

## Understanding the Stealth Properties of PEGylated lipids: A Mini-Review

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### Abstract

PEGylation is a well-established strategy for improving the target specificity, circulation time and stability of liposomes, thereby improving their stealth properties. This brief review provides an insight on the composition of PEGylated liposomes and the characteristics that dictate the functionality of PEGylated liposomes such as surface density, molecular weight, presence of linkers and acyl groups. Physicochemical techniques used to characterize the PEG liposomes and test their stability are also discussed along with their clinical implications. This review provides the readers with a broad range of understanding of various PEGylated lipids, techniques to access their stability in liposomal formulations and state-of -the-art development of PEGylated liposomal formulations.

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**Keywords:** PEG (Polyethylene Glycol) , liposomes, Differential scanning calorimetry (DSC) , dynamic light scattering (DLS) , drug delivery

**Received:** Jun 24, 2020

**Accepted:** Aug 14, 2020

**Published:** Aug 24, 2020

**Editor:** Xiaoyong Lu, Department of Chemistry and Biochemistry, Ohio University, Athens, Ohio, 45701 USA.

## Review of PEGylated Lipids

The lipid molecules comprise of a hydrophilic head group and hydrophobic tail region, in presence of an aqueous medium the lipids self-assemble to form liposomes. [1–6] The presence of hydrophilic aqueous core and hydrophobic lipid bilayer region enables them to encapsulate both hydrophobic and hydrophilic molecules, making them ideal candidates for vaccines [7–9] and drug carriers [10, 11] compared to metal [12, 13], polymer [14–16], and dendrimer [17–19]-based carries. The properties of liposomes can be tuned by inclusion of polymers [20–22], cholesterol [23–25] or membrane proteins [26, 27], choice of lipids and sizing methods to improve their biodegradability, biocompatibility and versatility [7, 8, 28] for potential applications in encapsulation of drugs used in cancer therapy. Besides, liposomes can be customized according to size, charge and number of lamellae depending on the applications [29, 30]. The classification of liposomal drug delivery is broadly divided into conventional and long circulation liposomes.

### *Conventional and Long Circulating Liposomes*

Conventional or the first-generation liposomes were developed commercially in the beginning of 1980s for the delivery of hydrophobic doxorubicin and amphotericin [31, 32]. These liposomes comprised of a combination of cationic, anionic and zwitterionic phospholipids in conjunction with cholesterol. However, conventional liposomes were found to attract plasma proteins, also called as opsonin proteins, from the blood stream. The opsonins serve as identification markers for macrophages [33, 34]. Opsonins attach themselves electrostatically to the surface of the lipids. The presence of opsonins on the liposomal surface makes them susceptible to macrophage attack and eventually, their removal through the reticuloendothelial system (RES). This phenomenon is also known as liposomal opsonization [35–37]. A comprehensive review on opsonization mechanisms of conventional liposomes is previously presented [34]. Therefore, various strategies have been exploited to induce stealth-ness in liposomes to prevent their opsonization and impart longer circulation in the bloodstream [32, 38, 39]. One of the commonly used techniques is introduction of steric stabilization by augmenting the liposomal surface with hydrophilic polymers as shown in Fig 1 [35, 40, 41]. Fig

1 represents the effects of steric stabilization on RES clearance; opsonins (shown in green) adhere only to conventional liposomes and not to the sterically stabilized liposomes (polymer chains shown in pink). This results in attack of conventional liposomes by the macrophage cells and subsequent clearance by RES. However, sterically stabilized liposomes have longer circulation times and successfully reach the tumor sites. Stealth nature of the liposomes is responsible for longer circulation times. This stealth nature is affected by the polymer's hydrophilicity, spatial conformation, density and molecular weight [42–44].

Some commonly reported polymers include PLA (poly lactic acid) [42], polystyrene (PSt) [43, 44], polyvinyl alcohol (PVA), polyacrylamide [43], polysaccharides [43, 45, 46], and Polyethylene glycol (PEG). However, PLA and PSt are highly anionic with zeta potentials ranging between  $-20$  and  $-76$  mV, due to the presence of carboxy groups, and these coatings are easily attacked by macrophages [47–51], polysaccharide coatings on the other hand activate the immune system, due to the presence of hydroxyl groups, resulting in elimination [52, 53]. Liposomes coated with PVA and polyacrylamide copolymers have shown to suffer mechanical degradation and subsequent leaking of contents [54].

Amongst the various polymers examined, PEG molecules being hydrophilic, biocompatible, non-immunogenic and uncharged [36, 55–59] has demonstrated potential in steric stabilization. Doxil™ is the benchmark formulation demonstrating the stealth nature of PEG [60, 61].

The stealth nature of PEG is attributed due to its hydrophilicity and uncharged surfaces. The inclusion of PEG increases the hydrophilicity and reduces the overall charge or the zeta potential on the surface of liposomes preventing opsonization [62–64]. This phenomenon has been demonstrated in previous studies, where coating of the PEG on anionic liposomes reduced the zeta potential to a near-neutral value, thereby increasing the circulation [65–67].

### *PEG (Polyethylene Glycol) Linked Lipids*

PEG (Poly-ethylene glycol) is non-ionic [68], bio-compatible [69], hydrophilic [63], and easy to synthesize [70] which makes it more favourable. PEGylated lipids consist of PEG chains, a linker, and a

hydrophobic anchor [70]. One of the ends of the PEG chain in PEGylated lipids is attached to the hydrophobic anchor through the linker.

#### *Components in a PEGylated Lipid*

The molecular weight of the PEG chains is usually in the range of 50kDa to 400 Da and can be functionalized by covalent conjugation with folate, biotin, amine, azide, carboxylic acid [71, 72]. The functionalization of PEG lipids which is vital to increase its target specificity has been reported extensively [73] and is beyond the focus of this review.

The molecular weight and grafting density affect the conformation of PEG chains on the lipid bilayer surface. At lower grafting density, the PEG molecules assume a lesser interacting mushroom regime with conformed chains, and increasing grafting density, the PEG chains extend and branch out to interact with neighbouring molecules [74, 75]. Brush regime is preferred to improve the stealth properties of a liposome as in this regime the interaction of the particle is less and diffuses faster through the tissues than the mushroom regime[60]. Moreover, opsonins bind predominantly through hydrophobic and electrostatic interactions. Non-ionic PEG chains in brush conformation render the surface of the liposome hydrophilic, reducing the interactions. Furthermore, some adsorbed opsonins tend to compress the brush like chains to mushroom like configuration. This change in energy impedes the attractive nature of opsonins from reaching the surface of the liposomes[37, 76].

According to Alexander-deGennes theoretical model of polymer regimes, the transition from mushroom to brush regime depends on the distance ( $D$ ) between the grafting sites and the length of the random PEG coils ( $R_f$ ).  $R_f$  or Flory dimension is mathematically represented as  $aN^{3/5}$ , where  $N$  is the degree of polymerization and  $a$  is the monomer size [77]. The conditions for each of the configuration depicted in Fig 2 is as follows: (i)  $D > R_f$ , the PEG chains have very little interaction and follow interdigitated mushroom configuration (ii)  $R_f < D < 2R_f$ , a mushroom packing is observed (iii)  $D \ll 2R_f$ , chains are closer and a brush like extended conformation is observed[36, 40, 77]. In all conformations, PEG chains form a fixed aqueous layer thickness (FALT) around the liposomes. This FALT value

controls the regime type and circulation time of liposomes. It is reported that a combination of shorter and long PEG chain lengths help in lengthening the circulation time[70].

The PEG chains are further attached to the hydrophobic acyl anchor through linker moieties. Common linker moieties include phosphate ester, ether, disulfide, carboxyl ester, amide, and peptide linkages[78–82]. The linker moieties affect the surface charge and binding properties of the liposomes. The conventional linker has been the phosphate linker, based on its presence or absence the PEGylated lipids are categorized as PEG-phospholipids and PEG-non-phospholipids [70]. The third component of PEG lipids is the hydrophobic anchor comprising of acyl groups that associate into the lipid bilayer with PEG chains branching into the aqueous region. The acyl groups determine the lamellar or micellar morphology of the lipid assemblies[20] and the extent of inclusion of PEGylated lipid in the liposome [83, 84]. In case of PEG-phospholipids, the acyl group is usually a fatty acid chain such as distearoyl, dipalmitoyl or dimyristoyl that is covalently bonded to the polar phospholipid head group[70]. These head groups are then attached to a linear chain methoxy-PEG (mPEG) as shown in Fig 3a. It is found that the length of the mPEG chains which is directly related to the molecular weight influences the FALT values, thereby determining the circulation time. Shorter m-PEG chains (350-750 Da) have shown relatively limited effects to avoid macrophage clearance due to their inherent mushroom configuration. Increasing the mPEG chain length has shown to increase the circulation time[85–90]. Additionally, mPEG phospholipids having a mixture of PEG chain lengths in the same molecule (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG<sup>2</sup>) elongates the circulation time and has increased tumor contact time because of its differing hydrophilic and hydrophobic properties[88]. It is also been found that concentration of mPEG-phospholipids in a liposome influence the rate of release of drugs. The higher molecular weight mPEG-phospholipids transform a diffusion-controlled drug release to an interfacial-controlled drug release[91].

Conventional phosphate linkers are anionic in nature that can activate the complement system, which

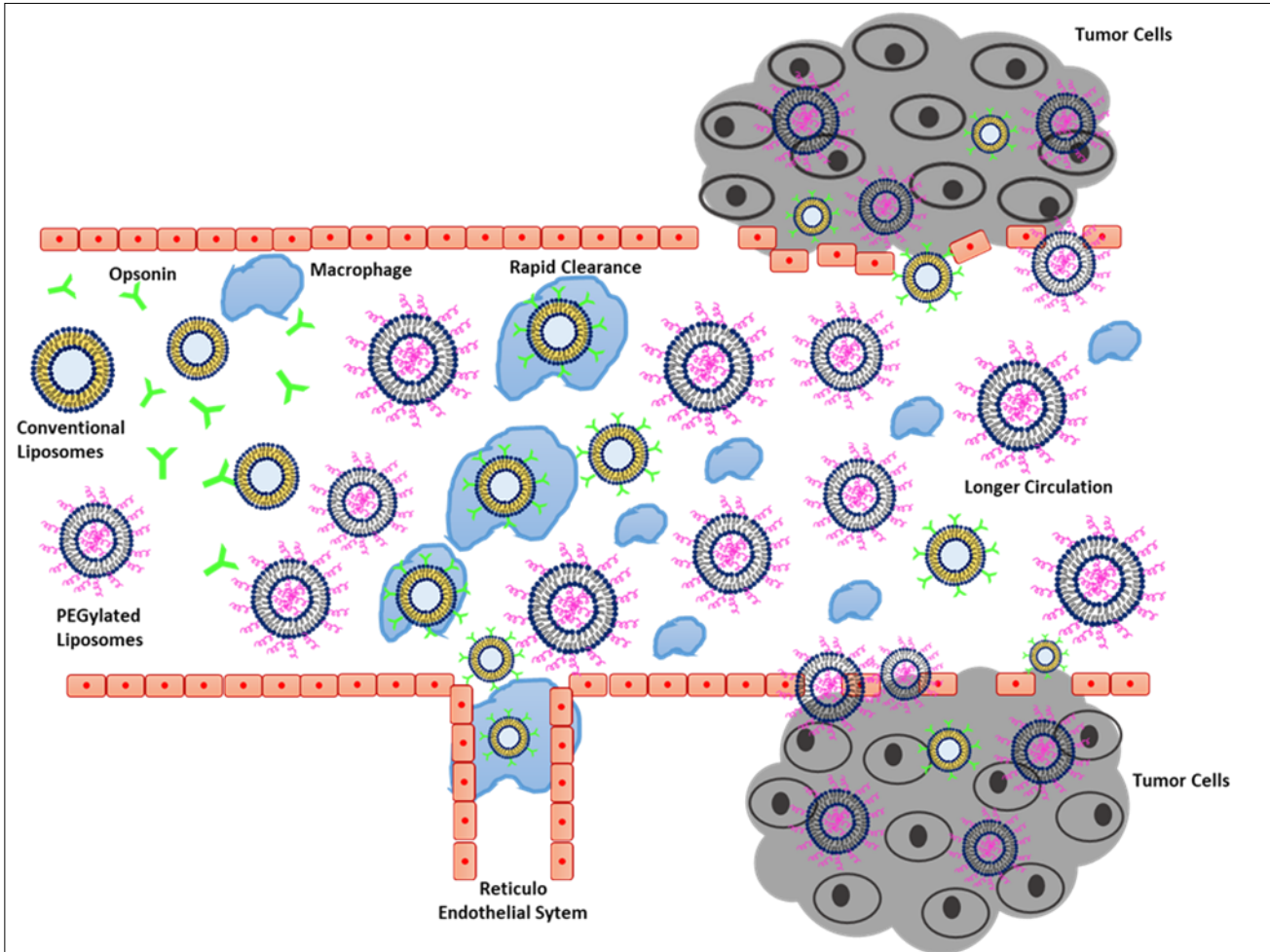


Figure 1. Conventional vs long circulating liposomes and its effect on macrophage clearance

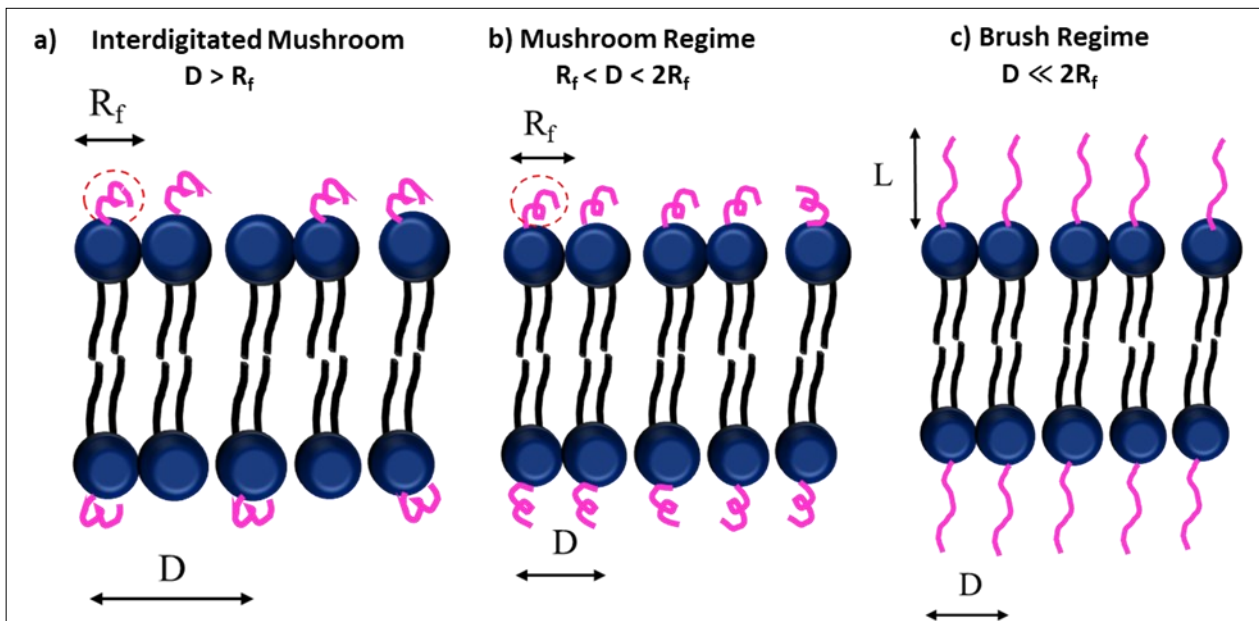


Figure 2. Regimes in a PEG coated liposome a) Interdigitated Mushroom; b) Mushroom Regime; c) Brush Regime

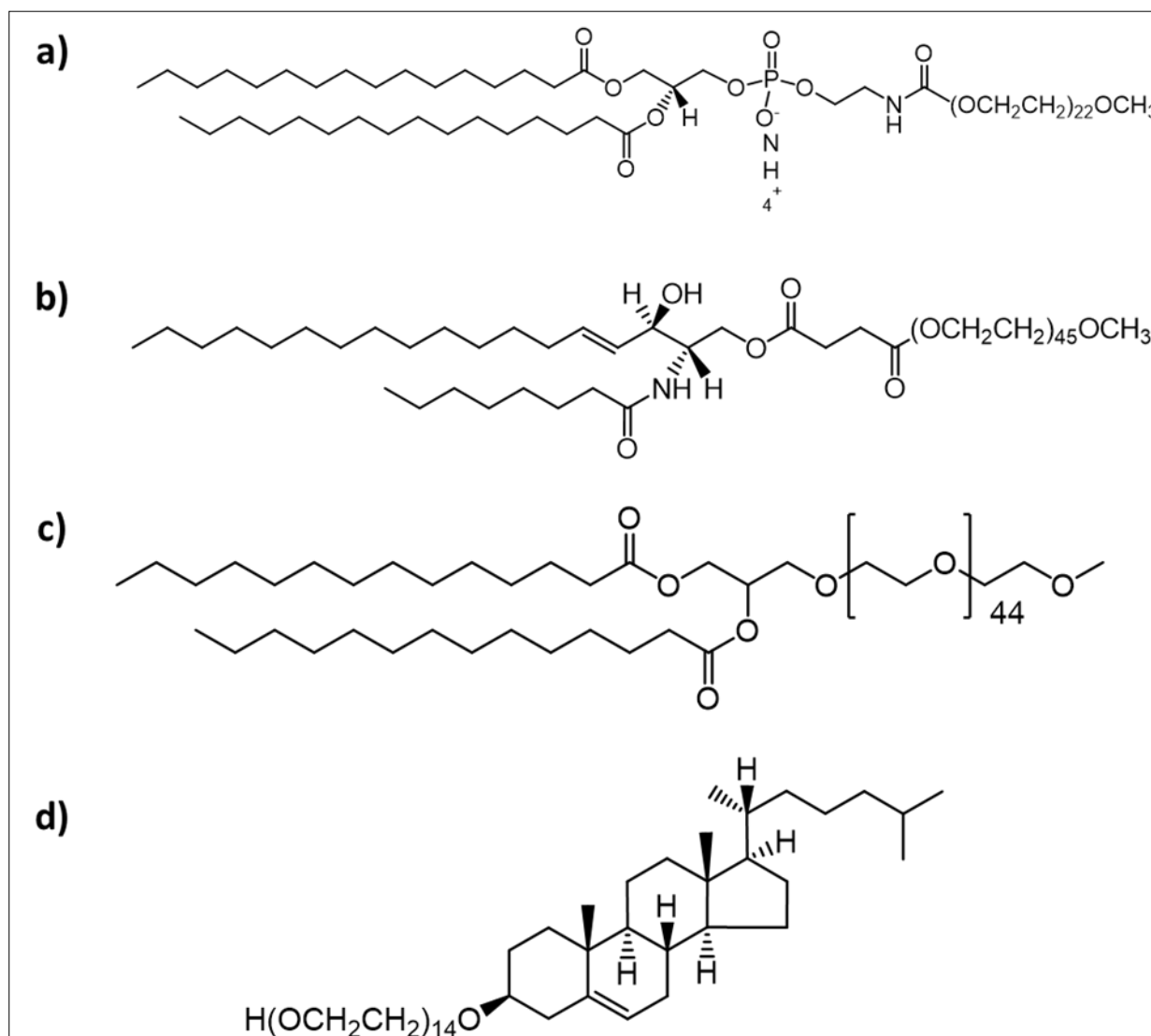


Figure 3. Structure of Phospholipid PEG a) DSPE-PEG2000 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; Non-Phospholipid PEG b) C8 PEG2000 Ceramide N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]}; c) DMG-PEG 2000 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000; d) Chol-PEG600 Cholesterol-(polyethylene glycol-600)

is responsible for innate immunity, leading to hypersensitivity reactions, however neutralizing these anionic charges, with cationic groups or methylation has shown to thwart this activation [92]. The costs associated with production of phospholipids is not justified due to proneness to enzymatic degradation by lipases and phospholipases [93–97] that results in their rapid clearance. Consequently, PEG chains conjugated to glycerolipids, sphingolipids and cholesterol shown in Figs 3b-d have been explored as alternatives to PEG-phospholipids. A study investigating the different lengths of PEGylated ceramides on the circulation times of liposomes concluded that longer acyl chained ceramides (C<sub>24</sub>) compared to shorter chain ceramides (C<sub>8</sub>), had stronger anchoring properties that resulted in longer circulation time and higher drug release rates[98, 99].

Cholesterol linked PEG or Cholesteryl-PEG (Chol-PEG) includes cholesterol as the hydrophobic anchor. Due to the lipophilicity, compatibility with other lipids and stabilizing properties, cholesterol is a favourable choice for anchoring PEG chains through ester bonds[5, 93, 100]. Chol-PEG is found to regulate the membrane fluidity, which helps in adding stability to the bilayer and prevents the leakage of drugs [70]. Additionally, the drug loading efficiency of Chol-PEG is found to be dependent on the percentage of Chol-PEG. Lower Chol-PEG ratios in the formulation could encapsulate more amount of drugs[101–103]. Studies have reported the synthesis of pH cleavable PEG chains by linking Cholesterol through succinate and carbamate linkers[104, 105]. Despite its advantages, Chol-PEG demonstrated shorter circulation time than PEG phospholipids. This is because cholesterol anchors deep into the hydrophobic zone of the lipids, counteracting the advantages offered by PEG chains. The addition of extra linkers is proposed to overcome this limitation, however complicates the synthesis process.[25, 70].

Furthermore, it is necessary to cleave the PEG chains after the circulation time is achieved to increase the absorption of drugs[25, 105]. This is accomplished by attaching linker moieties that either cleave the PEG chains upon the reaching the target site or when exposed to a suitable stimulus such as change in pH, temperature, or in the presence of an enzyme in the cellular microenvironment[106, 107]. Some prominent

moieties that are used as cleavers include vinyl ether bond, hydrazine bond, disulphide, peptide and ester bonds[108]. Vinyl ether bonds are non-reactive in near neutral and basic conditions that makes them labile in acidic conditions especially under pH < 5 [109]. Vinyl ether linked PEG to dioleoyl phosphatidyl ethanolamine (DOPE) liposomes have demonstrated efficacious release of therapeutic contents post the cleavage of PEG moiety under varying pH conditions [109]. It is also observed these PEG conjugated vinyl ether bonds are cleavable by reactive singlet oxygen generated encapsulated by photosensitizers [105]. Peptide bonds, are cleaved only in the presence of tumor specific enzymes such as matrix metalloproteinases (MMPs)[110–112]. In-vitro studies have shown that peptide conjugated PEG lipids have longer circulations times and prefer to accumulate on tumor sites due to the enhanced permeability and retention effect (EPR), which arise from lack of draining in the tumor tissue[110]. For intracellular delivery specific, PEGylated liposomes conjugated with disulfide bonds are desirable [113–115] because the disulfide linkage in nanocarriers gets cleaved in the presence of glutathione, an antioxidant overexpressed in the cytoplasm of cancerous cells. Furthermore, many studies indicate that antibody delivery by cleavable PEG linked by disulfide bond can potentially be used in cancer diagnosis and treatment[113, 116, 117].

#### *Experimental Techniques for the Stability Assessment of PEGylated Liposomes*

Thermal and physical stability are the important factors to consider in liposome-based vaccine and drug carrier design systems. A variety of analytical and optical methods are being used to characterize the physicochemical properties and assess the stability of PEGylated liposomes for in vivo studies and clinical trials as summarized in Fig 4. Studies from our laboratory have used a combination of techniques such as dynamic light scattering (DLS), differential scanning calorimetry (DSC), Nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FTIR), and cryogenic electron microscopy (EM) to elucidate the structure and properties of the liposomes. These studies provide insights on their steric and thermal stability of the liposomes. However, techniques such as NMR, FTIR and EM are very sample specific and require an elaborate and specific set up. On the other hand, DSC

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and DLS are relatively simple techniques which still provide desired information about the sample stability. A brief review on DLS and DSC studies is as follows.

DLS provides hydrodynamic diameter, polydispersity index, and zeta potential of the liposomal solution as shown in Fig 4. A monochromatic light reflected in the liposomal solution gets scattered due to the Brownian motion of the liposomes in the solution [4, 5, 118, 119]. The motion is related to the diffusion coefficient of the liposomes. Temperature and viscosity of the liposomal solution also influences the size measurement. The diffusion coefficient ( $D_T$ ) is related to the hydrodynamic radius ( $R_H$ ) is given by the Stokes-Einstein equation [120]:

$$D_T = \frac{k_B T}{6\pi\eta R_H} \quad \dots(1)$$

Where  $k_B$  is Boltzmann coefficient ( $1.380 \times 10^{-23} \text{ kg.m}^2. \text{ s}^{-2}.\text{K}^{-1}$ ),  $T$  is an absolute temperature, and  $\eta$  is the viscosity of the aqueous medium. In addition to the hydrodynamic radius measurements, this technique also generates the poly dispersity index (PDI) of the liposomes which determines the size uniformity of the liposomes.

Another important parameter that determines the circulation ability of the PEG liposomes is the FALT, which is derived from the zeta potential or surface charge of the liposomes [121–123]. The Zeta potential is measured using the Laser Doppler electrophoresis technique. When PEGylated liposomes are dispersed in water and when voltage is applied, a layer of ions from the solution strongly bind to the liposome surface forming a stern layer. This charged layer induces loose adhesion of ions of opposite charge called the diffuse layer. The two layers combined is called the electrical double layer. The electrical potential measured at the surface of the electrical double layer is called the zeta potential.

When an electric field is applied to a liposomal suspension, the liposomes move towards the oppositely charged electrodes of zeta potential measurement cell. The ratio between the velocity of motion of the particles and the electric field is called the electrophoretic mobility ( $\mu_e$ ) which is related to zeta potential ( $z$ ) by the Henry equation,

$$\mu_e = \frac{2\epsilon z f(k,\alpha)}{3\eta} \quad \dots(2)$$

Where  $\epsilon$  and  $\eta$  are the dielectric constant and the absolute zero-shear viscosity of the aqueous medium.  $f(k,\alpha)$  is known as “the Henry function”, where  $\alpha$  is the radius of the particle and  $k$  is known as the Debye-Huckel parameter, which represent the thickness of the electrical double layer [119, 124].

The zeta potential can be calculated from the electrophoretic velocity of the particles using Helmholtz-Smoluchowski equation

$$\mu_e = V_p E = \zeta \epsilon \eta \quad \dots(3)$$

where  $V_p$  is the electrophoretic velocity,  $\zeta$  is the zeta potential,  $\epsilon$  is the permittivity, and  $\eta$  is the viscosity of the medium.

The FALT measurement is calculated from the slope of the graph of the zeta potential vs Debye-Huckel parameter

The influence of PEG chains on the orientation of the lipids in the liposome and other morphological characteristics can be obtained by cryo-transmission electron microscopy (cryo-TEM), phase contrast microscopy (PCM), atomic force microscopy (AFM) and fluorescence microscopy (FM). Sample preparation in the case of EM and AFM and presence of other dyes in FM can generate spatial and resolution artefacts [125, 126]. The use of X-Ray to obtain information on the structural properties is constrained because of the difficulty arising in crystallizing the samples resulting in lack of long-range order [127, 128].

Our previous works have extensively used Differential scanning calorimetry (DSC) to gain an insight on the thermal stability and stressed induced in the lipid bilayer due to annealing and presence of peptides, and porphyrin, non-porphyrin amphiphilic fluorophores [2–5]. DSC measures change in c heat capacity ( $C_p$ ) as a function of temperature. PEGylated lipid molecules have characteristic pretransition and melting temperature that is accompanied during their thermal transition from gel to fluid crystalline transition which is endothermic in nature. The nature of the thermal transition is affected in the presence of molecules in the lipid bilayer. Change in enthalpy of transition is measured from the area under the melting transition and mathematically represented by the following equation [2–6, 119, 129]:

Table 1. FDA approved Formulations involving PEGylated Liposomes

Formulation	Active Ingredient	Composition	Stage	Application	Company
Doxil [132–134]	Doxorubicin	HSPC:Cholesterol:PEG 2000-DSPE (56:39:5 molar ratio)	Approved	Ovarian/Breast Cancer	Sequus Pharmaceuticals (1995)
Onivyde [135, 136]	Irinotecan	DSPC:MPEG-2000: DSPE (3:2:0.015 molar ratio)	Approved	Metastatic adenocarcinoma of the pancreas	Merrimack Pharmaceuticals Inc. (2015)
Thermodox [142]	Doxorubicin	DPPC, Myristoyl stearyl phosphatidylcholine and DSPE-N-[amino (polyethylene glycol)-2000]	Phase 3	Hepatocellular carcinoma	Celsion Corporation
Lipoplatin [143, 144]	Cisplatin	DPPG, soy phosphatidyl choline, mPEG-distearoyl phosphatidyl-ethanolamine lipid conjugate and cholesterol	Phase 3	Non-small cell Lung Cancer	Regulon Inc.
S-CKD602 [145, 146]	Potent topoisomerase I inhibitor	Phospholipids covalently bound to mPEG	Phase 2	Cancer	Alza Corporation
2B3-101 [141]	Doxorubicin	Glutathione PEGylated liposomes	Phase 1	Solid Tumors	2-BBB therapeutic
MCC-465 [147]	Doxorubicin	DPPC, cholesterol and maleimide palmitoyl phosphatidyl ethanolamine; immunoliposomes tagged with PEG and the F(ab) <sub>2</sub> fragment of human monoclonal antibody GAH	Phase 1	Metastatic Stomach Cancer	Mitsubishi Tanabe Pharma Corporation

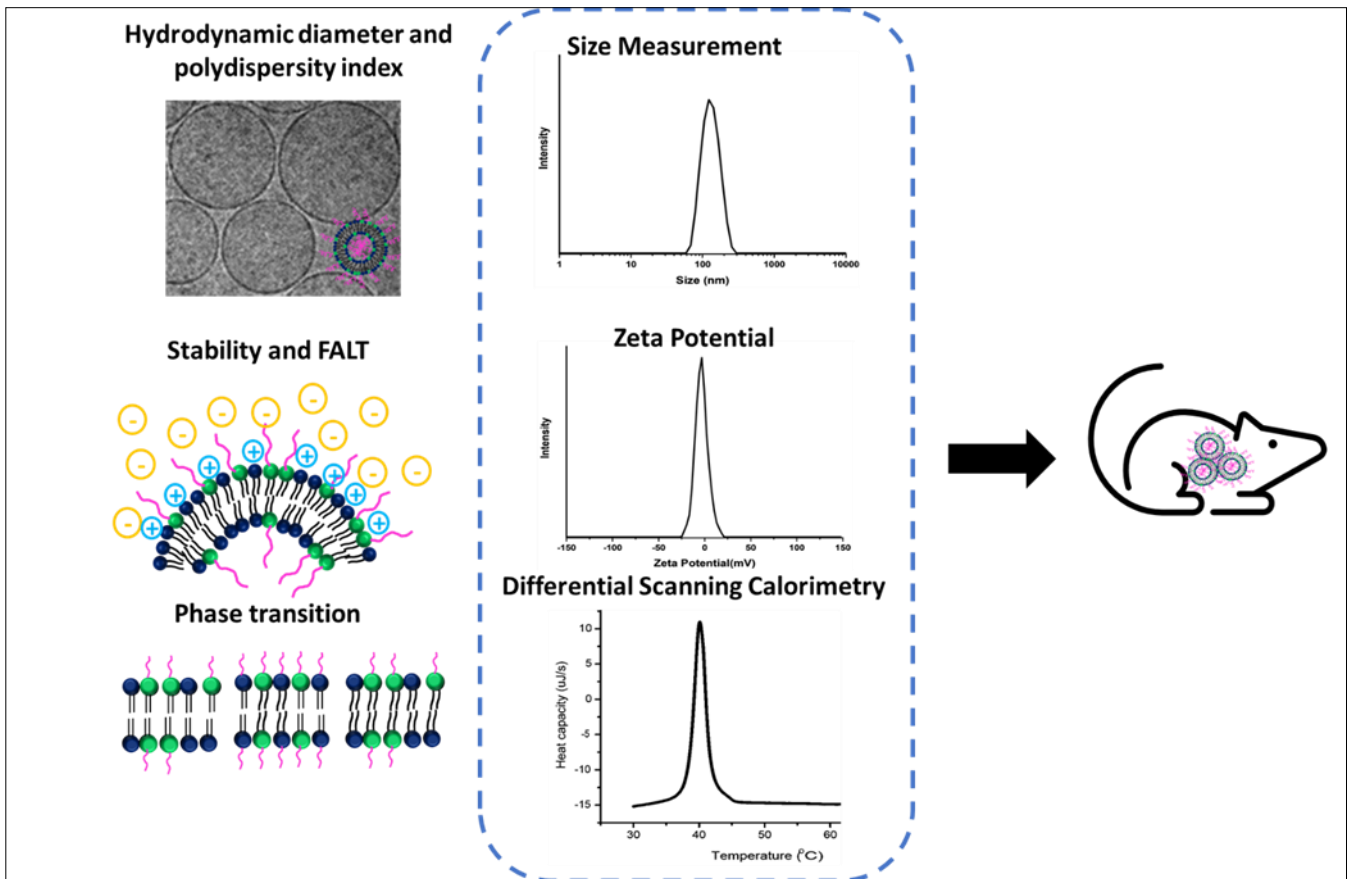


Figure 4. Assessment of physicochemical properties of PEGylated Liposome

#### Calorimetric Enthalpy

$$\Delta H_c = \int C_p dT \dots(4)$$

The change in shape of the melting peak of the lipids imply the presence of surface bound or encapsulated therapeutic molecules. Additional information on the cooperativity of these molecules can be obtained by computing thermodynamic parameters of the lipid-molecule system. [130, 131].

#### Benefits of PEGylated Liposomes in the Clinics

Based on previous discussions on the composition, properties, and advantages offered by PEGylated lipid molecules, it is clear that PEGylated formulations provide shielding effects from macrophages, offer longer circulation time, and allow preferential accumulation to sites of interests (such as tumors). Due to the substantial research in stealth technology, several PEGylated liposomes have been tested for clinical trials and approved by the FDA for treatment of patients. PEGylated lipids are typically used in combination with regular phospholipids and

cholesterol in various formulations. The first approved PEGylated liposomal formulation was Doxil®. Doxil®, is an intravenous formulation developed by Sequus Pharmaceuticals, USA in 1995 for the delivery of DOX (doxorubicin) to treat ovarian and breast cancer. This formulation has 5 mol% of phospholipid DSPE-PEG 2000, 39 mol% cholesterol and 59 mol % hydrogenated soy phosphatidylcholine (HSPC) [125–127]. Subsequently, Onivyde™, which has another anticancer agent, irinotecan, was approved by the FDA for the treatment of metastatic adenocarcinoma. Onivyde™ contains distearoyl-sn-glycero-phosphoethanolamine (DSPC), cholesterol and distearoylphosphatidyl ethanolamine (DSPR-PEG2000) in the ratio of 3:2:0.015 [128, 129].

Several other formulations which contain PEGylated phospholipids and Chol-PEG are currently in different phases of clinical trials. The active ingredient and composition of the formulations has been tabulated below in Table 1 [130].

Thus, the stealth nature of PEGylated liposomes makes them suitable for encapsulation of variety of

therapeutics. A very interesting recent application of PEGylated lipids is to use it in combination with photopolymerizable lipids (for example, DC<sub>8,9</sub>PC (1,2 bis (tricoso-10, 12-diyonol)-sn-glycero-3-phosphocholine)). However, these studies are relatively recent, and their potential is still not fully explored. Therefore, we chose to summarize these observations to abreast and update readership in the field of lipids (see section below).

DC<sub>8,9</sub>PC is a polymeric lipid having a tubular morphology. Previously, DC<sub>8,9</sub>PC has been successively used in combination with DPPC for light-triggered delivery of a photosensitizer (HPPH, currently in clinical trials. This patented technology [132] is being developed by Nano-Red™ for various therapeutic interventions [133]. In our recent studies, we have described utilization of the PEGylated phospholipid DSPE-PEG2000 to render tubule-forming photopolymerizable lipid, DC<sub>8,9</sub>PC to a stable vesicular morphology [131]. Therefore, the presence of hydrophilic PEG chains can induce formation of lamellar structure, thus resulting in eliminating the need for the presence of DPPC and therefore resulting in binary lipid stealth nanosystem. The DC<sub>8,9</sub>PC formulations could incorporate up to 20 mol% of and DSPE-PEG-2000 and encapsulated HPPH at very high efficiency [131]. To our knowledge, inclusion of such high concentration of PEG lipids in a lipid formulation has not been shown previously. Similarly, another group also investigated the encapsulation of dexamethasone, a potent rheumatoid arthritis drug [134] in this formulation. Both these studies have demonstrated the application of DC<sub>8,9</sub>PC to achieve longer circulation, stealth nature, and preferential accumulation of these formulations in the respective sites of interest in animals.

## Conclusions

Stealth nature and long circulation properties of PEGylated liposomes has made them an attractive choice for drug delivery applications. However, certain studies have indicated certain unexpected immunological response to PEGylated liposomes. One such response is the accelerated blood clearance (ABC), the periodic administration of PEGylated liposomes results in production of antibodies against the carrier [148–150]. This has reduced the effectiveness of the course of the treatment and caused hypersensitive immune reactions. Therefore, more research is required to understand the

intrinsic mechanisms responsible for these responses. This would support in designing PEGylated liposomes that can be used throughout a course of the treatment.

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