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Topical transport of hydrophilic compounds using water-in-oil nanoemulsions

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Abstract

A variety of water-in-oil nanoemulsions were prepared using sorbitan monooleate (Span®80), polyoxyethylene 20 sorbitan monooleate (Tween®80), olive oil and water. The nanoemulsions were tested for their ability to facilitate transport of a model hydrophilic solute, inulin, across hairless and hairy mouse skin and hairy rat skin following topical in vitro application. The transport of inulin incorporated in water-in-oil nanoemulsions was found to be significantly higher (5- to 15-fold) than that obtained with micellar dispersions or aqueous controls. The rate and extent of inulin transport across hairy mouse skin was found to be highly dependent on the hydrophile–lipophile balance (HLB) of the surfactant mixture in the nanoemulsion. Nanoemuslions prepared using mixtures with lower HLB exhibited significantly higher rate and extent of transport. It was also found that nanoemulsion-mediated transport was independent of molecular size of the hydrophilic solute and the nature of the aqueous phase. More importantly, transport of inulin from nanoemulsions was independent of animal skin characteristics such as stratum corneum thickness and follicle-type. The combined results suggest that water-in-oil nanoemulsions that are compatible with the lipophilic sebum environment of the hair follicle facilitate efficient transport of incorporated hydrophilic solutes and imply that such transport is predominantly transfollicular in nature.

Keywords: Nanoemulsions; Skin transport; Hydrophilic drug; HLB; Transfollicular; Sebum

1. Introduction

The importance of the transfollicular pathway to overall transport of a variety of drugs, including macromolecular compounds, into and across skin has gained increasing attention over the past few years. Numerous studies have provided sup-

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port for the transfollicular pathway in topical delivery for a wide variety of compounds (Wepierre et al., 1990; Illel et al., 1991; Hueber et al., 1994; Niemiec et al., 1995) and has been extensively reviewed (Lauer et al., 1995). We recently reported that the incorporation of plasmid DNA in water-in-oil nanoemulsions not only facilitates topical delivery of DNA into the skin but also enhances the extent of transfection of an exogenous reporter gene in hairless mouse skin following its topical application (Wu et al., 2000). However, the pathways of transport of organic macromolecules into skin afforded by these water-in-oil nanoemulsions remain to be determined. It is possible that these compounds are co-transported by the water-in-oil nanoemulsion via a transfollicular pathway or that their delivery across the transepidermal pathway is achieved via surfactant-mediated perturbations of the stratum corneum permeability. The transepidermal pathway appears highly unlikely for large molecular weight entities such as the plasmid even with partial disruption of the stratum corneum. It is more reasonable to expect co-transport of proteins and plasmid DNA by water-in-oil nanoemulsions into the follicles based on their size as well as the rationale that an oil external nanoemulsion would be quite compatible with the sebum-filled environment of the follicles.

Nanoemulsions are transparent, liquid isotropic dispersions composed of water, oil and surfactants and are thermodynamically stable (Osborne et al., 1988). At precise compositions of the ingredients their formation is spontaneous and high shear energies are not required for their preparation, essentially reducing the likelihood of protein or plasmid DNA degradation during formulation processing. Since they are thermodynamically stable, they can be prepared with low shear methods and are capable of encapsulating defined amounts of water. Water-in-oil nanoemulsions have been reported to improve absorption of water-soluble peptides following intraduodenal administration (Constantinides et al., 1995, 1996). There are relatively fewer studies that address the feasibility of topical delivery from nanoemulsions (Osborne et al., 1991; Trotta et al., 1994, 1996; Delgado-Charro et al., 1997; Schmalfub et al., 1997; Trotta et al., 1997; Bolzinger et al., 1998; Ktistis and Niopas, 1998). Although nanoemulsions possess several advantages with regards to their ease of preparation, high stability and clarity, the most commonly examined systems include short-chain alcohols such as butanol, pentanol and hexanol as well as non-polar phases such as hexane that make them unsuitable for pharmaceutical purposes. Further, when considering formulations for plasmids, it would be essential to employ alcohol-free systems in order to avoid flocculation effects. Thus, it would be desirable to use nanoemulsions that can be prepared on a large scale with oils and surfactants that are safe, non-toxic, non-irritating and with components that are generally regarded as safe (GRAS). A variety of water-in-oil nanoemulsions were therefore prepared using ingredients such as olive oil and combinations of the nonionic surfactants, sorbitan monooleate (Span®80) and polyoxyethylene 20 sorbitan monooleate (Tween®80), that have been widely used in topical products. In this report we describe the physicochemical characterization of these nanoemulsions and the results of studies on the permeation kinetics of water-soluble markers incorporated in select water-oil-nanoemulsions across a variety of skin models in an attempt to determine the mode of action of these nanoemulsions. The marker of choice was inulin since it is a non-metabolized polysaccharide used as a diagnostic aid for renal function and is sufficiently large and water soluble (molecular weight of 5 kDa). The effects of formulation variables on the permeation kinetics of inulin across hairy rat, hairy mouse and hairless mouse skin were therefore examined in an effort to establish pathways of transport facilitated by water-in-oil nanoemulsion formulations.

2. Materials and methods

2.1. Materials

The nonionic surfactants polyoxyethylene 20 sorbitan monooleate (Tween®80) and sorbitan monooleate (Span®80), as well as olive oil (OLO), inulin (MW = 5 KDa), *N*-[2-hydroxyethyl] piper-

azine-*N*′-[2-ethanesulfonic acid] (HEPES free acid) and (methoxy-^{3}H)-inulin (sp.act. 159 mCi/g) were purchased from Sigma Chemical Company (St. Louis, MO). Methylamine-^{14}C-tranexamic acid (sp.act. 54 mCi/mmol) was purchased from Amersham Life Science, Inc., Arlington Heights, IL. The water used was double-distilled and deionized using a Millipore Milli-Q® Water System, Millipore Corporation, Bedford, MA. All other chemicals were of analytical grade or better. Male, 8–12 week old Sprague Dawley hairy rats and male, 60 day old Skh-hr-1 hairless mice were purchased from Charles River Breeding Laboratories, Wilmington, DE. Male hairy albino mice, Hsd:ICR (CD-1®), 8 weeks old were obtained from Harlan, Indianapolis, IN.

2.2. Pseudo-ternary phase diagrams

Phase diagrams were constructed by titration of a series of olive oil/surfactant mixtures with water at ambient temperature. Typically, the surfactants were mixed at the desired ratio and allowed to equilibrate overnight. 2.5 ml of the surfactant mixture was placed in a 20 ml scintillation vial with a positive displacement pipet (Gilman). The aqueous phase was then added to the surfactant mixture within the vial in small aliquots of 25 μl. Following addition of the aliquot of water, the vial was capped and vortexed for 2 min to accelerate equilibration. Following vortexing, the mixture was visually examined for clarity. Titration was carried out until the mixture became hazy or turbid to establish the region of clear isotropic mixtures along the water–surfactant axis in the pseudo-ternary diagram. At this juncture, small aliquots of olive oil were added to the surfactant–water mixture to establish isotropic regions along the axis from the surfactant–water baseline towards the oil apex. If the mixtures appeared hazy, small aliquots of the surfactant mixture were added till it became clear. This process was continued to determine the entire domain of clarity from an oil-poor isotropic phase to an oil-rich isotropic one. The above process was carried out using surfactant combinations containing 1:1, 2:1, and 3:1, by volume of sorbitan monooleate and polyoxyethylene 20 sorbitan monooleate. No attempt was made to distinguish between micelles, swollen micelles, oil-in-water nanoemulsions, water-in-oil nanoemulsions, bicontinuous nanoemulsions or liquid crystalline phases.

2.3. Selection of isotropic mixtures for detailed studies

We hypothesized that water-in-oil nanoemulsions with oil-rich compositions would be most compatible with sebum and would therefore be the most effective in facilitating transfollicular transport of entrapped hydrophilic macromolecules. Select mixtures within the isotropic regions defined in the pseudo-ternary diagrams were therefore examined for their physicochemical characteristics as well as their ability to facilitate transport of water-soluble markers across skin. The systems chosen were well within the isotropic regions defined in the phase diagrams. Additionally, in order to evaluate the effects of surfactant ratio on transport characteristics of inulin, nanoemulsions containing identical compositions of olive oil, total surfactant and aqueous content but with different ratios of sorbitan monooleate to polyoxyethylene 20 sorbitan monooleate were also examined. Several systems containing no oil were also selected to determine if the nature of the isotropic mixture has any effects on transport characteristics across skin. The systems selected along with the compositions are shown in Table 1.

2.4. Evaluation of physicochemical properties

The desired nanoemulsion system was placed in an eppendorf tube and centrifuged at 5000 rpm for 15 min at room temperature to determine its stability as an isotropic single phase system. The particle size of the clear formulation was also determined using a Nicomp 370 Submicron Particle Sizer (HIAC-Royco). A water-soluble dye was used to qualitatively determine whether the isotropic systems were water-external or oil-external systems. Dilution tests with water or olive oil were also carried out to further qualitatively characterize the nature of the isotropic mixtures.

Table 1
Summary of compositions of nanoemulsion and micellar formulations and normalized transport rates of inulin following their topical in vitro application to hairy mouse skin

Formulation	Span 80 (v%)	Tween 80 (%v)	Olive oil (%v)	Aqueous phase (%v)	Normalized permeation rate (%/h)	HLB of surfactant mixture
Aqueous control					0.035	
3:1 aq nanoemulsion	18.7	6.2	72.8	2.3	0.519	1.734
2:1 aq nanoemulsion	21.5	10.8	64.5	3.2	0.321	2.545
1:1 aq nanoemulsion	15.1	15.1	66.6	3.2	0.176	2.914
1:1 oil-poor aq nanoemulsion	30.0	30.0	30.0	10.0	0.083	5.790
3:1 aq nanoemulsion	22.5	7.5	67.3	2.7	0.713	2.093
2:1 aq nanoemulsion	20.0	10.0	67.3	2.7	0.459	2.360
1:1 aq nanoemulsion	15.0	15.0	67.3	2.7	0.290	2.895
3:1 PG/aq nanoemulsion	23.8	7.9	62.5	5.8	0.666	2.208
2:1 PG/aq nanoemulsion	21.1	10.6	62.5	5.8	0.458	2.497
1:1 PG/aq nanoemulsion	15.8	15.8	62.5	5.8	0.200	3.049
2:1 micelle	64.5	32.3	–	3.2	0.076	7.619
1:1 micelle	42.5	42.5	–	15.0	0.073	8.203

2.5. Preparation of inulin and tranexamic acid formulations

Appropriate amounts of olive oil, sorbitan monooleate and polyoxyethylene 20 sorbitan monooleate were pipetted into a scintillation vial and mixed with an aqueous solution of inulin or tranexamic acid containing trace amount of ^{3}H-inulin or ^{14}C-tranexamic acid, respectively, and vortexed for 1 min to obtain clear isotropic systems. The ratios of sorbitan monooleate to polyoxyethylene 20 sorbitan monooleate used in the preparation of a variety of nanoemulsions were 1:1, 2:1, and 3:1 by volume. The aqueous phase consisted of the following:

1. tranexamic acid (3 μg/ml) dissolved in isotonic 0.05 M HEPES buffer, pH 7.4,
2. inulin (5 or 500 μg/ml) dissolved in distilled water,
3. inulin (5 μg/ml) dissolved in isotonic 0.05 M HEPES buffer, pH 7.4, and
4. inulin (5 μg/ml) dissolved in propylene glycol/isotonic 0.05 M HEPES buffer, pH 7.4 (50/50 v/v).

Appropriate solutions of inulin and tranexamic acid in isotonic 0.05 M HEPES buffer, pH 7.4, or in distilled water were also prepared and served as controls. All nanoemulsions were stored at ambient temperature in tightly capped scintillation vials until used in the experiments.

2.6. In vitro diffusion studies

Male hairy rats (Sprague Dawley, 8–12 weeks old) or male hairy mice (ICR, 8 weeks old) or male hairless mice (Skh-hr-1, also 8–12 weeks old) were sacrificed by a lethal dose (200 mg/kg) intraperitoneum injection of sodium pentobarbital. For hairy rat or mouse studies, the hair was clipped with animal clippers (Oster A5). Full thickness dorsal skin was carefully excised and subcutaneous fat was removed with a dull scalpel. Appropriate sized pieces of skin were then mounted on Franz diffusion cells with a surface area of 1.77 cm^2 and a receiver capacity of 8 ml (Crown Glass, Somerville, NJ). The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed by 0.05 M isotonic HEPES buffer, pH 7.4. The receiver solution was stirred continuously using a small Teflon-covered magnet. Care was exercised to remove any air bubbles between the underside of the skin and the receiver solution. The temperature of the receiver solution was maintained at 37°C. Following mounting of the skin, 200 μl of the test formulations were applied to the epidermal surface of the skin and carefully spread to achieve complete surface coverage. A minimum of three cells using skin from at least three different animals was used. All experiments were carried out under non-occluded conditions. 2 milliliter aliquots of the receiver solution were withdrawn at predetermined times in order to monitor the kinetics of marker transport across skin. In these experiments, percutaneous absorption was monitored for total period of 24–48 h. The receiver solutions were then assayed for radiolabeled marker using a scintillation counter after addition of 15 ml of Ecolite^{+} (ICN Biomedicals, Inc., Irvine, CA) to each system.

3. Results and discussion

3.1. Pseudo-ternary phase diagrams

The pseudo-ternary phase diagrams of the systems investigated are shown in Fig. 1A–C. Optically isotropic regions at low water content exhibited mean particle sizes of approximately 25 nm in diameter, values that are in agreement with size ranges previously reported for water-in-oil nanoemulsions (Muller and Muller, 1984; Wines et al., 1999). No attempts were made to identify regions at high water and low oil contents that were optically isotropic in nature. As the sorbitan monooleate:polyoxyethylene 20 sorbitan monooleate ratio is increased from 1:1 to 3:1, the region along the surfactant/water axis is significantly affected. Thus, at a 1:1 ratio, it was possible to obtain a mixture containing 17% water and 83% surfactant mix that is optically clear and isotropic. The amount of water that could be included with a 2:1 ratio of sorbitan monooleate:polyoxyethylene 20 sorbitan monooleate was severely reduced to around 5%

water and 95% surfactant mix. For the 3:1 ratio, the maximum amount of water that could be included was further reduced to about 3%. Dye solubility tests indicated that all isotropic mixtures along the surfactant/water axis were water-external systems. These systems might be rightly characterized as normal micellar dispersions.

Titrations of the surfactant mixtures at the volume ratios of 1:1, 2:1 and 3:1 with olive oil and vice-versa indicated varying degrees of immiscibility along the oil/surfactant axis. The total amounts of the surfactant mixtures required to obtain clarity increased from around 25% (v/v) for a 3:1 (v/v) sorbitan monooleate/polyoxyethylene 20 sorbitan monooleate mixture to 31% for the 2:1 (v/v) mixture. For the 1:1 (v/v) sorbitan monooleate/polyoxyethylene 20 sorbitan monooleate system the total surfactant required for clarity was about 45% (v/v). This is consistent with the requirement of a threshold amount of sorbitan monooleate required to offset the less oil-soluble polyoxyethylene 20 sorbitan monooleate. Thus, the total amount of the mixture required for clarity increases with increasing polyoxyethylene 20 sorbitan monooleate fraction in the surfactant mixture. Indeed, plots of sorbitan monooleate or polyoxyethylene 20 sorbitan monooleate content or HLB of the surfactant

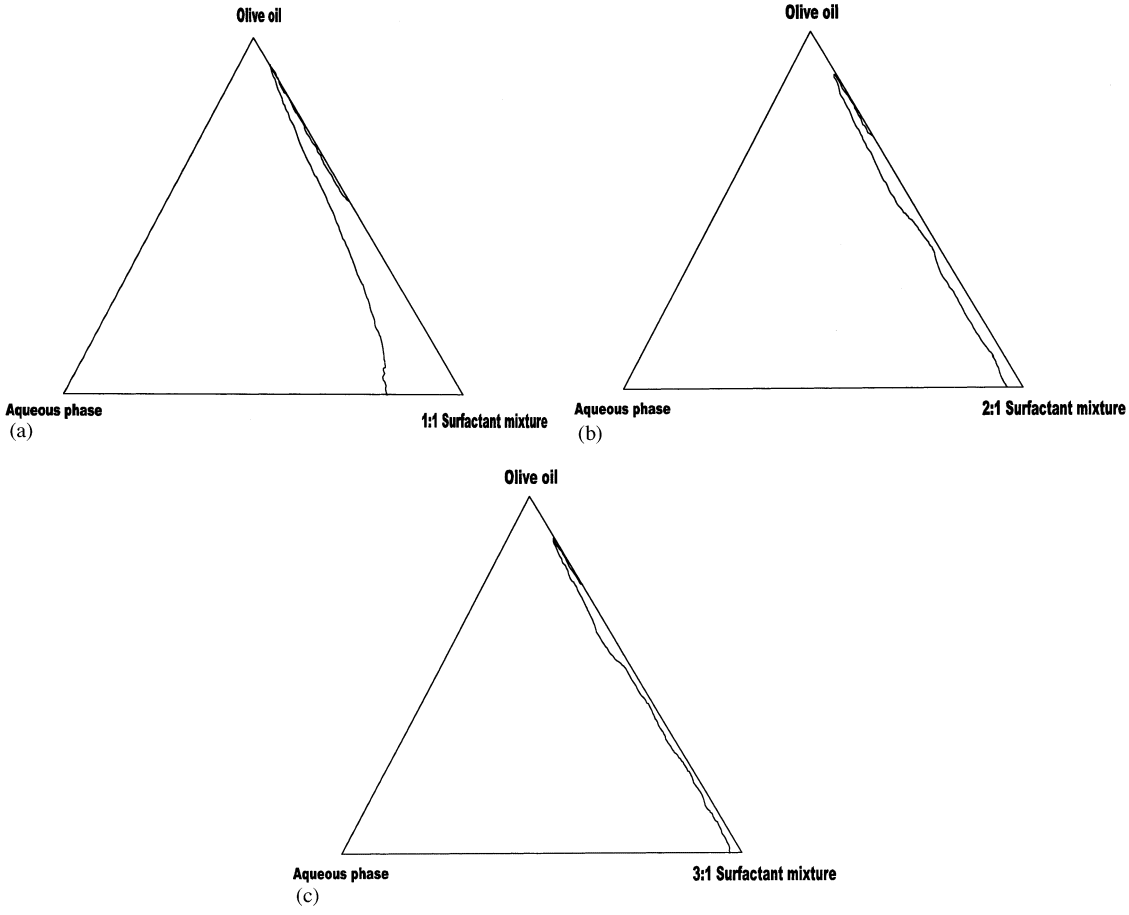

Fig. 1. (A) Pseudo-ternary phase diagram of the quaternary system containing olive oil, water, Span 80 and Tween 80. Span 80:Tween 80 volume ratio fixed at 1:1. (B) Pseudo-ternary phase diagram of the quaternary system containing olive oil, water, Span 80 and Tween 80. Span 80:Tween 80 volume ratio fixed at 2:1. (C) Pseudo-ternary phase diagram of the quaternary system containing olive oil, water, Span 80 and Tween 80. Span 80:Tween 80 volume ratio fixed at 3:1.

mixture were linearly related (directly or inversely) to both the limits of water solubilization in the respective surfactant mixture and the total amounts of surfactant mixture required to obtain clarity in mixtures with olive oil. All other mixtures from an oil content of 0% to the immiscibility values indicated above were optically clear.

In general, the isotropic regions tended to narrow down with increasing sorbitan monooleate: polyoxyethylene 20 sorbitan monooleate ratios in the oil-poor part of the pseudo-ternary diagram. Thus, it was possible to incorporate over 10% by volume of the aqueous phase with 1:1 ratios at olive oil content around 30%. This is reduced to around 3.5% (v/v) with a 2:1 ratio and to about 2% (v/v) with a 3:1 ratio, in mixtures containing 30% olive oil. For the 1:1 system the phase boundary appears to be almost a straight line connecting the limit on the surfactant/water axis with the limit on the surfactant/oil axis. For the 2:1 and 3:1 systems, however, higher amounts of water could be incorporated when the oil:surfactant mixture ratios were close to unity ($\sim 50\%$ oil). Thus, apex water solubilization capacity values of around 5 and 3.5% (v/v) could be obtained with the 2:1 and the 3:1 systems, respectively. Dye tests with a variety of formulations from oil-poor and oil-rich regions indicated the inability of the water-soluble dye to diffuse freely into the mixtures. This observation suggested that all of these formulations even those containing only around 30% olive oil, had olive oil as the external phase and could be termed water-in-oil nanoemulsions. Similar compositions for water-in-oil nanoemulsions prepared using mixtures of Captex®355/Capmul®MCM/Tween®80/aqueous at a ratio of 65/22/10/3 (w/w%) were reported (Constantinides et al., 1994). These observations also appear to be in agreement with the findings of Aboofazeli et al. who reported similar behavior with water-in-oil nanoemulsions using lecithin or lecithin mixtures with alcohols and triglycerides such as soybean oil or Miglyol®812 as the oil phase (Aboofazeli et al., 1995). These authors further reported the absence of isotropically clear regions in water-rich areas and that maximum water uptake was around 20% when using the two triglycerides as the oil phase.

It is quite clear from the phase diagrams that it is not possible to obtain high water incorporation in the absence of ethanol or short-chain alcohols. Ho et al. reported water contents of around 12–13% by weight using mixtures of nonionic surfactants containing roughly 8–13% by weight of short-chain alcohols such as ethanol, propanol and butanol (Ho et al., 1996). Trotta et al. reported nanoemulsions prepared using lecithin and 2-acyl lysolecithin derivatives that are capable of solubilizing high water contents of around 40–50% in the presence of high ethanol levels in the mixture (Trotta et al., 1999). We observed that it is possible to obtain isotropically clear formulations by lowering the polarity of the aqueous phase. This could be achieved, for example, by using 1:1 (v/v) mixtures of water with propylene glycol. Thus, with a 2:1 sorbitan monooleate: polyoxyethylene 20 sorbitan monooleate mixture a total of 20% of a 1:1 water:PG phase could be incorporated that resulted in a clear isotropic system.

3.2. Comparison of permeation profiles of inulin from nanoemulsion and micellar systems

The hypothesis that the delivery of water-soluble macromolecules into and across skin via a transfollicular pathway would be facilitated by olive oil-based water-in-oil nanoemulsions was tested by comparison of inulin transport from water-in-oil nanoemulsions, micellar dispersions and aqueous solutions. The three sorbitan monooleate:polyoxyethylene 20 sorbitan monooleate ratios tested, 1:1, 2:1 and 3:1, were chosen to reflect equal variation around the original volume ratio of 2:1 used in our earlier plasmid expression studies (Wu et al., 2000). Also, either an isotonic 0.05 M HEPES buffer, pH 7.4, or a 50/50 (v/v) mixture of propylene glycol/ isotonic 0.05 M HEPES buffer, pH 7.4 was used in the preparation of the aqueous phase as opposed to the use of distilled water. The latter aqueous phase was examined since the total aqueous phase incorporated could be doubled by the inclusion of 50% by volume of propylene glycol in the buffer. The inulin concentration in all the formulations tested was maintained constant at 5 μg/ml.

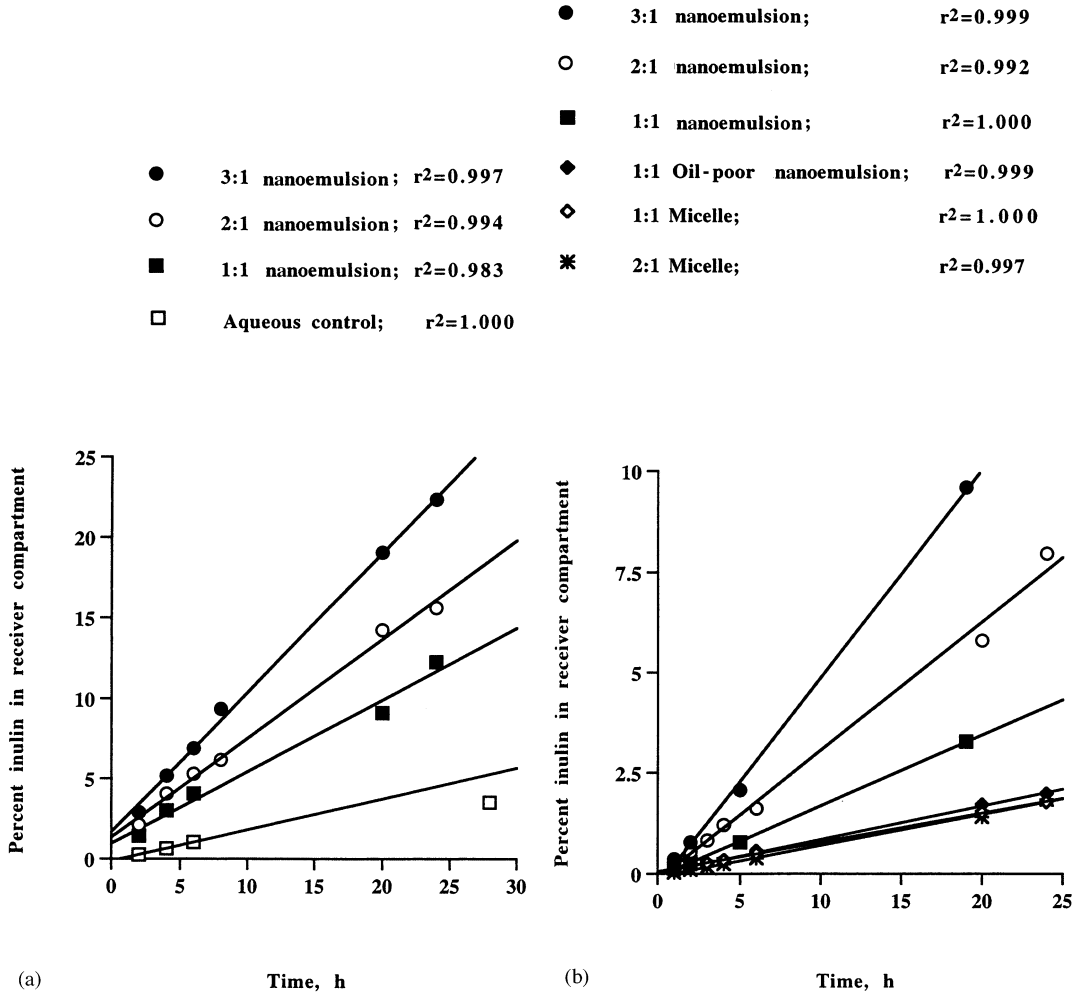

Fig. 2. (A) Comparison of permeation of inulin across hairy mouse skin following topical in vitro application of various water-in-oil nanoemulsions: ($n = 4–8$). Span 80:Tween 80 volume ratios of 3:1, 2:1, and 1:1. Total surfactant, 30% (v); Olive oil, 67.3% (v); Aqueous volume, 2.7% (v) isotonic HEPES, pH 7.4; inulin concentration, 5 μg/ml. Applied volume, 200 μl; aqueous control, 200 μl 5 μg/ml inulin solution in isotonic HEPES buffer, pH 7.4. (B) Comparison of permeation of inulin across hairy mouse skin following topical in vitro application of various water-in-oil nanoemulsions. ($n = 3–6$). Span 80:Tween 80 volume ratios of 3:1, 2:1, and 1:1. 3:1 nanoemulsion: total surfactant, 24.9% (v); olive oil, 72.8% (v); aqueous phase, 2.3% (v), pH 7.4. 2:1 nanoemulsion: total surfactant, 32.3% (v); olive oil, 64.5% (v); aqueous phase, 3.2% (v), pH 7.4. 1:1 nanoemulsion: total surfactant, 30.2% (v); olive oil, 66.6% (v); aqueous phase, 3.2% (v), pH 7.4. 1:1 oil-poor nanoemulsion: total surfactant, 60.0% (v); olive oil, 30.0% (v); aqueous phase, 10.0% (v), pH 7.4. 1:1 micelle: total surfactant, 85.0% (v); aqueous phase, 15.0% (v), pH 7.4. 2:1 micelle: total surfactant, 96.8% (v); aqueous phase, 3.2%(v), pH 7.4. inulin concentration, 5 μg/ml; applied volume = 200 μl.

Typical plots of permeation of inulin across hairy mouse skin from the various formulations are shown in Fig. 2A and B. The transport rate data obtained were normalized to concurrently run aqueous controls to account for animal skin variability among sets of experiments carried out at different periods of time. Table 1 shows normalized transport data from various formulations along with their compositions. Normalized transport data from nanoemulsions containing similar volume ratios of the surfactants (Table 1) were pooled (Table 2) and the averages were analyzed for differences using two-tailed Student's t-test. The results indicate that transport of inulin across

hairy mouse skin was significantly higher from nanoemulsions prepared with a 3:1 volume ratio than that obtained with nanoemulsions containing volume ratios of 2:1 or 1:1 ($P = 0.041$ and 0.004, respectively). Further, the transport of inulin was also significantly higher from a 2:1 nanoemulsion than that from a 1:1 nanoemulsion ($P = 0.029$). The results shown in Tables 1 and 2 and Fig. 2B also indicate that the permeation of inulin across hairy mouse skin is dependent on both the composition of the surfactants used in the preparation as well as the configuration of the colloidal system. Thus, nanoemulsions with higher sorbitan monooleate:polyoxyethylene 20 sorbitan monooleate ratios facilitate more rapid transport of inulin across hairy mouse skin. It is also seen that the rate of inulin permeation is dramatically lower from micellar formulations, regardless of the surfactant composition. It is possible that the high viscosities of these micellar systems could play a role in lowering overall permeation rates of inulin across hairy mouse skin into the receiver compartment. However, the near identical rates from micellar formulations with widely differing viscosity as well as the low permeation rate from an oil-poor nanoemulsion prepared using a 1:1 volume ratio of sorbitan monooleate and polyoxyethylene 20 sorbitan monooleate suggest that viscosity effects may not be the controlling factor in cutaneous transport. Sorbitan surfactants are more hydrophobic than polysorbate surfactants and may be expected to have a greater effect on the permeability of the stratum corneum (Lopez et al., 2000). However, co-administration of the various sorbitan monooleate:polyoxyethylene 20 sorbitan monooleate mixtures with aqueous inulin solutions gave rise to permeation rates that were not significantly different from aqueous control values (data not shown). Also, an examination of Tables 1 and 2 indicate a lack of correlation between permeation rates and sorbitan monooleate content. It is also clear that permeation rates do not correlate with total surfactant content in the formulations. On the contrary, an inverse correlation between permeation rates and polyoxyethylene 20 sorbitan monooleate content of the nanoemulsions was apparent (not shown).

The inverse correlation with polyoxyethylene 20 sorbitan monooleate (HLB = 15.0) content suggests that the HLB of the surfactant mixture in the nanoemulsion might determine the rate of inulin transport across hairy mouse skin. Indeed, a plot of transport rates as a function of HLB of the surfactant mixture, shown in Fig. 3, clearly illustrates the correlation. We believe that this data is consistent with a model wherein the degree of compatibility of the water-in-oil nanoemulsion with the sebum-rich lipophilic environment of the

Table 2
Average compositions ($\pm$SE) of various nanoemulsion and micellar formulations and pooled transport rates (expressed as average $\pm$ SE) of inulin following topical in vitro application to hairy mouse skin[a]

Formulation	Average permeation rate (%/h)	Average HLB of surfactant mixture	Average Span 80 in formulation (v%)	Average Tween 80 in formulation (v%)
3:1 Nanoemulsions	0.633 ± 0.058	2.01 ± 0.14	21.7 ± 1.5	7.2 ± 0.5
2:1 Nanoemulsions	0.413 ± 0.046	2.47 ± 0.06	20.9 ± 0.5	10.5 ± 0.2
1:1 Nanoemulsions	0.222 ± 0.035	2.95 ± 0.05	15.3 ± 0.3	15.3 ± 0.3
1:1 Oil-poor Nanoemulsions	0.083	5.79	30.0	30.0
2:1 Micelle	0.076	7.62	64.5	32.3
1:1 Micelle	0.073	8.20	42.5	42.5

[a] Two-tailed Student's t-tests: 3:1 Nanoemulsion vs 2:1 Nanoemulsion $P = 0.041$; 3:1 Nanoemulsion vs 1:1 Nanoemulsion $P = 0.004$; 2:1 Nanoemulsion vs 1:1 Nanoemulsion $P = 0.029$.

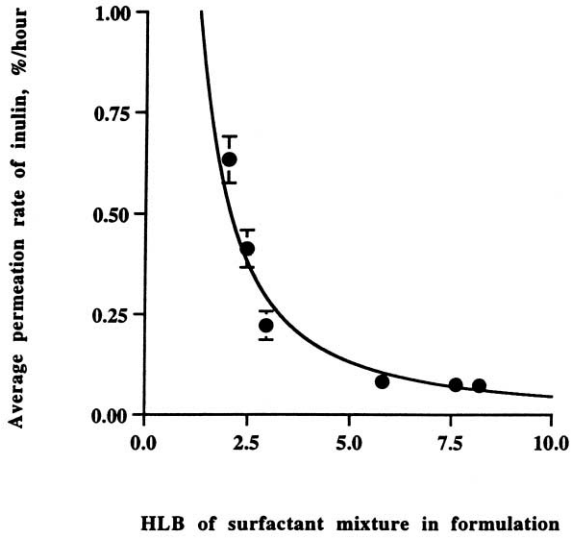

Fig. 3. Correlation between permeation rate of inulin across hairy mouse skin (expressed as percent of applied formulation per h ± SE) and HLB of surfactant mixture in nanoemulsion or micellar formulation. HLB of surfactant mixture = ((Volume percent Span 80 in formulation × 4.3) + (Volume percent Tween 80 in formulation × 15.0))/100.

hair follicle determines the rate and extent of permeation and transport of inulin entrapped within the aqueous core of the nanoemulsion. This model is also consistent with the extremely low rates of transport of inulin that were observed with oil-free micellar dispersions and surfactant-rich oil-poor water-in-oil nanoemulsions. Our results also argue against the hypothesis that sorbitan surfactants facilitate cutaneous transport of large hydrophilic molecules by disrupting the physical integrity of the stratum corneum. Rather, the data suggest that water-in-oil nanoemulsions that comprise a monodisperse distribution of an aqueous core dispersed within a lipid phase whose HLB is compatible with normal sebum would facilitate transport of large hydrophilic molecules that is mediated predominantly via a transfollicular pathway.

3.3. Comparison of inulin and tranexamic acid permeation across hairy mouse skin from water-in-oil nanoemulsions: effect of concentration and molecular weight

The permeation profiles of inulin across hairy mouse skin following topical in vitro application of water-in-oil nanoemulsions identical with respect to composition (sorbitan monooleate: polyoxyethylene 20 sorbitan monooleate volume ratio of 2:1, 32%; aqueous phase 4% distilled water, olive oil, 64%, all v/v) but with two widely differing concentrations of inulin (5 μg/ml and 500 μg/ml) were quite linear up to a period of around 20 h, although a slight curvature was evident at longer time periods in the high inulin concentration profile (plots not shown). The slope from the higher inulin (0.50 mg/ml) nanoemulsion, 0.172%/h/cm^2, was slightly higher than the value of 0.131%/h/cm^2 obtained with the 5 μg/ml system and may reflect possible changes in nanoemulsion stability or size or other characteristics. The similarity of the slopes, however, suggests that the percent of formulation applied that traverses skin is independent of inulin concentration within the aqueous interior of the nanoemulsion. Although, such behavior would also be expected for stratum corneum-mediated transport, it is likely that the similarity of profiles are due to the co-transport of the enclosed aqueous medium by the water-in-oil nanoemulsion. Under these circumstances, assuming minor changes in nanoemulsion properties such as size and stability, the amounts of nanoemulsion transported into and across skin should be similar and hence the percent aqueous medium transported would also be similar.

A more rigid test of the above hypothesis would be to incorporate water-soluble markers of widely different sizes in the water-in-oil nanoemulsions and monitor permeation profiles. Hence, tranexamic acid (4-(aminomethyl) cyclohexanecarboxylic acid; MW, 157.2 Da; water solubility, 1 g/6 ml) and inulin were incorporated in nanoemulsions of identical composition (sorbitan monooleate:polyoxyethylene 20 sorbitan monooleate volume ratio of 2:1, 32.3%; aqueous phase 3.2% isotonic HEPES pH 7.4, olive oil, 64.5%, all v/v). Inulin and tranexamic nanoemulsions containing 5 and 3 μg/ml inulin or tranexamic acid, respectively, with all other components exactly the same were examined for permeation kinetics with hairy mouse skin. The results indi-

cated that the permeation rates were not very different for the two markers; 0.283%/h/cm^2 for tranexamic acid and 0.233%/h/cm^2 for inulin (plots not shown). Tranexamic acid appears to be transported at a rate roughly 1.2 times faster than inulin across hairy mouse skin. The closeness of the permeation rates for the two markers indicates that transport of the water-soluble markers from the water-in-oil nanoemulsions is independent of marker molecular weight. More importantly, the similarity of the profiles suggests that the rate of permeation may be solely determined by the nanoemulsion.

It is worth noting that if perturbation effects on the stratum corneum permeability was chiefly responsible for the transport of the markers, a marked dependence on molecular weight should have been evident. Indeed, when 5% sodium lauryl sulfate, a potent permeation enhancer is co-administered with aqueous solutions of inulin and tranexamic acid, the permeation rates of the two markers were dramatically different. The permeation profiles (not shown) indicated a 5-fold difference in permeation rates in the presence of sodium lauryl sulfate(8.078 vs 1.63%/h/cm^2; controls 0.09 and 0.02%/h/cm^2, for tranexamic acid and inulin, respectively). It is remarkable, therefore, that the two markers, with dramatically different sizes, were transported by the water-in-oil nanoemulsions at very similar rates into and across the skin. Thus, it appears unlikely that the profiles obtained with the nanoemulsions can be explained based on perturbation effects by its components on stratum corneum permeability. Thus, the results are not consistent with the hypothesis that the markers are being transported independently across a stratum corneum barrier that has been modified by the components of the nanoemulsion. The permeation of water-soluble markers incorporated in water-in-oil nanoemulsions appears to be dictated by the nanoemulsion itself. Such dependency suggests that the markers are co-transported into the skin by the nanoemulsion and then released into the dermis and receiver compartment. The pathway that is implied is a follicular one with sebum acting as the thermodynamic deterrent. The olive oil-external nanoemulsion would be compatible with sebum and would preferably be transported into the follicles and sebaceous glands. As a consequence, the entrapped aqueous medium with its drug (marker) content would also be co-transported into these structures.

3.4. Comparison of nanoemulsion mediated inulin transport using different animal models

In vitro diffusion experiments with three different animal skin models, namely hairless mouse, hairy mouse and hairy rat were carried out in order to assess the effects of stratum corneum thickness and follicle type on the transport of inulin from water-in-oil nanoemulsions. The nanoemulsion used in these studies were prepared with an aqueous phase consisting of inulin in distilled water and a sorbitan monooleate: polyoxyethylene 20 sorbitan monooleate volume ratio of 2:1. The concentration of inulin in the nanoemulsion was 5 μg/ml. The results of permeation of inulin following topical in vitro application of this water-in-oil nanoemulsion to the three skin models are plotted in Fig. 4. For the sake of clarity, standard errors associated with the plots are not shown (in most cases, standard deviations were less than $\pm$ 30%). The plots show the percent of applied formulation found in the receiver compartment as a function of time.

It is noted that the plots are linear up to at least 24 h for all animal models examined with excellent linear regression coefficients ($r^2 = 0.99$). Interestingly, the slopes for hairless (0.144%/h/cm^2) and hairy mouse (0.131%/h/cm^2) are similar to that obtained with a hairy rat model (0.135%/h/cm^2). The similarity in permeation kinetics or flux for the three animal models suggests that the transport of the water-soluble inulin marker occurs via a common pathway. A transepidermal stratum corneum-mediated pathway for transport of inulin from the water-in-oil nanoemulsions does not appear likely. This is a fair conclusion since the thickness of the stratum corneum is vastly different in the two models ($\sim$ 5–8 μm for hairy and hairless mouse, and $\sim$ 18 μm for hairy rat stratum corneum) (Bronaugh et al., 1982). It is also possible that the presence of roughly 25–30%

by volume of a mixture of the nonionic surfactants sorbitan monooleate and polyoxyethylene 20 sorbitan monooleate in the nanoemulsion could act as enhancers by perturbation of the stratum corneum.

Fig. 4 also shows the permeation of inulin from a destabilized nanoemulsion. Destabilization was induced by adding an equal volume of a 5 μg/ml aqueous inulin solution to the 5 μg/ml nanoemulsion formulation and vigorously mixing the two in the donor compartment. The permeation rate with the destabilized non-nanoemulsion formulation was dramatically lower and similar to that obtained with aqueous controls (0.03%/h/cm^2)

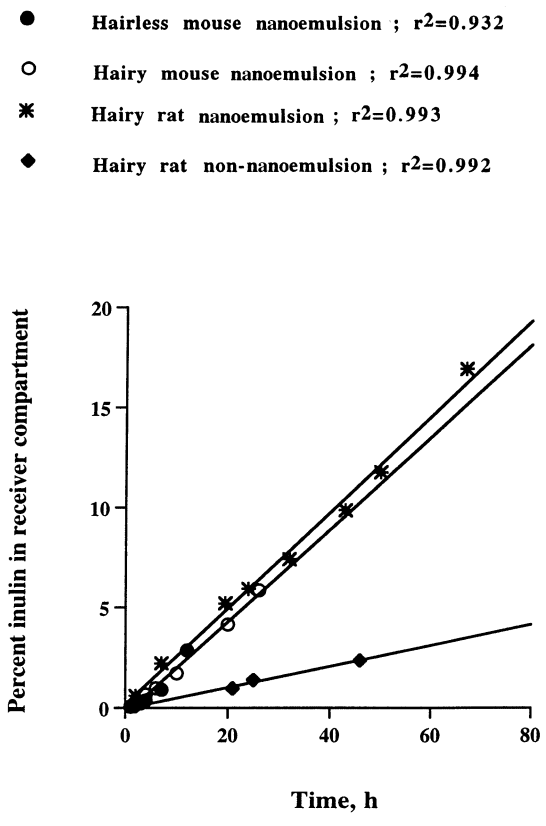

Fig. 4. Comparison of permeation of inulin following topical in vitro application of water-in-oil nanoemulsions to various animal skin models. Nanoemulsion formulation: total surfactant, 32.0% (v); olive oil, 64.0% (v); Aqueous phase, 4.0% (v); Span 80:Tween 80 volume ratio, 2:1. Inulin concentration, 5 μg/ml. Applied volume, 200 μl. Non-nanoemulsion formulation: 200 μl 5 μg/ml aqueous inulin + 200 μl 5 μg/ml 2:1 inulin water-in-oil nanoemulsion.

and suggests that a nanoemulsion configuration is required for effective transport. The lower permeation rate observed with the destabilized nanoemulsion also suggests that stratum corneum perturbation effects are probably not significant. It is also expected that such perturbation effects would be quite different with mouse and hairy rat stratum corneum, again due the differences in stratum corneum thickness. Such perturbations of the stratum corneum would have to be potent enough to exhibit a leveling effect with the two animal models. It appears that such effects are probably quite moderate based on transepidermal water loss (TEWL) studies reported earlier (Yang et al., 1995). The authors report that Tween®80 (at 50% by volume), and Span®20 (25% by volume) did not greatly disrupt hairless mice stratum corneum barrier function even with prolonged topical treatment.

4. Conclusions

The similarity of permeation profiles of inulin incorporated in water-in-oil nanoemulsions with hairy rat and hairless and hairy mouse skin strongly implies stratum corneum-independent transport. The near similarity of the permeation rates of inulin and tranexamic acid, two water-soluble markers with widely different molecular weights, from water-in-oil micromemulsions identical in all other respects appears to support such a supposition. The combined results suggest that nanoemulsions facilitate follicular transport of inulin and tranexamic acid, and by extension, of water-soluble compounds encapsulated within an oil external nanoemulsion droplet. The rate and extent of inulin transport across skin from water-in-oil nanoemulsions is highly dependent on the HLB of the surfactant mixture used in their preparation. Water-in-oil nanoemulsions with lower surfactant HLB values were far superior in facilitating transport of inulin solubilized within their aqueous cores. The combined results suggest that water-in-oil nanoemulsions prepared with a lipid phase whose HLB is compatible with normal sebum would efficiently facilitate skin transport of large hydrophilic molecules dissolved in the

aqueous core and that such transport is mediated predominantly via a transfollicular pathway.

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