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NYLON POLYETHYLENEIMINE MICROCAPSULES FOR IMMOBILIZING MULTIENZYMES WITH SOLUBLE DEXTRAN-NAD^+ FOR THE CONTINUOUS RECYCLING OF THE MICROENCAPSULATED DEXTRAN-NAD^+

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SUMMARY

Semipermeable nylon polyethyleneimine microcapsules were prepared to contain a multi-enzyme system of yeast alcohol dehydrogenase (EC 1.1.1.1) and malic dehydrogenase (EC 1.1.1.37) together with a soluble immobilized coenzyme, dextran-NAD^+. In the presence of the substrates ethanol and oxaloacetic acid dextran-NAD^+ was successfully recycled within the microcapsules by the sequential reaction of the included enzymes. Recycling rates of 72 cycles per hour were achieved. The microcapsules were also used in a continuous flow shunt where the rate of recycling of dextran-NAD^+ reaches a peak within 45 min. and remains at this maximal level throughout 4 hours of continuous reaction. The microcapsules have shown good storage stability, 50% of the activity was retained after 18 days of storage.

INTRODUCTION

Coenzyme regeneration has been investigated rather extensively in the recent years [1-9]. One of these approaches involves the use of multi-enzyme system immobilized within semipermeable microcapsules for the continuous recycling of coenzymes [6,9]. In biomedical applications where microencapsulated multi-enzyme systems are to act on external substrates, it will be advantageous to immobilize the coenzymes within the semipermeable microcapsules. There are various types of insoluble-matrix immobilized coenzymes [10-16]. However, coenzymes in the insoluble form, because of steric hindrance have limited activity. Recently, a number of groups have prepared soluble macromolecular derivatives of NAD^+ [17-21]. The present report is the first study to incorporate soluble dextran-NAD^+ together with soluble multi-enzyme systems within semipermeable microcapsules.

MATERIALS AND METHODS

Enzymes: Alcohol dehydrogenase (EC 1.1.1.1) from yeast (YADH)(304 U/mg); malic dehydrogenase (EC 1.1.1.37) from beef heart (MDH)(3845 U/mg); type I glutamate oxaloacetate transaminase (EC 2.6.1.1) from pig heart (235 U/mg)

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were obtained from Sigma Chemical Co. Malic dehydrogenase (EC 1.1.1.37) from porcine heart (MDH)(1130 U/mg) was obtained from Calbiochem Co.

Substrates and Coenzymes: The following substrates and coenzymes were used: grade III NAD^+ from yeast (Sigma Chemical Co.); grade I cis-oxaloacetic acid (Sigma Chemical Co.); reagent grade L-glutamic acid (Fisher Scientific Co.); and L-malic acid (Eastman Kodak Co.).

Chemicals: Chemicals used were as follows: dextran T-70 ($\bar{M}W$ 70,000) (Pharmacia Co.); 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl-p-toluene-sulfonate (ICN K&K Inc.); polyethyleneimine, 50% solution (ICN K&K Inc.); 1,6-Hexanediamine (Eastman Kodak Co.); terephathaloyl chloride (Metheson Coleman & Bell Co.); glutaraldehyde, 50% v/v (BDH Co.); all other solvents and reagents were from the highest available analytical grade.

Preparation of Water Soluble Dextran-NAD^+: NAD^+-N^6-[N-(6-aminohexyl)-acetamide] was prepared using the method of Mosbach et al [1,14]. This derivative of NAD^+ was coupled with cyanogen bromide activated soluble dextran following the method of Mosbach et al [18] with the following two modifications: dextran T-70 instead of dextran T-40 was used; and the activation of dextran was carried out in an ice bath for 4 min. The coupling of the coenzyme was carried out immediately by adding 2ml of NAD^+ derivative (50μmoles/ml) to a 10ml solution containing 500mg of activated dextran. The pH was kept at 8.5 by continuous addition of 1N NaOH for the first hour. The reaction was then continued for another 4 hours at room temperature and then 16 hours at 4°C.

The reaction mixture is diluted 5 folds with 0.02M LiCl and the pH was adjusted to 6.8. This solution was applied to Sephadex G-50 column (1.5 X 60cm) and eluted with 0.02M LiCl (pH 6.8). The effluent was monitored for absorbance at 254nm using a Pharmacia UV absorbance monitor attached to a recorder. The two peaks eluted corresponded to dextran-NAD^+ and free NAD^+ derivative respectively. The fractions containing dextran-NAD^+ were combined and concentrated to a final volume of 10ml using a rotary evaporator under vacuum at 30°C. The resulting concentrated solution was dialyzed against 1 liter of distilled water using Fisher cellulose dialysis tubing (12,000MW cut off) in order to remove salts and low molecular weight dextran molecules. The resulting solution was concentrated again on the rotary evaporator to 3ml of highly viscous solution containing 7μmole of immobilized NAD^+ in 400mg of soluble dextran-NAD^+. The dextran-NAD^+ solutions are highly stable and could be kept for months at 4°C.

Preparation of Semipermeable Microcapsules by Interfacial Polymerization (Nylon Polyethyleneimine Microcapsules): The microcapsules were prepared according to the updated standard procedure for nylon microcapsules [22-26] with the following two modifications: Terephthaloyl chloride is used instead of sebacyl chloride [27] and Polyethyleneimine is used instead of hemoglobin [28]. In a typical preparation, 10mg of yeast alcohol dehydrogenase (305 U/mg), 1.65mg of malic dehydrogenase (1,130 U/mg) and 200mg dextran-NAD^+ were used to prepare 4ml of microcapsules.

Measurements of the Recycling of Dextran-NAD^+ Within the Microcapsules: The recycling of the immobilized NAD^+ within the microcapsules was measured by using the enzymatic cycling method for NAD^+ determination as described by Kato et al [29] with modifications as described by Campbell and Chang [9]. 100μl of microcapsule suspension (50% v/v suspension in 0.1M phosphate pH 8.0) was added to 2.4ml of 0.1M Tris·chloride buffer pH 8.0 containing 200mM ethanol and 2.0mM oxaloacetate. The reaction was carried out in a Lab-Line Orbit Environ-Shaker 18 at 37°C and 200 RPM. After the desired reaction time, a sample of 0.5ml from the clear supernatant was removed and heated for 5 min. at 100°C to stop the reaction and destroy the unreacted oxaloacetate [28]. The amount of malic acid present in the 0.5ml supernatant was then assayed enzymatically [9,29].

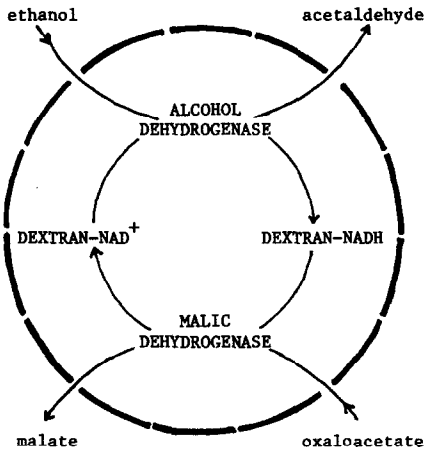

Figure 1

Schematic representation of the enzymatic recycling of dextran-NAD^+ within semipermeable microcapsules. The enzymes; yeast alcohol dehydrogenase and malic dehydrogenase and the soluble macromolecular dextran-NAD^+/NADH are retained within the microcapsules by the semipermeable membrane. On the other hand, the substrates and the products which are all small molecules, can equilibrate rapidly across the semipermeable microcapsule membrane.

RESULTS AND DISCUSSION

The recycling of soluble dextran-NAD^+ within the microcapsules could be initiated by adding the substrates ethanol and oxaloacetate to the microcapsule suspension. There were no free enzymes or free coenzymes present in the reaction mixture outside the microcapsules, since dextran-NAD^+, alcohol dehydrogenase and malic dehydrogenase are all immobilized as a solution within the microcapsules. The microencapsulated soluble dextran-NAD^+ is continuously recycled between its oxidized and reduced form by the sequential reaction of the two enzymes yeast alcohol dehydrogenase and malic dehydrogenase included in the same microcapsule. The cycling steps are shown in Figure 1.

Table 1 summarizes the coenzyme recycling activity of the microcapsules. Dextran-NAD^+ is recycled up to 72 cycles/hour. The microcapsules have good storage stability retaining 89% of its original recycling activity after 5 days, and 44% after 18 days (Table 1). The coenzyme recycling activity of the microcapsules was also measured in a continuous flow shunt system using a

TABLE 1

The recycling of dextran-NAD^+ within the microcapsules.

Storage[a] Time (days)	Recycling Rate in 1ml of microcapsules (cycles/hr)	(μM/hr)	(% of original activity)
Original[b]	72.0	59.0	100
1	72.0	59.0	100
2	72.0	59.0	100
5	64.0	52.5	89
10	45.2	37.0	63
18	32.0	26.2	44

[a]The microcapsules suspensions were stored at 4°C in 0.1M phosphate buffer pH 8.0.
[b]The original activity was determined by measuring the recycling activity immediately after the microcapsules were prepared.

small column (0.5 X 3.0cm) containing 0.5ml of microcapsules. A solution of 0.1M Tris·chloride buffer pH 8.0 containing the substrates (200mM ethanol and 2.5mM oxaloacetate) was perfused through the shunt at a flow rate of 0.1ml/min at 25°C. 0.5ml fractions of the effluent were collected and the recycling of dextran-NAD^+ was calculated from the amount of malate produced in the effluent (Fig. 1). The recycling activity reached a maximal level after 45 minutes and remained at this maximal level throughout 4 hours of continuous reaction (Table 2). When the same shunt was stored at 4°C and reused the next two days for 2 hours each time, it was found to retain the original activity (Table 2). After 10 days of storage, 50% of the original activity was still retained.

The approach of immobilizing inside semipermeable microcapsules, a solution containing multi-enzyme system and soluble coenzyme-macromolecular derivative, has a number of characteristics not shared by other immobilization approaches. Although enveloped within microscopic spherical ultrathin membranes, the enzymes and coenzymes remained in solution and are free to interact with one another in close proximity with no hindrance. What is also important is that microencapsulated enzymes are much more stable than enzyme in free solution

TABLE 2

The recycling of dextran-NAD^+ within the semipermeable microcapsules in a continuous flow shunt-system.

Time[a] (min)	Effluent fraction number	Abs. at 340nm (O.D.)	Malate produced/10 mins (μM X 10)
15	3	0.56 ± 0.01	1.20 ± 0.04
30	6	1.16 ± 0.02	3.35 ± 0.11
45	9	1.55 ± 0.02	5.20 ± 0.14
60	12	1.55 ± 0.01	5.20 ± 0.03
90	18	1.56 ± 0.04	5.27 ± 0.23
120	24	1.60 ± 0.04	5.50 ± 0.25
150	30	1.57 ± 0.02	5.33 ± 0.14
180	36	1.62 ± 0.06	5.70 ± 0.40
210	42	1.59 ± 0.02	5.42 ± 0.14
48[b] hr	-	1.56 ± 0.02	5.25 ± 0.14
72[b] hr	-	1.56 ± 0.02	5.25 ± 0.14

[a]The time passed since the initiation of the continuous perfusion of the substrates solution through the shunt.
[b]These measurements were done after the shunt was used for 4 hours and then the reaction was stopped, the shunt was stored at 4°C and reused the next 2 days for 2 hours each time.

[23-25]. Furthermore, an unlimited number of enzyme systems and coenzymes can be immobilized within each microcapsule at the desired concentration. This way, many extensions and modifications using other enzyme systems and other coenzymes can easily be carried out.

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