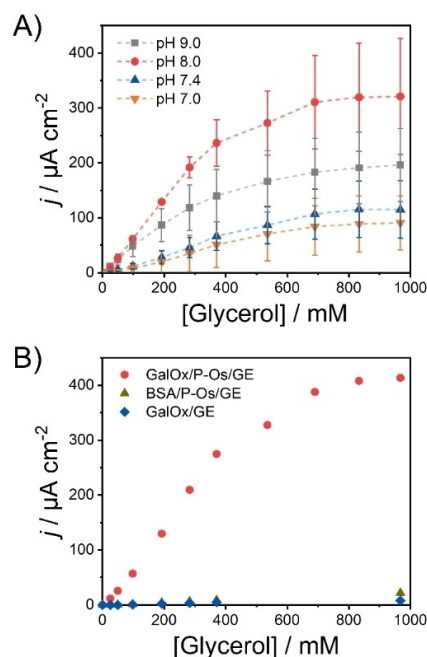


Figure 4. A) Catalytic currents recorded for glycerol conversion with the bioelectrode at different pH values. $E_{app} = 0.5$ V vs. Ag/AgCl/3 M KCl, $N = 3$ different electrodes. Error bars represent the standard deviation. The dashed lines are a guide for the eye only. B) Comparison of catalytic currents obtained for different glycerol concentrations with the modified bioelectrode of GalOx embedded in P-Os on graphite (GalOx/P-Os/GE) and control electrodes consisting of bovine serum albumin (BSA) embedded in P-Os (BSA/P-Os/GE) or GalOx adsorbed on the graphite electrode in the absence of P-Os (GalOx/GE). $E_{app} = 0.5$ V vs. Ag/AgCl/3 M KCl. Phosphate-citrate buffer, pH 8.0.

conversion was catalyzed by the enzyme in a mediated electron-transfer configuration, control experiments were performed using electrodes fabricated either in the absence of GalOx or in the absence of P-Os, which in both cases delivered negligible catalytic currents even in the presence of high glycerol concentrations (Figure 4B). The successful oxidation of other compounds of interest known to be converted by GalOx, such as dihydroxyacetone and 5-hydroxymethylfurfural (HMF), was also achieved using the modified bioelectrode (Figure S4).

An important aspect to consider in the development of biotechnological conversion systems is the reaction selectivity. The conversion of primary alcohols by GalOx is commonly assumed to be selective to the aldehyde product. However, as has been reported long ago, studies of the native enzyme have provided evidence that GalOx converts the C6 hydroxy group of galactose to an aldehyde group and can also further catalyze the oxidation to the carboxyl group.^[28] Recently, enzyme variants have been engineered to improve the aldehyde oxidase activity to attain the oxidation not only of alcohols but also of aldehydes, which is of relevance for biocatalytic applications.^[3]

The GalOx-based bioelectrode was applied to the electrocatalytic conversion of glycerol and HMF. Under optimal conditions, the maximum turnover frequency (TOF) for glycerol conversion was estimated to be 67 min^{-1} , while the maximum

TOF for HMF conversion was 60 min^{-1} . A circular flow-through setup,^[29,30] was used for enabling a detailed investigation of possible follow-up oxidation processes when the primary aldehyde product is accumulated in the reaction medium. It is important to note that in order to exclude possible products formed or converted at the counter electrode during electrochemical conversion, the counter electrode was placed in a separate compartment using an anion exchange membrane. Qualitative detection of the reaction products was performed by high-performance liquid chromatography (HPLC). The obtained results in the comparison of samples taken before starting the electrochemical conversion of glycerol and after different reaction times revealed the production of glyceraldehyde, but also the production of glyceric acid resulting from the subsequent oxidation of glyceraldehyde accumulated in the electrolyte (Figure 5A). In contrast, during the conversion of HMF, the main reaction product was 2,5-diformylfuran, with no noticeable production of other HMF-derived oxidation products (Figure S6A).

To confirm that the observed products were a consequence of the enzymatic reaction, a control electrode fabricated without GalOx was used for the flow-through electrochemical conversion experiments. In this case, no noticeable formation of glyceraldehyde or glyceric acid could be detected for the conversion of glycerol (Figure 5B), neither was 2,5-diformylfuran produced over time for the conversion of HMF (Figure S6B). Furthermore, a similarly prepared control electrode was also used to investigate whether the conversion of glyceraldehyde was catalyzed by the enzyme or as a consequence of reaction with the electrode material or the redox polymer. In this case, no perceivable formation of glyceric acid over time was observed (Figure 5C), indicating that the production of glyceric acid was indeed catalyzed by GalOx.

Conclusion

An Os-complex-modified redox polymer was used to wire native GalOx in mediated electron transfer with the electrode surface. Under these circumstances, an electrochemical regeneration of the enzyme is shown to support biocatalytic conversion with minimal influence from ambient O_2 . The implemented bioelectrode has the advantages of a heterogeneous catalytic system for a facile separation of the catalyst and redox mediator from the reaction medium and makes catalytic conversions possible without the necessity of introducing additional enzymes to remove H_2O_2 . The strategy is favorable for continuous processing and cascade reactions for future applications. Taking the oxidation of glycerol and HMF as an example for potential biotechnological applications, the conversion of substrates in a circular flow-through setup was investigated. Our results show that glyceraldehyde that accumulates in the reaction medium during glycerol conversion can be further oxidized to glyceric acid by GalOx. In contrast, 2,5-diformylfuran remains the main product in the conversion of HMF, even when the same circular-flow conditions are applied.

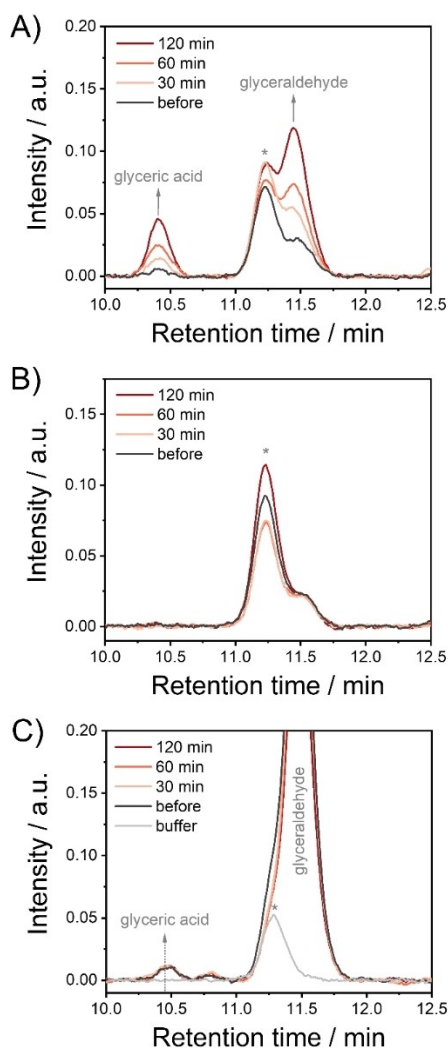


Figure 5. Chromatograms obtained from HPLC analysis of electrochemical conversion products. A) Glycerol conversion with a GalOx/P-Os bioelectrode. B) Glycerol conversion using a control electrode in the absence of GalOx (BSA/P-Os). C) Glyceraldehyde conversion with a BSA/P-Os control electrode. Results obtained for samples taken before or after different conversion times, as indicated. Product assignments are shown in the figure. System peaks are indicated with an asterisk. Refractive index detector; baseline corrected traces; for raw data, see Figure S5.

Experimental Section

Chemicals and materials: All chemicals were of analytical grade and used as received. Glycerol ($\geq 99\%$) was from Fisher Scientific. D-(+)-Galactose, DL-glyceraldehyde, dihydroxyacetone, glyceric acid, formic acid, 5-hydroxymethylfurfural, 2,5-furandicarboxylic acid, 2-formyl-5-furancarboxylic acid, 5-hydroxymethyl-2-furancarboxylic acid, 2,5-diformylfuran, pyruvic acid, sodium carbonate, sodium bicarbonate, bovine serum albumin (BSA), and galactose oxidase (GalOx, EC 1.1.3.9, $652 \text{ U mg}_{\text{protein}}^{-1}$) were from Sigma-Aldrich. Sulfuric acid (98%) was from Merck. Tris-HCl was from AppliChem. Citric acid monohydrate, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate trihydrate were from VWR Chemicals. Poly(ethylene glycol)diglycidyl ether (PEGDGE, $M_n=400 \text{ g mol}^{-1}$) was from Polysciences. All solutions were prepared with ultrapure deionized water ($\rho=18 \text{ M}\Omega \text{ cm}$).

The redox polymer poly(1-vinylimidazole-co-allylamine)-[Os(2,2'-bipyridine) $_2$]Cl (P-Os, Figure S1) was synthesized and purified as described previously.^[24]

Electrode modification: Home-made graphite electrodes were fabricated by encasing a graphite rod (3 mm diameter, Sigma-Aldrich) into a surrounding glass sheath. Before modification, the circular exposed graphite surface was polished using sandpaper of decreasing grits (P400, P1500, and P3000). After cleaning, rinsing, and drying, the electrode surface was modified with $4.0 \mu\text{L}$ of a mixture of the redox polymer P-Os (2.7 mg mL^{-1}), PEGDGE (0.06 mg mL^{-1}), and GalOx (2.8 mg mL^{-1}). For control experiments in the absence of GalOx, the electrodes were modified with $4.0 \mu\text{L}$ of a mixture of P-Os (2.7 mg mL^{-1}), PEGDGE (0.06 mg mL^{-1}), and BSA (1.0 mg mL^{-1}).

For electrochemical conversion at longer times using a flow-through setup, carbon paper (H23, Freudenberg) was used as the electrode substrate. A piece of $1.2 \text{ cm} \times 1.2 \text{ cm}$ was cut and modified in its central region (circular area of about 1.1 cm diameter) with $20 \mu\text{L}$ of a mixture of P-Os (2.7 mg mL^{-1}), PEGDGE (0.06 mg mL^{-1}), and GalOx (2.8 mg mL^{-1}). For control experiments, GalOx was replaced by BSA (1.0 mg mL^{-1}).

The graphite and carbon paper modified electrodes were stored at 4°C overnight until dry. Before measurement, the prepared bioelectrodes were incubated for 30 min in 50 mM Tris buffer solution (pH 9.0) to induce polymer collapse and crosslinking.^[31]

Electrochemical measurements: All experiments were performed in a three-electrode configuration, using the modified electrode as the working electrode, a Pt mesh as a counter electrode, and a Ag/AgCl/3 M KCl reference electrode. Batch measurements were performed using a CHI 1030 potentiostat. During amperometric measurements at a constant applied potential, the electrolyte solution was stirred with aid of a magnetic stirrer to ensure a homogeneous solution after the addition of different substrate concentrations. To investigate the pH effect, either phosphate-citrate buffer (pH 7.0, 7.4, and 8.0) or $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH 9.0) were used.

Long-term conversion experiments at a constant applied potential were performed using an Autolab PGSTAT12 (Metrohm Autolab) potentiostat and a flow-through cell.^[29,30] A circular flow was established for the accumulation of products in the electrolyte solution. The cell consisted of two compartments, enabling separation of the counter electrode from the other two electrodes by an anion exchange membrane (Fumasep FAA-3-PK-130, from Fumatech). A circular area of 0.95 cm^2 of the working electrode was exposed to the electrolyte solution during the electrochemical experiments. The system was filled with 4 mL of electrolyte, containing either 600 mM glycerol or 100 mM HMF in phosphate-citrate buffer, pH 8.0. For control measurements, $5 \mu\text{M}$ glyceraldehyde was used. The conversion was performed at an applied potential of 0.5 V vs. Ag/AgCl/3 M KCl.

All measurements were performed at room temperature and under ambient air conditions unless otherwise noted.

Turnover frequency: The TOF was calculated by assuming 100% Faradaic efficiency and using the following equation:

$$\text{TOF} = \frac{i}{zFn_{\text{cat}}} \quad (1)$$

where, i is the difference between the current measured after substrate addition and the background current [A], z is the number of electrons involved in the conversion ($2e^-$ in this case), F is the

Faraday constant ($96485.33 \text{ C mol}^{-1}$), and n_{cat} is the amount of immobilized GalOx (mol).

Product analysis: Samples of 400 μL were taken from the circular flow-through setup before electrochemical conversion and after 30, 60, and 120 min of conversion under applied potential. The samples were filtered using 0.22 μm syringe filters prior to analysis. HPLC measurements were performed using a Dionex ICS-5000+ system (Thermo Fisher Scientific) using a refractive index detector (RefractoMax 520, Knauer) and an ion-exclusion column and precolumn (Aminex HPX-87H, Bio-Rad). Following an optimized procedure for product separation,^[29] the column compartment was heated at 70 °C and 4 mM H_2SO_4 was used as eluent with a flow rate of 0.6 mL min^{-1} . For identification of the products according to their retention time, calibration was performed using commercially available reference compounds (Figures S8 and S9).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: bio-electrocatalysis · galactose oxidase · glycerol oxidation · modified electrodes · redox polymers

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