Expert Opinion

informa
healthcare

Active methods of drug loading into liposomes: recent strategies for stable drug entrapment and increased *in vivo* activity

Jerzy Gubernator
University of Wrocław, Faculty of Biotechnology, Laboratory of Lipids and Liposomes, Wrocław, Poland

Introduction: The use of liposomes increases the therapeutic index of many drugs, and also offers drug targeting and controlled release. The commercial impact of liposomes is strengthened by the invention of several active drug encapsulation methods, allowing the encapsulation of several weak base or weak acid drugs with very high drug-to-lipid ratios.

Areas covered: In recent years, there have been reports on several new approaches to retain more hydrophobic drugs inside liposomes, in the circulation. Most of these methods apply drug precipitation inside preformed liposomes, as low soluble complexes with ions or chemicals. In some cases, drug derivatization was applied to enable active encapsulation of hydrophobic drugs, previously not reported to encapsulate, by active or remote loading. This review presents and compares most of the existing methods of active drug encapsulation and outlines recent strategies to achieve stable drug encapsulation *in vivo*.

Expert opinion: At present, there is no single universal encapsulation method that offers stable encapsulation of most drugs; each drug requires a different approach to manage all of its properties. Now is the time to combine all these strategies to achieve the goal of a complex, but successful, anticancer therapy.

Keywords: active loading, anthracyclines, cancer treatment, ciprofloxacin, drug encapsulation, liposomes, pH gradient, remote loading

Expert Opin. Drug Deliv. (2011) 8(5):565-580

1. Introduction

At the start of the twentieth century, the German biochemist Dr Paul Ehrlich introduced his 'magic bullet' concept. In his times, medical treatments were based on therapies that were often toxic and given in overdose. After the discovery of antibodies, Ehrlich realized that they could represent an ideal drug thanks to their ability to affect only the place of action, bypassing healthy tissues [1].

His vision is now fulfilled by various drug delivery systems, including polymeric micelles, liposomes and other similar lipid-based systems. Of these systems, liposomes have the potential to be the best candidate for Ehrlich's magic bullet idea.

Liposomes are microscopic lipid vesicles with a cell bilayer structure, and they can be composed of several natural, semi-natural or purely synthetic molecules. Their size and fluidity can be fine-tuned across a wide range of values, giving researchers the opportunity to select the desired liposome diameter and properties to suit their goal. For example, they can be unilamellar (possessing only one bilayer) with a size of 20 nm (virus size) (small unilamellar vesicles [SUVs]) to even 5 μm (protozoan size) if required (large unilamellar vesicles [LUVs]), or they can be multilamellar (large multilamellar vesicles [MLVs]) with several internal water compartments

10.1517/17425247.2011.566552 © 2011 Informa UK, Ltd. ISSN 1742-5247

separated by a lipid bilayer of up to 20 μm or more in size [2]. Such rare characteristics make liposomes very useful drug delivery systems. The most important feature of liposomes is that hydrophilic drug solutions can be encapsulated inside the water compartment, which thus serves as a drug nanocontainer, while hydrophobic drugs can be incorporated into the liposome bilayer.

Shortly after the discovery of liposomes, it was realized that they meet the requirements to act as a drug carrier. However, this role could only be fulfilled after a huge improvement in the various techniques of liposome preparation and loading.

The first milestone was the observation that multilamellar liposomes, although biocompatible, are immediately cleared by the mononuclear phagocyte system (MPS), which is responsible for the clearance of foreign particles and organisms. By contrast, much smaller liposomes (with an average size of ~ 100 nm or less) are taken up more slowly by the MPS [3-10]. The second milestone was the discovery that cholesterol incorporation further increases the liposomes' lifespan in the blood. The cholesterol strengthens the mechanical bilayer properties that inhibit protein integration with the liposomes. Most of these proteins, called opsonins, act as signal marks for macrophages, leading to more rapid liposome elimination [5,6,10]. After this discovery, lipids with saturated (and thus more rigid) side chains were used, increasing liposome longevity yet further [11].

The next milestone was liposome production by means of the extrusion technique, which uses high-pressure liposome extruders equipped with polycarbonate filters. This technique gave a commercial aspect to liposomology as it yielded the potential to prepare high-volume liposome batches of the desired size (depending on the pore sizes of the polycarbonate filters) and offered a relatively high lipid concentration [12,13].

The last two milestones were achieved almost simultaneously. One was the invention of steric stabilization of liposomes via the incorporation of various hydrophilic polymers (the PEG-PE derivatives are the most commonly used in liposomal technology) into the liposome surface. This raised the liposomes' half-life to a level not observed for the other drug delivery systems. By incorporating usually 5% of the PEGylated phospholipids into the liposomal bilayer, a hydrophilic polymer shield is formed, protecting the liposome surface from penetration and disintegration by plasma proteins. Macrophage uptake also decreases remarkably [14-18]. In the case of small long-circulating liposomes, passive accumulation in the organs or tissues undergoing the inflammatory process was observed owing to leaky vasculature. The enhanced permeability and retention (EPR) effect caused considerable liposome and drug accumulation in the site of cancer tissues or other diseased tissues [19-22]. Therefore, the need for liposomes able to retain their encapsulated content seems to be crucial in terms of efficient passive or active drug targeting [23-26]. The other final milestone was the elaboration of active methods for drug loading. This resulted in the preparation and successful introduction of the first commercially available PEGylated liposome product, Doxil® (Alza, Palo Alto, CA) [27-31]. Active loading methodology is used to fill the preformed liposomes, which have some buffers or salt solutions inside, with drug molecules that are able to diffuse one way only. This process yields a very low level of drug loss, if any, and a very favorable drug-to-lipid ratio in most cases.

Advantages of liposomes:

- biocompatible and biodegradable
- non-toxic
- Reticuloendotelial system avoiding (polymer coated)
- targetable
- high drug/lipid ratio can be obtained.

2. Active drug encapsulation methods

2.1 Limitations of conventional drug encapsulation methods

A lipid bilayer with a thickness of ~ 8 – 10 nm is a natural barrier for many substances, including ions, charged molecules and larger non-charged water-soluble molecules, for example sugars and proteins. Substances such as water, gases, ammonia and glycerol can penetrate freely through the bilayer [2,32,33]. Therefore, many hydrophilic substances, among them amino acids, salts, antibiotics, proteins and sugars, can be encapsulated inside the liposome water compartment and retained with only negligible leakage over a prolonged period of time *in vitro*. Such water-soluble substances can be encapsulated inside the internal water compartment of the liposomes by means of several methods [34-37]. The most commonly used method is based on the so-called thin lipid film method. In thin lipid films, the lipid molecules are rather randomly orientated with a small degree of bilayer type caused by exposure to traces of water. On addition of water, the molecules self-organize to form bilayers. Then, water penetration causes the bilayers to swell and invaginations form. These are the so-called myelin figures, which are the basis of MLV liposomes [34]. At this point, a part of the water solution with water-soluble substances is passively encapsulated inside the formed vesicles. The advantage of this method is its simplicity, but only a very small percentage of a water-soluble drug can be encapsulated in this way.

The freezing-and-thawing technique (FAT), which uses a sequence of freezing and thawing a liposome suspension, increases the distances between the liposome layers. Ice crystals lead to transient hole formation, yielding better drug penetration and a greater increase in the liposome volume [38]. These techniques have several modifications. Using such a technique, only ~ 5 – 20% of the encapsulated molecules can be retained inside the liposomes. Most of the drug solution remains outside and must be removed by dialysis or molecular exclusion chromatography. Finally, the drug loss is high, and the drug-to-lipid ratio is often not optimal.

Other techniques use ethanol injections [39]. The lipid ethanol solution is rapidly injected into an excess of drug solution through a thin needle to form unilamellar liposomes with a diameter that is dependent on the lipid concentration, injection rate, lipid composition and temperature. The encapsulation efficiency is relatively low, and the liposome solution suffers because of dilution and the presence of ethanol. Recently, a new, commercial-scale methodology was introduced, offering more advantages compared with the laboratory version [40].

The reverse-phase evaporation method yields the highest achievable encapsulation efficiency by means of passive loading: ~ 50%. However, this technique uses organic solvents, which remain to some degree in the liposome suspension, and thus is probably not feasible for commercial production [41].

Passive drug encapsulation:

- low encapsulation efficiency in some of the cases
- non-encapsulated drug loss
- organic solvents sometimes remain
- rapid *in vivo* drug leakage of bilayer-permeable drug species.

2.2 Bilayer properties, partition coefficients and lipophilic drugs

The lipid bilayer is – as mentioned – a barrier to many chemical ingredients, especially ions and charged molecules. Unfortunately, most drugs are relatively lipophilic molecules containing primary, secondary, or tertiary amines (or sometimes acids) that can diffuse through the liposome bilayer in an unprotonated state. Indeed, the generality of this phenomenon argues that such characteristics are important for their function [42]. The equilibrium between free bases (bilayer diffusible) and those in a protonated state depends on the molecules' group(s) p*K*, which is in turn pH dependent. At a neutral pH, part of the population of molecules is in a charged (protonated) state and the remainder in an unprotonated (neutral) form. The more the pH differs from its neutral value, the more the balance between the charged and neutral molecules shifts towards neutral or charged species. For several weak bases, for example, at pH 4.0, most of the molecules are in the charged non-bilayer-permeable state. In addition, the diffusion rate of the free base through the bilayer correlates with the molecule oil/water partition coefficient (K_p), which is molecule dependent. Some migrate more rapidly, whereas others migrate more slowly [32,43-48]. The simple, passive encapsulation of such substances can result in very rapid molecule leakage owing to the rapid permeation of the non-charged drug population through the bilayer. From these physicochemical considerations and methodological problems, methods of active drug loading were born. The basic concept is based on two simple phenomena. First, a given lipophilic molecule can easily penetrate the lipid bilayer, but it will gain a charge while entering the internal compartment, provided the internal compartment possesses a low pH. Second, as an ion, that molecule will no longer be able to cross the bilayer freely. Thus, a pH gradient is the driving force to translocate and retain the amphiphilic weak bases and acids.

Thanks to the resulting accumulation, the intraliposomal concentration of a drug can markedly exceed a medium concentration. For example, a pH gradient of 3 units is predicted to lead to a 1000-fold higher concentration of a weak base within the vesicle as compared with the external solution [32,49]. Thus, this high concentration markedly exceeds the weak base or acid solubility and leads to drug precipitation inside the liposome water compartment (see **Figure 1**). The drug precipitation is usually increased by the presence of different ions inside the vesicles (citrates, sulfates, divalent metal ions, or others). These are used for pH/ion gradient generation, yielding the formation of various readily soluble precipitates. This in turn increases further the drug accumulation by decreasing the apparent drug concentration inside the vesicle, and decreases the osmolarity imbalance. The practical significance of this insolubility or low solubility of such precipitates is discussed in the following sections, because it is one of the most important factors by which the kinetics of, in particular, more hydrophobic drug release from the liposomes and the drug therapeutic index can be controlled.

2.3 An overview of active drug encapsulation methods

Nicols and Deamer were the first to demonstrate the active loading of amphiphilic amines, specifically catecholamines, in liposomes [50,51]. A basic solution was added to liposomes prepared in a low pH citrate buffer in order to elevate the pH of the external solution and create a pH gradient of ~ 3 units (5.0 inside and 8.0 outside the liposomes).

Later, Bally and co-workers observed the effect of the proton gradient generated when an intraliposomal solution was changed from a 300 mm citrate buffer (pH 4.0) to a HEPES buffer (pH 7.4) while the internal pH and composition remained unchanged. Accumulation of biogenic amines and anticancer doxorubicin was observed [50-52]. This so-called citrate method is still in use, and is widely selected for the encapsulation of various anthracyclines [53-57]. In this case, anthracycline citrate salts, which have a low solubility, precipitate inside liposomes, leading to the so-called coffee bean liposome appearance [58,59]. Doxorubicin, idarubicin or daunorubicin can be loaded in a drug-to-lipid ratio (D/L) of 0.3 w/w with an encapsulation efficiency of nearly 100% and excellent *in vitro* stability, especially when phospholipids with long and saturated fatty acid chains, such as DSPC or HSPC, are used for the liposome preparation. As PEGylated phospholipid species are utilized in most liposomes to achieve a long circulation effect, the drug release *in vivo* depends in this case on the hydrophobicity of the anthracycline molecule: doxorubicin is released more slowly than daunorubicin, and the most relatively rapid release is for idarubicin, which is

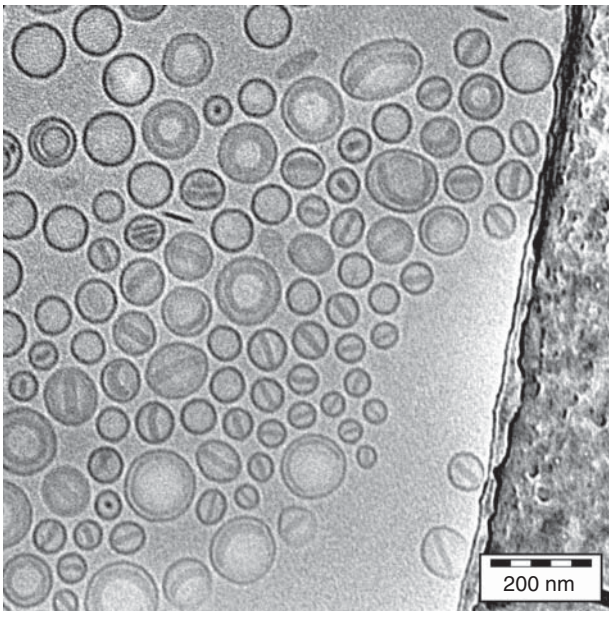

Figure 1. Idarubicin precipitates inside HSPC/Chol/DSPE-PEG 2000 liposomes visualized by the cryo-electron microscopy technique using the EDTA ion gradient method. This represents a typical appearance of the drug precipitates inside liposomes. Similar results can be achieved for all anthracyclines loaded by different pH gradient methods. In this case idarubicin was encapsulated by the EDTA ion gradient method in HSPC/Chol/DSPE-PEG 2000 liposomes.
Adapted from [67].

also the most hydrophobic of the three [45,54]. This method was proposed and successfully used to encapsulate doxorubicin and daunorubicin in two commercially available products, Myocet™ (Enzon Pharmaceuticals, Inc., USA) and DaunoXome® NeXtar Pharmaceuticals, inc. USA, respectively. Their efficacy, especially that of the latter, has been widely proved [56,57].

Haran *et al.* introduced an ammonium sulfate method for the generation of a pH gradient and the encapsulation of amines. The stability of the ammonium ion gradient is related to the low permeability of its counterion, the sulfate, which also stabilizes the anthracycline accumulation for a prolonged storage period owing to the aggregation and gelation of anthracycline salt [60]. In this case, the liposomes are usually prepared in a 300 mm solution of the ammonium sulfate salt with a (native) pH of 5.5. By means of extraliposomal ammonium sulfate exchange to the pH 7.4 buffer, a pH gradient is generated. The higher concentration of ammonium in the aqueous phase inside the liposomes causes the diffusion of the neutral ammonia molecules (permeability coefficient 1.3×10^{-1} cm/s). For every ammonia molecule that leaves the liposome, one proton is left behind. Thus, a pH gradient is formed. The internal aqueous solution is then more acidic. The permeability coefficient of the SO_4^- is 10^{-13} cm/s, so it is practically bilayer impermeable [33]. A schematic representation of the loading process is presented in **Figure 2A** [60,61]. The magnitude of this gradient is determined by the ratio $[NH_4^+]_{med}/[NH_4^+]_{lip}$. The acidification of the intraliposomal aqueous phase slows down the process by reducing the level of NH_3. Furthermore, accumulation of the protonated base inside the liposome leads to elevation of the internal pH, which increases the level of NH_3 and therefore again reduces the pH, enabling more of the drug to enter. The salting-out effect of the ammonium sulfate probably accelerates the flocculation and gelation of the drug, thus further improving the encapsulation and stabilizing it owing to the mass action law [60]. The solubility of the doxorubicin sulfate is relatively low in the pH range 4.0 – 7.5 (1.7 – 2.3 mg/ml), whereas in the case of doxorubicin citrate, the solubility is about three times higher in the same pH range [62]. This can influence the release rate and therapeutic index of the liposomes. In fact, the drug release rate seems to be too slow in the case of drugs such as doxorubicin with a relatively low rate of leakage *in vivo*. This resolves most of the problems associated with too fast drug leakage in the bloodstream (observed for many drugs) and inefficient drug accumulation in inflammatory tissue owing to the EPR effect. The low drug leakage rate in turn causes problems with low drug availability at the site of action, even after liposome internalization [63,64]. Therefore, several methods of active drug release have been established, including pH-sensitive liposomes, thermosensitive liposomes, and others, to release the contents of drug loaded liposomes at (usually) tumor tissue. For more information on this topic – active drug release at the site of action – see [65,66]. The ammonium sulfate method was used for the development of the first commercially available long-circulating liposomal doxorubicin formulation – Doxil [27-31]. In the case of doxorubicin, the method has proven applicability, but in the case of other drugs, especially hydrophobic anthracyclines, vinca alkaloids and ciprofloxacin, it seems to fail because of fast drug leakage characteristics *in vivo* [67-69]. These hydrophobic drugs require special approaches that are emerging from current modifications of the basic concept of drug loading by a pH gradient.

More recently, Barenholz's group published a paper summarizing their experience in terms of drug loading conditions. They proposed a new theoretical model of drug loading into liposomes by analyzing the results of both previously published and new experiments. The model is based on the drug's physicochemical properties and on loading conditions [70]. Factors such as external medium properties (electrolytic or non-electrolytic), external pH, gradient ions (mostly ammonium and acetate are taken into consideration), loading duration, process temperature and others were compared for their impact on drug loading. From the other side, drugs' properties were analyzed with the above variables. This resulted in a decision tree formation giving the possibility of selecting optimal drug loading conditions for a selected drug. Owing to space considerations, the reader should refer to the original papers for details.

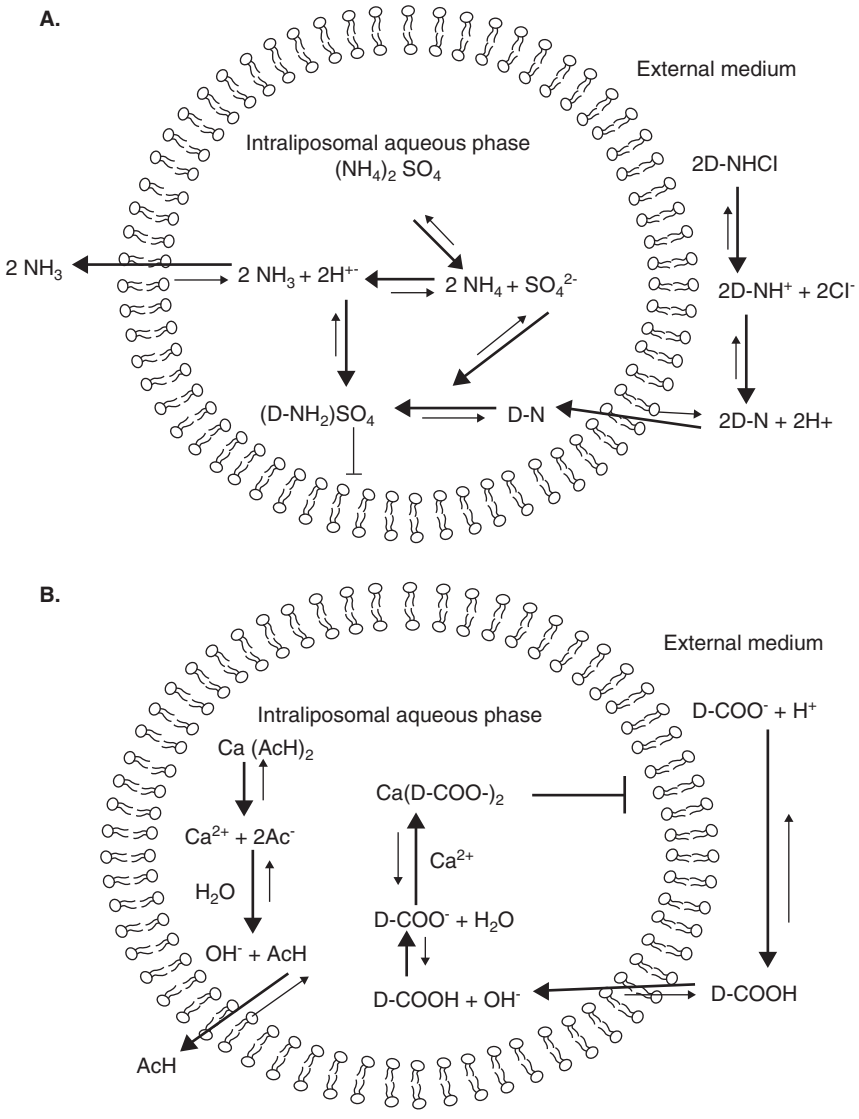

Figure 2. Schematic representation of the processes occurring during drug loading in the case of (A) the ammonium sulfate method and (B) the calcium acetate method. Detailed descriptions of the method and processes are given in the text.
Adapted with permission from [70].

The same principle of different permeability coefficients of weak acid salt species is the basis of the transmembrane calcium acetate method, which was developed in the same laboratory [71]. The bilayer permeability coefficient of acetic acids is 6.6×10^{-4} cm/s, whereas the calcium ion used for gradient generation is 2.5×10^{-11} cm/s. Whereas the calcium ions remain trapped inside the liposomes, the acetic acid molecules behave as proton shuttles. This generates a pH gradient (high inside) to trap the weak amphiphilic acid molecules inside the liposomes in a manner similar to that observed in the case of weak amphiphilic bases (see **Figure 2B**).

The ionophore-generated pH gradient method is another interesting example of a very versatile loading approach. This method is based on an observation first made by Deamer *et al.* that addition of nigericin to SUVs containing potassium salts led to the generation of a pH gradient (inside acidic) of ~ 2 units [72]. They applied this process to ionophores such as nigericin or A23187 to LUV, respectively, showing transmembrane gradients of K^+ or Mn^{2+} (Mg^{2+}). When the drug is added to the external medium, the uptake is initiated by the addition of an ionophore that couples the outward transport of the metal ion to the inward movement of H^+. This creates a pH gradient (inside acidic), which results in the uptake of molecules with weak base characteristics. The ionophore nigericin catalyzes a one-for-one exchange of K^+ for H^+, whereas A23187 transports $2H^+$ for every divalent metal cation Ca^{2+}, Mn^{2+}, or Mg^{2+} [73-76]. In the case of the divalent cations, the system also requires the presence of

EDTA as an external chelator to bind the cations that are released and to drive the uptake to completion and avoid liposome aggregation [73]. A schematic representation of the method is presented in **Figure 3**.

This method was applied for the first time for ciprofloxacin and vincristine loading. Both ionophores are able to generate a pH gradient of ~ 2 – 3 units, which is capable of driving the drug encapsulation with 95 – 100% efficiency at very high drug-to-lipid ratios. Taking into consideration that the A23187 ionophore generates a higher pH gradient, it is clear that this ionophore wins in a one-to-one competition. Both the encapsulation stability and the *in vivo* leakage rate are much superior when an A23187 ionophore is applied. By applying this technique, drug-to-lipid ratios as high as 1 w/w can be achieved for vincristine. Such high drug-to-lipid ratios have not been reported previously for any other drug or loading protocol [77].

An important aspect of the ionophore loading method is its universality in terms of the possibility of changing the internal ion to be transported by the ionophore. Depending on the drug to be encapsulated, one can select calcium or magnesium ions just to achieve a suitable pH gradient, or for example manganese or copper ions to form metal-drug complexes inside the liposomes [77-81]. This in turn changes the drug release kinetics, which can be modified in a simple way according to the treatment needs, because each drug-metal complex has different solubility and therefore liposome release characteristics.

During doxorubicin encapsulation via the ionophore loading method, the liposome color was observed to change from red to purple and back to red if manganese was used as the internal bivalent metal [78]. As anthracyclines possess the ability to form coordinate complexes with transit metal ions, it is clear that a complexation process occurs during drug loading in its initial stage (purple), and when the internal pH decreases because of ionophore action, the complex dissociates to release doxorubicin (red again), because the doxorubicin-manganese complex is not stable at low pH. If no ionophore is added to the incubation mixture, accumulation of the drug is also observed, even if no transmembrane pH gradient is established. This is because every drug molecule entering the liposome lumen encounters an ion with which a coordination complex is formed (two drug molecules by one Mn^{2+} in the case of doxorubicin). This complex has very low solubility and subsequently precipitates inside the liposomes. Therefore, the apparent doxorubicin concentration decreases below the concentration present in the external medium and, via equilibrium processes, further drug molecules enter to reduce the formed gradient. Then new complex molecules are formed and the drug precipitates as previously; because the drug coordinate complex has some minimal solubility, the loading process stops when the concentration of doxorubicin outside the liposomes equals the internal soluble complex concentration. The processes occurring during doxorubicin encapsulation are presented in **Figure 4**.

This method was recently applied successfully for the encapsulation of several difficult-to-retain molecules, such as topotecan [82,83], irinotecan (copper ions [84]) and mitoxantrone (by copper ions [80] and nickel ions [79]). This approach can also be very useful when pH-sensitive liposomes are prepared. The liposome structure becomes disorganized in low pH, making drug encapsulation by pH gradient at least difficult. When the transit metal gradient is applied, the drugs can be loaded in neutral pH with no liposome destabilization.

Another useful example of the active loading method is the transmembrane phosphate gradient, designed by Fritze *et al.* The basic concept is the same as in the case of other pH methods, as it utilizes the internal concentration of an acid ammonium phosphate solution [62]. They observed a near 100% doxorubicin accumulation within liposomes with D/L = 0.3, and drug precipitation was observed as in the case of other gradients. The interesting part of the method concerns the drug leakage characteristics, which are in this case sensitive to the external pH. At low pH the drug release is influenced by the extraliposomal ratio of positively charged doxorubicin to its uncharged population. When the external pH remains close to a physiological level, no drug leakage from the liposomes is observed. However, when the extraliposomal pH changes to acidic (for example in tumor tissue or inside the lysosomes), accelerated drug leakage is observed, increasing the local (and effective) drug concentration. This occurs more rapidly than in the case of the citrate or ammonium sulfate methods. This can be important in situations where shorter-lasting but elevated drug concentrations are required.

The last emerging active loading method is the EDTA ion gradient method, which is another example of an alternative to the pH gradient method. It was applied successfully to encapsulating the relatively hydrophobic anthracycline idarubicin [67]. The principle of the method relies on the formation of low-solubility precipitates of the drug with EDTA molecules at low pH. The dissolution rate of the drug precipitate should be slower than the drug portioning through the bilayer, therefore offering better hydrophobic drug release characteristics *in vivo*. The method was applied successfully for idarubicin encapsulation.

3. Barriers and solutions in the remote loading methodology

3.1 Drug precipitation and the encapsulation of hydrophobic drugs

To see how the encapsulation method can influence the drug-retaining ability of the liposomes, ciprofloxacin and idarubicin can be compared. It was previously shown that the efficacy of liposomal formulations of certain drugs can be extremely sensitive to drug release rates, with the most slow-releasing systems having the best efficacy profiles [85-88].

As mentioned, during drug loading the concentration inside the liposomes greatly surpasses its concentration in

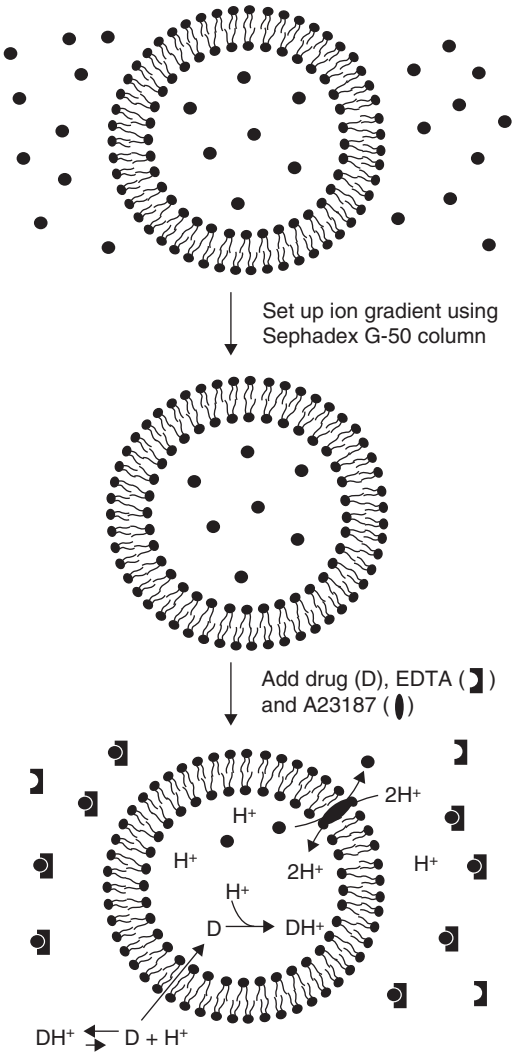

Figure 3. Processes occurring during drug loading in the case of the ionophore drug loading method. The divalent cations are first encapsulated inside vesicles and then the ionophore is added to translocate cation outside. Simultaneously two protons are translocated into the inner water compartment, generating the pH gradient. The EDTA molecules bind the translocated metal cations. A detailed method description is given in the text.
Adapted with permission from [73].

the solution, achieving about a 1000-fold increase relative to the external medium. This causes drug precipitate formation, leading to:

- a decrease in the apparent soluble drug concentration within the liposomes
- a decrease in the ionic strength across the bilayer
- a decrease in the rate of drug release *in vivo*.

The first and second points coordinate the entry of the next molecules and the further accumulation of the drug. In many situations, the formation of drug precipitates is observed [58,59,62,68,88-90].

It has been proved that the formation of drug precipitates with very low solubility is essential to govern the slow release of the drug *in vivo*, especially when the drug has a high bilayer affinity and is therefore pH gradient-sensitive. For drugs with a low bilayer affinity (e.g., doxorubicin), the drug leakage on pH gradient collapse is slow. For relatively hydrophobic bilayer-permeable drugs, the drug release is fast.

The nature of the precipitate is also very important. Its solubility can vary, and only very low solubility precipitates with a slow dissolution rate will serve as good candidates to achieve an ion gradient able to retain the hydrophobic drugs long enough for their practical use *in vivo*. Li *et al.* discussed different doxorubicin precipitate types produced using the citrate method and the monoanionic lactobionic acid method [59]. It was demonstrated that whereas the two organic acids have a similar loading ability, the polyvalent citric acid is able to produce denser precipitates, forming well-organized fibrous bundles. The monovalent lactobionic acid formed only randomly orientated single fibers filling the liposome interior. The randomly organized fibers of the drug precipitate influenced rapid drug release under *in vitro* conditions when compared with citrate liposomes.

Ciprofloxacin is a synthetic bacterial fluoroquinolone antibiotic with broad-spectrum efficacy against a wide variety of bacteria. At a neutral pH, it has poor solubility and requires a long infusion with the solution at a low concentration to avoid drug crystallization at the site of administration. As a zwitterion, it cannot be loaded simply by a citrate pH gradient [91]. Lasik *et al.* demonstrated the remote loading of ciprofloxacin for the first time by means of the ammonium sulfate method [68]. Using a methyl ammonium sulfate gradient, Webb *et al.* achieved nearly 100% encapsulation efficiency (D/L ratio 0.3) and obtained a very stable formulation *in vitro*, which retained 100% of the encapsulated drug over the course of 18 weeks at 4°C [91]. Although in general *in vivo* drug leakage was much slower than that for free drug pharmacokinetics, further approaches were undertaken to achieve slower drug release characteristics similar to those observed for Doxil. Lasic *et al.* suggested that the drug precipitates inside the liposomes and the relatively rapid drug release *in vitro* (observed when the ammonium sulfate method was applied) was a result of grooving of the ciprofloxacin crystals, leading to liposome rupture [68]. Maurer *et al.* observed liposomal formulations of ciprofloxacin to investigate the nature of ciprofloxacin encapsulation inside liposomes by means of the ammonium sulfate method. By applying ^{1}H-NMR spectroscopy, they were able to conclude that ciprofloxacin is located in the aqueous interior of the liposomes in the form of stacks consisting of a small number of ciprofloxacin molecules. The drug is not precipitated, although the intraliposomal ciprofloxacin concentration can exceed its solubility by orders of magnitude [69]. Speculation on possible massive drug–bilayer interactions or the crystals causing

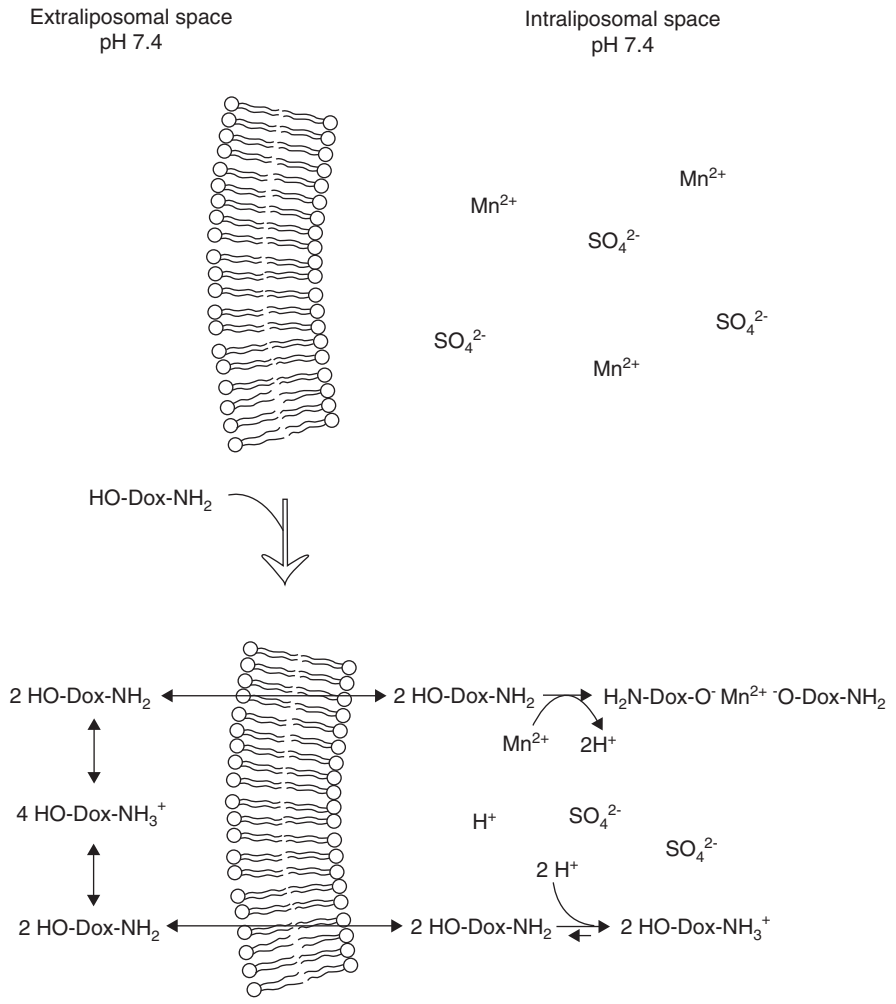

Figure 4. Schematic representation of the processes occurring during anthracycline (doxorubicin, Dox) loading by means of the transit metal ion method. Despite the lack of the proton (pH) gradient the drug is translocated throughout the bilayer and forming low soluble coordinate complex precipitate. Additionally the released protons protonize the doxorubicin molecules and arrest them inside of liposomes.
Adapted from [78].

bilayer rupture was not proved. The drug in its mostly soluble form was then released as a result of its faster portioning through the bilayer after pH gradient collapse.

This is a clear indication of the importance of drug precipitation: although the drug was encapsulated by means of the remote loading method, its plasma concentration was not optimal. The drug precipitation and the nature of the precipitate have a vital impact on the drug release kinetics *in vivo*. The same group published the results of a study where the formation of intraliposomal ciprofloxacin precipitates was finally achieved [88]. By applying the ionophore loading method in combination with arylsulfonate calcium salts, the authors were able to encapsulate stably ciprofloxacin (and vinorelbin) and finally show the formation of the drug precipitates inside the liposomes by applying cryo-electron microscopy (cryo-EM). In **Figure 5**, the influence of the state and dissolution rate of the precipitate on drug release kinetics is presented. The denser the precipitate and the slower the dissolution rate of the precipitate, the slower the drug release from the liposomes, even if the drug has strong bilayer interaction properties.

Idarubicin is an example of an anthracycline family drug that has been shown *in vitro* to be significantly more active than daunorubicin or doxorubicin. It is more lipophilic than daunorubicin and shows improved adsorption across the gastrointestinal mucosa [92].

The dynamics of encapsulation of idarubicin via the citrate or ammonium sulfate methods led to the conclusion that this anthracycline is rapidly bilayer permeable, and thus its rapid release from the liposomes can be observed [58,67]. Dos Santos's and the author's groups' approaches were different in terms of achieving slower idarubicin release properties

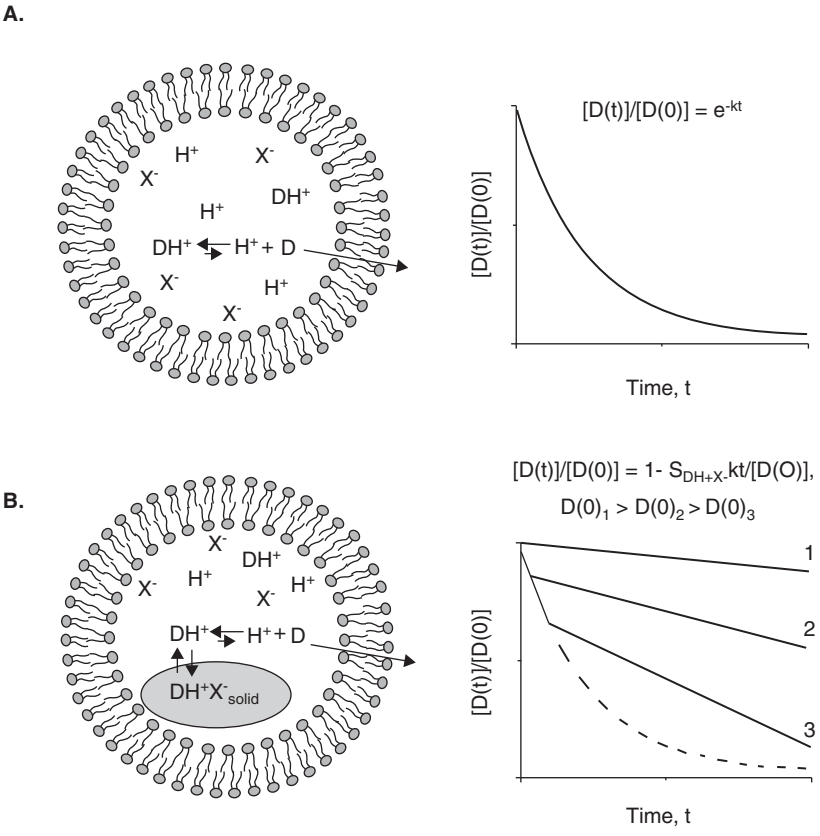

Figure 5. Scheme representing the difference of fast bilayer permeable drug release (proposed for relatively hydrophobic ciprofloxacin) in the case when the drug is (A) in the soluble form and (B) in the form of a low solubility precipitate. The greater the quantity of drug remaining in the form of the precipitate, the slower its release. A detailed scheme discussion is given in [88].

in vivo. As idarubicin has hydrophobic properties further enhanced by anthracycline–cholesterol interactions dos Santos *et al.* proposed a cholesterol-free formulation, which showed superiority over cholesterol-containing formulations. The drug leakage rate was slower, but only after 1 – 4 h post-liposome injection. The decrease of PEGylated lipid content from 5 to 2% and citrate ionic strength from 300 to 150 mm resulted in a further increase of the drug half-life in a murine system and increase of the drug activity in mice bearing murine P388 WT leukemia and LCC6/WT breast cancer xenografts in Rag2-M mice compared with free drug injection [55]. The author's group's approach was different: a cholesterol-containing PEGylated formulation was used to find out whether the nature of the drug precipitate and its dissolution rate can influence the drug release rate even if cholesterol-containing liposomes are applied. For this purpose, a new EDTA ion gradient method was developed, because the EDTA-idarubicin salts possess low solubility, and also because the salt dissolution rate seemed low *in vitro*. This approach gave better results compared with simple depletion of the cholesterol, but less pronounced than observed in the dos Santos experiments [67]. According to the citrate and EDTA-idarubicin salts solubility test, a large portion of the unprecipitated idarubicin should be present inside the citrate liposomes, whereas in the EDTA liposomes most of the drug forms a low solubility precipitate. What emerges from the two approaches is that a formulation of EDTA-loaded cholesterol-free liposomes should be examined in terms of the optimized drug release properties. As can be seen, optimal bilayer composition along with low solubility precipitate form can contribute to obtaining a suitable liposomal formulation, especially in the case of hydrophobic drugs such as idarubicin, where its liposome retention is still not optimal to accumulate efficiently in the tumor tissue by the EPR effect.

In the case of a cholesterol-free formulation, other anthracyclines can also be applied, but unlike idarubicin, doxorubicin cannot be loaded at 37°C [54]. For doxorubicin loading, 60°C is needed. This causes a problem connected with the sharp phase transition temperature of the pure liposome lipids. The high cholesterol content abolishes the transition where the bilayer permeability reaches its peak. For cholesterol-free liposomes, the encapsulation of liposomes near 60°C will cause rapid solute leakage and pH gradient

collapse. Therefore, an ethanol-induced loading method was developed by the same group to make the platform of cholesterol-free liposomes complete [54]. Dos Santos *et al.* examined the influence of the presence of 10% v/v ethanol in the incubation mixture on doxorubicin loading into liposomes. At 4 and 23°C, practically no drug accumulation was observed in the DSPC/DSPE-PEG 2000 95:5 mol/mol liposomes, but when the temperature was increased to 37°C, > 90% encapsulation efficiency of the doxorubicin was observed at the initial D/L ratio of 0.2 [54]. This platform seems to be a very useful tool in the case of thermosensitive liposomes where no cholesterol-containing vesicles are commonly in use. Recently this problem was solved by applying a different approach – drug loading at temperatures just below the phase transition temperature of the main lipid so the use of ethanol seems to be redundant [93].

3.2 Transition metal complexation

The transition metal ion encapsulation methodology seems to be not only interesting but also very helpful for achieving a high drug payload within liposomes in situations where other methods have failed. It is important to mention that this approach not only allows the encapsulation of the drug at a high D/L ratio, but also can be a method to obtain the proper drug release properties in the case of relatively hydrophobic drugs. As ciprofloxacin does not produce low solubility complexes with transition metal ions, other drugs were selected for this type of encapsulation. Besides doxorubicin (an anthracycline), which can be loaded using this technology, other drug classes also possess coordination sites capable of complexing transition metals. These drugs are: bleomycin, amikacin, non-steroidal anti-inflammatory drugs (NSAIDs) and camptothecins [83]. The main difference between the other encapsulation systems and the drug complexation with the transition metal ions is the lack of a pH gradient, which is the driving force in the other methods.

Amandeep Taggar *et al.* investigated copper-mediated topotecan encapsulation. They applied the DSPC/Chol 55:45 liposomal formulation to encapsulate the water-soluble analogue of the topoisomerase I inhibitor camptothecin [83]. Of several selected transit/transition metal gradients (Co^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+}), only the copper ions were able to drive the drug uptake with no pH gradient. Earlier investigations showed high drug loading in the presence of pH gradients, but the results did not seem to satisfy the research group [89]. A D/L ratio of 0.15 was achieved with > 80% encapsulation efficiency. The drug precipitation inside the drug loaded vesicles was observed as monitored by the cryo-EM technique.

Others have investigated mitoxantrone loading by applying this technique. The drug was efficiently loaded by complexation with copper and nickel ions with very high stability both *in vitro* and *in vivo*. Despite very good stability, a lower activity against L1210 murine tumor cells compared with the free drug was observed. It was concluded that faster drug leakage is needed when mitoxantrone is used in a liposomal system [79,80].

3.3 The drug-to-lipid ratio influences the drug therapeutic index

Besides liposomal drug accumulation at the site of cancer tissue mediated by EPR phenomena, another factor also emerges from several observations that drug release rates are also important at the tumor site. It was demonstrated that for the cell cycle-specific drug vincristine, the release rate can influence the liposome activity *in vivo* [94,95]. This arises from the prolonged exposure of cells to cell cycle-specific agents, resulting in greater cell death *in vitro* and enhanced antitumor activity *in vivo* [77]. Johnston *et al.* examined the influence of a vincristine liposomal formulation with the drug encapsulated at different D/L ratios [77]. As shown in **Figure 5**, the rate of bilayer-permeable drug release is dominantly correlated with its precipitate state. The precipitate can be dense with a single crystal appearance (bundle) or diffused as separates fibers inside the liposome [32]. The precipitate drug dissolution rate can then be slower or faster depending on the precipitate form and its solubility. The initial drug content (D/L ratio) influences the proportion of the free drug population inside the liposomes and the precipitated form of a slowly dissolved drug deposit. At a low D/L, most of the drug will be in the free form, but at high D/L the amount of free drug in the internal solution will potentially be the same, with a significantly increased portion of the precipitated drug. In the case of vincristine, the ionophore loading method was selected as offering the advantage of achieving very high drug-to-lipid ratios, promoting the formation of a dense drug precipitate [77]. The study showed that an increase in the D/L ratio from 0.1 to 0.3 w/w resulted in an increase in the half-life for the drug release of more than a factor of two. The ionophore loading gave the possibility of assessing the hypothesis that very high D/L results in the formation of internal drug precipitates. Indeed, at a D/L of 1.0, some structures with a grainy appearance were observed. One of the main conclusions arising from the study is that the half-life of vincristine release from the liposomes is linearly dependent on the drug-to-lipid ratio. This was observed both *in vitro* and *in vivo*, and it is a good example showing that the optimal liposomal formulation must be comprehensively elaborated to find all of the possible relationships between the drug encapsulation method, the optimal D/L ratio, the drug characteristics (the drug action mechanism) and the liposome formulation. In the case of very high D/L, only a low lipid dose is administered with a given drug amount. To overcome the problem of the rapid elimination of liposomes, as the rate of elimination is lipid dose-dependent, the MPS was pre-saturated with empty liposomes to avoid drug-containing liposomes being taken up by liver or spleen macrophages. Formulations with different D/L ratios were finally administered to the mice with a human MX-1 xenograft model at a dose of 1.5 mg/kg of both free

and liposomal (SM/Chol 55/45 mol/mol) vincristine. As expected, the liposomes showed increased antitumor activity over the free drug, but the optimal liposomal activity was not achieved for the higher D/L (0.6) used in the study but for a much lower one (0.1). This suggests that at least in this cancer model, the D/L ratio yielded slow release properties that were far from optimal [77].

Recently, Noble *et al.* proposed a new approach to retain the vincristine and vinblastine inside Doxil-like liposomes using a triethylammonium sucrose octasulfate that forms an electrostatically stabilized complex with these drugs. The formulation showed very good vincristine pharmacokinetics with drug retention similar to Doxil liposomes and good vinblastine retention. To increase drug internalization, both liposomal formulations were prepared as immunoliposome formulations with anti-HER2 antibodies attached to PEG terminals. These targeted versions showed much superior activity in mice bearing the human BT474-M2 tumor xenograft model. In this case a high drug-to-lipid ratio was not necessary to achieve the proper drug retention, but similarly to the above example, a low drug leakage rate was essential [96].

3.4 Active encapsulation of water-insoluble drugs

A totally new strategy is now emerging from the work published recently by Zhigaltsev *et al.* [90]. In the author's opinion, the next milestone of liposomology was achieved by the demonstration of active loading of water-insoluble drugs. The taxane representative docetaxel was selected as it possesses slightly more hydrophilic properties than paclitaxel. Docetaxel is a representative of the two-member taxane family, which has broad anticancer activity against a variety of cancers, and is administered as a micellar Tween 80 formulation as Taxotere® (Sanofi Aventis, USA).

Both drugs are totally water insoluble, and for their administration they require a hydrophobic carrier. Both drugs can be liposome formulated, but very low stability is observed for liposomal taxane formulations due to drug crystallization in the external solution [97]. The crystallization process can be fast or slow, depending on the bilayer properties. For liposomes with relatively fluid bilayers, the taxane stability can be extended up to several months, but with a relatively low D/L ratio. For rigid liposomal formulations, the overall stability, understood as the time before crystal formation, is very short and can be counted in days, hours or only minutes [98]. The drug crystallization is caused by dimer formation of the taxanes preceded by hydrogen bond formation between the two taxane molecules. Balasubramanian and Straubinger demonstrated that 2.8 mol% is the maximal bilayer taxane capacity [99]. Above that, the dimer formation accelerates and the drug is released from the bilayer to form crystal precipitates in the liposome suspension. In this light, achieving a therapeutically optimal drug concentration using liposomes seems impossible. Because of the above problems associated with the low stability of liposomes combined with a low D/L ratio and a rather fast drug release *in vivo*, the possibility of active encapsulation of taxanes is very welcome.

As neither taxane possesses ionizable groups and they cannot be directly loaded by a pH gradient, Zhigaltsev *et al.* prepared a weak base docetaxel derivative by derivatization of the hydroxyl group in the C-2′ position to form an *N*-methylpiperazinyl butanoic acid ester. The resulting docetaxel prodrug could then be described as water soluble (1.7 mg/ml at pH 7.4) and possessing amphiphilic properties, which is commonly required for active encapsulation. Indeed, the prodrug was actively loaded by an ammonium sulfate gradient into DSPC or DPPC or DMPC/Chol 55:45 mol/mol liposomes at 60°C. The encapsulation efficiency was nearly 100% at an initial drug-to-lipid ratio of 0.4. The liposomal formulation was stable during 4-month storage at 4°C. Inside the liposomes, the drug needles were visualized using cryo-EM. Direct comparison of the parent docetaxel formulation (Taxotere) with DSPC/Chol prodrug formulation showed drastically different drug pharmacokinetics with excellent drug retention for the liposomal formulation. Also, *in vivo* animal studies on the NDA435/LCC6 human breast carcinoma model (at a dose of 25, 40 or 88 mg/kg for the liposomal docetaxel prodrug and 25 mg/kg for micellar docetaxel) showed the superiority of the DSPC/Chol formulation versus Taxotere or other tested liposomal formulations [90].

This is the first evidence that hydrophobic drugs can be modified to form a labile prodrug and then actively loaded to achieve the high D/L ratio required for optimal drug activity.

3.5 Active drugs' co-encapsulation

A very interesting opportunity is emerging from the fact that two drugs can be co-encapsulated together within the structure of a single liposome. There is considerable evidence that more and more recent curative cancer treatments are using drug combinations to overcome the natural tendency of cancer cells to develop a resistance mechanism against a single medication. The possibility of achieving resistance against two drugs in combination is less likely, so the probability of successful treatment is higher. By introducing two or even three anticancer agents there is more room to overcome the natural lines of defense of the cancer cells. As mentioned earlier, liposomes have the potential to administer much higher drug amounts to the cancer tissue compared with other drug carriers, owing to passive vesicle accumulation at the site of the inflammatory process, always correlated with cancer progression. This is further enforced by a very high drug payload achieved by active drug loading. Tardi *et al.* investigated the appropriate dosing of the two anticancer agents floxuridine and irinotecan to achieve the optimal drug/drug balance having the most beneficial anticancer effect [84]. It was earlier discovered that for a given drug combination, the activity can be dramatically dependent on the molecular drug ratios. Some ratios of drug combination can be synergistic, whereas other ratios of the same drugs can be additive or even antagonistic [100,101].

This observation created the opportunity to elaborate a liposomal formulation of the optimal drug/drug molar ratio formulation combined with an equal drug *in vivo* release. By applying a modified transition metal encapsulation procedure, the researchers [84] were able to co-encapsulate irinotecan by its complexation with copper ion inside the vesicles with nearly 80% encapsulation efficiency and 0.18:1 drug-to-lipid ratio without a pH gradient being established. The floxuridine was passively loaded simultaneously with irinotecan encapsulation at 50°C. As a result of co-encapsulation ~ 80% of the initial irinotecan dose was encapsulated, while 50% encapsulation efficiency of the floxuridine (equilibrium of the drug concentration from both bilayer sides) was achieved. As the initial floxuridine medium concentration was higher than the irinotecan concentration, the inside of liposomes reached an almost equimolar concentration compared with irinotecan, as required for synergistic drug activity in the cancer cells [84]. As in the case of idarubicin, the cholesterol content was investigated in terms of the influence of drug retention *in vivo* [58]. It was then found that in the case of floxuridine, rapid drug leakage was noted for DSPC/Chol/DSPG 70:10:20 mol/mol liposomes, whereas when instead of cholesterol a 20 mol% of DSPG was used, the drug release was slow. In contrast to floxuridine, irinotecan required at least 10 mol% of cholesterol content to retain the drug inside the liposomes. Finally, the DSPC/Chol/DSPG 70:10:20 m/m formulation was the compromise needed to retain both drugs at the same optimal 1:1 molar level during *in vivo* circulation. Ultimately, if the trend presented in the cited papers generates wider feedback in the field of liposomology, then the need for more sophisticated methods of drug co-encapsulation will be essential. If this path is followed, the next step could be a similar approach utilizing other drug cocktails at optimized drug ratios, giving an optimized drug ratio at the tumor site but also possessing the properties of selective targeting of the active drug or its liberation on demand.

4. Expert opinion

Since their discovery, liposomes have become an important drug delivery system that offers rare characteristics, including small size, long circulation life, the so-called EPR effect and a very high drug-to-lipid ratio for drugs loaded via an active process. When the next generation of targeted liposomes finally reaches the production level, it will certainly cause a new revolution in cancer therapy, as was observed after the introduction of Doxil. Without doubt, these systems require further intensive research, but browsing the literature one can be certain that essential progress in liposome preparation techniques, drug encapsulation and targeting has already been achieved. Now is the time to wait for the first fruits of the next generation of liposomes.

The active loading techniques are very important in this liposomal revolution. Without high drug-to-lipid ratios the activity of the liposomes would be limited. From this review, one conclusion is clear: there is no single universal encapsulation method that offers stable encapsulation of most drugs. Each drug requires a different approach to manage all of its properties. The ammonium sulfate method is one of the best, as it is able to retain most of the weak bases, but in the case of ciprofloxacin and some vinca alkaloids, it seems to fail. The ionophore method allows the encapsulation of a similar drug range, but by juggling different metal ions, it is better able to withstand some lipophilic drug leakage. The highest D/L ratio (1.0) was observed for this method.

The author's personal candidate for future drug encapsulation is the methods based on the formation of insoluble (or rather low solubility) complexes inside the liposomes. Each drug requires the elaboration of a specific method or set of methods to maintain the desired drug leakage rate, but the final effect can be worth the effort of such an undertaking. As observed in the case of ciprofloxacin, which is difficult to retain long enough *in vivo*, it was not until arylsulfonate salts were applied that optimal retention capabilities were achieved. Active encapsulation required an extra driving force, ionophore loading, so the final methodology was rather complex. The transition metal ion complexation method belongs to this group of encapsulation methods, and it does offer these special features. Unfortunately, only a limited number of drug families can be encapsulated this way. The most important thing is that in most cases the drug should be precipitated in the form of a crystal-like structure to slow down its release.

For water-soluble substances, which have a structure that does not predestine them to be actively loaded, there is a way out, allowing the achievement of similar drug precipitation and thus slower release. In the case of at least some drugs, their solubility is pH dependent (e.g., methotrexate). This drug can be co-loaded by passive encapsulation (e.g., the new equilibrium method) with a specific salt solution that leaks counterions from the liposome interior, decreasing the pH value (e.g., ammonium sulfate or ammonium phosphate) [102]. Unless I am mistaken, the drug should be precipitated inside the vesicles as a response to the lowering of the environmental pH value. In the case of certain drugs, the ionophore loading method can be utilized, albeit in the opposite direction. The transition ions can be actively loaded (as already demonstrated) to form internal drug precipitates with molecules encapsulated using passive methodology. This may offer some of the advantages of the active loading methodology for water-soluble drugs [103]. As mentioned in the Section 3.4, the new drug range can now be loaded via gradient methods by simple derivatization. This will alter our way of approaching the liposome problem, leaving most of the recent problems concerning hydrophobic drugs behind. To follow this idea, the water-soluble drugs (at least some of them) can also be derivatized to form lipophilic cleavable derivatives able to accumulate inside in response to a pH or ion gradient. If this vision is successfully fulfilled, then most drugs can be loaded inside liposomes to achieve the optimal therapeutic level.

There is still one question, of whether the old and new methods of drug loading will fulfil the criteria of increased liposomal drug activity. A detailed look at the recent strategies and achievements clearly demonstrates that to 'accomplish the mission' the methods of simultaneous drug targeting and then release inside cancer cells are needed to make liposomal therapy complete. At present many liposomal formulations suffer from fast drug release, but at least in the case of liposomal doxorubicin, whose retention seems to be optimal, there is certainly a need for its increased internalization and then triggered release after accumulation by the EPR effect. Now is the time to combine all these strategies to achieve the goal of a complex but successful anticancer therapy. The new era of liposomology has just begun.

Declaration of interest

The author declares no conflict of interest and has received no payment for the preparation of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Ehrlich P. Die Seitenkettentheorie und ihre Gegner. Muenchener medizinische Wochenschrift 1901;52:2123-4
2. Cohen BE, Bangham AD. Diffusion of small nonelectrolytes across liposome membranes. Nature 1972;236:173-4
3. Devine DV, Bradley AJ. The complement system in liposome clearance: can complement deposition be inhibited? Adv Drug Deliv Rev 1998;32:19-29
4. Devine DV, Wong K, Serrano K, et al. Liposome-complement interactions in rat serum- implications for liposome survival studies. Biochim Biophys Acta 1994;1191:43-51
5. Ishida T, Harashima H, Kiwada H. Liposome clearance. Biosci Rep 2002;22:197-224
6. Liu DX. Biological factors involved in blood clearance of liposomes by liver. Adv Drug Deliv Rev 1997;24:201-13
7. Liu F, Liu DX. Amphipathic polyethylene glycol stabilized emulsions (o/w): Physical characterization and in vivo distribution. Int J Pharm 1995;125:73-80
8. Liu F, Liu DX. Serum independent liposome uptake by mouse liver. Biochim Biophys Acta 1996;1278:5-11
9. Liu SC, Ishida T, Kiwada H. Effect of serum components from different species on destabilizing hydrogenated phosphatidylcholine-based liposomes. Biol Pharm Bull 1997;20:874-80
10. Oja CD, Semple SC, Chonn A, et al. Influence of dose on liposome clearance: critical role of blood proteins. Biochim Biophys Acta 1996;1281:31-7
11. Senior J, Crawley JCW, Gregoriadis G. Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous-injection. Biochim Biophys Acta 1985;839:1-8
12. Hope MJ, Bally MB, Mayer LD, et al. Generation of multilamellar and unilamellar phospholipid vesicles. Chem Phys Lipids 1986;40:89-107
13. Mayer LD, Hope MJ, Cullis PR. Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim Biophys Acta 1986;858:161-8
14. Papahadjopoulos D, Allen TM, Gabizon A, et al. Sterically stabilized liposomes - improvements in pharmacokinetics and antitumor therapeutic efficacy. PNAS 1991;88:11460-4
15. Pashin YV, Bakhitova LM, Bentkhen TI. Antimutagenic activity of simple phenols and its dependence on the number of hydroxyl groups. Bull Exp Biol Med 1986;102:1121-3
16. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171-99
17. Woodle MC, Newman MS, Cohen JA. Sterically stabilized liposomes: physical and biological properties. J Drug Target 1994;2:397-403
18. Woodle MC, Newman MS, Collins LR, et al. Efficient evaluation of long circulating or stealth liposomes by studies of invivo blood-circulation kinetics and final organ distribution in rats. Biophys J 1990;57:A261
19. Allen TM, Hansen C, Martin F, et al. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim Biophys Acta 1991;1066:29-36
20. Drummond DC, Meyer O, Hong KL, et al. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev 1999;51:691-743
21. Proffitt RT, Williams LE, Presant CA, et al. Tumor-imaging potential of liposomes loaded with in-111-nta - biodistribution in mice. J Nucl Med 1983;24:45-51
22. Yuan F, Leuning M, Huang SK, et al. Microvascular permeability and interstitial penetration of sterically stabilized (Stealth) liposomes in a human tumor xenograft. Cancer Res 1994;54:3352-6
23. Loi M, Marchio S, Becherini P, et al. Combined targeting of perivascular and endothelial tumor cells enhances anti-tumor efficacy of liposomal chemotherapy in neuroblastoma. J Control Release 2010;145:66-73

•• **Important paper about significance of liposome targeting and increased activity when combined liposome targeting is applied.**

24. Pastorino F, Di Paolo D, Loi M, et al. Recent advances in targeted anti-vasculature therapy: the neuroblastoma model. Curr Drug Targets 2009;10:1021-7
25. Pastorino F, Marimpietri D, Brignole C, et al. Ligand-targeted liposomal therapies of neuroblastoma. Curr Med Chem 2007;14:3070-8
26. Sapra P, Allen TM. Ligand-targeted liposomal anticancer drugs. Progress Lip Res 2003;42:439-62

•• **A good review about liposome targeting.**

27. Barenholz Y, Amselem S, Goren D, et al. Stability of liposomal doxorubicin formulations - problems and prospects. Med Res Rev 1993;13:449-91

28. Barenholz Y, Bolotin E, Cohen R, et al. Sterically stabilized doxorubicin loaded liposomes (DOX-SL(TM)): from basics to the clinics. Phosphorus Sulfur Silicon Relat Elem 1996;109:293-6
29. Emanuel N, Kedar E, Bolotin EM, et al. Preparation and characterization of doxorubicin-loaded sterically stabilized immunoliposomes. Pharm Res 1996;13:352-9
30. Gabizon A, Catane R, Uziely B, et al. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res 1994;54:987-92
31. Gabizon A, Isacson R, Libson E, et al. Clinical-studies of liposome-encapsulated doxorubicin. Acta Oncol 1994;33:779-86
32. Cullis PR, Hope MJ, Bally MB, et al. Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. Biochim Biophys Acta 1997;1331:187-211
33. Lasic DD. Liposomes: from physics to aplications. Elsevier Science B.V., Amsterdam; 1993
34. Lasic DD. The mechanism of vesicle formation. Biochem J 1988;256:1-11
35. Szoka FC. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu Rev Biophys Bioeng 1980;9:467-508
36. Lichtenberg D, Barenholz Y. Liposomes - preparation, characterization, and preservation. Methods Biochem Anal 1988;33:337-462
37. Woodle MC, Papahadjopoulos D. Liposome preparation and size characterization. 1997. Academic Press, INC, San Diego; 1997
38. Mayer LD, Hope MJ, Cullis PR, et al. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochim Biophys Acta 1985;817:193-6
39. Batzri S, Korn ED. Single bilayer liposomes prepared without sonication. Biochim Biophys Acta 1973;298:1015-19
40. Wagner A, Platzgummer M, Kreismayr G, et al. GMP production of liposomes - A new industrial approach. J Liposome Res 2006;16:311-19
41. Szoka F, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. PNAS 1978;75:4194-8
42. Embree L, Gelmon KA, Lohr A, et al. Chromatographic analysis and pharmacokinetics of liposome-encapsulated doxorubicin in non-small-cell lung-cancer patients. J Pharm Sci 1993;82:627-34
43. Frezard F, Garnier-Suillerot A. Permeability of lipid bilayer to anthracycline derivatives. Role of the bilayer composition and of the temperature. Biochim Biophys Acta 1998;1389:13-22
44. Gregoriadis G, Panagiotidi C. Immunoadjuvant action of liposomes: comparison with other adjuvants. Immunol Lett 1989;20:237-40
45. Gallois L, Fiallo M,Garnier Suillerot A. Comparison of the interaction of doxorubicin, daunorubicin, idarubicin and idarubicinol with large unilamellar vesicles - Circular dichroism study. Biochim Biophys Acta 1998;1370:31-40
46. Harrigan PR, Wong KF, Redelmeier TE, et al. Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients. Biochim Biophys Acta 1993;1149:329-38
47. Madden TD, Harrigan PR, Tai LCL, et al. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient - a survey. Chem Phys Lipids 1990;53:37-46
• **A good review of active drug encapsulation theory and methodology.**
48. Regev R, Yeheskely-Hayon D, Katzir H, et al. Transport of anthracyclines and mitoxantrone across membranes by a flip-flop mechanism. Biochem Pharmacol 2005;70:161-9
49. Chakrabarti AC, Clarklewis I, Harrigan PR, et al. Uptake of basic-amino-acids and peptides into liposomes in response to transmembrane pH gradients. Biophys J 1992;61:228-34
50. Bally MB, Mayer LD, Loughrey H, et al. Dopamine accumulation in large unilamellar vesicle systems induced by transmembrane ion gradients. Chem Phys Lipids 1988;47:97-107
51. Mayer LD, Bally MB,Cullis PR. Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. Biochim Biophys Acta 1986;857:123-6
52. Mayer LD, Bally MB, Hope MJ, et al. Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane-potential. Biochim Biophys Acta 1985;816:294-302
53. Chou TH, Chen SC,Chu IM. Effect of composition on the stability of liposomal irinotecan prepared by a pH gradient method. J Biosci Bioeng 2003;95:405-8
54. Dos Santos N, Cox KA, McKenzie CA, et al. pH gradient loading of anthracyclines into cholesterol-free liposomes: enhancing drug loading rates through use of ethanol. Biochim Biophys Acta 2004;1661:47-60
55. Dos Santos N, Waterhouse D, Masin D, et al. Substantial increases in idarubicin plasma concentration by liposome encapsulation mediates improved antitumor activity. J Control Release 2005;105:89-105
•• **Interesting example of the influence of the bilayer composition on drug retention.**
56. Forssen EA. The design and development of DaunoXome(R) for solid tumor targeting in vivo. Adv Drug Deliv Rev 1997;24:133-50
57. Swenson CE, Perkins WR, Roberts P, et al. Liposome technology and the development of Myocet (TM) (liposomal doxorubicin citrate). Breast 2001;10:1-7
58. Dos Santos N, Mayer LD, Abraham SA, et al. Improved retention of idarubicin after intravenous injection obtained for cholesterol-free liposomes. Biochim Biophys Acta 2002;1561:188-201
59. Li XG, Hirsh DJ, Cabral-Lilly D, et al. Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient. Biochim Biophys Acta 1998;1415:23-40
60. Haran G, Cohen R, Bar LK, et al. Transmembrane ammonium-sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta 1993;1151:201-15
61. Barenholz Y. Liposome application: problems and prospects. Curr Opin Colloid Interface Sci 2001;6:66-77

62. Fritze A, Hens F, Kimpfler A, et al. Remote loading of doxorubicin into liposomes driven by a transmembrane phosphate gradient. Biochim Biophys Acta 2006;1758:1633-40

63. Seynhaeve ALB, Hoving S, Schipper D, et al. Tumor necrosis factor alpha mediates homogeneous distribution of liposomes in murine melanoma that contributes to a better tumor response. Cancer Res 2007;67:9455-62

•• **The method of increase of the EPR effect is given.**

64. Laginha KM, Verwoert S, Charrois GJR, et al. Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. Clin Cancer Res 2005;11:6944-9

65. Lindner LH, Hossann M. Factors affecting drug release from liposomes. Curr Opin Drug Discovery Dev 2010;13:111-23

66. Torchilin VP. Multifunctional nanocarriers. Adv Drug Deliv Rev 2006;58:1532-55

67. Gubernator J, Chwastek G, Korycinska M, et al. The encapsulation of idarubicin within liposomes using the novel EDTA ion gradient method ensures improved drug retention in vitro and in vivo. J Control Release 2010;146:68-75

68. Lasic DD, Ceh B, Stuart MCA, et al. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochim Biophys Acta 1995;1239:145-56

69. Maurer N, Wong KF, Hope MJ, et al. Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a H-1-NMR study. Biochim Biophys Acta 1998;1374:9-20

70. Zucker D, Marcus D, Barenholz Y, et al. Liposome drugs' loading efficiency: a working model based on loading conditions and drug's physicochemical properties. J Control Release 2009;139:73-80

•• **What are the best encapsulation conditions for a given drug? Find out in this paper.**

71. Clerc S, Barenholz Y. Loading of amphipathic weak acids into liposomes in response to transmembrane calcium acetate gradients. Biochim Biophys Acta 1995;1240:257-65

72. Deamer DW, Crofts AR, Prince RC. Response of fluorescent amines to ph gradients across liposome membranes. Biochim Biophys Acta 1972;274:323-35

73. Fenske DB, Wong KF, Maurer E, et al. Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients. Biochim Biophys Acta 1998;1414:188-204

74. Erdahl WL, Chapman CJ, Taylor RW, et al. Ca2+ transport-properties of ionophores a23187, ionomycin, and 4-bra23187 in a well-defined model system. Biophys J 1994;66:1678-93

75. Erdahl WL, Chapman CJ, Taylor RW, et al. Effects of pH conditions on Ca2+ transport catalyzed by ionophores A23187, 4-BrA23187, and ionomycin suggest problems with common applications of these compounds in biological systems. Biophys J 1995;69:2350-63

76. Pressman BC. Biological applications of ionophores. Ann Rev Biochem 1976;45:501-30

77. Johnston MJW, Semple SC, Klimuk SK, et al. Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations. Biochim Biophys Acta 2006;1758:55-64

78. Cheung BCL, Sun THT, Leenhouts JM, et al. Loading of doxorubicin into liposomes by forming Mn2+-drug complexes. Biochim Biophys Acta 1998;1414:205-16

79. Cui J, Li CL, Wang LF, et al. Ni2 +-mediated mitoxantrone encapsulation: Improved efficacy of fast release formulation. Int J Pharm 2009;368:24-30

80. Li CL, Cui JX, Li YG, et al. Copper ion-mediated liposomal encapsulation of mitoxantrone: the role of anions in drug loading, retention and release. Eur J Pharm Sci 2008;34:333-44

81. Abraham SA, McKenzie C, Masin D, et al. In vitro and in vivo characterization of doxorubicin and vincristine coencapsulated within liposomes through use of transition metal ion complexation and pH gradient loading. Clin Cancer Res 2004;10:728-38

82. Ramsay E, Alnajim J, Anantha M, et al. A novel liposomal irinotecan formulation with significant anti-tumour activity: use of the divalent cation ionophore A23187 and copper-containing liposomes to improve drug retention. Eur J Pharm Biopharm 2008;68:607-17

83. Taggar AS, Alnajim J, Anantha M, et al. Copper-topotecan complexation mediates drug accumulation into liposomes. J Control Release 2006;114:78-88

84. Tardi PG, Gallagher RC, Johnstone S, et al. Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo. Biochim Biophys Acta 2007;1768:678-87

85. Boman NL, Bally MB, Cullis PR, et al. Encapsulation of vincristine in liposomes reduces its toxicity and improves its anti-tumor efficacy. J Liposome Res 1995;5:523-41

86. Boman NL, Masin D, Mayer LD, et al. Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing p388 tumors. Cancer Res 1994;54:2830-3

87. Charrois GJR, Allen TM. Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer. Biochim Biophys Acta 2004;1663:167-77

88. Zhigaltsev IV, Maurer N, Edwards K, et al. Formation of drug-arylsulfonate complexes inside liposomes: a novel approach to improve drug retention. J Control Release 2006;110:378-86

89. Abraham SA, Edwards K, Karlsson G, et al. An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes. J Control Release 2004;96:449-61

90. Zhigaltsev IV, Winters G, Srinivasulu M, et al. Development of a weak-base docetaxel derivative that can be loaded into lipid nanoparticles. J Control Release 2010;144:332-40

•• **The new strategies for hydrophobic drug encapsulation.**

91. Webb MS, Boman NL, Wiseman DJ, et al. Antibacterial efficacy against an in vivo Salmonella typhimurium infection model and pharmacokinetics of a liposomal ciprofloxacin formulation. Antimicrob Agents Chemother 1998;42:45-52

92. Boccardo F, Cannata D, Cussotto M, et al. Intravesical idarubicin: a dose finding study. Cancer Chemother Pharmacol 1996;38:102-5

93. Hossann M, Wang TT, Wiggenhorn M, et al. Size of thermosensitive liposomes influences content release. J Control Release 2010;147:436-43

94. Mayer LD, Nayar R, Thies RL, et al. Identification of vesicle properties that enhance the antitumor-activity of liposomal vincristine against murine l1210 leukemia. Cancer Chemother Pharmacol 1993;33:17-24

95. Zhigaltsev IV, Maurer N, Akhong QF, et al. Liposome-encapsulated vincristine, vinblastine and vinorelbine: a comparative study of drug loading and retention. J Control Release 2005;104:103-11

96. Noble CO, Guo ZX, Hayes ME, et al. Characterization of highly stable liposomal and immunoliposomal formulations of vincristine and vinblastine. Cancer Chemother Pharmacol 2009;64:741-51

97. Hennenfent KL,Govindan R. Novel formulations of taxanes: a review. Old wine in a new bottle? Ann Oncol 2006;17:735-49

98. Crosasso P, Ceruti M, Brusa P, et al. Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. J Control Release 2000;63:19-30

99. Balasubramanian SV, Straubinger RM. Taxol-lipid interactions - taxol-dependent effects on the physical-properties of model membranes. Biochem 1994;33:8941-7

100. Mayer LD, Harasym TO, Tardi PG, et al. Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice. Mol Cancer Ther 2006;5:1854-63

101. Mayer LD, Janoff AS. Optimizing combination chemotherapy by controlling drug ratios. Mol Interv 2007;7:216-23

102. Woo J, Chiu GNC, Karlsson G, et al. Use of a passive equilibration methodology to encapsulate cisplatin into preformed thermo sensitive liposomes. Int J Pharm 2008;349:38-46

103. Wheeler JJ, Veiro JA,Cullis PR. Ionophore-mediated loading of ca2+ into large unilamellar vesicles in response to transmembrane ph gradients. Mol Membr Biol 1994;11:151-7

Affiliation

Jerzy Gubernator PhD
Professor Assistant,
University of Wrocław,
Faculty of Biotechnology,
Laboratory of Lipids and Liposomes,
Przybyszewskiego 63/77,
51-148 Wrocław, Poland
Tel: +4871 3756338; Fax: +4871 3756234;
E-mail: gubern@ibmb.uni.wroc.pl