CRITICAL ISSUES RELATED TO TRANSFERSOMES – NOVEL VESICULAR SYSTEM

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ABSTRACT

It has become increasingly apparent that vesicular drug delivery elicits modest possessions in drug targeting. Transfersomes are a form of elastic or deformable vesicle, which were first introduced in the early 1990s. Elasticity can be achieved by using an edge activator in the lipid bilayer structure. Molecules greater than 500 Da normally do not cross the skin. This prevents epicutaneous delivery of the high molecular weight therapeutics as well as non-invasive trans-cutaneous immunisation. Transdermal route will always remain a lucrative area for drug delivery. With the advent of new categories of drugs like peptides this route has captured more focus to combat the problems related to their delivery through oral route. But the transdermal route is equally filled with the hopes and disappointments as the transport of drug through this route faces many problems especially for the large molecules. To answer this problem many approaches were adopted. One of the very recent approaches is the use of ultra-deformable carrier systems (transfersomes). They have been used as drug carriers for a range of small molecules, peptides, proteins and vaccines, both *in vitro* and *in vivo*. Transfersomes penetrate through the pores of stratum corneum which are smaller than its size and get into the underlying viable skin in intact form. This is because of its deformable nature. The aim of this article is explanation the formation of micelle and vesicles, various types of vesicles, specifically focusing on transfersomes.

Key words: micelle, vesicles, transfersomes, sonicator, confocal scanning laser microscopy

INTRODUCTION

In the last few years, the vesicular systems have been promoted as a mean of sustained or controlled release of drugs. These vesicles are preferred over other formulations because of their specific characteristics such as lack of toxicity, biodegradation, capacity of encapsulating both hydrophilic and lipophilic molecules, capacity of prolonging the existence of the drug in the systemic circulation by encapsulation in vesicular structures, capacity of targeting the organs and tissues, capacity of reducing the drug toxicity and increasing its bioavailability. Vesicular systems such as liposomal formulations have been used as drug delivery vehicles for sustained release of proteins and peptides. These formulations have been used as carriers of cytotoxic drugs with the strategy based on reduction of toxicity and passive delivery to tumors. The propensity of the reticuloendothelial system (RES) uptake to liposomes from the circulation has thus far limited the prospect of targeting liposomes to tissues other than liver, spleen, and lung. Sterically stabilized liposomes,

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in particular, are considered promising carriers for therapeutic agents because they can facilitate controlled release and targeted delivery of drugs, thereby reducing drug-related toxicity [Arulsudar et al. 2004, Bhatia and Kumar 2004, Jadoul and Preat 1997, Jain et al. 2011, Mitragorti and Kost 2003, Popovici 1986, Porfire et al. 2009].

Vesicles are water-filled colloidal particles. The walls of these capsules consist of amphiphilic molecules (lipids and surfactants) in a bilayer conformation. In an excess of water these amphiphilic molecules can form one (unilamellar vesicles) or more (multilamellar vesicles) concentric bilayers. Hydrophilic drugs can be entrapped into the internal aqueous compartment, whereas amphiphilic, lipophilic and charged hydrophilic drugs can be associated with the vesicle bilayer by hydrophobic and/or electrostatic interactions. Most commonly, the vesicles are composed of phospholipids or non-ionic surfactants. The reason for using vesicles in transdermal drug delivery is based on the fact that they act as drug carriers to deliver entrapped drug molecules across the skin, as well as penetration enhancers because of their composition. In addition, these vesicles serve as a depot for the sustained release of active compounds in the case of topical formulations, as well as rate-limiting membrane barrier for the modulation of systemic absorption in the case of transdermal formulations [Hofland et al. 1994].

THEORIES AND APPROXIMATIONS IN MICELLE AND VESICLE FORMATIONS

Micelle formation

Law of mass action. We consider an aqueous solution of neutral amphiphilic molecules (i.e., non-ionic surfactants), each of which has a single alkyl chain as its hydrophobic tail. In general, amphiphiles can form aggregates of various sizes and shapes. We will assume each micelle is spherical and neglect the effects of fluctuations in micelle size and shape. Thus, we imagine that each surfactant molecule exists either as a monomer or as part of a spherical n-mer. We denote the number densities of the monomers and n-mers by $\rho 1$ and ρn, respectively, so that the total surfactant concentration is given by:

$$\rho = \rho 1 + \mathbf{n}\, \rho n \qquad (1)$$

The concentrations of monomers and micelles are related by the law of mass action [Chandler 1987]:

$$\rho \mathbf{n}\, \mathbf{a}^3 = (\rho \mathbf{1a}^3)\, \exp\, (-\beta\Delta G) \qquad (2)$$

where: β denotes inverse temperature (i.e., $\beta^{-1} = k_B T$), 'a' is a microscopic length that specifies the standard state convention, and is the driving force for assembly, namely, the free energy of the n-mer, f_n, relative to that of n monomers, nf_1. We take 'a' to be approximately the girth of a surfactant molecule.

$$\Delta G = fn - nf_1 \qquad (3)$$

For large n, equation 3 implies the existence of a threshold concentration of surfactant molecules ρ_{cmc}, at which the density of aggregates becomes significant. Because this crossover is precipitous, its location is almost independent of the specific definition of the threshold as long as it is physically sensible. Specifically, to within corrections of order $n^{-1} \ln n$.

$$\text{In } \beta cmc\ a^3 = \beta\, \Delta G\ In^* \qquad (4)$$

The driving force per surfactant, $\Delta G/n$, is a function of n, and it is to be evaluated at the most probable aggregation number, n*. This number is the value of n that minimizes $\Delta G/n$.

Driving force

The contributions to ΔG can be found in three steps.

1. Creation of a cavity. A micelle will fill a region vacated by water. Assuming the extent of the surface is at least 1 nm², the free energy to create this cavity is

$$\Delta G1 = \delta A \qquad (5)$$

Where A denotes the surface area of the cavity and δ is the water-vapour surface tension. In general, there is also pressure volume work for forming a cavity in a liquid. For water at standard conditions, pressure is sufficiently small that this contribution is negligible for cavities with diameters less than 5 nm. We will limit our consideration to sizes within this range.

2. Filling the hydrophobic core. Imagine disconnecting each hydrophobic tail in a surfactant from its respective hydrophilic head group and moving the hydrophobic tail from water into the micelle core. A total of *n* tails must be moved to fill the cavity formed in step 1. As such, one part of the free energy to fill the

cavity is $-n\Delta\mu$, where $-\Delta\mu$ is the free energy change in transferring the hydrophobic tail (e.g., an alkane chain) from water into the oily hydrophobic core. An additional part of the free energy for filling the cavity is an interfacial contribution due to the presence of Vander Waals attractions between oil and water. These interactions cause the oil-water surface tension, $\gamma_{o/w}$, to be lower than the water-vapour surface tension, γ. Thus, the free energy for filling the cavity is [Huand and Chandler 2002]:

$$\Delta G_2 = -n\Delta\mu - \Delta\gamma A \qquad (6)$$

where: $\Delta\gamma = \gamma - \gamma_{ow}$.

The interior of a micelle is densely packed and much like a hydrocarbon liquid. Thus, $\Delta\mu$ is close to the transfer free energy for moving the associated alkenes chain from oil into water. However, it is slightly smaller than this value because the environment of an alkane chain in a micelle interior is more confining than that in bulk oil [Tanford 1980]. To the extent that the micelle is spherical, $A = 4\pi L^2$, where L is the micelle radius. Since the interior is densely packed, L is given by $A = 4\pi L^{3/3} = n\delta a^2$, where ä is the mean length over which a polar head group is separated from an alkyl group within a surfactant molecule. From these considerations:

$$\Delta G_1 + \Delta G_2 = -n\Delta\mu + \mu g n^{2/3} \qquad (7)$$

$$g = (36\pi)^{1/3}(\gamma_{ow}a^2) \gg 4.8\,X(\gamma_{ow}a^2)(\gamma/a)^{2/3}$$

The right-hand side of the equation is essentially the free energy for nucleating oil clusters in water [Barrat and Hausen 2003]. It is the hydrophobic driving force identified in the Lum-Chandler-Weeks theory [Lum et al. 1999]. The first term is proportional to the volume of hydrophobic units. The second term is proportional to the area of the interface. The first term is extensive in n and dominates at large n. Thus, if only ΔG_1 and ΔG_2 were significant, the strength of the driving force would grow without bound leading to macroscopic clusters.

3. Placing hydrophilic head groups on the micelle surface. In the final step, the hydrophilic head groups are reconnected to the hydrophobic tails, placing them at the water-oil interface so as to maintain favourable solvation energy. This positioning is to be done while simultaneously enforcing the connectivity between heads and tails and while also maintaining the densely packed interior. These conditions result in an entropic cost that increases super-extensively with aggregate size. The form of this third contribution to the driving force is conveniently estimated from the electrostatic analogy of stoichiometric constraints [De Gennes 1979, Stillinger 1983, Woo et al. 1996]. The result is:

$$\Delta G_3 = hn^{5/3}/\beta \qquad (8)$$

where: $h = \dfrac{3}{(4\pi)^{2/3}}\left(\dfrac{96}{49}\right)\left(\dfrac{a}{\delta}\right)^{\frac{4}{3}} \approx 0.75\,X\left(\dfrac{a}{\delta}\right)^{\frac{4}{3}}$.

In employing this analogy, it is important to note that the micelle volume is essentially that of the densely packed alkyl chains.

Micelle size and critical micelle concentration

Combining the three contributions discussed above gives the driving force in units of k_BT.

$$\beta\Delta G \approx n\beta\Delta\mu + \beta g n^{2/3} + hn^{5/3} \qquad (9)$$

Minimization of $\Delta G/n$ therefore gives:

$$n^* \approx \frac{\beta g}{2h} = \left(\frac{49\pi}{48}\right)\beta\gamma\delta^2 \qquad (10)$$

With this aggregation number:

$$\ln \rho_{cmc}a^3 = c(\beta\gamma_{ow}a^2)^{2/3} - \beta\Delta\mu \qquad (11)$$

where: $c = \left(\dfrac{5832}{49}\right)^{\frac{1}{3}} \approx 4.9$.

The thermodynamic cycle of micelle formation can be observed in Figure 1.

Correlation of the amphiphile structure with its phase behavior could be understood with a simple geometric model, which defines a dimensionless critical packing parameter (CPP) to describe the relative bulkiness of the hydrophobic part and the hydrophilic part in an amphiphile. With the CPP increasing from a small value to a high value the amphiphile changes from hydrophilic to hydrophobic, its preferred phase structure from direct structures via lamellar structure to reverse structures. This model provides a basis for the molecular design of amphiphiles.

Molecules on the surface feels curvature when the size is small and molecules experience stress.
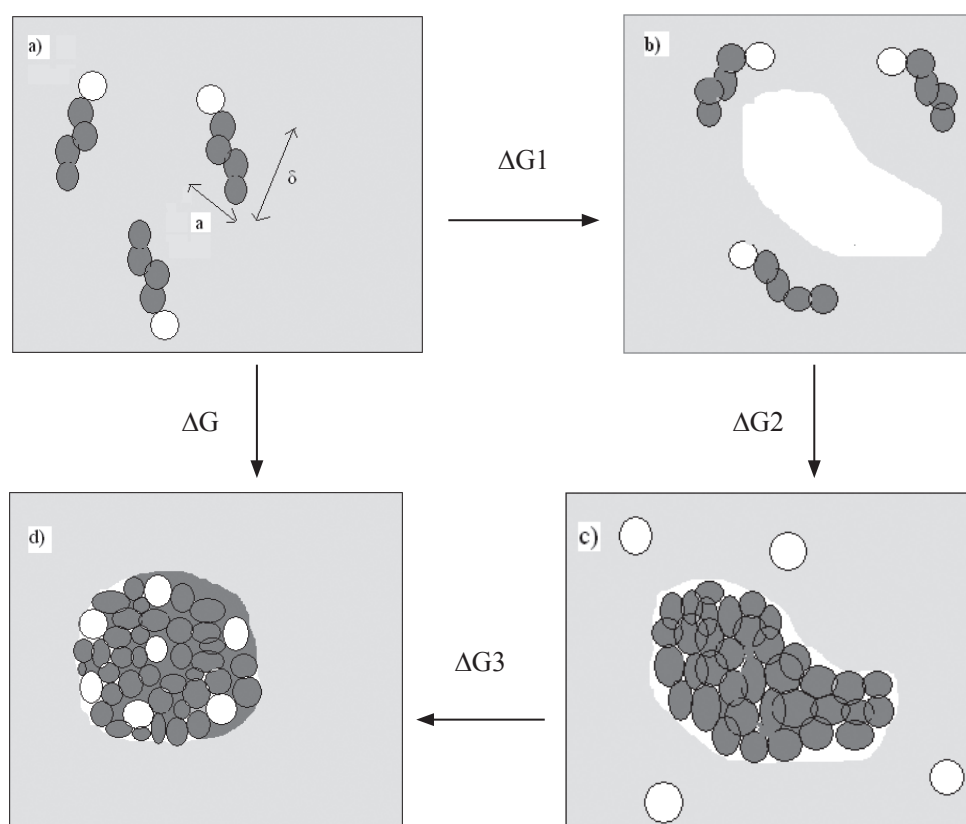
Fig. 1. Thermodynamic cycle of micelle formation. The process of assembling n separated amphiphiles (a) to a micelle (d) can be performed in three steps: 1 – creating a cavity in the solvent (light gray) (b), 2 – transferring the hydrophobic chains (dark gray) from the aqueous solution into the cavity (c), 3 – distributing the polar units (gray) over the surface of the cavity and reconnecting them to the hydrophobic groups (d)

The stress gets significant role when the size is below 100 nm. For bilayer formation, the molecules must be amphiphilic. Typical geometry for a vesicle formation is:

$$P = V / a0 \; Ic \qquad (12)$$

where:

P – critical packing parameter,
Ic – hydrophilic chain length,
V – volume of hydrophilic part,
a0 – optimum surface area/molecule at interface [Woo et al. 1996].

A pictorial representation of geometry and variants of packing arrangements are shown in Figure 2 and 3 respectively [Israelachvili 1991].

Vesicle formation

Formation of large vesicles from small, preformed vesicles. Small, uniform-sized egg lecithin vesicles were prepared as described. Any solute to be entrapped was added to the vesicle solution at this point and the vesicles were adjusted to 20 mM phospholipids. The solution was then warmed to 25°C and an aliquot of 250 mM sodium deoxycholate was rapidly added and mixed to give a final mixture containing a ratio of deoxycholate to phospholipids of 1:2. The large vesicles began to form almost immediately, as indicated by the increase in the light scattering of the solution, a change from nearly clear for the small vesicles to a transparent opalescence for the large vesicles. Vesicle formation was complete within 5-10 min
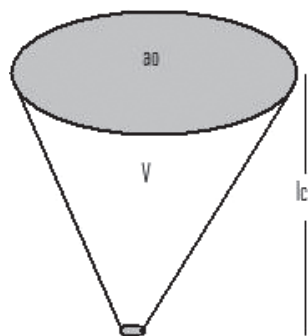
Fig. 2. Representation of typical geometry for a vesicle formation, P = V/ a0 Ic
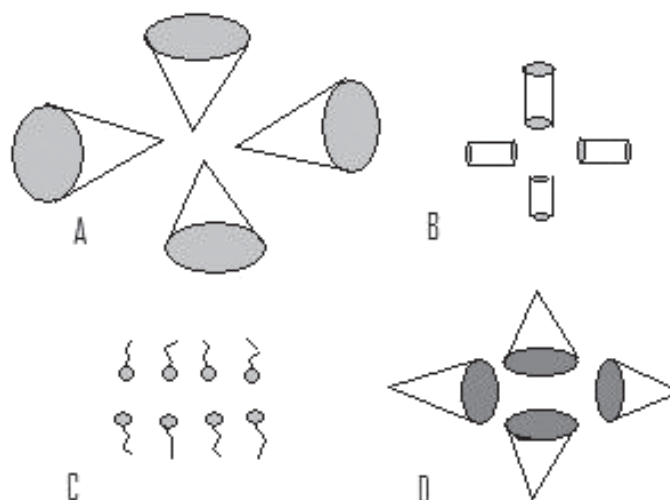
Fig. 3. A. P = 1/3, micelle formation, B. P = 1/2, cylindrical micelle, C. P = 1, planar bilayer, D. P > 1, inverted micelle

at 250°C (vesicles could also be formed at 40°C and required 15-30 min). The bulk of the detergent (96--98%) was then removed by passage of the sample over 60 vol of SephadexG-25 (medium porosity). The residual deoxycholate may represent detergent trapped within the vesicle which re-equilibrates with the outside volume after the removal of the bulk of the detergent; it was very readily removed by a second gel filtration column of 20-30 vol. The final preparation contained less than 1 deoxycholate molecule per 1000 phospholipid molecules. The vesicles are stable at this stage for several weeks, as indicated by the lack of change in the turbidity or the size when examined by electron microscopy. The stability appears to be limited by the chemical stability of the unsaturated phospholipid component [Strittmatter and Enoch 1978].

Formation of large vesicles from a phospholipid film. Egg lecithin (20 μmol) in chloroform was dried under reduced pressure to a thin film in a 15 ml Cortex tube. One millilitre of buffer containing 10 mM sodium deoxycholate plus any solute to be entrapped in the vesicles was added and the tube was swirled to suspend the lipid. This suspension was sonicated briefly in a bran sonic 12 sonifier bath at 25°C under N_2. After 1-2 min of sonication, the solution changed from an opaque, milky colour to a transparent opalescence. Continued sonication for 15 min brought no further change in the appearance of the solution and no further

decrease in the turbidity. At this point deoxycholate was removed.

Vesicles are classified into multi lamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs). General procedures for vesicular preparations are
- direct hydration
- hydration from organic solvent, and
- detergent removal.

Hydration from organic solvent method is used for producing multi lamellar vesicles (MLVs). MLVs extrusion through 0.1 μm polycarbonate filter and plus freeze-thaw technique, reverse phase evaporation and detergent removal procedures are used for producing large unilamellar vesicles (LUVs). Sonication is used for producing small unilamellar vesicles (SUVs).

Direct hydration procedure has certain advantages such as it is fast procedure. Its disadvantages include low trapped volume, low trapping efficiency and unequal distribution of solute.

The advantage of hydration from organic solvent procedure is high trapping efficiency and its disadvantages include technically complex and limited by lipid solubility in organic phase.

Detergent removal procedure has advantages such as reconstitution of proteins possible, high trapped volume and its disadvantages are its difficult to remove detergents completely, and low trapping efficiency

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[Bangham et al. 1965, Barenholzt et al. 1979, Hope et al. 1985, Enoch and Strittmatter 1979, Mayer et al. 1986, Ghada et al. 2010, Gruner et al. 1985, Deamer 1984, Allen 1984].

VESICLES AND ITS HYBRIDS

Liposomal formulations can be classified in two categories:
• rigid vesicles – liposomes and niosomes
• elastic or ultra deformable vesicles – transfersomes and ethosomes.

Depending on the structure, composition and methods vesicles can be modulated into several categories which have been described in Table 1.

TRANSFERSOMES AND ITS HISTORY

Transfersome is a term registered as a trademark by the German company IDEA AG, and used by it to refer to its proprietary drug delivery technology. The name means "carrying body", and is derived from the Latin word 'transferre', meaning 'to carry across', and the Greek word 'soma', for a 'body'. A Transfersome

Table 1. Hybrids of vesicles and their composition

| Type	Sub-type	Composition	References
1	2	3	4
Liposomes			
	conventional liposomes	neutral and or negatively charged phospholipids + cholesterol	Gregoriadis and Rynlan [1972]
	pH sensitive liposomes	phospholipid such as phosphotidyl ethanolamine with CHEMS	Encyclopedia... [1999]
	cationic liposomes	cationic lipids	Felgner and Ringlod [1994]
	long circulating liposomes (or) stealth liposomes	neutral high transition temperature, lipid cholesterol + 5-10% of PEG-DSPE, GMI, HPI	Targeting... [1998]
	immune-liposomes	conventional or long circulating liposomes with attached Ab or recognition sequence	Plautz [1993]
	magnetic liposomes	phosphotidylcholine, cholesterol, small amounts of a linear chain aldehyde and colloidal particles of magnetic iron oxide	Elmi and Sarbolouki [2001]
	temperature (or) heat sensitive liposomes	dipalmitoylphophatidylcholine	Sullivan and Huang [1986]
Virosomes		virus glycoprotein, incorporated into liposomal bilayers based on retro viruses derived lipids	Huckriede et al. [2003]
Ufasomes		vesicles enclosed by fatty acids	Babizhayev [1988]
Cryptosomes		lipid vesicles	Blume and Cevc [1993]
Emulsomes		bio adhesive nanoemulsion	Kretschmar et al. [2001]
Discomes		niosomes with non-ionic surfactant	Vyas et al. [1997]
Aquasomes		ceramic carbon nanocrystaline particulate core coated with glassy cellobiose	Khopade et al. [2002]
Ethosomes		phospholipid, ethanol and water	Godin and Touitou [2003]

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Table 1 – cont.

1	2	3	4
Genosomes		macromolecular complexes	Zhdanov et al. [2002]
Photosomes		photolase encapsulated in liposomes	Petit-Frere et al. [1998]
Erythrosomes		chemically cross linked human erythrocytes	Cuppoletti et al. [1981]
Hemosomes		haemoglobin containing liposomes	Gornicki [2003]
Proteosomes			Logan et al. [2004]
Vessome		nested bilayer	Kisak et al. [2004]
Archaeosomes		glycerolipids of archae	Krishnan et al. [2001]
Phytosomes		active ingredient is a herbal origin and is an integral part of the lipid membrane by chemical bond rather than occupying the centre cavity	Vinod et al. [2010]
Transferosomes		phosphotidylcholine, chloroform, methanol, phosphate buffer, drug	Shivanand et al. [2009]
Cubosomes		monoolein, poloxamer-407, phosphate saline buffer, chloroform	Di Bei et al. [2009]
Pharmacosomes		amphiphilic phospholipid and drug complex	Kavitha et al. [2010]

carrier is an artificial vesicle designed to be like a cell vesicle or a cell engaged in exocytosis, and thus suitable for controlled and, potentially targeted, drug delivery. Transfersomes are a special type of liposomes, consisting of phosphatidylcholine and a surfactant which act as an edge activator. The concept of transfersome was introduced in 1992 by Cevc and co-workers. These vesicular transferosomes are more elastic than the standard liposomes and thus well suited for the skin penetration [Cevc and Blume 1992, Dubey et al. 2006].

Transfersomes were developed in order to take the advantage of phospholipid vesicles as transdermal drug carrier. These self-optimized aggregates, with the ultra flexible membrane, are able to deliver the drug reproducibly either into or through the skin, depending on the choice of administration or application, with high efficiency. Transfersomes overcome the skin penetration difficulty by squeezing themselves along the intracellular sealing lipid of the stratum corneum. There is provision for this, because of the high vesicle deformability, which permits the entry due to the mechanical stress of surrounding, in a self-adapting manner. Flexibility of transfersomes membrane is achieved by mixing suitable surface-active components in the proper ratios. The resulting flexibility of transfersome membrane minimizes the risk of complete vesicle rupture in the skin and allows transfersomes to follow the natural water gradient across the epidermis, when applied under non-occlusive condition. Transfersomes can penetrate the intact stratum corneum spontaneously along two routes in the intracellular lipid that differ in their bilayers properties [Cevc 1991, Schatzlein and Cevc 1995].

The high and self-optimizing deformability of typical composite transfersomes membrane, which are adaptable to ambient tress allow the ultra deformable transferosomes to change its membrane composition locally and reversibly, when it is pressed against or attracted into narrow pore. The transfersomes components that sustain strong membrane deformation preferentially accumulate, while the less adaptable molecules are diluted at sites of great stress. This dramatically lowers the energetic cost of membrane deformation and permits the resulting, highly flexible particles, first to enter and then to pass through the pores rapidly and efficiently. This behaviour is not limited to one type of pore and has been observed
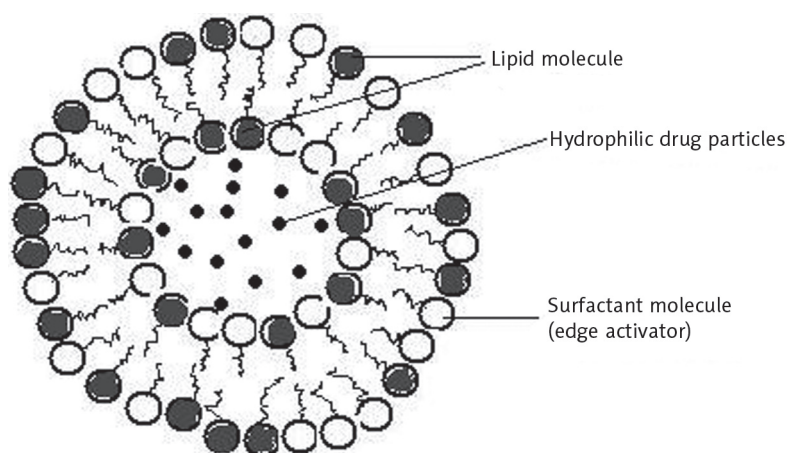
Fig. 4. Structural representation of one transfersome unit

in natural barriers such as in intact skin. The typical diagram of transfersome is shown in Figure 4.

Transfersomes in the thermodynamic equilibrium are normally large, $r_v > 40$ nm. Lipids mostly hydrophobic in nature accumulate in the membrane region with a positive surface curvature or at the site with a quasi planar geometry. Flexibility of transfersomes depends on the type of lipids, edge active substances or different lipid concentrations. The provison is that the transfersome becomes ultraflexible mixed lipid vesicles in a (quasi) metastable state by which it facilitates squeezing easily through pores of biological barriers much smaller than its own size (one-tenth of its own diameter) [Cevc et al. 2009, Cevc 1996].

SCOPE OF TRANSFERSOMES

Transfersome technology is best suited for non-invasive delivery of therapeutic molecules across open biological barriers. The Transfersome vesicles can transport across the skin, for example, molecules that are too big to diffuse through the barrier. Examples include systemic delivery of therapeutically meaningful amounts of macromolecules, such as insulin or interferon, across intact mammalian skin. Other applications include the transport of small molecule drugs which have certain physicochemical properties which would otherwise prevent them from diffusing across the barrier.

Another attraction of the Transfersome technology is the carrier's ability to target peripheral, subcutaneous tissue. This ability relies on minimisation of the carrier-associated drug clearance through cutaneous blood vessels plexus: the non-fenestrated blood capillary walls in the skin together with the tight junctions between endothelial cells preclude vesicles getting directly into blood, thus maximizing local drug retention and propensity to reach the peripheral tissue targets.

SALIENT FEATURES OF TRANSFERSOMES

At first glance, transfersomes appear to be remotely related to lipid bilayered vesicle, liposomes. However in functional terms, transfersomes differ vastly from commonly used liposomes in that they are much more flexible and adaptable. The extremely high flexibility of their membrane permits transfersomes to squeeze themselves even through pores much smaller than their own diameter.

Transfersomes possess an infrastructure consisting of hydrophobic and hydrophilic moieties together and as a result, can accommodate drug molecules with wide range of solubility. Transfersomes can deform and pass through narrow constriction (from 5 to 10 times less than their own diameter) without measurable loss. This high deformability gives better penetration of intact vesicles through tight junctions. They can act as a carrier for low as well as high molecular weight drugs e.g. analgesic, anesthetic, corticosteroids, sex

hormone, anticancer, insulin, gap junction protein, and albumin. They are biocompatible and biodegradable as they are made from natural phospholipids similar to liposomes. They have high entrapment efficiency, in case of lipophilic drug close to 90%. They protect the encapsulated drug from metabolic degradation. They act as depot, releasing their contents slowly and gradually. They can be used for both systemic as well as topical delivery of drug. Easy to scale up, as procedure is simple, do not involve lengthy procedure and unnecessary use or pharmaceutically unacceptable additives [Jain 2001].

PRODUCT DEVELOPMENT OF TRANSFERSOMES

Scope of transfersomes is mainly intended for topical application although other routes may be considered for further investigations. Drug should be selected in such a way that it fits in the criteria of topical delivery. It should have ideal limits for aqueous solubility, lipophilicity, molecular size, melting point and pH of the aqueous saturated solution.

METHOD OF PREPARATION

The most commonly used materials in the preparation of transfersomes are phospholipids, surfactants, alcohol, and buffering agents. Here, each material has its own importance. Phospholipids such as soya phosphatidyl choline, dipalmitoyl phosphatidyl choline, distearoyl phoshatidyl choline are vesicle forming components. Surfactants act as edge activators which are responsible for the flexibility of transfersomes. Sodium cholate, sodium deoxycholate, tween-80, Span-80 are used as surfactants. Alcohol is used as solvent. Dyes are used for confocal scanning micrscopy study. Here buffering agent (saline phosphate buffer) is used as hydrating medium.

All the methods of preparation of transfersomes are comprised of two steps. First, a thin film is prepared hydrated and then brought to the desired size by sonication; and secondly, sonicated vesicles are homogenized by extrusion through a polycarbonate membrane. The mixture of vesicles forming ingredients, that is phospholipids and surfactant were dissolved in volatile organic solvent (chloroform-methanol), organic solvent evaporated above the lipid transition temperature (room temp. for pure PC vesicles, or 500°C for dipalmitoyl phosphatidylcholine) using rotary evaporator. Final traces of solvent were removed under vacuum for overnight. The deposited lipid films were hydrated with buffer (pH 6.5) by rotation at 60 rpm at the corresponding temperature. The resulting vesicles were swollen for 2hr at room temperature. To prepare small vesicles, resulting LMVs were sonicated at room temperature or 500°C for 30 min using a B-12 FTZ bath sonicator or probe sonicated at 40°C for 30 min (titanium micro tip, Heat Systems W 380). The sonicated vesicles were homogenized by manual extrusion 10 times through a sandwich of 200 and 100 nm polycarbonate membrane [Fry et al. 1978].

CHARACTERIZATION AND EVALUATION OF TRANSFERSOMES

Transfersomes are characterized as follows using different methods.

Vesicle size, size distribution and vesicle diameter

Transfersomes can be visualized by transmission electron microscopy (TEM) and vesicle size and size distribution can be determined by dynamic light scattering (DLS) technique.

Vesicle diameter can be determined using photon correlation spectroscopy or dynamic light scattering (DLS) method. Samples were prepared in distilled water, filtered through a 0.2 mm membrane filter and diluted with filtered saline and then size measurement done by using photon correlation spectroscopy or dynamic light scattering measurements [Gamal et al. 1999].

Vesicle shape and type

Transfersomes vesicles can be visualized by using TEM, with an accelerating voltage of 100 kv. These vesicles can be visualized without sonication by phase contrast microscopy by using an optical microscope. Dynamic light scattering is also used for determining vesicle shape.

Number of vesicle per cubic mm

This is an essential parameter for optimizing the composition and other process variables. Transfersome

formulations (without sonication) can be diluted five times with 0.9% of sodium chloride solution and studied with optical microscopy by using haemocytometer.

Entrapment efficiency

Entrapment efficiency was determined by first separation of un-entrapped drug by the use of mini-column centrifugation method. After centrifugation, the vesicle was disrupted using 0.1% Triton X-100 or 50% n-propanol and then followed by suitable analytical technique to determine the entrapped drugs.

Surface charge and charge density, Turbidity measurement

Surface charge and charge density of transferosomes can be determined using zetasizer. Turbidity of drug in aqueous solution can be measured using nephelometer.

Degree of deformability or permeability measurement

The deformability study is done against the pure water as standard. Transfrsomes preparation is passed through a large number of pores of known size through a sandwich of different micropores filters with pore diameter between 50 nm and 400 nm, depending on the starting transferosomes suspension. Particle size and size distribution are noted after each pass by dynamic light scattering (DLS) measurements.

Skin interaction studies

Although interaction of deformable vesicles with skin is one of the most interesting current issues in vesicular science, it is also the most debated one. P. Loan Honeywell-Nguym et al. claims that this debate was initiated by a paper published in 1992, reported by Cevc G. et al. The author claims transfersomes could penetrate intact mouse skin and could travel as far as systemic circulation. The debate generated was whether transfersomes act in deep as carrier systems or act as penetration enhancers.

Several in-vivo studies were conducted to explore the pathway and fate of the vesicles of our issue within the tissue. Common *in-vivo* methods are confocal microscopy and tape stripping method.

Confocal scanning laser microscopy (CSLM) study

A confocal microscope illuminates and detects the scattered or fluorescent light within the vesicles. A set of conjugated apertures, one for illumination and one for detection of light, function as spatial filters. In confocal microscopy, lateral and axial resolutions are enhanced when compared to standard light microscopy. The axial resolution is responsible for identifying the lodging position of the vesicles ever deep within the tissues. The main advantage of confocal microscope is its ability to optically section thick specimens. Real time video frame can be captured with a low light video camera which inturn can be connected to a video recorder. Video frames give a live demonstration of the pathway and nature of the transportation of vesicles. Fluorescent dyes for detection used are Calcien AM (for green fluorescent), Rhodamine – 123, Rhodamine – DHPE, fluoroscien – DHPE, nile red.

Results indicated clearly that vesicles remarkably partitioned deep into the stratum corneum and could reach the layers as deep as the stratum corneum viable epidermal junction within one hour. Vesicles were found housed within the intercellular regions as channel like structures. No other abnormalities were detected within the intercellular lipid lamellae. The model drug was associated with vesicle material in the upper and central layers of stratum corneum. In the lower layers of stratum corneum, only vesicle material was there, not much of the drug. This clearly indicates that drug has been released from the vesicles before reaching the deeper layers. This indicates that the free drug molecules got released from the vesicles in to viable skin layers.

Using confocal laser scanning microscopy (CLSM), it is possible with a high degree of precision to locate and study transport phenomena of fluorescent chemical substances in different materials. This technique is often used in evaluation of biological phenomena and transport studies through various biological membranes, eg, the intestine and the skin. However, until now only a few studies using CLSM have focused on the application of technical pharmaceutical aspects. In this technique lipophilic fluorescence markers are incorporated into the transfersomes and the light emitted by these markers are used for the investigation of mechanism of penetration of transfersomes across the skin, for determining histological organization of

the skin and for comparison and differentiation of the mechanism of penetration of transferosomes with liposomes, niosomes and micelles. Penetration ability of transfersomes can be evaluated using fluorescence microscopy [Qvist et al. 2002].

Tape stripping method

Tape stripping technique is a widely accepted and it is used to examine the localization and distribution of substances within the stratum corneum. It is the simplest method for reducing the barrier imposed by the stratum corneum is to remove it. In this technique, an adhesive tape removes a layer of corneocytes. *In vivo*, removal of the stratum corneum by tape stripping is performed by the repeated application of adhesive tapes to the skin's surface. This can be used to investigate stratum corneum cohesion *in vivo* by quantifying the amount of stratum corneum removed. Tape stripping method can be studied in combination with electron microscopy and FT-IR. There are different parameters that can affect the quantity of stratum corneum removed by a piece of tape, and these include tape stripping mode, skin hydration, cohesion between cells, the body site and inter-individual differences [Bommannan et al. 1990, Higo et al. 1993, Lotte et al. 1993, Pershing et al. 1992, Rougier et al. 1986, Tojo and Lee 1989, Escobar-Chávez et al. 2005, Pinkus 1951, King et al. 1979, Sheth et al. 1987, Ohman and Vahlquist 1994].

In vitro drug release

The information from in-vitro studies is used to optimize the formulation before more expensive in vivo studies are performed. For determining in vitro drug release, beaker method is used in which transfersomes suspension is incubated at 32°C using cellophane membrane and the samples are taken at different times and then detected by various analytical techniques (UV, HPLC, HPTLC) and the free drug is separated by minicolumn centrifugation, then the amount of drug release is calculated [Fry et al. 1987].

In vivo fate of transfersomes and kinetics of penetration

After having penetrated through the outermost skin layers, transfersomes reach the deeper skin layer, the dermis. From this latter skin region they are normally washed out, via the lymph, into the blood circulation and through the latter throughout the body, if applied under suitable conditions. The kinetics of action of an epicutaneously applied agent depends on the velocity of carrier penetration as well as on the speed of drug redistribution and the action after this passage. The most important single factors in this process are:
1) carrier in-flow
2) carrier accumulation at the targets site
3) carrier elimination.

The onset of penetration-driving force depends on the volume of the suspension medium that must evaporate from the skin surface before the sufficiently strong trans-cutaneous chemical potential chemical potential or water activity gradient is established. Using less solvent is favorable in this respect. The rate of carrier passage across the skin is chiefly determined by the activation energy for the carrier deformation. The magnitude of the penetration driving force also plays a big role. This explains, for example, why the occlusion of an application site or the use of too strongly diluted suspension hampers the penetration process. Carrier elimination from the sub cutis is primarily affected by the lymphatic flow, general anesthesia or any other factor that affects this flow, consequently, is prone to modify the rate of trans-cutaneous carrier transport. While it has been estimated that approximately 10% of the cardiac blood flow pass through each gram of living skin tissue, no comparable quotation is available for the lymph. Further, drug distribution is also sensitive to the number of carrier used, as this may affect the rate of vehicle degradation and/or filtration in the lymph nodes.

The lag between the time of application and the time of drug appearance in the body, therefore, is always quite long, complex and strongly sensitive to the type of drug and formulation administration. In the best case, the skin penetration lag amounts to approximately 15 min if rapidly exchanging agents such as local analgesics are detected right under the skin permeability barrier. Less rapidly exchanging molecules or molecules measured in the blood compartment are typically detected with a lag time between 2 and 6 hr. depending on the details of drug formulation. Molecules that do not diffuse readily from the carriers or agents delivered with the suboptimal carriers normally fall in this category. The kinetics of vesicle penetration

into and across the skin can be controlled to a large extent by fixing the physicochemical characteristics of the drug carrier suspension. Kinetics of the transfersomes penetration through the intact skin is best studied in the direct biological assays in which the vesicle associated drugs exert their action directly under the skin surface. Local analgesics are useful for this purpose, for determining the kinetics of penetration, various Lidocaine loaded vesicles were left to dry out on the intact skin.

Corresponding subcutaneous injection is used as control. The animal's sensitivity to pain at the treated site after each application was then measured as a function of time. Dermally applied standard drug carrying liposomes or simple Lidocaine solution have never caused any analgesic effect. It was necessary to inject such agent preparations to achieve significant pain suppression. In contrast to this, the lidocaine-loaded transfersomes were analgesically active even when applied dermally. Maximum analgesic effect with the latter

type of drug application was typically observed 15 minutes after the drug application. A marked analgesic effect was still noticeable after a very long time. The precise reach as well as kinetics of transfersomes penetration through the skin are affected by: drug carrier interaction, application condition or form, skin characteristics, and the applied dose [Planas et al. 2008].

APPLICATIONS OF TRANSFERSOMES

Transfersomes have been widely used as a carrier for the transport of proteins and peptides. Proteins and peptide are large biogenic molecules which are very difficult to transport into the body, when given orally they are completely degraded in the GI tract. These are the reasons why these peptides and proteins still have to be introduced into the body through injections. Various approaches have been developed to improve these situations. The bioavailability obtained from transfersomes is somewhat similar to that resulting

Table 2. Drugs that can be accommodated in transfersomes

Drug	Indication	Remarks	Reference
Meloxicam	NSAID	improved skin permeation	Sureewan Duangjit et al. [2010]
Methotrexate	anti cancer drug	inceasing skin permeation	Trotta et al. [2004]
Oestradiol	estrogen	improved transdermal flux	Maghraby et al. [1998]
Curcumin	NSAID	improved bioavailability and permeability	Patel [2009]
Corticosteroids	vitiligo	improved site specificity and drug safety	Cevc et al. [1997]
Norgesterol	progestin	improved transdermal flux	Jain et al. [1998]
Tamoxifen	chemotherapy	improved transdermal flux	Jain et al. [1998]
Soluble proteins		non-invasive immunization through normal skin	Paul et al. [1995]
Human serum albumin			Paul and Cevec [1998]
Interferon-α	anti viral protective	efficient delivery means	Hafer et al. [1999]
Interlukin-2	regulators of lymphocytes	controlled release	Hafer et al. [1999]
Insulin	hypoglycemic	high encapsulation efficiency	Cevc et al. [1998]
Tetracaine	topical analgesic	suitable for the noninvasive treatment of local pain	Planas et al. [1992]
Lignocain	topical anesthetic	suitable for the noninvasive treatment of direct topical application	Planas et al. [1992]

from subcutaneous injection of the same protein suspension. The transfersomal preparations of this protein also induced strong immune response after the repeated epicutaneous application, for example the adjuvant immunogenic bovine serum albumin in transfersomes, after several dermal challenges is as active immunologically as is the corresponding injected proteo-transfersomes preparations. The drugs which can be incorporated in transfersomes are listed in Table 2.

LIMITATIONS OF TRANSFERSOMES

Like liposomes, transfersomes have certain limitations:
- transfersomes are chemically unstable because of their predisposition to oxidative degradation
- lack of purity of the natural phospholipids comes in the way of adoption of transfersomes as drug delivery vehicles and
- transfersomes formulations are expensive to prepare.

CONCLUSION

Transfersomes are specially optimized particles or vesicles, which can respond to an external stress by rapid and energetically inexpensive, shape transformations. Such highly deformable particles can thus be used to bring drugs across the biological permeability barriers, such as skin. When tested in artificial systems transfersomes can pass through even tiny pores (100 mm) nearly as efficiently as water, which is 1500 times smaller. The bio-distribution of radioactively labelled phospholipids applied in the form of transfersomes after 24 h is essentially the same after an epicutaneous application or subcutaneous injection of the preparations. When used under different application conditions, transfersomes can also be positioned nearly exclusively and essentially quantitatively into the viable skin region.

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TRANSFEROSOMY – NOWY SYSTEM TRANSPORTU LEKÓW

STRESZCZENIE

Wydaje się, że obecnie transport liposomowy nie jest zbyt wykorzystywany do celowego dostarczania leków. Transferosomy to rodzaj elastycznych i deformowalnych liposomów, które po raz pierwszy wprowadzono we wczesnych latach dziewięćdziesiątych ubiegłego wieku. Elastyczność struktury uzyskano, stosując aktywatory w zewnętrznej strukturze dwuwarstwy lipidowej. Cząsteczki o masie większej niż 500 Da nie przechodzą przez skórę. Zapobiega to dostarczaniu wysokocząsteczkowych leków przez naskórek oraz nieinwazyjnemu szczepieniu przezskórnemu. Bezpośredni transport przez skórę jest najlepszym sposobem dostarczania leków. Wraz z pojawianiem się nowych kategorii leków, np. peptydów, dostarczanie leków przez skórę budzi coraz większe zainteresowanie, między innymi dzięki uniknięciu problemów występujących w dawkowaniu doustnym. Dostarczanie przez skórę wiąże się z wieloma nadziejami i rozczarowaniami, szczególnie w odniesieniu do dużych molekuł. Aby przezwyciężyć problemy, podjęto wiele badań w celu ich rozwiązania. Jednym z ostatnio stosowanych sposobów jest wykorzystanie super deformowalnych systemów transportu – transferosomów. Używane są one jako transportery wielu małych cząsteczek, peptydów, białek i szczepionek, zarówno in vivo, jak i in vitro. Tranferosomy penetrują pory stratum corneum, które są mniejsze od wymiarów samych transferosomów, a dzięki elastycznej naturze dostają się do głębszych warstw skóry w formie niezmienionej. Celem pracy jest wyjaśnienie powstawania miceli oraz różnego rodzaju liposomów ze szczególnym uwzględnieniem transferosomów.

Słowa kluczowe: micela, liposomy, transferosomy, przyrząd ultradźwiękowy, konfokalny mikroskop skaningowy