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The Metabolism of Fatty Alcohols in Lipid Nanoparticles by Alcohol Dehydrogenase

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ABSTRACT Fatty alcohols are commonly used in lipid-based drug delivery systems including parenteral emulsions and solid lipid nanoparticles (NPs). The purpose of these studies was to determine whether horse liver alcohol dehydrogenase (HLADH), a NAD-dependent enzyme, could metabolize the fatty alcohols within the NPs and thus serve as a mechanism to degrade these NPs in the body. Solid nanoparticles (<100 nm) were engineered from oil-in-water microemulsion precursors using emulsifying wax NF as the oil phase and polyoxyethylene 20-stearyl ether (Brij 78) as the surfactant. Emulsifying wax contains both cetyl and stearyl alcohols. NPs were incubated with the enzyme and NAD^+ at 37°C for up to 48 h, and the concentrations of fatty alcohols were quantitatively determined over time by gas chromatography (GC). The concentrations of cetyl alcohol and stearyl alcohol within the NPs decreased to only 10–20% remaining after 15–24 h of incubation. In parallel, NP size, turbidity and the fluorescence intensity of NADH all increased over time. It was concluded that horse liver alcohol dehydrogenase/NAD^+ was able to metabolize the fatty alcohols within the NPs, suggesting that NPs made of fatty alcohols may be metabolized in the body via endogenous alcohol dehydrogenase enzyme systems.

KEYWORDS Cetyl alcohol, Gas chromatography, Surfactant, Enzyme, Polyoxyethylene 20-stearyl ether (Brij 78), β-nicotinamide adenine dinucleotide (NAD^+)

INTRODUCTION

Nanoparticles (NPs) have received considerable attention as potential drug delivery systems for over 30 years. There are some general advantages to the use of nanoparticles for drug delivery: (1) Due to their small size, nanoparticles help to solve many challenging problems in drug formulation. For example, nanoparticles are ideal carriers for poorly soluble drugs, especially when the drugs lack solubility in both aqueous and nonaqueous media. Reducing the particle size of poorly soluble drugs may enhance the dissolution rate and drug bioavailability since the available surface area per given mass of particles is increased (Nielloud, 2000). (2) The toxicity and side-effects of certain anticancer drugs such as doxorubicin (Franco, 1999) and cisplatin (Burger et al., 2002) may be considerably reduced by entrapping the drugs in nanoparticles.

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(3) In addition, nanoparticles have shown to provide benefit in enhancing the circulation time of drugs in the body (Kaul & Amiji, 2002; Nishiyama et al., 2003). (4) Finally, nanoparticles have been used to target specific cells and tissues in the body (Crispin, 2003) and to control the release rate of drugs (Zhao et al., 2004).

One of the major considerations in the use of nanoparticles as delivery systems is the selection of the composition of the components. Typically, nanoparticles are made from either biodegradable and/or biocompatible polymers or lipids (Dingler et al., 1999; Bondi et al., 2003). Recently, lipids such as fatty alcohols have been used to form colloidal carriers such as microspheres (Bodmeier et al., 1992; Maheshwari et al., 2003), liposomes (Sudimack et al., 2002; Cortesi et al., 2002), and nanoparticles (Ye et al., 2004). However, there are few or no reports on the metabolism of fatty alcohols within formed nanoparticles. It was previously shown by DeAngelis and Findlay that synthetic ^{14}C-cetyl alcohol was widely distributed in the body after intratracheal administration. ^{14}C-cetyl alcohol was oxidized to palmitic acid which was then used in the phospholipid biosynthesis pathway (DeAngelis & Findlay, 1993). Additionally, another report has shown that lipase was able to metabolize fatty acids used to make solid lipid nanoparticles (Olbrich et al., 2002).

Our laboratories have reported on a novel method to engineer stable nanoparticles from oil-in-water (o/w) microemulsion precursors. In this method, melted nonionic emulsifying wax NF which is composed of cetyl alcohol ($C_{16}H_{34}O$), stearyl alcohol ($C_{18}H_{38}O$), and polysorbate 60 was used to form the oil phase. These nanoparticles have been utilized for brain-targeting (Lockman et al., 2004), dendritic-cell–targeting (Cui et al., 2004; Mumper & Cui, 2003) and tumor-targeting (Koziara et al., 2004, Oyewumi et al., 2004; Oyewumi et al., 2003). In a recent study, it was demonstrated that these lipid-based nanoparticles did not cause in-vitro red blood cell lysis at concentrations up to 1 mg/mL (Koziara et al., 2005). In addition, these nanoparticles did not activate or aggregate platelets; however, the nanoparticles were shown to inhibit agonist-induced platelet activation and aggregation in a dose dependent manner. Although these NPs appear to have blood compatibility at clinically relevant doses, little is known about the metabolism of the NPs.

The purpose of the present studies was to investigate whether horse liver alcohol dehydrogenase (HLADH) (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) could metabolize cetyl and stearyl alcohol present in the lipid-based nanoparticles. The metabolism of the fatty alcohols in the nanoparticles was monitored by observing the increase in fluorescence intensity of NADH and by measuring the metabolized products using a quantitative gas chromatographic method.

EXPERIMENTAL

Materials

Cetyl alcohol (1-hexadecanol, 95%) was purchased from Sigma (St. Louis, MO). Stearyl alcohol (1-Octadecanol, 99%), stearic acid (99.5%), pyridinium chlorochromate (PCC), nonadecanoic acid (98%), diethyl ether, dichloromethane (CH_2Cl_2), NAD^+ (β-nicotinamide adenine dinucleotide) (99%), and horse liver alcohol dehydrogenase (HLADH) (0.89 units/mg lyophilized powder containing sodium phosphate buffer salt and NAD^+) were purchased from Sigma (St. Louis, MO.). Celite® 545, 1 N hydrochloric acid and 1 N sodium hydroxide were obtained from Fisher (Fairlawn, NJ.). Silica gel 60 was obtained from Macherey-Nagel (Düren, Germany). Hexane was purchased from EM (Darmstadt, Germany). Polyoxyethylene 20-stearyl ether (Brij 78) was purchased from Uniqema (New Castle, DE.). Emulsifying wax NF, palmitic acid, monobasic sodium phosphate and dibasic sodium phosphate were purchased from Spectrum (New Brunswick, NJ.).

Methods

Synthesis of Hexadecanal and Octadecanal

Hexadecanal and octadecanal were prepared by oxidation of the corresponding alcohols using pyridinium chlorochromate (PCC). The initial concentrations of pyridinium chlorochromate and fatty alcohols were 0.9 mM and 0.1 mM, respectively. Pyridinium chlorochromate was dissolved in 85 mL dichloromethane with Celite® 545 (PCC: Celite® 545, 1: 1 w/w). The fatty alcohol dissolved in 15 mL dichloromethane was slowly added to a well stirred PCC solution and reacted for 4 h. The reaction mixture was passed through a column (3 cm x 8 cm) packed with silica gel 60. The products were eluted

with dichloromethane, and the fraction was evaporated to dryness. The structures of hexadecanal and octadecanal were confirmed by GC-MS and NMR.

Preparation and Characterization of Nanoparticles from Microemulsion Precursors

Solid nanoparticles were engineered from oil-in-water microemulsion precursors using emulsifying wax as the oil phase and Brij 78 as the surfactant. Twelve (12) milligrams of emulsifying wax was accurately weighed into a glass vial and melted together with 20.7 mg Brij 78 at 60°C. Next, 6 mL of 50 mM phosphate buffer (pH 7.5) was added under magnetic stirring. After 30 min. of continuing stirring at 60°C, a homogeneous and clear oil-in-water microemulsion formed. The warm microemulsion was then cooled to room temperature while stirring to form a 6-mL solid nanoparticle suspension. After the nanoparticles formed, an additional 4 mL of 50 mM phosphate buffer (pH 7.5) was added to dilute the nanoparticles to 10 mL. Nanoparticle size was measured by photon correlation spectroscopy (PCS) using a Coulter N4 Plus Submicron Particle Sizer (Coulter, Miami, FL) by scattering light at 90° at 25°C for 1 min. Each 1-mL sample for PCS was prepared by diluting 100 μl nanoparticle suspension with 900 μl deionized and 0.22 μm filter water.

Incubation of Nanoparticles With Horse Liver Alcohol Dehydrogenase and NAD^+

Horse liver alcohol dehydrogenase and NAD^+ were added to nanoparticle suspensions so that the final concentrations were 1 mg/mL and 5 mg/mL, respectively. The amount of alcohol dehydrogenase and NAD^+ added was based on a previous reported study (Hinson & Neal, 1972.). The samples were incubated with shaking at 37°C for 48 h. Nanoparticle suspensions in the absence of alcohol dehydrogenase and NAD^+ were used as controls. To measure the metabolism of the fatty alcohols, 100 μl and 1000 μl aliquots were removed from the samples at different time points during the 48 h incubations. For the 100 μl aliquots, 900 μl water was immediately added to perform particle size, turbidity, and fluorescence intensity measurements (see below). For the 1000 μl aliquots, the enzymatic reaction was terminated by adding 100 μl of 1 N HCl. Next, 300 μl of the internal standard, nonadecanoic acid (1000 μg/mL), was added to the mixture. The mixture was then heated at 60°C for 30 min., cooled to room temperature, and then extracted with 1 mL diethyl ether (× 2) and 1 mL hexane (× 2). The combined extract was dried under N_2 and dissolved with 1 mL hexane for GC analysis.

Measurement of Particle Size, Turbidity and Fluorescence Intensity

The nanoparticle size was measured as described above. The turbidity of the nanoparticle suspensions was detected by UV spectroscopy using a Beckman DU 7500i Spectrophotometer at 320 nm using water as the blank. The fluorescence intensity of NADH was measured using a Hitachi Model F-2000 Fluorometer (excitation wavelength was 350 nm and emission wavelength was 460 nm). To obtain the corrected fluorescence measurement of each sample, the fluorescence intensity of a fresh mixture of 1 mg/mL alcohol dehydrogenase and 5 mg/mL NAD^+ was subtracted at each time point.

The Influence of Brij 78 on the HLADH/NAD^+ Enzyme Reaction of Fatty Alcohols

Cetyl alcohol (1 mg/mL) without Brij 78 and with 1 mM Brij 78 or 3 mM Brij 78 was incubated with 1 mg/mL horse liver alcohol dehydrogenase and 5 mg/mL NAD^+ at 37°C for 48 h. The enzymatic reactions were monitored by detecting NADH (as described above). As controls, the mixtures of alcohol dehydrogenase and NAD^+ in the absence and present of Brij 78 were used.

Quantitative Analysis of Cetyl Alcohol and Stearyl Alcohol by Gas Chromatography

GC analysis was performed using a Thermoquest Trace 2000 System equipped with a flame ionization detector (FID) and an AS 2000 Autosampler. Separation of samples was carried out on a 30 m column (0.53 mm i.d.) coated with SPB-1 phase (poly [dimethylsiloxane], 1.5 μm film thickness) from Supelco (Bellefonte, PA). The injector was used in the splitless mode, and the sample injection volume was 1 μl. The injector and detector temperatures were kept at 220°C and 270°C, respectively. The initial temperature of the column was 80°C and was held for 0.06 min., and then increased at 20°C/min. to 250°C. The temperature

was then held at 250°C for 6 min. The total run time was 14.56 min. Helium was used as the carrier gas. Data acquisition and processing were performed with a Chromquest (Version 2.53) software for GC system.

The identification of sample peaks was determined by comparing the relative retention time to that of known standards. Quantification was aided using nonadecanoic acid as an internal standard. The purity of each standard was determined by GC in triplicate. The revised concentration was used for subsequent calculations. The stock solutions of hexadecanal, octadecanal, cetyl alcohol, stearyl alcohol, palmitic acid, stearic acid, and nonadecanoic acid were prepared at a concentration of 1000 μg/mL in hexane. From the stock solutions of cetyl alcohol and stearyl alcohol, working standards of 600, 400, 300, 100, and 10 μg/mL containing 300 μg/mL nonadecanoic acid in hexane were made. Each sample was measured in triplicate, and average areas were used to obtain calibration curves of the standards. Consequently, quantitative linear ranges were determined and relative response factors (f_i) were calculated. The stock solutions of hexadecanal, octadecanal, palmitic acid, and stearic acid were used to identify the corresponding peaks. The concentrations of samples from the incubations were determined in triplicate and calculated as $W_i = (f_i \times A_i \times W_s)/A_s$: where W_i and W_s are the weighed amounts of sample (i) and internal standard (s), respectively; A_i is the peak area of sample, A_s is the peak area of the internal standard, and f_i is the relative response factor of sample (i).

Results and Discussion

Fatty alcohols present in the body can be incorporated into wax esters and ether lipids via the fatty-acid–fatty alcohol cycle (Rizzo et al., 1987). Fatty alcohols are oxidized to the corresponding fatty acids by fatty alcohol: NAD^+ oxidoreductase (FAO, EC 1.1.1.192). FAO is a complex enzyme that consists of at least two separate enzymes, fatty alcohol dehydrogenase (FADH) and fatty aldehyde dehydrogenase (FALDH). Fatty alcohol dehydrogenase (FADH) metabolizes fatty alcohols to fatty aldehydes, and fatty aldehyde dehydrogenase (FALDH) metabolizes fatty aldehydes to fatty acids (Lee, 1979; Rizzo et al., 1987; Ichihara et al., 1986). Previous studies of this enzyme system have been performed in a heterogeneous enzyme "cocktail" derived from the liver, and thus it was not an isolated enzyme system. However, in these present studies, a commercially available purified enzyme, horse liver alcohol dehydrogenase (HLADH), was utilized. The general reaction of HLADH is shown in Fig. 1 (Henehan & Oppenheimer, 1993). As a single enzyme, horse liver alcohol dehydrogenase catalyzes the reversible interconversion of fatty alcohols and their corresponding aldehyde/ketone products; it also catalyzes the dismutation and oxidation of the fatty aldehydes to the corresponding fatty acids. The activity of horse liver alcohol dehydrogenase for aldehyde oxidation is low due to the high K_m and low V_{max} of the reaction of fatty alcohols to fatty acids. Thus, alcohol dehydrogenase plays a more important role in the oxidation of alcohols to aldehydes. Related, aldehyde dehydrogenase may then convert the formed aldehydes to the corresponding acids (Hinson & Neal, 1975; Henehan & Oppenheimer, 1993).

In order to choose the appropriate operational parameters for the quantitative gas chromatographic method, several temperature programs were studied. The optimized chromatographic method balanced efficiency, sample capacity, and peak resolution. In initial GC method development studies, peaks of palmitic acid and stearyl acid showed some tailing. Thus, methods were adapted to increase resolution and resolve tailing. The final optimized temperature program was held at 80°C for 0.06 min. then increased to 250°C at a rate of 20°C/min. and held for 6 min. at 250°C. Seven compounds (hexadecanal, cetyl alcohol, palmitic acid, octadecanal, stearyl alcohol, stearic acid, and nonadecanoic acid) were fully separated with good resolution in the optimized operational condition. A representative GC chromatogram is shown in Fig. 2.

The purity of cetyl alcohol, stearyl alcohol, and nonadecanoic acid was determined by GC and is listed in Table 1. Nonadecanoic acid was chosen as the internal standard for quantitative measurement by

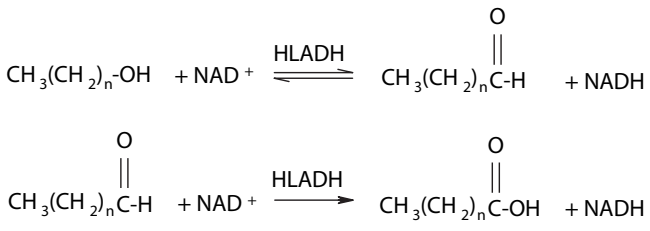

FIGURE 1 **The Reaction of Fatty Alcohol and Fatty Aldehyde With Horse Liver Alcohol Dehydrogenase (HLADH)/NAD^+ Enzyme System.**

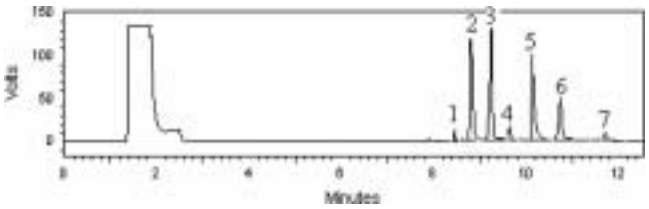

FIGURE 2 **Gas Chromatography (GC) Chromatogram of Seven Standards: (1) Hexadecanal, (2) Cetyl Alcohol, (3) Palmitic Acid, (4) Octadecanal, (5) Stearyl Alcohol, (6) Stearic Acid and (7) Nonadecanoic Acid.**

TABLE 1 **The Average Purity of Each Standard as Measured by Gas Chromatography (n=3)**

Standard	Cetyl Alcohol	Stearyl Alcohol	Nonadecanoic acid
Purity (%)	99.37	98.29	98.52
R.S.D. (%)	0.222	0.031	0.178

GC. All three compounds had purity in the range of 98.3–99.4%. Table 2 shows the calibration parameters of both cetyl and stearyl alcohol. A minimum of five concentrations were examined for each calibration curve (see Fig. 3). The results demonstrate that the calibration curves for both cetyl and stearyl alcohol were linear from 10 μg/mL to 600 μg/mL. The R^2 values for cetyl alcohol and stearyl alcohol were 0.9969 and 0.9941, respectively.

The in-vitro metabolism of lipid-based NPs is shown in Fig. 4 as a function of time. The GC assay was used to quantify the loss of both cetyl and stearyl alcohol from the NP suspension incubated with the HLADH/NAD^+ system at 37°C for up to 48 h. The concentrations of cetyl alcohol and stearyl alcohol decreased by more than 80% for each fatty alcohol after 15–24 h. However, from 24–40 h, the concentrations of both cetyl alcohol and stearyl alcohol increased by 2–3 fold. This phenomenon was very reproducible and was repeated in multiple experiments. Most likely, the subsequent increase in alcohol concentration at approximately 24 h could be explained by the unique property of the enzyme (see Fig. 1) whereby the equilibrium supports the regeneration of the alcohol from the aldehyde by the reversible reaction (Henehan & Oppenheimer, 1993; Olson, et al., 1996).

Experiments were also performed to quantify the corresponding fatty aldehydes (hexadecanal and octadecanal) and fatty acids (palmitic acid and stearic acids). In fact, the presence of these metabolic products could be identified by GC. However, due to extraction difficulties and stability limitations of the fatty aldehydes, additional studies were not performed to develop a quantitative GC method for these compounds.

Increase in Size, Turbidity and Fluorescence Intensity When NPs were Incubated With HLADH/NAD^+

In addition to quantitative studies assessing the loss of the primary NP components, cetyl and stearyl alcohol, evidence to prove NP metabolism was sought. It was hypothesized that NPs exposed to HLADH/NAD^+ would undergo an expansion in particle size coupled with an increase in turbidity due to the presence of metabolic products. As shown in Fig. 5, HLADH/NAD^+ caused the NPs to more than double in size over 48 h at 37°C versus a NP control. In addition, as shown in Fig. 6, there was a corresponding increase in the turbidity of the metabolized NP suspension. It was also noted that the metabolized NP suspension exhibited an increase in foaming, likely a result of free Brij 78 that was liberated from the NPs over time.

As shown in Fig. 1, the conversion of a fatty alcohol by HLADH results in the reduction of NAD^+ to NADH. NADH is fluorescent and thus its concentration over time would be expected to increase upon fatty-alcohol metabolism. As shown in Fig. 7, NADH

TABLE 2 **Calibration Parameters of Cetyl and Stearyl Alcohol in the Quantitative Gas Chromatographic Assay**

Standard	Calibration curve	Linear range (μg/mL)	R^2	Relative response factor (f_i)
Cetyl Alcohol(n=3)	y = 3.2438x – 0.1284	10–600	0.9969	0.30828
Stearyl Alcohol(n=3)	y = 3.4693x – 0.1838	10–600	0.9941	0.28824

Nonadecanoic acid was used as an internal standard. y= A_i/A_s and x= C_i/C_s; where A_i and A_s are the peak areas of the fatty alcohols and internal standard, respectively, and C_i and C_s are the concentrations of the fatty alcohols and internal standard, respectively.

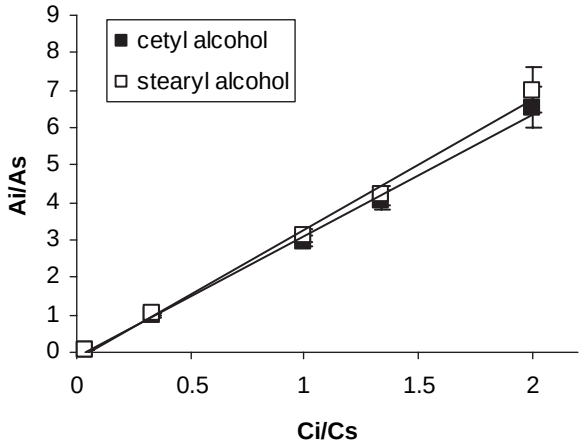

FIGURE 3 **Calibration Curves (C_i/C_s vs A_i/A_s) of Cetyl Alcohol and Stearyl Alcohol as Measured by Gas Chromatography. A_i and A_s Are the Peak Areas of the Fatty Alcohols and Internal Standard, Respectively. C_i and C_s Are the Concentrations of the Fatty Alcohols and Internal Standard, Respectively. Each Point Is the Mean ± SD (n=3). The Internal Standard was Nonadecanoic Acid.**

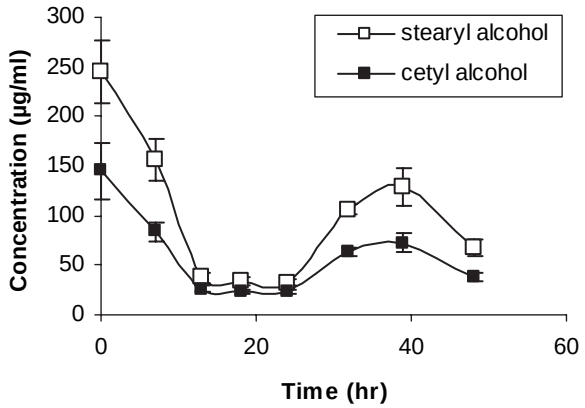

FIGURE 4 **In Vitro Metabolism of Cetyl Alcohol and Stearyl Alcohol in Lipid Nanoparticles as a Function of Time When Nanoparticles were Incubated With Horse Liver Alcohol Dehydrogenase and NAD^+ at 37°C for 48 h. Each Point Is the Mean ± SD (n=3).**

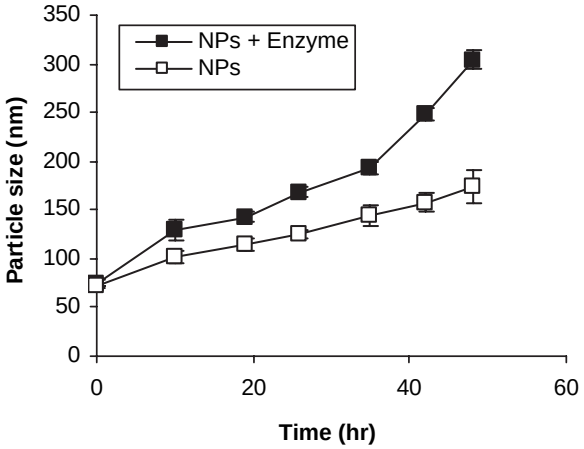

FIGURE 5 **The Change in Particle Size When Lipid Nanoparticles Were Incubated With Horse Liver Alcohol Dehydrogenase and NAD^+ at 37°C for 48 h. Each Point Is the Mean ± SD (n=3).**

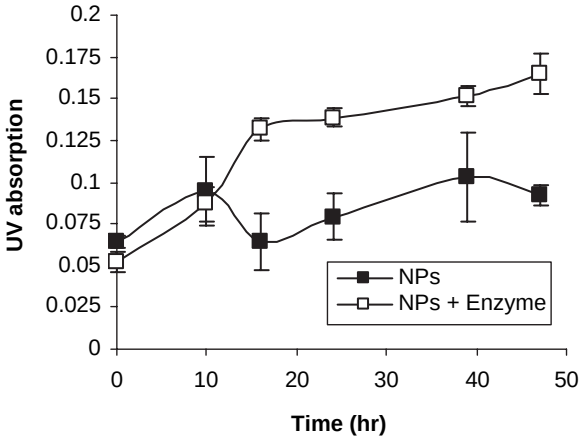

FIGURE 6 **The Change in Turbidity When Lipid Nanoparticles Were Incubated With Horse Liver Alcohol Dehydrogenase and NAD^+ at 37°C for 48 h. Each Point Is the Mean ± SD (n=3).**

fluorescence in the suspension substantially increased over the first 24 h, providing additional evidence of the enzymatic reaction. Notably, the rate and extent of NADH fluorescence very closely matched the rate and extent of the loss of fatty alcohols as shown in Fig. 4.

Effect of the Brij 78 Surfactant on the Enzyme Reaction

During the engineering of the nanoparticles, the Brij 78 surfactant was added to form the oil-in-water microemulsion at an elevated temperature. It is known that the addition of even low concentrations of surfactants influences the structure and activity of enzymes (Rubingh, 1996; Longo & Combes, 1997). No previous literature reporting the effect of Brij-based surfactants on the HLADH/NAD^+ enzyme system was identified. Thus, studies were undertaken to assess the effect of Brij 78 on the activity of this enzyme system. In these studies, cetyl alcohol was incubated with HLADH/NAD^+ in the presence and absence of Brij 78. The increase of NADH fluorescence was monitored over time. As shown in Fig. 8, the incubation of HLADH/NAD^+ with or without 2 mM Brij 78 resulted in no increase in NADH fluorescence intensity. The fluorescence intensity increased when HLADH/NAD^+

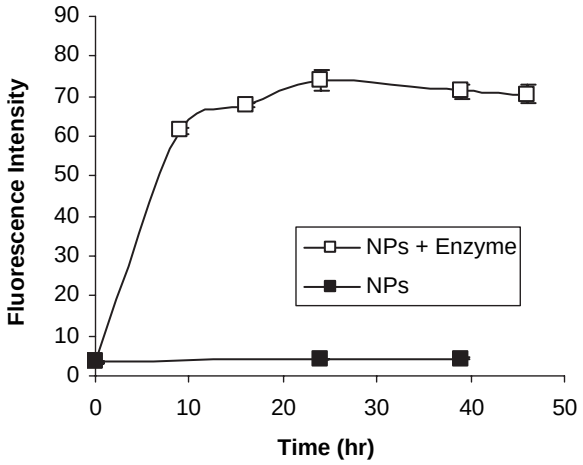

FIGURE 7 **The Change in Fluorescence Intensity when Lipid Nanoparticles Were Incubated With Horse Liver Alcohol Dehydrogenase and NAD^+ at 37°C for 48 h. Each Point Is the Mean ± SD (n=3).**

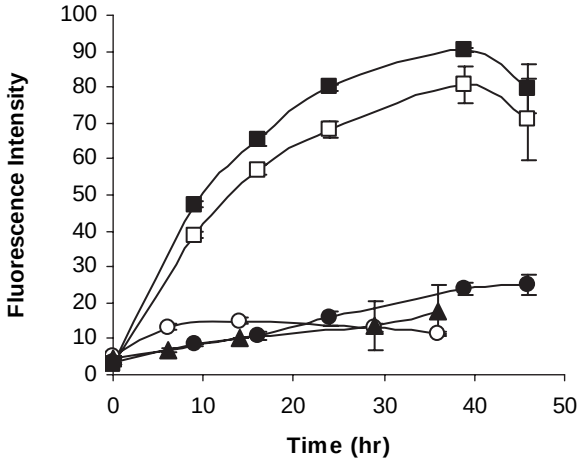

FIGURE 8 **The Effect of Brij 78 on the Enzymatic Reaction of Cetyl Alcohol With Horse Liver Alcohol Dehydrogenase (HLADH) and NAD^+ for 48 h. (○) 2 mM Brij 78 + HLADH/NAD^+, (▲) HLADH/NAD^+, (●) Cetyl Alcohol + HLADH/NAD^+, (□) 1 mM Brij 78 + Cetyl Alcohol + HLADH/NAD^+, (■) 3 mM Brij 78 + Cetyl Alcohol + HLADH/NAD^+. For All Samples, the Concentrations of HLADH, NAD^+, and Cetyl Alcohol Were 1 mg/mL, 5 mg/mL, and 1 mg/mL, Respectively. Each Point Is the Mean ± SD (n=3).**

was incubated with 1 mg/mL cetyl alcohol. However, the rate of increase of fluorescence intensity was considerably lower than the rate when HLADH/NAD^+ was incubated with lipid-based NPs (as shown in Fig. 7). This suggested that Brij 78 may be enhancing the activity of HLADH. In fact, when HLADH/NAD^+ was incubated with both Brij 78 and cetyl alcohol, the fluorescence intensity markedly increased similar to that of the NPs shown in Fig. 7. Moreover, the enhancement of fluorescence intensity was related to the concentration of Brij 78. These results indicate that the activity of HLADH was enhanced in the presence of Brij 78, and this enhancement accelerated metabolism of fatty alcohols present as either free fatty alcohols (cetyl alcohol alone) or fatty alcohols entrapped in a solid nanoparticle.

CONCLUSIONS

The results of these studies demonstrate that an alcohol dehydrogenase enzyme metabolized fatty alcohols present in solid lipid-based nanoparticles. In fact, entrapping the fatty alcohols into solid nanoparticles did not hinder the reaction of the fatty alcohols with the enzyme. Under the conditions tested, over 80% of cetyl alcohol and stearyl alcohol in the NP suspension were metabolized within 15 h. The results also showed that Brij 78 increased the activity of the enzyme. These studies suggest that lipid-based systems comprised of fatty alcohols are likely to be readily metabolized in the body by endogenous fatty alcohol dehydrogenases.

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