Liposomal delivery of proteins and peptides

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Introduction: A number of delivery issues exist for biotech molecules including peptides, proteins and gene-based medicines that now make up over 60% of the drug pipeline. The problems comprise pharmaceutical ad biopharmaceutical issues. One of the common approaches to overcome these issues is the use of a carrier and liposomes as carriers have been investigated extensively over the last decade.

Areas covered: The review has been discussed in terms of formulation and preclinical development studies and *in vivo* studies encompassing different delivery routes including parenteral, oral, buccal, pulmonary, intranasal, ocular and transdermal involving liposomes as carriers. Important research findings have been tabulated under each side heading and an expert opinion has been summarised for each delivery route.

Expert opinion: The conclusion and expert opinion – conclusion sections discuss in detail troubleshooting aspects related to the use of liposomes as carriers for delivery of biopharmaceutical moieties and scrutinises the aspects behind the absence of a protein/peptide-containing liposome in market.

Keywords: biopharmaceuticals, calcitonin, insulin, interleukins, lipid vesicles, vasoactive intestinal peptide

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1. Introduction

A number of delivery issues exist for biotech molecules including peptides, proteins and gene-based medicines that now make up over 60% of the drug pipeline [1]. The problems comprise pharmaceutical issues such as instability during processing and storage and biopharmaceutical issues including poor permeability across non-injected routes of delivery, lack of local targeting to retain the cargo at the site of action as well as instability in biological fluids and poor intracellular delivery. One of the possible approaches to overcome these delivery problems is the use of a carrier system. In this context, lipid-based drug delivery systems have unique advantages over other carriers in offering flexibility to form different types of delivery vehicles by varying lipid types and ratios to suit the payload requirements. In a nutshell lipid delivery includes liposomes, solid lipid nanoparticles, oily suspensions, submicron lipid emulsions, lipid implants, lipid microbubbles, inverse lipid micelles, cochlear liposomes, lipid microtubules and lipid microcylinders of which liposomes have been explored to a great extent [1].

2. Liposomes as carriers for protein/peptide delivery

Liposomes are concentric bilayered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer composed mainly of natural or synthetic phospholipids in the presence or absence of cholesterol [2]. Classification, formulation, preparation and cargo loading methods, properties and characterisation

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techniques of liposomes are discussed elaborately in other review articles [2-6]. Liposomes can be administered through parenteral, oral, pulmonary, nasal, ocular and transdermal routes. This is further explained in **Figure 1**.

The intent of this review is to reference/list all studies (to the best of our knowledge) that have been published to date, taking examples of four peptides and proteins (insulin, calcitonin, VIP and interleukins) encapsulated in/associated with liposomes. The criterion for choosing these peptides has been the extensive research that has happened with them over the last decade.

3. Formulation and pre-clinical development studies

Initially, commonly encountered formulation issues concerning liposomal encapsulation of proteins and peptides will be reviewed.

3.1 Advantages of encapsulation

Advantages of using a liposomal carrier over a free peptide have been established in studies by Stark *et al.* [7] and Adibzadeh *et al.* [8]. The former group highlighted the superior stability of liposomal VIP in bronchoalveolar lavage fluid, whereas the latter suggested that liposomal IL-2 was as active as free IL-2 for cloning and culture of both helper and cytotoxic alloreactive T cells.

1) Liposomal charge. Liposomal surface charge is an important parameter which contributes in the manner liposomes interact with cells or constituents of body fluids [9-11]. According to Kato *et al.* a positive surface charge showed better resistance to trypsin digestion in comparison to negative and neutral surface charged insulin-loaded liposomes [9]. Corona-Ortega *et al.* suggested improved adhesion to cells by cationic/positively charged liposomes in comparison to neutral/negative IL-2-encapsulated liposomes [10]. An elaborate investigation was performed by Law *et al.* on formulation development parameters with liposomal charge as an important parameter under study in salmon calcitonin-loaded liposomes where a negative surface charge facilitated increased encapsulation of the cargo in comparison to the neutral and positive surface charges [11].
2) Drug loading methods: Drug loading method decides the encapsulation efficiency of the payload and thus is an important parameter. Hwang *et al.* reported that interaction of insulin with liposomal membrane is facilitated in the presence of transmembrane gradient and this is not suitable for insulin loading [12]. Same research group reported a twofold increase in encapsulation of insulin into liposomes when prepared by reversed phase evaporation method in comparison to transmembrane gradient method [13].
3) PEG versus non-PEG: PEGylation has several advantages including prevention of opsonisation (*in vivo*) and improved encapsulation of payload in some cases (formulation related) [14]. PEGylated liposomes showed higher encapsulation of insulin compared to its non-PEG counterpart as demonstrated by Park *et al.* [15]. *In vitro*, IL-2-encapsulated PEG liposomes showed improved interaction with cells compared to its non-PEG counterpart as reported by Kedar *et al.* [16].
4) Association with lipid membrane: Some payloads show association with lipid membrane because they are either lipophilic or amphiphilic. Peptides belong to the latter category and studies showing their association with lipid membrane are explained in references 17–20. Arien *et al.* reported re-encapsulation of 50% of sCT following cholate-induced disruption of sCT liposomes, suggesting the formation of lipid-sCT complexes and concluded this as the reason behind hypocalcaemic effect [17]. Stark *et al.* reported the interaction of VIP with lipid bilayer [18] and Neville *et al.* reported the interaction of IL-2 with lipid bilayer as confirmed by freeze-fracture microscopy [19]. Enhanced proliferation of cytotoxic T-cells following administration of IL-2 liposomes was an outcome of IL-2 interaction with liposomal bilayers as postulated by Joffret *et al.* [20].
5) Permeation enhancer: Presence of permeation enhancers in liposomal formulations has showed a positive impact on *in vitro* studies. Degim *et al.* and Maitani *et al.* showed enhanced permeation of insulin (on treatment with insulin-loaded liposomes and presence of permeation enhancers, sodium taurocholate and sodium glycocholate) across Caco-2 cell monolayers and nasal mucosa of rabbit attached to diffusion cells [21,22]. Song *et al.* demonstrated enhanced permeation of sCT across Caco-2 cell monolayers in the presence of sodium taurodeoxycholate [23].
6) Special factors: Khanna *et al.* suggested liposomes to be stable to nebulisation in terms of reduced cargo loss following the process [24]. The work was performed with IL-2-loaded liposomes. Kim *et al.* and Mayer *et al.* reported successful treatment of atopic dermatitis of skin and gene transfection with IL-2-loaded liposomes [25,26].

Expert opinion: From the above mentioned examples it is clear that many factors contribute towards the formulation of stable, efficient and bioactive liposomes. In some cases, however, the superiority and advantage of using a carrier needs to be established. Surface charge is an important parameter and cationic/positive surface charge shows improved interaction with cells; however, a highly positive surface charge (greater than +30 mV) can be cytotoxic. Although PEGylation comes with added advantages, the

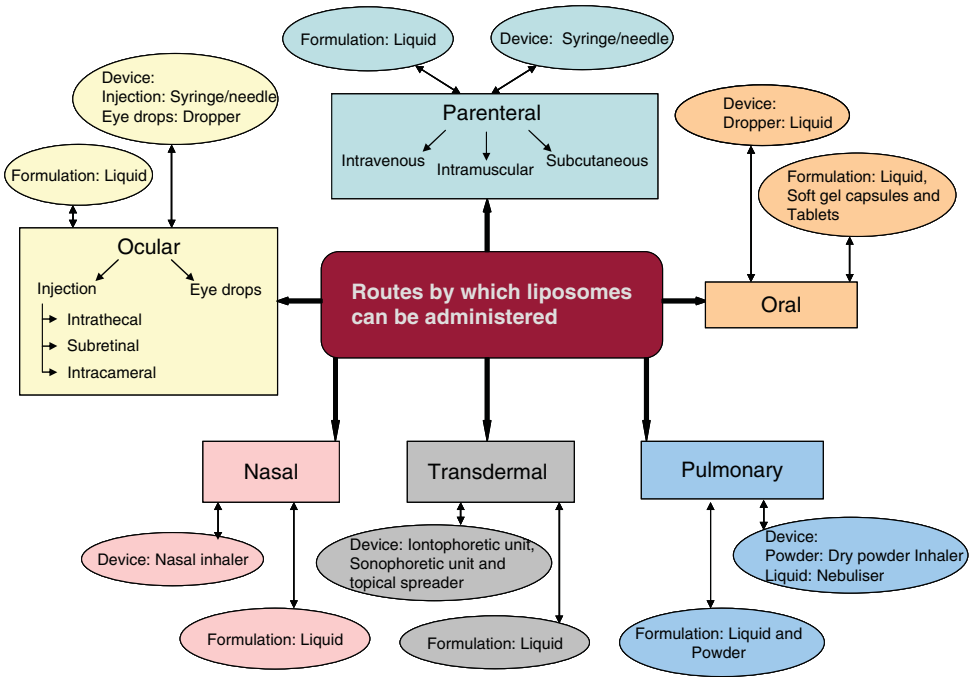

Figure 1. Routes of administration of liposomes. Various pathways by which liposomes containing biopharmaceuticals can be administered, including the nature of formulation and device used for administration.

cost associated with using a PEG moiety should be kept in mind and the need for the same should be carefully weighed out. Despite improved permeation with the use of permeation enhancers, the toxicity associated with these should be kept in mind before including the same in the liposomal formulation.

4. *In vivo* studies associated with commonly investigated peptides/proteins

4.1 Liposomes containing protein/peptide cargos for parenteral delivery

Expert opinion: From the above mentioned studies in **Table 1**, it is clear that protein payloads encapsulated in liposomes show excellent activity following parenteral delivery. Despite showing enhanced bioavailability and least hepatic first pass, the biggest issue of patient compliance remains associated with this route. Liposomes show improved PK/PD in comparison to free payload as evident from the examples but the expense involved in the formulation of liposomes needs to be kept in mind. Moreover, a liposomal formulation has to be lyophilised to improve its stability and this involves additional process optimisation which increases the duration of producing a stable formulation and the overall cost associated. Bioactivity of the sensitive payload – peptide – needs to be kept in mind following lyophilisation. Scale-up can be a major issue and the competence of the technology transfer team would be tested if lab scale batches have to be reproduced to macroscale.

Another important aspect of preparing a liposomal formulation containing a sensitive payload of parenteral delivery would be sterility associated with the same especially for administration in humans. Sterilisation techniques could compromise on the potency and bioactivity of the payload or even destabilise it. It is essential to come up with a suitable sterilisation technique. These issues have to be critically examined during scale-up and mass production as the examples mentioned only produce liposomes for laboratory scale and administer it to animals immediately after preparation.

Route of parenteral administration, that is, intravenous or subcutaneous shows no difference in PK/PD in some cases while in others i.v. shows enhanced activity due to the ability to get to the target site/elicit its activity immediately after administration. Vesicle size is an interesting parameter. Following i.v administration, the MLVs show pronounced activity due to the higher ability of being recognised by the reticuloendothelial system compared to SUVs. On the other hand, SUVs show better PK/PD following s.c. or intratumoural delivery due to smaller size of these vesicles that allows them to extravasate into tumour or elicit a local action following administration.

PEGylation increases circulation time of a liposomal formulation due to the ability to prevent opsonisation. The longer circulation time goes hand-in-hand with improved PK/PD in them. On the contrary, non-PEG liposomes stimulate the RES and act as co-adjuvants in vaccine formulations to trigger a potent immune response which would not happen if the free antigen is administered.

Table 1. Research studies on liposomes containing protein/peptide cargos for parenteral delivery.

Scope of research	Important research finding(s)	Ref.
Free payload versus encapsulation into liposomes	• Stevenson *et al.* demonstrated prolonged hypoglycaemic effect that lasted 24 h with insulin-loaded liposomes compared to 8 h with free insulin and empty liposomes in dogs • Gao *et al.* reported prolonged hypotensive effect in hamsters following administration of VIP liposomes in hamsters in comparison to the free peptide • Refai *et al.* published an enhanced uptake of VIP liposomes by the rat lung in comparison to free VIP using iodine-labelled VIP • Konno *et al.* reported a significant increase in survival time of rats with hepatoma following treatment with IL-2 liposomes which was not observed with free payload • An improvement in therapy was reported by Cao *et al.* who observed increased inhibitory effect on tumour growth in mice with liposomes containing IL-2 and IL-6 plasmids in comparison to the administration of free form of the same • Kanaoka *et al.* reported an increased residence time of IL-2 encapsulated in liposomal carriers compared to free IL-2 in rats • Rangel-Corona *et al.* reported significant reduction of tumour masses in mice with cervical cancer cell line following treatment with cationic liposomes containing IL-2. This was not pronounced with free IL-2 • Kabay *et al.* reported a 100% mortality in mice groups treated with free IL-10 while it was 75% with liposome-mediated IL-10 treatment	[42-49]
Route of administration	• Petkowick *et al.* published a similar hypoglycaemic effect in rats following both i.v and s.c administration of insulin liposomes suggesting the absence of impact with the parenteral route of administration • Kanaoka *et al.* reported an increase in the residence time of IL-2 in mice when encapsulated in liposomes following administration through both i.v. and s.c. routes while enhanced liver uptake was observed following i.v. administration	[50,51]
Vesicle size and route of administration	• Fukunaga *et al.* reported improved hypocalcaemia in rats following i.v. administration of salmon calcitonin liposomes (multilamellar vesicles; MLV) in comparison to small unilamellar vesicles (SUV) while following i.m. the activity was the same regardless of vesicle size • Cabanes *et al.* studied the anti-tumour activity of IL-2 liposomes in mice and reported SUVs being more potent in i.v. model and MLVs in i.p. model	[52,53]
PEG vs Non-PEG liposomes	• Kim *et al.* reported a threefold increase in encapsulation of insulin in PEG based dipalmitoylphosphatidylcholine liposomes in comparison to its non-PEG counterpart, and the PEG liposomes induced the lowest plasma glucose level in rats • Rubinstein *et al.* reported a decrease in mean arterial pressure (MAP) in hamsters following administration of sterically stabilised VIP liposomes that was not pronounced with non-PEG liposomes • Kedar *et al.* demonstrated a 2 – 6-fold longer survival time with sterically stabilised IL-2 liposomes in mice with metastatic carcinoma in comparison to free or PEGylated IL-2	[54-56]
Miscellaneous: Co-adjuvant effect	• Gursel *et al.* investigated the co-adjuvant effect of IL-2 in a liposomal formulation with tetanus toxoid and reported significant secondary immune responses when the moieties were present in the same vesicle rather than on administration of free form of them • Johnston *et al.* suggested that antibody, $CD8^+$ T cell and tumour protective immune responses markedly enhanced in mice (EG7-OVA cell treated) immunised with OVA + IL-2 liposomes compared to controls (ovalbumin injection in PBS or ovalbumin encapsulated in liposomes). • Duits *et al.* studied the immunoadjuvant activity of free antigen, antigen in liposomes and IL-6 in liposomes in mice and observed significantly higher antibody titres with the liposomal formulations in comparison to free antigen • Gursel *et al.* demonstrated the immunological co-adjuvant activity of IL-15 and tetanus toxoid (TT) antigen in mice and reported a 10-fold higher immunological response when both moieties were co-entrapped in the same vesicle rather than a physical mixture of different vesicles containing these • Dhillon *et al.* explored the effects of IL-4 complexed with liposomes in macaques and found a significant reduction of viral loads in the lymph nodes along with increase in secondary antibody titres • Simpson-Abelson *et al.* published local and sustained release of IL-12 from liposomes, reactivation of effector memory T-cells and release of interferon-gamma that killed tumour cells following intra-tumoural injection of IL-12 liposomes • Charoensit *et al.* compared the growth inhibition of metastatic lung tumour following i.v. injection of all trans retinoic acid (ATRA)-cationic liposome (CL) IL-12 plasmid DNA (pDNA) complex with CL-IL-12 pDNA and ATRA-CL and reported prolongation of survival time with the first formulation	[57-63]

4.2 Liposomes containing protein/peptide cargos for oral delivery

Expert opinion: Liposomes show enhanced PK/PD following oral administration and the results discussed above (**Table 2**) would seem that a liposomal formulation for oral delivery would promise significant market profit, though the case could be otherwise. From the research studies discussed above, positive surface charge shows improved biological activity which might be due to better interaction of positive surface charge with gastrointestinal mucosa. Inclusion of excipients such as surfactants or special lipids comes with an advantage of better PD but the toxicity and the cost of these should be weighed before inclusion in the formulation. Mucoadhesive polymers are those that interact with the gastrointestinal mucosa and adhere to the same, thus increasing the retention time in stomach or intestine. This depends on the type of polymer used. Commonly used polymers include chitosan, Carbopol, cellulose derivatives and alginate. One should be very careful when choosing the polymer type and its molecular weight before using it in the formulation. Polymers with very high molecular weights hinder the release of peptide due to steric hindrance and stability could be another issue with high molecular weight polymers. Generally these polymers interact/insert themselves into lipid bilayer to elicit their function. Fusogenic and double liposomes have evolved as novel liposomes due to the complexity of their design. Although significant differences in PK/PD have been reported, complexity and cost must be evaluated before attempting to produce these on large scale.

This being the scenario in oral delivery route, there are issues that need to be resolved before one can expect a peptide containing liposome for oral delivery in market. The primary issue is to determine if one would be interested in liquid or solid dosage form and if one decides on the former, stability would be an issue on large scale and the liquid liposome has to be lyophilised or spray dried to maintain stability. The powders can be reconstituted before consumption. On reconstitution, multilamellar vesicles would be obtained and the outcome due to these need not be similar to the results obtained in rodents with unilamellar vesicles on lab scale. If one decides on solid dosage forms like tablets or capsules, granulation of liposomes would be an issue. One of the possible ways would be to granulate a spray dried or freeze dried sample. Wet granulation would not be suitable because introduction of water and harsh drying temperatures would interrupt with the lipid dynamics. Dry granulation could be an option though bioactivity of the payload must be kept in mind. Coating with mucoadhesive polymers could be possible on the punched tablet or on pellets. If one decides on controlled release of payload, then pellets can be coated with different polymers of similar molecular weight or same polymer of different molecular weights to achieve different levels of coating. These polymers then dissolve based on the type of polymer or thickness of coating to release the payload over time.

Carrier poses lot of issues while oral forms of non-carrier containing sensitive payloads are Phase III clinical trials. Salmon calcitonin oral tablet (Ostora) marketed by Tarsa therapeutics has showed positive safety and efficacy results in the Phase III ORACAL trial evaluating its OSTORA tablet for the treatment of postmenopausal osteoporosis [27]. Novartis Pharma was also working on salmon calcitonin oral delivery using Emisphere's novel carrier technology. Emisphere is a company that specialises in preparing oral forms of payloads that are delivered otherwise. They use synthetic carriers and the payload that can be delivered has specifications: should be BCS class III drugs (that are water soluble and have low permeability issues) and should have a molecular weight of less than 10 kDa [28]. However in the end of 2011, Novartis said that the second Phase III study of oral calcitonin did not meet the end points. The data monitoring committee members did not see any safety issues to discontinue while they saw no efficacy issues to continue the project as well. This could be interpreted as an example of complication caused due to introduction of a carrier [29].

Similarly liposomes might not end up with safety issues but certainly the cost/complicated formulation-efficacy benefit needs to be understood before coming up with a large-scale research proposal for oral liposomes containing peptide payload.

4.3 Liposomes containing protein/peptide cargos for buccal delivery

Expert opinion: From the research studies discussed in **Table 3**, it is very clear that buccal administration has been researched only with VIP. The other model payloads have not been investigated for buccal delivery. Moreover very few examples are available for buccal delivery of VIP and only couple of research groups are working on the topic.

Although buccal delivery has advantages for patient compliance, ability to enter the systemic circulation quickly after administration and avoidance of hepatic first pass thus circumventing bioavailability problems, there are also associated issues that have to be considered before choosing this delivery route. The research groups have been using a liquid formulation that they administer into hamster cheek pouch. However, this cannot be done in bigger rodents or humans following success of the product on lab scale. The only dosage form for buccal administration would be a tablet and the success of the tablet in buccal studies has to be established. Moreover there would be tableting issues as discussed under oral delivery section. Thus all the above mentioned factors need to be kept in mind before deciding on buccal dosage forms.

4.4 Liposomes containing protein/peptide cargos for pulmonary delivery

Expert opinion: The examples mentioned above (**Table 4**) show that lungs have evolved as a new target organ and that not all payloads have been investigated for pulmonary delivery yet. Although pulmonary peptide delivery comes with advantages like large surface area of lungs, proximity to systemic

Table 2. Research studies on liposomes containing protein/peptide cargos for oral delivery.

Scope of research	Important research finding(s)	Ref.
Impact of surface charge	• Hashimoto *et al.* reported a significant reduction in blood glucose in rats following oral administration of insulin liposomes with positive surface charge in comparison to neutral and negatively charged liposomes • Kisel *et al.* published hyperinsulinaemia that was attended by a decrease in blood glucose in rats following administration of liposomes with positive surface charge	[64,65]
Inclusion of excipients		[66]
Inclusion of surfactant	• Chowdhari *et al.* published prolonged glucose lowering effect in rabbits following oral administration of insulin liposome containing 1% v/v Tween and the action was comparable to 1 IU/kg of insulin administered subcutaneously	[67-74]
Inclusion of special lipids	• Muramutsu *et al.* demonstrated that absolute pharmacological activity of soybean sterol containing insulin liposomes was 31.6% and the blood glucose level was reduced up to 21 h post administration in rats	
Inclusion of mucoadhesive polymers	• Takeuchi *et al.* proved an enhanced hypoglycaemic effect following administration of chitosan coated insulin liposomes in normal rats • Iwanaga *et al.* compared the mean transit time (MTT) of polyethylene glycol 2000 (PEG) and mucin-coated liposomes in rat small intestine and published that PEG liposomes had longer MTT in intestine while mucin-coated liposomes were retained in stomach much longer • Ramadas *et al.* described prolonged hypoglycaemia following the oral administration of insulin-loaded liposome encapsulated in alginate-chitosan gel capsules that delivered insulin in neutral environment of intestine in diabetic rats • Wu *et al.* demonstrated enhanced hypoglycaemic efficacy with liposomes coated with 0.2% chitosan of molecular weight 1,000 kDa in healthy mice • Degim *et al.* published most effective lowering of blood glucose level in both rats and mice following oral administration of methyl cellulose-containing insulin liposomes • Takeuchi *et al.* published enhanced and prolonged reduction in blood calcium level with carbopol- and chitosan-coated multilamellar vesicles with a consequent 2.4 and 2.8 times higher area under the curve compared to non-coated liposomes loaded with sCT in rats • Thongborisute *et al.* reported that oral administration of dodecylated chitosan-coated (DC) liposomes to rats produced greater reduction in blood calcium concentration compared to chitosan-coated liposomes	
Liposomal uptake following oral administration	• Das *et al.* (1988) reported that the percentage radioactivity associated with portal vein plasma samples was 60% due to liposomes and 20% due to free insulin while no radioactivity was detected from heart plasma samples following oral administration of ^{125}I-labelled insulin-encapsulated liposomes in rats suggesting better uptake of liposomes by the liver	[75]
Advances in liposomal design	• Goto *et al.* prepared fusogenic liposomes prepared by fusing liposomes loaded with insulin and p-chloromercuribenzoate with inactivated Sendai virus particles and administered into ileal, caecal and rectal loops in rats and a significant hypoglycaemia was observed which was further enhanced by increasing insulin concentration in liposomes and co-encapsulating insulin degrading enzyme inhibitor • Katayama *et al.* suggested remarkable hypoglycaemic effects following intragastric administration of positively charged double liposomes (DL) as carriers for insulin in combination with aprotinin, a protease inhibitor in male Wistar rats • Yamabe *et al.* reported an increase in bioavailability of sCT following oral administration in rats of neutral surface charged double liposomes (DL) prepared by mechanochemical method compared to positive surface charge/coatsome method • Ebato *et al.* published a similar work as Yamabe *et al.* on DL where a cationic inner liposome produced better hypocalcaemic efficacy in rats	[76-79]

circulation, circumvention of hepatic first pass and patient compliance, there are also issues such as presence of pulmonary metabolising enzymes, pulmonary macrophage and pulmonary surfactant in addition to the ability of a peptide to cross pulmonary epithelial barrier to enter the systemic circulation. Moreover, pulmonary administration would refer to inhalation by means of a dry powder inhaler or metered dose inhaler, aerosolisation or nebulisation using a nebuliser or intratracheal instillation. The first case involves the administration of a solid formulation while the others involve a liquid formulation.

Generally liquid formulations such as liposomes are administered using a nebuliser, and there are extensive varieties of nebulisers available in the market to choose from beginning

Table 3. Research studies on liposomes containing protein/peptide cargos for buccal delivery.

Bibliography	Important research finding(s)	Ref.
Liposome versus free drug	• Suzuki *et al.* demonstrated significantly greater vasorelaxant effects of VIP-encapsulated liposomes compared to free VIP following suffusion in hamster cheek pouch • Suzuki *et al.* reported significant vasodilatation following administration of free VIP and significantly prolonged vasodilatation with VIP liposomes in normotensive/ spontaneously hypertensive hamsters indicating the impairment of VIP-induced vasodilatation *in situ* in essential hypertension that is restored by encapsulation into liposomes	[80-81]
Studies with sterically stabilised liposomes (SSL)	• Sejourne *et al.* published significant and prolonged concentration-dependent vasodilatation following suffusion of VIP-sterically stabilised liposomes (VIP-SSL) in hamster cheek pouch • Ikezaki *et al.* confirmed that suffusion of monoclonal anti-VIP antibody significantly attenuates VIP-induced vasodilatation in the *in situ* hamster cheek pouch while suffusion of VIP-SSL restores the vasorelaxant effects of VIP even in the presence of anti-VIP antibody • Ikezaki *et al.* also reported that VIP-SSL significantly attenuates the vasoconstriction response obtained by suffusion of phenylephrine and ANG-II in hamster cheek pouch	[82-84]
Interaction of VIP with lipid bilayer	• Onyuksel *et al.* studied the interactions between VIP and rigid liposomes and reported a significantly potentiated vasodilatation following administration of liposomes (that were at stored at 4°C overnight with VIP) in hamster cheek pouch (in comparison to saline) confirming interactions between VIP and liposomal bilayers	[85]

Table 4. Research studies on liposomes containing protein/peptide cargos for pulmonary delivery.

Bibliography	Important research finding(s)	Ref.
Liposome versus free drug	• Huang *et al.* demonstrated prolonged effective hypoglycaemia following pulmonary delivery of insulin-loaded liposomes which was not observed with a combination free insulin and empty liposomes in rat lung • Bi *et al.* reported that a spray-freeze-dried dry powder inhalation of insulin-loaded liposomes produced normoglycaemia in diabetic rats on intratracheal instillation • Khanna *et al.* reported a significant increase in bronchoalveolar lavage (BAL) leukocyte count post inhalation of IL-2 liposomes in comparison to free IL-2 in normal dogs	[86-88]
Novel liposomal carriers	• Karathanasis *et al.* developed carrier particles consisting of insulin-loaded liposomes cross-linked through chemical bridges cleavable by cysteine which on endotracheal instillation in rats led to rapid lowering of blood glucose • Same research group later investigated microparticle agglomerate of colloidal liposomal particles cross-linked through chemical bridges cleavable by glucose following intratracheal instillation in rats that produced similar results	[89,90]
Feasibility and toxicity studies	• Khanna *et al.* evaluated the potential of aerosol IL-2 liposome therapy in dogs with pulmonary metastases and primary lung carcinoma with 2/4 animals showing complete regression of metastases, one showed stabilisation of disease while the fourth had a disease progression. Toxicity was minimal in all cases • Skubitz *et al.* performed a Phase I clinical trial to check the feasibility and toxicity of IL-2 liposome in patients with pulmonary metastases administered through nebulisation using a Puritan twin jet nebuliser. No significant toxicity was observed and delivery of IL-2 liposomes to lung was well tolerated • Ten *et al.* demonstrated the immune potential of IL-2-encapsulated liposomes in individuals with immune deficiency and hepatitis C administered through the aerosol route. IL-2 liposomes were well tolerated, no changes in chest X-ray and pulmonary function were seen and they showed significantly improved immune activity compared to placebo • Chono *et al.* compared the efficacy of various insulin-loaded liposomal preparations with different membrane rigidities following aerosolisation in rat lungs and reported dipalmitoylphosphatidylcholine (DPPC) liposomes performed best following *in vivo* tests which could have been due to compatibility of DPPC with lung surfactant because lung surfactant comprises 85% DPPC	[91-95]

with the air-jet and ultrasonic nebulisers that were developed initially to the latest breath actuated and breath-enhanced nebulisers where the loss of formulation is very less to almost nil. Nebulisers developed initially had disadvantages like loss of formulation to the exterior due to incompetent breathing pattern, loss of liquid in the formulation and concentration of payload in the reservoir of the nebuliser due to presence of baffles in nebuliser design, loss of bioactivity of payload due to increase in the temperature of reservoir chamber and large size that did not make the device patient friendly. The latest nebulisers guide the user regarding inhalation and exhalation cycles and also indicate the amount of formulation that was delivered/deposited [30]. Whatever be the advancement in nebuliser technology, it is still very challenging to deliver intact liposomes following nebulisation because the vesicles undergo stress during nebulisation process leading to leakage of the payload. The payload released in some cases gets re-encapsulated (depending on the nature of the payload and type of lipids that go into liposomal formulation) while in most others remains in its free form. The idea of using a carrier is to prolong the activity of the formulation thus reducing its dosing frequency. The free form that is released is readily available and the idea of controlled release is defeated.

Last but not least, the cost associated with both development and use of pulmonary drug delivery is enormous. If one could bring about a formulation that can solve the mentioned issues, pulmonary peptide delivery would hold a big market.

Yet another important issue is acceptability and costs of formulation. This has become a paramount concern from the time of discontinuation of Exubera, a dry powder inhaler of insulin that was produced by Pfizer and Nektar Therapeutics. Exubera was withdrawn from market after disappointing sales and the other inhaled insulin players like NovoNordisk/Aradigm and Eli Lilly/Alkermes backed out of their respective projects as well [31].

4.5 Liposomes containing protein/peptide cargos for intranasal delivery

Expert opinion: The examples above (**Table 5**) show that liposomes as carriers have a positive impact in drug delivery except in Chen *et al*. Chen *et al*. have indicated toxicity to nasal mucosa due to ultraflexible liposomes. This is of primary concern in drug delivery to any target organ and safety needs to be established with liposomes. Because liposomal products with peptide payloads are not available for delivery to any target organ, this information becomes exceedingly important for gaining marketability. Intranasal drug delivery offers advantages of patient compliance and avoidance of hepatic first pass but the issues of small nasal mucosa and presence of nasal secretion that would interfere with formulation administered still remain.

Moreover, salmon calcitonin, one of the payloads under discussion, is marketed as a nasal spray by Novartis as Miacalcin. It is available in a 3.7 mL glass bottle which contains sufficient volume for 30 doses. Active ingredient is salmon calcitonin in a concentration of 2,200 IU per mL corresponding to 200 IU per 0.09 mL. Usual dose for postmenopausal osteoporosis is 200 IU/day. Inactive ingredients in the formulation include sodium chloride, benzalkonium chloride, hydrochloric acid and purified water. The bioavailability of Miacalcin nasal spray relative to intramuscular administration is between 3 and 5% [32]. With a small relative bioavailability, the success of the product could be attributed to its ease of administration and patient compliance. This being the case, if a liposomal formulation has to hit the market, it needs to resolve the issues of low bioavailability, lesser dosing frequency in comparison to the already available nasal spray and ease of use/patient compliance at an affordable cost.

4.6 Liposomes containing protein/peptide cargos for ocular delivery

Expert opinion: From the above discussed research findings (**Table 6**), it is clear that payload following encapsulation in liposomes performs significantly better than its administration in free form. With this being the scenario, there is only one research group that has been working on the ocular delivery and only one payload – VIP has been studied for administration in the eye. Some of the following could be the reasons behind this: only small volume of formulation can be administered into the eye, eye is used as a target organ generally for local delivery of payloads rather than systemic delivery although the latter has been proved in Camelo *et al*. (presence of VIP in lymphoid organs following ocular administration), larger sized peptides cannot cross the ocular barrier to enter into systemic circulation if that is of concern and smaller sized peptides perform better and preparation of a formulation that is patient compliant (eye drop that does not cause stinging in the eye and that is not very viscous in order to cause blurring).

The references sited above use either an injectable or a gel form of the liposomal formulation for ocular administration and these might not be the best case scenarios for marketability as both forms might lack patient compliance. Liquid formulation of liposome that can be instilled into the eye as an eye drop would be most patient complaint but would lack stability and the formulation has to be lyophilised and reconstituted prior to use. However, following reconstitution the product might not be stable for prolonged storage and to preserve stability 'single use' packages need to be manufactured. This will increase the overall cost making the product expensive. Moreover, the necessity of a carrier needs to be established and if a solution of the peptide can achieve similar results, then the essentialness of a carrier is defeated as seen with the example of Miacalcin solution for intranasal delivery.

Another important parameter if a carrier proves beneficial is the safety profile of lipids in the ocular tissue. This needs to be established as all of the eye drops available in the market are simple solutions of drugs with preservatives, buffers and tonicity modifiers. Sterility is of paramount concern for instillation into the eye and ophthalmic products like parenterals

Table 5. Research studies on liposomes containing protein/peptide cargos for intranasal delivery.

Bibliography	Important research finding(s)	Ref.
Rigidity of liposomal membrane	• Muramatsu *et al.* reported that insulin liposomes with more fluid membrane (made from dipalmitoylphosphatidylcholine) showed improved absorption and increased hypoglycaemia in rabbits • Chen *et al.* demonstrated that ultraflexible liposomes performed significantly better (irrespective of surface charge associated) than sCT solution following intranasal administration in rats. However, results of toxicity study revealed ultraflexible liposomes to be slightly toxic to nasal mucosa	[96,97]
Surface charge	• Law *et al.* published an increase in absolute bioavailability of sCT from positively charged liposomes in rabbits due to accumulation of these on the negatively charged nasal mucosal surface compared to negatively charged liposomes or sCT solution	[98]
Mucoadhesive liposomes	• Jain *et al.* formulated mucoadhesive multivesicular liposomes for transmucosal insulin delivery and the effect produced by mucoadhesive and conventional liposomes were statistically insignificant, however, prolonged effect was observed with the former	[99]
Novel liposomes/liposomal vaccines	• Plessis *et al.* formulated a novel lipid-based colloidal delivery system called Pheroid vesicles (liposome-like bilayer vesicles) for improving the absorption of nasally administered sCT that performed significantly better than sCT solution in terms of higher C_{max} • Abraham *et al.* investigated intranasal immunisation in mice with liposomes containing bacterial polysaccharide (BPS), with and without IL-2 and reported a significant increase in polysaccharide-specific pulmonary plasma cells with IL-2-containing liposome in comparison to control	[100,101]

Table 6. Research studies on liposomes containing protein/peptide cargos for ocular delivery.

Scope of research	Important research finding(s)	Ref.
Intravitreal injection (IVT)	• Lajavardi *et al.* reported a more effective down-regulation of endotoxin-induced uveitis (EIU) following IVT of VIP liposomes compared to free VIP in rats. Moreover, EIU clinical severity, ocular infiltration of leukocytes as well as inflammatory cytokines was significantly reduced following administration of VIP liposomes compared to free VIP • Camelo *et al.* suggested excellent ocular (posterior segment of the eye, episclera, conjunctiva) and systemic bio-distribution (cervical lymph nodes) of rhodamine-conjugated VIP liposomes (VIP-Rh-lip) following IVT injection in Lewis rats • Camelo *et al.* investigated the effect of a single intravitreal injection of VIP-Rh-lip on experimental autoimmune uveoretinitis (EAU) and reported the presence of VIP in intraocular macrophages and lymphoid organs. Intraocular levels of interleukin-2 (IL-2), IL-4, IL-7 and interferon-γ were reduced while IL-13 was increased	[102-104]
Hyaluronic acid gel	• Lajavardi *et al.* evaluated the activity of rhodamine-conjugated VIP liposomes within hyaluronic acid (HA) gel (Gel-VIP-Rh-lip) and VIP-Rh-lip in the treatment of EAU and published that Gel-VIP-Rh-lip significantly reduced the number of polymorphonuclear cells infiltrating the eye and the clinical score. This suggested that formulation if VIP liposome into gel produced significantly improved results	[105]

need to be sterile. Sterilisation can be an issue with peptide formulations as the bioactivity would be lost following steam heat sterilisation (autoclaving), peptide conformation could be affected following UV/ozone sterilisation and the peptide could be lost during filtration (using membrane filters). Thus, it would be effective to continue research on liposomal formulation containing peptide payloads (small sized) for local rather than systemic targeting.

4.7 Liposomes containing protein/peptide cargos for transdermal delivery

Expert opinion: Transdermal delivery research on laboratory scale has proved that liposomes are efficient cargo carriers, however, the feasibility issues need to be considered when one has the idea of large-scale manufacture and marketability in mind. Payloads that have been investigated for transdermal delivery include insulin and interleukins as discussed in **Table 7**.

Table 7. Research studies on liposomes containing protein/peptide cargos for transdermal delivery.

Scope of research	Important research finding	Ref.
Transdermal application of liposomes	• Guo *et al.* compared the carrier potential of conventional and flexible liposomal vesicles for transdermal delivery of insulin when applied to abdominal skin of mice and reported flexible vesicles showed a significant drop in percentage of blood glucose • Li *et al.* investigated the anti-psoriatic efficacy following the transdermal delivery of ultradeformable cationic liposome containing murine IL-4 in transgenic mouse model. Plasmid DNA expression was detected in ear skin. Twenty-four h after topical application, pDNA was not detected in serum and liver while histological analysis using Baker scoring system revealed an anti-psoriatic effect	[106,107]
Novel liposome delivery methods	• Kajimoto *et al.* studied the iontophoretic delivery of charged liposomes loaded with insulin to skin of diabetic rats and reported significant drop in insulin concentration in plasma 18 h post administration which was maintained for 24 h • Suzuki *et al.* demonstrated more efficacious gene delivery by a combination of ultrasound and novel ultrasound-sensitive liposomes (bubble liposomes) that contained an IL-12-corded plasmid DNA. This system when tested in mice dramatically suppressed tumour growth which was T-cell dependent	[108,109]

Generally larger molecules can also be delivered through the skin if appropriate carriers or permeation enhancers are used. Skin has advantages of enormous surface area, avoidance of hepatic first pass, patient compliance (by the use of topical creams and lotions or using novel techniques such as transdermal patches, needle-less injections, microneedles and so on that have been under extensive research over the last 10 years), diversity of permeation enhancement techniques (like ultrasound aided delivery, iontophoretic delivery in addition to those mentioned above), versatility of payload selection (small to large payloads) and flexibility of local and systemic targeting.

Transdermal patches have evolved as the most patient compliant method for drug delivery and the number of transdermal products in the market proves this. Transdermal patches that contain nitroglycerin, scopolamine, nicotine, clonidine, oestrogen, oestradiol, testosterone and fentanyl are available in the market for angina treatment, motion sickness, smoking cessation, treatment of hypertension, hormone replacement therapy, post menstrual syndrome, hypogonadism in males and pain cessation [33,34]. In addition there are also patches in the market for weight reduction (Slim weight patch), appetite suppression (Hoodia patch) and antioxidant patch (Acai patch) [35]. With success of transdermal patches that contain solution form of the drug (reservoir patch) or with the drug embedded in matrix (matrix patches), it would be pointless to use liposomes. The controlled release offered by the carrier (liposome) is achieved by using polymers in these transdermal patches.

To use liposomes in skin delivery, novel techniques like iontophoresis (where a mild electric current of the order of 0.5 mA is used to enhance permeability into stratum corneum) and ultrasound (where ultrasonic waves are used) can be investigated. The downside would be patient compliance and cost because healthcare provider needs to administer the same as of today. However, iontophoretic patches that can be self administered are under investigation and this can gain good market if successful.

Microneedle patches are also under extensive research and these are self administration patches containing micron-sized needles that are coated with the formulation (solid needles) or filled with the formulation (hollow needles) for administration. Use of liposome as a carrier for the payload that needs to be administered using microneedles makes the system highly complicated and expensive. This might not be the best option.

If liposomes need to be delivered through the skin, the best ways would be: using a permeation enhancer, iontophoresis or sonophoresis to aid in better permeation through stratum corneum. If a stable formulation that can be scaled up and made available at affordable cost for self administration can be formulated, transdermal route can offer the best route for administration of any type of payload.

5. Conclusions

Liposomes have been used as carriers in protein and peptide delivery over the last 30 years, particularly, in cases where the payload needed to be protected from deleterious effects of surroundings or where a sustained release was essential. Although lot of investigation has been performed on using liposomes as carriers for proteins and peptides, liposome-containing drugs have made it to the market and clinical trials with Doxil® being probably the best example. Liposomal doxorubicin is also marketed under other trade names – Myocet, Lipodox, Thermodox. Other liposomal formulations available in the market include Daunoxome (Daunorubicin), Ambisome (Amphotericin B), Visudyne (Verteporfin), Depocyt (Cytarabine), Marqibo (Vincristine), LEP-ETU (Paclitaxel) and Epaxal (Hepatitis A vaccine). Arikace (Amikacin) is undergoing Phase III clinical trials. As far as biopharmaceutical (protein and peptide) payloads are

concerned, liposomes have shown promise regardless of the route of administration. In almost all studies reviewed in this paper, liposomes have produced a positive impact on delivery of the payload. In general, all payloads discussed with an exception of interleukins have been investigated for oral delivery to a large extent. This could be due to better compliance and use of well-established means of administration. Parenteral delivery has been the most preferred means of interleukin delivery. Transdermal and ocular delivery routes have not been exploited to the fullest despite the available potential in them. Pulmonary delivery has evolved as one of the most investigated administration routes recently and has produced promising results.

It should be noted that the majority of data was generated *in vitro* or in experimental models of rodent origin. Although most of these studies show liposomes to be efficient carriers, extrapolating from these animals to the human situation sometimes is challenging. In addition, the formulation of liposomes has advanced from using simple saturated and unsaturated lipid moieties to PEGylated lipids, mucin/chitosan/carbopol-coated liposomes, lectin/transferrin modified liposomes, pro-liposomes and lyophilised liposomes. Recently, liposomes have been loaded into alginate-chitosan capsules or hyaluronic acid gels to further prolong the release of the payload. Another state of the art development was the invention of fusogenic liposomes for gene delivery and the use of Sendai virus particles for colonic targeting.

In future liposome research, it will be important to focus on studies in higher animals and primates followed by human volunteers. Moreover, development of scale-up procedures will be paramount to bring the technology from the bench to bedside.

6. Expert opinion

Although lot of research has been conducted in the field of protein and peptide delivery using liposomes as carriers, there are no liposomal products (encapsulating protein/peptide) in market to date. This could be due to some associated issues with the protein/peptide payload considered in spite of the advantages liposomes offer. Presence of large number of liposomal drugs in market further confirms the versatility of liposomes as delivery systems and the promise they hold as excellent carriers. However, in some cases the use of a delivery system might not be essential and it is important to consider the advantages a carrier has to offer.

The primary issue with protein and peptide delivery using liposomes as carriers is the stability of the protein/peptide themselves. Proteins/peptides can sometimes be amphiphilic which causes them to associate with both lipid vesicle and aqueous interior thus leading to enhanced leakage. A good example for this is salmon calcitonin [36,37]. In general, proteins/peptides are sensitive cargos leading to denaturation and unfolding causing loss of their bioactivity. Other associated formulation problems include their adsorption and precipitation on use of techniques involving heat or organic solvents. Sometimes use of an appropriate preparation method that is mild and efficient for sensitive payloads like peptides/proteins might lead to success of a liposomal formulation – dehydration rehydration technique is one such technique that has been explored [38-41].

The next major issue is the cost associated with protein/peptide liposomal products. This is due to both the inherent cost of the protein/peptide itself and lesser encapsulation efficiency or retention in liposomes leading to wastage of non-encapsulated protein. This loss leads to increase in overall manufacturing cost. Recovery of non-encapsulated protein/peptide in most cases is equally expensive or is not feasible.

In the initial stages of formulation, one needs to decide if a solid powder or a liquid would be the formulation of choice. One of the other associated issues is the stability of the liquid formulation post preparation. Although lyophilisation is an option, formula should be optimised to avoid aggregation or coagulation of proteins post reconstitution. In general, lyophilisation increases the overall cost in addition to the already expensive payload.

Equally important would be to choose the perfect characterisation techniques. Although physicochemical characterisation will be important in the initial stages, *in vitro* and *in vivo* analysis would give a better idea of the formulation performance, bioactivity and pharmacokinetics. Choosing the right medium to conduct *in vitro* drug release is of paramount importance and the medium chosen has to be a stimulant of the fluid present in the body compartment where the formulation needs to be delivered (e.g., release studies need to be conducted in artificial plasma for parenteral liposomes and in artificial lung surfactant for pulmonary liposomes). For studying the bioactivity, apt cell models that contain specific receptors for the protein/peptide studied should be used. It is best to check the pharmacokinetics of the formulation in animal models as early as possible. The animal models can also reveal the necessity of a carrier. Only if the *in vitro* and *in vivo* studies show a significant advantage of using a carrier for a protein/peptide over the administration of the stand alone payload, it will be essential to use a delivery system.

If the above mentioned issues are addressed, many liposomal protein/peptide products can be expected in the market. The future still holds promise for liposomes as drug delivery systems.

Declaration of interest

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