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The co-immobilization of P450-type nitric oxide reductase and glucose dehydrogenase for the continuous reduction of nitric oxide *via* cofactor recycling

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ABSTRACT

The co-immobilization of enzymes on target surfaces facilitates the development of self-contained, multi-enzyme biocatalytic platforms. This generally entails the co-immobilization of an enzyme with catalytic value in combination with another enzyme that performs a complementary function, such as the recycling of a critical cofactor. In this study, we co-immobilized two enzymes from different biological sources for the continuous reduction of nitric oxide, using epoxide- and carboxyl-functionalized hyper-porous microspheres. Successful co-immobilization of a fungal nitric oxide reductase (a member of the cytochrome P450 enzyme family) and a bacterial glucose dehydrogenase was obtained with the carboxyl-functionalized microspheres, with enzyme activity maintenance of 158% for nitric oxide reductase and 104% for glucose dehydrogenase. The optimal stoichiometric ratio of these two enzymes was subsequently determined to enable the two independent chemical reactions to be catalyzed concomitantly, allowing for near-synchronous cofactor conversion rates. This dual-enzyme system provides a novel research tool with potential for *in vitro* investigations of nitric oxide, and further demonstrates the successful immobilization of a P450 enzyme with potential application towards the immobilization of other cytochrome P450 enzymes.

1. Introduction

Nitric oxide reductase is of significant interest as it has a variety of potential applications including the quantification of nitric oxide (NO) in solution [1], and the development of biosensors or research tools for elucidating the various biological roles of NO [2–6]. Nitric oxide is a small diatomic gaseous molecule with a relatively short half-life, which interacts with numerous targets in biological systems and is associated with various cellular signaling pathways [2,5–12]. Current *in vitro* research methodologies used to investigate the biological effects of NO are limited to the use of NO scavengers [13–15] or inhibitors of NO-synthesizing enzymes [14,16–20]. The use of inhibitors such as L-arginine analogues has facilitated the elucidation of NO as a signaling molecule and its contribution to pathogenesis [18,21–23]. However, L-arginine analogues have adverse effects on arginine metabolic pathways and may thus obscure the full biological function of NO [24,25]. NO scavengers are considered toxic chemical compounds, which may also result in possible masking of NO function. An enzymatic system for the reduction of NO therefore has the potential to avoid such challenges, thereby aiding investigations of this important cell signaling molecule.

Several NO-reducing enzymes (NOR) belonging to the P450 family have been identified in denitrifying bacteria [26–29] and fungi [30–34]. Fungal NOR is particularly interesting as it comprises a monomeric, single-domain structure that accepts electrons directly from its cofactor, NADH/NADPH [35–37], in contrast to the multi-

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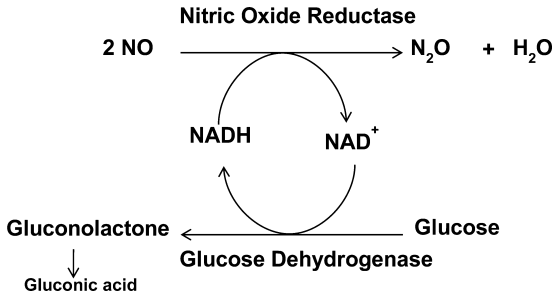

Fig. 1. Stoichiometric model of the proposed dual-enzyme system for the continuous reduction of nitric oxide. Two molecules of nitric oxide are reduced by NOR to form nitrous oxide and water using one reducing equivalent of NADH cofactor. The cofactor recycling reaction then reduces the NAD^+ back to NADH through the GDH-catalyzed conversion of glucose to gluconolactone.

domain bacterial NORs [28,38–40]. Reports on the immobilization of NOR enzymes have been limited to voltammetric studies on bacterial NOR adsorbed to graphite-based electrodes for further investigations into the catalytic mechanism of this enzyme, and minimal data were provided on enzyme activity maintenance or stability [40–42]. As for fungal NORs specifically, no immobilization data are currently available, with the majority of studies to date having focused on the NO reduction mechanism [36,43,44].

For many enzymes, solid supports have been shown to provide enhanced enzyme stability through multipoint covalent attachment [45–48], with improvements in enzyme specificity and selectivity also frequently observed [49–52]. However, several drawbacks of enzyme immobilization have been noted including diffusion limitations within partially porous supports [53], distortion of the enzyme structure via the multiple attachment points, the blocking of active sites [49,50,53] and enzyme inactivation through conformational changes [53,54].

Cofactors such as NADH are essential for redox reactions and several studies describe the co-immobilization of cofactors with their enzymes [53,55]. Immobilized cofactor recycling systems have also been reported [56–60], through either the attachment of the cofactor to a solid surface, or to hollow-fiber or packed bead reactors [56,57,59,61,62]. One of the main advantages of the co-immobilization of sequentially acting enzymes within close proximity, is that this increases catalytic efficiency due to significant reductions in substrate and/or cofactor diffusion time, and the high local concentrations of substrates/recycled cofactor generated can lead to increased rates of enzyme turnover and reduced reagent costs [63,64]. In this study, we describe a novel cofactor recycling system that entails the covalent co-immobilization of a fungal NOR with a tetrameric enzyme, glucose dehydrogenase (GDH). This redox system facilitates the recycling of NAD^+/H powered by two independent catalytic reactions: NO reduction by NOR and glucose oxidation by GDH (see stoichiometric model in Fig. 1). Such a dual-enzyme system allows for the continuous and quantitative reduction of NO for a duration that can be controlled by the researcher, without the detrimental effects of existing methods, provided that glucose is in excess in the micro-environment. For the development of this research tool, we chose to co-immobilize the fungal NOR from *Aspergillus oryzae*, Anor [65], and the bacterial GDH from *Bacillus megaterium* [66], on ReSyn polymer microspheres. ReSyn is a relatively new microsphere technology with comparatively high binding capacity due to its hyper-porous matrix, which translates into high volumetric activity and minimal diffusion limitations [67,68].

The demonstration of efficient and continuous reduction of nitric oxide with the concomitant regeneration of NADH by microsphere-immobilized enzymes was therefore the primary aim of this study. This comprised the following objectives: selection of a suitable microsphere conjugation chemistry for the immobilization of the NOR and GDH enzymes; activity and stability determination of the immobilized enzymes with varying buffer pH and temperature; synchronization of the two enzyme reactions; the determination of successful cofactor recycling by the dual-enzyme system, and lastly, evaluation of the system's potential as a novel research tool for *in vitro* investigations of the many biological roles of nitric oxide and for application towards the immobilization of other cytochrome P450 enzymes of interest.

2. Materials

ReSyn epoxide- and carboxyl-functionalized microspheres were sourced from ReSyn Biosciences (Pty) Ltd. (South Africa). These microspheres comprise a hyper-porous polymer matrix and were supplied at a custom functional group density of 1200 μmoles g^{-1}. The *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) required for the activation of the carboxyl microspheres were purchased from Sigma–Aldrich (Germany), as were the triethanolamine (TEA), 2-(N-morpholino) ethanesulfonic acid (MES), 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES), phorbol 12-myristate 13-acetate (PMA), enzyme cofactors (NAD^+ and NADH), glucose, NaOH, NaCl and the reagents for the Britton–Robinson buffer and phosphate-buffered saline (PBS). NOC-5 (3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene), Eppendorf LoBind tubes and Pall Acrodisc 0.8/0.2 μm syringe filters were purchased from Calbiochem (Merck-Millipore; Germany). Roswell Park Memorial Institute cell culture medium (RPMI), fetal bovine serum (FBS), S-nitroso-N-acetylpenicillamine (SNAP), 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM), and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) were purchased from Invitrogen (Life Technologies, USA). The CellTiter 96 AQueous One Solution Cell Proliferation Assay and Quick Start Bradford Protein Assay reagents were purchased from Promega (USA). The transparent and black 96-well microtiter plates were purchased from Greiner Bio-One (USA), while the 384 well microtiter plates were purchased from Genetix (now Molecular Devices, England). The human myeloid cell line, U937, was purchased from the European Collection of Cell Cultures (Public Health England) and the tissue culture flasks and microtiter plates for culturing were from TPP (Switzerland).

3. Methods

3.1. Immobilization of enzymes

Recombinant NOR enzyme based on *A. oryzae*, Anor (EC 1.7.99.7), was expressed and purified from *Escherichia coli* BL21 cells cultured in our laboratory as described by Garny et al. [1]. Briefly, the recombinant Anor comprised the coding sequence only from the full-length Anor (*nicA* gene from *A. oryzae*), which was ligated between *SalI* and *Not*I restriction sites on the pET-28a vector such that a C-terminal His-tag sequence configuration was obtained [1]. A commercial preparation of GDH was purchased from Codexis, Inc. (GDH-102; USA). GDH-102 is based on a GDH isozyme from *B. megaterium* (EC 1.1.1.47), which is recombinantly expressed in *E. coli* [66,69,70]. This isozyme has been engineered for improved catalytic activity and thermostability [66].

3.2. Activation of ReSyn carboxyl microspheres

Epoxide microspheres are spontaneously reactive [71,72] and were used as supplied for bioconjugation. Carboxyl microspheres were activated immediately prior to enzyme coupling using