NANOPARTICLE-CELL LIPID MEMBRANE BIOPHYSICAL INTERACTION
AND ITS ROLE IN DEVELOPING TUMOR TARGETED NANOPARTICLES

RADHIKA C. BHAVE

Bachelor of Engineering in Metallurgy
Government College of Engineering, Pune
July 2004

Master of Science in Material Science and Engineering
Clemson University
May 2007

submitted in partial fulfillment of requirement for the degree
MASTER OF SCIENCE IN BIOMEDICAL ENGINEERING
at the
CLEVELAND STATE UNIVERSITY
May 2015
We hereby approve this thesis for

(Radhika C. Bhave)

Candidate for the Master of Science in Biomedical Engineering degree for the

Department of Chemical and Biomedical Engineering

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies

Vinod Labhasetwar, Ph.D. (Chairperson)
Department of Biomedical Engineering; 9th December 2014

Nolan Holland, Ph.D.
Department of Biomedical Engineering; 9th December 2014

Mekki Bayachou, Ph.D.
Department of Chemistry; 9th December 2014

Kiril Streletzky, Ph.D.
Department of Physics; 9th December 2014

Xue-Long Sun, Ph.D.
Department of Chemistry; 9th December 2014

Maciej Zborowski, Ph.D.
Department of Biomedical Engineering; 9th December 2014

Student Date of Defense: December 9, 2014
To my family,

Sharmila and Chandrashekhar Bhave, Varsha and Chandrashekhar Sabnis,

Rupali Deo, Rashmi Joshi, Mocha and Amit Sabnis.

Thank You for believing in me.
ACKNOWLEDGEMENTS

I would like to express my gratitude to countless people without whose help and support this thesis would not have been possible.

First and foremost, I would like to express my sincere gratitude to my advisor Dr. Vinod Labhasetwar for his continuous support during my thesis study and research. A special thanks to him for the patience, sincerity, and immense knowledge with which he guided me throughout my graduate work. My sincere thanks to my academic advisor Dr. Nolan Holland for his guidance throughout my coursework and studies. I extend my gratitude to rest of the committee members: Dr. Mekki Bayachou, Dr. Maciej Zborowski, Dr. Xue-Long Sun, and Dr. Kiril Streletzky for their valuable feedback, suggestions, and insightful comments. I would also like to thank our BME program director Dr. Joanne Belovich for her help and guidance.

I acknowledge the help and guidance of my fellow labmates during the course of my doctoral studies. I thank Dr. Chiranjeevi Peetla, Dr. Sivakumar Vijayraghavalu, and Dr. Isaac M. Adjei for their help and feedback with various experiments. I would also like to thank all the past and present members of Dr. Labhasetwar’s lab. Many thanks to Rebecca Laird and Darlene Montgomery for their help with all the administrative services.
I would like to acknowledge the financial support: Graduate fellowship from Cellular and Molecular Medicine Specialization (CMMS) program between CSU and Cleveland Clinic; Doctoral Dissertation Research Expense Award, CSU; and Funds from Cleveland Clinic.

This work would not have been complete without the encouragement from my friends in Cleveland and Clemson that have been like a family. I would like to thank my parents Mrs. Sharmila Bhave, Mr. Chandrashekhar Bhave, Mrs. Varsha Sabnis and Mr. Chandrashekhar Sabnis, and Mrs. Rashmi Bhave-Joshi and Mrs. Rupali Sabnis-Deo for their support and encouragement throughout every step of my life. Finally, I would like to thank my loving husband Mr. Amit Sabnis, who has been patiently by my side through all the good and bad times. This work would not have been possible without him.

Thank You.
Nanoparticle-Cell Lipid Membrane Biophysical Interaction and its Role in Developing Tumor Targeted Nanoparticles

Radhika C. Bhave

ABSTRACT

Tumor targeted nanoparticles could improve drug delivery of the encapsulated cancer therapeutics to the tumor while reducing their non-specific side effects. However, complex conjugation chemistry, weak antibody-nanoparticle binding, and finite number of receptors available for nanoparticle binding could limit the efficacy of tumor targeted nanoparticles. Therefore, there is need for a new approach to improve nanoparticle localization in tumors.

Physical properties of nanoparticles particularly their surface properties have shown to influence in vitro cellular uptake, in vivo biodistribution and tumor localization of nanoparticles. Apart from physical characteristics of nanoparticles, their uptake has also been shown to depend on the cell type. Additionally, progression of disease such as cancer can cause changes in the cell membrane lipid composition, and thereby influence nanoparticle-cell membrane interactions and cellular uptake of nanoparticles. The research described in this thesis explores an interesting approach that explores the differences in the cell membrane lipid composition as well as modification in nanoparticle surface characteristics to design nanoparticles that would preferentially target tumors.
In our study, biophysical interactions between nanoparticles and endothelial cell model membrane demonstrate the effect of surface chemistry of nanoparticles on such interactions. Nanoparticles with sulfate and amine surface chemistry show higher interactions with model membrane as compared to nanoparticles with carboxyl and amidine surface chemistry.

Biophysical characteristics of cell membrane lipids extracted from normal endothelial and cancerous cells demonstrate the fluidic nature of cancerous cell membrane as compared to the rigid and condensed nature of normal endothelial cell membrane. Nanoparticle-cell membrane lipid interactions demonstrate more selective interactions between nanoparticles with sulfate surface chemistry and cancer cell membrane lipids than with normal cell membrane lipids. On the other hand, nanoparticles with amine surface chemistry demonstrate non-selective interactions with both cancerous and endothelial cell lipid membranes. Nanoparticles loaded with a hydrophobic near infrared dye were used to quantitatively determine biodistribution and tumor localization of nanoparticles \textit{in vivo}, using an optical imaging technique. Surface chemistry of nanoparticles was shown to influence nanoparticles biodistribution and tumor localization. Nanoparticles with sulfate groups demonstrate higher tumor localization and retention as compared to nanoparticles with amine and carboxyl groups.

The results demonstrate that selectivity of nanoparticle with sulfate surface chemistry towards cancerous cell lipid membrane translates in greater tumor localization \textit{in vivo}. 
We further studied effect of surface of PLGA-based biodegradable nanoparticles and their interactions with model membranes. These biodegradable nanoparticles when formulated using emulsion-solvent evaporation method retains a fraction of emulsifier, polyvinyl alcohol (PVA) associated with the surface, commonly referred as residual PVA. In this study, nanoparticles were formulated with PVA of different molecular weight and degree of hydrolysis. Our findings illustrated that surface associated residual PVA significantly influences biophysical interactions of nanoparticles with endothelial cell model membrane.

Biophysical interactions between nanoparticles and cell lipid membranes could potentially be explored to understand the effect of surface characteristics of nanoparticles on cellular uptake, biodistribution and targeting.
TABLE OF CONTENTS

DEDICATION........................................................................................................... iii

ACKNOWLEDGEMENTS ......................................................................................... iv

ABSTRACT ............................................................................................................... vi

LIST OF TABLES ..................................................................................................... xii

LIST OF FIGURES .................................................................................................. xiii

ABBREVIATIONS ................................................................................................... xvi

CHAPTERS

I. INTRODUCTION

1.1 Introduction .......................................................................................................1

1.2 Nanocarriers for Cancer Therapy ......................................................................2
   1.2.1 Liposomes .................................................................................................2
   1.2.2 Micelles ......................................................................................................3
   1.2.3 Dendrimers ................................................................................................4
   1.2.4 Nanoparticles .............................................................................................5

1.3 Tumor Targeting Approaches ..........................................................................6
   1.3.1 Passive Targeting ......................................................................................6
   1.3.2 Active Targeting ........................................................................................7
      1.3.2.1 Tumor Targeting Peptides .................................................................8
      1.3.2.2 Tumor Targeting Antibodies ............................................................10
      1.3.2.3 Tumor Targeting Co-factors ............................................................11

1.4 Limitations of Current Nanocarrier Targeting Strategies ...............................12

1.5 Influence of Physical Characteristics of Nanocarriers on Tumor Localization ..13
   1.5.1 Effect of Size and Shape of Nanocarriers on Tumor Localization ............13
   1.5.2 Effect of Surface Hydrophilicity of Nanocarriers on Tumor Targeting ....14
   1.5.3 Effect of Surface Charge of Nanocarriers on Tumor Targeting ..............16
   1.5.4 Effect of Surface Functionalization of Nanocarriers on Tumor Targeting .................................................................18

ix
1.6 Biophysical Characterization ................................................................. 19
  1.6.1 Influence of Lipids on Membrane Biophysics and Intracellular
        Nanocarrier Accumulation ................................................................. 20
  1.6.2 Changes in Cell Membrane Characteristics during
        Disease Progression .............................................................................. 23
1.7 Model Membrane Systems to Investigate Nanocarrier-Cell Membrane
        Interactions ............................................................................................ 24
  1.7.1 Supported Lipid Bilayers ................................................................. 25
  1.7.2 Liposomes ......................................................................................... 25
  1.7.3 Lipid Monolayers ............................................................................... 26
1.8 Nanocarrier-Cell Membrane Interactions for Development of Nanocarrier-based
        Drug Delivery Systems ............................................................................ 29
1.9 Statement of Problem ............................................................................. 29
1.10 Hypothesis ............................................................................................ 30
1.11 Specific Aims ......................................................................................... 30
1.12 General Overview of the Dissertation .................................................. 31

II. BIOPHYSICS OF NANOPARTICLE-ENDOTHELIAL CELL MODEL
    MEMBRANE INTERACTIONS: EFFECT OF SURFACE CHEMISTRY OF
    NANOPARTICLES
  2.1 Introduction ............................................................................................ 32
  2.2 Materials ................................................................................................. 33
  2.3 Methods .................................................................................................. 33
  2.4 Results ..................................................................................................... 38
  2.5 Discussion ............................................................................................... 59
  2.6 Conclusions ............................................................................................ 67

III. EFFECT OF NANOPARTICLE SURFACE CHEMISTRY ON BIOPHYSICAL
    INTERACTIONS WITH CELL LIPID MEMBRANES
  3.1 Introduction ............................................................................................ 68
3.2 Materials ..................................................................................................................70
3.3 Methods....................................................................................................................70
3.4 Results......................................................................................................................78
3.5 Discussion................................................................................................................93
3.6 Conclusions..............................................................................................................98

IV. EFFECT OF NANOPARTICLE SURFACE CHEMISTRY ON
BIODISTRIBUTION AND TUMOR TARGETING
4.1 Introduction..............................................................................................................99
4.2 Materials..................................................................................................................101
4.3 Methods..................................................................................................................101
4.4 Results.....................................................................................................................103
4.5 Discussion...............................................................................................................112
4.6 Conclusions............................................................................................................116

V. THE EFFECT OF RESIDUAL POLY(VINYL ALCOHOL) ON
BIOPHYSICAL INTERACTION OF NANOPARTICLES WITH
ENDOTHELIAL MODEL MEMBRANE
5.1 Introduction..............................................................................................................117
5.2 Materials..................................................................................................................119
5.3 Methods..................................................................................................................120
5.4 Results.....................................................................................................................126
5.5 Discussion...............................................................................................................136
5.6 Conclusions............................................................................................................137

VI. SUMMARY AND FUTURE DIRECTIONS .........................................................138
6.1 Summary...............................................................................................................138
6.2 Future Studies ......................................................................................................140

REFERENCES..............................................................................................................142
LIST OF TABLES

Table 2.1  Physical Characteristics of Polystyrene Nanoparticles with Different Surface Chemistry..................................................................................................................39

Table 2.2  Percent Trough Area at Different EMM SPs of Isotherm in Presence of NPs........................................................................................................................................47

Table 2.3  Relative Adsorption/Dissociation Coefficient of Nanoparticles estimated from Nanoparticle-EMM Interactions ...........................................................................51

Table 3.1  Relative Concentration of Different Phospholipids in Total Lipids ..........81

Table 3.2  Physical Characterization of dye-loaded NPs with different surface chemistry....................................................................................................................................90

Table 5.1  Characteristics of PVA used to formulate NPs........................................128

Table 5.2  Characterization of Nanoparticle Formulation with Different Type of PVA ........................................................................................................................................129
LIST OF FIGURES

Figure 1.1 Representation of Langmuir Balance to measure the differences in monolayer surface pressure using Wilhelmy plate ........................................27

Figure 1.2 Schematic representation of Langmuir isotherm and the stages of monolayer compression until complete collapse is attained.................................28

Figure 2.1 Schematic to prepare LS films for AFM Imaging ................................37

Figure 2.2 Intrinsic surface activity of NPs with different surface chemistry for NPs suspended in water and FBS .................................................................41

Figure 2.3 Changes in surface pressure following interaction with NPs with different surface chemistry .................................................................................44

Figure 2.4 Changes in EMM surface pressure for NPs with different surface chemistry for NPs suspended in water and FBS ..............................................45

Figure 2.5 Surface pressure-area isotherms of EMM in presence of NPs .............48

Figure 2.6 Variation of surface pressure of EMM after injection of NPs with different surface chemistry as function of time ½ ..............................................52

Figure 2.7 Changes in EMM LS film morphology following interaction for NPs suspended in water ..................................................................................55

Figure 2.8 Changes in EMM LS film morphology following interaction for NPs suspended in FBS ......................................................................................56

Figure 2.9 Changes in EMM surface pressure after interaction with NPs of different sizes and surface chemistry, for NPs suspended in water .....................58

Figure 2.10 Changes in EMM surface pressure after interaction with NPs of different sizes and surface chemistry .........................................................58
Figure 3.1  Phospholipid separation of lipids extracted from HIAECs and PC-3 cells analyzed by thin layer chromatography .............................................80

Figure 3.2  Biophysical characterization of normal endothelial and prostate cell lipid membranes ..................................................................................................................83

Figure 3.3  NP-cell lipid membrane biophysical interaction studies for NPs suspended in water ................................................................................................................86

Figure 3.4  NP-cell lipid membrane biophysical interaction studies for NPs suspended in FBS ................................................................................................................87

Figure 3.5  NIR dye was loaded in NPs with different surface chemistry ..........89

Figure 3.6  NIR dye release from NPs with different surface chemistry ..........89

Figure 3.7  Endothelial and Prostate cancer cell uptake of NPs at different time points ..................................................................................................................92

Figure 4.1  Circulation and Biodistribution of NPs with different surface chemistry loaded with the NIR dye 5491 in mice with a xenograft prostate tumor ....105

Figure 4.2  Biodistribution of nanoparticles after intravenous delivery ............106

Figure 4.3  *In vivo* and *Ex vivo* tumor localization of NIR dye-loaded NPs with different surface chemistry .................................................................................................108

Figure 4.4  Biodistribution of NPs in the harvested organs 4 days post intravenous injection of NPs ........................................................................................................109

Figure 4.5  Quantitative *Ex vivo* Biodistribution and Tumor localization of NPs in Homogenized Tissues .........................................................................................110

Figure 4.6  Signal Intensity of Excreted Feces .........................................................................111

Figure 5.1  Surface activity of NPs; NPs, nanoparticles; PVA Poly(vinyl alcohol) .....131
Figure 5.2  Isotherms of NPs with EMM; NPs, nanoparticles; EMM, endothelial cell membrane; PVA, Poly(vinyl alcohol) ..........................133

Figure 5.3  NP interaction with EMM; NP, nanoparticle; EMM, endothelial cell model membrane; PVA, poly(vinyl alcohol); NP, nanoparticle..........................135
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-(sn)-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>DPPE</td>
<td>1,2-dipalmitoyl-(sn)-glycerol-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DPPS</td>
<td>1,2-dipalmitoyl-(sn)-glycerol-3-phospho-L-serine</td>
</tr>
<tr>
<td>EMM</td>
<td>Endothelial Cell Model Membrane</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
</tr>
<tr>
<td>HIAEC</td>
<td>Human Iliac Arterial Endothelial Cell</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Lipid Chromatography</td>
</tr>
<tr>
<td>LS</td>
<td>Langmuir Schaeffer</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate Cancer Cell</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>RES</td>
<td>Reticulo-endothelial System</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SP</td>
<td>Surface Pressure</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
</tbody>
</table>

xvi
CHAPTER I
INTRODUCTION

1.1 INTRODUCTION

According to the Centers for Disease Control (CDC) and Prevention, cancer remains the second leading cause of mortality, next to the heart diseases (1). Within the past few decades, different therapeutic agents have been developed for the treatment of cancer, ranging from low molecular weight drugs to various biological molecules such as proteins and nucleic acid-based drugs (2, 3). However, despite these advances in therapy, many of the newly developed therapeutics have one or more drug delivery issue/s such as their 1) insolubility in aqueous medium limits oral bioavailability or inject intravenously to overcome bioavailability issue, 2) instability in biological environment resulting in very short-half life, 3) rapid clearance from circulation following intravenous injection, and/or 4) non-specific interactions that cause toxicity. These problems result in inefficient dosages reaching the target site to illicit the therapeutic response. On the other hand, hydrophilic nature of many drugs hinders their efficient transport across cell membrane which is critical for drugs with intracellular targets (4).
Nanocarriers as drug delivery systems can potentially address some of these issues by sustaining drug release rate, protecting drugs from degradation, and/or preventing their rapid elimination from circulation. In addition, use of nanocarriers can alter biodistribution of therapeutics and minimize nonspecific toxicity (5). Further, conjugation of targeting ligands to nanocarrier surface can improve the target specific delivery of therapeutics.

In this chapter, we review different nanocarrier-based drug delivery systems for delivery of anti-cancer therapeutics. In addition, we review a new approach, based on modulation of physical characteristics of nanocarriers and biophysical interactions of nanocarriers with membrane lipids, for developing target specific drug delivery systems.

1.2 NANOCARRIERS FOR CANCER THERAPY

1.2.1 Liposomes

Liposomes are artificially produced spherical vesicles prepared from natural phospholipids and cholesterols, which makes them biodegradable and bioacceptable. The main advantage of this system is that hydrophilic drugs can be incorporated in the aqueous core, and amphiphilic and hydrophobic drugs can be embedded into the phospholipid bilayer (6). In addition, liposome characteristics can be modified by using charged lipids or varying lipid composition to alter their biodistribution and pharmacokinetics, and release rate of the encapsulated drugs (7). However, high raw
materials cost, increased lipid-mediated toxicity at high dose, and relatively short shelf-life are some of the limitations of liposomal drug delivery system.

Currently, Doxil and DuanoXome are the US Food and Drug Administration (FDA) approved liposomal formulations used for the treatment of cancer (8).

1.2.2 Micelles

Micelles are amphiphilic block copolymers self assembled wherein the hydrophilic heads are in contact with the aqueous solvent and the hydrophobic tails are sequestered into the core. The advantages of polymeric micelles are their relatively narrow size distribution range compared to other nanocarriers, their ability to deliver hydrophobic drugs, and their improved thermodynamic stability and lower critical micellar concentration (CMC) as compared to conventional surfactant micelles (9). CMC is the minimum concentration of amphiphilic polymer chains required for formation of micelles. A lower CMC is desirable for stability of micelles; since at high CMCs, micelles dissociate into monomers and precipitate the encapsulated content in blood (10). However, the main disadvantage of micelles is their relatively low drug loading capacity as compared to nanoparticles (NPs).

Currently, micellar systems that are in clinical trial for cancer treatment are: Genexol-PM which is a polymeric micelle formulation of paclitaxel in monomethoxy poly(ethylene-glycol)-block-poly(ε-lactide), NK105 which is paclitaxel incorporated in poly(ethylene-glycol)-poly(aspartic acid), and cisplatin incorporated PEG and poly (glutamic acid) called as NC-6004 (11).
1.2.3 Dendrimers

Dendrimers are hyper branched, globular polymers with nanometer scale dimensions (12). Variation in central core and interior dendritic structure controls the shape and size of dendrimers; whereas surface functional groups on the exterior surface influence the adsorption capacity and drug release characteristics of dendrimers. Dendrimers have certain advantages such as their relatively low polydispersity index, multiple attachment sites (polymer branches) to cell receptors, controlled modifiable size and structure, and easily modifiable chemical properties (13). Drug molecules can either be encapsulated in the interior core of dendrimers or conjugated to the exterior surface groups (14). However, premature release of encapsulated drug as a result of hydrolytic degradation of the dendrimer in aqueous conditions within few hours of administration is a major drawback of drug loaded dendrimer complexes, as it reduces the drug payload reaching the intended site of action (15, 16).

In 2014, Starpharma in Australia received approval for conducting clinical trials on their dendrimer-docetaxel (DEP-docetaxel) chemotherapeutic product (17). Pre-clinical studies demonstrated significant improvement in efficacy of DEP-docetaxel as compared to free docetaxel in breast cancer model. This improvement in efficacy is found to be due to the longer circulation time, sustained release of docetaxel, and targeting of the DEP-docetaxel complex to the tumor tissue as compared to free docetaxel.
1.2.4 Nanoparticles (NPs)

NPs are defined as solid, colloidal particles that are in the size range of 10 nm to 1000 nm. Typically, NPs used for drug delivery applications are smaller than 300 nm. NPs have been investigated as a vehicle for delivery of anti-cancer drugs wherein drugs are encapsulated into NP core, entrapped or chemically conjugated to the NP surface (18). Properties of NPs such as their high stability and long shelf life, high drug carrying capacity as compared to other systems like micelles, liposomes and dendrimers, controlled drug release rate, and prolonged circulation make it a good candidate as drug delivery system (19).

The US FDA has approved Abraxane, which are paclitaxel albumin-stabilized NPs for treatment of patients with breast cancer, ovarian cancer, and metastatic lung and pancreatic cancer (20, 21). Cyclodextrin NPs called Cyclosert-Camptothecin are commercially available NPs used for the treatment of primary and metastatic tumors (22). The Cyclodextrin NPs overcome the limitations encountered upon drug association with free cyclodextrin such as high drug dose as well as negative impact on the pharmacokinetics of the drug (23).

**PLGA-PLA NPs**

NPs formulated from poly (DL-lactide co-glycolide) (PLGA) and polylactide (24) are biodegradable and biocompatible in nature (25). Advantages of these NPs are the sustained release of drugs over weeks (26), low toxicity, protection of drug from degradation, ability to modify surface properties to improve biodistribution, and
possibility to target NPs to tumors by conjugation of tumor-specific targeting ligands (27). PLGA/PLA polymers are being used in FDA approved products like Lupron Depot, Trelstar Depot, Decapeptyl, Pamorelin, and Zoladex for treatment of prostate cancer (28, 29). Several studies at the pre-clinical stage are investigating PLGA/PLA NPs for the tumor targeted delivery of anti-cancer drug (30).

Poly(D,L-lactide), poly(lactic acid) PLA, poly(D,L-glycolide) PLG, and poly(cyanoacrylate) PCA are few other biodegradable polymers that have been used in synthesis of NPs for drug delivery applications. However, higher production cost, and toxic effects of NPs while interacting with cells are few of the problems that may limit their use as drug delivery systems.

1.3 TUMOR TARGETING APPROACHES

The main objective of developing nanocarrier-based drug delivery system for cancer therapy is to enhance therapeutic efficacy while reducing the toxic effects of anticancer drugs. This can be partially accomplished by targeting of nanocarriers specifically to tumor site. In this section, various methods of nanocarrier tumor targeting are discussed.

1.3.1. Passive Targeting

Solid tumors possess certain unique characteristics such as extensive angiogenesis caused by rapid vascularization of blood vessels, and impaired lymphatic drainage from the tumor interstitium that differentiate them from normal tissues (31, 32). Leaky tumor
vasculature facilitates extravasation of the intravenously injected nanocarriers into the tumor, and impaired lymphatic drainage retains them in the tumor (33, 34). This phenomenon is commonly referred to as the enhanced permeability and retention (EPR) effect. Nanocarriers within size range of 10-500 nm have shown to avoid renal clearance (< 10 nm) as well as rapid opsonization and clearance by the organs of the RES (> 500 nm), and extravasate into the tumor interstitium due to the wide fenestrations in the tumor vasculature (29). For example, smaller size doxorubicin loaded PEG liposomes (100 nm) have shown to accumulate in tumor via the EPR effect as opposed to large-sized liposomes (500 nm) which are rapidly cleared from circulation (35).

One of the drawbacks of the EPR effect is that it works under the assumption that all tumors behave identically. Heterogeneity of tumor vasculature (36, 37) leads to variation in vascular density within tumors which causes variability in nanocarrier distribution within the tumor (38). The EPR effect has also shown to be ineffective in poorly vascularized tumors such as pancreatic tumors or small sized tumors (39) due to lack of extensive angiogenesis in these tumors which results in lower vascular permeability and hence lower nanocarrier localization. In addition, the necrotic core of tumor is poorly vascularized which limits the nanocarrier localization to the peripheral region of the tumor (29, 31, 33).

1.3.2 Active Targeting

Tumor specific ligands like tumor homing cell-penetrating peptides, antibodies or co-factors are conjugated to the nanocarrier surface which then binds to the antigens
and/or receptors over-expressed on the tumor cells/tumor vasculature to achieve efficient nanocarrier localization in the tumor tissue. These targeting methods are discussed below.

1.3.2.1 Tumor Targeting Peptides

Cell surface and extracellular peptides (40) come under category of the molecular markers whose receptors are over-expressed by the tumor cells and tumor vasculature but are expressed at very low levels by the normal cells and blood vessels of normal tissue (41, 42). Peptides that mediate delivery of drug-loaded nanocarriers can be divided as (43): (i) tumor homing peptides that deliver the conjugated nanocarriers around the tumor cells/tumor vasculature, but do not have cell penetrating properties, (ii) cell-penetrating peptides that cause nanocarrier internalization via endocytosis or pore formation but do not posses selectivity towards tumor cells/tumor vasculature, and (iii) tumor homing cell-penetrating peptides that can specifically target tumor cells/tumor vasculature and get internalized into the cells.

Tumor Homing Peptides (HPs)

Arginine-glycine-aspartic acid (RGD) and asparagine-glycine-arginine (NGR) are the two of the most common HPs that target towards tumor vasculature. For instance, Zitzmann et al. have illustrated RGD peptide specificity towards tumor endothelial cells as well as breast cancer tumor cells (44). On the other hand, tumor specificity of NGR peptide is due to binding of NGR cyclic peptide cysteine-asparagine-glycine-arginine-cysteine (CNGRC) specifically to CD13 receptors expressed on tumor vasculature rather than other CD13 expressing tissues (45). However, main drawback of most HPs is that
they localize around the targeted tissue but are unable to internalize inside the cells without any external penetrating agent (46).

**Cell-Penetrating Peptides (CPPs)**

CPPs internalize into cells with or without receptor mediated endocytosis (47) and hence have been used for intracellular drug delivery of NPs (48). For example, trans-activating transcriptionor (TAT) peptide conjugated doxorubicin (DOX) loaded micelles have been investigated as CPPs for delivery of therapeutic drug into the ovarian carcinoma SKOV3 cells (49). They show higher cell uptake as compared to DOX loaded micelles demonstrating cell-penetrating ability of the TAT peptide. This has been attributed to the positive residues present in the TAT peptide sequence which interact with the negative lipids from the cell membrane and form pores for passage of CPP conjugated nanocarriers into the cells (50). Apart from TAT-peptide, SynB1 and MPG-cholesterol are few other examples of CPPs that have been investigated for intracellular delivery of cancer therapeutics (47, 51, 52). Although CPPs penetrate into the cell, their inability to distinguish between neoplastic and non-neoplastic cells lowers their potential to be used for targeted cancer therapy.

**Cell-Penetrating Tumor Homing Peptides (CPHP)**

Cell-penetrating tumor homing peptides (CPHP) have the ability to target tumor cells/tumor vasculature as well as penetrate into the tumor cells. CPHPs could either be synthesized or obtained by conjugating a CPP with a tumor homing peptide. For instance, internalizing RGD peptide (iRGD) bound micelles spread across the tumor parenchyma
following intravenous injection as compared to RGD peptide bound micelles that remained at the surface of targeted blood vessels, demonstrating usefulness of such peptides for targeted therapeutic delivery to tumor tissues (53). Sugahara et al. have suggested that the RGD motif is proteolytically cleaved such that it binds to integrins expressed on cancer cell surface and thereby internalizes into the cancer cells (53).

1.3.2.2 tumor Targeting Antibodies

Antibodies have gained recognition as cancer therapeutics due to their ability to inhibit cancer cell growth by inducing cell toxicity as well as intercepting cellular pathways of tumorigenesis such as triggering apoptosis, hindering angiogenesis, and inhibiting tumor cell proliferation (54, 55). Interestingly, certain antibodies have also shown to target specifically to the antigens expressed on neoplastic cells and hence have been investigated as tumor targeting ligands for delivery of drug loaded nanocarriers to the tumor site (56-58). For example, antibody conjugated paclitaxel loaded PLGA NPs have shown high affinity towards lung cancer cells (59). Further, intravenous administration of these NPs demonstrated significantly higher inhibition of tumor growth and increased survival rates. Similarly, monoclonal antibody TRC105 conjugated nanographene oxide NPs have shown to specifically target CD105 antibody marker expressed on neovasculature of breast cancer tumor cells in vivo demonstrating potential of antibody conjugated NPs for tumor targeting (60).
1.3.2.3 Tumor Targeting Co-factors

Apart from antibodies and peptides, there are certain ligands whose receptors are over-expressed on the cancer cells, and have been found to be effective in targeting drug loaded nanocarriers to the tumor site. Transferrin and folate are two of the most common co-factors that have been investigated to achieve tumor targeted delivery of nanocarriers (61, 62).

For example, folate conjugated PEG functionalized gold NPs have demonstrated higher cell uptake efficiency in folate receptor positive cervical cancer cells (30) as compared to folate receptor negative lung cancer cells (A549) (63). The higher uptake of NPs in cervical carcinoma cells has been attributed to the over-expressed folate receptors which improve endocytosis of these folate conjugated NPs as compared to the A549 cells that do not express folate receptors (30).

Conjugation of gold NPs with transferrin has shown to lower NP uptake in hepatocytes as compared to cancer cells, demonstrating the specificity of these NPs towards cancer cells (64). Similarly, specific targeting of transferrin conjugated paclitaxel loaded NPs to the over-expressed transferrin receptors on prostate cancer cells has also been demonstrated (65).

Apart from folate and transferrin co-factors, long circulating chitosan conjugated NPs have exhibited tumor specific targeting due to their ability to penetrate through angiogenic vessels in the tumor tissues (66). Conjugation of hyaluronic acid to NPs has
also shown to increase the uptake of NPs by tumor cells in vivo as compared to unconjugated NPs due to interaction of hyaluronic acid with CD44 receptors expressed on surface of cancer cells (67).

**1.4 Limitations of Current Nanocarrier Targeting Strategies**

Although synaphic i.e. affinity based targeting of nanocarriers can enhance the nanoparticle localization into the tumor tissue (66), saturation of receptors and low affinity of ligand for its receptor have limited the binding efficiency and effective delivery of nanocarriers to the tumor site (68). Binding site barrier, wherein ligand modified nanocarriers strongly bind to the receptors but do not internalize into the cell, also limits the penetration of ligand-modified nanocarriers into the tumor cells (37).

Physical characteristics of nanocarriers such as size, shape, charge, and surface functionalization have shown to influence their circulation time as well as tumor localization via the EPR effect. The changes in nanocarrier characteristics have also shown to influence their interaction with cancer cell membrane and intracellular uptake. Therefore, modulation of physical characteristics is a promising approach that should be further explored for passive tumor localization of nanocarriers and has been discussed in the next section.
1.5 INFLUENCE OF PHYSICAL CHARACTERISTICS OF NANOCARRIERS ON TUMOR TARGETING

Changes in physical characteristics of nanocarriers, e.g. size (69), shape (70), charge (71), hydrophobicity and surface functionalization (72) have shown to influence their cellular uptake, cytotoxicity, blood circulation half-life, and/or biodistribution, and hence have been explored as drug delivery systems for efficient delivery of therapeutics to the tumor tissue.

1.5.1 Effect of Size and Shape of Nanocarriers on Tumor Localization

The size of nanocarriers has shown to influence the cell internalization pathway and the rate of uptake. For example, Rejman et al. have shown the effect of size on internalization pathway wherein NPs with size smaller than 200 nm internalized via clathrin-mediated endocytosis whereas NPs between 200 to 500 nm internalized via caveolae-mediated internalization (73). Higher intracellular uptake of gold NPs with diameter of 30 and 50 nm by prostate cancer cells as compared to larger size NPs (100 nm) has been demonstrated (74). Similarly, Chithrani et al. observed higher uptake of 50 nm size NPs as compared to NPs with 74 nm in cervical cancer cells (75).

In vivo studies demonstrate that circulation half-life of nanocarriers and their clearance by the RES organs such as spleen and liver depends upon their size. Typically, nanocarrier size governs the amount and conformation of protein adsorbed on NP surface (76), and hence influences nanocarrier biodistribution as well as their clearance via the RES (77, 78). For instance, nanocarriers smaller than 10 nm are removed via renal
filtration while nanocarriers greater than 100 nm are rapidly cleared by the liver (69, 79). In addition, nanolatex NPs with 20 nm size have shown wide organ distribution with longer circulation time as compared to 60 nm size NPs (80).

Particularly, the size of nanocarriers (10 nm to 600 nm) governs the extravasation of NPs into the tumor interstitium to take advantage of the EPR effect and hence influences tumor localization of nanocarriers.

In addition to size, shape has also shown to impact nanocarrier attachment to cells and internalization by macrophages. For example, ellipsoid particles are taken up by macrophages more readily than spherical and cylindrical particles (81, 82). In addition, circulation of nanocarriers has shown strong dependence on shape. For example, cylindrical shaped filomicelles showed about ten times longer circulation as compared to their spherical counterparts (83). Shape of nanocarriers has also shown to influence their biodistribution with accumulation of spherical nanocarriers in non-RES organs, cylindrical NPs mostly in liver and discoidal nanocarriers in most organs other than liver (84).

1.5.2 Effect of Surface hydrophilicity of Nanocarriers on Tumor Targeting

Prolonged circulation of nanocarriers can be achieved by modifying their surface with hydrophilic polymers such as poly(ethylene glycol)(PEG) as they are not readily recognized by the immune system (69). PEG is one of the most common hydrophilic polymers investigated due to its properties like low polydispersity, low rate of
opsonization due to hydrophilic nature of the polymer, and easy modulation of the molecular weight corresponding to the biomedical application. For example, DNA-lipid nanocapsules coated with PEG show prolonged \textit{in vivo} circulation than uncoated DNA-lipid complex (85). Similarly, micelles coated with PEG have shown to achieve longer circulation as compared to non-coated micelles (86).

Although, PEG coating has shown to improve the stealth properties of nanocarriers, recent studies have recognized few of its disadvantages (87). Specifically, PEG coating attached to the nanocarrier surface has shown to hinder the nanocarrier-cell membrane interactions prior to intracellular localization as well as vesicular transport following nanocarrier internalization. For example, PEGylated liposomal doxorubicin showed lower tumor accumulation as compared to liposomal doxorubicin without PEG, indicating interference of PEG in the cell-liposomal doxorubicin interactions due to the steric hindrance (88). Intracellular fate of oligonucleotides delivered by PEGylated and non-PEGylated lipoplexes demonstrate different intracellular trafficking pathways, wherein PEG interferes with the endosomal escape of oligonucleotides and results in their degradation (89). PEG coated complexes have also shown to exhibit immunogenicity under certain conditions (90). For example, dose of PEG-coated liposome induces anti-PEG-IgM production in the spleen. PEG specific IgM then binds to the PEG coated liposome injected in the second dose, triggering activation of the complement cascade and resulting in accelerated blood clearance of PEG lipoplexes (91).
Potentially unfavorable properties of PEG have led to search for alternate polymers that provide stealth characteristics. Polymers like poly (vinyl alcohol) (PVA), polysaccharides, polyglycerols, N-(2-hydroxypropyl)methacrylamide (HPMA) etc. have shown promising results. In addition, some studies have suggested conditional removal of PEG triggered by cellular clues like pH or enzymatic stimuli to attain higher cellular uptake along with longer circulation (92). Recent reports have also used bio-mimetic stealth coating to improve circulation of NPs via adhesion to the red blood cells; wherein NPs remain in circulation as long as they are attached to the red blood cells (93).

**Role of Poly (vinyl alcohol) (PVA) on NP Physical Characteristics**

PVA is used as an emulsifier during the process of NP synthesis particularly using solvent evaporation method. Previous studies have demonstrated that differences in PVA characteristics such as the degree of hydrolysis could influence the amphiphilic property of PVA and lead to modulation in size as well as the surface characteristics of NPs. The influence of PVA on NP characteristics and their biophysical interactions with model membrane has been investigated. In Chapter V, we have demonstrated influence of adsorption of PVA molecules on NP surface and its influence on their biophysical interactions with model membrane.

1.5.3 Effect of Surface Charge of Nanocarriers on Tumor Targeting

The charge of nanocarriers has shown to influence their interaction with cell membrane, cell uptake and *in vivo* circulation (94, 95). For instance, higher cell membrane penetration of cationic nanocarriers has been observed as compared to anionic
nanocarriers. The difference in NP penetration is mostly due to the electrostatic interactions between negatively charged cell membrane and the positively charged particles, which causes pore formation in the cell membrane that serve as passage for entry of cationic NPs into the cells (72, 96). In addition, it has been demonstrated that cationic and neutral NPs internalize in both normal and lung cancer cell lines, whereas anionic cerium oxide NPs mostly localize in lung cancer cells illustrating specificity of anionic NPs towards lung cancer cells as compared to cationic NPs which show non-specific internalization in both cell lines (97). Uptake of cationic particles in both cell lines is accounted to be due to electrostatic interactions between cationic particles and anionic cell membrane. It is however known that zeta potential of A549 lung cancer cells is about – 10 mV, which means there are fewer cationic sites available for adsorption of anionic particles (98). In addition, adsorption of particles further reduces negative charge density and favors adsorption of other free anionic particles to form NP clusters thereby enhancing their cell uptake (99).

Charge of nanocarriers has shown to influence the type of protein adsorbed on the nanocarrier surface which in turn influences opsonization of particles (100). For instance, IgG and albumin adsorb on the cationic particle surface whereas apolipoprotein H (ApoH) adsorb on the anionic particles (101). The adsorption of IgG protein promotes phagocytosis and clearance of particles via RES, and thereby influence the circulation time of these particles (102). Although the role of ApoH has not been clarified, it has shown to participate in lipid metabolism, as well as regulator of complement system (103), Yan et al. have shown that ApoH binding to negatively charged liposomes inhibit
rather than enhance liposome uptake by liver cells (104). Furthermore, 3-D in vitro tumor
model revealed significantly faster uptake of cationic gold NPs as they are taken up by
proliferating cells, whereas anionic gold NPs diffuse faster into the tumor tissue resulting
in deeper penetration of these particles (105).

1.5.4 Effect of Surface Functionalization of Nanocarriers on Tumor Targeting

Recent studies have shown that surface functionalization and not just surface
charge of nanocarriers influences nanocarrier-cell membrane interactions, cell uptake and
toxicity (106, 107). For instance, NPs with alternate striations of hydrophobic and anionic
moiety showed membrane penetration without bilayer disruption by directly passing
through the bilayer; whereas NPs with random distribution of these same moieties
internalize via endocytosis and are mostly trapped in the endosomes (108). The effect of
molecular structure of surfactants adsorbed on NP surface and their influence in NP-cell
membrane interactions and cell uptake have been investigated (109). Differences in the
NP-membrane interactions have been attributed to the chain length and hydrophobicity of
the surfactants. In addition, higher interaction and cell uptake of di-chained surfactant
didodecyldimethylammonium bromide (DMAB) has been suggested to be due to
adsorption of one chain of DMAB to the particle surface while the other free chain of
surfactant interacts and penetrates into the cell membrane. Furthermore, in vivo studies
have demonstrated higher tumor localization of DMAB modified NPs as compared to
cetyltrimethylammonium bromide (CTAB) modified NPs illustrating influence of NP
surface functionalization on tumor targeting (110). This improved tumor localization of
DMAB modified NPs has been attributed to the selective biophysical interaction of
DMAB modified NPs with prostate cancer cells (PC-3) as opposed to normal endothelial cells.

Nanocarrier surface functionalization has shown to influence the amount and type of proteins adsorbed on nanocarrier surface which thereby regulates their clearance via RES, in vivo circulation, and tumor localization (111, 112). For instance, NPs with similar charge but different surface functional groups showed higher adsorption of albumin on NPs with carboxyl functional groups as compared to NPs with sulfate functional groups which demonstrated higher adsorption of ApoH (101). Albumin has been shown to facilitate clearance of adsorbed NPs via RES while ApoH tends to inhibit clearance of NPs. These results support the findings that clearance of NPs with carboxyl functional groups is faster as compared to NPs with sulfate functional groups, demonstrating influence of surface functionalization on protein adsorption and hence clearance from circulation (101, 102).

The above literature suggests that the surface characteristics of nanocarriers influence their cell uptake as well as biodistribution. Therefore, the influence of surface chemistry of nanocarriers on their cell uptake and biodistribution should be further explored to design nanocarriers for targeted delivery of therapeutics to tumor site.

1.6 BIOPHYSICAL CHARACTERIZATION

Cancer therapeutic drugs mostly have their site of action inside the cell. Therefore, intracellular delivery of drug-loaded nanocarriers improves the efficacy of the
drug. However, nanocarriers need to first interact with the cell membrane for their passage into the cell either passively via cell permeation or get engulfed into cell via endocytosis. Recent studies have shown demonstrated the effect of cell membrane lipid composition on biophysical characteristics of cell membrane (113-115), nanocarrier-cell membrane interactions, and nanocarrier uptake (116). Therefore, it is important to understand effect of differences in cell membrane lipid composition from one cell type to another on biophysical characteristics of cell membrane as well as nanocarrier uptake.

In this section, differences in cell lipid composition and its influence on biophysical characteristics of cell membrane, differences between normal and cancerous cell membrane lipids, and the application of these cell specific biophysical characteristics in developing nanocarrier-based drug delivery systems are discussed.

**1.6.1 Influence of Lipids on Membrane Biophysics and Intracellular Nanocarrier Accumulation**

The cell membrane is a sheet-like structure which mainly consists of lipids that are held together by hydrophobic interactions between their acyl chains (tails) (117). Generally, hydrophilic headgroups are oriented towards the exterior and the hydrophobic tails towards the interior core of the lipid bilayer. Until recently the lipid bilayer was recognized as homogeneous passive structural backbone for insertion of proteins and carbohydrates. However, recent studies have shown the heterogenic nature of lipids and its influence on the cell membrane characteristics (118). Lateral domain structure of lipids like lipid rafts, caveolae and coated pits demonstrate the complexity of cell
membrane structure (119). In addition, physical characteristics of lipids, i.e., the
cellular shape of lipids, termed as ‘lipid polymorphism’ has shown to influence the
dynamic shape of membrane (120). In other words, changes in cell membrane lipid
composition and lipid polymorphism can explain various cell membrane functions like
signaling and apoptosis.

The molecular shape of major lipids can be categorized into three groups:
cylinder-shaped lipids, cone-shaped lipids, and inverted-cone-shaped lipids (121). These
differences in shape of lipids arise from relative differences in lipid’s headgroup size and
tail. For example, major membrane lipids like phosphatidylcholine (PC) and
phosphatidylinerine (PS) have a cylindrical shape due to headgroup and tail with similar
cross-sectional area resulting in lamellar bilayer structure. On the other hand,
sphingomyelin (SM) has cone structure due to its larger headgroup compared to tail
resulting in non-lamellar structure like micelles, whereas phosphatidylethanolamine (PE)
has inverted-cone structure due to its smaller headgroup and prefers a non-lamellar
spherical vesicle structure.

The type and arrangement of lipids within the lipid bilayer has been associated
with the affinity of lipids towards one another (120, 122). For example, affinity of SM is
higher towards cholesterol as compared to that with PE (123). Higher affinity of SM
towards cholesterol has been attributed to the cone shape of SM lipids which cover
hydrophobic headgroup of inverted-cone shape of cholesterol better than cone shape of
PE.
Changes in membrane lipid composition have shown to influence membrane curvature and membrane fluidity due to differences in the molecular shape of lipids (124-126). For example, intrinsic negative curvature of PE has shown to induce budding and membrane fusion (120, 127). The cone shape of PE has also shown to increase the fluidity of membrane due to the smaller size of headgroups as compared to fatty acyl chains (120). On the other hand, association of cholesterol with SM together gives rigid characteristic to the membrane and thereby decreases membrane permeability (24). Fatty acyl chain lengths and chain saturation of lipids also influence membrane rigidity. For instance, saturated fatty acyl chain lipids in cell membrane have shown to increase membrane rigidity as compared to unsaturated fatty acyl chain lipids because of an increase in the lipid order within intracellular membrane (128, 129). Cholesterol has also shown to induce membrane rigidity because of its rigid ring structure and ability to fill interstitial spaces (130).

Differences in membrane lipid composition as well as membrane characteristics such as curvature and permeability are important factors that have shown to influence the NP-cell membrane interactions and NP internalization into the cell. For instance, charge of membrane lipids influences their interactions with charged NPs. Higher uptake of cationic NPs can also be attributed to formation of holes in the membranes due to strong electrostatic interaction of cationic NPs with anionic lipids (108, 131, 132). Apart from the electrostatic interactions between NPs and cell membrane lipids, membrane lipid composition also influences membrane curvature and thereby control membrane budding.
and hence endocytosis of NPs into the cell (124, 133). Transmembrane diffusion of NPs into the cells has shown to be related to the membrane fluidity of cells; wherein greater diffusion of NPs is observed for highly fluidic membrane as compared to a rigid membrane (134).

1.6.2 Changes in Cell Membrane Characteristics During Disease Progression

During disease progression, cell transformation from normal to disease condition has shown to alter membrane lipid composition (130). For example, an increase in the phospholipid content, such as phosphatidylinositol (PI), PS, PE, or PC contributing to the anionic charge of cancer cell membrane has been observed (135). In another study, PS and PE lipids have shown to redistribute from inner to outer membrane leaflet due to oxidative stresses induced within tumor microenvironment (136, 137). On the other hand, decrease in free unsaturated fatty acid content such as linoleic acid (LA), α-linoleic acid (ALA) and palmitoleic acid (PA) and increase in arachidonic acid (AA) content has been observed in tumor large intestine tissue as compared to normal large intestine tissue (135, 138).

Changes in membrane lipid composition have shown to modify membrane biophysics like fluidity and thereby influence cellular functions. For example, characterization of normal vs cancerous neck tissues has illustrated the transformation of membrane from rigid to fluid nature (139). In addition, changes in lipid composition of drug sensitive and drug resistant cancer cells have also been observed. In our recent work, we observed rigid characteristics of drug resistant breast cancer cell membrane as
compared to fluid nature of sensitive breast cancer cell membrane (140). Mainly, characteristics of cell lipid membrane like the rigidity and compressibility have been attributed to the differences in lipid composition of the drug sensitive and drug resistant breast cancer cells. Further, these differences in membrane biophysical characteristics between drug sensitive and resistant cancer cells have shown to influence their interaction with chemotherapeutic drug doxorubicin, and hence their cell uptake. Similar results have been reported by Preetha et al., wherein differences in biophysical characteristics of normal cervical tissues as opposed to cancerous cervical tissues have shown to alter penetration of paclitaxel in the biological membranes (141).

Cell lipid composition has been explored as a marker to detect disease conditions. For example, plasma samples obtained from breast cancer patients showed lower levels of lysophosphatidylcholine and higher levels of sphingomyelin as compared to healthy controls (142). In another study, plasma samples with higher proportion of palmitic acid have been associated with greater risk of prostate cancer (143).

1.7 MODEL MEMBRANE SYSTEMS TO INVESTIGATE NANOCARRIER-CELL MEMBRANE INTERACTIONS

Biological membranes, composed of lipids as their basic structural unit, act as a barrier for passage of nanocarriers into the cell cytoplasm as well as between cytoplasm and different cell organelles. In order to enter into cell cytoplasm nanocarriers need to interact with cell membrane. Hence, nanocarrier-cell membrane interactions would provide insight into nanocarrier penetration into cell membrane as well as their
internalization into cells. However, complex and dynamic processes of nanocarrier-cell membrane interaction and cellular uptake makes it difficult to study nanocarrier-cell membrane interactions in real time. Hence, different model membrane systems like liposomes (144), supported lipid bilayers, and Langmuir monolayers have been developed to investigate the nanocarrier-cell membrane interactions, predict nanoparticle uptake and cytotoxicity of different nanocarrier formulations under controlled conditions (145-147).

1.7.1 Supported Lipid Bilayers (SLB)

SLBs are lipid bilayers formed on a smooth solid substrate such as silicon or mica. SLB model membrane systems allow investigation of interactions between lipid headgroups and different drugs or drug delivery systems (148). The changes in structure, morphology and surface characteristics of SLBs following interaction with drugs have been investigated using techniques like X-ray scattering, atomic force microscopy (AFM), or Fourier transform infrared spectroscopy (FTIR).

1.7.2 Liposomes

Liposomes are spherical lipid vesicles which contain aqueous solution (149). Liposomes have been used as model membrane system to investigate permeability of drugs and drug delivery systems along with use of various spectroscopic techniques like Fluorescence and Raman spectroscopy. For example, drug permeability was investigated from interactions between cationic drugs and anionic liposomes (150). A majority of cationic drug enters the liposomal bilayer via electrostatic interactions between charged
regions of drug and charged lipid headgroups forming holes and hence influencing drug permeability.

1.7.3 Lipid Monolayers

Langmuir balance has been used to form lipid monolayers on air/water interface (Figure 1.1). Lipid monolayers have been widely used as model membrane system since various parameters like lipid composition, subphase, and temperature can be controlled (151). Drug interactions with lipid monolayers can be investigated by two methods (152). First, lipid monolayers are compressed to surface pressure (SP) of 30 mN/m by applying lateral pressure. SP of 30 mN/m is selected as at this SP lipid monolayer mimics outer surface of cell membrane and the lipid packing density is expected to be similar to that exhibited in a cell membrane. Changes in lipid monolayer SP upon addition of drugs or drug delivery systems to the subphase are recorded. In the second method, lipid/drug or lipid/drug delivery system mixture is spread over the subphase to form a monolayer. Monolayer is then compressed, and the changes in SP-area isotherms for pure lipid monolayer and lipid/drug or lipid drug delivery system are compared (Figure 1.2).
Figure 1.1 Langmuir Balance used to measure the differences in monolayer surface pressure using Wilhelmy plate.
Figure 1.2 Schematic representation of Langmuir isotherm and the stages of monolayer compression until complete collapse is attained.
1.8 NANOCARRIER-CELL MEMBRANE INTERACTIONS FOR
DEVELOPMENT OF NANOCARRIER-BASED DRUG DELIVERY SYSTEMS

Nanocarrier-model membrane biophysical interactions have been previously explored as an initial screening process for selecting nanocarrier formulations that interact with model membrane for further investigations like in vitro cell uptake and in vivo biodistribution. Previously, biophysical interactions between surfactant modified polystyrene NPs and endothelial model cell membrane (EMM) have been investigated in our laboratory (109). Greater interaction of DMAB-modified NPs has shown to correlate with higher cellular uptake of DMAB modified NPs in endothelial cells. Further studies demonstrated correlation between biophysical interactions of DMAB modified NPs with prostate cancer cell lipid membranes and in vivo tumor accumulation of these NPs (110). These studies illustrate the potential of NP-cell membrane biophysical interactions as a tool to optimize NP characteristics for their targeted delivery to tumor site.

1.9 STATEMENT OF PROBLEM

Nanocarrier-mediated delivery of cancer drugs to the tumor site is hindered by problems like: 1) rapid clearance of nanocarriers via RES organs which results in decrease in their in vivo circulation half-life, and 2) highly toxic side effects as a result of their non-specific localization in normal cells as well as cancerous cells. Modulation in surface characteristics of nanocarriers like size, charge and, hydrophilicity have shown to improve their in vivo circulation and influence their cellular internalization, and hence overcome the above mentioned limitations to a certain extent.
Apart from physical characteristics of nanocarriers, differences in cell membrane characteristics have also shown to influence cellular uptake of nanocarriers (69). Changes in membrane lipid composition have shown to affect the cell membrane characteristics, and hence could influence nanocarrier interactions with cell membrane and uptake. These differences between nanocarrier-cell membrane biophysical interactions could be further explored to determine nanocarrier selectivity towards cancer cell membrane lipids, and thereby design nanocarriers for tumor targeted drug delivery applications.

1.10 HYPOTHESIS

We hypothesize that surface chemistry modified NPs that preferentially interact with cancer cell membrane lipids will demonstrate an improved tumor localization and retention of NPs in vivo.

1.11 OBJECTIVES

The objectives of thesis are:

(1) To study the effect of surface chemistry of NPs on their biophysical interactions with EMM.

(2) To evaluate selectivity of NPs to interact with cancer cell membrane lipids vs. normal cell membrane lipids.

(3) To determine tumor localization of NPs based on their surface chemistry, and its correlation to type of NPs that preferentially interacted with cancer cell membrane lipids.
1.12 GENERAL OVERVIEW OF THE THESIS

This thesis is divided into different chapters to cover the above mentioned specific aims. Chapter II details studies that describe the effect of surface chemistry of NPs on their biophysical interactions with model membrane (Aim 1). Chapter III covers the NP-cell lipid membrane interactions wherein the specificity of NPs towards cancer cell lipids vs. normal cell membrane lipids is described (Aim 2), and cellular uptake of NPs with different surface chemistry. Chapter IV describes biodistribution and tumor localization of NPs with selected surface chemistry (Aim 3). Chapter V describes the effect of residual PVA on NP surface and NP biophysical interactions with model membrane. An overall summary of the study and the potential applications of NP-cell lipid membrane biophysical interactions in developing tumor-specific NPs are discussed in Chapter VI.
CHAPTER II

BIOPHYSICS OF NANOPARTICLE-ENDOTHELIAL CELL MODEL MEMBRANE INTERACTIONS: EFFECT OF SURFACE CHEMISTRY OF NANOPARTICLES

2.1 INTRODUCTION

Understanding NP-cell membrane interactions is an important factor in the design of NPs for drug delivery applications, especially for drugs that have intracellular site of action. Physical characteristics of NPs such as their size, surface charge, and surface functional groups have shown to regulate nanoparticle transport into the cells (75, 79).

In this chapter we focused on investigating the effect of surface chemistry of NPs on their NP-cell membrane interactions. However, complex and dynamic nature of cell membrane hinders study of these interactions in real time. Therefore, model membrane systems that mimic the cell membrane conditions have been developed to investigate interactions between NPs and cell membrane. In our previous work, we have illustrated the NP-EMM interactions to predict cell uptake of NPs, wherein EMM mimics phospholipid headgroup composition on an endothelial cell (109, 153). Therefore, in this study we used EMM as endothelial model membrane system. Polystyrene NPs were used
as model particles since they are available in variety of sizes and surface chemistry. The changes in EMM surface pressure (SP) following interaction with NPs were used as a measure to determine differences in interaction of NPs based on their surface chemistry. Reports have demonstrated a correlation between the higher adsorption coefficient of NPs and greater cellular uptake of NPs (154). Therefore, we propose a method to determine NP adsorption into the lipid monolayer to understand NP penetration into cell membrane (155). Further, NP penetration into the membrane was validated from the Atomic Force Microscopy (AFM) images of EMM following interaction with NPs.

2.2 MATERIALS

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Dulbecco’s phosphate buffered saline (D-PBS) was purchased from Central Cell Services’ Media Laboratory of our institution. Polystyrene NPs with size 20 nm and 100 nm were purchased from Invitrogen (Carlsbad, CA). All other solvents used in this study were purchased from Fisher Scientific (Pittsburgh, PA) and were of high performance liquid chromatography (HPLC) grade.

2.3 METHODS

2.3.1 Physical Characterization of NPs

Size and zeta potential of polystyrene NPs were measured by diluting the NP stock in (i) Milli-Q water, and (ii) 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY). NP size was measured by diluting 100 µL of NP suspension (5 mg/mL) to 3 mL of MilliQ water or 10% FBS solution. For measurement of zeta
potential, NP stock solution (20 mg/mL) was sonicated (Misonix Incorporated, Farmingdale, NY) for 30 s and then adjusted to concentration of 5 mg/mL by addition of either Milli-Q water or 10% FBS solution. Mean particle size was measured using dynamic light scattering (DLS) technique, and zeta potential was measured using phase analysis light scattering technique (PSS/NICOMP 380/ZLS, Santa Barbara, CA).

2.3.2 Endothelial Cell Model Membrane (EMM) Lipid Solution

A lipid mixture was prepared by mixing individual lipid solutions with composition as follows: DPPC (56%), DPPE (24%), PI (8.0%), DPPS (4.3%), SM (6.0%) and CL (1.7%). Individual lipid solutions were prepared with different solvents based on lipid solubility. DPPC, PI, SM and CL were dissolved in chloroform; whereas DPPE and DPPS were dissolved in a 4:1 chloroform and methanol mixture as they do not dissolve in chloroform alone. EMM lipid composition was selected because it represents the headgroup chemistry of phospholipids of native arteries in the endothelial cell membrane (156). EMM was formed using a Langmuir balance (Minimicro 2, KSV Instruments, Helsinki, Finland) (Figure 1.1).

2.3.3 Effect of Surface Chemistry of NPs on NP-EMM Interactions

A 100 µl of 0.5% NP suspension was injected into the subphase below the membrane and the changes in SP were monitored over period of 20 min. In a separate set of experiments, changes in SP of EMM were determined for NP suspension prepared in 10% FBS, to study the effect of serum proteins bound to NPs on interactions with
membrane. For the above experiments, controls were recorded by injecting equal volumes of deionized water or 10% FBS into subphase.

The intrinsic surface activity of NPs was analyzed by addition of 100 µL of 0.5% NP suspension to the subphase in absence of the EMM.

To investigate the influence of size of NPs, NPs with different sizes were added to the subphase and changes in SP were recorded.

2.3.4 Diffusion Kinetics of NPs into the EMM

NP adsorption into the EMM was analyzed from the changes in the membrane SP measured over time. The adsorption/dissociation coefficient of NPs was calculated from equation-1 (155):

\[ \Pi = \frac{2RTC_b}{\pi^{1/2}} D^{1/2} t^{1/2} \]

Adsorption and dissociation coefficient of NPs with different surface chemistry was calculated from the slope of the change in SP (ΔΠ) vs t^{1/2} plots. Here, the gas constant (R), absolute temperature (T) and the molar concentration of NPs (C_b) are constant for all NPs. Hence, the adsorption/dissociation coefficient is given by:

\[ \frac{\Pi}{t^{1/2}} = \text{Constant} \times D^{1/2} \]

Where, Constant = \[ \frac{2RTC_b}{\pi^{1/2}} \]
2.3.5 Langmuir Schaeffer (LS) Membrane Preparation for AFM Imaging

Following interaction with NPs at SP of 30 mN/m, EMM was transferred onto hydrophobic silicon substrate (Ted Pella, Inc., Redding, CA) by using Langmuir-Schaeffer (LS) technique, by horizontal uplifting of the EMM at rate of 5 mm/min (Figure 2.1). Transfer ratio denotes the transfer efficiency of the LS films and ranged between 0.85 to 1.1. The LS films were dried in vacuum desiccator for 24 hr prior to imaging.

The LS films were imaged using a Bioscope atomic force microscope (AFM, Veeco Metrology, Inc., Santa Barbara, CA) in tapping mode using 125 µm long silicon probe with resonance frequency of 300 Hz and tip radius of <10 nm (Ted Pella, Inc.). The acquired AFM images were flattened using a second order flattening process using Nanoscope software, version 7.3, (Veeco Metrology, Inc.).
Figure 2.1 Schematic to prepare LS films for AFM Imaging. Schematic illustration of preparation of LS films by horizontal uplifting of the lipid monolayer for AFM imaging to determine the surface morphology of the LS films.
2.4 RESULTS

2.4.1 Physical Characterization of NPs

The mean NP size measured by DLS was higher than the size listed by the manufactures (Table 2.1). These differences in size could be due to the discrepancy in the method/technique used by the manufacturers i.e. Transmission Electron Microscopy (TEM) as opposed to DLS method used in our laboratory. NP size measured in 10 % FBS did not show significant change from that measured in water, except for NPs with amidine and aldehyde/amidine surface chemistry which showed significant increase in their size when suspended in 10% FBS.

Zeta potential of NPs suspended in water showed similar charge for NPs with sulfate and carboxyl/sulfate surface chemistry (- 30 mV); and epoxy/sulfate, carboxyl and chloromethyl (- 40 mV) surface chemistry. In general, zeta potential of NPs in FBS was higher than that measured in water (Table 2.1). For instance, negative zeta potential of NPs in water changed to positive (0 – 6 mV), while that of positively charged NPs lowered when NPs were suspended in 10 % FBS.
<table>
<thead>
<tr>
<th>Surface Chemistry of NPs</th>
<th>TEM Size of NPs (in nm)</th>
<th>Size of NPs in Water (in nm)</th>
<th>Zeta Potential of NPs in Water (in mV)</th>
<th>Size of NPs in FBS (in nm)</th>
<th>Zeta Potential of NPs in FBS (in mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfate</td>
<td>20</td>
<td>41.8</td>
<td>-31.5</td>
<td>54.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Aldehyde/Sulfate</td>
<td>20</td>
<td>38.2</td>
<td>-83.8</td>
<td>52.4</td>
<td>-1.4</td>
</tr>
<tr>
<td>Epoxy/Sulfate</td>
<td>60</td>
<td>65.8</td>
<td>-38.6</td>
<td>73.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Carboxyl/Sulfate</td>
<td>20</td>
<td>44.8</td>
<td>-30.1</td>
<td>57.9</td>
<td>6.5</td>
</tr>
<tr>
<td>Carboxyl</td>
<td>20</td>
<td>46.1</td>
<td>-39.9</td>
<td>35.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Aliphatic amine</td>
<td>20</td>
<td>150.1</td>
<td>21.8</td>
<td>206.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Amidine</td>
<td>20</td>
<td>57.9</td>
<td>43.9</td>
<td>915.2</td>
<td>9.4</td>
</tr>
<tr>
<td>Aldehyde/Amidine</td>
<td>20</td>
<td>75.8</td>
<td>57.8</td>
<td>1674.3</td>
<td>-1.4</td>
</tr>
<tr>
<td>Chloromethyl</td>
<td>20</td>
<td>41.8</td>
<td>-40.7</td>
<td>27.2</td>
<td>-1.6</td>
</tr>
<tr>
<td>Sulfate</td>
<td>100</td>
<td>129.6</td>
<td>-23.6</td>
<td>153.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Carboxylate</td>
<td>100</td>
<td>130.2</td>
<td>-25.8</td>
<td>173.2</td>
<td>8.0</td>
</tr>
<tr>
<td>Amine</td>
<td>100</td>
<td>130.5</td>
<td>23.9</td>
<td>265.4</td>
<td>24.0</td>
</tr>
</tbody>
</table>
2.4.2 Intrinsic Surface Activity of NPs

Generally, the surface activity of NPs varied with surface chemistry of NPs, and was not solely dependent on the surface charge of NPs (Figure 2.2). For instance, NPs with sulfate surface chemistry showed higher surface activity as compared to NPs with carboxyl/sulfate surface chemistry even though both NPs have similar charge. The order of surface activity of NPs was: sulfate > carboxyl/sulfate > amine > chloromethyl > carboxyl > aldehyde/amidine (Figure 2.2 a).

Typically, NP suspension in 10 % FBS caused an increase in surface activity of NPs from that observed when NPs were suspended in water (Figure 2.2 b). The order of surface activity of NPs suspended in 10 % FBS (Figure 2.2 b) was different as compared to that observed for NPs suspended in water (Figure 2.2 a). The order of surface activity of NPs was: sulfate > amine > carboxyl/sulfate > carboxyl > chloromethyl > aldehyde/amidine.
Figure 2.2 Intrinsic Surface Activity of NPs with different surface chemistry for NPs suspended in (a) water, and (b) 10 % FBS. A 100 µL aliquot of NP suspension (5 mg/mL) was injected into 50 mL subphase, and the changes in SP over time were recorded for NPs with different surface chemistry. (Key: 1, Sulfate; 2, carboxyl/sulfate; 3, carboxyl; 4, chloromethyl; 5, amine; 6, aldehyde/amidine). Figure shows representative data obtained from set of three repeats.
2.4.3 Effect of Surface Chemistry of NPs on NP-EMM Interactions

Changes in EMM SP following interaction with NPs have shown to depend on the surface chemistry of NPs (Figure 2.3 and 2.4). NP-EMM interactions can be categorized as those resulting in: 1) an increase in membrane SP as observed for NPs with amine surface chemistry, 2) decrease in membrane SP as observed for NPs with sulfate surface chemistry, and 3) negligible SP changes as observed for NPs with carboxyl surface chemistry due to no interaction with the cell membrane lipids.

Changes in EMM SP varied for NPs with similar charge but different surface chemistry, illustrating influence of surface chemistry of NPs on NP-EMM interactions (Figure 2.3). For instance, NPs with epoxy/sulfate and chloromethyl surface chemistry caused decrease in the membrane SP, while NPs with carboxyl surface chemistry did not cause any change in membrane SP although all three NPs have similar charge. These results are similar to the surface activity of NPs which depend on their surface chemistry (Figure 2.2).

In addition, the changes in SP following interaction with EMM was found to depend on the surface chemistry of NPs (Figure 2.4 a). For instance, NPs with sulfate surface chemistry showed rapid increase in EMM SP and reached its maximum SP within 5 min followed by gradual decrease in EMM SP over time, resulting in negative SP change in EMM post 20 min. On the other hand, NPs with amine surface chemistry showed gradual increase in EMM SP with positive change in SP over 20 min.
The medium or subphase in which NPs were dispersed also affected the interactions studied (Figure 2.3 and 2.4). For instance, NPs with sulfate surface chemistry suspended in 10 % FBS showed an increase in membrane SP as opposed to decrease in membrane SP as observed for NPs suspended in water. NPs with sulfate surface chemistry rapidly increased membrane SP to reach maximum value within first 5 min followed by plateau region over 20 min; whereas, NPs with aldehyde/amidine surface chemistry caused gradual increase in EMM SP. The control experiment showed decrease in EMM SP following addition of 100 µL of 10 % FBS solution (as compared to EMM control after addition of 100 µL of water).
Figure 2.3 Changes in surface pressure of EMM following interaction with NPs with different surface chemistry. A 100 µL of NP suspension was injected into the subphase when EMM SP of 30 mN/m was attained and changes in EMM SP were recorded 20 min following interaction. NP size is 20 nm. Zeta potential values are measured in water. (n=3).
Figure 2.4 Changes in surface pressure of EMM following interaction with NPs with different surface chemistry when NPs were suspended in (a) water, and (b) 10 % FBS. A 100 µL of NP suspension was injected into the subphase when EMM SP of 30 mN/m was attained and changes in EMM SP were recorded over 20 min following interaction. NP size is 20 nm. (Key: 1, Sulfate; 2, carboxyl/sulfate; 3, carboxyl; 4, chloromethyl; 5, amine; 6, aldehyde/amidine). Figure shows representative data obtained from set of three repeats.
2.4.4 Isotherm of EMM in presence of NPs

The initial SP of the EMM isotherm in the presence of NPs was dependent on the surface chemistry of NPs. The SP in the presence NPs with aldehyde/amidine surface chemistry was \( \sim 0 \) mN/m, approximately 5 mN/m for NPs with carboxyl and chloromethyl surface chemistry, and above 10 mN/m for NPs with sulfate, amine and carboxyl/sulfate surface chemistry (Figure 2.5).

Upon compression, shape of the isotherm in presence of NPs remained unchanged as compared to EMM control, except for shift in trough area (Figure 2.5, Table 2.2). For instance, at SP of 20 mN/m isotherms in presence of all type of NPs showed shift towards higher trough area as compared to EMM control; whereas at SP of 30 mN/m isotherm in presence of NPs with carboxyl, epoxy/sulfate and aldehyde/amidine surface chemistry seem to merge with the control EMM isotherm. At SP of 40 mN/m, isotherm in presence of NPs is merged with the control isotherm except for NPs with amine surface chemistry which showed shift towards higher trough area; while presence of NPs with carboxyl and epoxy/sulfate surface chemistry shifted EMM isotherm towards lower trough area as compared to the control isotherm.
Table 2.2 % Trough Area at Different EMM SPs of Isotherms (Figure 3a)

<table>
<thead>
<tr>
<th>NP Surface Chemistry</th>
<th>20 mN/m</th>
<th>30 mN/m</th>
<th>40 mN/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMM Control</td>
<td>50.20</td>
<td>44.19</td>
<td>40.04</td>
</tr>
<tr>
<td>Aldehyde/Sulfate</td>
<td>67.93</td>
<td>50.08</td>
<td>41.59</td>
</tr>
<tr>
<td>Sulfate</td>
<td>70.00</td>
<td>49.58</td>
<td>40.86</td>
</tr>
<tr>
<td>Carboxyl/Sulfate</td>
<td>67.82</td>
<td>49.88</td>
<td>40.35</td>
</tr>
<tr>
<td>Epoxy/Sulfate</td>
<td>52.78</td>
<td>44.40</td>
<td>38.69</td>
</tr>
<tr>
<td>Carboxyl</td>
<td>52.43</td>
<td>44.01</td>
<td>38.26</td>
</tr>
<tr>
<td>Chloromethyl</td>
<td>59.83</td>
<td>48.32</td>
<td>41.38</td>
</tr>
<tr>
<td>Amine</td>
<td>65.97</td>
<td>51.34</td>
<td>44.08</td>
</tr>
<tr>
<td>Aldehyde/Amidine</td>
<td>53.21</td>
<td>45.85</td>
<td>40.23</td>
</tr>
<tr>
<td>Amidine</td>
<td>54.46</td>
<td>46.15</td>
<td>38.69</td>
</tr>
</tbody>
</table>
Figure 2.5 The SP-area isotherm of the EMM in presence of NPs with different surface chemistry. A 100 µL of 0.5% NP suspension in water was injected into the subphase and allowed to interact with the membrane for 20 min followed by constant rate compression of barriers at 5 mm/min. (Key: 1, Sulfate; 2, carboxyl/sulfate; 3, carboxyl; 4, chloromethyl; 5, amine; 6, aldehyde/amidine; 7, EMM). Figure shows representative data obtained from set of three repeats.
2.4.5 Diffusion Kinetics of NPs analyzed from NP-EMM Interactions

Diffusion kinetics of NPs as measured from the change in SP (ΔΠ) vs square root of time (t^{1/2}) plots varied with the surface chemistry of NPs and showed two regions: 1) NP adsorption, and 2) saturation or dissociation of NPs into the subphase (Figure 2.6a). In general, ΔΠ- t^{1/2} plots showed positive slope in region 1, which illustrates NP diffusion into EMM; whereas region 2 has negligible slope due to NP saturation, or negative slope due to NP dissociation from the EMM (Table 2.3). In region 1, the order of adsorption of NPs into the EMM was: aldehyde/sulfate, sulfate > carboxyl/sulfate > amine, chloromethyl > epoxy/sulfate, carboxyl, amidine, aldehyde/amidine; whereas in region 2, the order of dissociation of NPs from EMM was sulfate > aldehyde/sulfate > carboxyl/sulfate, chloromethyl > epoxy/sulfate, amine > carboxyl, aldehyde/amidine.

The adsorption of NPs into EMM as observed from ΔΠ- t^{1/2} plots shows negligible increase for NPs with carboxyl and aldehyde/amidine surface chemistry, or deviates from the linear behavior and either i) plateaus as in case of NPs with amine surface chemistry, or ii) decreases as observed for NPs with sulfate, carboxyl/sulfate, and chloromethyl surface chemistry. In addition, it should be noted that adsorption coefficient of NPs with similar charge varied with their surface chemistry. For example, NPs with sulfate surface chemistry showed higher adsorption coefficient in region 1 as well as higher dissociation from the EMM in region 2 as compared to the NPs with carboxyl/sulfate surface chemistry, although both have similar charge.
Adsorption coefficients of NPs were higher when NPs were suspended in 10% FBS as compared to NPs suspended in water (Figure 2.6 b, Table 2.3), except for NPs with amine surface chemistry which showed similar adsorption coefficient irrespective of dispersion medium. NPs with carboxyl and aldehyde/amidine surface chemistry showed negligible diffusion. Region 2 shows that NPs did not dissociate from the membrane in the subphase.
Table 2.3 Relative Adsorption/Dissociation Coefficient Estimated from NP-EMM Interactions

<table>
<thead>
<tr>
<th>Surface Chemistry of NPs</th>
<th>NPs suspended in water</th>
<th>NPs suspended in 10 % FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adsorption Region 1</td>
<td>Dissociation Region 2</td>
</tr>
<tr>
<td>Aldehyde/Sulfate</td>
<td>0.21</td>
<td>-0.06</td>
</tr>
<tr>
<td>Sulfate</td>
<td>0.19</td>
<td>-0.21</td>
</tr>
<tr>
<td>Carboxyl/Sulfate</td>
<td>0.12</td>
<td>-0.04</td>
</tr>
<tr>
<td>Epoxy/Sulfate</td>
<td>0.02</td>
<td>-0.01</td>
</tr>
<tr>
<td>Carboxyl</td>
<td>0.00</td>
<td>-0.00</td>
</tr>
<tr>
<td>Chloromethyl</td>
<td>0.06</td>
<td>-0.04</td>
</tr>
<tr>
<td>Amine</td>
<td>0.06</td>
<td>-0.01</td>
</tr>
<tr>
<td>Aldehyde/Amidine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Amidine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Figure 2.6 Variation of surface pressure of EMM after injection of NPs with different surface chemistry as function of time$^{1/2}$. A 100 µL of NP suspension was injected into the subphase when EMM SP of 30 mN/m was attained and changes in EMM SP were recorded over time$^{1/2}$. The adsorption and dissociation coefficients of NPs were calculated from the positive and negative slopes of the curves, respectively. (Key: 1, Sulfate; 2, carboxyl/sulfate; 3, carboxyl; 4, chloromethyl; 5, amine; 6, aldehyde/amidine). Figure shows representative data obtained from set of three repeats.
2.4.6 AFM Imaging of EMM in Presence of NPs

The surface morphology of EMM prior to and post interaction with NPs varied with surface chemistry of NPs (Figure 2.7). The 2-D and 3-D height images showed NP penetration in EMM following interaction. NPs with sulfate, carboxyl/sulfate and amine surface chemistry penetrated into the EMM; whereas aggregates of NPs with chloromethyl and aldehyde/amidine surface chemistry were embedded in the EMM. NPs with carboxyl surface chemistry did not penetrate into the EMM.

Section analysis of height images provided further insight into NP penetration in EMM. It showed that height range following interaction of NPs with chloromethyl and aldehyde/amidine surface chemistry was 120-130 nm, while that of NPs with sulfate surface chemistry was 20-30 nm, NPs with carboxyl/sulfate surface chemistry was 85-100 nm, and NPs with amine surface chemistry was 20 to 90 nm. Height analysis for membrane alone was < 3 nm which is significantly lower than that for membrane following NP interaction.

The 2-D and 3-D height images of LS films after interactions between NPs suspended in 10 % FBS with EMM demonstrate that all type of NPs penetrated into the EMM (Figure 2.8). The number of NPs that penetrated into the EMM varied depending on surface chemistry of NPs. The order of NP penetration was sulfate > carboxyl/sulfate, chloromethyl > amine > aldehyde/amidine. NP penetration into the EMM is also evident from the section analysis of LS films where height range for NPs with sulfate, carboxyl/sulfate and sulfate surface chemistry was 20-50 nm, while that for NPs with
amine, chloromethyl and aldehyde/amidine surface chemistry was 75-100 nm. Section analysis also demonstrates that aggregates of NPs with chloromethyl and aldehyde/amidine surface chemistry penetrated into the EMM. Height analysis of membrane alone was < 5 nm, where 10 % FBS solution was used as control.
Figure 2.7 Changes in EMM Langmuir-Schaeffer film morphology following interaction with NPs with different surface chemistry, for NPs suspended in water. LS films were transferred onto a silicon substrate after interaction with NPs for period of 20 min and dried for 24 hours before imaging. Images were obtained by using tapping mode AFM in air. (a) EMM transferred at SP of 30 mN/m. Panels b-g show images of EMM following interaction with NPs with sulfate, carboxyl/sulfate, carboxyl, chloromethyl, amine and aldehyde/amidine surface chemistry respectively. Height scale for all the images was 150 nm; scan size was 5 µm and section analysis was carried out on AFM height images across the white line. At least three samples were scanned to obtain a height image.
Figure 2.8 Changes in EMM Langmuir-Schaeffer film morphology following interaction with NPs with different surface chemistry, for NPs suspended in 10% FBS. LS films were transferred onto a silicon substrate after interaction with NPs for period of 20 min and dried for 24 hours before imaging. Images were obtained by using tapping mode AFM in air. (a) EMM transferred at SP of 30 mN/m. Panels b-g show images of EMM following interaction with NPs with sulfate, carboxyl/sulfate, carboxyl, chloromethyl, amine and aldehyde/amidine surface chemistry respectively. Height scale for all the images was 150 nm; scan size was 5 μm and section analysis was carried out on AFM height images across the white line. At least three images were scanned to obtain a height image.
2.4.7 Effect of Size of NPs on NP-EMM Interactions

In general, the changes in EMM SP after interaction with NPs demonstrated that smaller size NPs (20 nm) showed higher interaction with EMM as compared to larger NPs (100nm). As observed from the changes in EMM SP-time plots, the magnitude and pattern of interaction was dependent on surface chemistry of NPs (Figure 2.9). In case of NPs suspended in 10 % FBS, similar trend was observed with higher interaction of smaller size NPs as compared to larger NPs (Figure 2.10).
Figure 2.9 Changes in EMM surface pressure after interaction with NPs of different sizes and surface chemistry were investigated for NPs suspended in water. A 100 µL of 0.5% NP suspension was injected into the subphase when EMM SP of 30 mN/m was attained and the changes in membrane SP were recorded. (Key: Red = 100 nm, Blue = 20 nm)

Figure 2.10 Changes in EMM surface pressure after interaction with NPs of different sizes and surface chemistry were investigated for NPs suspended in 10% FBS. A 100 µL of 0.5% NP suspension was injected into the subphase when EMM SP of 30 mN/m was attained and the changes in membrane SP were recorded. (Key: Red = 100 nm, Blue = 20 nm)
2.5 DISCUSSION

In this chapter, we studied biophysical interactions of NPs with different surface chemistry with EMM using a Langmuir balance. Our data show that biophysical interactions significantly depend on surface chemistry and particle size of NPs as well as the presence of serum proteins in the NP suspension medium used for the interaction studies. This is evident from (i) the changes in EMM SP following interaction with NPs, (ii) EMM isotherms in presence of NPs, and (iii) AFM images collected from the NP-EMM interacted LS films.

Biophysical interactions of NPs with EMM were investigated by injecting NPs into the subphase and monitoring the changes in membrane SP over time at a constant trough area. Typically, any change in membrane SP indicates NP-EMM interactions (157, 158). For instance, positive changes result either from NP penetration into the membrane which cause membrane lipid condensation or as a result of electrostatic interactions between membrane lipids (159), whereas membrane destabilization or loss of membrane lipids into the subphase induces negative changes in EMM SP (160). On the other hand, negligible changes in EMM SP represent minimal interactions between NPs and EM, as observed for NPs with carboxyl surface chemistry, which showed no change in membrane SP post interaction (Figure 2.3 and 2.4 a).

Comparison of interaction studies demonstrated greater penetration of cationic NPs as compared to anionic NPs which could be attributed to either the (i) strong electrostatic interactions between positively charged NPs and negatively charged lipids,
(ii) pore formation within the lipid membrane which allows cationic NP penetration into the membrane, and/or (iii) steric repulsion between negatively charged NPs and anionic cell lipids and/or two negatively charged NPs which results in loss of lipids into the subphase and thereby decrease in membrane lipid packing causing drop in membrane SP (Figure 2.3 and 2.4 a) (161). Molecular simulation study by Li et al. suggested that the SP increase in presence of cationic NPs could be due to their interaction with phosphate terminus of the lipid which causes an increase in the tilt angle of lipid headgroup and results in membrane condensation (162, 163). On the other hand, anionic NPs form clusters around positively charged lipid headgroups and result in decrease in tilt angle of lipid headgroup and hence results in lower membrane lipid packing (162).

In our study, an interesting observation was that although NPs have similar zeta potential their interaction with EMM varied corresponding to their surface chemistry (Figure 2.3 and 2.4). For instance, NPs with sulfate surface chemistry demonstrate greater interaction with EMM as compared to NPs with carboxyl/sulfate surface chemistry, although both have similar surface charge (Figure 2.4 a). This difference in interaction could be as a result of variation in the surface activity of NPs based on their surface chemistry, wherein NPs with sulfate surface chemistry showed higher surface activity as compared to NPs with carboxyl/sulfate surface chemistry (Figure 2.2 a). Similar findings have been reported by Peetla et al. where they have demonstrated higher interaction of TAT-NPs with EMM as compared to scrambled TAT peptide conjugated NPs, due to the higher surface activity of TAT-NPs as compared to scrambled TAT-NPs which do not impart surface activity (153).
However, it should be noted that changes in EMM SP following NP-EMM interaction were different as compared to changes in SP of air-buffer interface after addition of NPs to the subphase in absence of EMM (surface activity of NPs), and hence suggest that these NP-EMM interactions are not merely dependent on the intrinsic surface activity of NPs (Figure 2.2 and 2.4). For instance, NPs with epoxy/sulfate, carboxyl and chloromethyl surface chemistry have similar charge, with higher surface activity of NPs with chloromethyl surface chemistry as compared to NPs with epoxy/sulfate surface chemistry; however, NPs with epoxy/sulfate surface chemistry show higher interaction with EMM as compared to NPs with chloromethyl surface chemistry. Similarly, NPs with carboxyl/sulfate surface chemistry have higher surface activity as compared NPs with amine surface chemistry; however, NPs with amine surface chemistry show higher interaction with EMM.

Above findings suggest that surface activity alone does not influence NP-EMM interactions. Apart from the surface activity of NPs, we speculate that the molecular structure of adsorbed surface groups influences NP-model membrane interactions (Figure 2.2 and 2.4). Similar findings have been reported by Peetla et al. where they have illustrated the effect of molecular structure of adsorbed surfactants on NP surface (109). Higher cell membrane penetration and cell uptake of DMAB loaded NPs as compared to CTAB NPs was observed, although both NPs have positive charge. This difference was suggested to be due to presence of an extra chain on the DMAB as compared to single chain CTAB; wherein, one chain of DMAB adsorbed to the NP surface while the other
chain penetrated into the lipid membrane. The results from above report and our findings from NP-EMM interaction study suggest that the molecular structure of chemical groups adsorbed on NP surface along with surface activity and charge of NPs influence their interaction with cell membrane.

NP-EMM interaction studies may not provide sufficient information whether the increase in EMM SP is a result of NP penetration into the membrane or due to electrostatic interactions between NPs and phospholipid headgroups. In our previous work, EMM isotherms in the presence of NPs have shown the distinction between the different patterns of NP-EMM interactions (109, 152). Therefore, we investigated changes in the EMM isotherm in presence of NPs for better understanding of the NP-EMM interaction studies (Figure 2.5).

The isotherm experiments demonstrated that all type of NPs facilitate NP penetration into the lipid monolayer at lower lipid densities (SP of ~10 mN/m); however, after lipid monolayer compression at higher lipid densities (30 mN/m), only NPs with aldehyde/sulfate, sulfate, carboxyl/sulfate, and amine surface chemistry seem to remain in the monolayer as demonstrated from the shift in trough area of EMM in presence of these NPs (Figure 2.5, Table 2.2). On the other hand, NPs with carboxyl, amidine, aldehyde/amidine and chloromethyl surface chemistry seem to squeeze out of the monolayer at SP of 30 mN/m, and hence trough area in presence of these NPs is similar to that of control EMM alone (Figure 2.5, Table 2.2).
The mechanism of NP penetration into the monolayer could be further explained based on the diffusion-penetration-rearrangement model (164). This model can be summarized in following steps: (i) NPs diffuse from subphase to the monolayer interface; (ii) NPs penetrate into the lipid monolayer; and (iii) adsorbed NPs rearrange at the monolayer interface. According to this model, the adsorption of NPs is initially diffusion controlled. This leads to penetration of NPs into the monolayer which results in an increase in membrane lipid packing and leads to membrane condensation and causes an increase in membrane SP. However, at higher surface coverage, ability of NPs to penetrate and rearrange on the surface is rate-determining.

In our present study, the SP (ΔΠ) vs time$^{1/2}$ plots demonstrate NP adsorption into or dissociation from the EMM (Figure 2.6 a and Table 2.3). Initially, NPs diffuse into the membrane and cause membrane condensation and hence an increase in EMM SP is observed (154). Whereas, loss of lipids into the membrane or steric repulsions between charged NPs and lipids results in rearrangement of lipids that lowers the membrane lipid packing and hence decrease in EMM SP (154). In our study, the diffusion of NPs through the subphase into the monolayer is governed by the charge of NPs as well as the steric interference caused by NPs based on their surface chemistry. Furthermore, adsorption of NPs into the membrane causes an increase in membrane NP concentration and therefore increases the probability of NPs to squeeze out of the monolayer into the subphase (154) (Figure 2.6 a). For instance, higher adsorption coefficient of NPs with sulfate surface chemistry is due to higher membrane lipid packing (region 1, Table 2.3). However, over time increase in the number of NPs at the interface decreases the membrane stability.
It has also been reported that macromolecular crowding of NPs in the subphase can cause steric restrictions on diffusion of NPs and thereby influence diffusion via weak intermolecular interactions (166, 167). In our study, macromolecular crowding and steric repulsion between NPs with sulfate surface chemistry may cause rearrangement of lipids and/or loss of lipids into the membrane resulting in loss of membrane integrity and hence dissociation of NPs from EMM (region 2, Table 2.3). In comparison, NPs with amine surface chemistry have lower adsorption coefficient (region 1, Table 2.3), but negligible dissociation from EMM (region 2, Table 2.3) which results in an increase in membrane lipid packing and hence positive adsorption coefficient of NPs.

Height images of EMM LS films following interaction with NPs confirmed the NP-EMM interaction studies and NP penetration into the EMM (Figure 2.7). For example, the average height of NPs with sulfate surface chemistry was lower than NPs with amine surface chemistry, which suggests that NPs with sulfate surface chemistry were embedded in the EMM; whereas NPs with amine surface chemistry were anchored to the EMM which may be due to attractive electrostatic interactions between NPs and lipids. These findings demonstrate the variation in the NP penetration into the EMM monolayer based on the surface chemistry of NPs and further confirm our observations from the SP-area isotherm, NP-EMM interaction and NP diffusion experiments.

EMM control after injecting 100 µL of 10% FBS solution demonstrates a decrease in SP. Since EMM controls after injecting 100 µL of de-ionized water show stable model membrane at SP of 30 mN/m over time, we attribute this destabilization to
ionic interactions between phospholipid headgroups of EMM lipids with the variety of positively and/or negatively charged proteins present in FBS solution. The above findings suggest that FBS alone does not influence the changes in EMM SP following NP-EMM interactions for NPs suspended in 10% FBS solution.

Influence of protein adsorption on NP surface has been reported by Ehrenberg et al. wherein they have demonstrated the effect of protein adsorption on NPs binding to cell membrane as well as their cell uptake (168). In our present study, NP suspension in FBS resulted in variation of NP interactions with EMM as compared to NPs suspended in water, which could be caused by adsorption of serum proteins on NP surface (Figure 2.3 and 2.4 b). For example, NPs with sulfate surface chemistry caused positive increase in EMM SP change (Figure 2.4 b) as compared to the negative changes in EMM SP for these NPs suspended in water (Figure 2.4 a).

Our study also demonstrated that NP-EMM interaction in the presence of serum varied with surface chemistry of NPs (Figure 2.4 b). For example, NPs with sulfate surface chemistry showed greater interaction as compared to NPs with amine surface chemistry. We speculate that these differences in interaction are due to the amount, type and conformation of proteins adsorbed on the NP surface depending on their surface chemistry (169, 170). In addition, the lesser interactions of NPs with chloromethyl and aldehyde/amidine surface chemistry with EMM (Figure 2.3 and 2.4 b) could be as a result of their aggregation following their suspension in 10% FBS (Table 2.1).
Adsorption coefficient of NPs demonstrated that NP penetration in EMM in presence of serum was greater than when NPs were suspended in water (Figure 2.6 b and Table 2.3). For example, NPs with sulfate surface chemistry showed relative adsorption coefficient of 0.64 in presence of serum as opposed to 0.19 when NPs were suspended in water (Table 2.3). In addition, the penetrated NPs stayed at the interface and did not dissociate into the subphase over time (Figure 2.6 b). We speculate that the differences in NP penetration could be as a result of protein adsorption on NP surface that stabilizes the NPs such that they stay at the EMM.

Height images of EMM LS films following interaction with NPs showed higher number of NPs penetrating into the membrane in presence of serum proteins (Figure 2.8) as compared to NPs suspended in water (Figure 2.7), and illustrate the influence of protein adsorption on NP penetration into EMM. The aggregates of NPs with chloromethyl and aldehyde/amidine surface chemistry embedded in EMM LS films following interaction and demonstrate the effect of proteins on size of NPs (Figure 2.8).

We found that NP-EMM interactions also depend on the size of NPs (Figure 2.9) with greater interaction for smaller NPs (20 nm) as compared to larger NPs (100 nm). The variation in interactions suggest that small NPs can permeate the membrane leading to higher membrane condensation and cause an increase in EMM SP as opposed to larger NPs (Figure 2.9). We speculate that larger surface area per weight of small NPs exposes greater amount of surface groups available for interaction with lipid monolayer which improves their interactions with EMM. Our study also demonstrated that serum proteins
influence the interaction of both small and large NPs with EMM, although the magnitude of interaction was higher for smaller NPs (Figure 2.10).

The current study demonstrated the influence of surface chemistry of NPs on their interactions with EMM model membrane. It would be interesting to determine how the surface chemistry of NPs influences their interactions with cell lipid membranes. Therefore, in the next chapter we concentrate on developing lipid monolayer membrane which mimics the lipid composition of different cells and study NP interactions with these cell lipid membranes.

2.6 CONCLUSIONS

We have demonstrated that surface chemistry of NPs significantly influences biophysical interactions of NPs with EMM. In addition, NP suspension in serum also influences the magnitude of NP penetration into the EMM. Our findings suggest that biophysical interactions between NPs and lipid monolayer membrane have potential to be employed as a tool to optimize NP characteristics for their efficient intracellular delivery.
CHAPTER III
EFFECT OF SURFACE CHEMISTRY OF NANOPARTICLES ON THEIR SPECIFICITY TOWARDS CANCEROUS CELL MONOLAYER MEMBRANE AND CELL UPTAKE

3.1 INTRODUCTION

Interactions between NPs and cell membrane lipids are critical for cellular uptake of NPs. A cell membrane is a complex and dynamic structure, which makes it difficult to study the process of NP-cell membrane interactions in live cells. The results described in the previous chapter (Chapter II) illustrate how surface chemistry of NPs influences biophysical interactions of NPs with model membranes. Apart from NP characteristics, it has been recognized that biophysical properties of cell membrane lipids may also influence NP uptake and trafficking into cells. The differences arise due to multitude of factors such as the presence of caveoles or noncaveolar rafts which are involved in the process of endocytosis (171). Alterations in membrane lipid composition have been reported in various disease conditions, including cancer (140) and certain neurological (172) and inflammatory conditions (173) and may alter the cell membrane properties and hence membrane interaction with NPs. Recently, we have shown that the rigid nature of resistant breast cell membrane influences the endosomal functions that inhibit the drug uptake when cells were treated with a liposomal formulation of doxorubicin (Doxil) (140). Similar findings have been reported by Preetha et. al., where they demonstrated
higher penetration of paclitaxel drug through cancerous cervical monolayer membrane as compared to normal cervical monolayer membrane due to the fluidic characteristics of cancerous membranes, illustrating cell specific effect of drug (141).

In this chapter, we aim to investigate the a) effect of surface chemistry of NPs and their biophysical interactions with non-cancerous Human Iliac Arterial Endothelial Cells (HIAECs) and prostate cancer cells (PC-3) to determine if NPs with specific surface chemistry shows selective interaction with PC-3 cell lipid membrane over HIAEC lipid membrane, and b) determine how the selective biophysical interaction of NPs with cancer cell lipid membranes translate into their specificity towards cells for uptake.

We selected PC-3 cells as a model cancer cell line because prostate cancer is the most diagnosed and second most leading cause of cancer deaths in men after lung cancer (174). Following intravenous drug delivery, therapeutics/drug delivery systems first come in contact with normal arterial endothelial cells; hence, HIAECs were selected as normal endothelial cells. Normal and cancerous cell lipid membranes were formed from the lipids extracted from HIAECs and PC-3 cells respectively to study selectivity of NP interactions towards cancer cell membrane lipids vs. normal cell membrane lipids. Polystyrene NPs with 20 nm size with sulfate, amine and carboxyl surface chemistry were selected based on the variation in their pattern of interaction with EMM (Chapter II). For instance, in chapter II the interaction of NPs with amine surface chemistry caused positive increase in EMM SP; whereas, NPs with sulfate surface chemistry show maximum interaction with EMM, although the changes in EMM SP were negative; and
NPs with carboxyl groups show negligible interaction with EMM. NPs have been loaded with a near infrared (12) dye to measure their cellular uptake, and cell uptake has been quantified using Maestro Imaging system (12).

3.2 MATERIALS

Polystyrene NPs with a size of 20 nm and sulfate, carboxyl and amine surface chemistry were purchased from Invitrogen (Carlsbad, CA). Dulbecco’s phosphate buffered saline (D-PBS) was purchased from Central Cell Services’ Media Laboratory of our institution. All other solvents used were purchased from Fisher Scientific (Pittsburgh, PA) and were of high performance liquid chromatography (HPLC) grade.

3.3 METHODS

3.3.1 Cell Lipid Membranes

Lipids from HIAECs and PC-3 cells were isolated and purified to remove membrane proteins. Purified lipids were used to form lipid membranes for biophysical interaction studies, using Langmuir balance. The method of lipid isolation and purification has been described below.

3.3.1.1 Cell Culture of HIAEC and PC-3 Cells

HIAECs purchased from Lonza (Walkersville, MD) were cultured and grown in endothelial basal medium with growth factors (bovine brain extract with heparin), human epidermal growth factor, hydrocortisone, GA-1000 (Gentamycin Sulfate, Amphotericin B), and FBS supplied by Lonza (Walkersville, MD) in a T-162 cell culture flask (Becton
Dickinson Labware, Fanklin Lakes, NJ) in an incubator at 37 °C in a 5% CO\textsubscript{2} environment. PC-3 cells purchased from American Type Culture Collection (ATCC, Rockville, MD) were cultured and grown in RPMI media containing Earle’s salts, L-glutamine, 10 % FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin. Medium in both cell lines was changed every alternate day until the cells reached 80-90% confluency.

3.3.1.2 Lipid Extraction from HIAECs and PC-3 Cells

Lipids were extracted from HIAECs and PC-3 cells by using a modified Bligh and Dyer method of lipid separation as described in our previous study (140, 175). Briefly, cells were scraped using Corning cell scraper (Lowell, MA) from five T-162 cell culture flasks when they reached 80-90% confluency, resuspended in 10 mL of deionized water, lyophilized for 48 hr at -45 ºC (FreeZone 4.5, Labconco Corp., Kansas City, MO) and weighed. The lyophilized cell mass was suspended in 3 mL of nitrogen purged water, followed by addition of 10.2 mL chloroform:methanol:1 M HCl (10:23:1 v/v) mixture. The above mixture was vortexed thoroughly and then kept on an ice bath for 15 min to obtain a cell mass suspension to which 3 mL of 0.1 M HCl and 3 mL of chloroform were added and the suspension was vortexed and centrifuged (Sorvall Legend RT, Thermo Electron Corporation, Waltham, MA) at 3,500 rpm at 0 ºC for 5 min. The cell mass suspension separates into aqueous phase and organic phase. The organic phase was collected using Hamilton syringe (Hamilton Co., Reno, NV) in a glass vial (Fisher Scientific) and was mixed with 3 mL of buffer mixture (0.1 M sodium chloride, 0.05M Tris, 0.1 M ethylenediaminetetraacetic acid, pH ~ 8.2). The above procedure was
repeated one more time and separated organic phase was added to the above buffer mixture. The buffer-organic phase mixture was further separated by first vortexing followed by centrifugation as explained above. The separated organic phase was collected in a glass vial, mixed with isopropanol (Fisher Scientific) (1 mL isopropanol for 15 mL of organic phase) and stored at -80 °C.

3.3.1.3 Hydrophobic Protein Separation from HIAEC and PC-3 Cell Lipid Extracts

Lipids extracted from the cell mass contain some hydrophobic proteins. These proteins were removed from the lipid extract by column chromatography as described in our previous study (140). Briefly, 2 g of silica gel (Polygosyl-60, Macherey-Nagel, Inc., Bethlehem, PA) was used as the stationary phase and packed in a glass column (Sigma-Aldrich, St. Louis, MO). The column was first rinsed with 45 mL of chloroform:methanol (1:1 v/v) mixture containing 1% NH₄OH to remove traces of hydrochloric acid (HCl) and then with 10 mL of 1.5μmol egg-phosphatidylcholine (PC) (Avanti Polar Lipids) to saturate the binding sites of silica to PC, such that PC present in lipid extract was eluted. The lipid extract was added to the top of the column and the 45 mL of chloroform and chloroform:methanol (1:1 v/v) were passed through the column, followed by collection of eluted fractions at the bottom. Organic solvents were then evaporated using Rotavapor rotary evaporator at pressure of 318 mbar in water bath with temperature of 50 °C (R-215, Buchi Corp., New Castle, DE). The lipid residues were weighed, dissolved in nitrogen purged chloroform:methanol (4:1 v/v) mixture and stored at -20°C until further used. Lipid extracts were analyzed by Fourier Transform Infrared
Spectroscopy (Spectrum 100 FTIR spectrometer, PerkinElmer, Shelton, CT) to ensure that hydrophobic proteins were eliminated from the extract.

3.3.1.4 Estimation of Lipid Composition of HIAEC and PC-3 Cell Lipid Extracts

Composition HIAEC and PC-3 cell lipid extracts was analyzed using a high performance thin layer chromatography (HPTLC) technique as described in our previous study (140). In brief, TLC plates (10 cm × 10 cm, Sigma-Aldrich) were dried at 140 °C for 30 min followed by addition of 5 µL of 5 mg/mL of lipid mixture at the distance of 1 cm from the bottom of the HPTLC plate. TLC chamber was first saturated with 50 mL mobile phase, followed by placing the TLC plate in a chamber containing mobile phase. Mobile phase was allowed to run until a distance of 1 cm to top of the TLC plate was left. For phospholipid separation, chloroform: methanol: water: ammonia (120:75:6:2 v/v) mixture was used as mobile phase. Later, plates were dried in fume hood in the presence of nitrogen gas for 15 min. Phospholipids were stained and identified by immersing the TLC plates in copper sulfate solution for 5 s, followed by heating the plates at 140 °C for 30 min. Copper sulfate solution was prepared by adding 20 g of copper sulfate pentahydrate to a mixture of 200 mL methanol, 8 mL sulfuric acid and 8 mL of 85% orthophosphoric acid. Lipids purchased from Avanti Polar Lipids Inc. served as standards. HPTLC plate was further analyzed using ImageQuant TL software (ImageQuant 300, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) to quantify the lipid composition of HIAEC and PC-3 cells.
3.3.1.5 Formation of Cell Lipid Monolayer Membrane

HIAEC and PC-3 cell membrane lipids were formed using Langmuir balance (Minimicro 2, KSV Instruments, Helsinki, Finland) as described in chapters II. In brief, HIAEC or PC-3 cell lipid membrane was formed by addition of 10 µL of 5 mg/mL of cell lipid mixture. Organic solvents were allowed to evaporate for 10 min, and then barriers were compressed till the membrane SP of 30 mN/m was reached. NP interactions with cell lipid membrane were investigated at SP of 30 mN/m, since it is similar to the lateral SP of human erythrocyte cell membrane and therefore arrangement of phospholipids at this SP would mimic outer leaflet of cell membrane (176).

3.3.2 HIAEC and PC-3 Lipid Membrane SP-Area (π-A) Isotherm

To obtain a SP-area (π-A) isotherm, 5 µL of 5 mg/mL of cell lipid mixture was added drop wise to the subphase such that SP did not increase and was maintained between ~ 0-1 mN/m. Organic solvent was allowed to evaporate for 10 min followed by compression of the barriers until complete membrane collapse was observed. Due to the limited trough area of our system, the isotherm could not be collected in a single experiment; therefore, different parts of the isotherm were collected and combined to obtain the complete isotherm.

The compression modulus was calculated from the SP-area isotherm data using the equation: $C_s = -A(d\pi/dA)$; where A is the trough area and $\pi$ the corresponding SP.
3.3.3 Biophysical Interactions of NPs with HIAEC and PC-3 Cell Lipid Membranes

After compression of cell lipid membranes to a SP of 30 mN/m, a 100 µl aliquot of 0.5% NP suspension in water was injected into the subphase and changes in SP were monitored over 20 min. In a separate set of experiments, changes in SP of cell lipid membrane were determined for NPs suspended in 10% FBS. For all the above experiments, controls were recorded by injecting equal volumes of deionized water or 10% FBS into subphase.

3.3.4 Dye Loading in NPs

NPs were loaded with near-infrared dye, SDB 5491 (H.W. Sands Corporation, Jupiter, FL) to measure their cellular uptake (12). The SDB 5491 NIR dye has been successfully used in our previous study to evaluate the biodistribution of magnetic NPs in breast tumor model (177). The dye loading was achieved by addition of the dye solution (1 mg/ml) in methanol to 4 mL of 1% NP suspension prepared in Mannitol Citrate Buffer, with continuous stirring at room temperature for 12 hr (109). To achieve consistent signal for each type of NPs, the amount of dye solution used for dye loading was adjusted as follows: 432 µL of stock dye was used for NPs with sulfate surface chemistry, and 720 µL for NPs with amine and carboxyl surface chemistry (Figure 4.5). The dye-loaded NP suspension was centrifuged at 4,000 rpm (Sorvall Legend RT Centrifuge, Thermo Electron Corporation, Waltham, MA) for 10 min to remove excess dye, and the supernatant containing NPs was collected and the sediment containing the excess dye was discarded. The NP suspension was dialyzed against 500 mL water, using a dialysis membrane with molecular weight cutoff of 10,000 (Sigma-Aldrich) for 24 hr to
remove methanol. The dye-loaded NPs were characterized for size and zeta potential as described above. The dye-loaded NP suspension was stored in dark at 4 ºC until used.

Dye loading and imaging protocols were optimized such that a linear correlation between NP concentration and average signal intensity was attained. Briefly, 100 µL of NP suspension containing different concentration of NPs was added into white 96 well-plates (Fisher Scientific), and the plates were imaged using Maestro EX fluorescence imaging system (Cambridge Research and Instrumentation, Woburn, MA) with NIR filter set with exposure time of 300 ms. The regions of interest (ROI) were created along each well and total signal (scaled counts/s) was recorded. NPs with known concentration were suspended in cell lysate of untreated cells and added in 96 well-plate to obtain a standard plot. The standard plot was used to determine cellular uptake of NPs.

Dye release from NPs was determined in 1 % bovine serum albumin solution in PBS (release buffer). For this purpose, 2 mg/mL NP suspension in the release buffer loaded into a 1 mL Float-A-Lyzer (Spectrum Laboratories) with molecular weight cutoff of 500 D and was dialyzed against 5 mL release buffer at 37 ºC with continuous shaking. The amount of dye released was determined by comparing the absorbance of NP suspension collected at each time point to the absorbance of initial NP suspension using UV-Vis spectroscopy (Perkin Elmer, Waltham, Massachusetts).
3.3.5 Physical Characterization of NPs

To study the effect of dye loading on NP characteristics, 100 µL of dye-loaded NP suspension was added in 3 mL of 10 % FBS solution. Size and zeta potential of NPs were measured as per the method described in chapter II.

3.3.6 Uptake of NPs by HIAEC and PC-3 cells

Cells (1 ×10^5 cells/well/mL) were seeded in 24-well plates (BD Biosciences, San Jose, CA) and cultured for 2 days as described above, until they reached 80-90 % confluency. NP suspension was prepared by addition of 250 (1 mg/mL) of NP suspension to serum containing RPMI medium for PC-3 cells and endothelial basal medium with growth factors for HIAECs such that the total volume was 10 mL, and final NP concentration in wells was 25 µg/mL. Cells were washed twice with D-PBS before addition of NP suspension. Cells were incubated either for 5 or 15 min at 37 °C in a CO₂ incubator after addition of NP suspension, washed twice with ice-cold PBS and then lysed in 200 µL of cold radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) consisting of 1× protease inhibitor cocktail. Typically, 200 µL of RIPA buffer was added to each well and cells were scrapped and collected. Cell lysate (6 µL) was used to determine the total protein content using the Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Standard curve for protein assay was obtained using bovine serum albumin solution with different concentrations.

The remaining cell lysate was lyophilized for 24 h at - 45 °C (FreeZone 4.5 liter Benchtop 115, Labconco Corp., Kansas City, MO), and the lyophilized cell lysates were
used to analyze the NIR dye content as described in our previous study (140). Briefly, cell lysate was incubated at 37 °C for 48 hr in 500 µL of methanol. The samples were centrifuged at 14,000 rpm using a microcentrifuge (Eppendorf, Westbury, NY) for 10 min at 4 ºC. Hundred micro liter of the supernatant was collected from each sample and added to a white 96 well plate (Nunc Brand, Fisher Scientific, Pittsburg, PA) and analyzed using Maestro EX fluorescence imaging system using NIR filter set with exposure time of 2500 ms. Exposure time was adjusted such that even small amount of NPs in the cell lysate could be detected, without saturation of the signal. A standard plot was obtained under identical conditions (filter set, exposure time) for each formulation of NPs in range of 10 ng/mL to 10 µg/mL. Samples were normalized with respect to the total protein content for each sample.

3.3.7 Statistical Analysis

Statistical analysis was performed using Student’s t-test. The difference between groups was considered significant for $p$ values of < 0.05.

3.4 RESULTS

3.4.1 Composition of HIAEC and PC-3 Cell Lipids

The HPTLC analysis showed that the phospholipid composition of the normal endothelial and cancerous cells vary (Figure 3.1). Mainly, sphingomyelin (SM) spot was slightly lower in intensity and phosphatidylethanolamine (PE) spot was darker for PC-3 cells as compared to HIAECs. The phosphatidylcholine (PC) spot also seems to be darker for PC-3 cells as compared to HIAECs. Quantitative analysis of the HPTLC plates
provided relative concentrations of phospholipids in both cells. The amount of PE and PC was nearly 1.5 fold in PC-3 cells as compared to HIAECs (Table 3.1).
Figure 3.1 Phospholipid separation and quantification of lipids extracted from HIAEC and PC-3 cells analyzed by high performance thin layer chromatography. Lipids were separated by addition of 5 µL of lipid extracts to the bottom of the TLC plate, and mobile phase was allowed to run till the top of the TLC plate. Figure shows representative data from three different lipid extracts for each cell line.
Table 3.1 Relative Concentration of Different Phospholipids in Total Lipids*

<table>
<thead>
<tr>
<th>Lipids</th>
<th>HIAEC</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>11.4</td>
<td>8.6</td>
</tr>
<tr>
<td>PC</td>
<td>13.3</td>
<td>17.7</td>
</tr>
<tr>
<td>PI</td>
<td>6.8</td>
<td>6.3</td>
</tr>
<tr>
<td>PS</td>
<td>16.4</td>
<td>17.0</td>
</tr>
<tr>
<td>PE</td>
<td>11.0</td>
<td>13.9</td>
</tr>
<tr>
<td>PA</td>
<td>4.8</td>
<td>4.4</td>
</tr>
<tr>
<td>CL</td>
<td>7.9</td>
<td>5.1</td>
</tr>
<tr>
<td>NL</td>
<td>28.4</td>
<td>26.9</td>
</tr>
</tbody>
</table>

Lipids marked in red can affect membrane packing and fluidity
* SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatic acid; CL, cardiolipin; NL, neutral lipid.
3.4.2 HIAEC and PC-3 Cell Lipid Membrane Isotherms

The isotherm of the PC-3 cell lipids showed increase in membrane SP at about 98% trough area, whereas that of HIAEC lipids began to increase membrane SP at about 35% of trough area (Figure 3.2 a). The PC-3 cell lipid isotherm showed a gradual increase in SP till SP of 15 mN/m was reached, followed by a pseudoplateau region until 20 mN/m, and then a rapid increase in SP with further compression until collapse at SP of 45 mN/m was observed. On the other hand, HIAEC lipid isotherm showed a rapid increase in SP until the collapse at SP of 42 mN/m. In addition, at any given SP, the trough area covered by HIAEC lipids was lower than PC-3 lipids (Figure 3.2 a).

The compression modulus of each monolayer was plotted against the SP (Figure 3.2 b). At lower membrane SP both the cell lipid membranes showed similar compression modulus of < 15 mN/m. However, at 20 mN/m PC-3 cell lipid membrane showed significantly lower compression modulus (10 mN/m) as compared to HIAEC lipid membrane (30 mN/m). This trend continued at 30 mN/m where PC-3 cells showed slightly lower compressibility modulus. The maximum compression modulus for HIAEC (70 mN/m) and PC-3 (40 mN/m) cell lipid membrane was observed prior to their respective membrane collapse SP.
Figure 3.2 Biophysical characterization of lipids isolated from normal endothelial and prostate cancer cells. a) Compression Isotherm (\(\pi-A\)) of lipids isolated from HIAEC and PC-3 cells. To obtain a complete isotherm, lipid extract was spread on the subphase at different initial SPs and the different parts of the isotherm were compiled with merging the overlapping regions to obtain a complete isotherm. Lipid monolayers were compressed at rate of 5 mm/min. until complete membrane collapse was observed. b) Compression modulus of Langmuir monolayers for normal and cancerous cell lipids. Compression modulus was calculated from the \(\pi-A\) isotherm data using equation \(\frac{1}{Cs} = -\frac{dA}{d\pi}\).
3.4.3 Effect of Surface Chemistry of NPs on NP-Cell Lipid Membrane (HIAEC and PC-3) Interactions

Biophysical interactions between NPs and cell membrane lipids have shown to depend on the surface chemistry of NPs, and also varied between HIAEC and PC-3 lipid membranes (Figure 3.3). NPs with amine surface chemistry demonstrated an increase in HIAEC lipid membrane SP over time; whereas, NPs with sulfate surface chemistry showed a slight increase followed by gradual decrease in membrane SP (Figure 3.3a). On the other hand, NPs with amine surface chemistry showed an increase in PC-3 lipid membrane SP within first few min. followed by a plateau over time (Figure 3.3b). NPs with sulfate surface chemistry showed maximum increase in PC-3 membrane SP which was attained within first few min after NP injection and was followed by a gradual drop in membrane SP over time. NPs with carboxyl surface chemistry showed minimal interaction with PC-3 cell and HIAEC lipid membranes resulting in negligible changes in membrane SP.

At the endpoint of the interaction study i.e. 20 min post interaction, NPs with amine surface chemistry caused increase in HIAEC lipid membrane SP (Figure 3.3a), whereas interaction with NPs with sulfate surface chemistry caused drop in SP such that HIAEC lipid membrane SP was similar to the control HIAEC lipid membrane, and NPs with carboxyl surface chemistry caused a decrease in HIAEC lipid membrane SP. Although, the pattern of NP interaction with PC-3 lipid membranes was similar to HIAEC lipid membranes, magnitude of SP changes 20 min post interaction varied. It was
lower (positive SP change) following interaction of NPs with amine surface chemistry, and higher (negative SP change) for NPs with sulfate and carboxyl surface chemistry.

For NPs suspended in 10 % FBS, interaction of NPs with HIAEC lipid membrane caused an increase in membrane SP within 5 min post NP injection followed by gradual drop in membrane SP, such that membrane SP 20 min post interaction is similar to the control HIAEC lipid membrane (Figure 3.4a). On the other hand, NPs showed rapid increase within first few min. post interaction with PC-3 lipid membrane, followed by plateau region over time (Figure 3.4b). Here, the magnitude of interaction was dependent on the surface chemistry of NPs; wherein, NPs with sulfate surface chemistry demonstrate greater interaction with PC-3 cell lipid membrane as compared to NPs with amine and carboxyl surface chemistry.

Pattern of NP interaction varied with HIAEC as well as PC-3 lipid membranes for NPs suspended in 10 % FBS as compared to NPs suspended in water (Figure 3.3 and 3.4). Mainly, NPs with amine surface chemistry showed positive changes in HIAEC and PC-3 lipid membrane for NPs suspended in water (Figure 3.3 a); whereas, NPs with sulfate surface chemistry showed positive changes in PC-3 cell lipid membranes for NPs suspended in 10 % FBS (Figure 3.4 b). In addition, NPs with carboxyl surface chemistry showed minimal interactions with either cell lipid membranes in either NP suspension medium.
Figure 3.3 NP-lipid biophysical interaction studies for NPs suspended in water. The change in SP was monitored over time for a) HIAEC and b) PC-3 cell membrane lipids after interaction with NPs. To study the interactions, 100 µL of 0.5% NP suspension in water injected into the subphase, using a Hamilton digital micro-syringe, below the membrane after the cell lipid membrane SP of 30 mN/m was attained, and changes in membrane SP were recorded over period of 20 min (Key: Black = Control, Green = Sulfate, Red = Carboxyl, Blue = Amine).
Figure 3.4 NP-lipid biophysical interaction studies for NPs suspended in 10 % FBS. The change in SP was monitored over time for a) HIAEC and b) PC-3 cell lipid membrane after interaction with NPs. To study the interactions, 100 µL of 0.5% NP suspension in 10 % FBS was injected into the subphase, using a Hamilton digital micro-syringe, below the membrane after the cell lipid membrane SP of 30 mN/m was attained, and changes in membrane SP were recorded over period of 20 min (Key: Black = Control, Green = Sulfate, Red = Carboxyl, Blue = Amine).
3.4.4 Dye Loading of NPs

The dye loaded NPs demonstrate linear correlation between the increase in average signal intensity and the concentration of NPs (Figure 3.5). Dye loading did not significantly change the mean particle size or surface charge of NPs (Table 3.2). Furthermore, minimal level of dye (~3%) is released from NPs under *in vitro* conditions (Figure 3.6).
Figure 3.5 NIR dye was loaded in NPs with different surface chemistry. Dye loading was analyzed using Maestro EX imaging system, with NIR filter and exposure time of 300 ms. Dye loading was attained such that signal intensity was similar for all type of NPs at all concentrations. (n=3)

Figure 3.6 NIR dye release from NPs with different surface chemistry over time. Dye release was analyzed over period of 24 hr, using UV-Vis spectroscopy.
Table 3.2 Physical Characterization of dye-loaded NPs with different surface chemistry.

<table>
<thead>
<tr>
<th>Polystyrene NP surface chemistry</th>
<th>Size of NPs in FBS (nm)</th>
<th>Size of Dye-loaded NPs in FBS (nm)</th>
<th>Zeta potential of NPs in FBS (mV)</th>
<th>Zeta potential of Dye-loaded NPs in FBS (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfate</td>
<td>54.8</td>
<td>55.8</td>
<td>2.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Carboxyl</td>
<td>35.4</td>
<td>54.4</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Amine</td>
<td>206.2</td>
<td>273.9</td>
<td>3.6</td>
<td>5.3</td>
</tr>
</tbody>
</table>
3.4.5 Uptake of NPs by HIAECs and PC-3 Cells

Uptake was evaluated 5 and 15 min post incubation, corresponding to time-frame used for biophysical interaction studies. Cell uptake of NPs in PC-3 cells was comparatively higher than in HIAECs (Figure 3.7). In addition, cell uptake of NPs doubled from 5 to 15 min for both HIAECs and PC-3 cells (Figure 3.7). Following NP incubation with PC-3 cells (Figure 3.7), NPs with amine surface chemistry demonstrated significantly higher uptake as compared to NPs with sulfate surface and carboxyl surface chemistry; while NPs with sulfate surface chemistry showed significantly higher uptake as compared to NPs with carboxyl surface chemistry. On the contrary, NP uptake by HIAECs did not show any significant differences between NPs based on surface chemistry.
Figure 3.7 Cell Uptake of NPs. Uptake of NPs (25 µg/mL) by HIAECs and PC-3 cells after 5 min (a) and 15 min (b) of incubation. Cells were seeded with density of $1 \times 10^5$ cells/well in a 24-well plate. Data expressed as mean ± s.e.m. (n = 3). * p < 0.05
3.5 DISCUSSION

The goal of our study was to determine the influence of surface chemistry of NPs on their specificity towards cancer cell (PC-3) lipid membrane vs. normal cells (HIAEC) lipid membrane and whether such selective interactions translate into greater cellular uptake of NPs in cancer cells as opposed to normal healthy cells. Although there is some discrepancy between the biophysical interactions and cell uptake studies, our findings demonstrate that biophysical interaction studies are helpful in prediction of cell uptake of NPs. Biophysical interactions between NPs and PC-3 cell lipid membrane is a promising approach that should be further explored for developing tumor cell targeted NPs.

Cell lipid composition of normal and malignant cells differs as reported in previous studies (138). This difference has shown to influence cell membrane characteristics such as fluidity and elasticity (178, 179), which have in turn suggested to control the nanoparticle penetration into the membrane (140, 141). Therefore, the phospholipid composition of cells was quantified using TLC to understand the variation in lipid composition and its influence on biophysical characteristics of cell membranes. The TLC results show that membrane lipids of PC-3 cells have significantly different composition as compared to HIAECs (Figure 3.1). Particularly noticeable was the higher concentration of PE and PC lipids and lower concentration of SM lipids in PC-3 cells as compared to HIAECs (Table 3.1).

Isotherms were used to study the biophysical properties of HIAEC and PC-3 cell lipid membrane; wherein the membrane condensation of lipids and compression modulus
of cell lipid membrane isotherms provide insight into the membrane lipid packing density and membrane fluidity, respectively (180). Differences in membrane properties such as membrane curvature and permeability could be attributed to the affinity of lipids towards other lipids during the formation of lipid monolayer/bilayer based on the differences in their molecular shape of lipids due to relative differences in the size of lipid tail and headgroup (120, 181). For instance, SM has been suggested to cause membrane condensation due to its close lipid packing in the membrane caused by structural ordering of the acyl chains in membrane due to formation of SM-cholesterol complexes (18). In our study, at same membrane SP, lower trough area of HIAEC lipid isotherm as compared to PC-3 cell lipid isotherm reflects greater condensation of HIAEC lipid membrane as compared to PC-3 cell lipid membrane (Figure 3.2 a). Membrane condensation of HIAEC lipid membrane could be associated with their higher SM content as compared to PC-3 cells (Table 3.1).

Further, compression modulus calculated from the lipid membrane isotherm reflects the elasticity of acyl chains within the membrane and thereby provides information about the resistance of lipid monolayer to compress (182). Therefore, compression modulus can be related to the membrane fluidity of the cell membrane (140). Lower compression modulus of PC-3 cell lipid membrane (30 mN/m) compared to HIAEC lipid membrane (40 mN/m) measured at SP of 30 mN/m (Figure 3.2 b) further support our finding from the isotherms that PC-3 cell lipid membrane are fluid compared to HIAEC lipid membrane. Higher fluidity of PC-3 cell lipid membrane could be
attributed to their higher PE content as compared to HIAECs (69); wherein the conical shape of PE results in loose lipid packing of membrane (Table 3.1).

The interaction between NPs with amine surface chemistry and HIAEC and PC-3 lipid membrane could be as a result of electrostatic interactions between the positively charged NPs and negatively charged phospholipids which cause NPs to adhere strongly to lipid monolayers (Figure 3.3) (21). Adhesion of cationic NPs has shown to be independent of lipid composition, indicating non-specific electrostatic interactions (183), and explain the non-specific interactions observed between NPs with amine surface chemistry and HIAEC and PC-3 cell lipid membrane. On the other hand, penetration of NPs with sulfate surface chemistry into the lipid membrane tend to cause membrane condensation and hence an increase in membrane SP; however, over time steric repulsions between negatively charged NPs and anionic lipids result in lipid dissociation from membrane into subphase and hence result in decrease in EMM SP (21). The above findings suggest non-specific interactions between NPs with amine surface chemistry and HIAEC and PC-3 cell lipid membranes as compared to specific interactions between NPs with sulfate surface chemistry with PC-3 cell lipid membrane. The effect of surface chemistry on NPs is illustrated from the differences in interaction of NPs with sulfate and carboxyl surface chemistry. Although both type of NPs have similar charge (Figure 3.3), NPs with sulfate surface chemistry interact with PC-3 cell lipid membrane while NPs with carboxyl surface chemistry do not interact with either HIAEC or PC-3 cell lipid membrane.
Proteins adsorb on the NP surface as soon as NPs enter any biological environment and form a ‘protein corona’ (168). In addition, cell uptake of NPs is generally estimated in serum containing media. Hence, NP interactions with cell membrane are better represented when NPs are suspended in FBS. Therefore, we investigated NP-cell lipid membrane interactions for NPs suspended in 10 % FBS. Our findings demonstrate that following NP interaction with PC-3 cell lipid membrane, NPs penetrate into the lipid membrane and cause an increase in membrane SP; however, the membrane condensation limits further NP penetration into the membrane and hence results in a plateau region over time (Figure 3.4). The difference in the magnitude of interaction could be attributed to the amount and type of protein adsorbed on NP surface depending on their surface chemistry. On the other hand, lesser interaction of NPs with HIAEC lipid membrane could be due to its higher lipid condensation and membrane rigidity as compared to PC-3 cell lipid membrane which causes a decrease in NP penetration into the membrane.

Cell uptake of NPs demonstrates greater NP uptake by PC-3 cells as compared to HIAECs at both incubation time points and could be attributed to the higher PE content in PC-3 cells; wherein, PE has shown to influence the membrane curvature due to its conical shape, and hence facilitate membrane invagination which results in faster endocytosis (126). In addition, we speculate that the increased interaction of NPs with PC-3 lipid membrane as well as membrane fluidity of PC-3 cell membrane play an important role in the higher cellular uptake of NPs as compared to HIAEC.
Interaction of NPs with HIAEC and PC-3 cell correlated with the cell uptake studies in respect to NPs with amine and carboxyl surface chemistry (Figure 3.4, 3.7). However, interaction of NPs with sulfate surface chemistry did not correlate with the observed cell uptake of these NPs, especially for PC-3 cells. Since, PC-3 cell lipid membrane has higher anionic lipids as compared to normal endothelial cell membrane, higher uptake of NPs with amine surface chemistry as compared to NPs with sulfate surface chemistry could be attributed to greater electrostatic interactions between cationic NPs with amine surface chemistry and anionic cell membrane lipids (184), and further results in pore formation in the membrane and/or cause membrane disruption and leads to greater transport of NPs into the cells (107). On the other hand, PC-3 cell uptake of NPs with sulfate surface chemistry has been suggested to be due to interactions between NPs and cationic lipids that form NP clusters because of their repulsion towards the negative lipid domains, which are later engulfed into the cells (98). The above findings cannot explain the discrepancy observed between biophysical interaction and cell uptake results. However, greater uptake of NPs with amine surface chemistry may be due to NP binding to the membrane instead on NPs endocytosed into the cells. Further experiments like confocal microscopy will provide insight and detail whether NPs have adsorbed to the cell membrane and are in the exterior of the cell or have been endocytosed into the cells.

Our findings suggest that apart from the physical characteristics of NPs biophysical characteristics of cell lipid membranes like fluidity and lipid packing influence NP-cell lipid membrane interactions (141, 182). Biophysical interactions between NPs and endothelial model membranes have been previously investigated to
predict cellular uptake of NPs. However, NP-EMM interactions do not mimic the effect of lipid composition of different cell membrane on NP-cell lipid membrane interactions. In this study, we have demonstrated the role of selective NP interaction with cell lipid membrane in predicting cellular uptake of NPs. Our findings suggest that exploiting cancer cell lipid membrane characteristics and NP interactions with cancer cell lipid membrane may be an effective method for developing NPs targeted specifically towards tumor cells.

3.6 CONCLUSIONS

We have demonstrated that biophysical properties of cell lipid membranes are different, wherein PC-3 cell lipid membrane shows fluidic characteristic as compared to rigid nature of HIAEC lipid membrane. The fluidic nature of PC-3 cell lipid membrane causes greater biophysical interactions with NPs and also results in higher cell uptake of NPs than HIAECs. Furthermore, NPs with sulfate and carboxyl surface chemistry with similar charge demonstrated differences in their NP-cell lipid membrane biophysical interactions and cell uptake illustrating the importance of surface chemistry of NPs in their interactions with cell lipid membrane. Although the biophysical interaction studies did not exactly correlate with the cell uptake results, overall they show potential in estimating cell uptake of NPs and hence could be employed for developing NPs with specificity towards tumor cells.
CHAPTER IV
EFFECT OF SURFACE CHEMISTRY OF NANOPARTICLES ON THEIR BIODISTRIBUTION AND TUMOR LOCALIZATION

4.1 INTRODUCTION

Diverse array of parameters have been investigated to improve tumor localization of intravenously injected NPs. The conventional approach has been to formulate NPs with stealth characteristics by modifying NP surface with hydrophilic polymers such as PEG to avoid their clearance by the RES (87), prolong circulation time, and thereby improve tumor localization by EPR effect (69). Though PEGylation prolongs the circulation time of NPs, it has shown to hinder NP-cell membrane interaction and lower cell uptake of NPs. Apart from PEGylation, conjugating ligands like peptides, antibodies or co-factors to NP surface whose receptors are over-expressed on tumor cells or tumor vasculature has shown to enhance tumor localization of NPs (185). However, several factors limit the efficacy of this tumor targeting approach such as the limited number of cell surface receptors available for binding, and low affinity of conjugated ligand to receptors expressed on cancerous cells (68).
Modification of physical characteristics of NPs such as size, shape, and surface functionalization is an alternate approach that is being explored to enhance tumor localization of NPs (169). For instance, size of NPs has shown to influence the protein adsorption and hence circulation and tumor localization of NPs; whereas, shape of NPs has shown to influence NP binding to macrophages and hence their clearance from circulation. Recent studies have shown that NPs with similar size and charge demonstrated variation in biodistribution and tumor localization depending on surface functionalization of NPs. Therefore, in our study we focused on investigating the influence of surface chemistry of NPs on their biodistribution and tumor localization.

In the previous chapters (chapter II and III), we have investigated the influence of surface chemistry of NPs on their biophysical interactions with model membrane system as well as cell membrane lipids, and cell uptake. The results illustrated that membrane properties of PC-3 and HIAEC lipid membrane depend on the lipid composition of the membrane, and influence NP-lipid membrane interactions. Interaction studies demonstrated that NPs with sulfate surface chemistry preferentially interact with PC-3 cell lipid membrane; whereas NPs with amine surface chemistry non-specifically interacted with both the cell lipid membrane. In the present chapter, our objective is to determine how surface chemistry of NPs influences their biodistribution and tumor localization in vivo.
4.2 MATERIALS

Polystyrene NPs of size 20 nm were purchased from Invitrogen (Carlsbad, CA). The size of 20 nm was selected based on our previous findings, wherein we observed that 20 nm size NPs caused higher interaction with EMM as compared to 100 nm size NPs (Chapter II). NPs with sulfate, carboxyl and amine surface chemistry were selected because of the variation in their interaction with EMM as well as cell lipid membranes and cell uptake (Chapter II and III). SDB 5491 NIR dye was purchased from HW Sands Corporation (Jupiter, FL).

4.3 METHODS

4.3.1 Tumor Inoculation

Male athymic nude mice (4-6 weeks, nu/nu) were purchased from Charles River Laboratories (Wilmington, MA). Animal procedures performed were approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Prior to tumor inoculation, mice were anesthetized by an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. One million PC-3 cells, suspended in 200 µL of 50:50 PBS and Matrigel (Growth Factor Reduced, BD Biosciences, San Jose, CA), were injected subcutaneously into the left flank of the mouse (186). Tumor size was measured every other day using a digital caliper (Starrett, Athol, MA) and tumor volume was calculated as Volume = (length × width²/2). Animals with tumor volume ~ 300 mm³ were used to study biodistribution of NPs.
4.3.2 Evaluation of Biodistribution and Tumor Localization of Dye-Loaded NPs

4.3.2.1 In vivo Biodistribution and Tumor Localization of NPs

Mice anesthetized with isoflurane were injected via tail vein 50 µL of NP suspension (2 mg/mL) prepared in Mannitol Citrate Buffer. Animals were imaged before and after injection at different time points in two positions with mouse lying on back and sideways, using the Maestro EX Blue and NIR filter sets with exposure time of 730 ms and 2500 ms, respectively. Maestro Blue filter set was used to acquire mouse auto-fluorescence whereas the NIR filter was used to detect the 5491 dye-loaded NPs. The fluorescence image displaying the biodistribution of dye-loaded NPs was obtained by unmixing the imaged cube. Changes in the signal intensity over time were measured by drawing the regions of interest (ROIs) over tumor, liver, and spleen by inferring to approximate positions of these organs in vivo.

4.3.2.2 Ex vivo Biodistribution and Tumor Localization of NPs

Four days post-injection, animals were euthanized with a lethal dose of sodium pentobarbital (100 mg/kg), and were perfused via intracardiac injection of saline to remove circulating NPs. Tumors and other organs (heart, liver, spleen, kidneys and lungs) were harvested, weighed, and imaged ex vivo using Maestro EX by drawing ROIs around each tissue. Later, each tissue was homogenized in DI water (0.1 g/mL), the tissue homogenates were serially diluted to the final concentration of 1 mg/mL, and 100 µL of sample was added to white 96 well plate, and the signal intensity was measured using Maestro EX imaging system. The dye-loaded NPs were suspended in tissue homogenate of control mice to obtain a standard plot. The averages of the diluted samples were
compared to the standard plot to determine the amount of NPs present per weight of tissue sample.

Excreted animal feces were imaged at different time points by drawing ROIs around the feces, and the differences in signal intensity were used to compare the NP excretion from circulation. The data were normalized with respect to the number of feces.

4.3.3 Statistical Analysis

Statistical analysis was performed using Student’s t-test. The difference between groups was considered significant for p values of ≤ 0.05.

4.4 RESULTS

4.4.1 In vivo Biodistribution and Tumor Localization of NPs

The in vivo images show that NPs remain in circulation until the end point of our study i.e. 96 hr post injection (Figure 4.1 a). However, the differences in the signal intensity demonstrate that NPs with amine and carboxyl surface chemistry were rapidly cleared from circulation as compared to NPs with sulfate surface chemistry which showed prolonged circulation (Figure 4.1 c).

In vivo images show NP accumulation in the tumor within few minutes post NP injection (Figure 4.2 a). Based on the differences in signal intensity, accumulation of NPs with amine and carboxyl surface chemistry is greater in liver and spleen up to 24 hrs. However, NPs with sulfate surface chemistry show greater accumulation in these organs
24 hrs post injection (Figure 4.2 d and e). Tumor accumulation of NPs with sulfate surface chemistry is greater as compared to NPs with amine and carboxyl surface chemistry throughout the study (Figure 4.2 c). According to the differences in signal intensity of NPs, accumulation of NPs in tumors increases upto 24 hrs and is highest 24 hrs post injection followed by decrease in NP retention in tumor over time until the end point of study (Figure 4.2 c). Together, the in vivo images and the variations in signal intensity of NPs demonstrate the differences in their tumor retention depending on surface chemistry of NPs (Figure 4.2 c). NPs with sulfate surface chemistry showed greater tumor retention as compared to NPs with amine and carboxyl surface chemistry.
Figure 4.1 Distribution of NPs with different surface chemistry loaded with the NIR dye 5491 in mice with a xenograft prostate tumor. A) Mice were injected with 50 µL of 2 mg/mL suspension of NPs with different surface chemistry and imaged over 4 days post injection. B) The circulation of NPs was determined from ROIs drawn from skin (Sk) region away from tumor. C) Quantification of the NIR signal in skin region over time. Figure is representative for one animal from each group (n=3). Arrows indicate the tumors.
Figure 4.2 Biodistribution of nanoparticles after intravenous delivery. A) Side view Images from one animal for NPs with different surface chemistry at various time points post-intravenous injection with NPs containing NIR dye. The arrows indicate the location of tumor. Red indicates higher fluorescence, blue indicates comparatively lower fluorescence signal from the NPs. B) The signal intensity of NPs in the tumor (T), liver (L), and spleen (S) was determined from ROIs drawn over the area of each organ/tissue. Quantification of NIR signal in (C) Tumor, (D) Liver, and (E) Spleen over time (n=3 animals) for NPs with different surface chemistry.
4.4.2 Ex vivo Biodistribution and Tumor Localization of NPs

*Ex vivo* images of harvested organs are in congruence with the *in vivo* images, wherein NPs with sulfate surface chemistry show greater tumor localization as compared to NPs with amine and carboxyl surface chemistry (Figure 4.3 and 4.4). Based on the normalized signal intensity of tissue homogenate, NPs with sulfate surface chemistry have significantly greater tumor localization as compared to NPs with amine and carboxyl surface chemistry (Figure 4.5). For all type of NPs studied, higher amount of NPs accumulated in liver than in tumor. Apart from liver and tumor, biodistribution of NPs demonstrates that NPs with sulfate surface chemistry show greater localization in all other organs as compared to NPs with carboxyl surface chemistry.

The signal intensity of the excreted feces showed significantly higher excretion of NPs with amine surface chemistry as opposed to NPs with sulfate surface chemistry (Figure 4.6) within the first 24 hr post injection. In general, similar trend continued until the endpoint of the study.
Figure 4.3 Tumor localization of NIR dye-loaded NPs with different surface chemistry. Mice bearing PC-3 xenograft prostate tumors were injected with 50 µL of 2 mg/mL dye-loaded NP suspension with different surface chemistry. At 96 hrs post injection, mice were imaged in vivo; arrows point tumor. Animals were euthanized and perfused with saline, and tumors were extracted and imaged ex vivo. Blue indicates low fluorescence, red is for high fluorescence.
Figure 4.4 Biodistribution of NPs in the harvested organs 4 days post intravenous injection of NPs. A) For measurement of NP signal intensity, ROIs were drawn over harvested organs: tumor (T), liver (Lr), spleen (S), kidneys (K), heart (H), and lungs (Lg). B) Fluorescence signal was measured from ROI drawn around the harvested organs. Data are shown as mean ± SEM (n=3). Statistical analysis performed using Student’s t-test, *p≤0.05
Figure 4.5 *Ex vivo* Biodistribution and Tumor localization of NPs in Homogenized Tissues. Mice were injected with 50 µL of 2mg/mL NP suspension via tail vein injection in mouse xenografts prostate cancer, and euthanized 4 days post injection. Harvested organs were homogenized and normalized with respect to the tissue mass. Fluorescence signal was measured from a ROI drawn around the homogenized tissues in a 96 well plate. Data are shown as mean ± SEM (n=3). Statistical analysis performed using Student’s t test, *p≤0.05
Figure 4.6 Signal Intensity of Excreted Feces. Mice were injected with NIR dye loaded NPs. Feces excreted from mice were collected and imaged over 96 hours. Fluorescence signal intensity was measured from the ROIs drawn over feces. The data were normalized with respect to 10 feces per animal per time point. (n=3) Statistical analysis performed using Student’s t test, *p≤0.05
4.5 DISCUSSION

Physical characteristics of NPs such as size and shape, surface charge, and surface functionalization have shown to influence the in vivo circulation half-life, biodistribution and tumor localization of NPs (84, 187, 188). The studies that have investigated the effect of surface functionalization of NPs on biodistribution have mainly focused on surface modification of NPs by PEGylation or surface charge (189). However, rapid clearance of these NPs from circulation have hindered applicability of such NPs in drug delivery applications (190). This has led to investigation of other strategies like modification of surface chemistry of NPs to achieve improved biodistribution and tumor localization of NPs.

The focus of this chapter was to study the effect of surface chemistry of NPs on their biodistribution and tumor localization. The NP circulation in vivo was tracked by loading NPs with NIR dye. Our findings demonstrate that circulation, biodistribution and tumor localization of NPs depends on their surface chemistry, and the prolonged circulation time of NPs leads to their greater tumor retention. In addition, these findings illustrate role of NP-cell lipid membrane biophysical interactions in predicting tumor localization of NPs.

Importance of longer circulation time of NPs is evident from the greater tumor localization of NPs that demonstrate prolonged circulation time as compared to NPs that are rapidly cleared from circulation (85). In our study, all type of NPs remain in
circulation 96 hrs post injection (Figure 4.1 a), which could be a result of the smaller size of NPs (20 nm) (191, 192). However, differences in signal intensity of NPs suggest the variability in clearance rate of NPs based on their surface chemistry (Figure 4.1 c). Therefore, we propose that as NPs with sulfate surface chemistry have higher signal intensity they remain in circulation for longer time as compared to NPs with amine and carboxyl surface chemistry.

The difference in circulation time of NPs has been suggested to be due to amount of type of proteins adsorbed on NP surface based on their size, charge and surface functionalization (93). For instance, proteins like IgG adsorb on surface of cationic NPs such as NPs with amine surface chemistry (101) which causes their rapid uptake by macrophages (193, 194), and hence results in their clearance from circulation via RES organs (111, 195). On the other hand, ApoH protein adsorbs on surface of anionic particles such as NPs with sulfate surface chemistry (101), and inhibits macrophage uptake which results in their prolonged circulation (104). Based on the above reports we speculate that opsonins like IgG adsorb on surface of NPs with amine and carboxyl surface chemistry which results in their accumulation in RES organs like liver and spleen, followed by their rapid clearance from circulation; whereas NPs with sulfate surface chemistry resist phagocytosis and hence cause prolonged circulation time (Figure 4.2 d and e). Analysis of the excreted feces from mice at different time points also demonstrated rapid clearance of NPs with amine and carboxyl surface chemistry as opposed to NPs with sulfate surface chemistry which remained in circulation for longer time (Figure 4.6).
The longer circulation of NPs with sulfate surface chemistry (Figure 4.1) is further reflected from in vivo images that demonstrate greater tumor localization of these NPs as compared to NPs with amine and carboxyl surface chemistry (Figure 4.2 c). Similar results have been reported by Gabizon et al., wherein they have demonstrated higher tumor localization of NPs with longer circulation time (196). In addition, our findings from the in vivo biodistribution studies correlated well with the in vitro biophysical interaction studies (Chapter III). We observed greater tumor localization and retention of NPs with sulfate surface chemistry than NPs with amine and carboxyl surface chemistry similar to higher biophysical interaction of PC-3 cells with NPs with sulfate surface chemistry.

Our results illustrate the correlation between in vivo and ex vivo biodistribution of NPs (Figure 4.3, 4.4 and 4.5). Ex vivo biodistribution studies demonstrated significantly higher accumulation of NPs with sulfate surface chemistry in most organs as compared to NPs with carboxyl surface chemistry; however the difference in accumulation was not significantly higher as compared to NPs with amine surface chemistry. We speculate that within first 24 hrs post NP injection there is rapid clearance of NPs with amine and carboxyl surface chemistry, which results in decreased circulation half-life of these NPs and hence lower accumulation in tissues 4 days post injection (Figure 4.5) as compared to NPs with sulfate surface chemistry that showed greater circulation half-life of these NPs.
Generally, it has been reported that surface charge (zeta potential) of NP plays a significant role in their in vivo circulation time, biodistribution and tumor localization (188, 191). Interestingly, our study demonstrated that surface charge of NPs alone cannot entirely explain the differences observed in the in vivo circulation, biodistribution and tumor localization of NPs and these differences can be explained based on the differences in the surface chemistry of NPs. For instance, NPs with sulfate surface chemistry demonstrated higher tumor localization as compared to NPs with carboxyl surface chemistry, although both have negative charge (Figure 4.1 and 4.2).

Our findings suggest that surface chemistry of NPs is an important factor that governs the circulation as well as tumor localization of NPs in vivo. We observed greater tumor localization and retention for NPs with sulfate surface chemistry. NPs accumulate into the tumors mostly due to the EPR effect. Once inside the tumor interstitium, interactions with tumor cell membranes is required to facilitate NP uptake, as most chemotherapeutic drugs have intercellular site of action. The in vivo biodistribution studies showed correlation with the biophysical interaction studies. Based on our findings, we believe that NPs with sulfate surface chemistry have improved tumor targeting due to greater tumor localization and their enhanced biophysical interactions with PC-3 cell lipid membrane, resulting in improved cell uptake compared with other NPs. Although there is discrepancy in the cell uptake results, detailed studies like confocal microscopy need to be conducted to understand the process of cell uptake of these NPs before coming to any conclusion. In general, we believe that biophysical
interactions serve as an excellent tool to optimize NP characteristics for their enhanced tumor specific delivery in vivo.

4.6 CONCLUSIONS

Our data show that surface chemistry of NPs influence their circulation time; wherein NPs with sulfate surface chemistry demonstrated prolonged circulation as compared to NPs with amine and carboxyl surface chemistry which tend to rapidly clear from circulation. The prolonged circulation of NPs with sulfate surface chemistry leads to their higher tumor localization via the EPR effect. The findings from our study demonstrate that surface chemistry of NPs could be further explored to optimize NP characteristics for designing tumor targeted delivery systems.
CHAPTER V

THE EFFECT OF RESIDUAL POLY(VINYL ALCOHOL) ON BIOPHYSICAL INTERACTION OF NANOPARTICLES WITH ENDOTHELIAL CELL MODEL MEMBRANE

ACKNOWLEDGEMENTS

Reproduced from article “The Effect of Residual Poly (vinyl alcohol) on Biophysical Interaction of Nanoparticles with Endothelial Cell Model Membrane”, with permission from Bhave et al., International Journal of Nanoscience, Vol. 10 Nos. 4 and 5, Copyright 2011 World Scientific (211).

5.1 INTRODUCTION

In previous chapters (II, III and IV), we studied the influence of surface chemistry of polystyrene NPs on their biophysical interactions with model membranes, selectivity towards cell lipid membranes, in vivo biodistribution and tumor localization. However, biodegradable nanoparticles (NPs) formulated using poly(D,L-lactide co-glycolide) (PLGA) and polylactide (PLA) based polymers are commonly used in drug/gene delivery
applications because of their biodegradable nature and biocompatibility (197). To understand the influence of molecular structure of residual PVA adsorbed on NP surface, we studied the interactions between endothelial model membrane and NPs that have different hydrophilicity and amount of residual PVA adsorbed on NP surface.

The emulsion solvent-evaporation method is commonly used to formulate NPs comprising these polymers with polyvinyl alcohol (PVA) as an emulsifier (198). NPs formulated using PVA are usually uniform in size and easy to re-disperse in an aqueous medium (199). Typically, the oil phase is an organic solvent (chloroform or methylene chloride) containing a polymer and the drug of interest, which is emulsified in an aqueous phase containing PVA to form oil-in-water (o/w) emulsion. For the encapsulation of water soluble therapeutics, typically an aqueous solution of drug is emulsified first into the organic phase to form w/o emulsion which is then emulsified into the PVA phase to form a double, water-in-oil-water (w/o/w) emulsion. Following evaporation of the organic solvent, NPs are recovered via ultracentrifugation or filtration.

PVA is partially hydrolyzed poly(vinyl acetate). Polyvinyl acetate is hydrophobic, whereas the partially hydrolyzed part of the molecule is hydrophilic, thus imparting to PVA an amphiphilic property. The poly(vinyl acetate) portion of the PVA molecule anchors at the oil-water interface during emulsification and integrates with the NP polymer matrix once the organic solvent evaporates. Therefore, a fraction of PVA (termed residual PVA) remains associated with the NPs, even after their repeated washing with water (200). We have previously shown that the amount and type of this
residual PVA influences the cellular uptake of NPs and gene transfection with DNA-loaded NPs, whereas others have shown changes in the physical characteristics of the NPs (201, 202).

PVA is available in different molecular weights and degrees of hydrolysis. Usually, no consideration is given to the characteristics of the PVA used in the formulation of NPs. We hypothesize that residual PVA could influence the interfacial properties of NPs and hence their biophysical characteristics. In this study, we analyzed biophysical interactions of NPs formulated using PVA of different molecular weights and PVA of the same molecular weight but obtained from different batches. The goal is to understand how residual PVA influences the biophysical properties of NPs and whether the biophysical characterization of NPs can be used as a useful tool to optimize NP properties for consistent results.

5.2 MATERIALS

Lipids 1,2-dipalmitoyl-<i>sn</i>-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-<i>sn</i>-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-<i>sn</i>-glycero-3-phospho-<i>L</i>-serine (DPPS), L-<i>α</i>-phosphatidylinositol (PI), sphingomyelin (SM) and cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL). Performance liquid chromatography grade chloroform and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Poly(L-lactide) with inherent viscosity = 0.4 dL/g and molecular weight of 40,000 was purchased from Durect Corporation (Pelham, AL). Different types of PVA were purchased from Sigma-Aldrich (St. Louis, MO). The different PVA used were with
the same degree of hydrolysis (87-89%) (203) but varied in molecular weight (Table 5.1). PVA with the same molecular weight but obtained from different lots was also used. Dulbecco’s phosphate-buffered saline (D-PBS) (1X) obtained from our in-house Cell Services Core was used as the subphase in Langmuir study.

5.3 METHODS

5.3.1 Endothelial Cell Model Membrane Lipid Solution

A lipid mixture was prepared by mixing individual lipid solutions with composition as follows: DPPC (56%), DPPE (24%), PI (8.0%), DPPS (4.3%), SM (6.0%) and CL (1.7%). Individual lipid solutions were prepared with different solvents based on lipid solubility. DPPC, PI, SM and CL were dissolved in chloroform; whereas DPPE and DPPS were dissolved in a 4:1 chloroform and methanol mixture as they do not dissolve in chloroform alone. This composition of lipids was selected because it represents the headgroup chemistry of phospholipids of native arteries in the endothelial cell membrane (156).

5.3.2 Formulation of NPs

NPs were prepared using an emulsion-solvent evaporation method. In a typical procedure, 90 mg of PLA polymer was dissolved in 3 mL of chloroform for 1 hour with intermittent shaking. A 2% w/v PVA solution was prepared by slowly sprinkling the desired amount of PVA over cold water at 4°C while stirring on a magnetic stir plate. The stirring was continued for 2 hours, and then the PVA solution was centrifuged at 1000 rpm for 10 min at 4 °C (Sorvall Legend RT Centrifuge, Thermo Electron Corporation,
Waltham, MA). The supernatant was used for the NP formulation. The polymer solution prepared as above was emulsified into 18 mL of 2% w/v aqueous PVA solution using a staged microtip probe sonicator set at an energy output of 55 W (XL 2015 Sonicator ultrasonic processor, Misonix, Inc., Farmingdale, NY) for 3 min over an ice-bath to form an oil-in-water emulsion. The resulting emulsion was stirred continuously overnight on a magnetic stir plate under a chemical hood to evaporate chloroform and to form NPs. Additionally, the NP suspension was kept in a vacuum desiccator with magnetic stirring to ensure that all chloroform had evaporated. The formed NPs were recovered by ultracentrifugation at 30,000 rpm (Beckman Optima LE-80K, Beckman Instruments, Inc., Palo Alto, CA) using 50.2 Ti rotor, for 30 min at 4 °C. NPs were washed three times with distilled water to remove excess PVA. The pellet was resuspended in distilled water, and the suspension was sonicated for 2 min as above and centrifuged at 1000 rpm for 10 min at 4 °C to remove any large aggregates. The supernatant was frozen at -70 °C in a freezer, and then lyophilized for 48 hours to obtain dry powder (FreeZone 4.5 liter Benchtop 115, Labconco Corp., Kansas City, MO). NPs with different PVA were formulated using the identical protocol (Table 5.1).

We determined the insoluble fraction of the different PVA types in cold water. For this, 2% w/v PVA solution for each type (20 mg/mL) was prepared in cold water at 4 °C with stirring as above and centrifuged. The supernatant was carefully removed and the bottom portion (~5 mL) containing an insoluble fraction of PVA was carefully transferred in different pre-weighed vials and lyophilized as above. The difference in weight of the empty vial and that after lyophilization was taken as the insoluble fraction
of PVA. PVA5 was more soluble in hot water (70° C) than in cold water; hence, NPs using this PVA were formulated using the PVA solution prepared in hot water.

5.3.3 Particle Size and Zeta Potential Measurements

A suspension of NPs was prepared in distilled water at 100 µg/mL concentration by sonication for 30 s using a probe sonicator as above. The mean hydrodynamic diameter and zeta potential of NPs were measured using the dynamic light scattering and phase analysis light scattering techniques, respectively (PSS/NICOMP 380/ZLS particle analyzer, Particle Sizing Systems, Santa Barbara, CA).

5.3.4 Intrinsic Properties of NPs

The intrinsic surface properties of different formulations of NPs were determined using a Langmuir balance (Minimicro 2, KSV Instruments, Ltd., Helsinki, Finland). “Intrinsic surface property” reflects the propensity of NPs to localize at the interface. NPs with higher surface activity tend to come to the interface and increase SP. The Langmuir balance contains a Teflon trough, two hydrophilic Delrin barriers and a platinum Wilhelmy plate. The trough contains 50 mL D-PBS buffer, which acts as a subphase. The temperature of the trough was maintained at 37 °C by using a circulating water bath.

To measure the intrinsic surface property, the change in SP of the subphase was measured for each formulation of NPs over a period of 20 min. For this, a 500 µL (5 mg/mL) of NP suspension was injected using a Hamilton digital microsyringe (Hamilton
Company, Reno, NV) into the trough with minimal disturbance. This experiment was carried out without the lipids added onto the subphase.

5.3.4.1 Langmuir Balance

Langmuir monolayers have been used as a model system to study NP-cell lipid interactions (119). Studies have shown application of such model systems in predicting NP cellular uptake as well as cell toxicity (204). For instance, cell toxicity of different sized silica NPs was determined by studying their interactions with supported lipid bilayers using AFM (205). In another study, course-grained model was developed to study gold NP interactions with model lipid membranes (206). The simulation results show influence of surface charge as well as surface charge density on adherence to and penetration through the lipid bilayer.

In the Langmuir balance technique, differences in the lipid monolayer properties and monolayer interactions with different materials are measured by the differences in the surface tension of the monolayer. When lipid mixture solution is added to the interface, the solution spreads across the interface to cover the entire trough area and a monolayer is formed after evaporation of organic solvent. Initially, in the two dimensional gaseous phase of the lipid monolayer, the trough area is large with lipid molecules randomly oriented and away from each other leading to weak interactions between the lipid molecules. However, as the lipid monolayer is compressed, the lipid molecules start to exert repulsive forces on each other, reducing the surface tension of the subphase. The
difference in the surface tension of subphase caused by re-arrangement of lipid molecules is called as the surface pressure (SP), \( \Pi \), and is given by the relationship

\[ \Pi = \Gamma - \Gamma_0 \]

Where, \( \Gamma \) is surface tension of subphase in absence of monolayer and \( \Gamma_0 \) the surface tension in presence of monolayer.

Generally, SP-area isotherm of lipid monolayer is recorded by constant rate compression of the monolayer, at constant temperature. SP-area isotherm of the lipid monolayer show many distinct regions which are called as phases. As the monolayer is compressed, it passes through various phases which are associated with discontinuities in the isotherm. The phase behavior of the monolayer depends on the physical and chemical characteristics of the material as well as the subphase composition, temperature and pH. Monolayer phase also depends on the hydrocarbon chain length as well as the interactions between hydrophilic head groups. For example, increasing the chain length causes increase in the attraction between hydrophobic tails, resulting in condensation of the monolayer, which is seen from increase in SP of isotherm after compression.

Isotherms provide information about the lipid membrane characteristics like rigidity or fluidity, elasticity and resistance to compression and could be used to determine biophysical characteristics of lipid monolayer.

Previous reports have shown that lateral SP of human erythrocyte cell membrane is about 30 mN/m, and therefore the arrangement of phospholipids mimics the outer leaflet of cell membrane. Hence, the NP-model membrane interactions at the SP of 30
mN/m would better represent the NP adsorption or penetration into the cell membrane. In our previous work, we have demonstrated the influence of NP physical characteristics on NP-EMM interactions and its significance in predicting the cell uptake of the NPs (145). For example, TAT peptide conjugation to NP surface increased NP interaction with EMM as opposed to plain unconjugated NPs, and correlated well with the cell uptake results (153). In a similar study, NP-EMM interactions were investigated for surfactant modified NPs, elucidating the influence of surface characteristics of the surfactants absorbed on NP surface on biophysical interactions with EMM, and its correlation to the cellular uptake (109).

5.3.5 $\pi$-A isotherms of EMM in the Presence of NPs

The detailed methods by which to obtain an isotherm have been described in our previous studies (109, 207). These experiments were carried out to investigate how well NPs penetrate into the EMM. Briefly, 5 µL (1.17 mg/mL) of EMM lipid mixture was added dropwise on the subphase at SP of 0 mN/m. The organic solvents used to dissolve lipids were allowed to evaporate for 10 min. A 500 µL (5 mg/mL) of NP suspension was injected into the buffer subphase below the EMM lipid mixture. NPs were allowed to interact with the EMM lipid mixture for 20 min and then the barriers were compressed at a constant rate of 5 mm/min until the film collapsed.
5.3.6 Interaction of NPs with EMM

The EMM was formed on the subphase as described in our previous studies (109, 207). Briefly, 10 µL of EMM lipid mixture was added on the air-buffer interface, and organic solvents were allowed to evaporate for 10 min. The barriers were compressed until the SP of the membrane reached 30 mN/m. This SP is equivalent to the lateral surface pressure of a red blood cell membrane; hence the arrangement of the phospholipid monolayer would resemble the outer leaflet of the cell membrane. A suspension of NPs (500 µL, 5 mg/mL) was injected into the subphase below the membrane. The changes in SP of the EMM were recorded as a function of time for 20 min at a constant mean molecular area.

5.4 RESULTS

5.4.1 Solubility of PVA, Particle Size and Zeta Potential

There was a difference in cold-water solubility of different PVA types, but all PVA showed a >96% soluble fraction. Despite having the same molecular weight, PVA5 showed the most cold-water insoluble fraction of the PVA3, PVA4 and PVA5 (Table 5.1). Furthermore, it was noticed that PVA5 took a longer time to dissolve than PVA4 whereas PVA3 dissolved quickly. This was based on visual observation of changes in turbidity of each PVA solution with stirring time. However, PVA5 was almost completely soluble in hot water. As anticipated, PVA with higher molecular weight showed relatively low cold-water solubility (PVA1 vs. PAV2).
The NP characterization shows that the mean hydrodynamic diameter is slightly higher for the NPs formulated with PVA5, which is less soluble in cold water than those formulated with PVA that is more cold-water soluble (PVA3 vs. PVA5; Table 5.2). The zeta potential of NPs varied slightly, but there was no particular trend, and all formulations of NPs showed negative values.
Table 5.1 Characteristics of PVA used to formulate NPs

<table>
<thead>
<tr>
<th>PVA</th>
<th>Molecular weight (kDa)</th>
<th>Lot number</th>
<th>% PVA undissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA1</td>
<td>30-50</td>
<td>JO06615EO</td>
<td>0.33</td>
</tr>
<tr>
<td>PVA2</td>
<td>85-146</td>
<td>LO9418HO</td>
<td>0.57</td>
</tr>
<tr>
<td>PVA3</td>
<td>30-70</td>
<td>124K0052</td>
<td>0.50</td>
</tr>
<tr>
<td>PVA4</td>
<td>30-70</td>
<td>089K0037</td>
<td>1.20</td>
</tr>
<tr>
<td>PVA5</td>
<td>30-70</td>
<td>039K0147</td>
<td>4.02</td>
</tr>
<tr>
<td>PVA5*</td>
<td>30-70</td>
<td>039K0147</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Solubility in hot water; NPs, nanoparticles; PVA, poly(vinyl alcohol).
<table>
<thead>
<tr>
<th>Type of PVA used</th>
<th>Mean particle size (nm)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mV)</th>
<th>Surface Activity of NPs (mN/m)</th>
<th>Change in SP at 30 mN/m SP (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA1-NPs</td>
<td>352.35 ± 11</td>
<td>0.13 ± 0.04</td>
<td>-23.46 ± 1.18</td>
<td>13.5</td>
<td>0.84</td>
</tr>
<tr>
<td>PVA2-NPs</td>
<td>355.30 ± 39</td>
<td>0.46 ± 0.04</td>
<td>-26.15 ± 1.14</td>
<td>16.0</td>
<td>0.93</td>
</tr>
<tr>
<td>PVA3-NPs</td>
<td>341.90 ± 22</td>
<td>0.06 ± 0.03</td>
<td>-18.52 ± 0.39</td>
<td>16.0</td>
<td>1.25</td>
</tr>
<tr>
<td>PVA4-NPs</td>
<td>366.90 ± 8</td>
<td>0.05 ± 0.01</td>
<td>-22.60 ± 0.86</td>
<td>17.5</td>
<td>1.21</td>
</tr>
<tr>
<td>PVA5-NPs</td>
<td>380.30 ± 5</td>
<td>0.05 ± 0.03</td>
<td>-24.32 ± 1.16</td>
<td>19.0</td>
<td>0.41</td>
</tr>
</tbody>
</table>

NP, nanoparticle; PVA, poly(vinyl alcohol); SP, surface pressure; D-PBS, Dulbecco’s phosphate-buffered saline; EMM, endothelial cell model membrane.
5.4.2 Intrinsic Properties of NPs

There appears to be a trend between cold-water solubility of PVA and intrinsic property of NPs. NPs formulated with the least cold-water soluble PVA5 showed the highest intrinsic surface activity, as evidenced by an increase in SP of the buffer (Figure 5.1). This finding suggests that the NPs formulated with PVA5 have a hydrophobic surface and hence show a greater propensity to come to the interface as compared to other formulations of NPs. However, there appears to be no particular trend between the molecular weight of each of the PVA types and the increase in surface activity of NPs (Tables 5.1 and 5.2). These results clearly suggest that the residual PVA significantly influences the intrinsic surface properties of NPs.
Figure 5.1 Surface activity of NPs; NPs, nanoparticles; PVA Poly(vinyl alcohol).
5.4.3 $\pi$-A Isotherms of EMM in Presence of NP

The $\pi$-A isotherm of the EMM with and without NPs was studied (Figure 5.2). The EMM isotherm without injecting any NP suspension was used as a control. The control EMM isotherm starts in a gaseous phase, but the isotherm with NP suspension shows an initially higher value, suggesting the presence of NPs at the interface. NPs come to the air-buffer interface and interact with the lipids of the EMM, causing an increase in SP. NPs remain at the air-buffer interface with the EMM membrane until the membrane lipids reach a solid phase, characterized by a close packing of lipids. Isotherms merge and become identical to isotherms prepared in the absence of NPs, suggesting that at a point beyond a certain SP, the NPs squeeze out of the membrane into the subphase.

The isotherm for NPs formulated with PVA4 merges at a higher SP, suggesting these NPs have a higher level of interaction with the lipids of the EMM than other formulations of NPs. The data suggest that there is variation in the interaction of different formulations of NPs with membrane lipids.
Figure 5.2 Isotherms of NPs with EMM; NPs, nanoparticles; EMM, endothelial cell model membrane; PVA, Poly(vinyl alcohol).
5.4.4 Interaction of NPs with EMM

For this study, the EMM was formed first, the suspension of NPs was added into the subphase, and the change in SP of the EMM was determined over 20 min. The change in SP of the EMM following interaction with NPs demonstrates that NPs interact with EMM membrane (Figure 5.3). The increase in SP was attributed to the interaction of NPs with the EMM; any decrease in SP was held to indicate the loss of phospholipids into the subphase; no change in SP indicated that NPs do not interact with the EMM.

NPs formulated with PVA3 and PVA4 showed a greater increase in SP, whereas NPs formulated with PVA5 showed the least increase in SP. Despite the highest intrinsic surface activity of the NPs formulated with PVA5 (Figure 5.1), these NPs showed lower interactions with the EMM, indicating that they have lower penetration into the EMM. These results suggest that the intrinsic surface property of NPs does correlate to the interactions of NPs with membrane lipids and their penetration into the EMM.
Figure 5.3 NP interaction with EMM; NP, nanoparticle; EMM, endothelial cell model membrane; PVA, poly(vinyl alcohol); NP, nanoparticle.
5.5 DISCUSSION

PVA solubility experiments show that each type of PVA had a different degree of cold-water solubility, reflecting the difference in characteristics among all types. Although we have not determined the cause, this difference in cold-water solubility could occur because of the variation in the degree of hydrolysis of each PVA type (208). Differences in the characteristics of PVA, particularly the degree of hydrolysis could influence amphiphilic property of PVA (198), which leads to difference in particle size of the formulated NPs (209).

Differences in the characteristics of each PVA type could influence the localization of PVA at the oil-water interface and emulsion stabilization property (203, 210). It is clear that PVA with lower solubility in cold water has less emulsion stabilization effect than PVA that is more soluble in cold water. The ability of the emulsifier to reduce the emulsion drop-let size and the stability of the emulsion prior to solidification of the polymer phase controls the size of NPs.

The differences in the intrinsic surface activity of NPs, EMM isotherms in presence of NPs and NP interactions with EMM at SP of 30 mN/m for NPs formulated with different PVAs suggest that the residual PVA at the NP interface can significantly influence the biophysical properties of NPs and their interactions with the membrane lipids. From the above findings, NPs formulated with PVA3 and PVA4 seem to have similar physical properties (mean particle size and zeta potentials) and also biophysical properties (intrinsic surface activity and change in SP of the EMM).
Recently, we have shown that (a) the molecular structure of the surfactant at the NP interface significantly influences the biophysical interactions of NPs with membrane lipids and (b) these biophysical interactions correlate to cellular uptake of NPs. NPs that demonstrated higher biophysical interactions with membrane lipids also showed higher cellular uptake. The above results thus suggest that interfacial properties of NPs influence the biophysical interactions of NPs with model membrane lipids and are critical for successful cellular delivery of encapsulated therapeutics.

The variation in the biophysical properties of NPs formulated with different types of PVA could influence their drug delivery properties (210). Although in this study we focused only on PVA, modulating the interfacial characteristics of NPs with different surface chemistry could be explored as a promising tool for drug delivery applications. Thus, biophysical interactions studies could be used to optimize the properties of NPs for drug/gene delivery applications.

5.6 CONCLUSIONS

The overall results show that (a) residual PVA influences intrinsic properties of NPs and their interactions with membrane lipids and that (b) despite similar molecular weight and degree of hydrolysis, PVA shows variations in cold-water solubility that seem to influence the characteristics of NPs. In addition to physical characterization, biophysical characterization of NPs could ensure better consistency in the applications of NPs for drug/gene delivery. The variations in the interfacial properties of NPs could be further explored as a promising tool for drug delivery applications.
CHAPTER VI
SUMMARY AND FUTURE STUDIES

6.1 SUMMARY

The overall goal of this study was to understand the role of biophysical interactions between NPs and cell membrane lipids in depicting the tumor localization of NPs based on their surface chemistry. To attain this goal, we divided our study into smaller objectives which are, to: a) evaluate effect of surface chemistry of NPs on their biophysical interactions with cell membrane, b) investigate the role of surface chemistry in NP specificity towards cancer cell membranes, and c) evaluate the role of surface chemistry on NP specificity towards tumor cells and improve tumor localization of NPs. These objectives were discussed and evaluated over different chapters in this thesis.

In chapter II, we studied the effect of surface chemistry of NPs on their interactions with EMM. NPs with sulfate and amine surface chemistry demonstrated higher interaction with EMM as compared to the other type of NPs investigated. Apart
from surface chemistry of NPs, the size and the medium in which NPs are dispersed also influenced NP interactions with EMM.

In chapter III, we investigated the differences in biophysical characteristics of normal endothelial (HIAEC) and cancerous (PC-3) cell membrane lipids, and NP selectivity towards cancerous cell membrane based on the surface chemistry of NPs. NP interactions with PC-3 cell lipid membranes were greater as compared to HIAEC lipid membrane due to the fluidic nature of PC-3 lipid membranes as opposed to rigid and condensed nature of HIAEC lipid membrane. NPs with sulfate surface chemistry showed selective interactions with PC-3 cell lipid membrane; whereas NPs with amine and carboxyl surface chemistry showed non-specific interactions with both cell lipid membranes.

In chapter IV, we studied effect of surface chemistry of NPs on *in vivo* biodistribution and tumor localization of NPs. NPs with sulfate surface chemistry showed prolonged circulation *in vivo*, greater tumor localization and tumor retention over time as compared to NPs with amine and carboxyl surface chemistry.

The studies described in this work (chapter II, III and IV) have been investigated using model polystyrene NPs. Typically, NP drug delivery systems are prepared using biodegradable polymers. Therefore, in chapter V we studied effect of residual PVA adsorbed on NP surface with EMM as an example to demonstrate how surface modifications influence biophysical interactions with EMM, using biodegradable
PLGA/PLA NPs. We found that differences in hydrophilicity as well as the amount of residual PVA adsorbed on the NP surface influence their interactions with EMM and demonstrate the influence of surface functionalization on NP-EMM interactions.

Overall, in this study we observed that surface chemistry of NPs influences NP interactions with cell lipid membrane, and NP specificity towards cancer cell lipid membranes. NPs with sulfate surface chemistry showed higher interaction with cancer cell lipid membranes as compared to NPs with amine and carboxyl surface chemistry. The in vivo results correlated well with the biophysical interaction studies and demonstrated greater circulation, tumor localization and tumor retention of NPs with sulfate surface chemistry as compared to NPs with amine and carboxyl surface chemistry.

In conclusion, our study demonstrates the role of NP-lipid biophysical interactions in optimizing NP characteristics for tumor targeting of NPs. Characterization of the biophysical interactions between NPs and lipid membranes of tumors or other infected tissues may be a promising approach for developing tissue specific drug delivery systems.

6.2 FUTURE STUDIES

The biophysical interactions of NPs should be further analyzed with an acidic subphase pH to physiologically represent the acidic tumor cell environment. Polystyrene NPs were used in this study as these NPs were available in different sizes and surface
chemistry. Future studies should involve synthesizing biodegradable NPs with different surface chemistry to investigate their biodistribution and tumor localization.
REFERENCES


51. Walker L, Perkins E, Kratz F, & Raucher D. Cell Penetrating Peptides Fused to a Thermally Targeted Biopolymer Drug Carrier Improve the Delivery and


153. Peetla C, Rao KS, & Labhasetwar V. Relevance of Biophysical Interactions of Nanoparticles with a Model Membrane in Predicting Cellular Uptake: Study with


204. Maget-Dana R. The Monolayer Technique: A Potent Tool for Studying the Interfacial Properties of Antimicrobial and Membrane-Lytic Peptides and Their


