Molecular design of layer-by-layer functionalized liposomes for oral drug delivery

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Abstract

Liposomes are small spherical vesicles composed mainly of phospholipids and cholesterol. Over the years, a number of liposomal formulations have shown clinical promise, but the use of liposomes in oral drug delivery is still limited. This is partly due to the vulnerability of conventional liposomes to the detrimental effect of gastrointestinal destabilizing factors and also to the poor efficiency in intestinal absorption of liposomes. Some of these issues can be ameliorated using the layer-by-layer (LbL) assembly technology, which has been widely applied in surface modification of various nanoparticulate systems. Discussions on LbL functionalization of liposomes as oral drug carriers, however, are scant in the literature. To fill this gap, this article presents an overview of the roles of LbL functionalization in the development of liposomes, followed by a discussion of major principles of molecular design and engineering of LbL functionalized liposomes for oral drug delivery. Regarding the versatility offered by LbL assembly, it is anticipated that LbL functionalized liposomes may emerge as one of the important carriers for oral drug administration in the future.

Keywords

Liposome; drug delivery; oral administration; layer-by-layer assembly; intestinal absorption; surface modification

Running Title

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1. Introduction

Liposomes are small spherical vesicles composed mainly of phospholipids and cholesterol. They can be classified into multiple types [including multivesicular vesicles (MVV), multilamellar vesicles (MLV), and unilamellar vesicles (ULV)] based on the lipid bilayer structure (**Fig. 1**).[1] Various liposomal formulations have entered clinical trials and have shown good therapeutic performance.[2] For instance, compared to free doxorubicin (DOX), liposomal DOX has been shown to be more well-tolerated at myelosuppressive doses and to produce less venous sclerosis in cancer treatment.[3] Its promising therapeutic effect has been tested in patients with metastases from primary gastric or colonic tumors[4] and in patients with hepatoma.[4] In addition to DOX, other liposomal formulations have been tested clinically. Examples include liposomal paclitaxel,[5-6] and

liposome-encapsulated all-trans retinoic acid.[7] This exciting potential for clinical use has rendered liposomes attractive in the drug delivery research.

Despite the general success in drug delivery, the use of liposomes in oral drug delivery is limited. In fact, delivery via the oral route has advantages over other conventional routes (such as intravenous and intramuscular injection) because oral administration is less invasive and simple, thereby being able to avoid the risks led by parenteral delivery and resulting in higher patient compliance. Compared other routes, oral delivery, however, will subject the carriers to more extreme variations in pH (from the acidic environment in the stomach to the neutral or alkaline environment in the intestine) and to highly concentrated enzymatic activity in the gastrointestinal tract. Taking the case of liposomes as an example, upon administration via the oral route, they are subjected to the action of gastric acid, bile salts and pancreatic lipases in the gastrointestinal tract,[8] resulting in not only a drastic reduction in the number of intact liposomes to be transported to intestinal epithelia for absorption,[9] but also undesired payload leakage during the delivery process.[8] Due to the comparatively large size of liposomes and the presence of gastrointestinal mucus that trap liposomes via hydrophobic interactions,[10] oral absorption of liposomes is further reduced. Diverse strategies have been reported to enhance the physical stability of liposomes in the gastrointestinal tract so as to facilitate trans-epithelial absorption (**Table 1**).[11-22] Among them, applying a suitable surface coating on liposomes is one of the promising strategies, partly due to ease of operation as well as the high tunability of the coating properties. This article will focus on the application of the layer-by-layer (LbL) assembly technology, which is a well-established surface coating technique that involves a sequential assembly of polymers onto a core,[23-24] in the functionalization of liposomes as oral drug carriers. It is hoped that by revisiting related advances in the field, not only will the opportunities and challenges for the design and use of LbL functionalized liposomes in oral drug delivery be illuminated, but insights into the optimization and engineering of liposomal formulations for other routes of administration can also be attained for future research.

2. Historical development of liposomes as oral drug carriers

Development of liposomes was initiated by the discovery made in the late 1960s when phospholipid molecules were found to generate closed bilayer vesicles spontaneously in water.[25] After that, liposomes have been extensively studied as drug carriers. Research on the use of liposomes in oral drug delivery was reported as early as the late 1970s when liposomes were adopted to deliver insulin via the oral route.[26-27] The delivery efficiency, however, was far from satisfactory at that time, with only 54% of the normal rats and 67% of the diabetic rabbits responding to the treatment.[28]. Later, liposomal entrapment was found not to be able to enhance the uptake of polyethylene glycol (PEG).[29] The excretion patterns of hydrocortisone and salicylic acid, when delivered orally by using egg lecithin/cholesterol or L-α-phosphatidylcholine distearoyl/cholesterol liposomes as carriers, were also shown to be the same as those of free drugs.[29] Such disappointment of expectations on the use of liposomes as oral drug carriers has led to a short period of quiescence in research interest at that time.

Because the poor efficiency of liposomes in oral drug delivery stems partly from the physical instability of liposomes in the gastrointestinal environment,[30] some efforts have addressed this problem by directly manipulating the chemical composition of the liposome membrane. The workability of this approach has been partly demonstrated by the fact that, upon incorporation of

sodium deoxycholate (SDC) into the liposome membrane, the efficiency of liposomes in oral delivery of cyclosporine A (CyA) has been enhanced.[31] Compared with conventional soybean phosphatidylcholine (SPC)/cholesterol liposomes, SPC/SDC liposomes have been shown to be more effective in enhancing the absorption of CyA upon oral administration.[31] The use of SDC in manipulating the composition of the liposome membrane for enhanced oral drug delivery has also been reported by another study, which has found that liposomes containing SDC are more effective than conventional liposomes in enhancing the oral bioavailability of fenofibrate.[32] All these studies confirm that the chemical composition of the liposome membrane is one of the factors that can be manipulated for enhanced performance in oral drug delivery. Apart from manipulating the membrane composition, other strategies (e.g., incorporation of an outer lipid bilayer,[14] interior thickening,[15-17] and entrapment in other systems[18]) have been exploited to enhance the stability of liposomes as oral drug carriers (**Table 1**). Most of these methods, however, either involve a tedious and time-consuming developmental process, or provide poor flexibility for surface functionalization. Their wide applications in liposome research have been impeded.

Compared to the methods mentioned above, surface coating provides a rapid track for the development of oral liposomal formulations. Not only does it allow existing liposomes to be used so that the length of the developmental process can be shortened, but diverse functionalities [such as targeting ligands (which enable more effective internalization of liposomes into specific cells), protective elements (which can enhance the biocompatibility and stability of liposomes in the gastrointestinal tract), and imaging tags (which enable real-time monitoring of the location and fate of the administered liposomes)] can also be incorporated during the coating process. Till now, a large variety of polymers (including carbopol,[33] chitosan,[34-35] pectins,[36-37] hydroxyethyl cellulose,[38] and hydroxypropylmethyl cellulose[39]) have already been exploited to coat liposomes. Recently, the use of Eudragit as a coating material has been reported.[40] Eudragit is a polyanionic copolymer of methacrylic acid and methyl methacrylate. It can be dissolved above pH 7.0 but is insoluble at gastric pH. After being coated with this copolymer, liposomes can be protected from the gastric environment by the Eudragit coating. Not only can this reduce the impact of the gastric environment on the stability of liposomes,[40] but this also enables controlled release of the payload in the gastrointestinal tract.[40] The latter has been demonstrated by Barea and coworkers,[41] who have observed that, upon coating liposomes with Eudragit, drug release from the liposomes is remarkably reduced at pH 1.4 (stomach) and pH 6.3 (small intestine), with release at pH 7.8 (ileocecal region) being significantly enhanced. This, along with the success of surface coating in enhancing the oral bioavailability of drugs delivered by liposomes as reported by other studies,[42-47] has made surface coating one of the important techniques in research and development of oral liposomal formulations.

3. Roles of LbL functionalization in liposome-mediated oral drug delivery

The idea of LbL assembly was first proposed in 1966 by Iler,[48] who reported the generation of multilayered films by alternating deposition of oppositely charged colloidal particles. Soon after that, the LbL technology emerged as a widely used method of fabricating multilayered polymer films. Later in the 1990's, LbL deposition of coatings on a colloidal template, followed by the removal of the colloidal template, was adopted to generate capsules with a follow structure.[49-50] This has extended the use of LbL technology from two-dimensional, film-based systems to colloidal, three-dimensional ones, and has greatly expanded research on the LbL techniques since then.[51] Compared to single-layer coating, the multilayer one enables the surface, chemical and

mechanical properties of liposomes to be more precisely and flexibly tuned by changing the deposition conditions and coating materials.[52] As an important technique for multilayer coating, the LbL technology has already been applied to manipulate different nanoparticulate systems as carriers for a wide range of chemical entities (**Table 2**).[53-62] In the case of liposomes, the space formed between coating layers can provide extra room, on top of the aqueous core and the lipid bilayer, for drug molecules to be loaded.[60] This facilitate co-delivery of both hydrophilic and lipophilic drugs.

LbL functionalization can enhance the efficiency in oral drug delivery by increasing the physical stability and the efficiency in intestinal absorption, too. This has been demonstrated by an earlier study, which applied LbL deposition to liposomes for oral delivery of sorafenib.[19] Upon LbL coating, electrostatic repulsion caused by the surface charge helps minimize aggregation of liposomes. Changes in the particle size of the liposomal suspension have, therefore, been found to be negligible during storage at 4 °C and 25 °C.[19] Drug retention provided by the LbL functionalized liposomes has also been shown to be significantly higher than the uncoated ones.[19] In rats, compared to the uncoated liposomes, the area under the plasma concentration-time curve of the LbL functionalized ones is around one fold higher.[19] This demonstrates the efficiency of LbL functionalization in enhancing liposomal absorption and transportation in the gastrointestinal tract.

LbL functionalization can also be applied to achieve controlled release of the payload from liposomes.[63-65] The technical possibility of this has been shown by Hashemi and coworkers, who coated DOX-loaded cationic liposomes with graphene oxide (GO) and poly(L-lysine) (PLL)-functionalized GO (GO-PLL) using the LbL technique.[54] GO can adsorb on the surface of zwitterionic liposomes.[66] Such interactions have been found to show negligible influence on drug leakage and the phase transition temperature of lipids in the liposome membrane.[67] Owing to its effective NIR absorption via the delocalization of its electron states,[68] along with the rapid transformation of absorbed light into thermal energy, GO has already been widely studied for photothermal therapy.[69-73] In LbL functionalized liposomes, both GO and GO-PLL can absorb NIR light.[54] Conversion of the light to heat by these components enables the activation of the liquid phase transition of the phospholipid membrane,[54] and has successfully been adopted to achieve controlled release of DOX.[54] Although this method of achieving controlled release may not be suitable for applications in order administration, it sheds light on the technical feasibility of exploiting LbL deposition to attain stimuli-responsive oral drug delivery. Such feasibility has been supported by recent studies which incorporate pH responsiveness into the LbL coating to control the site of drug release from liposomes in the intestinal tract.[40, 74] All these point to the effectiveness of the LbL technology in enhancing the versatility and functionality of liposomes in oral drug formulation.

4. Fabrication of LbL functionalized liposomes

To generate LbL functionalized liposomes, the first step is to fabricate the liposomal core. The core can be generated using several methods. One method is sonication, which has been adopted to prepare drug-loaded liposomes obtained from a dispersion containing phospholipon 90G, stearylamine and the drug molecules.[40] The generated liposomes had a diameter of around 80 nm, with positive zeta potential owing to the charge carried by stearylamine.[40] Apart from being used alone, sonication can be used along with thin film hydration. This combined use has been reported

to fabricate quercetin-loaded liposomes, which were fabricated by hydrating a lipid thin film with a quercetin-containing PEG solution prior to sonication and extrusion.[75] Apart from the methods mentioned above, other methods (e.g., detergent dialysis,[76] reversed phase evaporation,[77] solvent-injection techniques,[78] high pressure extrusion,[79] microfluidization,[80] supercritical anti-solvent method,[81] dual asymmetric centrifugation,[82] and membrane contactor technology[83]) may be employed for the preparation of liposome cores.

After fabrication of the core, LbL functionalization can be performed.[84] Over the years, different methods have been developed for LbL coating. For instance, by using the heat-up strategy (in which nanoparticles with a multi-shelled structure are generated by either repeating the same synthetic protocol in multiple times or by combining dissimilar synthetic procedures to deposit different shells onto the same core[85]), epitaxial shells have been successfully incorporated into the particle surface. Such strategies, however, are applied mainly to metal cores. Because liposomes are soft matters, they are less stable compared to metal cores. The use of the strategies adopted to coat metal cores in coating soft matters is, therefore, limited. In liposomes, LbL coating is performed mainly via electrostatic interactions between oppositely charged polyelectrolytes (**Fig. 2**). Other interactions (e.g., hydrogen bonds,[86] hydrophobic interactions,[87] and van der Waals forces[88]) may also be adopted; however, compared to the electrostatic forces, they are less commonly mentioned in the literature on LbL functionalized liposomes. This is attributed partly to the wide availability of polyelectrolytes (e.g., chitosan, xanthan, alginate, dextran sulfate, and hyaluronic acid) for biomedical use. Use of these polyelectrolytes in LbL coating may enable better control of the biocompatibility of the functionalized liposomes generated. Moreover, the ease of operation and the straightforward design of the electrostatic interaction-based LbL deposition protocol plays a role.

Upon LbL coating, the surface phospholipids in the liposome membrane can be protected from the outer gastrointestinal environment. Owing to the electrostatic nature, the stability of the LbL layer would, however, be hampered in media with high polarity or high salt concentration. To enhance the stability of the coating, incorporation of additional covalent crosslinking has been adopted in a recent study,[89] which first performed LbL deposition to generate a multilayer film via non-covalent interactions. After that, infiltration of 4, 4'-diazostilbene-2, 2'-disulfonic acid disodium salt (DAS) into the film was carried out, followed by crosslinking of the film under UV irradiation. The film has been reported to be more stable upon covalent crosslinking, and to increase the release sustainability of loaded molecules.[89] This evidences the possible combination of multiple types of interactions for enhancing the stability and performance of the LbL layer.

5. Principles of molecular design of LbL-functionalized liposomes

LbL functionalization is technically easy to be performed, particularly when physical interactions are employed for LbL assembly; however, it does not necessary imply that the oral delivery performance of the generated functionalized liposomes will be achieved even without proper optimization of the functionalization process. In the following part of this section, important parameters to be considered during molecular design of LbL functionalized liposomes will be discussed (**Fig. 3**).

5.2 Size and size distribution

When LbL functionalized liposomes are designed, one of the important parameters to be considered is the size of the core, which should have a diameter less than the expected diameter of the final product. This is because, upon LbL functionalization, some increase in the size of the liposomes takes place, as was shown for the case of dioctadecyldimethyl ammonium bromide (DODAB) liposomes.[59] The size of those liposomes increased from around 60 nm to 130 nm after coated with xanthan.[59] Upon further incorporation of a galactomannan layer, the size of the liposomes increased further to around 165 nm.[59] Because particles with the size below 1 μm showing mucoadhesive properties can be efficiently taken up by M cells,[10] controlling the ultimate diameter below this range may also be desired for LbL functionalized liposomes. To counteract the increase in size, it is possible to modify the procedure for liposome preparation. One strategy is to control the length of sonication time and hence the energy input during liposome fabrication.[90] An increase in the energy input can lead to a decrease in the size of the generated liposomes.[90] This method, however, can only reduce the diameter of the liposomes to a limited extent because the increase in the surface tension of liposomes may impose a limit in the size of liposomes attained.[31] An alternative method to control the size of the liposomes produced is to use the microfluidic techniques.[91] The feasibility of this has been demonstrated by an earlier study,[92] in which a microfluidic chip is designed to hydrodynamically focus a stream of lipids (which have been pre-dissolved in alcohol) between two sheathed aqueous streams for formation of liposomes via self-assembly. By changing the ratio of the alcohol-to-aqueous volumetric flow rate, liposomes of a tunable mean diameter (from 50 to 150 nm) have been successfully fabricated.[92] Similar success in controlling the size of liposomes has also been reported recently by Lou and coworkers,[93] who have injected a tris(hydroxymethyl)aminomethane buffer simultaneously with a lipid solution [prepared by dissolving cationic lipids with 1,2-dioleoyl-*sn*-3-phosphoethanolamine (DOPE) in methanol] into a Y-shaped staggered herringbone micromixer. Liposomes with a wide size range (40-750 nm) have been generated by simply manipulating the concentration of the aqueous buffer adopted.[93] The use of microfluidic technologies have enabled the production of monodisperse liposomes with controlled sizes for subsequent LbL functionalization.

Besides the size *per se*, size distribution is another parameter to be noted. Although various post-processing procedures (e.g., membrane extrusion, and sonication) are available for size control during liposome preparation, solely relying on these procedures can hardly generate liposomes with a narrow size distribution. With recent advances in microfluidics, production of highly monodisperse liposomes has been facilitated. For instance, an earlier study has adopted a microfluidic device, in which an ethanolic solution of lipids was hydrodynamically focused between two sheathed streams of deionized water, to control the size and size distribution of the liposomes generated.[92] The laminar flow in microchannels enabled diffusive mixing at the liquid interface, allowing the lipids to undergo self-assembly to form vesicles.[92] By changing the ratio of the alcohol-to-aqueous volumetric flow rate, the size of the liposomes can be tuned.[92] The size of the liposomes formed in a microfluidic device can be further reduced upon the incorporation of sonication into microfluidic production of liposomes,[94] with the smallest mean size reported being less than 70 nm. Although the use of microfluidics in the fabrication of LbL functionalization liposomes is still limited in the literature till now, taking the versatility and promising capacity of manipulating droplet properties into account, it is expected that the role played by microfluidic technologies in LbL functionalization of liposomes will become increasingly significant.

5.2 Surface charge and surface geometry

Proper control of the surface charge is vital to the design of LbL functionalized liposomes because it is a major factor that potentiates LbL deposition. To form liposomes with positive surface charge, the simplest method is to incorporate cationic lipids [e.g., 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), didodecyldimethylammonium bromide (DDAB), DODAB, and trimethylhexadecyl ammonium bromide (CTAB)] into the liposome membrane.[59] On the other hand, anionic lipids [e.g., 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), dihexadecyl phosphate, and 1,2-dipalmitoyl-sn-glycero-3-phospho-l-serine (DPPS)] may be used for the generation of liposome cores with negative charge.[95] Selection of charged lipids to be incorporated into the liposome membrane is largely based on the procedural design of LbL functionalization. Apart from potentiating LbL deposition, the ultimate surface charge of the liposomes upon functionalization should be properly optimized because it determines the efficiency in the uptake by Peyer′s patches.[96] As the M cell pathway contributes largely to the absorption of orally administered liposomes,[97-98] adjusting the overall surface charge to negative may be helpful in facilitating the intestinal absorption of the LbL functionalized liposomes upon oral administration.[96]

When the performance of LbL functionalized liposomes in oral drug delivery is to be optimized, the effect of surface geometry should not be overlooked. The importance of this has been highlighted by Verma and coworkers,[74] who applied the LbL technique to coat liposomes by using sodium alginate and vitamin B_{12}-conjugated chitosan as a polyanion and a polycation, respectively.[74] The 4-layered coated liposomes were found to be spherical in shape when they were put at pH 1.2 and pH 6.8, but showed an increase in surface roughness when they underwent acidification followed by neutralization.[74] Such an increase in surface roughness significantly enhances the surface contact area between liposomes and the plasma membrane of target cells.[74] In intestinal epithelial HT-29 cells, LbL coated liposomes have been shown to undergo cell internalization more effectively than the uncoated ones.[74] Owing to the higher effectiveness of coming in contact with the lipid bilayer membrane of M cells, the rough surface on the LbL coated liposomes has also enhanced the efficiency in mucus penetration, and has improved the oral bioavailability of the payload in Peyer's patches.[74] In fact, it is common for a rough surface to be attained upon LbL deposition. This is because owing to the heterogeneous distribution of charges, full saturation of the charged surface by polymer coating can hardly be achieved.[99] This leads to the formation of a heterogeneously rough surface upon LbL deposition of multiple polyelectrolyte layers. The impact of changes in the roughness of the liposome surface before and after LbL coating to the ultimate performance of LbL functionalized liposomes in oral drug delivery, however, may vary from case to case.

5.4 Surface properties

Because the surface the liposomes is the interface where the liposomes meet and interact with the external biological environment, its properties, along with other factors (**Fig. 4**), can largely influence the ultimate fate of the liposomes in the body upon oral administration. One strategy is to enhance intestinal absorption of LbL-coated liposomes by incorporating specific ligands (e.g., mannose,[100] folic acid,[101-102] and biotin[103-104]) into the surface coating. The viability of this concept has been evidenced by an earlier study,[74] in which vitamin B_{12} was conjugated to chitosan via carbodiimide chemistry and was used, along with sodium alginate, for LbL functionalization of liposomes. Although the ligand adopted was used to render the coating pH-sensitive, by replacing the ligand with targeting moieties for receptor-mediated endocytosis, the functionalized liposomes

generated can on one hand be able to adhere and accumulate at the site of absorption via ligand-receptor interaction and on the other hand can undergo pinocytosis/phagocytosis by both the antigen-presenting cells in the gastrointestinal tract and the M cells in the follicle-associate epithelia of Peyer′s patches. In fact, although M cells only represent approximately 1% of the total intestinal epithelial cell population,[105-106] the M cell pathway is one of the preferable absorption mechanisms for liposomes because absorption through this pathway involves less membrane hydrolases, fewer lysosomes, and less glycocalyx.[107] In addition, M cells do not secrete mucus. They are exposed to chime, and are highly accessible for liposomal absorption via endocytosis and phagocytosis.[108] The M cell pathway is, therefore, one of the most important routes of liposomal absorption upon oral administration, and should always be taken into consideration when the LbL coating is engineered for enhanced delivery performance.

The efficiency in oral delivery can be enhanced by incorporating polymers with mucoadhesive and mucus penetrating properties into the LbL coating so that mucoadhesion of liposomes to intestinal epithelia can be enhanced, leading to an extension of the elimination half-life of the liposomes in small intestines. Mucoadhesion can be achieved by ionic interactions of positively charged polymers with the negatively charged constituents (including the sialic and sulfonic acid residues) of mucus,[109-110] some polycations (e.g., chitosan, and PLL) can, therefore, be adopted as the outer layer of the LbL functionalized liposomes, although overall negative surface charge has been reported to facilitate subsequent intestinal absorption.[96] Here it is worth noting that, simply showing mucoadhesive properties is not sufficient for effective oral drug delivery because the intestinal permeability of the LbL functionalized liposomes is still restricted by the turnover time of the mucus layers at the end.[111] It is, therefore, desired for the liposomes to be able to undergo mucus penetration rather than mucus entrapment so that direct contact of liposomes with epithelia can be facilitated, thereby increasing the chance of uptake of the liposomes by clathrin- or caveolae-mediated endocytosis. Some commonly used polymers that show mucus penetrating ability include Pluronic F127,[112-113] and PEG.[114] These polymers, however, are largely neutral, and can hardly be used directly for LbL deposition where the assembly process is mediated by ionic interactions. Despite this, modification of one of the polycations or polyanions involved in LbL functionalization may be a feasible way to incorporate these mucus penetrating moieties into the LbL coating.

5.5 Other factors

Apart from the aforementioned parameters, few other factors should be determined during the molecular design process. One of these is the composition of the liposome membrane. Although the liposome membrane in LbL coated liposomes is protected by an LbL coating and hence may not interacts directly with the plasma membrane of target cells as conventional liposomes do, changes in its composition may ultimately lead to alternations in the particle size, polydispersity index, and drug encapsulation efficiency of the liposome core fabricated.[90] This has been reported by an earlier study,[75] in which changing the molar ratio of egg phosphatidylcholine (EPC)/cholesterol in liposomes from 1:1 to 9:1 has led to an increase in the mean size of the liposomes from around 210 nm to over 600 nm, with the surface charge changing from -32 mV to -13 mV.[75] The influence of lipid composition on the properties of the liposome core, however, is complex and can hardly be predicted. At the moment, optimization of the composition has to be done largely on a trial-and-error basis.

In addition to the composition of the liposome membrane, the procedure of the liposome preparation affects the properties and drug encapsulation efficiency of the liposome core. For instance, by changing the order of incorporating quercetin into liposomes (i.e., either incorporating quercetin into the lipid phase before the lipid thin film formation, or incorporating quercetin into the film during liposome preparation), the encapsulation efficiency attained has been reported to vary in a large range, from 60% to 80%.[75] In addition, in liposomes treated with LbL deposition of polyacrylic acid (PAA) and polyallylamine hydrochloride (PAH), the size, zeta potential and polydispersity index (PDI) have been shown to be changed continuously as the number of deposited layers has increased, with those changes further affected by the concentrations of the polymer solutions used.[90] Proper determination of the number of deposition cycles is vital because it can directly affect the physical stability of the final product, too. This has been suggested by Jeon *et al.*,[115] who coated anionic liposomes first with chitosan followed by sodium hyaluronate. Liposomes with an odd number of layers have shown high polydispersity and high susceptibility to flocculation;[115] whereas those having an even number of layers have been found to be stable and comparatively monodisperse.[115] This phenomenon is partly caused by the variation in the strength of electrostatic interactions inside the coating when the number of polymer layers changes, thereby affecting the ultimate stability of the functionalized liposomes generated.[115] All these corroborate that the properties of the generated liposomes are subjected to the procedural design and experimental conditions adopted during the preparation and LbL functionalization of the liposome core.[75]

Last but not least, the properties of drugs to be delivered may have to be taken into consideration during the design process as the encapsulation efficiency of liposomes may vary from drug to drug. This can be exemplified by the case of flavonoids, whose encapsulation in EPC liposomes can be affected by the position of hydroxyl groups, as well as the presence and absence of a sugar moiety, on the flavonoid structure.[116] In general, owing to the strong interactions between aglycones and EPC acyl chains, aglycones can interact more strongly with the lipophilic region of liposomes, leading to greater incorporation efficiency.[116] On the other hand, due to the comparatively weak interactions of flavonoid glycosides with the liposome membrane, the incorporation efficiency is low in general.[116] While the structure of the payload can affect the encapsulation efficiency of liposomes, liposomal formulation *per se* may affect the biological activity of the payload in return. The effect of the latter, however, vary from drug to drug as well. While the cytotoxicity of liposomal quercetin has been found to be lower than that of free quercetin in SF268, MCF7 and H460 cells,[116] liposomal isoscutellarein has shown higher cytotoxic effects to those cancer cell lines than the free form.[116] Because of this, the process of designing LbL functionalized liposomes for oral drug delivery has to be tailored, when necessary, to the specific needs and properties of the payload to be carried. Reported cycles of optimization for different parameters may be needed before an ideal liposome prototype is attained for oral drug delivery.

6. Conclusion and outlook

Over the years, LbL technology have already shown its application potential in diverse areas, ranging from the fabrication of multilayered reactors[117] and conducting electrodes[118] to the development of stimuli-responsive drug release systems.[119] LbL technology has a great promise in enhancing both the versatility and functionalities in the development of drug delivery systems, including liposomes. Despite the promising potential brought about by the LbL technology in research and development of oral liposomal formulations, before related scientific studies can be

translated into routine clinical practice, the safety profile of the LbL functionalized liposomes has to be fully characterized. At the moment, electrostatic interactions constitute the most predominately adopted mechanisms for LbL deposition. Production of charged liposomes, therefore, becomes a precondition. Safety concerns, however, have been raised on the use of some of the charged agents in the drug delivery. For instance, some studies have reported that stearylamine, which is a commonly used positive charge-inducing agent for liposome preparation is stearylamine,[120-122] may induce apoptosis by generating reactive oxygen species, activating protein kinase C, and stimulating the release of apoptosis-dependent proteins (e.g., cytochrome c, caspase 3, and caspase 8).[123-124] This concern has been further supported by the observation that, upon a 24-h exposure of HT-29 cells to stearylamine-incorporated liposomes, the viability of the cells may drop to 30-40%.[40] Despite the importance of toxicological evaluation for clinical translation, right now studies examining the toxicity of LbL functionalized liposomes have been performed predominately in the *in vitro* context. Detailed evaluation of the short-term and long-term toxicity of the liposomes before and after LbL coating is lacking. To streamline clinical translation of LbL functionalized liposomes for oral drug delivery, clarification of the toxic effects both *in vitro* and *in vivo* should be taken as a prioritized direction for future research.

Apart from toxicity, the M cell pathway is the major pathway for intestinal absorption of LbL functionalized liposomes at the moment. Regarding the upper limit on oral absorption of liposomes imposed by the M cells residing in the follicle-associate epithelia of Peyer's patches,[105, 125] development of LbL functionalized liposomes that can get absorbed via multiple pathways is, therefore, another promising avenue for future research. This goal may partly be achieved by using elastic liposomes, whose development was triggered in the 1990s when liposomes with high deformability were generated by associating phosphatidylcholine with sodium cholate or sodium deoxycholate.[126] Compared to conventional liposomes, elastic liposomes can squeeze between cells despite their large vesicle size, and show high adaptability.[127-128] If such deformability can be provided to LbL functionalized liposomes, it is projected that those liposomes may get through enterocytes and undergo intestinal absorption more easily.

As a summary, conventional liposomes show poor stability, poor intestinal absorption efficiency, and low resistance to the detrimental effect of gastrointestinal destabilizing factors. This has drastically hindered the applications of liposomes in oral drug delivery for many years, but the LbL technology may have to potential to bring the prospect of an end to this predicament. At the moment the development of LbL functionalized liposomes for oral drug administration is still in the beginning, and many technical challenges (e.g., toxicity concerns, high polydispersity of LbL functionalized liposomes, and possible aggregation of liposomes during the coating process) have to be overcome before the wide clinical use of LbL functionalized liposomes in oral drug administration can be attained. Regarding the rapid advances in LbL techniques and liposomal technologies, the design and engineering of LbL functionalized liposomes will continue to be improved in a more sophisticated manner. At that time, not only will the translation of liposomes from research to clinical practice in oral drug delivery be facilitated, but research on the design, optimization, and engineering of liposomal formulations for other routes of administration may also be inspired.

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References

1. Daraee, H.; Etemadi, A.; Kouhi, M.; Alimirzalu, S.; Akbarzadeh, A., Application of liposomes in medicine and drug delivery. *Artificial cells, nanomedicine, and biotechnology* **2016,** *44* (1), 381-91.
2. Lai, W. F.; Tang, R.; Wong, W. T., Ionically Crosslinked Complex Gels Loaded with Oleic Acid-Containing Vesicles for Transdermal Drug Delivery. *Pharmaceutics* **2020,** *12* (8).
3. Rahman, A.; Treat, J.; Roh, J. K.; Potkul, L. A.; Alvord, W. G.; Forst, D.; Woolley, P. V., A phase I clinical trial and pharmacokinetic evaluation of liposome-encapsulated doxorubicin. *J Clin Oncol* **1990,** *8* (6), 1093-100.
4. Owen, R. R.; Sells, R. A.; Gilmore, I. T.; New, R. R.; Stringer, R. E., A phase I clinical evaluation of liposome-entrapped doxorubicin (Lip-Dox) in patients with primary and metastatic hepatic malignancy. *Anticancer Drugs* **1992,** *3* (2), 101-7.
5. Wang, X.; Zhou, J.; Wang, Y.; Zhu, Z.; Lu, Y.; Wei, Y.; Chen, L., A phase I clinical and pharmacokinetic study of paclitaxel liposome infused in non-small cell lung cancer patients with malignant pleural effusions. *Eur J Cancer* **2010,** *46* (8), 1474-80.
6. Zhang, Q.; Huang, X. E.; Gao, L. L., A clinical study on the premedication of paclitaxel liposome in the treatment of solid tumors. *Biomed Pharmacother* **2009,** *63* (8), 603-7.
7. Boorjian, S. A.; Milowsky, M. I.; Kaplan, J.; Albert, M.; Cobham, M. V.; Coll, D. M.; Mongan, N. P.; Shelton, G.; Petrylak, D.; Gudas, L. J.; Nanus, D. M., Phase 1/2 clinical trial of interferon alpha2b and weekly liposome-encapsulated all-trans retinoic acid in patients with advanced renal cell carcinoma. *J Immunother* **2007,** *30* (6), 655-62.
8. Hu, S. W.; Niu, M. M.; Hu, F. Q.; Lu, Y.; Qi, J. P.; Yin, Z. N.; Wu, W., Integrity and stability of oral liposomes containing bile salts studied in simulated and ex vivo gastrointestinal media. *Int J Pharm* **2013,** *441* (1-2), 693-700.
9. Liu, W. L.; Ye, A. G.; Liu, W.; Liu, C. M.; Han, J. Z.; Singh, H., Behaviour of liposomes loaded with bovine serum albumin during in vitro digestion. *Food Chem* **2015,** *175*, 16-24.
10. Ensign, L. M.; Cone, R.; Hanes, J., Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. *Adv Drug Deliver Rev* **2012,** *64* (6), 557-570.
11. Richards, M. H.; Gardner, C. R., Effects of bile salts on the structural integrity of liposomes. *Biochim Biophys Acta* **1978,** *543*, 508-522.
12. Parmentier, J.; Becker, M. M. M.; Heintz, U.; Fricker, G., Stability of liposomes containing bio-enhancers and tetraether lipids in simulated gastro-intestinal fluids. *Int J Pharm* **2011,** *405* (1-2), 210-217.
13. Kato, Y.; Hosokawa, T.; Hayakawa, E.; Ito, K., Influence of Liposomes on Tryptic Digestion of Insulin. *Biol Pharm Bull* **1993,** *16* (5), 457-461.
14. Katayama, K.; Kato, Y.; Onishi, H.; Nagai, T.; Machida, Y., Double liposomes: Hypoglycemic effects of liposomal insulin on normal rats. *Drug development and industrial pharmacy* **2003,** *29* (7), 725-731.

15. Zhang, B.; Lu, Y.; Chen, J. A.; Wu, W., Effects of Interior Gelation on Pharmacokinetics and Biodistribution of Liposomes Encapsulating an Anti-Cancer Drug Cytarabine. *J Biomed Nanotechnol* **2010,** *6* (6), 704-709.
16. Ng, C. C.; Cheng, Y. L.; Pennefather, P. S., One-step synthesis of a fluorescent phospholipid-hydrogel conjugate for driving self-assembly of supported lipid membranes. *Macromolecules* **2001,** *34* (17), 5759-5765.
17. Kazakov, S., Liposome-Nanogel Structures for Future Pharmaceutical Applications: An Updated Review. *Current pharmaceutical design* **2016,** *22* (10), 1391-1413.
18. Pantze, S. F.; Parmentier, J.; Hofhaus, G.; Fricker, G., Matrix liposomes: A solid liposomal formulation for oral administration. *Eur J Lipid Sci Tech* **2014,** *116* (9), 1145-1154.
19. Zhao, M. J.; Lee, S. H.; Song, J. G.; Kim, H. Y.; Han, H. K., Enhanced oral absorption of sorafenib via the layer-by-layer deposition of a pH-sensitive polymer and glycol chitosan on the liposome. *Int J Pharm* **2018,** *544* (1), 14-20.
20. Minato, S.; Iwanaga, K.; Kakemi, M.; Yamashita, S.; Oku, N., Application of polyethyleneglycol (PEG)-modified liposomes for oral vaccine: effect of lipid dose on systemic and mucosal immunity. *J Control Release* **2003,** *89* (2), 189-197.
21. Mohanraj, V. J.; Barnes, T. J.; Prestidge, C. A., Silica nanoparticle coated liposomes: A new type of hybrid nanocapsule for proteins. *Int J Pharm* **2010,** *392* (1-2), 285-293.
22. Dwivedi, N.; Arunagirinathan, M. A.; Sharma, S.; Bellare, J., Silica-Coated Liposomes for Insulin Delivery. *J Nanomater* **2010**.
23. Such, G. K.; Johnston, A. P.; Caruso, F., Engineered hydrogen-bonded polymer multilayers: from assembly to biomedical applications. *Chemical Society reviews* **2011,** *40* (1), 19-29.
24. Ok, S.; Kozlovskaya, V.; Sousa, A.; Libera, M.; Sukhishvili, S. A., Hydrogen-bonded polymer capsules formed by layer-by-layer self-assembly. *Abstr Pap Am Chem S* **2004,** *227*, U531-U531.
25. Bangham, A. D.; Standish, M. M.; Watkins, J. C., Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of molecular biology* **1965,** *13* (1), 238-52.
26. Dapergolas, G.; Gregoriadis, G., Hypoglycaemic effect of liposome-entrapped insulin administered intragastrically into rats. *Lancet* **1976,** *308*, 824-827.
27. Patel, H.; Ryman, B. E., Oral administration of insulin by encapsulation within liposomes. *FEBS Lett* **1976,** *62*, 60-63.
28. Arrieta-Molero, J.; Aleck, K.; Sinha, M.; Brownscheidle, C.; Shapiro, L.; Sperling, M., Orally administered liposome-entrapped insulin in diabetic animals. *Horm Res* **1982,** *16*, 249-256.
29. Chiang, C. M.; Weiner, N., Gastrointestinal Uptake of Liposomes .2. Invivo Studies. *Int J Pharm* **1987,** *40* (1-2), 143-150.
30. Sulkowski, W. W.; Pentak, D.; Nowak, K.; Sulkowska, A., The influence of temperature, cholesterol content and pH on liposome stability. . *J Mol Struct* **2005,** *744* (3), 737-47.
31. Guan, P.; Lu, Y.; Qi, J.; Niu, M.; Lian, R.; Hu, F.; Wu, W., Enhanced oral bioavailability of cyclosporine A by liposomes containing a bile salt. *International journal of nanomedicine* **2011,** *6*, 965-74.
32. Chen, Y.; Lu, Y.; Chen, J.; Lai, J.; Sun, J.; Hu, F.; Wu, W., Enhanced bioavailability of the poorly water-soluble drug fenofibrate by using liposomes containing a bile salt. *International journal of pharmaceutics* **2009,** *376* (1-2), 153-60.
33. Karn, P. R.; Vanic, Z.; Pepic, I.; Skalko-Basnet, N., Mucoadhesive liposomal delivery systems: the choice of coating material. *Drug development and industrial pharmacy* **2011,** *37* (4), 482-8.

34. Quemeneur, F.; Rinaudo, M.; Pepin-Donat, B., Influence of polyelectrolyte chemical structure on their interaction with lipid membrane of zwitterionic liposomes. *Biomacromolecules* **2008,** *9* (8), 2237-43.
35. Mady, M. M.; Darwish, M. M.; Khalil, S.; Khalil, W. M., Biophysical studies on chitosan-coated liposomes. *European biophysics journal : EBJ* **2009,** *38* (8), 1127-33.
36. Klemetsrud, T.; Kjoniksen, A. L.; Hiorth, M.; Jacobsen, J.; Smistad, G., Polymer coated liposomes for use in the oral cavity - a study of the in vitro toxicity, effect on cell permeability and interaction with mucin. *Journal of liposome research* **2018,** *28* (1), 62-73.
37. Nguyen, S.; Alund, S. J.; Hiorth, M.; Kjoniksen, A. L.; Smistad, G., Studies on pectin coating of liposomes for drug delivery. *Colloids and surfaces. B, Biointerfaces* **2011,** *88* (2), 664-73.
38. Meland, H. G.; Rov-Johnsen, A.; Smistad, G.; Hiorth, M., Studies on surface coating of phospholipid vesicles with a non-ionic polymer. *Colloids and surfaces. B, Biointerfaces* **2014,** *114*, 45-52.
39. Gutierrez de Rubalcava, C.; Rodriguez, J. L.; Duro, R.; Alvarez-Lorenzo, C.; Concheiro, A.; Seijo, B., Interactions between liposomes and hydroxypropylmethylcellulose. *International journal of pharmaceutics* **2000,** *203* (1-2), 99-108.
40. Caddeo, C.; Gabriele, M.; Fernandez-Busquets, X.; Valenti, D.; Fadda, A. M.; Pucci, L.; Manconi, M., Antioxidant activity of quercetin in Eudragit-coated liposomes for intestinal delivery. *International journal of pharmaceutics* **2019,** *565*, 64-69.
41. Barea, M. J.; Jenkins, M. J.; Gaber, M. H.; Bridson, R. H., Evaluation of liposomes coated with a pH responsive polymer. *International journal of pharmaceutics* **2010,** *402* (1-2), 89-94.
42. Lee, C. M.; Lee, H. C.; Lee, K. Y., O-palmitoylcurdlan sulfate (OPCurS)-coated liposomes for oral drug delivery. *Journal of bioscience and bioengineering* **2005,** *100* (3), 255-9.
43. Cao, J.; Sun, J.; Wang, X.; Li, X.; Deng, Y., N-trimethyl chitosan-coated multivesicular liposomes for oxymatrine oral delivery. *Drug development and industrial pharmacy* **2009,** *35* (11), 1339-47.
44. Werle, M.; Makhlof, A.; Takeuchi, H., Carbopol-lectin conjugate coated liposomes for oral peptide delivery. *Chemical & pharmaceutical bulletin* **2010,** *58* (3), 432-4.
45. Li, C.; Zhang, Y.; Su, T.; Feng, L.; Long, Y.; Chen, Z., Silica-coated flexible liposomes as a nanohybrid delivery system for enhanced oral bioavailability of curcumin. *Int J Nanomedicine* **2012,** *7*, 5995-6002.
46. Kowapradit, J.; Apirakaramwong, A.; Ngawhirunpat, T.; Rojanarata, T.; Sajomsang, W.; Opanasopit, P., Methylated N-(4-N,N-dimethylaminobenzyl) chitosan coated liposomes for oral protein drug delivery. *Eur J Pharm Sci* **2012,** *47* (2), 359-66.
47. Gradauer, K.; Barthelmes, J.; Vonach, C.; Almer, G.; Mangge, H.; Teubl, B.; Roblegg, E.; Dunnhaupt, S.; Frohlich, E.; Bernkop-Schnurch, A.; Prassl, R., Liposomes coated with thiolated chitosan enhance oral peptide delivery to rats. *J Control Release* **2013,** *172* (3), 872-8.
48. Iler, R. K., Multilayers of colloidal particles. *J Colloid Interface Sci* **1966,** *21*, 569-594.
49. Caruso, F.; Caruso, R. A.; Mohwald, H., Nanoengineering of inorganic and hybrid hollow spheres by colloidal templating. *Science* **1998,** *282* (5391), 1111-1114.
50. Donath, E.; Sukhorukov, G. B.; Caruso, F.; Davis, S. A.; Mohwald, H., Novel hollow polymer shells by colloid-templated assembly of polyelectrolytes. *Angew Chem Int Edit* **1998,** *37* (16), 2202-2205.
51. Zhao, S.; Caruso, F.; Dahne, L.; Decher, G.; De Geest, B. G.; Fan, J.; Feliu, N.; Gogotsi, Y.; Hammond, P. T.; Hersam, M. C.; Khademhosseini, A.; Kotov, N.; Leporatti, S.; Li, Y.; Lisdat, F.; Liz-Marzan, L. M.; Moya, S.; Mulvaney, P.; Rogach, A. L.; Roy, S.; Shchukin, D. G.; Skirtach,

A. G.; Stevens, M. M.; Sukhorukov, G. B.; Weiss, P. S.; Yue, Z.; Zhu, D.; Parak, W. J., The Future of Layer-by-Layer Assembly: A Tribute to ACS Nano Associate Editor Helmuth Mohwald. *ACS Nano 13* (6), 6151-6169.
52. Chen, J.; Kozlovskaya, V.; Goins, A.; Campos-Gomez, J.; Saeed, M.; Kharlampieva, E., Biocompatible shaped particles from dried multilayer polymer capsules. *Biomacromolecules* **2013,** *14* (11), 3830-41.
53. Zhao, M.; Lee, S. H.; Song, J. G.; Kim, H. Y.; Han, H. K., Enhanced oral absorption of sorafenib via the layer-by-layer deposition of a pH-sensitive polymer and glycol chitosan on the liposome. *Int J Pharm* **2018,** *544* (1), 14-20.
54. Hashemi, M.; Omidi, M.; Muralidharan, B.; Tayebi, L.; Herpin, M. J.; Mohagheghi, M. A.; Mohammadi, J.; Smyth, H. D. C.; Milner, T. E., Layer-by-layer assembly of graphene oxide on thermosensitive liposomes for photo-chemotherapy. *Acta biomaterialia* **2018,** *65*, 376-392.
55. Jeon, S.; Yoo, C. Y.; Park, S. N., Improved stability and skin permeability of sodium hyaluronate-chitosan multilayered liposomes by Layer-by-Layer electrostatic deposition for quercetin delivery. *Colloids and surfaces. B, Biointerfaces* **2015,** *129*, 7-14.
56. Chen, M. X.; Li, B. K.; Yin, D. K.; Liang, J.; Li, S. S.; Peng, D. Y., Layer-by-layer assembly of chitosan stabilized multilayered liposomes for paclitaxel delivery. *Carbohydr Polym* **2014,** *111*, 298-304.
57. Fukui, Y.; Fujimoto, K., The preparation of sugar polymer-coated nanocapsules by the layer-by-layer deposition on the liposome. *Langmuir* **2009,** *25* (17), 10020-5.
58. Chen, W. H.; Luo, G. F.; Qiu, W. X.; Lei, Q.; Liu, L. H.; Wang, S. B.; Zhang, X. Z., Mesoporous silica-based versatile theranostic nanoplatform constructed by layer-by-layer assembly for excellent photodynamic/chemo therapy. *Biomaterials* **2017,** *117*, 54-65.
59. Kaminski, G. A.; Sierakowski, M. R.; Pontarolo, R.; Santos, L. A.; de Freitas, R. A., Layer-by-layer polysaccharide-coated liposomes for sustained delivery of epidermal growth factor. *Carbohydr Polym* **2016,** *140*, 129-35.
60. Haidar, Z. S.; Hamdy, R. C.; Tabrizian, M., Protein release kinetics for core-shell hybrid nanoparticles based on the layer-by-layer assembly of alginate and chitosan on liposomes. *Biomaterials* **2008,** *29* (9), 1207-15.
61. Lin, M.; Gao, Y.; Diefenbach, T. J.; Shen, J. K.; Hornicek, F. J.; Park, Y. I.; Xu, F.; Lu, T. J.; Amiji, M.; Duan, Z., Facial Layer-by-Layer Engineering of Upconversion Nanoparticles for Gene Delivery: Near-Infrared-Initiated Fluorescence Resonance Energy Transfer Tracking and Overcoming Drug Resistance in Ovarian Cancer. *ACS Appl Mater Interfaces* **2017,** *9* (9), 7941-7949.
62. Santos, J. L.; Nouri, A.; Fernandes, T.; Rodrigues, J.; Tomas, H., Gene delivery using biodegradable polyelectrolyte microcapsules prepared through the layer-by-layer technique. *Biotechnology progress* **2012,** *28* (4), 1088-94.
63. Simovic, S.; Barnes, T. J.; Tan, A.; Prestidge, C. A., Assembling nanoparticle coatings to improve the drug delivery performance of lipid based colloids. *Nanoscale* **2012,** *4* (4), 1220-1230.
64. del Mercato, L. L.; Rivera-Gil, P.; Abbasi, A. Z.; Ochs, M.; Ganas, C.; Zins, I.; Sonnichsen, C.; Parak, W. J., LbL multilayer capsules: recent progress and future outlook for their use in life sciences. *Nanoscale* **2010,** *2* (4), 458-467.
65. De Cock, L. J.; De Koker, S.; De Geest, B. G.; Grooten, J.; Vervaet, C.; Remon, J. P.; Sukhorukov, G. B.; Antipina, M. N., Polymeric multilayer capsules in drug delivery. *Angewandte Chemie* **2010,** *49* (39), 6954-73.

66. Wang, F.; Liu, B. W.; Ip, A. C. F.; Liu, J. W., Orthogonal Adsorption Onto Nano-Graphene Oxide Using Different Intermolecular Forces for Multiplexed Delivery. *Adv Mater* **2013,** *25* (30), 4087-4092.
67. Wang, F.; Liu, J. W., Nanodiamond decorated liposomes as highly biocompatible delivery vehicles and a comparison with carbon nanotubes and graphene oxide. *Nanoscale* **2013,** *5* (24), 12375-12382.
68. Compton, O. C.; Nguyen, S. T., Graphene oxide, highly reduced graphene oxide, and graphene: versatile building blocks for carbon-based materials. *Small* **2010,** *6* (6), 711-23.
69. Yan, M. M.; Liu, Y. J.; Zhu, X. H.; Wang, X. L.; Liu, L. X.; Sun, H. F.; Wang, C.; Kong, D. L.; Ma, G. L., Nanoscale Reduced Graphene Oxide-Mediated Photothermal Therapy Together with IDO Inhibition and PD-L1 Blockade Synergistically Promote Antitumor Immunity. *Acs Appl Mater Inter* **2019,** *11* (2), 1876-1885.
70. Roy, S.; Sarkar, A.; Jaiswal, A., Poly(allylamine hydrochloride)-functionalized reduced graphene oxide for synergistic chemo-photothermal therapy. *Nanomedicine-Uk* **2019,** *14* (3), 255-274.
71. Park, J. H.; Yoon, J. K.; Kim, Y. J.; Lee, T. J.; Jeong, G. J.; Kim, D. I.; Bhang, S. H., Enhancing therapeutic efficacy of photothermal therapy using poloxamer-reduced graphene oxide and mesenchymal stem cells. *J Ind Eng Chem* **2019,** *80*, 846-853.
72. Liu, Z.; Zhang, J.; Tian, Y.; Zhang, L.; Han, X.; Wang, Q.; Cheng, W., Targeted delivery of reduced graphene oxide nanosheets using multifunctional ultrasound nanobubbles for visual and enhanced photothermal therapy (vol 13, pg 7859, 2018). *Int J Nanomed* **2019,** *14*, 2449-2450.
73. Zhang, L. M.; Xia, J. G.; Zhao, Q. H.; Liu, L. W.; Zhang, Z. J., Functional Graphene Oxide as a Nanocarrier for Controlled Loading and Targeted Delivery of Mixed Anticancer Drugs. *Small* **2010,** *6* (4), 537-544.
74. Verma, A. K.; Sharma, S.; Gupta, P.; Singodia, D.; Kansal, S.; Sharma, V.; Mishra, P. R., Vitamin B-12 Grafted Layer-by-Layer Liposomes Bearing HBsAg Facilitate Oral Immunization: Effect of Modulated Biomechanical Properties. *Mol Pharmaceut* **2016,** *13* (7), 2531-2542.
75. Priprem, A.; Watanatorn, J.; Sutthiparinyanont, S.; Phachonpai, W.; Muchimapura, S., Anxiety and cognitive effects of quercetin liposomes in rats. *Nanomedicine* **2008,** *4* (1), 70-8.
76. Zumbuehl, O.; Weder, H. G., Liposomes of Controllable Size in the Range of 40 to 180 Nm by Defined Dialysis of Lipid-Detergent Mixed Micelles. *Biochimica Et Biophysica Acta* **1981,** *640* (1), 252-262.
77. Szoka, F.; Papahadjopoulos, D., Procedure for Preparation of Liposomes with Large Internal Aqueous Space and High Capture by Reverse-Phase Evaporation. *Proceedings of the National Academy of Sciences of the United States of America* **1978,** *75* (9), 4194-4198.
78. Deamer, D. W., Preparation and properties of ether-injection liposomes. *Ann N Y Acad Sci* **1978,** *308*, 250-258.
79. Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R., Production of Large Unilamellar Vesicles by a Rapid Extrusion Procedure - Characterization of Size Distribution, Trapped Volume and Ability to Maintain a Membrane-Potential. *Biochimica Et Biophysica Acta* **1985,** *812* (1), 55-65.
80. Pradhan, P.; Guan, J.; Lu, D.; Wang, P. G.; Lee, L. J.; Lee, R. J., A facile microfluidic method for production of liposomes. *Anticancer Res* **2008,** *28* (2A), 943-947.
81. Lesoin, L.; Crampon, C.; Boutin, O.; Badens, E., Preparation of liposomes using the supercritical anti-solvent (SAS) process and comparison with a conventional method. *J Supercrit Fluid* **2011,** *57* (2), 162-174.

82. Massing, U.; Cicko, S.; Ziroli, V., Dual asymmetric centrifugation (DAC)--a new technique for liposome preparation. *J Control Release* **2008,** *125* (1), 16-24.
83. Laouini, A.; Jaafar-Maalej, C.; Sfar, S.; Charcosset, C.; Fessi, H., Liposome preparation using a hollow fiber membrane contactor-Application to spironolactone encapsulation. *Int J Pharm* **2011,** *415* (1-2), 53-61.
84. Ciobanu, M.; Heurtault, B.; Schultz, P.; Ruhlmann, C.; Muller, C. D.; Frisch, B., Layersome: development and optimization of stable liposomes as drug delivery system. *International journal of pharmaceutics* **2007,** *344* (1-2), 154-7.
85. Wang, F.; Deng, R.; Wang, J.; Wang, Q.; Han, Y.; Zhu, H.; Chen, X.; Liu, X., Tuning upconversion through energy migration in core-shell nanoparticles. *Nature materials* **2011,** *10* (12), 968-73.
86. Wang, L. Y.; Wang, Z. Q.; Zhang, X.; Shen, J. C.; Chi, L. F.; Fuchs, H., A new approach for the fabrication of an alternating multilayer film of poly(4-vinylpyridine) and poly(acrylic acid) based on hydrogen bonding. *Macromolecular rapid communications* **1997,** *18* (6), 509-14.
87. Lojou, E.; Bianco, P., Buildup of polyelectrolyte-protein multilayer assemblies on gold electrodes. Role of the hydrophobic effect. *Langmuir : the ACS journal of surfaces and colloids* **2004,** *20* (3), 748-55.
88. Sato, M.; Sano, M., van der Waals layer-by-layer construction of a carbon nanotube 2D network. *Langmuir : the ACS journal of surfaces and colloids* **2005,** *21* (24), 11490-4.
89. Li, Y. L.; An, Q.; Hu, Y. M.; Luan, X. L.; Zhang, Q.; Zhang, T. H.; Zhang, Y. H., A facile method for the construction of covalently cross-linked layered double hydroxides layer-by-layer films: Enhanced stability and delayed release of guests. *Chem Phys Lett* **2015,** *631*, 118-123.
90. Jain, S.; Kumar, D.; Swarnakar, N. K.; Thanki, K., Polyelectrolyte stabilized multilayered liposomes for oral delivery of paclitaxel. *Biomaterials* **2012,** *33* (28), 6758-68.
91. Lai, W. F., Microfluidic Methods for Non-Viral Gene Delivery. *Curr Gene Ther* **2015,** *15* (1), 55-63.
92. Jahn, A.; Vreeland, W. N.; DeVoe, D. L.; Locascio, L. E.; Gaitan, M., Microfluidic directed formation of liposomes of controlled size. *Langmuir* **2007,** *23* (11), 6289-6293.
93. Lou, G.; Anderluzzi, G.; Woods, S.; Roberts, C. W.; Perrie, Y., A novel microfluidic-based approach to formulate size-tuneable large unilamellar cationic liposomes: Formulation, cellular uptake and biodistribution investigations. *Eur J Pharm Biopharm* **2019,** *143*, 51-60.
94. Huang, X.; Caddell, R.; Yu, B.; Xu, S.; Theobald, B.; Lee, L. J.; Lee, R. J., Ultrasound-enhanced microfluidic synthesis of liposomes. *Anticancer research* **2010,** *30* (2), 463-6.
95. Benne, N.; van Duijn, J.; Lozano Vigario, F.; Leboux, R. J. T.; van Veelen, P.; Kuiper, J.; Jiskoot, W.; Slutter, B., Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. *J Control Release* **2018,** *291*, 135-146.
96. Tomizawa, H.; Aramaki, Y.; Fujii, Y.; Hara, T.; Suzuki, N.; Yachi, K.; Kikuchi, H.; Tsuchiya, S., Uptake of Phosphatidylserine Liposomes by Rat Peyers-Patches Following Intraluminal Administration. *Pharmaceut Res* **1993,** *10* (4), 549-552.
97. Ling, S. S. N.; Magosso, E.; Khan, N. A. K.; Yuen, K. H.; Barker, S. A., Enhanced oral bioavailability and intestinal lymphatic transport of a hydrophilic drug using liposomes. *Drug development and industrial pharmacy* **2006,** *32* (3), 335-345.
98. Shukla, A.; Mishra, V.; Kesharwani, P., Bilosomes in the context of oral immunization: development, challenges and opportunities. *Drug Discov Today* **2016,** *21* (6), 888-899.

99. Chun, J. Y.; Choi, M. J.; Min, S. G.; Weiss, J., Formation and stability of multiple-layered liposomes by layer-by-layer electrostatic deposition of biopolymers. *Food Hydrocolloid* **2013,** *30* (1), 249-257.
100. Wang, T.; Zhen, Y. Y.; Ma, X. Y.; Wei, B. A.; Li, S. Q.; Wang, N. N., Mannosylated and lipid A-incorporating cationic liposomes constituting microneedle arrays as an effective oral mucosal HBV vaccine applicable in the controlled temperature chain. *Colloid Surface B* **2015,** *126*, 520-530.
101. Anderson, K. E.; Stevenson, B. R.; Rogers, J. A., Folic acid-PEO-labeled liposomes to improve gastrointestinal absorption of encapsulated agents. *J Control Release* **1999,** *60* (2-3), 189-198.
102. Anderson, K. E.; Eliot, L. A.; Stevenson, B. R.; Rogers, J. A., Formulation and evaluation of a folic acid receptor-targeted oral vancomycin liposomal dosage form. *Pharmaceut Res* **2001,** *18* (3), 316-322.
103. Zhang, X. W.; Qi, J. P.; Lu, Y.; Hu, X. W.; He, W.; Wu, W., Enhanced hypoglycemic effect of biotin-modified liposomes loading insulin: effect of formulation variables, intracellular trafficking, and cytotoxicity. *Nanoscale research letters* **2014,** *9*.
104. Zhang, X. W.; Qi, J. P.; Lu, Y.; He, W.; Li, X. Y.; Wu, W., Biotinylated liposomes as potential carriers for the oral delivery of insulin. *Nanomed-Nanotechnol* **2014,** *10* (1), 167-176.
105. Giannasca, P. J.; Giannasca, K. T.; Leichtner, A. M.; Neutra, M. R., Human intestinal M cells display the sialyl Lewis A antigen. *Infect Immun* **1999,** *67* (2), 946-953.
106. Lopes, M. A.; Abrahim, B. A.; Cabral, L. M.; Rodrigues, C. R.; Seica, R. M. F.; Veiga, F. J. D. B.; Ribeiro, A. J., Intestinal absorption of insulin nanoparticles: Contribution of M cells. *Nanomed-Nanotechnol* **2014,** *10* (6), 1139-1151.
107. Agrawal, A. K.; Harde, H.; Thanki, K.; Jain, S., Improved Stability and Antidiabetic Potential of Insulin Containing Folic Acid Functionalized Polymer Stabilized Multi layered Liposomes Following Oral Administration. *Biomacromolecules* **2014,** *15* (1), 350-360.
108. Buda, A.; Sands, C.; Jepson, M. A., Use of fluorescence imaging to investigate the structure and function of intestinal M cells. *Adv Drug Deliver Rev* **2005,** *57* (1), 123-134.
109. Han, H. K.; Shin, H. J.; Ha, D. H., Improved oral bioavailability of alendronate via the mucoadhesive liposomal delivery system. *Eur J Pharm Sci* **2012,** *46* (5), 500-507.
110. Chen, H. L.; Wu, J.; Sun, M.; Guo, C. Y.; Yu, A. H.; Cao, F. L.; Zhao, L. Y.; Tan, Q.; Zhai, G. X., N-trimethyl chitosan chloride-coated liposomes for the oral delivery of curcumin. *J Liposome Res* **2012,** *22* (2), 100-109.
111. Jones, D. S.; Bruschi, M. L.; de Freitas, O.; Gremiao, M. P. D.; Lara, E. H. G.; Andrews, G. P., Rheological, mechanical and mucoadhesive properties of thermoresponsive, bioadhesive binary mixtures composed of poloxamer 407 and carbopol 974P designed as platforms for implantable drug delivery systems for use in the oral cavity. *Int J Pharm* **2009,** *372* (1-2), 49-58.
112. Li, X. Y.; Chen, D.; Le, C. Y.; Zhu, C. L.; Gan, Y.; Hovgaard, L.; Yang, M. S., Novel mucus-penetrating liposomes as a potential oral drug delivery system: preparation, in vitro characterization, and enhanced cellular uptake. *Int J Nanomed* **2011,** *6*, 3151-3162.
113. Zhu, Q. L.; Guo, T.; Xia, D. N.; Li, X. Y.; Zhu, C. L.; Li, H. Y.; Ouyang, D. F.; Zhang, J. W.; Gan, Y., Pluronic F127-modified liposome-containing tacrolimus-cyclodextrin inclusion complexes: improved solubility, cellular uptake and intestinal penetration. *J Pharm Pharmacol* **2013,** *65* (8), 1107-1117.

114. Yuan, H.; Chen, C. Y.; Chai, G. H.; Du, Y. Z.; Hu, F. Q., Improved Transport and Absorption through Gastrointestinal Tract by PEGylated Solid Lipid Nanoparticles. *Mol Pharmaceut* **2013,** *10* (5), 1865-1873.
115. Jeon, S.; Yoo, C. Y.; Park, S. N., Improved stability and skin permeability of sodium hyaluronate-chitosan multilayered liposomes by Layer-by-Layer electrostatic deposition for quercetin delivery. *Colloid Surface B* **2015,** *129*, 7-14.
116. Goniotaki, M.; Hatziantoniou, S.; Dimas, K.; Wagner, M.; Demetzos, C., Encapsulation of naturally occurring flavonoids into liposomes: physicochemical properties and biological activity against human cancer cell lines. *J Pharm Pharmacol* **2004,** *56* (10), 1217-24.
117. Sun, J.; Zhu, Z. Y.; Lai, J. P.; Luo, J.; Liu, X. Y., Layer-by-layer Assembled Graphene/Polyaniline Nanocomposite Film and Applied in Electrochemical Sensing. *Chem J Chinese U* **2015,** *36* (3), 581-588.
118. Cho, I.; Song, Y.; Cheong, S.; Kim, Y.; Cho, J., Layer-by-Layer Assembled Oxide Nanoparticle Electrodes with High Transparency, Electrical Conductivity, and Electrochemical Activity by Reducing Organic Linker-Induced Oxygen Vacancies. *Small* **2020**.
119. Huang, J.; Shu, Q.; Wang, L. Y.; Wu, H.; Wang, A. Y.; Mao, H., Layer-by-layer assembled milk protein coated magnetic nanoparticle enabled oral drug delivery with high stability in stomach and enzyme-responsive release in small intestine. *Biomaterials* **2015,** *39*, 105-113.
120. Sharma, S.; Rajendran, V.; Kulshreshtha, R.; Ghosh, P. C., Enhanced efficacy of anti-miR-191 delivery through stearylamine liposome formulation for the treatment of breast cancer cells. *International journal of pharmaceutics* **2017,** *530* (1-2), 387-400.
121. Vhora, I.; Lalani, R.; Bhatt, P.; Patil, S.; Patel, H.; Patel, V.; Misra, A., Colloidally Stable Small Unilamellar Stearyl Amine Lipoplexes for Effective BMP-9 Gene Delivery to Stem Cells for Osteogenic Differentiation. *AAPS PharmSciTech* **2018,** *19* (8), 3550-3560.
122. Kim, J. H.; Shin, D. H.; Kim, J. S., Preparation, characterization, and pharmacokinetics of liposomal docetaxel for oral administration. *Archives of pharmacal research* **2018,** *41* (7), 765-775.
123. Takano, S.; Aramaki, Y.; Tsuchiya, S., Physicochemical properties of liposomes affecting apoptosis induced by cationic liposomes in macrophages. *Pharmaceutical research* **2003,** *20* (7), 962-8.
124. Kelly, C.; Jefferies, C.; Cryan, S. A., Targeted liposomal drug delivery to monocytes and macrophages. *Journal of drug delivery* **2011,** *2011*, 727241.
125. Wu, W.; Lu, Y.; Qi, J. P., Oral delivery of liposomes. *Ther Deliv* **2015,** *6* (11), 1239-1241.
126. Cevc, G.; Blume, G., Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochim Biophys Acta* **1992,** *1104* (1), 226-32.
127. Gillet, A.; Grammenos, A.; Compere, P.; Evrard, B.; Piel, G., Development of a new topical system: drug-in-cyclodextrin-in-deformable liposome. *Int J Pharm* **2009,** *380* (1-2), 174-80.
128. Karande, P.; Mitragotri, S., Enhancement of transdermal drug delivery via synergistic action of chemicals. *Biochim Biophys Acta* **2009,** *1788* (11), 2362-73.

Tables

Table 1 Major strategies adopted to enhance the stability of liposomes for oral drug administration.

Strategy	Working principle	Strength	Limitation	Ref.
Modification of lipid compositions	Modifying the composition of the lipid bilayer by incorporating specific lipids or sterols (e.g., stearylamine, glycerylcaldityl tetraether, and phospholipids with the phase transition temperatures above 37 °C) into the liposome membrane	• Be the most direct method of confronting membrane instability • Relatively fewer variables and parameters to be considered during the design process • Maintenance of the cell-like membrane structure	• Time-consuming for liposome design • Limited capacity to enhance stability • Less flexibility to couple with site-specific ligands as compared to other strategies • Less flexibility to control surface geometry	11-13
Incorporation of an outer lipid bilayer	Incorporating an additional outer lipid bilayer to liposomes so as to protect the liposomes from damage caused by intestinal enzymes	• Potentially supported by the emergence of microfluidic technologies • Multiple compartments are available for codelivery of both hydrophilic and lipophilic drugs • Maintenance of the cell-like membrane structure	• Difficult to maintain monodispersity • Fail to provide extra surface functionalities to liposomes • Tedious synthetic procedures • Potentially instable • Phospholipid may undergo oxidation and hydrolysis-like reactions • Less flexibility to control surface geometry	14
Interior thickening	Thickening the interior aqueous phase of the liposome (e.g., by increasing the viscosity of the aqueous phase, by using a lipid bilayer to enclose a hydrogel bead, or by inducing *in situ* gelation of the aqueous phase using physical stimuli) to improve the physical stability of liposomes and to increase the rigidity of the lipid bilayer.	• Enable sustained release of the loaded drug • Can take advantage of works performed on existing and developed liposomes for carrier development • Maintenance of the cell-like membrane structure • No limitation on the shape or size of the particle core	• Less flexibility to couple with site-specific ligands as compared to other strategies • Less flexibility to control surface geometry	15-17
Entrapment in another systems	Embedding liposomes in other materials (e.g., hydrogels) to stabilize the lipid bilayer and to achieve sustained release in the gastrointestinal tract	• Enable sustained release of liposomes • Supported by the accumulated efforts of research on hydrogels • No limitation on the shape or size of the particle core	• Less flexibility to couple with site-specific ligands as compared to other strategies • Changes in size and shape after the coating process	18
Surface coating	Coating the surface of liposomes by using either organic materials [e.g., poly(ethylene glycol) and polysaccharides] or inorganic materials (e.g., silica and silica nanoparticles) so as to protect the liposomes from the gastrointestinal environment	• Enable sustained release of the loaded drug • Provide an easy route for functionalization of liposomes • Easy to operate • Can take advantage of studies performed on existing and developed liposomes for carrier development • Supported by the accumulated efforts of research on polymers • Possible manipulation of the surface geometry • No limitation on the shape or size of the particle core	• Difficult to maintain monodispersity • Changes in size and shape after the coating process • Multiple variables and parameters to be considered during the design process	19-22

Table 2 Types of agents reported to be successfully delivered by LbL functionalized nanoparticulate systems

Type of agent	Coating agents	Description	Ref.
Small molecular compound	Glycol-chitosan, and Eudragit S100	After an oral administration to rats, LbL coated liposomes successfully enhance the oral bioavailability and absorption of sorafenib, as compared to the uncoated ones.	[53]
	Graphene oxide-conjugated poly(L-lysine), and graphene oxide	The LbL coated liposomes generated enable intracellular DOX delivery and can respond to either acidic environments or NIR excitation for photo-chemotherapy.	[54]
	Chitosan, and sodium hyaluronate	Multilayered liposomes properly coated with polyelectrolytes show improved stability and higher release sustainability of quercetin.	[55]
	Chitosan, and dextran sulfate	The LbL-coated liposomes improved the release sustainability of 1-hydroxy pyrene-3,6,8-trisulfonic acid, alendronate, and glucose.	[57]
	Chitosan, and polyacrylic acid	LbL coated liposomes showed higher drug release sustainability, and lead to higher paclitaxel-induced cytotoxicity in human cervical cancer cell culture experiments as compared to the uncoated ones.	[56]
	Chitosan, and deoxyribonucleic acid	The LbL-coated liposomes improved the release sustainability of 1-hydroxy pyrene-3,6,8-trisulfonic acid, alendronate, and glucose.	[57]
Inorganic salt	5,10,15,20-Tetrakis(4-sulfonatophenyl)-porphyrin, and per-O-methyl-β-cyclodextrin-grafted-hyaluronic acid	LbL coated nanoparticles generated by using aminated mesoporous silica nanoparticles as the core were adopted to carry gadolinium-III (Gd^{3+}), along with other therapeutic agent, for photodynamic/chemo therapy	[58]
Protein	Xanthan, and galactomannan	The bilayer-coated liposomes showed a significant increase in the release sustainability of epidermal growth factor	[59]
	Chitosan, and alginate	The LbL coated liposomes showed enhanced release sustainability of bovine serum albumin, and also enhance the efficiency in protein encapsulation	[60]
Nucleic acid	Dextran sulphate, and poly-L-arginine	Biodegradable LbL-coated microcapsules were adopted to deliver DNA plasmids to NIH 3T3 cells	[62]
	Poly(ethylenimine), and poly(acrylic acid) (PAA)	LbL engineered upconversion nanoparticles were adopted for near-infrared (NIR)-initiated tracking and for delivery of small interfering RNA (siRNA) to resensitize resistant ovarian cancer cells to chemotherapy.	[61]
	Poly(styrene sulfonate), and poly(allylamine hydrochloride)	Biodegradable LbL-coated microcapsules were adopted to deliver DNA plasmids to NIH 3T3 cells	[62]

Figure Legends

Fig. 1 An overview of different types of liposomes.

Fig. 2 A schmetaic diagram depicting the procedures for LbL functionalization of liposomes via electrostatic interactions between oppositely charged polyelectrolytes.

Fig. 3 Important parameters to be considered for the molecular design of LbL functionalized liposomes.

Fig. 4 Properties of LbL functionalized liposomes affecting the ultimate performance as effective oral carriers