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Molecular Dynamics Simulations of Drug Delivery Liposomes and Their Interactions With Bloodstream Elements

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ACADEMIC DISSERTATION

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Cover: Cartoon representation of all atom model of polyethylene glycol coated drug delivery membrane bilayer in water with ions.

Abstract

Drug delivery is a vital issue in pharmaceutical research; once a drug candidate molecule is identified, it must be delivered to the target area of the body where it can take effect. In addition, non-specific distribution of drug molecules to areas other than the drug target must be decreased to avoid unwanted side effects. To achieve this, nanotechnological drug delivery systems can be used. Nanotechnological drug delivery systems come in a wide variety of forms, including liposomes, dendrimers, nanoparticles, and polymeric micelles. Of these, our research is focused on drug delivery liposomes.

Drug delivery liposomes are composed of a membrane that forms a closed spherical sack, with a diameter of approximately 100 nm that can contain drug molecules. The criteria for effectiveness of these drug delivery liposomes (DDLs) are structural stability, its lifetime in the bloodstream, the release rate of the encapsulated content and site specific targeting. Cholesterol is one of the crucial lipid components of the DDL known to increase its stability. They also can have a protective polymer coating such as polyethylene glycol (PEG) that protects the DDL from the body's defense mechanisms. Also the DDL can posses targeting moieties, able to direct the PEGylated liposomes to the specific target. In this study we have investigated surface structure of the DDL and its interactions with elements of the bloodstream.

While it is difficult to determine an accurate picture of the DDL surface and its interactions with ions and bloodstream proteins with atomistic resolution by experiments alone, computational molecular modelling techniques can provide insights into it. Hence, we have used computational modelling and molecular dynamics simulations to understand the role of each component of the DDL in its structure.

The three of the five reported studies in this thesis (I, II, III) are focused on how surface charge plays an important role in the liposome, how it is affected by various components of the DDLs, and how the specific interactions of DDLs and ions present in the bloodstream influence it. The chapter IV deals with understanding the properties by systematically varying components such as cholesterol and PEG. Also we have produced the first ever model of the first FDA approved drug delivery liposome (DOXIL ®) at atomistic resolution details. The last study (V) deals with the application of molecular dynamics in targeted drug delivery research. In this study we could identify the reason for failure of specific novel targeting peptide (AETP), which is used to functionalize the DDL, by identifying its interactions with the protective PEG polymer.

The insights obtained by these studies can be used to improve the design of PEGylated or other polymer coated liposomes and will have the potential to lead to breakthroughs in drug delivery efficacy as these techniques can be applied to a wide range of therapies that involve delivery through the DDL.

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Helsinki, August 2014

Aniket Magarkar

Dedicated to,

I would like to dedicate this thesis to my parents Ms. Jyoti Magarkar and Mr. Suresh Magarkar who believed in me, have been there for me all the time and with their support making me strong throughout my life. Thank you for all the love and support.

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List of original publications

This thesis is based on the following publications:

I Cholesterol level affects surface charge of lipid membranes in saline solution
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doi: 10.1038/srep05005.

II Molecular Dynamics Simulation of Inverse-Phosphocholine Lipids
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III Molecular dynamics simulation of PEGylated bilayer interacting with salt ions: a model of the liposome surface in the bloodstream
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IV Molecular Dynamics Simulation of PEGylated Membranes with Cholesterol: Building Toward the DOXIL Formulation
Magarkar A., Róg T. and Bunker A. J. Phys. Chem. C, 2014, 118 (28), pp 15541–15549. doi: 10.1021/jp504962m

V Analysis of cause of failure of new targeting peptide in PEGylated liposome: molecular modelling as rational design tool for nanomedicine.
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The publications are referred to in the text by their roman numerals.

* Indicates equal contribution

Abbreviations

aa	all atom
AETP	activated endothelium-targeting peptide
BSA	bovine serum albumin
cg	coarse grained
Chol	cholesterol
CPe	phosphatidylcholine group reversed
DDL	Drug Delivery Liposome
DLCPe	1,2-dilinoleoyl-sn-glycero-3-phosphatidylcholine
DLPC	1,2-dilinoleoyl-sn-glycero-3-phosphatidylcholine
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-amino (polyethylene glycol)
EGFR	epidermal growth factor receptor
EPR	enhanced permeability and retention
fs	femtosecond
HSA	human serum albumin
HSPC	fully hydrogenated phosphatidylcholine
HUVEC	human umbilical vein endothelial cell
MD	molecular dynamics
ns	nanosecond
PEG	polyethylene glycol
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
ps	picosecond
RES	reticuloendothilial system
RGD	arginine, lysine and aspartic acid containing peptide (Phosphatidylcholine group reversed)
etc.	et cetera
i.e.	id est
e.g.	exempli gratia

1. Introduction

Drug delivery is a vital issue in pharmaceutical research; once a drug candidate molecule is identified, it must be delivered to the desired biological target where it can take its effect. (Farokhzad & Langer 2009; Lasic 1998) In addition, non specific distribution of drug molecules to areas other than the drug target must be decreased to avoid unwanted side effects (Lasic 1998; Torchilin 2005a; Kang et al. 2010). Also in many cases, the potential drug molecule has unfavourable biochemical properties such as poor water-solubility and poor pharmacokinetic distribution. Encapsulation of the drug molecules in nano-scale drug delivery systems has shown particular promise in overcoming these limitations. (Cattel et al. 2003; Sapra et al. 2005). Nanotechnological drug delivery systems come in a wide variety of forms, including liposomes, dendrimers, nanoparticles, and polymeric micelles (Orive et al. 2003). Of these, our research is focused on drug delivery liposomes. Alec Bangham discovered liposomes in the 1960s. The first polymer-coated liposome was formulated by Peter Speiser in the 1970. So far there are more than twenty thousand research articles with keywords "liposome" and "drug delivery". The research has been directed towards understanding of the properties of the DDL and characterizing their interactions with the constituents of blood plasma. Once these properties are understood in greater detail, efforts can be made towards increasing the half-life of the DDL in the bloodstream and designing better DDLs.

Drug delivery liposomes are composed of a membrane bilayer that forms a closed spherical sack, with a diameter of approximately 100-200 nm that can contain drug molecules (Cattel et al. 2003). The membrane bilayer of the DDL consists of phospholipids, with a hydrophilic headgroup and two hydrophobic hydrocarbon chains. The composition of the DDL determines its biophysical properties. For example, altering the lengths of the hydrophobic chains and the extent of saturation alters the phase behaviour of the lipid. The phospholipid headgroups are responsible for interactions of the DDL with the blood plasma constituents. Drug delivery liposomes can also have a protective polymer coating, the most common being polyethylene glycol (PEG) that protects the liposome from the body's defense mechanisms. The biochemical properties of the polymer, molar fraction of polymer in DDL composition and the length of the polymer determine the structure and properties of the protective polymer on the DDL. In most cases of clinically approved DDLs cholesterol is included. The addition of cholesterol is known to alter the biophysical properties of the bilayer such as increase in mechanical strength and decrease in permeability across the membrane bilayer.

The interaction of drug delivery liposomes with bloodstream proteins has been the subject of many experimental studies. The results of these studies are, however, as of yet, unclear. While it is difficult to directly determine an accurate picture of the

liposome surface and its interactions with bloodstream proteins experimentally at nano-scale resolution and nano-second time scale, computational molecular modelling is capable of providing insight into this.

In the studies presented here, we have used molecular modelling as a tool to study the effect of varying the formulation of the DDL on the surface structure of the DDL and its interaction with ions present in the bloodstream. The results discussed in this study are expected to provide insight that can be used in the rational design of improved drug delivery liposomes.

2. Literature review

One of the major challenges faced today in DDL based drug delivery is to understand the physicochemical properties, which are responsible for the performance of the DDL *in vivo*. Liposomes and liposome based drug delivery is an active field of research since the 1960s and there exists a considerable amount of knowledge concerning its properties gained from *in vitro*, *in vivo* and *in silico* studies. In this section, we discuss and summarize the available results from the research reported to date, regarding the topics covered in our studies (and which are not covered in original articles included in this thesis).

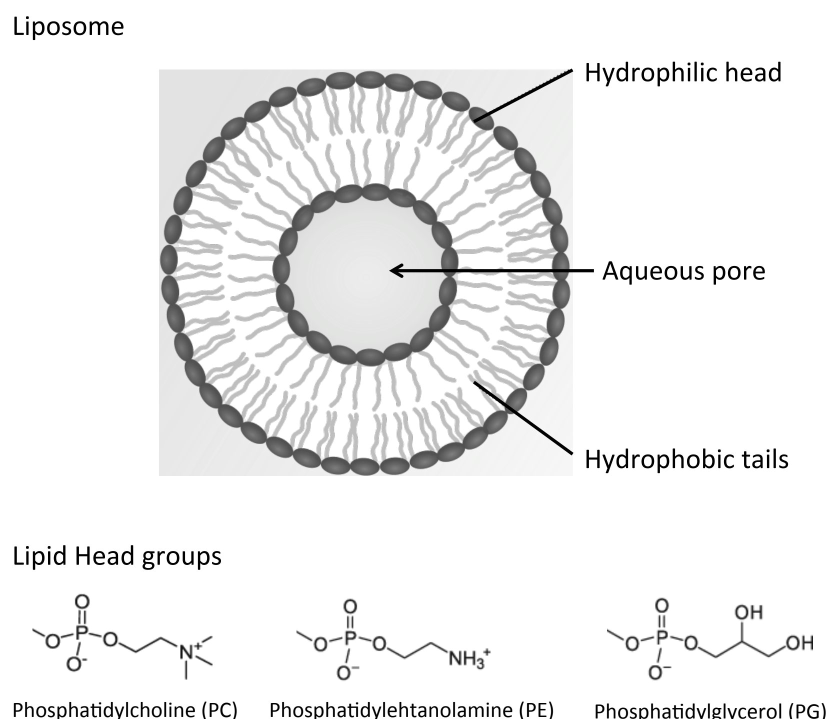
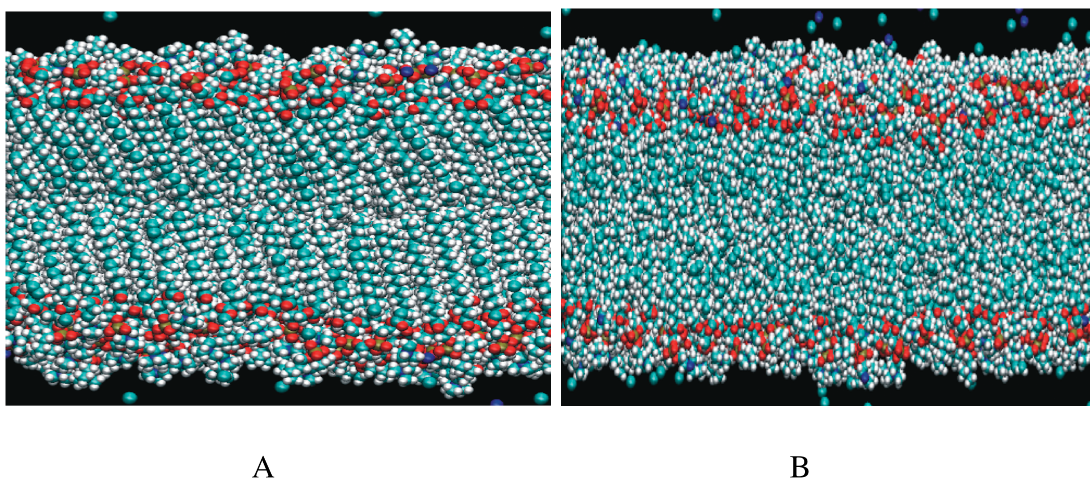


Figure 1: Generic structure of liposome formed with membrane bilayer, showing hydrophilic head groups, hydrophobic acyl tails and aqueous pore of the liposome.

2.1 Liposomes

Liposomes are self-assembling spherical lipid bilayer vesicles with an internal aqueous core. (Figure 1) The lipid bilayer of the liposome is composed of amphiphilic natural or synthetic phospholipid molecules. The lipid molecule has one hydrophilic head group attached to two hydrophobic lipid tails consisting of acyl chains. The length of the hydrocarbon tails of the phospholipid ranges from 8 to 18

carbons, which can be either saturated or unsaturated. The longer saturated acyl chain results in the gel phase of the membrane (Figure 2), in contrast, a shorter hydrocarbon chain imparts liquid crystalline structure to the membrane bilayers (Figure 2). Depending on the chemical composition of the lipid headgroup it can either be zwitterionic, e.g. phosphatidylcholine (PC) and phosphatidylethanolamine (PE), or positively charged, e.g. 3-trimethylammonium-propane (TAP), or negatively charged, e.g. phosphatidylglycerol (PG). Apart from phospholipids, cholesterol is included in the DDL membrane bilayer, and is known to play a role in increasing the mechanical strength of the membrane bilayer and decreasing the permeability of the membrane. Due to the amphiphilic nature of the DDL, it can encapsulate both hydrophobic and hydrophilic drug molecules. The aqueous core of the DDL can be loaded with hydrophilic drug molecules and hydrophobic drugs can reside in the lipid tail regions of the membrane bilayers (Sadzuka et al. 2002).



A B

Figure 2: Effect of acyl chain length on the nature of membrane bilayer.
(A) DSPC membrane bilayer in the gel state
(B) DLPC membrane bilayer in the liquid-crystalline state

2.2 Liposome based DDLs

Liposome based nanoparticles have proven to reduce the side effects of toxic anti-cancer drugs. Liposome based DDLs are one of the four available nanoparticle based FDA approved therapeutics. The Doxil®, DaunoXome®, and Marqibo® are the DDL therapeutics have so far been approved by FDA. The Doxil® formulation reduces the cardiotoxicity associated with free doxorubicin. Doxil® has been used in the treatment of AIDS-related Kaposi's sarcoma (FDA approval: 1995), ovarian cancer (FDA approval: 1999), and for multiple myeloma (FDA approval: 2007) (Sadzuka et al. 2006). DaunoXome and Marqibo are used for the treatment of Kaposi's sarcoma and leukaemia respectively. Doxil contains DSPE-PEG-2000, which is known to prolong its bloodstream half-life up to 3-4 days (Jiang et al. 2011). From *in vivo* experiments it is known that PEGylated liposomes accumulate at the tumor site due to the enhanced permeability retention (EPR) effect (discussed further) (Li et al. 1998; Schiffelers et al. 2005), however the exact mechanism of

drug release from the liposomes and uptake by tumor cells is not currently well understood.

Due to a lack of understanding of the exact mechanism of the drug release from the DDL and its interactions with blood plasma, all available FDA approved DDL therapeutics rely on passive accumulation at the target site via the EPR effect. Although there have been many attempts made already for the active targeting of DDLs to the specific target site, so far there are no FDA approved targeted DDL therapeutics available (Immordino et al. 2006; Maruyama et al. 1997).

2.3 Passive targeting and active targeting of the DDL

2.3.1 Passive targeting via the EPR effect

For the case of cancer, the targeting of the DDL to the desired location is achieved through passive targeting. As the tumor continues to grow (up to 1-2 mm^3), in order to meet increased demand from the growing mass of cells for oxygen and nutrients, there begins the formation of new blood vessels termed as angiogenesis. The process of angiogenesis can be up regulated by vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and tumor necrosis factor-α (TNFα), and down regulated by angiogenic inhibitors, such as thrombospondin-1. The morphology of this new tumor vasculature differs from the normal tissue, as it is inherently leaky. Thus, due to a drastic increase in permeability in vasculature at tumor site, there is higher accumulation of the DDL. This effect is termed as the EPR effect (Fang et al. 2011; Maeda 2012; Jain & Stylianopoulos 2010; Torchilin 2011). The EPR effect has been demonstrated to achieve 10–50 fold local concentrations of nanoparticles in the tumor in comparison to normal tissues (Iyer et al. 2006) (Fang et al. 2011; Maeda 2010; Jain 1987; Maeda et al. 2009). In order for the DDL to be accumulated at the tumor site, increased half-life in the bloodstream is essential. Also the size of the nanoparticle is a crucial factor, it should have a diameter less than 100 nm to prevent accumulation in the liver and more than 10 ns to prevent filtration by the kidneys (Danhier, Feron & Preat 2010).

2.3.2 Active targeting

In order to increase the accumulation of DDLs at specific targeting sites, the target specific moieties (peptide or antibodies) are attached on the surface of the liposome or attached at the end of the protective polymer of the DDL. (Sapra, Allen 2003). The targeting moieties can be antibodies (ElBayoumi, Torchilin 2009, Pastorino et al. 2003b, Iyer et al. 2011), peptides (Moreira et al. 2001, Temming et al. 2005, Xiong et al. 2005), small molecule ligands (Gabizon et al. 1999, Voinea et al. 2002, Riviere et al. 2011) or specific sugar molecules (David et al. 2004). The advantage of the active targeting includes the accumulation of DDLs and release-encapsulated drug from the DDL only at the targeted site. Despite recent efforts to

achieve this, *in vitro* and *in vivo*, to date there are no FDA approved active targeted DDLs achieved yet.

2.4 Surface charge

Surface charge of the DDL is known to affect its half-life in the bloodstream and its tissue distribution as well as cellular uptake at the target site (Capriotti, Caracciolo, Cavaliere, Foglia, et al. 2012). Also surface charge of the DDL plays a major role in its interactions with opsonin proteins which initiate the process of internalization of DDL by the macrophages, and thus accelerate elimination from bloodstream (Yan et al. 2005, Patel et al. 2011).

It has been noted that neutrally charged liposomes have exhibited significantly enhanced accumulation at the tumor site and tumor vasculature in comparison to the charged ones (Krasnici et al. 2003). Also, several studies have indicated that charged DDLs have shorter half-life in the bloodstream as they activate the complement pathway of the immune system strongly in comparison t bloodstream bloodstream o neutral DDLs (Krasnici et al. 2003). An extensive *in vivo* study showed that charged DDLs containing phosphatidylglycerol (PG), phosphatidic acid (PA), cardiolipin (CL), phosphatidylinositol (PI), or phosphatidylserine (PS) activate the classical immune pathway, with promoting interaction with C1q protein. (Bradley et al., 1999a,b; Chonn et al., 1991a; Devine et al., 1994). Also, the DDLs containing cationic lipids such as DOTAP, have been shown to promote the activation of the alternate immune pathway *in vivo* (Chonn et al., 1991a). Also an interesting study by Ishida et al. (2001) demonstrated that the cholesterol content in DDL plays a key role in the decision of which immune pathway will be activated (classical or alternate pathway). They showed that, 33-mol% or less cholesterol activated complement via the classical pathway, while liposomes with 44-mol% cholesterol activated the complement system through the alternative pathway. They could not, however explain the reason for this observation. With our *in silico* and *in vitro* studies, we have shown that cholesterol content in the DDL affects its surface charge and thus provide a possible explanation for this observation.

2.5 Effect of PEGylation on the DDL

Polyethylene glycol is one of the predominant protective polymers used for coating the DDL. Although it is known to increase the bloodstream lifetime, the exact mechanism through which it achieves this, is still unclear. The results from the reported studies so far have been conflicting, which makes it difficult to derive any heuristics on the possible mechanism through which PEG can prolong the half-life of the DDL in circulation.

On incorporating PEG in anionic liposomes at 5-10% molar concentration, Bradley et al. (1998) noticed inhibition of complement activation mediated through C1q protein binding to the DDL; in another study by Szebeni et al. (2002), they

demonstrated activation of the complement pathway after inclusion of PEG in the DDL formulation; and in contrast Price et al. (2001) observed no effect of PEGylation of the DDL at all on either activation or prevention of the complement pathway.

The deposition of complement pathway proteins and opsonins on the PEGylated DDL surface leads to uptake of the DDL by macrophages (Moghimi and Szebeni, 2003). These findings were attributed to the negative charge on the phosphodiester group of DSPE. It has been reported that upon repeating the dose of PEGylated DDL *in vivo*, its blood half-life decreases significantly, and its uptake by the liver increases suggesting involvement of soluble serum factors in the process. (Bendas et al., 2003; Dams et al., 2000; Ishida et al., 2003a,b; Laverman et al., 2001a,b). This phenomenon is termed as accelerated blood clearance (ABC). (Dams et al., 2000; Laverman et al., 2001b) PEGylation is not responsible for this effect though, as non-PEGylated DDLs shows similar response to the ABC effect.

Cullis et al. (1998) showed that, the formulation density of the PEGylated lipid is an important factor dictating its interactions with protein in the bloodstream. This finding was also confirmed by Ishida et al (2004), where they showed only when the PEG formulation density is increased to 10% in the DDL, its interactions with proteins was minimized with serum proteins.

Other than immune system proteins, PEGylated DDLs can also weakly bind to the serum albumin, which is predominantly present in blood. Johnstone et al. (2001) demonstrated that incubating PEGylated DDLs with serum reduced uptake by macrophages for the case of neutral, cationic and anionic DDLs. They concluded that, interaction with serum albumin might have altered the DDLs properties, which have provided protection against the proteins of the immune system. It has been shown that one of the ways that PEG enhances circulation time of the DDL is by preventing their aggregation. (Allen et al., 2002, Ahl et al. 1997) These studies question the widely accepted phenomenon of prevention of opsonization by PEG, by providing a steric barrier alone to the interactions of proteins with DDL. Thus a clear picture is required with advanced analytical experiments and modelling to understand the above results.

2.6 Protein corona of DDL

Upon introduction of the DDL into circulation *in vivo*, different protein molecules present in bloodstream rapidly adsorb to DDL. These loosely bound proteins on the DDL is known as the “protein corona”; and it varies from DDL to DDL based on its physicochemical characteristics such as size, surface charge and lipid composition (Chonn et al., 1992; Johnstone et al., 2001; Moghimi and Patel, 1988). This protein corona dictates the fate of the DDL. (Since 2002, there have been efforts going on to characterize this protein corona of the DDL. Understanding constituent proteins of corona will help understand the mode of action of DDL in

details. Typically this protein corona consists of complex mixture of opsonins, fibrinogens, immunoglobulins and complement proteins. All of these proteins are recognized by the mononuclear phagocyte system and thus cleared rapidly from the blood circulation (Monopoli et al. 2012). It has been shown that PEGylation helps reduce the protein binding to the DDL, but does not prevent it completely. Thus the bio-distribution and targeting of the PEGylated DDL is also dependent on the adsorbed protein corona in the bloodstream. (Thus the precise knowledge of the constituents of the protein corona will help understand it better. (Barrán-Berdón et al. 2013; Capriotti, Caracciolo, Cavaliere & Foglia 2012a) Recent studies from Wolfram et al. revealed that, when PEGyated as well as non-PEGylated DDLs where incubated with serum, the changes in the zeta potential was observed in both cases (Wolfram et al. 2014). The characteristics of PEGylated and non-PEGylated DDLs differed significantly due to the different protein corona content of the both. Lundqvist et al. have reported that the nature and component of the protein corona can change according to the surface properties and size of the DDL despite being composed of same material. (Lundqvist et al. 2008) This will result in an entirely different biological fate of the two DDLs formulated with same material but having different size.

Advanced analytical techniques are being implemented such as isothermal titration calorimetry, surface plasmon resonance and size exclusion chromatography for studying the affinity and stoichiometry of protein binding to nanoparticles. Coupling these experimental studies with molecular modelling techniques will certainly help understand the specific interactions of DDLs with protein corona in detail.

2.7 Computational simulations of lipids and ions

Lipids are the predominant component of the cell, as it constitutes membranes, lipoproteins and lipid droplets. (Sackmann 1995; Mouritsen 2005; van Meer 2005) Lipids mediate or facilitate varied molecular interactions such as protein functions, signaling and transfer of molecules across them (Simon 2006; Simons 2010). For these reasons, lipids have been studied extensively *in vitro*, *in vivo* as well as *in silico*. As with experiments alone it is difficult to access atomic resolution and the timescales of less than a microsecond, coupling them with MD simulations have helped gain an understanding of the biological phenomena involving ion channels and membrane protein interactions (VR, Bjelkmar 2009; Bucher et al. 2010; Fan et al. 2010).

Kox et al. reported the first simulation of lipids in 1980, consisting of 32 lipids for 80 ps. Since then there have been many more reported studies exploring various aspects of the membrane bilayers and their interactions with ions as well as biological macromolecules. Czaplewski et al. presented the first extensive study encompassing MD simulations of different hydrated PC lipid bilayers with and without sterols. (Czaplewski 2000) Later in 2004, Mukhopardhyay et al. reported the

interactions of ions with the hydrated membrane bilayers (Mukhopadhyay et al. 2004). The exact mechanism of the interactions of the Na^+ with PC membrane were reported by Vácha et al. (VAcha et al. 2009; VAcha et al. 2010).

Recently reported MD simulations of membranes, consisting of ~1000 lipids are performed over a microsecond timescale at atomistic resolutions (Dror et al. 2012). Also, coarse-grained MD simulations allow to access longer timescales (discussed in the MD simulations chapter), by approximating the set of atoms or functional group to a bead, which essentially reduces the number of particles under consideration in MD simulation (Marrink et al. 2004, 2007; Monticelli et al. 2008; Lopez et al. 2009;Ayton and Voth 2009; Murtola et al. 2009).

In our MD simulation studies we build upon this existing knowledge of hydrated membrane bilayer and ion interactions in order to answer the question relevant to pharmaceutical drug delivery.

3. Aims of the study

The general objective of the study was to understand the surface structure and the properties of the DDL with and without PEG and its interactions with the ions in the bloodstream. We have used molecular dynamics to study the DDL model for all cases. These computational simulations were coupled with *in vitro* and *in vivo* experimental validations in studies in I and V respectively.

The specific aims were as follows:

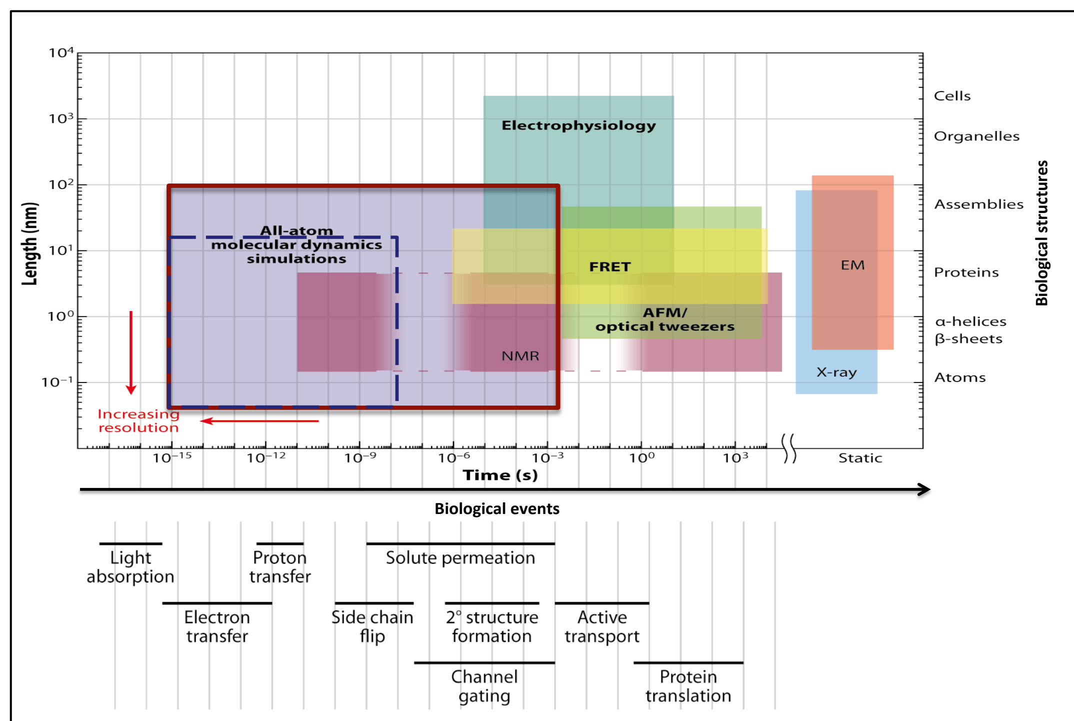
1. Investigate the role of cholesterol in drug delivery liposomes structure
2. Investigate surface structure and properties of the membrane bilayer composed of novel synthetic DLCPe lipids, where the positions of the phosphate and choline groups are exchanged from their positions in regular phospholipids.
3. Study of the effects of different salts present in bloodstream such as NaCl, KCl and $CaCl_2$ and effect of salt concentration on the surface structure of the PEGylated liposome.
4. Understand the interactions at the PEGylated liposome structure containing cholesterol and build the first model for the Doxil® formulation with atomistic level resolution.
5. Investigate the reason for the failure of a new cancer targeting peptide in the PEGylated liposome through computational molecular dynamics and molecular docking.

4. Overview of the methods

4.1 Molecular dynamics simulations

All atom molecular dynamics simulation implemented through the Gromacs package 4.5 (Pronk et al. 2013) was used for all the studies to look at the structure and interactions of the liposome (I – V).

4.1.1 Overview



Adapted from Dror RO et al, 2012

Figure 3: Available biophysical techniques for analysing biomolecules and their interactions spatiotemporally. The x-axis denotes the time scales of events occurring in cells and the y-axis denotes, the size of the biological structures. Each colored box in the plot shows a technique which can be used to examine the specified biological event and at the specific length scale (Dror et al. 2012).

The biomolecules themselves and their interactions are highly dynamic in nature and their motions are often critical to their function. Molecular dynamics simulations can examine these dynamic motions and interactions of macromolecules with atomic resolution. In some way it can be looked at as a computational microscope, capable of revealing biomolecular mechanisms at spatial and temporal scales, which are difficult to observe by present experimental techniques. (The typical biochemical processes that can be viewed by MD simulations include, protein folding, drug

binding, membrane transport, and the conformational changes critical to protein function (Karplus 2002).

4.1.2 MD algorithms

The all-atom MD simulation includes a description of each atom in the simulated system. After describing the system, at each iterative step the forces acting on each atom are computed using Newton's laws of motion to update its position and velocity. The mathematical equation to calculate the physical force on the atom is called a force field. It has three components:

1. Bonded interaction forces - interactions between small groups of atoms connected by covalent bonds
2. van der Waals forces - short range interactions among all pairs of atoms in the system
3. Electrostatic forces - interactions among all pairs of atoms, this fall off slowly with distance.

For nearby pairs of atoms, electrostatic interactions are computed explicitly. However the long-range electrostatic interactions are calculated by approximate methods to speed up the calculations.

4.1.3 Force fields

In classical MD, interactions between the particles are modelled by a potential energy function called a force field, which calculates sums of multi-body potentials including bond stretching, angle bending, torsional twisting, out-of-plane bending, Lennard-Jones (LJ) interactions and Coulomb interactions.

A general force field can be written as follows (note: the particular force field may contain additional terms)

$$\begin{aligned} V(r) = & \sum_{bonds} k_b (b - b_0)^2 + \sum_{angles} k_\theta (\theta - \theta_0)^2 \\ & + \sum_{dihedrals} k_\phi \left(1 + \cos\left(n\phi - \phi_0\right)\right) + \sum_{impropers} k_\psi (\psi - \psi_0)^2 \\ & + \sum_{\substack{non\text{-}bonded \\ pairs(i,j)}} 4\varepsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] + \sum_{\substack{non\text{-}bonded \\ pairs(i,j)}} \frac{q_i q_j}{\varepsilon_D r_{ij}} \end{aligned}$$

where,
V= potential energy function
r = position vector (for all particles)
b, r_{ij} = inter-particle distance
θ = bond angle

ϕ = dihedral angle
ψ = improper dihedral angle
k_b, k_θ, k_ϕ, k_ψ = respective force constants
q = partial charge
ε_D = dielectric constant

The parameters for the force fields are derived from a combination of experimental data and quantum mechanical calculations.

The most commonly used force fields for all-atom simulations are CHARMM (MacKerell et al. 1998), AMBER (Case et al. 2005) and OPLS (Jorgensen & Maxwell 1996). All of these force fields have been validated with various experiments. In all of our studies we have used OPLS all atom force field. The specific details for the MD simulations are discussed in respective chapters.

4.1.4 Limitations of molecular dynamics simulations

Molecular dynamics simulations have been an active area of research for more than 40 years, and so far have faced two major challenges. The first is the "computational expense of the MD simulation" that is how fast the calculations can be performed; this limits the overall timescales for the biomolecular interaction. Second challenge is the development of force fields and the approximations considered in them. Together these two limit the length and their accuracy of MD simulations.

As described above, the relevant timescale for the events in the biological system ranges from the level of nanoseconds to seconds. The longest molecular dynamics simulation reported so far is few milliseconds and considered state of the art today. These simulations can model protein folding (for small and fast folding protein), drug binding, membrane transport, and the conformational changes critical to protein function. Though there are very few cases that involve millisecond long simulation due to their computational demands. As the molecular force fields available today involve appropriate modelling of the relevant underlying physics, they have been restricted in their accuracy (e.g. Non-polarizable force-fields which underestimate the amount of the dielectric response in low-dielectric protein environment and lipid membranes). (Monticelli & Tieleman 2012) Also most of the MD simulations do not yet completely capture the detailed molecular composition of biological systems, which consists of various types of molecules. Lastly, classical MD simulations treat covalent bonds as an assumed parameter of the simulation, as bonds are not able to break or make. Hence, chemical reactions involving breaking/making of covalent bonds cannot be simulated. There are other hybrid computational methods such as quantum mechanics/molecular mechanics (QM/MM) simulations, which can help solve this problem (Kamerlin & Warshel 2011; Senn & Thiel 2009). Since quantum mechanical simulations are computationally expensive, the time and length scale that can be examined with them is extremely limited.

4.2 Molecular Docking

4.2.1 Overview

The biological process involves communication between biomolecules by molecular interactions. Molecular docking is the method to predict these interactions by predicting the global minimum in the interaction energy between the small molecule/hit/lead/drug and the target molecules for e.g. protein, by exploring all available degrees of freedom for the system. By understanding these interactions, novel molecules can be designed which can be then used to control specific biological processes, by optimizing the required interactions.

4.2.2 Autodock

Autodock is one of the several available molecular docking packages, which was used in our study (chapter V), to understand the comparison between interactions of HSA with different component of the PEGylated liposome with targeting moieties. Autodock has automated procedure for predicting the interaction of ligands with bio-macromolecular targets. The Autodock utilizes the Lamarckian Genetic Algorithm and empirical free energy scoring function, to provide docking results for ligands with approximately 10-20 flexible bonds. It uses semi-empirical free energy force field (Autodock force field) to evaluate conformations during docking simulations. This force field was parameterized using a large number of protein-inhibitor complexes for which both structure and inhibition constants, or Ki, are known.

4.2.3 Force field for scoring the interactions

The force field can evaluate binding of the biomolecules in two steps.

1. Intra-molecular energetics is estimated for the transition-unbound states to the conformation of the ligand and target protein in the bound state.
2. Evaluation of the intermolecular energetics of combining the ligand and protein in there bound conformation.

The force field includes six pair-wise evaluations (V) and an estimate of the conformational entropy lost upon binding (ΔSconf) by:

$$\Delta G = (V_{bound}^{L-L} - V_{unbound}^{L-L}) + (V_{bound}^{P-P} - V_{unbound}^{P-P}) + (V_{bound}^{P-L} - V_{unbound}^{P-L} + \Delta S_{conf})$$

where,
L = ligand
P = protein

Each of the pair-wise energetic terms includes evaluations for repulsion, hydrogen bonding, electrostatics, and desolvation:

$$V = W_{vdw}\sum_{i,j}\left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}}\right) + W_{hbond}\sum_{i,j}E(t)\left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}}\right) + W_{elec}\sum_{i,j}\frac{q_i q_j}{e(r_{ij})r_{ij}} + W_{sol}\sum_{i,j}\left(S_i V_j + S_j V_i\right)e^{(-r_{ij}^2/2\sigma^2)}$$

Dispersion/repulsion interaction; Hydrogen bonds; Electrostatic interaction; Desolvation potential

In the equation X, W represents weighting constant, which is optimized to calibrate the empirical free energy based on a set of experimentally determined binding constants.
First term = typical 6/12 potential for dispersion/repulsion interactions. (Based on Amber force field) (Anon 2014)
Second term= H-bond term based on a 10/12 potential (C and D are assigned to give a maximal well depth of 5 kcal/mol at 1.9Å for hydrogen bonds with oxygen and nitrogen, and a well depth of 1 kcal/mol at 2.5Å for hydrogen bonds with sulphur). E(t) provides directionality based on the angle t from ideal H-bonding geometry.
Third term = Coulomb potential for electrostatics
Fourth term = Desolvation potential based on the volume of atoms (V) that surround a given atom and shelter it from solvent, weighted by a solvation parameter (S) and an exponential term with distance-weighting factor σ=3.5Å (Morris et al. 2009).

4.2.4 Limitations of molecular docking

The docking protocols are improving significantly in force field parameters (Karaca & Bonvin 2013). Molecular docking is able to correctly predict the molecular pose of interactions, however, the main issue remains scoring and ranking of the various obtained poses. Due to this, the amount of false positives obtained is significantly higher (Dror 2012), hence one needs to validate the results obtained by molecular docking by complementary experiments. Also the entropic contribution in ligand-receptor interactions is an important factor in binding energy calculations and is very difficult to be considered in docking protocols. Kongsted et al. and Coutinho et al. and have attempted to factor in the entropic component in the docking calculations which have increased the accuracy of the scoring functions. These approached include, taking into account the interactions from water shell (4Å) around the protein molecules to minimize the changes in the protein geometry or calculating the loss in torsional, vibrational, rotational and translational free energies of the ligand upon binding with the receptor.

In this section, the brief details of the molecular modelling methodologies and their limitations are summarized. The exact details and parameters of the methodologies used in each of the study are mentioned in the respective chapters I-V in the next section.

10. Summary of the main results

in silico /in vitro	Property	Materials	Result	Publication
in silico	Ion binding to membrane headgroups	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	Na+ is highest in the absence of cholesterol ~48% and decreases with increase in cholesterol to ~20%	I
		POPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	Na+ is highest in the absence of cholesterol ~45% and decreases with increase in cholesterol to ~16%	
in silico	Charge density	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	Charge density decreases as cholesterol content increases	I
		POPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	Charge density decreases as cholesterol content increases	
in vitro	ζ potential	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	Zeta potential is maximum in absence of choleasterol, wiz ~2mV and drops down to ~-6 with increase in cholesterol in presence of saline	I
		POPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	Zeta potential is maximum in absence of choleasterol, wiz ~1mV and drops down to ~-2 with increase in cholesterol in presence of saline	
in silico	Mass density profile	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1 in presence of NaCl at	With increase in cholesterol content, Na+ peak is shifted away	I

		125 mM	from the membrane bilayer	
		POPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	With increase in cholesterol content, Na+ peak is shifted away from the membrane bilayer	
in silico	Area per lipid of bilayer	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM	With increase in cholesterol content, area per lipid decreases	I
		POPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM		
in silico	Mass density plot	DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM NaCl	Na+ peak shifts away from the membrane bilayer in case of DLCPe as compared to DLPC	II
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM KCl	K+ does not absorb to the membrane bilayer in both DLPC and DLCPe membranes	
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM CaCl2	Ca2+ peak shifts away from the membrane bilayer in case of DLCPe as compared to DLPC	
in silico	Water ordering	DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM NaCl	Comparing the DLPC with DLCPe, in case of all systems, water ordering is plot shows reversal in the orientation of water molecules with respect to membrane normal	II
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM KCl		
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM CaCl2		
in silico	Electrostatic potential across membrane	DLPC membrane bilayer and DLCPe membrane bilayer with 125	In cases of all 3 salts (NaCl, KCl and CaCl2) . The peak at the	II

	bilayer	mM NaCl	potential in the headgroup region is shifted towards the bilayer center and is roughly 0.15 mv higher for the case of the CPe lipids than for the PC lipids.	
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM KCl		
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM CaCl2		
in silico	Rotational motion of headgroups	DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM NaCl	The rotation of CPe shows negligible dependence on the variety of salt present, while for the case of the PC bilayer, the type of salt affects the rotational motion of the headgroup.	II
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM KCl		
		DLPC membrane bilayer and DLCPe membrane bilayer with 125 mM CaCl2		
in silico	Mass density profile	DSPC membrane bilayer with PEG at 10% formulations density with 125nM NaCl and 0mM NaCl (only counter ions)	In the presence of the Cl− anions shifts the Na+ in the PEG layer further out into the layer. The presence of salt can also be seen to expand the PEG layer slightly, for the liquid crystalline (DLPC) case also increasing the depth of its penetration into the membrane interior.	III
in silico *in silico*	Mass density profile Ion binding to PEG oxygens	DLPC membrane bilayer with PEG at 10% formulations density with 125nM NaCl and 0mM NaCl (only counter ions)	In the presence of the Cl− anions shifts the Na+ in the PEG layer further out into the layer. The presence of salt can also be seen to expand the PEG layer slightly,	
		Comparison of Na+, K+, Ca2+ ions		III

		mass density profiles at 125 mM with DSPC membrane bilayer at 10% PEG formulation density	for the liquid crystalline (DLPC) case also increasing the depth of its penetration into the membrane interior. Na+ strongly interact with PEG oxygens (66.4%), K+ moderately interact with PEG oxygens(25%) and its non existent for the Ca2+ ions	
in silico	Area per lipid of bilayer	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM with 10% PEG formulation density	With increase in cholesterol content at 10% PEG formulation density, area per lipid decreases from 0% to 16.67% of cholesterol then increases with increase in cholesterol till 50%	IV
in silico	Visualization	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM with 10% PEG formulation density	PEG enters the membrane bilayer in presence of cholesterol while when it is absent, it doesn't enter DSPC membrane bilayer	IV
in silico	Visualization	DSPC:Chol membrane bilayer with 6:0, 5:1, 4:1, 3:1, 2:1, 1:1 in presence of NaCl at 125 mM with 10% PEG formulation density	PEG interacts with cholesterol in a specific way interacting with β side of cholesterol	IV
			Cellular affinity for AETP liposomes did not increase of liposomes in HUVEC-cells.	V
	Pharmacokinetics		Cytotoxic efficacy of doxorubicin-loaded AETP liposomes did not increase in HUVEC or SVEC4-10 cells.	V

in vivo	Pharmacokinetics Uptake in tumor		AETP-targeted liposomes showed similar elimination half-life (~7–10 h) to non-targeted liposomes (~6–7 h).	V
			No significant differences in co-localization of liposomes and endothelial cells observed between the AETP-targeted and non-targeted liposomes in confocal microscopy.	V
			No significant difference in tumoral uptake between AETP-targeted and non-targeted liposomes could be seen.	V
in silico	Solvent accesable surface area	DLPC and DSPC membrane bilayer with 10% PEG formulation density at X% targeting moeities RGD and AETP	Computational modeling revealed that both AETP and RGD peptides located in the PEG region of the PEGylated liposomes. However, AETP was more covered by the PEG chains, while RGD was more exposed to the solvent.	V
in silico	Molecular docking	HAS with PEG, AETP, and RGD peptide	Protein-ligand docking showed HSA having stronger binding affinity to PEG and to AETP than to RGD peptide.	V

11. General discussion

11.1. Surface charge of the DDL

Surface charge of the DDL is one of the important properties responsible for its interactions with the proteins in the bloodstream. Hence, in order to maximize the DDLs' blood circulation time by minimizing its non-specific interactions with proteins in the bloodstream and immune system components, the surface charge of the DDL is important to take into account while formulating it.

We altered the levels of several elements of the DDL formulation (cholesterol, DSCP and PEGylated lipids) and studied their role on the effective surface charge of the DDL resulting from its interactions with salts present in the bloodstream (NaCl, KCl and $CaCl_2$).

11.1.1 Role of cholesterol in the DDL and biological membrane

Cholesterol is not only an important component of biological membranes (Van Meer et al. 2008; g et al. 2009), it is also a component of most FDA approved DDL formulations (Dawidczyk et al. 2014). Cholesterol plays a role in many of the membrane properties, including mechanical strength, elasticity, packing density and permeability. Here we report a novel role of cholesterol in the lipid membrane, it contributes to the surface charge of membrane bilayer. With our MD simulation studies we show that the presence of cholesterol results in a decrease in Na^+ binding for the typical neutral (zwitterionic) phospholipid membranes (DSPC and POPC). The ζ-potential measurements carried out in parallel with our simulations showed decrease in its surface charge with increase in its cholesterol content. While cholesterol has been shown to alter several properties of the phospholipid membrane, this specific effect of altering the membrane charge is novel and has both biological and pharmaceutical relevance.

It is known that surface charge strongly influences the interactions of biological membranes and DDLs with proteins (Townson et al. 2013; Pozzi et al. 2014), however these interactions certainly involve more factors than surface charge alone. A significant amount of further study is required to understand this in full detail.

11.1.2 DDLs composed of synthetic lipids

Surface properties of DDLs govern their interactions with bloodstream proteins and also the release profile of encapsulated drug molecules. (Torchilin 1996) While so far the majority of DDLs have been composed of standard phospholipids, recently DDLs have also been formulated from novel synthetic

phospholipids with the aim of imparting specific desired properties to the DDL. (Zhao et al. 2012; Perttu et al. 2012).

One such novel synthetic lipid is inverse phosphatidylcholine (CPe) (Perttu et al. 2012). The inverse phosphatidylcholine (CPe) lipids contain a phosphate group and choline group as PC lipids but the places of these groups are interchanged such that the phosphate group is at the membrane interface oriented out into the water and the choline group is directly attached to the lipid tails.

When the physicochemical properties of the CPe formulated liposome was compared to the PC formulated ones, they were similar, except CPe formulated liposomes differed significantly in their surface charge and release profile of the encapsulated molecules. To understand the possible cause of these differences, we performed MD simulations of the membrane bilayers formulated with DLPC and DLCPe lipids with three salts found in the bloodstream, NaCl, KCl and $CaCl_2$.

The simulations studies revealed that CPe formulated membrane bilayers have a significantly larger area per lipid in comparison to the PC membrane bilayer. Also, there is a marked difference between the electrostatic potentials along the membrane normal of the CPe formulated bilayer as compared to the PC formulated bilayer. The difference in electrostatic potential of the PC and CPe formulated membrane bilayer is due to difference in the membrane-cation (Na^+ and Ca^{2+}) interactions. As indicated from the proportion of ions bound to the membrane and the mass density profile along the membrane normal, we could see that Na^+ ions bind strongly to the PC headgroups of the membrane bilayer in comparison to the case for the CPe membrane bilayer. The K^+ ions, however, do not show significant binding for both PC and CPe membrane headgroups. The Ca^{2+} ions binding to the lipid headgroups are observed for the cases of both the PC and CPe formulated liposomes.

Together, size and surface charge of the DDL affect the release rate of encapsulated drug molecules. Hence, depending on biochemical characteristics of the drug, specific formulations of the DDL can be designed to control its release rate from the DDL.

11.2 PEGylated DDLs

DDLs are often PEGylated in order to reduce uptake by the RES (Harris & Chess 2003) and increase uptake at tumor sites (Fang et al. 2011). The PEG polymer forms a protective polymer corona surrounding the DDL, which influences its surface properties. This in turn affects its interactions with proteins in the bloodstream. Hence obtaining a correct picture of the mechanisms involved in the PEG stealth sheath becomes crucial. We have examined the PEGylated DDL membrane bilayer with atomistic level resolution using MD simulation. To obtain

the surface structure of the PEGylated liposomes and factors affecting it we varied the concentration of different components of the DDL that are lipids, cholesterol and PEG and observed the interactions with salt ions in the bloodstream.

11.2.1 Effect of PEG formulation density on surface structure of the DDL

We systematically varied the PEG formulation density of the DDL from 5 to 10 %, in both the absence and presence of NaCl at physiological concentration. Through MD simulations we demonstrated that at physiological salt concentration, the PEG layer expands due to Na^+ binding to PEG oxygens. At 5 % PEG formulations density, Na^+ ions bind to the membrane bilayer in addition to the PEG oxygen, and Cl^- ions collocate to the PEG layer, but do not show any binding. This binding of Na^+ to PEG oxygen and co-localization of Cl^- ions in the PEG layer results in a neutral charge layer in the PEG layer. However, when the PEG formulation density is increased to 10 %, Na^+ ions do show binding to the PEG oxygens but Cl^- ions do not co-localize with the PEG layer as the membrane structure is tighter and the Cl^- ions are expelled from the PEG layer. Due to this exclusion of Cl^- ions from the PEG layer, a negatively charged layer is formed above the PEG layer and the PEG layer is now positively charged due to association with the Na^+ ions. This results in an effective charged double layer on the DDL surface.

We also compared the extent to which Na^+, K^+, and Ca^{2+} ions bind to the membrane headgroups and PEG oxygens. We observed that Na^+ interacts strongly with PEG oxygen in comparison to the K^+ ions, whereas the Ca^{2+} ions do not interact with the PEG oxygen at all. They however interact very strongly with the membrane headgroups and the Cl^- ions.

These results provide a possible explanation for the results of the *in vitro* studies reported by Bronich et al. and Holland et al. The Ca^{2+} ions crosslink the headgroups of two separate liposomes, and thus induce liposome fusion. In both experimental studies it was observed that crosslinking between the liposomes is inhibited in the presence of Ca^{2+} ions when they are PEGylated. In the presence of PEG, Ca^{2+} ions do not bind to PEG oxygen at all and are located outside of the PEG layer, acting as a steric barrier to fusion.

11.2.2 Role of cholesterol in PEGylated DDL

To study the role of cholesterol in the PEGylated DDL, we incorporated cholesterol into the PEGylated membrane bilayer and varied its concentration systematically from 0 to 50 % molar fraction.

We observed that the PEG polymer enters the DSPC membrane bilayer only when cholesterol is included in the formulation. This results in an increase in the area per lipid of the DDL with increase in cholesterol. Also, the PEG polymer enters the lipid bilayer in the presence of cholesterol in a very specific way, such that it interacts only with the β-side of the cholesterol. This shows that the PEG polymer

plays a role in the overall membrane bilayer structure and will affect its properties including membrane permeability and stability.

Results obtained from MD simulations provided a possible explanation for the previously reported *in vitro* studies. Nikolova et al studied the effect on permeability of encapsulated D-glucose from the liposome by varying its PEG formulation density (Nikolova & Jones 1996). They reported that with an initial increase in the PEG formulation density up to 5 %, the permeability decreases, however in the range of 5-7 % PEG formulation density, there is an increase in the permeability for D-glucose. In another study, Garbuzenko et al. studied the effect of PEG formulation density on liposome size (Garbuzenko et al. 2005). They observed that liposome size is inversely proportional to the PEG formulation density up to a level of ~7 %. There is, however an anomalous increase in liposome size with further increase in the PEGylated lipid content.

Both of the above mentioned observations were previously attributed to the transition in the structure of the PEG corona from the mushroom regime to the polymer brush regime, resulting from the increasing density of bound polymer on the surface. As evident from our MD simulation studies of PEGylated bilayers, we consistently observed PEG to enter in membrane bilayers in the presence of cholesterol. Also as PEG enters the membrane bilayer it interferes with membrane structuring and, hence, will influence the release rate of the encapsulated molecules in the DDL.

In addition, Janout et al. observed that for the case of the PEGylated liposomes with cholesterol, PEG is capable of stimulating the release of cholesterol from the liposomes (Janout et al. 2012). In our simulations we did not observe this mechanism directly, however our results suggest this might be possible, as PEG enters the DDL membrane bilayer and interacts specifically only with cholesterol in all the cases. As indicated by mass density profile, we observed that due to this interaction, cholesterol shifts toward the membrane water interface in comparison to non-PEGylated liposome bilayers.

We studied the effect of cholesterol on the surface structure of the PEGylated DDL and provided the first model at atomistic resolution for the Doxil® formulation that is the first FDA approved PEGylated DDL. This model can be used in future MD studies to investigate interactions with proteins.

11.3 Targeted DDL

In order to reduce off target effects and increase the concentration of the PEGylated DDL at the specific target site, the PEGylated DDL can be conjugated with a targeting moiety. The targeting moiety achieves this by binding to specific receptors present on the surface of target cells.

The novel neovasculature endothelium targeting moiety, AETP, was identified through phage display experiments. The AETP moiety is a polypeptide, predominantly consisting of hydrophobic amino acids. The AETP moiety was conjugated with the PEGylated DDL to achieve an increase in target cell affinity and greater accumulation at the tumor site as compared to non-targeted PEGylated DDL. However, as observed in both *in vitro* and *in vivo* experiments, this was not the case. Thus, the AETP moiety was not able to provide the desired targeting efficacy. As the AETP moiety is hydrophobic in its biochemical nature, it was thought to be interacting with the hydrophobic lipid tails of the DDL membrane bilayer. As this was difficult to validate with the experiments alone, we performed molecular dynamics simulations of the conjugated AETP moiety with the PEGylated DDL and molecular docking studies of the AETP moiety with HSA to understand possible promiscuous interactions. Molecular dynamics simulations revealed that AETP does not enter into the membrane core but it is instead covered by PEG polymer. This excessive coverage of the AETP moiety with PEG polymer could potentially mask its interaction with the intended target site. Furthermore molecular docking studies suggest that the AETP moiety has a higher binding affinity towards HSA (predominantly present in blood plasma). This binding of AETP to the HSA could be an additional factor in not achieving targeted drug delivery.

Thus molecular modelling studies showed that PEG might not be a suitable choice for AETP targeted DDLs and alternative protective polymer could be used to address the issue. The search for possible alternatives to PEG is an active field of research and Knop et al. have already reported some of the possible alternatives for the PEG polymer. The combined approach of molecular modelling methods coupled with *in vitro* and *in vivo* studies is capable of characterizing the potential interactions of these alternative protective polymers and components of the DDL and can help construct effective DDLs in the future.

12. Conclusions

1. With increasing cholesterol concentration from 0 to 50 % in lipid bilayer (DSPC and POPC) in presence of saline, *in silico* we find that the number of Na^+ ions binding to the membrane headgroups decreases. The *in vitro* validation by zeta potential measurements confirmed these results by showing the same trend; the zeta potential of the DSPC and POPC liposomes decreased with increase in cholesterol content.

2. By reversal of the phosphatidylcholine group to CPe (where the phosphate group faces the membrane interface instead of the choline), with all-atom MD simulations we report that binding of Na^+, K^+ and Ca^{2+} ions is reduced. This affects the membrane zeta potential and thus surface charge of the membrane bilayer. These results provide an explanation to the experiment that showed a drop in zeta potential of the CPe liposomes when compared with PC liposome in presence of Ca^{2+} ions.

3. Increasing the formulation density of PEGylated lipid from 5 to 10 % decreased the extent to which the Cl^- ions penetrated the PEG layer. This makes the PEG layer effectively positively charged. The interaction of the PEG with the K^+ ions was weaker than for the Na^+ ions, and non-existent for the Ca^{2+} ions. Our results provide an explanation for the experimental observation of the mechanisms through which PEG may inhibit uptake of the liposome by the RES and also a possibility that calcium induced membrane fusion is inhibited.

4. In presence of cholesterol, PEG enters into the lipid bilayer in a very specific orientation; the PEG winds along the β face of the cholesterol. Also, when the membrane is PEGylated, the area per lipid increases rather than decreases, due to interactions with cholesterol. This study provides mechanistic explanations for the existing experimental results concerning the effect of adding cholesterol to the PEGylated liposome.

MD simulations show that novel AETP targeting moieties were located deep in the PEG layer of the liposomes, and thus affect the interactions with target receptors. Also, molecular docking shows that peptide binding to HSA may further inhibit target binding

13. Future prospects

Drug delivery liposomes: Integration of *in vitro* and *in vivo* with *in silico* methods

Liposome based drug delivery approaches have already demonstrated great promise to tackle diseases such as cancer (Fonseca et al. 2014). Recent studies are moving toward targeted DDLs and nanoparticles in general. The research is increasing in this area, reflected in the number of publications in the field (>20000), however only few of these potential drugs make it into clinical trials.

The development of novel DDLs faces many technical challenges, which are described previously. These include structural stability of the DDL, short half-life in the bloodstream and limited accumulation at the target site. As the composition of DDLs and their surface structure are responsible for the nature of its interactions with proteins in the blood plasma, there is a need to understand the physicochemical properties of the DDLs (Townson et al. 2013).

It has recently been established that once liposomes enter into the bloodstream, they loose their synthetic identity (Pozzi et al. 2014). With specific and non-specific interactions of the DDL surface with elements of the bloodstream such as ions, serum proteins etc. This new bio–nano interface around the DDL is termed as a protein corona(Caracciolo et al. 2010). This protein corona decides the fate of the DDL in the bloodstream. (Cedervall, Lynch, Lindman, et al. 2007b; Nel et al. 2009; Lundqvist et al. 2008; Lindman et al. 2007; Cedervall, Lynch, Foy, et al. 2007a).

Recent studies with respect to charecteriztion of the protein corona include understanding of nanoparticle surface properties and size (Lundqvist et al. 2008), binding with HSA (Lindman et al. 2007), identification of specific plasma proteins (Nel et al. 2009) and characterization of these interactions as specific or non-specific ones (Cedervall, Lynch, Lindman, et al. 2007). Most recent work along these lines includes the effect of PEG chain length on bio-nano interface formation in cancer cells (Wolfram et al. 2014).

While these efforts are attempting to provide detailed kinetics and interactions of the DDLs with proteins in the bloodstream there have been some puzzling findings. One of the recent *in vitro* study reports that, upon incubation of serum with liposomes (PEGylated and non-PEGylated), the liposomes decreased in size (Wolfram et al. 2014). Also Townson et al. (Townson et al. 2013) showed for the first time that, despite having identical components, size and surface charge; the nanoparticle differing in only the spatial arrangement of surface groups, showed completely different *in vivo* results. Molecular dynamics simulations can attempt to answer these questions as all the interactions can be observed and the properties can be understood at atomic level of resolution.

As the current experimental efforts are on-going, they can be coupled with MD simulation studies with model membrane bilayers of PEGylated and non-PEGylated liposomes with proteins. These simulations can certainly help look at interactions of DDLs in greater details. For more comprehensive studies, however, more coarse grained simulations such as martini models of the membrane bilayer with proteins of interest can be used (Periole & Marrink 2013).

I hope that findings presented in our studies can help build an understanding of specific and non-specific interactions of DDLs with proteins of the blood plasma and immune system. Once these interactions are understood in greater details, the novel DDLs can be designed for specific drug delivery purposes and help move the research in targeted drug delivery forward, in a hypothesis driven way.

14. References

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