Polyelectrolyte Complex Capsules as a Material for Enzyme Immobilization

Catalytic Properties of Encapsulated Lactate Dehydrogenase

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ABSTRACT

The polyelectrolyte complex (PEC) membrane formed by cellulose sulfate and poly(dimethyldiallylammonium chloride) was used to encapsulate lactate dehydrogenase. The exclusion limit of the membrane was found to be low enough to secure irreversible entrapping of the enzyme. The obtained capsules were checked for their functionality in a stirred-batch reactor by following the kinetics of NADH oxidation. The data were fitted with an isotropic kinetic model including competitive product-inhibition phenomenon. The results of mathematical modeling demonstrated that the anisotropic system, like PEC capsules, could be satisfactorily described by the isotropic model.

Index Entries: Polyelectrolyte complex capsules; lactate dehydrogenase; encapsulation; intrinsic kinetics; diffusion limitation; batch reactor; isotropic model.

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NOMENCLATURE

Abbreviations

DMF	dimethylformamid
IEP	immobilized enzyme particle
IES	immobilized enzyme system
LDH	lactate dehydrogenase
PDMDAAC	poly(dimethyldiallylammonium chloride)
PEC	polyelectrolyte complex
PEG	poly(ethylene glycol)
SCS	cellulose sulfate

Symbols

C_b	concentration in bulk phase
C_e	enzyme concentration
C_0	initial concentration
C_P	product concentration
C_s	substrate concentration
C_{so}	initial substrate concentration
D_e	effective diffusion coefficient
K_m	Michaelis constant
K_P	product inhibition constant
k_2	rate constant
P	dimensionless product concentration
P_s	dimensionless substrate concentration
r	radial distance from IEP center
R	IEP radius
S_p	total external surface area of IEP in the reactor
t	time
v	reaction rate
V	volume of liquid phase in the batch reactor
V_m	maximum reaction rate

Greek Symbols

α	dimensionless product concentration
β	dimensionless substrate concentration
ϵ	void fraction in the batch reactor
ϵ_p	apparent porosity of IEP
η	internal diffusion effectiveness factor
$\eta_{1\%w/w}$	viscosity of 1% (w/w) solution
ρ	dimensionless radial distance from IEP center
τ	dimensionless time
ϕ_m	modified Thiele modulus

INTRODUCTION

In the 1980s Dautzenberg et al. developed a new method of encapsulation based on the formation of polyelectrolyte complex (PEC) using sodium cellulose sulfate (SCS) and poly(dimethyldiallylammonium chloride) (PDMDAAC) as polyelectrolyte components (*1*). With this polyanion/polycation combination, the capsule properties, such as mechanical strength, elasticity, and deformation, and capsule-wall properties, such as morphology, permeability, and transparency, can be widely varied by changing the parameters of the polyelectrolyte precursors as well as the conditions of capsule preparation (*1*).

The PEC capsules have been used to encapsulate single enzyme (urease) and multienzyme complex (cytochrome P-450 and/or cytochrome-b_5 reductase) in the form of cell fragments (*2*). In this way, immobilized microreactors performing detoxication (degradation of urea, *O*- and *N*-demethylation) should be constructed (*1,2*). However, the detailed investigation of the kinetic behavior of the enzyme system immobilized in PEC capsules is more needed.

The aim of the presented study was to investigate the intrinsic kinetics of an enzyme immobilized in PEC capsules in a stirred-batch minireactor. Theoretical model equations including the capsule internal mass-transfer limitation and reaction kinetics were designed and experimentally validated, and the intrinsic immobilized enzyme kinetics were compared with those of free enzyme. The bovine muscle lactate dehydrogenase (LDH) was used as a model of the enzyme.

Mathematical Model

Model of Reaction Catalyzed by Immobilized Enzyme

The following assumptions were taken into account:

1. The immobilized enzyme particles (IEP) are isotropic and of ideal spherical shape.
2. The capsule of PEC is electrically neutral—charges of membrane composed from polyelectrolytes are fully compensated.
3. The active enzyme (LDH) molecules are homogeneously distributed over the whole volume of IEP.
4. The intrinsic reaction kinetics can be expressed by Eq. (1).
5. External mass-transfer resistance has no significant influence on the reaction rate.
6. The effective diffusion coefficients of NAD and NADH have the same value, D_e.

The kinetic model of the pyruvate reduction with NADH catalyzed by LDH have been introduced by Duggleby and Morrison (3). The model is valid when the pyruvate concentration is high in comparison to both the Michaelis constant, K_m, and the concentration of NADH. In our work the still-more-simplified equation, taking into account only the competitive product-inhibition effect, is used:

$$v = k_2 C_e / [1 + (K_m/C_s)(1 + C_P/K_P) \tag{1}$$

where k_2, K_m, and K_P represent rate, Michaelis, and product-inhibition constant, respectively; and C_s, C_P, and C_e represent substrate, product, and enzyme concentration, respectively.

Under the introduced assumptions, the material balances for substrate and product in the IEP in dimensionless form are given as follows:

$$\frac{\partial^2 S}{\partial \rho^2} + \frac{2}{\rho}\frac{\partial S}{\partial \rho} - 9\phi_m^2 \frac{S}{S + \alpha(1 + P/\beta)} = \frac{\partial S}{\partial \tau} \tag{2}$$

$$\frac{\partial^2 P}{\partial \rho^2} + \frac{2}{\rho}\frac{\partial P}{\partial \rho} + 9\phi_m^2 \frac{S}{S + \alpha(1 + P/\beta)} = \frac{\partial S}{\partial \tau} \tag{3}$$

For a batch-stirred reactor initial and boundary conditions are in the form

$$\tau = 0 \quad S = P = 0$$
$$S_b = 1 \quad P_b = 0 \tag{4}$$

$$\rho = 0 \quad \frac{\partial S}{\partial \rho} = \frac{\partial P}{\partial \rho} = 0 \tag{5}$$

$$\rho = 1 \quad -\frac{\partial S_b}{\partial \tau} = \frac{3(1-\epsilon)\epsilon_p}{\epsilon}\frac{\partial S}{\partial \rho} \tag{6a}$$

$$-\frac{\partial P_b}{\partial \tau} = \frac{3(1-\epsilon)\epsilon_p}{\epsilon}\frac{\partial P}{\partial \rho} \tag{6b}$$

where dimensionless variables are defined as

$$S = C_s / C_{so};\ P = C_P / C_{so};\ \rho = \frac{r}{R};\ \tau = \frac{t\, D_e}{R^2 \epsilon_p}$$

$$\alpha = K_m / C_{so};\ \beta = K_P / C_{so};\ \phi_m = \frac{R}{3}\,(k_2 C_e / D_e C_{so})^{1/2} \tag{7}$$

The introduced partial differential equation system was solved by using the finite difference replacement of the equation according to the implicit method (4).

MATERIALS AND METHODS

Polyelectrolytes

Both of the polyelectrolytes used for PEC formation were laboratory samples. SCS was prepared by homogeneous reaction of cellulose (bleached and swollen cotton linters, cuoxam degree of polymerization, DP= 1400) in the SO_2/N_2O_4/DMF system (*5*), and PDMDAAC by radical polymerization of dimethyldiallylammonium chloride in aqueous solution (*6*).

The PDMDAAC sample was characterized by its average molecular weight, which was found to be 3.2×10^4, calculated from the $[\eta]$-M relationship (*6*), and the SCS sample by its degree of substitution (to 0.31) and the viscosity of the aqueous solution (to $\eta_{1\% w/w} = 152$ mPa, at 20°C determined in an Ubbelohde viscometer).

Enzymes and Substrates

Lactate dehydrogenase (LDH) (EC 1.1.1.27, L-lactate:NAD oxidoreductase), from bovine muscle, activity ≤400 U/mg, was isolated by means of dye–ligand affinity chromatography (*7*) and stored at 4°C in 50% glycerin diluted with the mixture of 0.1*M* $(NH_4)_2SO_4$ and 0.1*M* KH_2PO_4 of pH 7. NAD ex yeast was obtained from Koch-Light (Colnbrook, England); β-NADH from yeast, Grade III; disodium salt from Sigma (St. Louis, MO); and NADH, disodium salt, minimum assay 80%, from Reanal (Budapest, Hungary). Pyruvic acid, sodium salt, analytical grade, was purchased from Lachema (Brno, CSFR).

Encapsulation of LDH and Preparation of Empty Capsules

Capsules were formed by introducing droplets of aqueous SCS solution (2% w/w) through a needle with a diameter of 0.5 mm into an aqueous solution of PDMDAAC (2% w/w) and allowing the components to react at the surface of the droplets in the permanently stirred precipitation bath for about 15 min.

For preparing IEP by encapsulation of LDH both of the polyelectrolyte solutions contained NaCl (0.1*M*). LDH was added to the SCS solution as a concentrated solution (0.33 mg/mL) and adjusted to a total catalytic activity in the solution of 0.7 U. The capsules were removed from the precipitation bath and washed with sodium chloride solution (0.154*M*) for usage or temporary storage.

Physical Characterization of PEC Capsules

Mechanical strength of capsules was determined as the force and time required for an irreversible collapse of the spherical shape (*8*). The size (particle diameter) and geometric shape were measured and/or controlled by microscopy. The thickness of the capsule wall was estimated optically with the aid of electron-microscopy photomicrographs (*9*). Dry weight was determined by two different methods (*8*).

Permeability and Diffusion Characteristics of PEC Capsules

Permeability of capsules was measured in solutions containing polyethylene glycol (PEG) of different molecular weights (1500–45,000) obtained from Serva Feinbiochemica (Heidelberg, FRG). To study diffusion properties the NAD diffusion rate was measured.

Empty capsules were equilibrated within PEG (1% w/w) or NAD solution (13.94 m*M*) in aqueous NaCl solution (0.154*M*) for 24 h or more. Then the capsules were placed in the NaCl solution, and the PEG or NaCl concentration in the solution was measured polarographically using the electrochemical measuring device ECM 700 (Zentrum für wissenschaftlichen Gerätebau der AdW DDR) and the program DC Pol. Experimental details and processing of data have been published recently (*10*). The initial amount of PEG in the capsules was determined from material balance in equilibrium.

The determination of the effective diffusion coefficient of NAD was based on the kinetic measurement of NAD release from PEC capsules. In a mathematical description of this dependence derived by Crank (*11*), the effective diffusion coefficient D_e was the only unknown parameter. For each of six concentration–time curves D_e was determined by the least-squares sum method. The mean value of D_e was 1.1×10^{-6} (SD $= 0.2 \times 10^{-6}$ dm^2/s.

Assay of Free-LDH Kinetics

Kinetic properties of free LDH were studied by the progress-curve method. The reaction was performed in quartz cuvets located in blocks thermostatted at 25°C. The reaction mixture contained 3.17 μg/mL LDH and 732 μM pyruvate in 50 m*M* phosphate buffer solution of pH 7.5 containing 0.154*M* NaCl. Initial NADH concentration varied within the range 42–160 μ*M*. NADH consumption was followed spectrophotometrically at 340 nm and recorded.

Resulting progress-curve data were fitted to Eq. (1). To optimize parameter values a modified form of Gauss-Newton nonlinear regression was employed (*3*). The initial concentrations of NADH were close to saturation concentration. Therefore, the value of K_m constant was estimated separately by the initial rate measurements. Six different initial NADH concentrations in the range of 26–130 μM were used and the initial rate of the reaction was determined from the change of NADH concentration at the beginning of the reaction (2 min). In this case the product inhibition could be neglected and the value of K_m was calculated from the Michaelis-Menten equation by nonlinear regression.

Kinetics of NADH Oxidation Catalyzed with Entrapped LDH

The oxidation of NADH with pyruvate catalyzed by entrapped LDH was performed in a small glass stirred-batch reactor equipped with a thermostatted jacket. The reaction conditions, as well as the analytical method, were the same as those in the study of free-LDH kinetics. Reactions were run using a constant number of capsules (420) containing LDH in 15 mL of 732 μM pyruvate solution. Solutions were agitated, ensuring independence of the reaction rate on the external mass-transfer rate. Samples of the solution were taken in certain time intervals for NADH concentration analysis.

RESULTS AND DISCUSSION

Characteristics of PEC Capsules

The IEP and the empty PEC capsules represent systems consisting of a liquid core containing a residual amount of nonreacted cellulose sulfate, covered by a semipermeable membrane functioning as capsule wall. In the case of the IEP used for kinetic measurements, capsule diameter was in the range of 1.8–1.9 mm (wet mass $\cong$ 4.2 mg, dry mass $\cong$ 70 μg). The thickness of the capsule wall was approx 15 μm.

Capsules are deformable and flexible. They can be loaded with forces up to 1 N without rupture. During storage over a period of some weeks no remarkable changes are observed. No damage of capsules occurs in a stirred-batch reactor (up to 400 rpm of the mechanical stirrer).

Figure 1 gives an impression of the morphology of the capsule wall. Asymmetry is evident. Inside (right part) are very porous regions; the layer outside (left part), being responsible for controlling the molecule permeation to the interior of the capsule, shows a compact structure. Further details about capsule properties in dependence on the conditions of their preparation were described elsewhere (*1*).

Figure 2 shows the permeability of the capsule wall measured with PEG in different media (water, physiological saline solution). As can be seen from the onset of permeation, pore width ranges up to about 12 nm in water and up to 6 nm in saline solution, with a sharper permeation curve in the latter case. According to these data, LDH (hydrodynamic diameter is estimated to 5 nm [*12*]) should be reliably immobilized at least at sufficiently high salt concentration. Hence no leakage of LDH takes

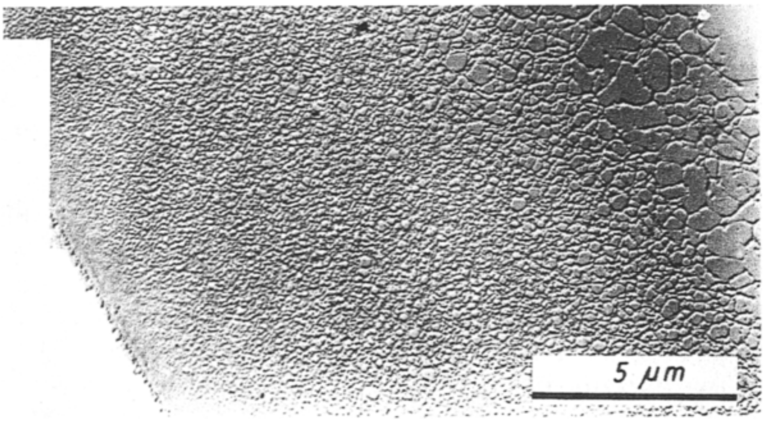

Fig. 1. Electron microscopy photomicrograph of the cross-section of the capsule membrane (ultrathin section).

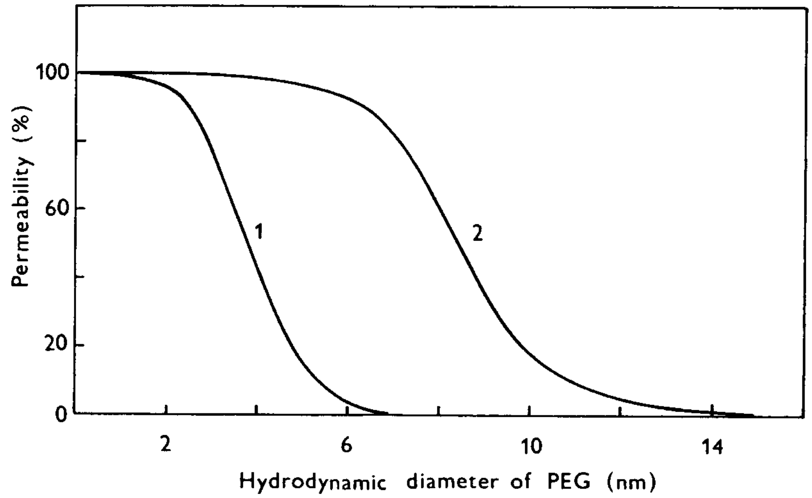

Fig. 2. Permeability of capsule membrane measured with the aid of PEG in water (1) and saline (0.154*M* NaCl) solution (2).

place when IEP is stored for 1 wk. The membrane is permeable for all molecules with diameter <2 nm, which means that capsule interior is accessible for both substrates: NADH and pyruvate.

Kinetics of Free LDH

A typical run of NADH oxidation catalyzed by free LDH is presented in Fig. 3. The parallel course of the curves indicates nonsignificant dependence of the initial rate on the substrate concentration, suggesting that the value of K_m for NADH was substantially below the range of experimental NADH concentrations. The progressive curvature of the lines,

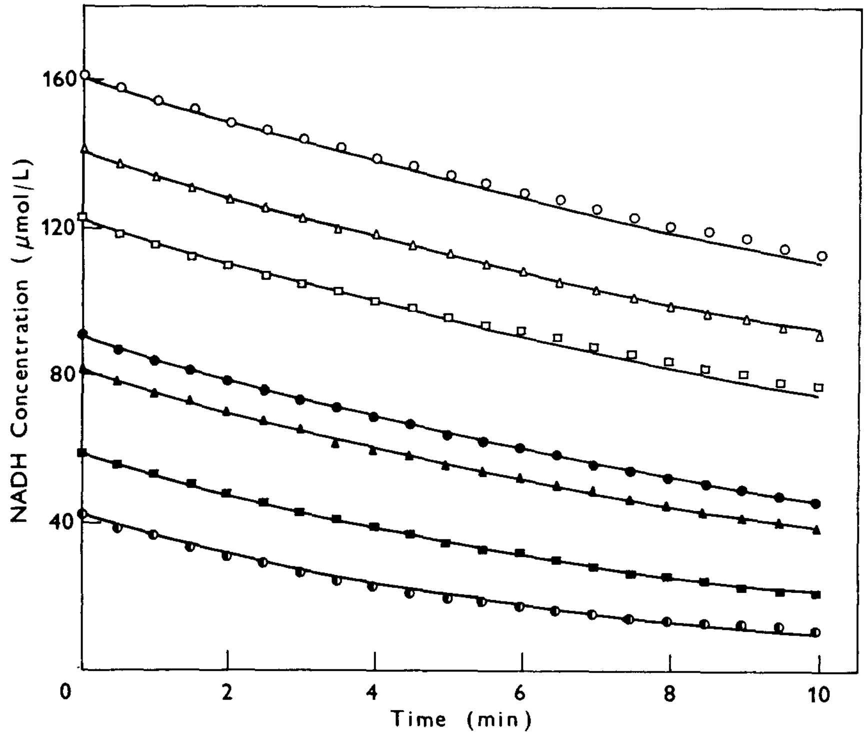

Fig. 3. Course of the reaction catalyzed by free LDH. Initial NADH concentrations (μmol/L): ◐, 42; ■, 59; ▲, 81; ●, 90; □, 122; △, 140; ○, 160.

however, does not correspond to zero-order kinetics. In accordance with published data (*3,13*) this is caused by a product-inhibition phenomenon and thus is considered in the kinetic equation used for describing the experimental data. Taking all seven progress curves, i.e., altogether 140 experimental points, we obtained the following parameter values: $k_2 = 285$ μmol/min/mg, $K_P = 12.1$ μM. The kinetic model obtained in that way fitted the data, giving a coefficient of correlation of 0.999. The low K_m value, confirmed by the independent initial rate measurement using lower substrate concentrations, was 11.3 μM.

Kinetics of Entrapped LDH

The stoichiometry of the membrane precipitates is 1:1 in the broad range of the initial PDMDAAC/SCS ratio (*14*). This means that the charges of the polyelectrolytes in the membrane are fully compensated and do not influence the mass transfer. Nevertheless, the exact description of the diffusion-reaction process in the IES used is rather complicated. The real particle can be characterized as an environment whose physical properties (density, viscosity, porosity) change gradually from outside to the center of the particle so that the membrane area cannot be clearly separated from

Table 1
Parameters Used for Modeling of Reaction
Catalyzed by LDH Entrapped Within PEC Capsules

Parameter	Symbol	Physical unit	Value
Effective diffusion coefficient of NAD	D_e	dm^2/min	1.1×10^{-6}
Rate constant	k_2	μmol/mg/min	285
Michaelis constant	K_m	μ*M*	11.3
Product-inhibition constant	K_P	μ*M*	12.1
Particle enzyme concentration	C_e	mg/dm^3	1.65
Number of particles in the reactor	n	-	420
Particle diameter	$2R$	dm	1.84×10^{-2}
Reactor liquid-phase volume	V	dm^3	1.525×10^{-2}

the internal solution. Hence the particle should not be effectively considered either isotropic or a capsule with a discrete membrane. That is why the applicability of the model of an isotropic particle was investigated.

In Table 1 the kinetic parameters of Eq. (1), valid for free LDH and other parameters characterizing the immobilized enzyme system, are presented. The parameters were used to predict the run of NADH oxidation in the stirred-batch reactor. The comparison of the computed results to the experimental data is presented in Fig. 4. Good agreement between the model and the experiment was reached when the value of $V_m = k_2 C_e$ was adjusted to a value smaller (0.73 V_m) than that estimated for the free enzyme. The values of K_m and K_P determined for the free enzyme were not changed.

Conclusion

From the results of the calculation of the course of the reaction catalyzed by the immobilized LDH, three main conclusions can be made:

1. The environment inside PEC capsules does not cause significant changes in enzyme affinity against NADH and the product inhibitory effect, since parameters K_m and K_P were not evidently changed.
2. The comparison of the maximum reaction rate for the free and encapsulated LDH pointed out that, in IEP, ~27% of the enzyme is not effective for substrate conversion. One explanation for this could be incorporation of some amount of LDH into capsule wall, causing either inactivation of LDH and/or its inaccessibility for substrate.
3. The diffusion behavior of the capsule wall is of minor importance and the overall kinetics of the reaction in the IEP can be described by an isotropic model.

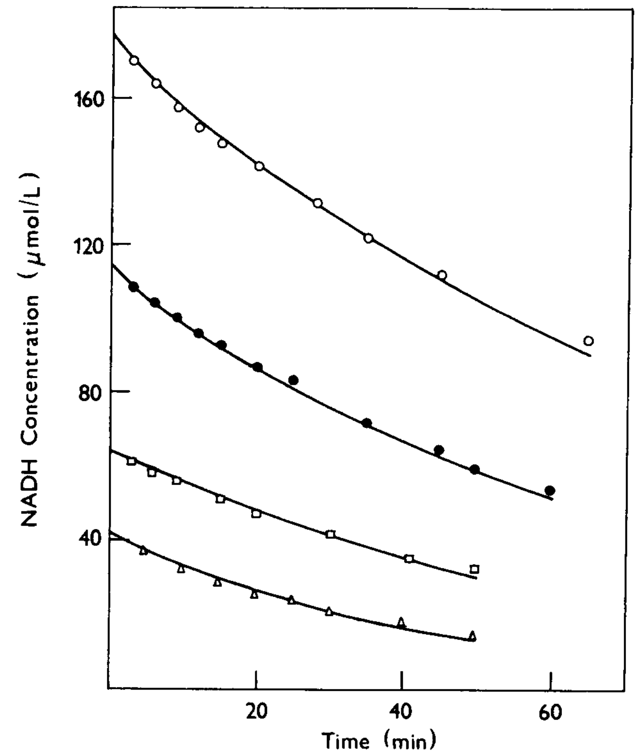

Fig. 4. Comparison of experimental and computed courses of NADH oxidation catalyzed by PEC entrapped LDH in the stirred-batch reactor. Initial NADH concentrations (μmol/L): △, 43; □, 65; ●, 116; ○, 179.

The microencapsulation by PEC formation using SCS and PDMDAAC is suitable for immobilization of enzymes, at least down to a mol wt of about 100,000. In addition, the mild conditions of PEC formation allow the encapsulation also of sensitive enzymes. By choosing a selected polyanion/polycation combination and adequate PEC-formation conditions, the pore width of the capsule wall under working conditions can be adjusted, in a range of 2–6 nm. Within the interior of the IEP, the enzyme is freely movable and can catalyze reaction in quasi-free state. The pore width of the capsule wall, adjustable in the range from 2 to 6 nm, guarantees the enzyme immobilization without its leakage, on the one hand, and the free access for low-soluble-weight compounds, including such substances as NADH and/or pyruvate, on the other hand.

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