TARGETED DELIVERY OF IMMUNO-NANO-COMPOSITE BASED DELIVERY SYSTEMS: POTENTIAL IN TREATMENT AND PROPHYLAXIS OF CANCER

ABSTRACT

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOTECHNOLOGY

BY

MOHD. ASIF SHERWANI

INTERDISCIPLINARY BIOTECHNOLOGY UNIT
ALIGARH MUSLIM UNIVERSITY
ALIGARH-202002 (INDIA)

2014
Abstract

Development of nanocomposites is currently in vogue, in order to harness the desirable properties of the components used for their synthesis in a single delivery system. Moreover, selective delivery of nanoparticles to tumors, specifically antibody mediated targeted delivery, for avoiding off-target effects of the payload is being immensely investigated. Considering these, the work presented in this thesis is focused on development of novel immuno-nanocomposites and evaluation of their anti-tumor efficacy both in vitro as well as in vivo.

In the first part of the study (chapter two), anti-cancer activity of dextran-doxorubicin complex encapsulated anti-Her2 mAb functionalized immuno-stealth-liposomes was evaluated on SKBR3 cancer cell line. Doxorubicin (DXR) commonly used in cancer therapy produces undesirable side effects such as cardiotoxicity. To minimize these attempts have been made to couple the drug with dextran but the complexes are degraded in a short span of time. Therefore, in the present study, we encapsulated dextran-doxorubicin complex in PEGylated liposomes which have longer circulation tendency than non-PEGylated liposomes. The size of in-house synthesized dual-core nanocomposites as determined by TEM was found to be in the range of 200-300nm, which can favour the EPR effect as observed in most solid tumors. In order to facilitate targeted delivery of these drug conjugates, the conjugates were encapsulated in immuno-stealth liposome and the efficacy of anti-Her2 mAb functionalized and dextran-doxorubicin complex encapsulated immuno-stealth liposomes on Her2 overexpressed SKBR3 breast cancer cell line has been evaluated. Surface functionalization of dextran-doxorubicin complex encapsulated PEGylated liposomes improved the therapeutic efficacy of the nanocomposites. Antibody tagging on lipid polymer composite was confirmed by protein estimation assay and also with fluorescence microscope. The results of this chapter suggest that encapsulation of the dextran-doxorubicin complex in lipid shell not only reduces the side effects of doxorubicin, but also improves its therapeutic efficacy.

The second part of the study (chapter three) demonstrates development of liver cancer specific antibody conjugated PAMAM-dendrimer/siRNA complex functionalized and cisplatin loaded PLGA nanocomposites. PAMAM dendrimers being cationic polymers facilitate binding of negatively charged siRNA. In this nanocomposite system (Ab@PAMAM-siRNA@Cis-PLGA NC), liver cancer specific antibody (Ab) targets the nanocomposite to the cells expressing liver cancer antigens against which
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2014
Declaration

I hereby declare that the thesis entitled “Targeted delivery of immuno-nano-composite based delivery systems: Potential in treatment and prophylaxis of Cancer” submitted to the Aligarh Muslim University, Aligarh, in fulfilment of the requirement for the award of the degree of Doctor of Philosophy, embodies the original research work carried out by me under the guidance and supervision of Prof. Mohammad Owais. To the best of my knowledge, the data included in the thesis have not been fabricated and plagiarized. The work has not been submitted elsewhere in part of full for the award of any other degree of this or any other university.

Mohd. Asif Sherwani  
Senior Research Fellow (DBT)  
Interdisciplinary Biotechnology Unit  
Aligarh Muslim University  
Aligarh-202002
Certificate

Certified that the thesis entitled "Targeted delivery of immuno-nano-composite based delivery systems: Potential in treatment and prophylaxis of Cancer" submitted to the Aligarh Muslim University, Aligarh, in fulfilment of the requirement for the award of the degree of Doctor of Philosophy, embodies the original research work carried out by Mr. Mohd. Asif Sherwani, under my guidance and supervision. The work has not been submitted elsewhere in part of full for the award of any other degree of this or any other university.

4/4/2014
Nov 10, 2014

Prof. Mohammad Owais, Ph.D.
(Supervisor)
Certificate

This is to certify that the thesis entitled "Targeted delivery of immuno-nano-composite based delivery systems: Potential in treatment and prophylaxis of Cancer" submitted to the Aligarh Muslim University, Aligarh for the award of the degree of Doctor of Philosophy, is an authentic record of the research work carried out by Mr. Mohd. Asif Sherwani, under the supervision of Prof. Mohammad Owais. The work has not been submitted elsewhere in part or full for the award of any other degree of this or any other university.

Prof. Asad U Khan, Ph.D.
(Co-ordinator)
Course work/Comprehensive examination/Pre-submission seminar completion certificate

This is to certify that Mr. Mohd. Asif Sherwani has satisfactorily completed the course work, passed the comprehensive examination and successfully delivered the pre-submission seminar, which are parts of his Ph.D. programme.

Prof. Asad U Khan, Ph.D.
(Co-ordinator)
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### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>µl</td>
<td>Microliter</td>
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<td>µm</td>
<td>Micrometer</td>
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<td>Micromolar</td>
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<td>Ab</td>
<td>Antibody</td>
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>ALT</td>
<td>Alanine transaminase</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>Br-dUTP</td>
<td>Bromo deoxyuridine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's adjuvant</td>
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<tr>
<td>CHOL</td>
<td>Cholestrol</td>
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<td>Cisplatin</td>
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<td>CLR</td>
<td>C-type lectin receptors</td>
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<td>CTAB</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<td>DEN</td>
<td>Diethylnitrosamine</td>
</tr>
<tr>
<td>DEX</td>
<td>Dextran</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12 Dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DSPE</td>
<td>1,2-Distearoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DXR</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HPMA</td>
<td>N-(2-hydroxypropyl) methacrylamide</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund's adjuvant</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Kda</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LFT</td>
<td>Liver function test</td>
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<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>Mal-PEG</td>
<td>Maleimde polyethylglycol</td>
</tr>
<tr>
<td>MBS</td>
<td>m-maleimidobenzoyl-N-hydroxysuccinimide ester</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimeter</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NC</td>
<td>Nanocomposite</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
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<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>p53mut</td>
<td>p53 mutant</td>
</tr>
<tr>
<td>p53wt</td>
<td>p53 wildtype</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amido amine)</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine (egg derived)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLK-1</td>
<td>Polo like kinase-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>-------------</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute,</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SL</td>
<td>Stealth liposome</td>
</tr>
<tr>
<td>SPACE</td>
<td>Skin penetrating and cell entering</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor associated antigens</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell Receptor</td>
</tr>
<tr>
<td>Tdt</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper cells</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td></td>
<td>dUTP nick end labelling</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
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<tr>
<td>XRD</td>
<td>X ray Diffraction</td>
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Chapter One

Review of literature
1.1. BACKGROUND

Cancer, also known as malignancy, is an abnormal growth of cells with the potential to invade or spread to other parts of the body. It is caused by genetic instability and multiple molecular alterations, and remains the leading cause of numerous deaths across the globe (Boyland, 1952; Kinzler and Vogelstein, 2002; Stein and Pardee, 2004). According to the World Cancer Report 2014, released on 3 February by the World Health Organization’s International Agency for Research on Cancer (WHO, IARC), by 2025, there will be 20 million new cancer cases per year, compared with 14.1 million in 2012 (Figure 1.1). By 2030 and 2035, the number of global cancer cases arising per year is predicted to increase to 22 million and 24 million respectively (Press Release ‘N’ 224’, IARC, WHO, 2014). It is anticipated that the greatest impact will be on low- and middle-income countries as a consequence of growing and ageing populations. More than 60% of the world’s total cases occur in Africa, Asia and and Central and South America, and these regions account for about 70% of the world’s cancer deaths. Asia alone experienced 46% of global cancer cases in 2012 but a larger number of cancer mortalities, 53% (Figure 1.2).

**Figure 1.1. Predicted global cancer cases according to World Cancer Report 2014.**
(Source: WHO GloboCan)
THE BURDEN OF CANCER

Asia experienced around 46% of global new cancer cases in 2012, but 53% of cancer mortalities.

**INCIDENCE: 14.1 million new cases**

<table>
<thead>
<tr>
<th>Region</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>North America</td>
<td>12.7%</td>
</tr>
<tr>
<td>Oceania</td>
<td>1.1%</td>
</tr>
<tr>
<td>East and central Asia</td>
<td>16.6%</td>
</tr>
<tr>
<td>Middle East/N. Africa</td>
<td>3.6%</td>
</tr>
<tr>
<td>Europe</td>
<td>24.4%</td>
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<tr>
<td>Latin America/Caribbean</td>
<td>7.8%</td>
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<td>China</td>
<td>21.6%</td>
</tr>
<tr>
<td>India</td>
<td>7.2%</td>
</tr>
<tr>
<td>Sub-Saharan Africa</td>
<td>4.4%</td>
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**MORTALITY: 8.1 million deaths**

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<td>North America</td>
<td>8.4%</td>
</tr>
<tr>
<td>Oceania</td>
<td>0.7%</td>
</tr>
<tr>
<td>East and central Asia</td>
<td>17.3%</td>
</tr>
<tr>
<td>Middle East/N. Africa</td>
<td>4.1%</td>
</tr>
<tr>
<td>Europe</td>
<td>21.4%</td>
</tr>
<tr>
<td>Latin America/Caribbean</td>
<td>7.4%</td>
</tr>
<tr>
<td>China</td>
<td>26.3%</td>
</tr>
<tr>
<td>Sub-Saharan Africa</td>
<td>8.3%</td>
</tr>
<tr>
<td>Africa</td>
<td>5.5%</td>
</tr>
</tbody>
</table>

Figure 1.2. The burden of cancer in 2012 according to World Cancer Report 2014. (Source: IARC World Cancer Report 2014)

As can be analyzed from the data presented in Figure 1.1, in less developed countries like India, the number of cancer cases are predicted to increase from 7.5 million in 2012 to 15 million by 2035 in contrast to more developed countries where the number of cases arising every year were 6 million and are anticipated to grow to only 8 million. The latest statistics on trends in cancer incidence and mortality worldwide emphasizes the urgent need for prevention complemented with improved treatments in order to address the alarming rise in cancer burden globally.

1.2. CANCER TREATMENT AND PROPHYLAXIS

1.2.1. Cancer treatment

The traditional strategies for cancer treatment, includes surgery, radiation, and chemotherapy or combined strategies of these treatments. These are supplemented by some more specialized therapies such as immunotherapy or hormone therapy which can be applied only to some tumor types (Miller et al., 1981).
Review of Literature

1.2.1.1. Surgery
The oldest form of cancer treatment is surgery. It renders the greatest chance of cure, mainly for solid tumors; especially those which have not yet metastasized to other parts of the body. It is and will remain in future one of the most important weapons against cancer.

1.2.1.2. Radiotherapy
Radiotherapy is the second major weapon against cancer. Radiation therapy involves use of high-energy particle beams or waves (radiation), such as X-rays, gamma rays, neutrons or pimesons for treating cancer (Israel, 1978). Radiotherapists often implant radioisotopes into tumors (Thorell and Larson, 1978). The radioactive material transfers its energy into highly energetic electrons which ionize the matter they hit, such as water and/or proteins or other molecules of the cell cytoplasm, or RNA and DNA. This ionization changes the molecules and hence leads to variations or inactivation in their biological properties; e.g. causing cell death or inhibits cell division. Radiation is more harmful for cancerous cells than for normal cells because cancerous cells are more unstable in all respects and thus more vulnerable to the damaging radiations. However, the cellular repair mechanism is also not prominently active in the highly dividing cells like cancer cells. However, due to proper functionally active cellular repair mechanism normal cells can recover from the effects of radiation more easily. One of the major drawbacks of radiotherapy is that it is impossible to treat only tumor cells, without affecting the surrounding healthy cells.

1.2.1.3. Chemotherapy
Chemotherapy uses chemicals to treat cancer, especially suitable for those cancers that have been spread out (metastasised) and cannot be treated any longer by localized methods such as surgery and radiation. One common characteristic of most cancer cells is their rapid rate of cell division. Anticancer drugs like taxol (interferes with the depolarization of microtubules and hyperstabilizes their structure), doxo- (is thought to intercalates in DNA) or daunorubicin (intercalates, with its daunosamine residue directed toward the minor groove), all adversely affects the process of cell division. Thus are aimed to destroy aggressive cancers. Nevertheless, chemotherapeutics have the same disadvantage like radio-therapeutics. They are unspecific and therefore do
not distinguish between healthy and cancerous cell and hence damage also healthy
cells as well.

1.2.1.4. Hormone therapy
Hormone therapy which is also known as androgen deprivation therapy or androgen
suppression therapy changes the internal environment and prevents the growth of
cancers that are hormone-dependent, such as some cases of breast or prostate cancers
(Vrbanec et al., 1998; Torri and Floriani, 2005). Hormone therapy lowers androgen
levels (either by surgery or drugs such as pituitary down-regulators and anti-
androgens) and slows down the tumor growth or reduces the tumor volume.

1.2.1.5. Photodynamic therapy
PDT which is a very local and highly tumor specific treatment has greatly reduced the
side-effects as the photosensitizers is accumulating mainly (intravenous or intratumoral
injection) in the tumor tissue and then becomes activated by a local application of
nontoxic infrared light. This irradiation produces highly toxic singlet oxygen which
irreversibly damages the cancer cells so they undergo apoptosis or necrosis (Chen et
al., 1997; Chen et al., 1996; D'Cruz et al., 2004; Dolmans et al., 2003).
Unfortunately this technique can be used exclusively for superficial tumors such as
melanomas, head and neck cancer, tumors of the bladder because of the limited light
penetration of the tissue and accessibility by a light source.

1.2.1.6. Immunotherapy
Immunotherapy has emerged as a more specific cancer treatment method for cancers
that affect the immune system, like leukemia (cancer of the leukocytes) and
lymphoma (cancer of the lymphatic tissues). These cancers weaken the immune
system and make it harder for the body to fight off disease. Immunotherapy stimulates
a patient's immune system so it is better able to fight disease by administration of
cytokines or monoclonal antibodies (Rosenberg, 1999). Cytokines bind to cancer
cells, making them easily recognizable and more susceptible to the action of other
immune cells. Other cytokines enhance the killing action of immune cells and help the
natural ability of the body to repair cells damaged by radiation or chemotherapy
(Dranoff, 2004; Kim-Schulze et al., 2007). Interferon-alfa (INF-α), interleukin-2
(IL-2) and colony-stimulating factor (CSFs) have shown promise as cancer therapies.
INF-α slows the growth of cancer cells, and promotes more normal cell activity and
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stimulates the body's natural immune system to fight cancer cells, according to the National Cancer Institute (NCI). Monoclonal antibodies specifically target cancer cells and then block their activation (therapeutic antibodies) or inhibit tumor growth by delivering covalently bound radioactive chemicals (targeting antibodies) (Weiner, 1999). Compared to the side effects of standard chemotherapy, the side effects of naked mAbs are often "allergic" reactions which rarely can be life-treating but often prevents a second treatment (Gruber et al., 1996).

As our current regimens combining chemotherapy, radiation therapy, and surgery for the treatment of cancer continue to expand, the mechanisms underlying tumor biology are becoming better understood. The continued study of tumor biology and natural history through controlled trials focusing not only on efficacy endpoints but also on biologic markers in tissue and serum will help develop detailed risk models.

1.2.2. Cancer prophylaxis
Cancer prophylaxis or cancer prevention lowers the risk of cancer or slows its development, although this strategy is not used to treat cancer. Cancer prophylaxis can be differentiated into cancer chemoprophylaxis and cancer immuno-prophylaxis.

1.2.2.1. Chemoprophylaxis
Cancer chemoprophylaxis or cancer chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer (Tsao et al., 2004). The success of several recent clinical trials in preventing cancer in high-risk populations suggests that chemoprevention is a rational and appealing strategy.

The FDA's approval of tamoxifen for breast cancer prevention was a landmark achievement that crowned over 20 years of progress in chemoprevention research (Tsao et al., 2004). Since then chemoprevention of cancer has been widely studied. Several newer agents with potentially less toxicity have shown promise. Enzyme inhibitors, natural products like green tea polyphenols and essential oils, hormone agonists and vitamins etc. have shown promise in prevention of various forms of cancer. In fact, many high-quality clinical trials recommend the use of chemoprevention in defined circumstances (Wattenberg, 1985). For example, selective estrogen receptor modulators such as tamoxifen and raloxifene have been extensively used to prevent breast cancer in high-risk women (Vogel et al., 2006).
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resembles the human disease (Gingrich et al., 1996, Lollini et al., 2006). Treatment of the prostate tumor cells with 15-deoxy-812,14- prostaglandin J2 induced expression of heat-shock protein (Hsp) 70, and vaccination of TRAMP C57/BL transgenic mice with Hsp 70 from these tumors has been reported to elicit tumor-specific CTLs and protected from tumor challenge (Vanaja et al., 2000). Moreover, Andreasson et al. have demonstrated that prophylactic and therapeutic protection could be obtained with murine pneumotropic virus chimeric Her2/neu virus-like particles and also established cross-reactivity between human and rat Her2/neu (Andreasson et al., 2009).

Although most antigen-discovery methods employed tumors as sources of antigen, many TAAs have been found to be expressed very early in the process of transformation and are frequently expressed in premalignant dysplasia and metaplasia. Several human tumors, such as colon, breast, prostate and lung, yield themselves to early detection and patients with familial history or other risk factors for these malignancies are under regular monitoring. Surgery is an option for removal of a small tumor but, with the exception of polyp removal for colon cancer prevention, there are no strategies for dealing with premalignant changes (Finn and Forni, 2002).

Immune prevention could also be considered for individuals whose tumors have been eradicated by standard therapy. Experimental data in mice immunized after tumor surgery (Finn and Forni, 2002; Bolli et al., 2011) show that prevention of recurrences in conditions of minimal residual disease is a permissible immunological challenge. However, since the immune system has been imprinted by a previous encounter with a progressing tumor, tolerance to TAAs may need to be overcome in order to elicit an efficient response.

1.3. NANOMEDICINES FOR CANCER

Cancer therapeutics including drugs, gene or siRNA often have undesired physiochemical and pharmacological properties, such as low solubility, rapid degradation and narrow therapeutic index (Langer, 1998) which limit their applications for clinical cancer treatments. Two strategies have emerged over the past several decades to address these intrinsic drawbacks (Li and Wallace, 2008). One approach is to design and develop new derivatives of chemotherapeutics with improved physiochemical and pharmacological properties so that they can be used to
precisely and specifically modulate the molecular processes and pathways associated
with tumor progression (Collins and Workman, 2006). Alternatively, existing
anticancer agents can be made more effective by using nanocarriers that bring more
payload to the tumor site, compared with the conventional formulation, while
reducing exposure of normal tissues to the drug (Duncan, 2006; Ferrari, 2005;
Peer et al., 2007). Most drug delivery vehicles of cancer therapeutics possess
dimensions of 1-1,000 nm and usually contain many therapeutic agents per vehicle
(Peer et al., 2007). Nowadays these new anticancer modalities are often also termed
nanomedicine. Drug incorporation in nanoscale drug carriers protects drugs from
premature degradation and controls drug tissue distribution. Owing to the nanoscale
size, these anticancer drug carriers can be administered in the bloodstream where
they selectively localize therapeutic drug payload to metastatic tumors by
incorporating defined biological rationales in the formulation design (Li and
Wallace, 2008).

The family of nanocarriers includes liposomes where highly ordered lipid molecules
in lamellar arrangement encapsulate drugs either inside or between the bilayers
(Torchilin, 2005); polymeric nanocarriers where nanoparticles are constructed from
natural or synthetic polymers with an either dense or core-shell structure (Sutton et
al., 2007, Allen et al., 1999); polymer-drug conjugates where water-soluble polymers
are covalently coupled with anticancer drugs along the chains or amphiphilic
polymers having drugs coupled on the hydrophobic segments self-assemble into core-
shell nanoparticles (Duncan, 2006); and dendrimers where synthetic branched
macromolecules encapsulate drugs/gene/siRNA physically (Patri et al., 2005).
Recent development of new chemistry (e.g. click chemistry (Brannon-Peppas and
Blanchette, 2004)) and fabrication technologies (Sutton et al., 2007) is expected to
allow for unprecedented, precise control of nanomedicine formulation, making it
possible to evaluate nanomedicine with the variation of one parameter (e.g. size,
surface property, and shape) at a time (Iyer et al., 2006). In the construction of
nanomedicine, lipid and polymer-based systems are amongst the most extensively
explored, accounting for more than half of the nanomedicines approved for clinical
disease treatment. Therefore, here lipid and polymer based nanomedicines are mainly
focused.
1.3.1. Liposomes

Liposomes are frontrunners among the nanomedicine systems developed so far (Bangham et al, 1965; Gregoriadis, 1976). Liposomes are self-assembling colloid structures composed of lipid bilayers surrounding (an) aqueous compartment(s), and can encapsulate a wide variety of (chemo)therapeutic agents. Myocet (Doxil in the United States) was among the first of such lipid self-assemblies to be approved by the regulatory authorities. This formulation contains doxorubicin entrapped liposomes. The pharmacokinetic benefits of liposomal drug encapsulation can be illustrated as follows: for free doxorubicin, an elimination half-life time of 0.2 h and an AUC (area under the curve) of 4 μg/ml were found in patients, as compared with 2.5 h and 45 μg/ml for Myocet (Hofheinz et al, 2005). Both in animal models and in patients, such (liposome-mediated) improvements in AUC have been shown to result in significant improvements in EPR mediated drug targeting to tumors (Lammers et al., 2008; Drummond et al, 1999; Torchilin, 2005). However, thus far, the primary justification for approving liposomal anthracyclines has been their ability to attenuate drug-related toxicity (e.g., cardiomyopathy, bone marrow depression, alopecia and nausea), rather than to enhance antitumor efficacy. A phase III head-to-head comparison of free doxorubicin vs Myocet in patients with metastatic breast cancer, for instance, demonstrated in this regard that at comparable response rates (RR: 26% for both) and progression-free survival times (PFS: 4 months for both), the incidence of cardiac events (29 vs 13%) and of congestive heart failure (8 vs 2%) were significantly lower for Myocet (Harris et al, 2002). Besides Myocet, several other non-stealth liposomal nanomedicines have been evaluated over the years, including, for example, liposomal daunorubicin (Daunoxome) and vincristine (Onco-TCS), and lurtotecan (OSI-211), and lipoplexes, such as Allovecitin and LEraFAON, in which cationic lipids are used to complex, carry, protect and deliver genetic material, such as plasmid DNA and antisense oligonucleotides. Thermodox, a temperature sensitive version of liposomal doxorubicin has also entered clinical trials. At the preclinical level, numerous additional liposomal nanomedicines have been tested, aiming not only to establish novel carrier–drug combinations (Schiffelers et al, 2006), but also to improve the efficacy of already existing formulations, for example, by optimising the composition of the lipid bilayer (Drummond et al, 1999).
1.3.2. Polymeric nanomedicines

Ten years after the first report on liposomes (Lammers et al., 2008; Bangham et al., 1965), and coinciding with the appreciation of their clinical potential (Gregoriadis, 1976), natural and synthetic polymers started to attract attention as drug delivery systems. Conceptualised by Ringsdorf (1975), it was envisioned that polymeric macromolecules can be conjugated to pharmacologically active agents by means of linkers that are stable in blood, but labile in the acidic and/or enzymatic conditions typical of, for example, the tumor microenvironment or certain intracellular compartments. These so-called ‘polymer-therapeutics’ have been shown to passively accumulate in tumors by means of the EPR effect, and to be able to beneficially affect the therapeutic index of attached lowmolecular-weight agents (Duncan, 2006). The biodegradable and biocompatible features of synthetic polymeric NPs constructed from PLA and PLGA, largely account for their widespread use in biomedical applications and justify the approval of PLGA micro- and nanoparticles for human use in drug delivery by the FDA (McCarron et al., 2008; Moreno et al., 2010; Su et al., 2012). PLGA and PLANPs slowly degrade via surface erosion in an aqueous environment due to hydrolysis of the ester linkages in the (co)polymer backbone, to produce the original monomers lactic acid and glycolic acid. The latter can be further metabolized in the human body, which explains the favorable toxicity profile of these polyesters.

In 1994, a conjugate called PK1 was the first tumor-targeted polymeric prodrug to enter clinical trials. In PK1, doxorubicin is conjugated to the prototypic polymeric drug carrier PHPMA (poly(N-(2-hydroxypropyl)methacrylamide)) through an enzymatically cleavable tetrapeptide spacer (GFLG). Like Myocet, PK1 primarily improved the therapeutic index of doxorubicin by attenuating its (cardio)toxicity (Duncan, 2006). This is exemplified by the remarkably high maximum tolerated dose observed for PK1 in clinical trials, being 45 times higher than that determined for free doxorubicin (320 vs 60 mg m⁻²; Vasey et al, 1999). Following this proof of principle, PK1 progressed into phase II evaluation, and several additional polymer therapeutics entered clinical trials. In 2005, Abraxane (i.e., albumin-bound paclitaxel) was the first passively tumor-targeted polymeric nanomedicine to gain FDA approval. Evidence for an advantage of Abraxane over the standard, Cremophor-formulated version of paclitaxel has been provided by a large phase III trial in which 4400 women with
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metastatic breast cancer were randomized to receive either Abraxane (260 mg m\(^2\) given as a 30-min infusion, without premedication) or the free drug (i.e., Taxol; 175 mg m\(^2\) given as a 3-h infusion, with standard steroid and antihistamine premedication). As compared with Taxol, Abraxane significantly improved both the response rate (33 vs 19%) and the PFS time (23 vs 17 weeks) of systemic taxane treatment (Gradishar et al, 2005), and at the same time, it also attenuated its toxicity: the incidence of grade 4 neutropenias was significantly lower for Abraxane (9 vs 22%), despite the 50% higher dose, and no hypersensitivity reactions were observed, despite the absence of premedication (Gradishar et al, 2005). Besides Abraxane and PK1, a number of additional polymeric nanomedicines have been evaluated clinically. Oncaspar, for instance, in which the polymer PEG is conjugated to the protein L-asparaginase (to decrease allergic reactions and frequency of administration), has been used for treating patients with acute lymphoblastic leukemia for 410 years; Zinostatin, that is, prostate specific membrane antigen (PSMA) bound neocarcinostatin, has been approved in Japan for the treatment of liver cancer; and Xyotax, that is, PLGA-conjugated paclitaxel, is in phase III evaluation for ovarian and non-small-cell lung cancer. In addition to such conventional polymer–drug and polymer–protein conjugates, several novel types of polymeric nanomedicines have also recently entered clinical trials, including cationic polyplexes for DNA and siRNA delivery (De Smedt et al, 2001; Schifflers et al, 2004), dendrimers (Bai et al, 2006), and polymeric micelles (Nishiyama and Kataoka, 2006).

1.3.3. Nanocomposites

Both polymer-based and lipid-based nanocarriers have demonstrated immense clinical benefit, but still suffer from inherent shortcomings. In spite of the many favorable characteristics they exhibit, liposomes may experience low loading efficiencies, especially for hydrophobic drugs. In addition, depending on their composition, liposomes may suffer from poor stability in vivo, smaller half life resulting in unwanted burst drug release (Raemdonck et al., 2014; Hadinoto et al., 2013; Torchilin, 2005). Polymeric NPs provide a valuable alternative to overcome some of the above-mentioned limitations, as they generally demonstrate outstanding drug loading capacity for drugs and/or contrast agents with diverging physicochemical properties. Moreover, progress in polymer chemistry enables the design of NPs with ample control over their nano-architecture and biophysical properties which facilitates
application in controlled and triggered drug release strategies. On the other hand, issues have been raised regarding the biocompatibility of certain polymeric materials and the potential heterogeneity and low density of chemical surface functionalization (Raemdonck et al., 2014; Kamaly et al., 2012). To address the multifaceted drug delivery challenges outlined above, several research groups have turned to the design of ‘hybrid nanoparticles’ or ‘nanocomposites’ with the primary aim to combine the most valuable features of both the components forming nanocomposites (Raemdonck et al., 2014; Hadinoto et al., 2013). In a broad sense the word “composite” means “combination of two or more different materials that are mixed in an effort to blend the best properties of both.” A nanocomposite is a composite material, in which at least one of the components is in nanodimension. Lipid-polymer and polymer-polymer nanocomposites are currently in vogue for drug/gene/siRNA delivery to combat various untamed diseases including cancer and hence would be discussed in the following section.

1.3.3.1. Lipid-polymer nanocomposites

Multiple functionalities can be incorporated into polymeric NP design (Raemdonck et al., 2014) to assist in drug encapsulation, drug release, in vivo bio-distribution, cellular targeting and intracellular trafficking, which has recently been comprehensively reviewed by others (Raemdonck et al., 2014; Kamaly et al., 2012; Nicolas et al., 2013). One particular strategy to modulate the surface of polymeric NPs, and hence also influence their behaviour at the nano–bio interface, is by depositing a stabilized (phospho)lipid layer onto the hydrophobic NP core. Moreover, lipid envelope of liposomes can be made ‘stealth’ by depositing a layer of polymer PEG. The motivation to invest in the development of these more complex lipid–polymer hybrids lies within their anticipated improved in vivo stability, increased drug loading and added control over the drug release process as with the case of PEGylated liposomes which have longer shelf life and hence remain in circulation for longer periods (Drummond et al., 1999; Torchilin, 2005; Kamaly et al., 2012; Mandal et al., 2013).

The consequential advantages of combining lipids and polymers in a hybrid drug delivery platform are situated at many levels (Hadinoto et al., 2013; Mandal et al., 2013; Tan et al., 2013). Efficient drug encapsulation can be achieved both in the polymeric component and in the lipid envelope. Drug release can be controlled by
polymer degradation but also modulated by the presence of the lipid envelope that acts as a diffusional barrier. In this way, unwanted drug leakage from the nanocarrier can be prevented. Furthermore, the lipid layer may hinder the influx of water, tempering polymer hydrolysis and slowing down drug release. The polymer matrix core may also contribute to the structural integrity of the lipid coat.

In this section, lipid-polymer composite based nanomedicines for treatment of cancer are revealed. The earliest form of lipid polymer composite approved by FDA is Caelyx (doxorubicin encapsulated PEGylated liposomes). The pharmacokinetic benefit of Caelyx can be illustrated as follows: for free doxorubicin, an elimination half-life time of 0.2 h and an AUC (area under the curve) of 4 μg/ml were found in patients, as compared with 55 h and 900 μg/ml for Caelyx (Hofheinz et al., 2005). Also for Caelyx, significantly reduced cardiomyopathy was observed, whereas its response rates, its PFS times and its overall survival times were always at least comparable with those of the free drug (Drummond et al., 1999; Hofheinz et al., 2005; Torchilin, 2005). In certain specific cases, for example in patients suffering from AIDS-related Kaposi’s sarcomas, which are characterized by a dense and highly permeable vasculature, Caelyx not only reduced the toxicity of the intervention but also substantially improved its efficacy: as compared with the formerly standard combination regimen ABV (i.e., adriamycin (doxorubicin), bleomycin and vincristine), which produced a partial response in 31 out of 125 patients (RR=42.5%), Caelyx achieved 1 complete response and 60 partial responses (RR=44.6%; Northfelt et al., 1998). Caelyx has consequently been approved for Kaposi’s sarcoma, and is currently also marketed for metastatic breast cancer, advanced ovarian cancer and multiple myeloma. PEGylated liposomal cisplatin (SPI-77) is another lipid-polymer nanocomposite which has been clinically investigated.

Many pre-clinical trials have also established the efficacy of newer lipid-polymer nanocomposites, some of them which have been recently conducted are discussed here. Aryal et al. (2010) reported on the synthesis of amphiphilic drug conjugates of two widely used chemotherapeutic agents, i.e. the hydrophobic paclitaxel and hydrophilic gemcitabine hydrochloride, which were subsequently encapsulated into lipid-coated PLGA NPs. The drug conjugate loaded NPs demonstrated markedly enhanced cytotoxicity compared to the free conjugate in a human pancreatic carcinoma cell line. The intracellular combinatorial release of both chemotherapeutics
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is believed to occur via PLGA degradation and concurrent acid catalyzed hydrolysis of the drug conjugate ester linkage in acidified endolysosomal vesicles (Aryal et al., 2010). In a similar report, the same authors also demonstrated enhanced ovarian cancer cell killing by encapsulating a hydrolysable paclitaxel–cisplatin drug conjugate in lipid–polymer hybrid NPs (Raemdonck et al., 2014). However, no details are provided on the functional role of the lipid monolayer in the cellular drug delivery process.

Sengupta et al. (2005) also elegantly demonstrated the benefit of a lipid–polymer nanoarchitecture to gain better spatiotemporal control over the delivery of two distinct anticancer drugs. These authors proposed PLGA NPs that covalently encapsulate doxorubicin allowing a slow and degradation-controlled release of the chemotherapeutic agent. In addition, the PLGA NPs were enclosed in a PEGylated lipid bilayer in which the hydrophobic anti-angiogenesis drug combretastatin was inserted. Following systemic administration, the PEGylated lipid shell ensures prolonged blood circulation times and increased extravasation in the tumor interstitium. The physical segregation of both therapeutic agents allowed a temporal drug release profile with a fast initial release of the combretastatin, causing angiogenesis inhibition, preceding a slow release of the cytotoxic doxorubicin. This sequential exposure of the tumor to two distinct types of drugs synergistically acting on both the cancerous cells and the tumor vasculature allows the accumulation of effective doxorubicin concentrations in the tumor mass (Raemdonck et al., 2014).

In another strategy, Wang et al. (2010) translated the concept of chemoradiotherapy, i.e. the synchronized treatment with both chemotherapy and radiotherapy, to lipid–PLGA nanomedicines. The utilization of a lipid–polymer hybrid NP platform for chemoradiation, which they fittingly dubbed ChemoRad NPs, enabled the encapsulation of radioisotopes without affecting the NP surface characteristics or interfering with drug encapsulation efficiency and drug release kinetics. The latter was accomplished by the integration of phospholipids, modified with the chelator diethyleneetriaminepentacacetate (DTPA), in the PEGylated lipid monolayer that surrounds the docetaxel loaded PLGA core. By employing the A10 RNA aptamer as a targeting ligand, ChemoRad NPs demonstrated selective delivery of docetaxel and yttrium90 (90Y) to prostate-specific membrane antigen (PSMA) overexpressing
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prostate cancer cells and showed synergistic cell killing when compared with targeted monotherapy (Raemdonck et al., 2014; Wang et al., 2010).

Clawson et al. (2011) synthesized a PEGylated lipid with a pH-sensitive succinate merging the dipalmitoyl phospholipid and the PEG2000 moiety. Lipid–polymer nanohybrids were constructed with a PLGA core and a fusogenic lipid monolayer consisting of DOPE and oleic acid. The interspersed PEGylated lipids ensure steric stabilization of the NPs at neutral pH, yet drive lipid fusion and NP aggregation upon succinate hydrolysis and PEG shedding at acidic pH. The NP destabilization as a function of pH and incubation time can be tuned by adjusting the molar ratio of pH-sensitive PEGylated lipids incorporated in the lipid monolayer. It is anticipated that this pH-dependent behavior would be of particular interest in cancer drug delivery by taking advantage of the acidified tumor interstitium (Danhier et al., 2010). Selective removal of the stabilizing PEG layer in the tumor microenvironment can thus induce fusion with the tumor cell membrane. In addition, gradual acidification of endosomal compartments following endocytosis of PEGylated NPs in tumor cells can trigger lipid fusion with the limiting membrane of late endosomes or lysosomes. Both mechanisms could greatly enhance cytosolic drug delivery (Clawson et al., 2011).

1.3.3.2. Polymer-polymer nanocomposites

Various polymers such as PLGA, PLA, PGA, PAMAM dendrimers and PEI etc. have been used for drug/gene/siRNA delivery in tumors. However, each bears certain pros and cons and none is free from limitations. Despite the favorable biocompatibility and biodegradability of PLGA, PLA and PGA nanoparticles, a major problem encountered with the use of these colloidal carriers is that the particles are amenable to rapid clearance from circulation by macrophages of the MPS immediately after their injection into the bloodstream. To circumvent this problem, the PLGA, PLA and PGA based polymers are conjugated to another polymer PEG and these hybrid nanoparticulate conjugates are being extensively investigated for drug delivery and relevant applications (González-Aramundiz et al., 2012; Hu et al., 2013; Wang et al., 2014b; Chun, 2002). Surface modification with PEG enables the nanoparticles to evade the MPS attack, with a concomitant increase in their plasma half-life, and in turn the area under the curve (AUC) concentration time. Moreover, cationic polymers like dendrimers and PEI bear toxicities and hence they are conjugated to PEG, PLGA or both which facilitates reduction in their toxicities (Intra and Salem, 2010).
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Here, several pre-clinical, clinical trials and some in vitro studies where polymer-polymer nanocomposites have been exploited for cancer treatment are discussed. Cisplatin-loaded PLGA-mPEG was prepared by Grypa et al. (2007). These investigators tested the dose tolerance of blank and cisplatin-loaded (2% w/w) PLGA-mPEG nanoparticles on BALB/c mice and assessed the in vivo anticancer activity on SCID mice xenografted with colorectal adenocarcinoma. They found that both blank and cisplatin-loaded, PEGylated nanoparticles could be administered in high doses (10 mg/kg) in normal BALB/c mice. HT 29 tumor-bearing SCID mice treated with cisplatin-loaded PLGA-mPEG nanoparticles showed higher survival rate and delayed tumor growth compared with animals treated with the free drug. In another study, in vitro degradation of the PLGA-mPEG nanoparticles in phosphate-buffered saline (pH 7.4) was found to increase the mPEG content (i.e., the mPEG:PLGA ratio) of the nanoparticles. As expected, PEGylation was found to prolong the circulation time of PLGA nanoparticles in BALB/c mice (Jain et al., 2011). Senthilkumar et al. (2008) investigated the potential of PEGylated PLGA nanoparticles for the delivery of docetaxel to solid tumors. PEGylated, docetaxel-loaded PLGA nanoparticles enhanced the biological half-life of the drug while imparting considerable solid tumor accumulation. The success of PEGylated PLGA as a long-circulating drug carrier has prompted researchers to exploit this polymer for the targeted delivery of a wide range of anticancer drugs. Moreover, a PLGA-b-methoxyPEG NP encapsulating paclitaxel, has received regulatory approval in South Korea for clinical use and is currently undergoing phase II clinical trials for a number of cancer indications in the United States.

Interestingly, another polymer polyglutamic acid (PGA) has been conjugated to PEG and PEG-PGA conjugate has been proposed as anti-cancer drug delivery vehicle when presented in the form of micelles or polymer conjugate (González-Aramundiz et al., 2012; Chun, 2002). The success of this formulation is exemplified by the fact that PGA-PEG micelles, are under clinical development (Xyotax®/Opaxio® and NC-6004) (Song et al., 2012; Singer, 2005; Plummer et al., 2011). In the case of micelles, the PEGylation of PGA has been reported to positively influence its inherent shielding properties (Bae and Kataoka, 2009). Very recently, Lollo et al. 2014 have reported polyglutamic acid-PEG nanocapsules for the delivery of docetaxel. PGA-PEG nanocapsules were loaded with docetaxel and their antitumor efficacy was
evaluated in a xenograft U87MG glioma mouse model. The results showed that the survival rate for mice treated with docetaxel-loaded nanocapsules was significantly increased over the control Taxotere®, while the antitumoral effect of both formulations was comparable (60% tumor growth inhibition with respect to the untreated mice).

Cationic polymer and PEG conjugated nanocomposites have also been reported for various small molecule anticancer drugs and siRNA/DNA. Recently, PEG-appended dendrimer conjugate with α-cyclodextrin has been reported as novel cancer cell-selective siRNA delivery carrier (Arima et al., 2012). The siRNA complex with PEG-dendrimer conjugate tended to show the in vivo RNAi effects after intratumoral injection and intravenous injection in tumor cell-bearing mice. The FITC-labeled siRNA were found to actually accumulate in tumor tissues after intravenous injection in the mice. In another very recent study, PEGylated PAMAM dendrimer-doxorubicin conjugate-hybridized gold nanorods have been reported for combined photothermal-chemotherapy (Li et al., 2014). In this study, PEG-attached PAMAM G4 dendrimers (PEG-PAMAM) were first covalently linked on the surface of mercaptohexadecanoic acid-functionalized gold nanorod (AuNR), with subsequent conjugation of anti-cancer drug doxorubicin (DXR) to dendrimer layer using an acid-labile-hydrazone linkage. In vitro drug release studies demonstrated that DXR released from PEG-DXR-PAMAM-AuNR was negligible under normal physiological pH, but it was enhanced significantly at a weak acidic pH value and hence facilitating drug release in acidic lysosomes. Furthermore, the combined photothermal-chemo treatment of cancer cells using PEG-DXR-PAMAM-AuNR for synergistic hyperthermia ablation and chemotherapy was demonstrated both in vitro and in vivo to exhibit higher therapeutic efficacy than either single treatment alone, underscoring the great potential of PEG-DXR-PAMAM-AuNR particles for cancer therapy.

Another cationic polymer PEI has been conjugated with PEG and PLGA time and again for delivery of anionic nucleic acids (DNA or siRNA). Chumakova et al., 2008 have developed PLGA/PEI/DNA nanoparticles for improved ultrasound-mediated gene delivery in solid tumors in vivo. It was found that at least an 8-fold increase of the cell transfection efficacy was obtained in irradiated tumors compared to non-irradiated controls, while little to no cell death was produced by ultrasonication. Similarly, Su et al. coated paclitaxel loaded PLGA NPs with cationic PEI enabling it
to carry stat3 siRNA through electrostatic interactions. They found that silencing stat3 by siRNA made cancer cells more sensitive to paclitaxel. Huang et al., 2010 demonstrated successful in vitro transfection of human gastric cancer SGC7901 cell line with (PEI-PEG)/siRNA nanocomposites and the subsequent down-regulation of gene expression of the adherence factor CD44v6. PEI-PEG based nanoparticles have also been reported for the co-delivery of DNA and doxorubicin to tumor cells (Liu et al., 2013).

Altogether, as judged by the emerging research output on lipid–polymer and polymer–polymer nanocomposites, these hybrid nanocarriers evoked much enthusiasm among the drug delivery community. With further research and clinical assessment, we are confident that these multifaceted drug delivery systems could have broad clinical impact.

1.4. CANCER TARGETING
Targeted delivery of anticancer drugs, which provides therapeutic concentrations of anticancer agents at the desired sites of actions and spare normal tissues, promises reduced systemic toxicity and enhanced therapeutic efficacy (Allen, 2002; Marcucci et al., 2004; Ashley et al., 2011). There are a wide range of strategies available for drug delivery in cancer therapy, among which systemic delivery using nanoscale drug carriers (e.g., liposomes, polymeric nanoparticles or nanocomposites) has been demonstrated to be efficacious (Peer et al., 2007; Torchilin, 2005; Duncan, 2006; Sutton et al., 2007). Having a passive and/or active targeting mechanism, these nanoscale drug carriers are able to selectively target cancer sites where they locally deliver the incorporated drugs.

1.4.1. Targeting Mechanisms
There are two main mechanisms through which nanoscale drug carriers achieve tumor targeting, namely passive targeting and active targeting. Passive targeting is based on the prolonged circulation time due to the hydrophilic outer shell thus avoiding phagocytic and renal clearance, and selective tumor accumulation via EPR effect (Iyer et al., 2006). Active targeting builds on passive targeting and adds specific interaction of the targeting ligands on delivery systems with the receptors of cancer cells/tissues (Allen, 2002). Characteristics of an ideal tumor-targeted nanomedicine are listed in Table 1.1.
Table 1.1. Characteristics of an ideal tumor-targeted nanomedicine

(1) Increase drug localisation in the tumour through:
   (a) Passive targeting
   (b) Active targeting
(2) Decrease drug localisation in sensitive, non-target tissues
(3) Ensure minimal drug leakage during transit to target
(4) Protect the drug from degradation and from premature clearance
(5) Retain the drug at the target site for the desired period of time
(6) Facilitate cellular uptake and intracellular trafficking
(7) Biocompatible and biodegradable

Note that not all characteristics apply to all types of nanomedicines.

1.4.1.1. Passive Tissue Targeting

It has been demonstrated that vascular endothelium increases its permeability in tumor sites, have a leaky vasculature when compared to the normal (Yuan et al., 1995; Jain, 1987). Therefore, relatively large macromolecular drugs or nanoscale drug carriers with size ranging from 100 to 780 nm (Wahl et al., 1991; Bremer et al., 2005; Montet et al., 2007), can extravasate and accumulate inside the interstitial space. In contrast to normal tissues, cancer tissues do not have a well-defined functioning lymphatic network, which prevents the penetrated particles from being cleared rapidly and promotes their accumulation (Jain, 1987). The ability of nanoscale particles to accumulate selectively and be retained for a prolonged period in cancer tissues has been termed as the “enhanced permeability and retention” (EPR) effect (Iyer et al., 2006; Jain 1987) (Figure 1.3). Interestingly, the EPR effect does not apply to free drugs with low molecule weights due to their rapid diffusion back into the circulating blood and being cleared from the circulation by renal filtration. Therefore, the EPR effect is a key mechanism for selective solid tumor targeting of nanoscale drug carriers and considered a basis for novel drug carrier design (Peer et al., 2007; Duncan, 2006).
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To achieve selective tissue accumulation via the EPR effect, nanoscale drug carriers should demonstrate the ability to circulate long in the bloodstream to provide a sufficient level of target accumulation. The ability to bypass the recognition of the reticuloendothelial system (RES) is crucial for achieving prolonged circulation time in blood compartments. The RES or mononuclear phagocyte system (MPS), which is a class of cells, including monocytes and macrophages, are responsible for engulfing and clearing old cells, miscellaneous cellular debris, foreign substances, and pathogens from the bloodstream. Non-biocompatible foreign substances are recognized by the RES via the complement activation followed by elimination from the circulation. For colloidal nanoparticles, proteins will often absorb to the surface of the nanoparticles within only the first few minutes of its exposure, especially if the material is charged or hydrophobic. Surface adsorption of opsonins will enhance RES clearance of the nanoparticles from the system preventing the vehicle from fulfilling its role. The recognition of nanoparticles by RES is largely determined by their physical and biochemical properties such as particle sizes and surface interaction with blood components (Moghimi et al., 2001; Storm et al., 1995; Mosqueira et al., 2001; Owens and Peppas, 2006). Nanoparticles with size less than 200 nm are demonstrated to be less susceptible to RES clearance (Ishida et al., 1999). Furthermore, the presence of a biocompatible and hydrophilic corona, such as PEG corona, will sterically stabilize the nanoparticles by creating entropic and osmotic forces which outweigh the protein-surface attractive force such as Van der Waals or hydrophobic interactions, thereby resisting protein adsorption and reducing RES uptake and prolonging blood circulation of nanoparticles (Storm et al., 1995; Mosqueira et al., 2001; Owens and Peppas, 2006; Vlerken et al., 2007; Litzinger et al., 1994). Prolonged circulation increases the probability that nanoparticles reach the tumor leaky vasculature, where, mediated by the EPR effect, nanoscale drug carriers accumulate selectively allowing for significantly elevated drug concentration. The process has been described as "passive targeting" (Peer et al., 2007; Moghimi et al., 2001). Passive targeting is a prerequisite for the specific binding of drug carriers to a localized recognition moiety (i.e., active targeting).
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Figure 1.3. Overview of the drug targeting strategies. (A) Upon the intravenous injection of a low-molecular-weight (chemo)therapeutic agent, which is often rapidly cleared from blood, only low levels of the drug accumulate in tumors and in tumor cells, whereas their localisation to certain healthy organs and tissues can be relatively high. (B) Upon the implementation of a passively targeted drug delivery system, by virtue of the EPR effect, the accumulation of the active agent in tumors and in tumor cells can be increased substantially. (C) Active drug targeting to internalization-prone cell surface receptors (over)expressed by cancer cells generally intends to improve the cellular uptake of the nanomedicine systems, and can be particularly useful for the intracellular delivery of macromolecular drugs, such as DNA, siRNA and proteins. (Adapted from Lammers et al., 2008).

1.4.1.2. Active Cellular Targeting

Actively targeted delivery vehicles are the second generation of delivery vehicles for intravenous administration. The first comprises nanoscale drug carriers that are not derivatized with a targeting ligand. Non-targeted nanoparticle formulations demonstrate selective tumor targeting as a consequence of passive targeting but do not interact with cancer cells directly. By linking targeting ligands to the surface of the long-circulating nanoparticles, specific binding with receptor-expressing cancer cells can be designed to enhance the therapeutic activity of anticancer drugs (Allen, 2002; Peer et al., 2007; Sutton et al., 2007; Brannon-Peppas and Blanchette, 2004; Marcucci and Lefoulon, 2004). The rationale for the specific binding of ligand-nanoparticle with receptor-cancer cell targeting is based on i) the overexpression of specific antigenic receptors on the surface of cancer cells relative to cells in normal tissues; ii) the extreme specificity and high binding affinity of targeting ligands to receptors. Ligand-nanoparticles promise intracellular drug delivery via receptor-mediated endocytosis (Torchilin, 2005; Duncan, 2006; Kirpotin et al., 2006).
Review of Literature

Receptor-mediated endocytosis is the internalization of ligand receptor complexes upon the binding of ligand-nanoparticles with surface receptors. The internalized nanoparticles end up in small vesicles of endosomes which then undergo a rapid maturation to late endosomes and fuse with each other or lysosomes. Receptor mediated endocytosis allows for an increase of the intracellular concentration of drug carriers up to 1000-fold. By incorporating stimuli-response mechanism in drug carrier design where the physico-chemical properties response to the intracellular microenvironments (e.g., the acidic pH value or enzymes in endosomes/lysosomes), anticancer drugs can be released intracellularly for targeting to specific organelles (Allen, 2002; Oh et al., 2007; Twaites et al., 2005; Ulbrich et al., 2004; Ashley et al., 2011). For those DNA-interacting drugs such as doxorubicin and paclitaxel, the therapeutic efficacy can be increased dramatically due to receptor-mediated cellular uptake and intracellular drug release (Ulbrich et al., 2004; Yoo and Park, 2004; Dhankar et al., 2011; Huang et al., 2007).

The ligands that are employed to derivatize nanoparticles for active drug targeting include a broad class of synthetic and natural compounds of different chemical classes. Targeting agents can be broadly classified as proteins (e.g., antibodies and their fragments), receptor ligands (e.g., peptides, vitamins, and carbohydrates), and nucleic acids (aptamers). The active targeting strategy exploiting different tumor-specific ligands has been summarized in Figure 1.4. Antibodies and their antigen-binding fragments have dominated the “targeting” in targeted drug delivery systems over the past several decades (Firer and Gellerman, 2012; Schrama et al., 2006; Carter, 2006). Relative to antigen-binding fragments, whole antibodies offer the advantage of high binding activity due to the presence of more than one binding domain on the same molecules. Moreover, whole antibodies, having an Fc domain, can potentially trigger complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) leading to apoptosis and cell death (Firer and Gellerman, 2012; Sapra and Allen, 2003; Peer et al., 2007) which brings additional cytotoxicity benefit to antibody mediated drug delivery systems.
Figure 1.4. Ligands for active targeting in cancer (PSMA, ASGP, Her 2 and LH-RH stand for prostate specific membrane antigen, asialoglycoprotein, human epidermal growth factor receptor 2 and luteinizing hormone-releasing hormone respectively). (Adapted from Jain et al., 2011)
Targeted delivery of doxorubicin to breast cancer cells using anti-Her2 mAb functionalized dextran-doxorubicin nanocomplex encapsulated immuno-stealth liposomes
Chapter 2: Introduction

2.1. Introduction

Chemotherapy is a major therapeutic approach for the treatment of localized and metastasized cancers (Bisht and Maltra, 2009). Whereas potent chemotherapeutic agents seem promising in the test tube, clinical trials often fail due to unfavourable pharmacokinetics, poor delivery, low local concentrations, and limited accumulation in the target cell (Beardsley, 1994; Jang et al., 2003). The patho-physiology of the tumor vasculature and stromal compartment presents a major obstacle to effective delivery of agents to solid tumors. Poor perfusion of the tumor, arterio-venous shunting, necrotic and hypoxic areas, as well as a high interstitial fluid pressure work against favourable drug uptake. Thus, targeted drug delivery using long-circulating particulate drug carriers holds immense potential to improve the treatment of cancer by selectively providing therapeutically effective drug concentrations at the tumor site while reducing undesirable side effects (Yousefpour et al., 2011b). These targeted nanosystems can deliver drugs in a passive or active way. Passive targeted drug delivery takes advantage of the poor lymphatic systems of tumor tissues and their leaky vasculature with pore sizes ranging from 100 to 780 nm (Wahl et al., 1991; Bremer et al., 2005; Montet et al., 2007). These characteristics enable what is called the “enhanced permeability and retention” effect, which allows enhanced deposition of delivery nanovehicles at the site of a solid tumor. Active targeted drug delivery on the other hand is achieved via covalent conjugation of targeting molecules on the nanoparticle surface which can recognize and bind to specific ligands expressed specifically in cancer cells. One such ligand is human epidermal growth factor receptor 2 (Her2), the expression of which is amplified in about 30% of breast cancers and 20% of ovarian cancers, and this receptor is expressed weakly in normal adult tissues (Shukla et al., 2006; Olayioye, 2001; Slamon et al. 1989).

Trastuzumab (Herceptin®) is a humanized monoclonal antibody directed against the Her2 receptor, and is the only Her2-targeted therapy approved by FDA for the treatment of advanced breast cancer. Combination of trastuzumab with conventional chemotherapy leads to increased response rates in comparison with trastuzumab alone (Pegram et al., 2004; Montemurro et al., 2004; Merlin et al., 2002). In addition, according to some clinical trials, anthracycline-based chemotherapy demonstrated more successful results in Her2+ women (Ferretti et al., 2007). However, trastuzumab has been shown to aggravate anthracycline-induced cardiotoxicity, and
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thus cannot be given concomitantly with anthracyclines, including doxorubicin (Xu et al., 2004; Rayson et al., 2008). Conjugation of trastuzumab to doxorubicin-carrying nanoparticles allows transport of the chemotherapeutic agent specifically to tumor cells and reduces their adverse cardiotoxic effects. Furthermore, in such nanoparticulate formulations, trastuzumab is intended to act as a targeting ligand rather than as a therapeutic agent and thus its concentration is far below its therapeutic dose. Previous studies have shown promising results for either cancer therapy or imaging via trastuzumab decoration of such nanoparticles as dextran iron oxide nanoparticles (Chen et al., 2009), poly(lactide-co-glycolide)/montmorillonite nanoparticles (Sun et al., 2008), poly(-lactic acid) nanoparticles (Cirstoiu-Hapca et al., 2007), and human serum albumin nanoparticles (Anhorn et al., 2008; Steinhauser et al., 2006; Steinhauser et al., 2008).

Doxorubicin (DXR) is widely used in chemotherapy due to its efficacy in fighting a broad range of cancers (Qi et al., 2010). However, its therapeutic potential is restricted by its dose limited toxicity which often results in narrow therapeutic index owing to high levels of toxicity to healthy tissues. Several groups have conjugated/complexed DXR to macromolecules such as N-(2-hydroxypropyl) methacrylamide (HPMA), pyran, dextran etc. for reducing its toxicity while maintaining the therapeutic efficacy (Mitra et al., 2001). Dextran (DEX), a macromolecular drug carrier for chemotherapeutics, has several advantages owing to its non-digestible and inert nature. Yousefpour et al. (Yousefpour et al., 2011b) have reported development of DEX-DXR nanocomplex exploiting the electrostatic interaction between cationic DXR and anionic DEX. Although the lower molecular weight conjugate/complex of DEX bears advantage of reduced side effects, its therapeutic potential is not optimum because of its relatively short plasma half-life. The possibility of improving the plasma half-life of the drug-polymer conjugate/complex can be achieved through its encapsulation in long circulating liposomes. The development of stable and long-circulating liposomes requires their sterical stabilization for rendering them with ‘stealth property’ by coating polymeric PEG on their surface which avoids reticulo-endothelial recognition and subsequent elimination resulting in substantially prolonged drug circulation.

Keeping the aforementioned details into consideration, in the present work, we first developed DEX-DXR nanocomplex and encapsulated it in stealth liposomes which
were further surface functionalized with anti-Her2 monoclonal antibody. A schematic representation of the sequence of events in the synthesis of anti-Her2 mAb conjugated DEX-DXR nanocomplex encapsulated stealth liposome based nanocomposites (Ab@DEX-DXR-SL NCs) is depicted in Figure 2.1. The DEX-DXR nanocomplex was characterized by UV-Vis, fluorescence and FTIR spectroscopy and SEM analysis. The encapsulation of DEX-DXR nanocomplex into stealth liposomes was ascertained by transmission electron microscopy (TEM). Once the conjugation of anti-Her2 mAb on the surface DEX-DXR nanocomplex loaded stealth liposomes was ascertained, we evaluated the cytotoxic potential of as-synthesized Ab@DEX-DXR-SL NCs on SKBR3 breast cancer cell line since breast cancer cells overexpress Her2 receptors. The regulation of anti-/pro-apoptotic protein expression in cancer cells was also used as a parameter to establish efficacy of Ab@DEX-DXR-SL NCs. Our study also sheds light on the mechanism of apoptosis induction by Ab@DEX-DXR-SL NCs.

Figure 2.1. Schematic representation of the sequence of events in the synthesis of DEX-DXR nanocomplex loaded immuno-stealth liposomal nanocomposite
2.2. Materials and methods

2.2.1. Materials

SKBR3 cancer cell line was purchased from NCCS, Pune, India. Soybean phosphatidylcholine ($\text{S}_{100}$PC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine [methoxy(polyethylene glycol)-2000] (mPEG$_{2000}$-DSPE) and maleimide derivatized PEG$_{2000}$-DSPE (Mal-PEG$_{2000}$-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (CHOL), doxorubicin HCl, dextran, Rhodamine 123, NAC, MTT, RPMI 1640, PI, FCS, rabbit anti-mouse FITC labelled 2° antibody, 2-iminothiolane (Traut's reagent) and BCA protein assay kit were bought from Sigma Aldrich (St. Louis, MO, USA). Anti-Her2 mouse monoclonal antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Anti-p53 wt, anti-p53 mut, antibax, anti-Bcl2, anti-β-actin antibodies, non-fat dry milk and BrdU TUNEL Assay kit were purchased from BD Biosciences (San Diego, CA). PVDF membrane was purchased from Millipore India Pvt. Ltd. (Bangalore, India). All other reagents used were of analytical grade and procured from local suppliers.

2.2.2. Preparation of DEX-DXR nanocomplexes

DEX-DXR nanocomplex was prepared by following the protocol published elsewhere (Yousefpour et al., 2011a). Briefly, DXR HCl was dissolved in deionized water at a concentration of 0.05 mg/mL. To this solution, an equal volume of DEX solution (prepared in deionized water) was added to give final DXR-DEX solutions of 0.5 w/w. Solutions were left for 45 min in the dark while stirring. Nanocomplexes were collected by pelleting down the solution at 20,000 rpm which were further lyophilized and stored at 4°C. The synthesis of the nanocomplexes was ascertained by analyzing the UV-Vis, fluorescence and FTIR spectra of the DXR-DEX nanoassembly. The size and morphology of the DXR-DEX nanocomplexes were further measured by SEM. DXR has been reported to retain its anti-tumor activity even in nanocomplex form (Yousefpour et al., 2011a).

2.2.3. UV-Vis and fluorescence spectroscopic studies of DEX-DXR nanocomplexes

DXR solution was mixed with DEX in a ratio 1:2 and the absorbance of the resultant nano-assembly was measured at 480 nm using Perkin Elmer UV/VIS spectrometer model lambda 25 in a 1 cm path length cuvette at room temperature (Yousefpour et
Chapter 2: Materials and methods

al., 2011a). The fluorescence measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer at room temperature using a cell with a 1 cm light path. The excitation wavelength was set at 470 nm and the spectra were recorded between 500-700 nm (Yousefpour et al., 2011a). The slit length was kept at 5 nm to reduce the intensity of the signal depending on the experiment.

2.2.4. SEM analysis of DEX-DXR nanocomplex

SEM samples were prepared following protocol as published elsewhere (Farazuddin et al., 2012). Briefly, the synthesized nanocomplex was lyophilized and the vacuum dried powder was mounted on clear glass stub followed by coating with gold-palladium alloy and finally observed by SEM (JEOL, Japan) in high-vacuum mode at 20 kV.

2.2.5. Preparation of DEX-DXR nanocomplex encapsulated stealth-liposomes

Self-assembled DEX-DXR nanocomplexes were further co-entrapped in PEGylated liposomes using a freeze-thaw method (Yang et al., 2007). Briefly, PEGylated liposomes, composed of S100PC:CHOL:mPEG2000-DSPE (90:10:5 M% ratio), were prepared using the thin-film hydration method as described elsewhere (Yang et al., 2007). DEX-DXR nanoassemblies were then mixed with empty PEGylated liposomes, and subjected to several cycles of freeze and thaw. The suspension was then subjected to sonication for 5 min, in a water bath sonicator. Then centrifugation was carried out at 5,000×g for 5 min to separate the DEX-DXR nanocomplex encapsulated liposomes from empty liposomes.

2.2.6. TEM analysis of DEX-DXR nanocomplex encapsulated stealth-liposomes

DEX-DXR nanocomplex encapsulated stealth-liposome was characterized by TEM studies. TEM samples were prepared following the protocol as published elsewhere (Farazuddin et al., 2012) by placing a drop of synthesized nanoparticles over gold coated negative grid followed by evaporation of the solvent. TEM analysis was performed on JEOL model (1200 EX, JOEL Inc, Peabody, MA) which was operated at an accelerating voltage of 200 kV was used for imaging.

2.2.7. Preparation of DEX-DXR nanocomplex encapsulated immuno-stealth-liposome by covalent conjugation of anti-Her2 monoclonal antibody

Anti-Her2 mAb was first thiolated following the literature procedure with slight modification (Yang et al., 2007). Briefly, anti-Her2 mAb (10 mg/mL) was thiolated
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with Traut’s reagent at a ratio of 20:1 (mol/mol) in HEPES buffered saline (HBS, 25 mM HEPES, 140 mM NaCl, pH 8.0) for 1 hr. The thiolated anti-Her2 mAb was conjugated with Mal-PEG\textsubscript{2000}-DSPE by incubating overnight at 4°C with Mal-PEG\textsubscript{2000}-DSPE in HBS (pH7.4). The amounts of conjugated and free mAb were indirectly measured by BCA protein assay. The PEGylated immunoliposome, composed of S\textsubscript{100}PC, CHOL, mPEG\textsubscript{2000}-DSPE, Mal-PEG\textsubscript{2000}-DSPE at a molar ratio of 90:10:4:1, was prepared by overnight incubation of the mAb-PEG\textsubscript{2000}-DSPE with preformed DEX-DXR nanocomplex entrapped PEGylated liposome at 4 °C for 24 hrs.

2.2.8. Determination of anti-Her2 mAb binding on the surface of nanocomposites

The as-synthesized immuno-nanocomposites were characterized for antibody conjugation employing protein estimation using BCA Protein Assay Kit with BSA as protein standard and detection by FITC labelled anti-mouse 2′ antibody. To 50 μl of DEX-DXR nanocomplex encapsulated immuno-stealth-liposome solution, FITC tagged anti-mouse 2′ antibody was added in the ratio 1:2500 and incubated for 30 min. The incubated solution (10 μl) was dropped on a glass slide to be analysed under fluorescence microscope (Zeiss model, magnification 100X).

2.2.9. Cell targeting and internalization of immuno-nanocomposite

Cellular targeting and uptake of immuno-nanocomposites were investigated with a SKBR3 breast cancer cell line mono-culture over-expressing Her2 receptor. Samples (100 μl) containing 0.5 mg nanocomposites/ml of growth medium were added to the cells and incubated at 37°C for different time periods up to 24 hrs. Nanocomposite exposed SKBR3 cells were further incubated with FITC labelled anti-mouse 2′ antibody in the ratio 2500:1 of culture medium. Fluorescence microscopy was then performed using Zeiss inverted fluorescence microscope equipped with FITC filter set.

2.2.10. In vitro cell viability

The in vitro cytotoxicity of the various doxorubicin formulations was tested on SKBR3 cells using the MTT test following the published procedure with slight modification (Siddiqi et al., 2013). SKBR3 cells were seeded in 96-well plates (Costar, Chicago, IL) at the density of 1 × 10\textsuperscript{4} viable cells/well and incubated for 24 hrs to allow cell attachment. The medium was replaced by 100 μl of the formulation
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at concentrations of 1–200 nM for 48 hrs. The diluent for preparing the working solution for various formulations of DEX-DXR nanocomposites was RPMI-1640 culture medium. At designated time intervals, 20 μL MTT (5 mg/mL in phosphate-buffered saline) was added to each well, and the culture medium containing MTT solution was removed after 3–4 hrs. The formazan crystals were dissolved in 150 μl dimethyl sulfoxide and read at 570 nm by a microplate reader. Cell viability was calculated using the following equation:

\[
\text{Cell viability (\%)} = (\text{Int}_s/\text{Int}_{\text{control}}) \times 100
\]

where \(\text{Int}_s\) is the colorimetric intensity of cells incubated with the samples, and \(\text{Int}_{\text{control}}\) is the colorimetric intensity of cells incubated with the phosphate-buffered saline only (positive control).

2.2.11. Effect of DEX-DXR nanocomplex encapsulated immuno-stealth liposome on tumor cells analysed by fluorescence microscopy

The SKBR3 cell line was maintained in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum. The cells were plated at a density of \(1 \times 10^4\) cells on glass coverslips, and cultured for 24 hrs at 37°C. The cells were subsequently exposed to various DEX-DXR formulations. The plates were incubated for 18 hrs. The cells were washed with sterile HBSS and stained with Propidium iodide (50 μg/ml) for 30 min at 37°C. Cells were fixed by 2% paraformaldehyde for 2 hrs followed by washing with HBSS. The fixed cells were observed under fluorescence microscope.

2.2.12. Determination of caspase-9 level by fluorescence microscopy

The cells treated with various formulations were permeabilized with 0.1% Triton X100 in PBS at RT for 10 min followed by fixation in 4% paraformaldehyde for 2 hrs at room temperature, blocked with 2% FCS, stained with mouse monoclonal antibody which detects only 35 Kda cleaved caspase 9 and revealed with rabbit anti-mouse FITC tagged 2' antibody. Microscopy was performed on Ziess fluorescence microscopy (100X magnification).

2.2.13. Assay of mitochondrial membrane potential

Mitochondrial membrane potential was measured following the published procedures (Zhang et al., 2011b; Siddiqui et al., 2013). In brief, control and treated cells were
harvested and washed twice with PBS. Cells were further exposed with 10 mg/ml of Rhodamine-123 fluorescent dye for 1 hr at 37°C in dark. Again cells were washed twice with PBS. Then, fluorescence intensity of Rhodamine-123 was measured using upright fluorescence microscope (Zeiss) by capturing the images at 20X magnification.

2.2.14. Annexin V assay for apoptosis using fluorescence microscopy
In general, apoptosis is manifested by certain morphological features, including loss of plasma membrane asymmetry, condensation of the cytoplasm as well as nucleus, and intranucleosomal cleavage of DNA. The altered phospholipid distribution in the two leaflets of plasma membrane is analyzed with the help of Annexin V staining kit following the manufactures instructions. Briefly, cells treated with various DEX-DXR formulations were harvested after trysinization. Cells were further incubated with FITC Annexin V for 30 min at RT and visualized under the fluorescence microscope (100 X magnifications) after washing with PBS.

2.2.15. Effect of DEX-DXR nanocomplex loaded immuno-stealth-liposome on DNA fragmentation as analyzed by Apo-BRDU study (TUNEL Assay)
TUNEL assay was performed following the published procedure (Chauhan et al., 2014). Cells were harvested after tryspinization and pelleted at 2000 g for 10 min at 4°C from various experimental groups. Cells were re-suspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4). Cells were placed on ice for 30 min followed by centrifugation for 5 min at 300g and supernatant was discarded. The cell pellet was resuspended in 70% (v/v) ice-cold ethanol followed by washing. After keeping for 30 min in 70% ethanol, cells were washed 3 times and pellet was resuspended in 50 µl of DNA labelling solution (Reaction buffer+TdT enzyme and Br-dUTP). Cells were incubated in the DNA labelling solution for 60 min at 37°C in a temperature controlled water bath. Cell pellet was incubated with the FITC-labelled anti-BrdU antibody staining solution in the dark for 30 min at room temperature and visualized under the fluorescence microscope. For flow-cytometric analysis, after labelling the tumor cells with FITC-tagged anti-BrdU antibody, PI/RNase (50 µg/ml) was added to the cells which were analyzed by FACS (GUAVA, Millipore, MA, USA) followed by incubation for 30 min at 37°C.
2.2.16. Western blot analysis

Western Blotting was done to detect various pro and anti apoptotic factor such as p53 wild type, p53 mutant, Bax and Bcl2 expressed in the cell lines (Farazuddin et al., 2012). Cells were lysed after 48 hrs of exposure with various formulations as described earlier. Protein estimation was done using a BCA kit (Sigma, India) using BSA as standard. Equal amount of sample protein (30 µg) was resolved on 10% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was blocked by 5% non-fat dry milk (Bio-rad, USA.) followed by incubation with rabbit anti-p53 wild type antibody, rabbit anti-p53 mutant antibody, rabbit anti-bax antibody and rabbit anti-Bcl2 antibody. To quantify equal loading, membranes were probed with β-actin antibody, and rabbit anti-β-actin antibody (BD Pharmingen) was used correspondingly as the secondary antibody. The bands were detected using the ECL (Enhanced chemiluminescence) detection system. The intensity of the bands was quantified using Alpha Image Analysis software on Alpha Image Gel Documentation System.

2.2.17. Measurement of intracellular reactive oxygen species generation

The production of intracellular ROS was measured using 2,7- dichlorofluorescin diacetate (DCFH-DA) as described elsewhere with some modifications (Siddiqui et al., 2013). The DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). In brief, SKBR3 cells (5x10⁴) were seeded in 6-well plates and allowed for adherence. Following respective exposure, cells were washed twice with PBS and incubated for 30 min in dark in culture medium (without FBS) containing DCFH-DA (20 mM). The control and treated cells were visualized by use of a fluorescence microscope (Zeiss) by capturing the images at 20X magnification.

2.2.18. Statistical Analysis

Data were analyzed and two groups were compared with the Student t test, and one way ANOVA (Holm-Sidak method) to assess the differences among various groups, using Sigma-Plot version 10 software. P values <0.05 were considered to be significant.
2.3. Results

2.3.1 Characterization of DEX-DXR nanocomplex and stealth liposomes loaded with the nanocomplex

As shown in Figure 2.2A, DEX-DXR nanocomplex exhibits $\lambda_{\text{max}}$ at 495 nm in contrast to free DXR showing maximum absorption at 480 nm. No shift was observed in the fluorescence emission spectrum, but the intensity was lowered compared with the visible absorption spectrum (Figure 2.2B). FTIR analysis was also taken into consideration for characterizing DEX-DXR nanocomplex. Figure 2.2C reveals the FTIR spectrum of DXR and DEX-DXR nanocomplex. The DEX-DXR complex exhibited additional peaks in the region 3550-3200 cm$^{-1}$ as compared to the peaks in the spectrum of DXR. DEX-DXR spectrum exhibits overlapping peaks to that in DXR spectrum for amide N-H stretching vibrations and amide C=O stretching vibrations in the range 3700-3500 cm$^{-1}$ and 1690-1630 cm$^{-1}$ respectively. The SEM image of lyophilized DEX-DXR nanocomplex showed in Figure 2.2D establishes formation of nanocomplexes in the size range of approx. 100 nm. Figure 2.3 exhibits TEM micrograph clearly revealing the entrapment of the as-synthesized DEX-DXR nanocomplex within the stealth liposomes. The arrow heads in the inset of Figure 2.3 exhibit DEX-DXR nanocomplex encapsulated within the stealth liposomes. The DEX-DXR nanocomplex loaded stealth liposomal nanocomposites (DEX-DXR-SL NCs) are observed to be in the size range of 200-300 nm.

<table>
<thead>
<tr>
<th>Table 2.1. Size of various DXR formulations</th>
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<tr>
<td>Formulation</td>
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<td>DEX-DXR nanocomplex</td>
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<td>DEX-DXR-SL-NC</td>
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Figure 2.2. Characterization of DEX-DXR nanocomplex. A) UV-Vis spectroscopic analysis of DEX-DXR nanocomplex B) Fluorescence spectroscopic analysis of DEX-DXR nanocomplex C) FTIR spectra of dextran-doxorubicin nanoassembly D) SEM of dextran-doxorubicin nanocomplex. Data are representative of at least three independent experiments with similar observations.

2.3.2. Characterization of antibody tagging to DEX-DXR-SL NC and the interaction of antibody conjugated DEX-DXR-SL NCs with the tumor cells

Figure 2.4A shows representative fluorescence microscopic image of DEX-DXR-SL NC coated with anti-Her2 monoclonal antibody (mAb). Ab@DEX-DXR-SL NCs were incubated with anti-mouse FITC tagged 2° antibody. The green fluorescence shown by NCs establishes conjugation of mAb on their surface. The fluorescence localized to the periphery of the tumor cells shown in Figure 2.4B ascertains binding of the antibody tagged DEX-DXR-SL NCs (Ab@DEX-DXR-SL NC) to the surface of the Her2 expressing tumor cells. Figures 2.4C and D reveal internalization of antibody conjugated NCs by tumor cells after 6 hrs and 12 hrs of exposure.
respectively. Figure 2.4D also highlights that after 12 hrs of exposure of NCs, the DXR ensued in killing of the tumor cells.

Figure 2.3. TEM image of DEX-DXR nanocomplex loaded stealth liposome. Arrows represent encapsulation of DEX-DXR nanocomplex in stealth liposomes. Data are representative of at least three independent experiments with similar observations.
Chapter 2: Results

Figure 2.4. Assessment of anti-Her2 mAb conjugation to DEX-DXR-SL NCs and interaction of Ab@DEX-DXR-SL NC with SKBR3 cancer cells. A) Green fluorescence reveals conjugation of anti-Her2 mAb onto the DEX-DXR-SL NC surface. B) Fluorescence localized to the periphery of tumor cells highlights binding of Ab@DEX-DXR-SL NCs to the cancer cells. Interaction of Ab@DEX-DXR-SL NCs with SKBR3 cancer cells after 6 hrs of exposure C) and 12 hrs of exposure D). Data are representative of at least three independent experiments with similar observations.

2.3.3 Cytotoxicity of as-synthesized DEX-DXR-SL NCs on cancer cells

MTT assay was performed to assess the cytotoxicities of various DEX-DXR formulations on cancer cells after exposing them with increasing concentrations of various formulations (0-250ng/ml). Inhibition of cell growth was observed to be in a dose-dependent manner. As shown in Figure 2.5, Ab@DEX-DXR-SL NC formulation exhibited significant cancer cell killing as compared to plain DEX-DXR-SL NC formulation, \( p < 0.05 \).

![Graph depicting cell viability vs concentration of DEX-DXR](image)

Figure 2.5. Cytotoxicity of various DEX-DXR nanocomplex formulations on SKBR3 cancer cell line. Data are mean values ± standard deviation from three different experiments (Ab@DEX-DXR-SL NC vs DEX-DXR-SL NC, \( p < 0.05 \) at 200ng/ml conc.). ‘Sham’ refers to ‘empty’.
2.3.4. Effect of Ab@DEX-DXR-SL NC preparation on the morphology and population of cancer cells as determined by fluorescence microscopy
SKBR3 cells were exposed to various formulations and visualized under fluorescence microscope after propidium iodide staining. Microscopic images clearly reveal the significant decrease in number of cancer cells and alteration in morphological features of treated cells upon exposure of Ab@DEX-DXR-SL NC formulation as compared to control exhibiting healthy cells adhered to the substratum (Figure 2.6, Panel A). Cells exposed to DEX-DXR nanocomplex exhibited a feeble difference in morphology and number of cells as compared to control while cancer cells treated with naked (not conjugated to mAb) showed a marked difference in morphological changes as well as decrease in cancer cell population.

2.3.5. Effect of DEX-DXR encapsulated immuno-stealth liposome on the expression of caspase-9 level as determined by fluorescence microscopy
SKBR3 cells exposed to various DXR formulations (DEX-DXR nanocomplex, DEX-DXR nanocomplex entrapped in stealth liposome and DEX-DXR nanocomplex encapsulated immuno-stealth liposome) were evaluated for the expression of caspase-9 using fluorescence microscopy. Apoptosis induction was quite visible in DEX-DXR nanocomplex treated cells in comparison with untreated groups, where caspase-9 expression was almost negligible (Figure 2.6, Panel B). Apoptosis induction was found to be highest in cells treated with DEX-DXR nanocomplex entrapped immuno-stealth liposomes followed by cells exposed to naked DEX-DXR-SL NC which also induced marked apoptosis in treated cancer cells but the result was not as significant as observed for Ab@DEX-DXR-SL NCs (Figure 2.6, Panel B).

2.3.6. Ab@DEX-DXR-SL NCs decreased the mitochondrial membrane potential
It is well known that during apoptosis the mitochondrial membrane potential (MMP) decreases (Siddiqui et al., 2013). The effect of various DXR-DEX preparations on MMP was evaluated in SKBR3 cells. Cells were exposed to various formulations for 24 h and assayed for Rhodamine 123 uptake using fluorescence microscope. The brightness of the red fluorescence was remarkably reduced in cells exposed to DEX-DXR nanocomplex entrapped in immuno-stealth liposome that indicates a significant reduction of MMP in treated SKBR3 cells as compared to control untreated cells (Figure 2.6, Panel C).
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2.3.7. Annexin V apoptosis using fluorescence microscopy

In general, the apoptosis is manifested by certain morphological features, including loss of plasma membrane asymmetry, condensation of the cytoplasm as well as nucleus, and intra-nucleosomal cleavage of DNA (Chauhan et al., 2014). The altered phospholipid distribution in the two leaflets of plasma membrane is analyzed with the help of annexin V staining. Apoptosis induction was quite visible in DEX-DXR nanocomplex treated cells in comparison with cells belonging to untreated groups where PS level in the outer leaflet level was negligible (Figure 2.7, Panel A). Interestingly, there was marked annexin V binding in SKBR3 cells treated with naked DEX-DXR-SL NCs but was not as significant as observed for SKBR3 cancer cells exposed to Ab@DEX-DXR-SL NC, where annexin V binding was significantly higher than all other groups (Figure 2.7, Panel A).
Figure 2.6. Effect of Ab@DEX-DXR-SL NC preparation on cancer cell viability, induction of caspase-9 and mitochondrial membrane potential of cancer cells. Panel A) Fluorescence microscopic images revealing morphological changes and reduction in cell population after treatment with immune-stealth liposome. Panel B) Involvement of caspase-9 in doxorubicin bearing immuno-stealth liposome mediated
apoptosis of SKBR3 breast cancer cell line as revealed by fluorescence microscopy.

Panel C) DEX-DXR loaded immuno-stealth liposome induced MMP in SKBR3 cells. (a) Untreated control cells. (b) Treated with free DEX-DXR (c) Treated with DEX-DXR loaded stealth liposome. (d) Treated with DEX-DXR immuno-stealth liposome. Data are representative of at least three independent experiments with similar observations.

2.3.8. DNA fragmentation as analyzed by Apo-BrdU TUNEL assay using fluorescence microscopy and flow cytometry

DNA fragmentation during apoptosis results from the activation of endonucleases during the apoptotic program. The nuclease digests the higher order chromatin structure into fragments and subsequently into smaller DNA pieces (Chauhan et al., 2014). Cells were collected after treatment with various DXR formulations and were fixed using a DNA cross-linking chemical fixative (e.g. paraformaldehyde). Efficacy of DEX-DXR nanocomplex loaded immuno-stealth liposome was observed by BrdU TUNEL analysis using fluorescence microscopy as well as FACS. The enzyme TdT catalyzes a template dependent addition of Br-dUTP to 3-hydroxyl ends of double and single stranded DNA. After incorporation, DNA breaks at specific sites and is identified by a FITC-labeled anti-BrdU mAb. As revealed by TUNEL analysis employing fluorescence microscopy and flow cytometry (Figure 2.7, Panels B and C respectively), apoptosis induction was significantly enhanced in SKBR3 cells treated with Ab@DEX-DXR-SL NCs as compared to untreated control cells.
Figure 2.7. Ab@DEX-DXR-SL NC formulation induces marked apoptosis of cancer cells as revealed by annexin V and BrdU TUNEL assay. Panel A) Annexin-V staining of immuno-stealth liposome mediated apoptosis of SKBR3 cancer cells as revealed by fluorescence microscopy. Panels B and C) DNA fragmentations as analyzed by Apo-BrdU study using fluorescence microscopy and
flow cytometry respectively. Data are representative of at least three independent experiments with similar observations.

2.3.9. Impact of Ab@DEX-DXR-SL NCs on apoptosis as revealed by expression of various pro/anti-apoptotic factors

Treatment of SKBR3 breast cancer cells with free DXR resulted in a slight increase in the expression of p53wt (Figure 2.8A, lane 2). Significant upregulation of p53wt was observed when cancer cells were treated with Ab@DEX-DXR-SL NC preparation (Figure 2.8A, lane 5). DEX-DXR nanocomplex and stealth liposome encapsulated DEX-DXR nanocomplex exhibited higher expression of p53wt as compared to free DXR but trailed far behind the level of expression shown by antibody conjugated DEX-DXR-SL NC formulation (Figure 2.8A, lanes 3&4). As depicted in Figure 2.8B, a significantly upregulated expression of p53wt protein was recorded for cancer cells treated with Ab@DEX-DXR-SL NC formulation as compared to the cells exposed to free DXR (p < 0.01), DEX-DXR nanocomplex (p < 0.05) and DEX-DXR-SL NC (p < 0.05). Treatment with Ab@DEX-DXR-SL NC formulation resulted in a marked downregulation of p53mut in comparison to naked DEX-DXR-SL NC (Figure 2.8A, lane 5). As depicted in Figure 2.8B, the level of p53mut was significantly reduced in cells treated with Ab@DEX-DXR-SL NC compared with the control, p < 0.001. Treatment with naked DEX-DXR-SL NC was not as effective in downregulating p53mut compared with antibody conjugated DEX-DXR-SL NC (p < 0.05). Free DXR (p < 0.001) as well as DEX-DXR nanocomplex (p < 0.01) were found to downregulate the expression of p53mut significantly lower than Ab@DEX-DXR-SL NC. The effect of Ab@DEX-DXR-SL NC formulation on the expression of Bax was also recorded. Immunoblot analysis showed upregulated expression of Bax in SKBR3 cancer cells treated with various DXR formulations as compared to control. The level of Bax was found to be significantly upregulated in the cells treated with Ab@DEX-DXR-SL NC as compared to control and other DXR preparations (Figure 2.8A, lane 5). Densitometric analysis (Figure 2.8B) showed significant increase in Bax level in the cells that were treated with Ab@DEX-DXR-SL NC formulation when compared to other DXR formulations (Ab@DEX-DXR-SL NC vs DEX-DXR-SL NC, p < 0.05; Ab@DEX-DXR-SL NC vs DEX-DXR nanocomplex, p < 0.01; Ab@DEX-DXR-SL NC vs free DXR, p < 0.01). Expression of Bcl-2 was also analysed. Cells treated with naked DEX-DXR-SL NC and Ab@DEX-DXR-SL NC
formulations exhibited a marked downregulation of Bcl2 although the decrease in Bcl2 expression was higher for cells exposed to antibody conjugated NC formulation (Figure 2.8A, lanes 4&5). Free DXR and DEX-DXR nanocomplex did not exhibit much downregulation in the expression of Bcl2 (Figure 2.8A, lanes 2&3). As shown in Figure 2.8B, the level of Bcl2 was significantly reduced in cells treated with Ab@DEX-DXR-SL NC compared to those exposed to DEX-DXR-SL NC ($p < 0.05$), DEX-DXR nanocomplex ($p < 0.001$) and free DXR ($p < 0.001$).

Figure 2.8. Anti-Her2 mAb conjugated DEX-DXR-SL NCs cause upregulation of p53 wildtype and Bax and downregulation of p53 mutant and Bcl2 expression.
Chapter 2: Results

(A) Immunoblots showing the effect of Ab@DEX-DXR-SL NC on the expression of p53wt, p53mut, Bax and Bcl2 in SKBR3 breast cancer cells. (B) Densitogram showing relative pixel densities of p53wt, p53mut, Bax and Bcl2 after treatment with various DXR preparations. Results are mean values ± standard deviation in triplicates. (For p53 wt: Ab@DEX-DXR-SL NC vs DEX-DXR-SL NC, p < 0.05; Ab@DEX-DXR-SL NC vs DEX-DXR nanocomplex, p < 0.05; Ab@DEX-DXR-SL NC vs free DXR, p < 0.01. For p53 mut: Ab@DEX-DXR-SL NC vs DEX-DXR-SL NC, p < 0.05; Ab@DEX-DXR-SL NC vs DEX-DXR nanocomplex, p < 0.01; Ab@DEX-DXR-SL NC vs free DXR, p < 0.001. For Bax: Ab@DEX-DXR-SL NC vs DEX-DXR-SL NC, p < 0.05; Ab@DEX-DXR-SL NC vs DEX-DXR nanocomplex, p < 0.01; Ab@DEX-DXR-SL NC vs free DXR, p < 0.01. For Bcl2: Ab@DEX-DXR-SL NC vs DEX-DXR-SL NC, p < 0.05; Ab@DEX-DXR-SL NC vs DEX-DXR nanocomplex, p < 0.001; Ab@DEX-DXR-SL NC vs free DXR, p < 0.001)

2.3.10. Toxicity of DEX-DXR nanocomplex entrapped immuno-nanocomposite was mediated through oxidative stress

We evaluated the effect of various DXR formulations on ROS generation in the presence as well as absence of the anti-oxidant N-acetyl-cystein (NAC). Fluorescent microscopy data revealed that DEX-DXR nanocomplex entrapped immuno-nanocomposite induced the intracellular production of ROS (Figure 2.9). We further observed that co-exposure of NAC effectively prevented the ROS generation induced by DEX-DXR nanocomplex entrapped immuno-stealth liposome (Figure 2.9E). ROS level was reduced up to that of control for Ab@DEX-DXR-SL NC in the presence of NAC.
Figure 2.9. DEX-DXR nanocomplex encapsulated immuno-stealth liposome induced oxidative stress in SKBR3 cells. (A) Untreated control cells (B) ROS level in SKBR3 cells treated with DEX-DXR nanocomplex (C) SKBR3 cells exposed to DEX-DXR nanocomplex loaded stealth liposome (D) Cancer cells exposed to DEX-DXR nanocomplex encapsulated immuno-stealth liposome in the absence of NAC (E) SKBR3 cells treated with DEX-DXR nanocomplex encapsulated immuno-stealth liposome in the presence of NAC. Data are representative of at least three independent experiments with similar observations.
2.4. Discussion

Breast cancer is the fifth most common cause of cancer death and the second leading cause of cancer deaths after lung cancer in women (Wang et al., 2014a; Hutchinson, 2010). Among the therapeutic modalities currently available for breast cancer treatment, chemotherapy has been a primary option for managing advanced-stage breast cancer including metastatic (stage IV) or recurrent breast cancer. The major advantage of the clinical use of chemotherapy over surgery and radiation is its systemic action which kills cancer cells in both primary and metastatic tumors. Still, non-selective bio-distribution and dose-limiting toxicity lead to suboptimal therapeutic outcomes for conventional chemotherapy (Wang et al., 2014a). Doxorubicin, one of the most widely-used chemotherapeutics in the treatment of a wide range of cancers including breast cancer, has long been known to produce severe side-effects including cardiac toxicity and myelo-suppression (Singal and Ilikovic, 1998). Its dose-limiting toxicity has strongly influenced its clinical use. As a result, improvement of the therapeutic index of doxorubicin has been long sought. Targeted delivery of doxorubicin is one of the approaches to avoid its off-target effects. Antibodies that react with their target antigen in a specific manner potentially can be utilized to increase the maximal tolerated dose by increasing the specific delivery of doxorubicin to the tumor. Exploitation of Her2 receptors expressed on numerous cancerous cells including breast cancer cells is currently in vogue for targeted delivery of cytotoxic agents including doxorubicin.

The present study was aimed at developing a targeted delivery system for delivery of the chemotherapeutic drug, doxorubicin, to Her2-overexpressing SKBR3 breast cancer cell line. The as-synthesized doxorubicin-dextran (DEX-DXR) nanocomplex was further encapsulated in stealth liposomes in order to develop a dual-core delivery system so as to improve the half-life of the DEX-DXR nanocomplex and facilitate sustained release of doxorubicin. In order to establish the formation of DEX-DXR nanocomplex, we compared its absorption spectrum, fluorescence emission spectrum and FTIR spectrum with that of un-complexed DXR. As shown in Figure 2.2A, DEX-DXR nanocomplex exhibits λ_max at 495 nm in contrast to free DXR showing maximum absorption at 480 nm. This bathochromic shift in absorption spectrum in addition to hypochromism and broadening of spectrum is an attribute of complexation of DEX to DXR (Yousefpour et al., 2011b). No shift was observed in the
fluorescence emission spectrum, but the intensity was lower compared with the visible absorption spectrum (Figure 2.2B) revealing complexation of DEX with DXR as reported by Yousefpour et al., 2011b. Further the success of DEX-DXR complexation was analysed from concording results of FTIR analysis. Figure 2.2C shows the FTIR spectrum of our obtained product. The appearance of more peaks in DEX-DXR complex in the region 3550-3200 cm⁻¹ represents alcoholic O-H stretch vibrations owing to the presence of numerous O-H groups in DEX (Chauhan et al., 2011). DEX-DXR spectrum in addition harbours the peaks present in DXR spectrum for amide N-H stretching vibrations and amide C=O stretching vibrations in the range 3700-3500 cm⁻¹ and 1690-1630 cm⁻¹ respectively ascertaining the formation DEX-DXR complex (Dao et al., 2014). Figure 2.2D establishes the formation of nanocomplexes in the size range of approx. 100 nm. Once the synthesis of DEX-DXR nanocomplex was established employing various methods, the nanocomplexes were encapsulated within stealth liposomes. As shown in Figure 2.3, the as-synthesized nanocomplex was successfully encapsulated within the stealth liposomes bearing size in the range of 200-300 nm. Figure 2.4 depicts anti-Her2 antibody conjugation to as-synthesized DEX-DXR-SL NCs, targeting of the in-house prepared Ab@DEX-DXR-SL NCs to Her2 expressing tumor cell surface and tumor cell killing mediated by internalization of antibody conjugated NCs. Cytotoxicity of Ab@DEX-DXR-SL NCs on SKBR3 was found to be significantly higher than naked DEX-DXR-SL NCs, \( p < 0.05 \) (Figure 2.5).

Changes in cancer cell morphology and population upon treatment with Ab@DEX-DXR-SL NC were significant when compared to naked DEX-DXR-SL NCs and DEX-DXR nanocomplex (Figure 2.6, Panel A). Caspase-9, an apical caspase in the intrinsic pathway initiated by cytochrome c released from the mitochondrial membrane space into the cytosol, induces apoptosis formation during apoptosis (Chauhan et al., 2014). Caspase-9 activates the effector caspases-3 and -7, which cleave several cellular proteins, resulting in the characteristic biochemical and morphological features associated with apoptosis. Expression of caspase-9 was found to be enhanced upon treatment with Ab@DEX-DXR-SL NCs compared with the naked DEX-DXR-SL NCs (Figure 2.6, Panel B). In the case of untreated cells, no increase in the expression of caspase-9 was observed. It seems Ab@DEX-DXR-SL NC mediated targeted delivery of DXR has great potential in facilitating apoptosis of
cancer cells. During apoptosis, mitochondrial membrane potential has been reported to decrease (Siddiqui et al., 2013). On the same line of action, in the present study, the marked decrease in mitochondrial membrane potential in cells treated with Ab@DEX-DXR-SL NCs as compared to untreated control indicates apoptosis induction (Figure 2.6, Panel C). These results are indicative of higher level of apoptosis induction in cells treated with Ab@DEX-DXR-SL NCs which can be owed to the targeted delivery of DXR to SKBR3 cancer cells. Moreover, annexin V binding (Figure 2.7; Panel A) and the APO-BrdUTM (Molecular Probes, Inc., Invitrogen, OR, USA) study (Figure 2.7, Panels B&C) via fluorescence microscopy/FACS establish better apoptosis induction in cells treated with Ab@DEX-DXR-SL NCs.

Cancer cell apoptosis induced by various DXR formulations was also ascertained by analysing pro/anti apoptotic factors. As depicted in Figure 2.8, comparatively higher expression of p53wt and Bax proteins was recorded in the cells treated with Ab@DEX-DXR-SL NC formulation compared with naked DEX-DXR-SL NC formulation treated cells. Similarly, treatment with various formulations of DXR was also successful in aborting upregulation of p53mut and Bcl2 in cancer cells. The data clearly suggest that anti-Her2 antibody conjugation on the surface of DEX-DXR nanocomplex loaded stealth liposomes play a determining role in tumor selective delivery of doxorubicin and subsequent upregulation of pro-apoptotic factors and downregulation of anti-apoptotic factors.

Oxidative stress has been suggested to play an important role in the toxicity mechanisms of nanoparticles (Siddiqui et al., 2012; Siddiqui et al., 2013). This has been attributed to their tendency to generate ROS. Moreover, DXR has also been reported to induce ROS generation in various tumor cells (Wang et al., 2004). ROS such as superoxide anion (O$_2^-$), hydroxyl radical (HO) and hydrogen peroxide (H$_2$O$_2$) elicit a variety of physiological and cellular events including inflammation, DNA damage and apoptosis (Asharani et al., 2009; Ahamed et al., 2011). Several groups have reported that inhibiting DXR-induced intracellular oxidative stress by the overexpression of antioxidant enzymes prevented apoptosis in tumor cells, and that depleting endogenous antioxidants (e.g. glutathione) made tumor cells more susceptible to DXR (Siddiqui et al., 2013). In the present study, ROS levels were markedly higher in SKBR3 cells exposed to Ab@DEX-DXR-SL NCs as compared to cells treated with naked DEX-DXR-SL NCs. Moreover, antioxidant NAC reduced
Chapter 2: Discussion

cell death enormously indicating that oxidative stress plays an important role in Ab@DEX-DXR-SL NCs induced cytotoxicity (Figure 2.9). The higher ROS levels for Ab@DEX-DXR-SL NC exposed cells can be attributed to the targeted delivery of DXR which results in delivery of larger payload of DXR to tumor cells.

From the results of this study, it can be concluded that a successful DEX-DXR nanocomplex-loaded PEGylated immuno-stealth-liposome with high cellular uptake efficiency of DXR against Her2 over-expressing cancer cells was developed. This formulation could serve as a promising platform for future tumor specific cancer therapy of Her2-expressing breast cancers.
Chapter Three

Tumor-specific antibody mediated targeted delivery of siRNA and cisplatin employing multifunctional PLGA-dendrimer immuno-nanocomposite for treatment of murine hepatocellular carcinoma
3.1. Introduction

Chemotherapy as well as RNA interference (RNAi) mediated tumor-specific gene therapy remain the most promising platforms for cancer treatment (Dong et al., 2013). siRNA molecules mediate sequence-specific enzymatic cleavage of target mRNA through complementary base pairing. The therapeutic potential of siRNA, however, is restricted by its susceptibility to serum degradation, renal clearance, and poor cellular uptake due to its polyanionic nature (Whitehead et al., 2009). Therefore, considerable efforts have been made to develop carriers for improved pharmacokinetics, cellular delivery, and intracellular trafficking of siRNA. Similarly, several nanoscale delivery systems have been introduced for the delivery of anticancer chemotherapeutics as well in order to improve the efficacy of chemotherapeutics and circumvent their toxicity issues. (Xu et al., 2013; Zhang et al., 2011a; Farokhzad and Langer, 2009). However, despite delivering promising results in pre-clinical as well as clinical trials; neither chemotherapy nor siRNA therapy, owing to the molecular complexity of cancer, has been found free of limitations and sufficient to arrest the development of numerous cancers. Therefore, recently it has been suggested that combining chemotherapy with siRNA therapeutics could be a promising strategy for improving the efficacy of chemotherapy as well as siRNA therapy through additive or synergistic effects (Xu et al., 2013; Wang et al., 2009). Rather, several research groups have begun to explore the feasibility of combining chemotherapeutics with siRNA using a variety of nanocarrier platforms (Wang et al., 2006; Zhang et al., 2012). One of the earliest efforts using this therapeutic paradigm involved cancer treatment by targeted minicells containing specific siRNA followed by drug-loaded minicells, which efficiently reversed drug resistance in drug-resistant tumors and produced enhanced therapeutic efficacy in inhibiting tumor growth (MacDiarmid et al., 2009). However, to exert optimal synergistic effects, both the drug and siRNA may need to be temporally co-localized in the tumor cells. As a result, nanocarrier platforms that are capable of simultaneously delivering siRNA and anticancer drugs to the same tumor cells are emerging as a promising nanomedicine approach for improved cancer therapy (Sun et al., 2011; Xiong and Lavasanifar, 2011).

Polymeric nanoparticles prepared from PLGA, a FDA approved biodegradable and biocompatible polymer are attractive delivery vehicles because of their high stability,
low toxicity and controlled drug/DNA/siRNA release (Cheng et al., 2011). In general, loading of nucleic acids into PLGA nanoparticles can be achieved using two approaches: (i) Entrapment into the matrix of the nanoparticle and (ii) adsorption on the surface of prepared particles by electrostatic interactions (Zhang et al., 2007). Although the first approach allows sustained release of the nucleic acid but it bears certain drawbacks. The demerits of this approach include inefficient encapsulation of nucleic acid in hydrophobic PLGA nanoparticles owing to lack of electrostatic interaction of these particles with negatively charged (hydrophilic) nucleic acids. Moreover, the harsh manufacturing conditions such as exposure to organic solvents and shear forces associated with the homogenization and sonication steps may lead to inactivation of nucleic acids further decreasing the functional nucleic acid entrapped in these particles (Oster and Kissel, 2005; Tinsley-Bown et al., 2000; Walter et al., 1999). Several approaches have sought to overcome these limitations by incorporating cationic excipients into PLGA matrix that condense and protect the nucleic acid during entrapment (Ando et al., 1999). In the second approach, nucleic is adsorbed onto the surface of the nanoparticles after they have been prepared (Oster and Kissel, 2005; Kasturi et al., 2005; Oster et al., 2005). This method prevents nucleic acid damage during nanoparticle preparation and also facilitates nanoparticle loading along with drugs or proteins that complement or enhance the efficacy of the surface-bound nucleic acid. To facilitate binding of the nucleic acid to the surface of the nanoparticles, usually a cationic agent is incorporated into the formulation method to prepare nanoparticles bearing a net positive charge. The positively charged nanoparticles interact with negatively charged nucleic acids by electrostatic interactions. Cationic surfactants such as cetyltrimethylammoniumbromide (CTAB) or cationic polymers such as polyethyleneimine (PEI) have conventionally been used to prepare cationic nanoparticles (Zhang et al., 2007). PAMAM dendrimers in a manner similar to PEI polymers bear numerous amine groups and therefore also fall under the category of cationic polymers (Zhou et al., 2006; Liu et al., 2014). Therefore, for co-delivery of siRNA and a chemotherapeutic drug, entrapping the anticancer drug within the PLGA nanoparticle and facilitating siRNA binding to the cationic polymer attached to the PLGA nanoparticle surface seems to be a practical approach.
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Although the use of NP formulations has improved drug bioavailability at the tumor site whilst also reducing off-target effects by passive targeting owing to enhanced EPR effect, which arises as a consequence of leaky vasculature and inefficient lymphatic drainage in the tumor (Lu et al., 2013). However, not all solid tumors exhibit the EPR effect (Matsumura and Maeda, 1986; Maeda et al., 2000), and nanoparticles are no more efficacious than free drugs in the passive targeting in such cancers (Kohlschutter et al., 2008). Therapeutic efficacy and the regulation of drug release can be improved by using selective targeting drug delivery systems. This approach utilizes ligands that specifically interact with receptors expressed on cancer cells, in order to enhance binding and internalization of nanoparticle-based drugs (Svensen et al., 2012; Wu and Chang, 2010; Chang et al., 2009; Torchilin, 2008). Nanoparticles with targeting moieties on their external surface exhibit much higher affinity for cancer cells (Cho et al., 2012; Ashley et al., 2011; Park et al., 2009). Studies on targeted drug delivery have largely centred on using antibody linked nanoparticles (Tappertzhofen et al., 2014; Dhankar et al., 2011; McCarron et al., 2008; Yang et al., 2007; Weiner, 1999).

Herein, we report the development of cisplatin encapsulated PLGA NPs functionalized with PAMAM dendrimer-siRNA complex and liver cancer cell specific antibody. The in-house synthesized cisplatin/siRNA bearing PLGA-dendrimer nanocomposites were further evaluated for their anticancer potential in the treatment of liver cancer in Swiss albino mice. To fabricate these nanocomposites, PAMAM dendrimers were attached onto the surface of cisplatin encapsulated PLGA NPs. PAMAM dendrimers bear the capability of disrupting of intracellular organelles through a “proton sponge effect” which results in cytosolic delivery of the payload. For active targeting of the drug/siRNA payload to the tumor site, the PLGA-dendrimer nanocomposites were modified with liver cancer cell specific antibodies.
3.2. Materials and methods

3.2.1. Materials

All the reagents used in the study were of highest purity available. PLK-1 specific siRNA was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Anti-p53 wt, anti-p53 mut, anti-bax, anti-Bcl2, anti-β-actin antibodies, non-fat dry milk and BrdU TUNEL Assay kit were purchased from BD Biosciences (San Diego, CA). Cisplatin, PLGA, PAMAM dendrimer generation 4, Diethyl nitrosamine (DEN), Rhodamine 123, NAC, FCS, RPMI-1640 medium, CFA, IFA, EDC, Sulpho-NHS, Ninhydrin, MBS, rabbit anti-mouse FITC labelled 2⁺ antibody, 2-iminothiolane (Traut’s reagent) and BCA protein estimation kit were purchased from Sigma-Aldrich (St Louis, MO, USA). PVDF membrane was purchased from Millipore India Pvt. Ltd. (Bangalore, India). ECL kit was bought from Bio Rad Laboratories, Inc. (CA, USA). Protein A column was purchased from Genel Pvt. Ltd. (Bangalore, India). All others reagents were of analytical grade and procured from local suppliers.

3.2.2. Preparation and characterization of cisplatin loaded PLGA NPs

PLGA nanoparticles loaded with cisplatin were prepared by a water–oil–water (w/o/w) emulsion solvent evaporation method as described elsewhere (Moreno et al., 2010) with slight modification in our laboratory. A solution of cisplatin in Tris–HCl (1.67 mg/ml) was emulsified in 0.5 ml of chloroform containing 100 mg of PLGA, using a microtip probe sonicator (Vibra-Cell, Sonics & Materials, Inc., CT, USA) set at level 20 for 5 s. This primary (w/o) emulsion was added to 2 ml of 9% PVA containing 1 mg/ml of cisplatin and homogenized using a Silverson L4RT Homogenizer (Silverson Machines, East Longmeadow, MA, USA). The second w/o/w emulsion was transferred drop wise to 8 ml of 9% PVA saturated also with cisplatin (1 mg/ml) and agitated by magnetic stirrer for 3 hrs at RT until complete evaporation of the organic solvent. NPs were collected by centrifugation (15,000 g for 10 min), washed with water, freeze-dried and stored at -20°C until use. Entrapment efficiency of cisplatin entrapped PLGA NPs was calculated as described in the next sub-section. The morphological examination of the developed nanoparticles was performed using SEM. Briefly, the as-synthesized PLGA NP solution was lyophilized and the vacuum dried powder was mounted on clear glass stub followed by coating with gold-palladium alloy and finally observed by SEM (JEOL, Japan) in high-vacuum mode at 20 kV.
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3.2.3. Entrapment efficiency of cisplatin in PLGA nanoparticles

Entrapment efficiency of cisplatin in PLGA NPs was measured by dissolving 10 mg of freeze-dried formulations in 1.0 ml of 0.1 M methanolic sodium hydroxide (Farazuddin et al., 2014). This solution was vortexed, then incubated at 37°C for 1 hr. After incubation, the solution was centrifuged at 9,168 × g at 25°C for 10 min. The supernatant was then examined for its cisplatin content, using HPLC, according to a published procedure (Jayaprakasha et al., 2002). A standard curve for the drug was plotted, by determining the area under the curve corresponding to a known (increasing) quantity of the drug. The percent entrapment efficiency (% EE) was calculated using the following formula:

\[
\text{% EE} = \frac{\text{Amount of cisplatin entrapped}}{\text{Total amount of cisplatin used in the beginning}} \times 100
\]

3.2.4. Conjugation of PAMAM dendrimers onto PLGA NPs

A modified EDC/NHS chemistry was used to conjugate generation 4 (G4) PAMAM dendrimers to the surface of PLGA nanoparticles to obtain PLGA-dendrimer nanocomposites following the published procedure with slight modification (Zhang et al., 2007). Cisplatin entrapped PLGA nanoparticles (100 mg) prepared as described above were suspended in 10 ml of 0.1 M MES (2-(N-morpholino) ethanesulfonic acid) buffer, pH 6.2, EDC solution (1ml, 60 mM) in 0.1 M MES buffer and 1 ml of Sulfo-NHS solution (60 mM) in 0.1 M MES buffer was added dropwise to the PLGA nanoparticle suspension. EDC activation was carried out for 2 hrs at room temperature. A 10 molar excess of G4 PAMAM dendrimer was dissolved in 5 mL of 0.1 M MES buffer. Activated PLGA nanoparticles were added dropwise to the dendrimer solution with magnetic stirring and incubated for another 4 hrs at room temperature. Dendrimer-decorated PLGA nanoparticles were washed twice in 1 M NaCl to remove physically adsorbed polymer and twice with deionized water. The resulting nanoparticles were thereafter lyophilized and stored at -20°C until further use. Ninhydrin assay was performed to quantify the dendrimers associated with PLGA-dendrimer nanocomposites.

3.2.5. Ninhydrin Assay

The dendrimer content of various PLGA-dendrimer nanocomposites was measured by ninhydrin assay following the published protocol (Chan et al., 2008) as standardized
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in our laboratory. Ninhydrin reacts with primary amines in dendrimers to form a Ruheman Purple colored compound. Briefly, 2 mg of various nanocomposite formulations were taken in separate 1.5 ml microcentrifuge tubes. Further, to each tube was added 200 µl of deionized water and 1 mL of ninhydrin solution (one part of 4% (w/v) ninhydrin in 2-ethoxyethanol and one part 200 mM citric acid with 0.16% (w/v) stannous chloride, pH 5.0). The tubes were heated at 95 °C for 30 min in a waterbath. A deep blue or purple color chromophore (Ruhemann’s purple) was obtained. This mixture was vortexed, and the optical absorbance of the solution was recorded with a Perkin Elmer spectrophotometer at a wavelength of 570 nm. Cisplatin entrapped naked PLGA nanoparticles in ninhydrin solution were taken as blank. Glycine was used as standard.

3.2.6. Transmission Electron Microscopy
TEM samples were prepared following the protocol as published elsewhere (Farazuddin et al., 2012) by placing a drop of synthesized nanocomposites over gold coated negative grid followed by evaporation of the solvent. TEM analysis was performed on JEOL model (1200 EX, JOEL Inc, Peabody, MA) which was operated at an accelerating voltage of 200 kv.

3.2.7. Animals
Female Swiss albino mice of weight 20 ± 3 g were obtained from the institute’s animal house facility. The animals were housed in poly-propylene cages on wood powder bedding in an air-conditioned ambience. Animals were quarantined on equal light/dark cycles (12/12 hrs) and were kept on a pellet diet and water ad libitum. Animals were examined for their mortality and morbidity prior to commencement of the study, and only healthy animals were included in the experiments. The techniques used for administration of various formulations as well as sacrifice of the animals were strictly performed following mandates approved by the animal ethics committee (Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India).

3.2.8. Induction of liver cancer by diethylnitrosamine
Liver cancer in experimental models was induced following a method described elsewhere (Chauhan et al., 2014). Briefly, a dose of 2.4 mg DEN per animal was delivered by intraperitoneal injection, and animals were quarantined for 40 days.
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After this incubation period, cancer establishment was determined by histopathological study.

3.2.9. Isolation of tumor cell proteins and development of liver cancer cell specific antibodies

Tumor cells were isolated from the mice following a standard protocol (Chan et al., 2014). Firstly, tumor cell lysate was prepared following the published procedure (Chauhan et al., 2011). The protein content of each sample was estimated using BCA protein assay kit with BSA as protein standard. The animals were immunized with a total of three doses of antigen (100 μg/mouse in CFA on day 0, and 50 μg/mouse in IFA on days 14 and 21). On day 25, the animals were bled and serum was isolated for antibody collection. Protein A column was used to purify the antibodies. The eluted antibodies were pre-cleared by incubation with cell lysate prepared from normal healthy liver cells. For this, the mixture was incubated for 6 hrs followed by centrifugation at 3000 g to remove the agglutinated mass, and the supernatant was further processed using the following method to obtain the liver cancer cell specific antibodies.

3.2.10. Purification of liver cancer cell specific antibodies

Liver cancer cell specific antibodies were purified following the published procedure (Chauhan et al., 2011). For development of the liver cancer specific antibodies, we used a whole cell lysate that induced indiscriminate production of antibodies specific for the cytosol as well as the cell surface. To isolate the antibodies from the mixed population, the liver cells were brought into a single cell suspension and incubated with an equal volume of glutaraldehyde (6.25%) to form a stroma, following a previously published procedure standardized in our laboratory (Owais et al., 1995). The stroma was used to remove cytosolic antigen-specific antibodies of cancer cells by negative selection. The stroma-bound liver cancer cell specific antibodies were eluted using propionic acid (1 M, pH 2.4). The pH of the eluent was adjusted with Tris-NaOH (3 M, pH 8.6) and finally extensively dialyzed against 10 mM phosphate buffer at pH 7.4.
3.2.11. Conjugation of liver cancer specific antibodies onto dendrimers

The PLGA-dendrimer immuno-nanocomposites were prepared by slight modifications in the method reported by Dhankar et al., 2011. The preparation of immuno-nanocomposites included three steps:

1. Activation of NH₂ groups of dendrimers with MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester).
2. Thiolation of purified liver cancer cell specific antibody.
3. Conjugation of thiolated anti-liver cancer antibody on the surface of MBS activated dendrimer decorated PLGA nanocomposites.

In the first step, PLGA-dendrimer nanocomposites (5 mg) were dispersed in 1 ml PBS (pH 7.2) and incubated with 100 µl crosslinker MBS solution (3.14 mg MBS dissolved in 1 ml of DMSO) for 30 min at 25°C temperature in shaker incubator. The excess of MBS was separated from activated nanoparticles using sephadex G25. Secondly, for thiolation of anti-liver cancer antibody, 5.7 mg of 2-iminothiolane (Traut’s Reagent) was dissolved in 5 ml PBS (pH 7.4). From a stock solution of 10 mg/ml, 100 µl anti-liver cancer cell antibody was incubated with 4.2 µl of 2-iminothiolane for 2 hrs at 20°C under constant shaking. Finally, 100 µl of thiolated antibody was added in 100 µl MBS-activated nanoparticles for 30 min at 25°C under constant shaking for preparation of PLGA-dendrimer immuno-nanocomposites.

3.2.12. Assessing the conjugation of liver cancer specific antibodies onto PLGA-dendrimer nanocomposites

The as-synthesized PLGA-dendrimer NCs were characterized for antibody conjugation employing protein estimation using BCA Protein Assay Kit (Sigma, India) with BSA as protein standard and detection by FITC labelled anti-mouse 2° antibody. To 50 µl of PLGA-dendrimer immuno-nanocomposite solution, FITC tagged anti-mouse 2‘antibody was added in the ratio 1:2500 and incubated for 30 min. The incubated solution (10 µl) was dropped on a glass slide to be analysed under fluorescence microscope (Zeiss model, magnification 100X).
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3.2.13. Formation and characterization of siRNA complex of PLGA-dendrimer nanocomposite
Antibody conjugated PLGA-dendrimer NCs were further complexed with siRNA. It is to be noted that even after activation of PLGA-dendrimer nanocomposites by MBS, there were significant number of free amine groups left as ascertained by ninhydrin assay (data not shown). Antibody conjugated PLGA-dendrimer NCs were incubated with 50 nM of siRNA in PBS (pH 7.4) at 25°C for 20 min so as to facilitate siRNA complexation to the dendrimer decorated on the surface of PLGA NPs (Dutta et al., 2010).

3.2.14. Toxicity tests for various PLGA-dendrimer NC formulations
In vitro erythrocyte lysis test was carried out as a preliminary toxicity test of as-synthesized nanocomposite formulations, which is assessed by measuring the hemoglobin released as a result of membrane leakage or disruption caused by exposure to low doses of the nanoparticles. Briefly, fresh blood obtained from a healthy rabbit was collected in anticoagulant solution (EDTA) and centrifuged at 1000 g for 10 min at 4°C. Both buffy coat and plasma were discarded. Washed erythrocytes were diluted with isotonic buffer (20 mM PBS) to prepare 50% hematocrit. Extent of hemolysis was studied by incubating the RBC suspension with various PLGA-dendrimer NC formulations at a concentration of 100 μg/ml at 37°C for 1 hr. The incubated solutions were centrifuged at 1500 g after 1 hr and supernatant was collected and analyzed by ultraviolet-visible spectroscopy (λmax = 576 nm) for released hemoglobin. The percentage hemolysis was determined by the following equation:

\[
\% \text{ hemolysis} = \left( \frac{\{Abs(t) - Abs(c)\}}{\{Abs(100\%) - Abs(c)\}} \right) \times 100
\]

where Abs(t) is the absorbance of the supernatant from samples incubated with the particles, Abs(c) is the absorbance of the supernatant from controls (normal saline), and Abs(100%) is the absorbance of the supernatant of controls incubated in the presence of 1% Triton® X-100, which causes complete lysis of RBCs (total lysis).

Hepatic and renal toxicities were measured by applying multidose regimen (total 10 doses for consecutive 10 days) with various PLGA-dendrimer NC formulations for determining the biochemical profile of renal parameter serum creatinine and hepatic

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parameters such as ALP, ALT and AST by respective detection kits (COGENT, Span Diagnostics Ltd., India). Blood was collected by retro-orbital puncture from mice belonging to various treated groups. The blood was allowed to clot at RT and serum was separated for investigation of above mentioned renal and hepatic parameters as per respective guidelines provided by the manufacturer.

3.2.15. Treatment schedule and assessment of anti-cancer efficacy of various PLGA-dendrimer NC formulations
The animals with liver tumor were pooled and randomized into five groups each comprising of 10 animals as detailed in Table 3.1.

Table 3.1. Various groups of animals employed for the study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Formulations administered</th>
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<tbody>
<tr>
<td>Group 1.</td>
<td>Untreated control animals</td>
</tr>
<tr>
<td>Group 2.</td>
<td>Dendrimer decorated sham (empty) PLGA NCs (PAMAM@PLGA NC) administered animals</td>
</tr>
<tr>
<td>Group 3.</td>
<td>Free siRNA and free cisplatin mixture (siRNA+Cis) treated animals</td>
</tr>
<tr>
<td>Group 4.</td>
<td>Dendrimer-siRNA complex decorated and cisplatin loaded PLGA NCs (PAMAM-siRNA@Cis-PLGA NC) treated mice</td>
</tr>
<tr>
<td>Group 5.</td>
<td>Liver cancer specific antibody functionalized dendrimer-siRNA complex decorated and cisplatin loaded PLGA NPs (Ab@PAMAM-siRNA@Cis-PLGA NC) treated mice</td>
</tr>
</tbody>
</table>

Untreated cancerous mice represent ‘untreated control’ group. After 40 days of DEN treatment, free siRNA and cisplatin mixture (50 nM siRNA/0.1μM cisplatin/200μl of solution/animal), PAMAM-siRNA@Cis-PLGA NC (50 nM siRNA/0.1μM Cisplatin/200μl of NC formulation/animal) and Ab@PAMAM-siRNA@Cis-PLGA NC formulation (50 nM siRNA/0.1μM Cisplatin/200μl of nanocomposite formulation/animal) were administered intravenously once in a day for 10 days consecutively.

3.2.16. Preparation of nuclear fraction
Nuclear fraction was prepared following the published procedure (Farazuddin et al., 2014). The liver tissues were removed from experimental mice aseptically with sharp
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scalpel blades. The tissue samples were placed on ice and fat was scraped off before further processing. Finally, the samples were homogenized in the presence of a protease inhibitor cocktail, then a nuclear fraction was prepared following the method reported by Farazuddin et al., 2014.

3.2.17. Western blot analysis

Proteins in the nuclear fraction were estimated using a BCA Protein Assay kit with reference to BSA as protein standard (Fausther et al., 2014). The homogenate (30 µg protein) was resolved on 10% SDS-PAGE gel and electrobotted onto nitrocellulose membrane (Chauhan et al., 2014). Employing an enhanced chemiluminescence kit, the membrane was probed for the presence of p53 wt, p53 mutant (p53mut), PLK-1, Bax and Bcl2 using specific antibodies. Blots were re-probed with an antibody for β-actin and used as a control for equal protein loading and transfer. Densitometric values of protein bands were quantified using Alpha Image Analysis software (Proteinsimple, CA, USA) on Alpha Image Gel Documentation System.

3.2.18. Annexin V apoptosis using fluorescence microscopy

In general, apoptosis is manifested by certain morphological features, including loss of plasma membrane asymmetry, condensation of the cytoplasm as well as nucleus, and intranucleosomal cleavage of DNA. The altered phospholipid distribution in the two leaflets of plasma membrane was analyzed with the help of annexin V staining kit (BD Biosciences; San Diego, CA) following the manufactures instructions. Briefly, single cell suspension was prepared from isolated liver tumor tissues treated with various formulations of PLGA-dendrimer nanocomposites (Chauhan et al., 2014). Cells were incubated with FITC annexin V for 30 min at RT and were visualized under the fluorescence microscope (40X magnification) after washing with PBS.

3.2.19. Assay of mitochondrial membrane potential

Mitochondrial membrane potential was measured following the published procedures (Zhang et al., 2011a; Siddiqui et al., 2013). Cells in the single cell suspension prepared from isolated liver tumor tissues from various treated groups were exposed to 10 mg/ml of Rhodamine-123 fluorescent dye for 1 hr at 37°C in dark. Further, cells were washed twice with PBS followed by analysing the fluorescence intensity of Rhodamine-123 using fluorescence microscope (Zeiss) by capturing the images at 20X magnification.
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3.2.20. Determination of caspase-9 level by fluorescence microscopy
Single cell suspension of liver cancer cells was permeabilized with 0.1% Triton X100 in PBS at RT for 10 min followed by fixation in 4% paraformaldehyde for 2 hrs at room temperature. The fixed cells were blocked with 2% FCS followed by incubation with monoclonal antibody which detects only 35 Kda cleaved caspase 9. Finally, the cells were incubated with FITC tagged 2° antibody. Fluorescence microscopy was performed using Zeiss fluorescence microscope at 100X magnification.

3.2.21. Effect of various PLGA-dendrimer NC formulations on DNA fragmentation as analyzed by Apo-BrdU study
Single cell suspension of liver cancer cells was prepared by pelleting cells at 300 g for 10 min at 4°C from various experimental groups. Cells were re-suspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4). Suspension was placed on ice for 30 min followed by centrifugation for 5 min at 300 g and supernatant was discarded. The cell pellet was resuspended in 70% (v/v) ice-cold ethanol followed by washing. After keeping for 30 min in 70% ethanol, cells were washed 3 times and pellet was resuspended in 50 µl of DNA labelling solution (Reaction buffer + TdT enzyme and Br-dUTP). Cells were incubated in the DNA labelling solution for 60 min at 37°C in a temperature controlled water bath. Cell pellet was incubated with the FITC-labelled anti-BrdU antibody staining solution in the dark for 30 min at room temperature. After labelling the tumor cells with FITC-tagged anti-BrdU antibody, PI/RNase (50 µg/ml) was added to the antibody labelled cells which were analyzed by flowcytometer (GUAVA, Millipore, MA, USA) followed by incubation for 30 min at 37°C.

3.2.22. Histopathological studies
To examine the effect of PLGA-dendrimer NC formulations on treatment of cancer, liver tissue samples were collected from control and various experimental groups and histopathological studies were performed as described elsewhere (Chauhan et al., 2014).

3.2.23. Statistical Analysis
Data were analyzed and two groups were compared with the Student t test, and one way ANOVA (Holm-Sidak method) to assess the differences among various groups,
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using Sigma-Plot version 10 software. $P$ values $<0.05$ were considered to be significant.
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3.3. Results

3.3.1. Characterization of cisplatin loaded PLGA nanoparticles

The in-house synthesized PLGA nanoparticles exhibited an entrapment efficiency of 46.4±2.1 for cisplatin (Table 3.1). Figure 3.1A represents the morphology of cisplatin encapsulated PLGA nanoparticles obtained by SEM. The particles appear to be spherical. These results are in agreement with those previously reported by Moreno et al., 2010. These authors concluded that the particle size obtained with the method used for their synthesis was dependent on the homogenization system used in the second emulsion. In this study, the microtip probe sonicator was used for the homogenization of two emulsions similar to that used by Moreno et al., (2010) therefore, particle size obtained was also similar to that obtained by them (approx. 100-200 nm in our study and 180 nm reported by Moreno et al., 2010).

<table>
<thead>
<tr>
<th>Table 3.2. Size and entrapment efficiency of in-house synthesized NPs</th>
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<tbody>
<tr>
<td>Formulation</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>PLGA NP</td>
</tr>
<tr>
<td>PAMAM@Cis-PLGA NC</td>
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3.3.2. Assessing the conjugation of dendrimers onto PLGA NPs

Conjugation of PAMAM dendrimers on the surface of PLGA nanoparticles was ascertained by ninhydrin assay and transmission electron microscopy. The ninhydrin assay was used to quantitatively assess the amount of free amines present in the PLGA-dendrimer NCs. The results of the quantitative assessment of amine content using colorimetric ninhydrin assay are shown in Figure 3.1B. EDC/NHS cross-linking of dendrimer reduced the amount of free amine groups by amide bond formation with carboxyl groups present on PLGA NPs. Dendrimer only showed significantly higher free amine groups when compared to PAMAM@Cis-PLGA NCs (p < 0.05). The morphology of the particles decorated with dendrimers appears to be different from naked PLGA NPs. The rough surface of dendrimer decorated PLGA NPs (shown with arrow in Figure 3.1C) in contrast to smooth surface shown for naked PLGA NPs ascertains the conjugation of PAMAM dendrimers onto PLGA.
NPs. The increased size of the particles upon EDC/NHS crosslinking also supports the conjugation of dendrimer onto PLGA NPs.

![Graph](image)

**Figure 3.1.** Characterization of PLGA-dendrimer nanocomposites. (A) SEM micrograph of cisplatin loaded PLGA NPs. (B) Free amine content measurements in PLGA-dendrimer NCs by the ninhydrin assay. (C) TEM micrograph revealing conjugation of dendrimers on the surface of PLGA NPs. Arrows highlight the rough surface of dendrimer decorated PLGA NPs. Data are representative of at least three independent experiments with similar observations. For (B), data are mean values ± standard deviation from three different experiments.

### 3.3.3. Characterization of liver cancer cell specific antibody conjugated PLGA-dendrimer nanocomposites and siRNA complex formation thereof

Liver cancer cell specific antibodies were conjugated onto PLGA-dendrimer nanocomposites using the procedure described in materials and methods section. BCA protein assay was used to quantify the amount of antibodies conjugated onto the PLGA-dendrimer NCs. The significantly high concentration of protein in antibody conjugated PLGA-dendrimer nanocomposite formulation as compared to other control groups ascertains conjugation of antibody to the NCs (Figure 3.2A). Antibody conjugation to the PLGA-dendrimer nanocomposites was further supported by fluorescence microscopic studies. As shown in Figure 3.2B, liver cancer specific antibody conjugated PLGA-dendrimer NCs were found to fluoresce upon incubation with FITC tagged anti-mouse secondary antibodies. Dendrimers decorated onto the surface of PLGA-dendrimer NCs were incubated with siRNA. siRNA binding to the as-synthesized nanocomposites was ascertained by determining the absorbance at 260
Figure 3.2C reveals binding of siRNA to PLGA-dendrimer nanocomposite. The absorbance at 260 nm for the siRNA complexed nanocomposite was found to be significantly higher than uncomplexed nanocomposites although it was lower than that observed for free siRNA.

Figure 3.2. Assessment of antibody conjugation and siRNA complexation to PLGA-dendrimer NCs. (A) BCA protein assay to quantify antibody tagged to PLGA-dendrimer NCs. Data are mean values ± standard deviation from three different experiments. (B) Fluorescence microscopy image revealing conjugation of antibody to PLGA-dendrimer NCs owing to binding of anti-mouse FITC labelled 2° antibodies to liver cancer specific antibody tagged PLGA-dendrimer NCs. Data are representative of at least three independent experiments with similar observations. (C) Complex formation of siRNA with antibody conjugated PLGA-dendrimer NCs as
measured by determining absorbance at 260 nm. Data are mean values ± standard deviation from three different experiments.

![Graph showing percent hemolysis](image)

**Figure 3.3.** Erythrocyte lysis test. *In vitro* toxicity was measured by erythrocyte lysis, induced by various PLGA-dendrimer NC formulations. Hemolysis testing was performed. The data represented here are the means of three independent experiments, with standard deviations (Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, $p < 0.05$; PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$).

### 3.3.4. *In vitro* and *in vivo* toxicity of Ab@PAMAM-siRNA@Cis-PLGA NC formulation

In-house developed various PLGA-dendrimer nanocomposite formulations were evaluated for *in vitro* toxicity using erythrocyte lysis test. **Figure 3.3** shows the extent of cell lysis induced by various siRNA-based formulations. The complete (100%) lysis of RBCs caused by Triton-X-100 was considered to be total lysis. The lytic ability of empty dendrimer conjugated empty PLGA NCs (PAMAM@PLGA NC) was also examined. As shown in **Figure 3.3**, antibody conjugated PAMAM-siRNA@Cis-PLGA NC and naked PAMAM-siRNA@Cis-PLGA NC caused
negligible lysis of erythrocytes, compared with mixture of free siRNA and cisplatin (Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, $p < 0.05$; PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$).

*In vivo* toxicity of different PLGA-dendrimer formulations was examined by the estimation of hepatic and renal function parameters (Figure 3.4). Sera isolated from animals treated with various formulations were tested for the levels of creatinine and ALP, AST and ALT. Untreated animals served as a control for the comparison study. As shown in Figure 3.4, Ab@PAMAM-siRNA@Cis-PLGA NC formulation induced significantly lower toxicity as compared to naked PAMAM-siRNA@Cis-PLGA NC and mixture of free siRNA and cisplatin for renal as well as all the hepatic parameters tested (Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$; Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, $p < 0.01$ for creatinine, ALP, AST and ALT).
Figure 3.4. *In vivo* toxicity test of Ab@PAMAM-siRNA@Cis-PLGA NC formulation. Concentrations of creatinine, ALP, AST, and ALT in plasma of animals treated with various nanocomposite formulations. Data are mean values ± standard deviation from three different experiments (Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$; Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, $p < 0.01$ for creatinine, ALP, AST and ALT).
Figure 3.5. PLK-1 inhibition by Ab@PAMAM-siRNA@PLGA-Cis NC causes upregulation of p53 wildtype as well as Bax and downregulation of p53 mutant as well as Bcl2 expression. (A) Western blot of PLK-1 in various groups. lane 1:
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untreated control group; lane 2: animals treated with mixture of free siRNA and cisplatin; lane 3: PAMAM-siRNA@Cis-PLGA NC formulation administered group; lane 4: group treated with Ab@PAMAM-siRNA@Cis-PLGA NC formulation. Data are representative of at least three independent experiments with similar observations. (B) Immunoblots showing the effect of Ab@PAMAM-siRNA@Cis-PLGA NC on the expression of p53wt, p53mut, Bax and Bcl2 in a cancer-inflicted liver. Lane 1: healthy control group; lane 2: untreated control group; lane 3: animals treated with mixture of free siRNA and cisplatin; lane 4: PAMAM-siRNA@Cis-PLGA NC formulation administered group; lane 5: group treated with Ab@PAMAM-siRNA@Cis-PLGA NC formulation. Data are representative of at least three independent experiments with similar observations. Densitograms show relative pixel densities of PLK-1, p53wt, p53mut, Bax and Bcl2 after treatment with mixture of free siRNA and cisplatin, PAMAM-siRNA@Cis-PLGA NC and Ab@PAMAM-siRNA@Cis-PLGA NC formulations. Results are mean values ± standard deviation in triplicates (for PLK-1: Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, p < 0.01 and Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, p < 0.01; for p53 wt: Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, p < 0.05 and Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, p < 0.01; for p53 mut: Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, p < 0.01, Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, p < 0.01 and PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, p < 0.05; for Bcl2: Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, p < 0.05 and Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, p < 0.001; for Bax: Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, p < 0.05; Ab@PAMAM-siRNA@Cis-PLGA NC vs free siRNA and cisplatin mixture, p < 0.05).

3.3.5. Impact of various PLGA-dendrimer NC formulations on apoptosis

Exposure with DEN resulted in over-expression of PLK-1 in experimental animals (Figure 3.5A). Treatment with tumor-specific antibody tagged PAMAM-siRNA@Cis-PLGA NCs induced marked reduction of PLK-1 expression. Densitometric analysis of PLK-1 levels exhibit significant decrease in the expression of PLK-1 in Ab@PAMAM-siRNA@Cis-PLGA NC treated group as compared to animals treated with naked PAMAM-siRNA@Cis-PLGA NC (p < 0.01) and free
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siRNA and cisplatin mixture ($p < 0.01$). Downregulation of p53 wild-type (p53wt) was observed upon exposure to DEN (control untreated cancerous mice; Figure 3.5B, lane 2) compared with healthy (normal) control mice (Figure 3.5B, lane 1). Treatment with siRNA and cisplatin mixture resulted in a slight increase in the expression of p53wt (Figure 3.5B, lane 3), which was not only used as a parameter to establish induction of liver cancer in the experimental animals, but also offered as a tool to ascertain efficacy of various siRNA based nanocomposite formulations. Significant upregulation of p53wt was observed when cancerous mice were treated with Ab@PAMAM-siRNA@Cis-PLGA NC formulation in comparison to animals belonging to PAMAM-siRNA@Cis-PLGA NC administered group and siRNA+Cis group (Figure 3.5B, lane 5). As revealed from comparative study of densitometric results, for p53 wt, Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$ and Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, $p < 0.01$. Exposure of animals to DEN ensued in upregulation of p53mut (Figure 3.5B, lane 2). Treatment with Ab@PAMAM-siRNA@Cis-PLGA NC preparation resulted in its downregulation (Figure 3.5B, lane 5). As depicted in the densitogram of p53mut, the level of p53mut was significantly reduced in the animals treated with Ab@PAMAM-siRNA@Cis-PLGA NC compared with the animals treated with PAMAM-siRNA@Cis-PLGA NC ($p < 0.01$) and siRNA+Cis formulations ($p < 0.01$). Treatment with free siRNA and free cisplatin mixture was not as effective in downregulation of p53mut as compared with PAMAM-siRNA@Cis-PLGA NC formulation where both siRNA and cisplatin are in encapsulated form ($p < 0.05$). The effect of Ab@PAMAM-siRNA@Cis-PLGA NC formulation on the expression of Bax was also recorded. Immunoblot analysis showed downregulated expression of Bax in cancerous (untreated) mice, whereas its level was upregulated in the group treated with Ab@PAMAM-siRNA@Cis-PLGA NC (Figure 3.5B, lanes 2 and 5, respectively). From comparative analysis of densitograms of Bax, Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$; Ab@PAMAM-siRNA@Cis-PLGA NC vs free siRNA and cisplatin mixture, $p < 0.05$. In contrast to Bax, untreated mice exhibited upregulated expression of Bel2 while its level of expression was reduced in mice treated with Ab@PAMAM-siRNA@Cis-PLGA NC as compared to other formulations (Figure 3.5B) (Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$; Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, $p < 0.001$).
Figure 3.6. Ab@PAMAM-siRNA@Cis-PLGA NC formulation induces enhanced apoptosis in liver cancer cells as revealed by annexin V assay and assay for
mitochondrial membrane potential using fluorescence microscopy (magnification 40X). **Left Panel.** Apoptosis induction as revealed by Annexin-V staining in hepatocytes isolated from various PLGA-dendrimer nanocomposite administered group. **Right Panel.** Mitochondrial membrane potential in liver cancer cells isolated from different groups treated with various PLGA-dendrimer nanocomposite formulations. Apoptosis induction is visible in cells isolated from animals treated with various PLGA-dendrimer formulations. Among various formulations, apoptosis induction is significantly enhanced in cells isolated from Ab@PAMAM-siRNA@Cis-PLGA NC treated mice as revealed by reduced mitochondrial membrane potential in hepatocytes isolated from Ab@PAMAM-siRNA@Cis-PLGA NC administered animals. (A), (B), (C) and (D) in both left and right panels denote DEN exposed untreated group, mixture of free siRNA and cisplatin, PAMAM-siRNA@Cis-PLGA NC and Ab@PAMAM-siRNA@Cis-PLGA NC treated groups respectively. Data are representative of at least three independent experiments with similar observations.

3.3.6. Annexin V apoptosis using fluorescence microscopy

In general, apoptosis is manifested by certain morphological features, including loss of plasma membrane asymmetry, condensation of the cytoplasm, as well as nucleus, and intranucleosomal cleavage of DNA. The altered phospholipid distribution in the two leaflets of the plasma membrane was analyzed with the help of Annexin V staining. Apoptosis induction was quite visible in cells treated with free siRNA and free cisplatin mixture in comparison with cells belonging to untreated control group where the phosphatidyl-serine translocation (Annexin V binding) was negligible (**Figure 3.6A and 3.6B respectively, Left Panel**). Interestingly, Annexin V binding was markedly enhanced in liver cells from Ab@PAMAM-siRNA@Cis-PLGA NC treated mice (**Figure 3.6D, Left Panel**). There was no apoptosis in cells isolated from control animals (DEN exposed with no subsequent treatment) (**Figure 3.6A, Left Panel**).

3.3.7. Mitochondrial membrane potential was decreased in Ab@PAMAM-siRNA@Cis-PLGA NC treated group

It is well known that during apoptosis the mitochondrial membrane potential (MMP) decreases (Siddiqui et al., 2013). MMP of liver cancer cells isolated from various treated groups was evaluated. Cells were assayed for Rhodamine 123 uptake using
fluorescence microscope. The brightness of the red fluorescence was remarkably reduced in cells isolated from Ab@PAMAM-siRNA@Cis-PLGA NC treated group that indicates a significant reduction of MMP as compared to cells from control untreated group (Figure 3.6, Right Panel).

3.3.8. Effect of Ab@PAMAM-siRNA@Cis-PLGA NC formulation on the expression of caspase-9
Liver cancer cells were squeezed out from experimental animals and the efficacy of various formulations was evaluated by determining the expression of caspase-9 using fluorescence microscopy (Chauhan et al., 2014). Apoptosis induction was quite visible in free siRNA and cisplatin mixture-treated cells in comparison with untreated control group, where caspase-9 expression was almost negligible (Figures 3.7A and 3.7B respectively, Left Panel). However, expression of caspase-9 was significantly enhanced in cells isolated from Ab@PAMAM-siRNA@Cis-PLGA NC-treated mice (Figure 3.7D, Left Panel).

3.3.9. Effect of Ab@PAMAM-siRNA@Cis-PLGA NC on DNA fragmentation as analyzed by the APO-BrdU™ study
Activation of endonucleases during apoptotic events in the cell generally result in DNA fragmentation. Liver cancer cells were isolated from mice belonging to various experimental groups (untreated control, free siRNA and cisplatin mixture, PAMAM-siRNA@Cis-PLGA NCs and Ab@PAMAM-siRNA@Cis-PLGA NC) and fixed using paraformaldehyde. Efficacy of Ab@PAMAM-siRNA@Cis-PLGA NC was observed by 5-bromo-2'-deoxyuridine (BrdU) TUNEL analysis using FACS. The terminal deoxynucleotidyl transferase enzyme catalyzes a template-dependent addition of 5-bromo-2'-deoxyuridine 5'-triphosphate to 3'-hydroxyl ends of double- and single-stranded DNA. After incorporation, DNA breaks at specific sites and is identified by a FITC-labeled anti-BrdU monoclonal antibody. As revealed by TUNEL analysis, apoptosis induction was significantly enhanced in cells isolated from Ab@PAMAM-siRNA@Cis-PLGA NC treated mice compared with PAMAM-siRNA@Cis-PLGA NC or free siRNA and cisplatin mixture administered mice (Figure 3.7, Right Panel).
Figure 3.7. Ab@PAMAM-siRNA@Cis-PLGA NC preparation induces caspase-9 mediated apoptosis in liver cancer cells and causes marked DNA fragmentation
in treated cancer cells. Left Panel. Involvement of caspase-9 in various dendrimer-PLGA NC formulations mediated apoptosis of cancerous hepatocytes as revealed by fluorescence microscopy (magnification 100X). Hepatocytes isolated from (A) Untreated control (B) free siRNA and cisplatin mixture (C) PAMAM-siRNA@Cis-PLGA NC (D) Ab@PAMAM-siRNA@Cis-PLGA NC treated groups. Right Panel. DNA fragmentations as analyzed by Apo-BrdU study using flow cytometer. Liver cancer cells isolated from various treated groups. (A) Untreated control (B) free siRNA and cisplatin mixture (C) PAMAM-siRNA@Cis-PLGA NC (D) Ab@PAMAM-siRNA@Cis-PLGA NC. Data are representative of at least three independent experiments with similar observations.

3.3.10. Histopathological analysis

The efficacy of various forms of PLK-1-specific siRNA was established by histopathological studies. Liver tissue samples from mice belonging to various treated groups were used for histopathological analysis. Figure 3.8 shows sections of liver cancer cells after treatment with free siRNA and cisplatin mixture, PAMAM-siRNA@Cis-PLGA NC and Ab@PAMAM-siRNA@Cis-PLGA NC formulations. Control healthy liver tissue shows normal hepatic laminae, sinusoids and hepatocytes (Figure 3.8A). No congestion and infiltration was observed; whereas, cancerous liver (untreated control; Figure 3.8B) shows a loss of normal hepatic structure, including laminae and sinusoids, dilated central vein and widespread hepatocyte necrosis. The mixture of free siRNA and cisplatin treated group showed cancer regression, but was associated with poorly preserved hepatocellular architecture (Figure 3.8C). Most of the hepatocytes were accompanied with eosinophilic as well as abundant Kupffer cells. The treatment with PAMAM-siRNA@Cis-PLGA NC formulation resulted in mild recovery of cancerous liver (Figure 3.8D), showing enlarged hepatocyte contour, narrowing of sinusoidal space, mild apoptosis and Kupffer cell prominence was still predominantly seen. By contrast, in the mice treated with Ab@PAMAM-siRNA@Cis-PLGA NC formulation (Figure 3.8E), noticeable tumor regression in comparison with PAMAM-siRNA@Cis-PLGA NC formulation and free siRNA and cisplatin mixture was observed. Maintained hepatic microstructure, focal necrosis and apoptosis were also detected. Ab@PAMAM-siRNA@Cis-PLGA NC treatment showed the best regression where normal hepatic architecture was found to be maintained. Focal lytic apoptosis was also very much apparent.
Figure 3.8. Histopathological studies showing effect of Ab@PAMAM-siRNA@Cis-PLGA NC formulation on DEN exposed mice. Photomicrographs of mouse liver from (A) Healthy control; (B) Untreated control; (C) free siRNA and cisplatin mixture; (D) PAMAM-siRNA@Cis-PLGA NC formulation; (E) Ab@PAMAM-siRNA@Cis-PLGA NC formulation (H&E, 200X). In PAMAM-siRNA@Cis-PLGA NC formulation treated group, lobular architecture, hepatic laminae, contour and staining of hepatocytes is markedly visible. Ab@PAMAM-siRNA@Cis-PLGA NC formulation treated group had shown better recovery in DEN induced necrosis when compared to PAMAM-siRNA@Cis-PLGA NC formulation. Control groups highlight profound liver necrosis. Data are representative of at least three independent experiments with similar observations.
3.3.11. Effect of Ab@PAMAM-siRNA@Cis-PLGA NC formulation on survival of mice with liver cancer

The anticancer efficacy of Ab@PAMAM-siRNA@Cis-PLGA NC, PAMAM-siRNA@Cis-PLGA NC as well as free siRNA and cisplatin mixture against DEN-induced liver cancer at various time points in terms of animal survival is shown in Figure 3.9A. Ab@PAMAM-siRNA@Cis-PLGA NC treated animals showed 80% survival, whereas PAMAM-siRNA@Cis-PLGA NC formulation could offer 60% survival for the treated mice in 12 weeks duration ($p < 0.01$). No mice survived in the siRNA+Cis group after the eleventh week post-treatment, while untreated animals and animals administered PAMAM@PLGA NC succumbed to death by the seventh and sixth week post-treatment respectively ($p < 0.05$; Figure 3.9A). We have also used the weight gain of treated animals as a parameter to assess efficacy of Ab@PAMAM-siRNA@Cis-PLGA NC formulation. Exposure to the carcinogenic agent, DEN, resulted in gradual weight loss in mice as evident from the total weight determination of animals in control groups (untreated and PAMAM@PLGA NC treated animals) that went down to 7–8 g until the 50th day after exposure. The treatment with Ab@PAMAM-siRNA@Cis-PLGA NC prevented weight loss and also mitigated it to the original level to a larger extent by the 50th day of DEN exposure as compared to groups treated with PAMAM-siRNA@PLGA-Cis NC ($p < 0.01$) and free siRNA and cisplatin mixture ($p < 0.001$). PAMAM-siRNA@Cis-PLGA NC treated animals did exhibit gradual weight loss but significantly lower than the group treated with free siRNA and cisplatin mixture ($p < 0.05$, Figure 3.9B). Organomegaly of vital organs (i.e., liver, kidney and spleen) was also found to be retreated after treatment with Ab@PAMAM-siRNA@Cis-PLGA NC. Ab@PAMAM-siRNA@Cis-PLGA NC formulation was found to be more effective in normalizing the increased mass of organs than the PAMAM-siRNA@Cis-PLGA NC ($p < 0.05$) and siRNA+Cis ($p < 0.01$) formulations (Figures 3.9C–3.9E).
Figure 3.9. Effect of Ab@PAMAM-siRNA@PLGA-Cis NC formulation on survival, weight of various vital organs and overall weight of mice inflicted with DEN-induced liver cancer. (A) Survival curve showing effect of Ab@PAMAM-
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siRNA@PLGA-Cis NC and other formulations in terms of percentage of tumor-bearing animals at different time intervals (Ab@PAMAM-siRNA@PLGA-Cis NC vs PAMAM-siRNA@PLGA-Cis NC at week twelve, $p < 0.01$). The analysis was made on a weekly basis. (B) Animal weight total, (C) liver weight, (D) kidney weight and (E) spleen weight. The data are mean values ± standard deviation from three different experiments. (For liver, kidney as well as spleen: Ab@PAMAM-siRNA@Cis-PLGA NC vs PAMAM-siRNA@Cis-PLGA NC, $p < 0.05$; Ab@PAMAM-siRNA@Cis-PLGA NC vs siRNA+Cis, $p < 0.01$).
3.4. Discussion

Hepatocellular carcinoma (HCC) is believed to be the fifth most common solid tumor worldwide, and is one of the leading causes of cancer-related death (Farazuddin et al., 2014). Because it is generally diagnosed at an advanced stage, available chemotherapy modules fail to cure HCC. Moreover, available chemotherapeutic agents against cancer suffer from intrinsic toxicity issues when used in clinical settings. Therefore development of alternative approaches or improvement in the current therapeutic strategies is warranted. Gene therapy is emerging as a powerful strategy for treatment of various cancers including hepatocellular carcinoma (Raskopf et al., 2008; Li et al., 2013; Bogorad et al., 2014) because of the genetic link associated with tumor development and progression. With a deeper understanding of the genetic aberrations involved in cancer cells, the delivery of nucleic acid therapeutics (e.g. DNA, siRNA, shRNA, antisense oligonucleotides) to down-regulate or replace mutated genes, and to silence unwanted gene expression, is becoming a highly attractive approach to suppress tumor cell growth and invasion. There have been intensive efforts to develop safe and efficient gene delivery materials to provide high transfection efficiency at the desired site of action. While initial preclinical and clinical studies are promising, the molecular complexity of cancer suggests that the use of therapeutics alone may be insufficient to halt the progression of most cancers. Thus, combination therapies using nucleic acid and anticancer drugs represent a more promising approach (Saraswathy and Gong, 2014). In fact, several studies have demonstrated the efficacy of siRNA in combination with chemotherapeutics in combating cancer (Dong et al., 2013; Jung et al., 2012; Xu et al. 2013). Moreover, one promising approach to treating cancer is the use of ligand/antibody-conjugated nanoparticles that target tumor cells/blood vessels. Antibodies against the surface markers of cancers conjugated to drug loaded nanoparticles facilitate specific binding to tumor targets to precisely deliver chemotherapeutic agents to tumor cells. In this study, we synthesized cisplatin loaded PLGA nanoparticles decorated with PLK-1 (Polo-like kinase 1) specific siRNA complexed PAMAM dendrimer which were further functionalized with tumor specific polyclonal antibodies. Figure 3.10 depicts the schematic illustration of the synthesis of liver cancer specific antibody conjugated dendrimer-siRNA complex decorated cisplatin entrapped PLGA nanocomposites.
Figure 3.10. Schematic representation of the steps involved in the synthesis of liver cancer cell specific antibody conjugated dendrimer-siRNA decorated cisplatin entrapped PLGA nano-composite

PLK-1 owing to an important regulator of cell-cycle progression represents an ideal target for cancer drug development (Chauhan et al., 2014). Over-expression of PLK-1 has been shown to result in the formation of abnormal centrosomes and mitotic spindle poles, which have been correlated with aneuploidy and chromosomal instability leading to tumor development (Chauhan et al., 2014). The purpose behind selecting cisplatin for the synthesis of nanocomposite for the present study was that cisplatin being DNA adduct forming chemotherapeutic bears potential to cure a broad spectrum of malignancies (Xu et al., 2013). The multifunctional nanocomposite in the present study bears the potential to deliver the siRNA and cisplatin to the cytosol of target cells which is pre-requisite for their optimal anti-tumor activity. Since RISC resides within the cytosolic compartment of the cell, siRNA can exert its effect only when delivered to the cytosol. Moreover, as cisplatin makes DNA adduct to induce its cytotoxicity, it too needs to be delivered to the cytosol. The in-house synthesized
nanocomposite formulation was henceforth decorated with PAMAM-dendrimer as PAMAM-dendrimers being cationic polymers like PEI are able to disrupt intracellular organelles through a "proton sponge effect". Hyperbranched PAMAM dendrimer abounds in multivalent amine groups and has the ability to accept the protons, leading to an osmotic pressure build up across the organelle membrane. The osmotic pressure might cause a disruption of the acidic endosomes and the release of the entrapped materials into the cytoplasm (Tu et al., 2012). However, PAMAM exhibits the high cytotoxicity due to the excess of cationic charges. To reduce toxicity and improve the biocompatibility, PAMAM were conjugated to PLGA nanoparticles since it has been reported that PLGA PAMAM composite bears far less toxicity than PAMAM dendrimer alone (Intra and Salem, 2010).

The data of the present study establish higher efficacy of tumor-specific antibody conjugated PAMAM-siRNA decorated cisplatin entrapped PLGA nanocomposite formulation which was assessed on the basis of its ability to modulate pro/anti-apoptotic factors, induce caspase-9 production, augment DNA fragmentation and enhance survival of the treated animals. The increased efficacy could be attributed to the fact that Ab@PAMAM-siRNA@Cis-PLGA NC formulation may have delivered the payload (siRNA and cisplatin) to the tumor cells specifically facilitated by binding of tumor-specific antibody with the antigens expressed on the tumor cell surface. SEM micrograph of cisplatin entrapped PLGA nanoparticle (Figure 3.1A) in addition to confirming the synthesis of the particles indicates its size to be approx. 100-200 nm in concordance with a previous report (Moreno et al., 2010). The conjugation of PAMAM dendrimer to PLGA NP was confirmed by ninhydrin assay and TEM analysis as shown in Figure 3.1B and C respectively. Figure 3.2 ascertains the conjugation and complexation of tumor specific antibody and siRNA to dendrimer respectively.

Toxicity studies overruled the systemic, hepatic and renal toxicities of Ab@PAMAM-siRNA@Cis-PLGA NC formulation (Figures 3.3 and 3.4). Further, the study was extended to gain insight in Ab@PAMAM-siRNA@Cis-PLGA NC mediated modulation of various proteins involved in the apoptosis of tumor cells of various experimental groups. The Ab@PAMAM-siRNA@Cis-PLGA NC formulation was successful in downregulating the expression of target protein PLK-1 and therefore enhancing the expression of p53wt and bax and reducing the expression of p53mut.
and Bcl2 (Figure 3.5). On the other hand, free siRNA and cisplatin mixture or PAMAM-siRNA@Cis-PLGA NC could feebly modulate the expression of these proteins. In fact, tumor cells over-express PLK-1-mediated cyclin-dependent kinase 1 (Cdc2)/cyclin B1 that help in rapid proliferation of the cells. This ensues in less production of p53wt and upregulated production of p53mut. Earlier studies have demonstrated involvement of the suppressor p53 in PLK-1 depletion-induced apoptosis. In principle, the silencing of the PLK-1 gene should reinstate normal production of the p53wt protein. As depicted in Figure 3.5B, comparatively higher expression of p53wt protein was recorded in the cells treated with Ab@PAMAM-siRNA@Cis-PLGA NC compared with PAMAM-siRNA@Cis-PLGA NC administered group. The data clearly suggest that targeted delivery of siRNA and cisplatin plays a determining role in upregulation of p53wt expression. Moreover, apoptosis induction by Ab@PAMAM-siRNA@Cis-PLGA NC was further ascertained by increased annexin-V binding and reduced mitochondrial membrane potential in liver cells isolated from Ab@PAMAM-siRNA@Cis-PLGA NC treated group (Figure 3.6). An augmented apoptosis induction in the liver cancer cells by Ab@PAMAM-siRNA@Cis-PLGA NC in comparison to PAMAM-siRNA@Cis-PLGA NC and free siRNA and cisplatin mixture was further supported by caspase-9 level detection and APO-BrdU™ TUNEL assay employing FACS analysis (Figure 3.7).

Further, histopathological analysis of tissue from various treated groups of mice established higher efficacy of Ab@PAMAM-siRNA@Cis-PLGA NC. Histopathological studies of liver revealed prominent recovery and maintainance of general architecture of hepatic tissue upon treatment with Ab@PAMAM-siRNA@Cis-PLGA NC formulations (Figure 3.8). Administering Ab@PAMAM-siRNA@Cis-PLGA NC formulation resulted in an efficient suppression of tumor growth, significant mitigation of the weight loss of cancer inflicted animals and enhanced survival in comparison to the PAMAM-siRNA@Cis-PLGA NC formulation treated group (Figure 3.9).

Taken together, data of the present study suggest that Ab@PAMAM-siRNA@Cis-PLGA NC formulation provides a robust and modular platform for targeted and synchronous delivery of siRNA and cisplatin for treatment of hepatocellular carcinoma.
Immunization with dendritic cell targeted tumor antigens employing PLGA-PEG immuno-nanocomposite followed by dendroosome based topical siRNA therapy exerts highly effective anti-tumor response in skin papilloma mouse models
4.1. Introduction

Developing cancer vaccines is an attractive approach towards activating a robust antitumor immunity. For this promising anti-cancer strategy, efficient delivery of the tumor-associated Ags to the APCs and strong cross-presentation of spliced peptides on MHC class I molecules on APCs are crucial (Mukai et al., 2011; Banchereau and Steinman, 1998). Efficient MHC class I presentation enhances the production of tumor-associated Ag-specific CTL, the major effector cells in tumor immunity (Mukai et al., 2011; Huang et al., 1994). PLGA NPs have been reported to induce efficient cross presentation of the entrapped antigens and hence are useful toward designing vaccine carriers that induce Ag-specific CTL in vivo (Silva et al., 2013; Shen et al., 2006), a pre-requisite in cancer treatment. Choosing the delivery vehicle with coveted benefits is not enough, understanding the accessibility of the antigen to APCs especially DCs, the chief APCs, in vivo is of utmost importance in designing an antitumor vaccine. Dendritic cells utilize several surface receptors to internalize, process and present antigen to both CD4\(^+\) and CD8\(^+\) T cells (Tappertzhofen et al., 2014). Therefore, targeted delivery of antigen to DC has been proven to be a very efficient strategy for induction of antigen-specific T cell response (Trumpfheller et al., 2012). A number of C-type lectin receptors (CLR) have been explored as target receptors for antibody-mediated antigen delivery, including DEC205 (CD205), CD11c, Dectin-1 and -2, DNGR1 (Clec9A) and DCIR2 (Apostolopoulos et al., 2013; Caminschi et al., 2009). Targeted delivery of antigens to CLR leads to efficient induction of humoral and cellular responses and has been shown to be efficient in inducing anti-tumour immunity (Apostolopoulos et al., 2013). Although vaccines represent a strategic successful tool used to prevent or contain diseases with high morbidity and/or mortality and have proven to be effective in combating pathogenic microorganisms, based on the immune recognition of these foreign antigens, vaccines aimed at inducing effective antitumor activity are still unsatisfactory (Fishman, 2014). A possible approach may be to combine the benefits of tumor vaccine with a drug/gene/siRNA therapy so as to develop a potential anti-tumor strategy harboring the attributes of a vaccine and a therapeutic as well.

Selective silencing of gene expression using siRNA has been investigated as a potential therapeutic approach to cancer. However, as a result of its large molecular weight (MW 13 kDa) and polyanionic nature (40 phosphate groups), naked siRNA
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does not freely cross the cell membrane, and if however it somehow crosses the membrane, rapid enzymatic degradation, and substantial liver and renal clearance restrict the therapeutic applications of siRNA in vivo (Pan et al., 2014). Consequently, development of efficient delivery systems that protect siRNA from degradation and facilitate uptake by target cells is one of the major challenges in the therapeutic applications of RNAi. Several types of synthetic vectors have been investigated for gene silencing applications, including the development of cationic lipids, liposomes, or both; cationic polymers; cationic dendrimers (branchlike polymer structures); and cationic cell-penetrating peptides (Lee et al., 2013). Dendrosomes, the lipophilic vesicles encapsulating the dendrimer-oligonucleotide complex have recently begun to be exploited for various gene therapies including cancer (Dutta et al., 2010). They possess negligible hemolytic toxicity and higher transfection efficiency, and they are better tolerated in vivo than dendrimers. Dendrosome based delivery of E6 and E7 oncogene specific siRNA has been found to be effective against cervical cancer cell lines (Dutta et al., 2010). Therefore, dendrosomes can be said to hold potential for delivery of siRNA for treating cancer.

Of the various options available for the administration of medications, topical application is the most promising approach for treating skin tumors as it leads to a localized effect at the desired site with minimal side effects (Khan et al., 2007). However, topical application of naked siRNA in both normal as well as tumor afflicted tissues is marred by several challenges like lesser absorption into skin owing to cellular and tissue barriers, feeble retention and enzymatic degradation of naked siRNA due to the presence of nucleases in skin (Pan et al., 2014). However, recently a few reports have shown success in topical delivery of siRNA. SPACE peptide when conjugated to cargos such as small molecules and proteins, was able to facilitate their penetration into epidermis and dermis (Hsu and Mitragotri, 2011). Moreover, conjugation of the peptide to siRNA has led to their enhanced absorption into skin and knockdown of corresponding protein targets. In another study, it has been shown that spherical nucleic acid nanoparticle conjugates, gold cores surrounded by a dense shell of highly oriented, covalently immobilized siRNA, freely penetrate almost 100% of keratinocytes in vitro, mouse skin, and human epidermis within hours after application (Zheng et al., 2012).
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Keeping into consideration the above mentioned details, in the present study, model animals were first immunized with tumor associated antigen (TAA) entrapped anti-DEC 205 antibody functionalized PLGA PEG NCs so as to facilitate targeted delivery of TAAs to DCs. Prophylactic potential of delivered NCs was investigated against DMBA induced skin papilloma in model animals. Tumor regression efficacy of dendrosome mediated topical delivery of siRNA in pre-immunized animals was further evaluated. The siRNA based treatment study was performed on the premise that the leaky vasculature of skin papilloma owing to EPR effect (Khan et al., 2007) would capture the nanosized siRNA bearing dendrosomes upon their topical administration onto the tumor nodules and may bear potential to suppress skin cancer. The in-house developed antibody functionalized PLGA PEG NCs and dendrosomes were characterized for their size, entrapment efficiency of respective cargos and surface functionalization. Besides assessment of survival of cancer inflicted animals, the histopathological study and measurement of body weight, tumor volume and number were used as parameters to establish the efficacy of combined therapy.
4.2. Materials and methods

4.2.1. Materials

All the reagents used in the study were of highest purity available. DMBA, anti-DEC205 mouse monoclonal antibody and PLK-1 specific siRNA were purchased from Santa Cruz Biotechnology (USA). IFN-γ, IL-6, IL-12, TNF-α kits, anti-p53 wild type (wt), anti-bax, anti-cyclin and anti-β-actin antibodies and BrdU TUNEL Assay kit were purchased from BD Biosciences (San Diego, CA). Egg phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabaster, Alabama USA). FITC labelled 2' antibody, BCA Protein Assay kit, NHS, DCC, PEGdiamine, MBS, PLGA polymer, PAMAM dendrimer generation 4, HBSS, RPMI, MTT dye, antibody and cholesterol were purchased from Sigma Aldrich (St. Louis, USA). PVDF membrane was purchased from Millipore India Pvt. Ltd. (Bangalore, India). DCM, acetone and PVA were purchased from Merck, India. All others reagents were of analytical grade and procured from local suppliers.

4.2.2. Animals

Female Swiss albino mice of weight 20 ± 3 g were obtained from the institute’s animal house facility. The animals were housed in polypropylene cages on wood powder bedding in an air-conditioned ambience. Animals were quarantined on equal light/dark cycles (12/12 hour) and were kept on a pellet diet (Ashirwad, Chandigarh, India) and water ad libitum. Animals were examined for their mortality and morbidity prior to commencement of the study, and only healthy animals were included in the experiments. The techniques used for administration of various formulations as well as sacrifice of the animals were strictly performed following mandates approved by the animal ethics committee (Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India).

4.2.3. Methods for prophylactic study

4.2.3.1. Tumor induction and isolation of tumor cell proteins

Animals in the resting phase of hair cycle were taken for the study. First of all, tumors were induced in a few animals so as to prepare lysate from excised tumors for tumor antigen immunization following the procedure described elsewhere (Khan et al., 2007). Briefly, hair of the animals were removed from the inter-scapular region over an area of 2 cm² using electric clippers that were not lubricated with any oil or grease.
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The skin of the shaved dorsal portion of mice was exposed to DMBA (52 µg in 200 µl acetone) that was applied topically three times a week for 12 weeks. After 12 weeks, when average size of the tumors was about 120 mm$^3$, the mice were sacrificed and tumor was excised. A tumor cell lysate was prepared according to a previously published procedure (Farazuddin et al., 2012). The protein content of the lysate was estimated using BCA Protein Assay Kit (Sigma, India) with BSA as protein standard.

4.2.3.2. Synthesis of PLGA-PEG diblock copolymer

PLGA-PEG diblock copolymer was synthesized by DCC and NHS chemistry following the procedure as described elsewhere (Dhankar et al., 2011). The PLGA (100 mg) was dissolved in DCM (20 ml). After that DCC (0.24 g) and NHS (0.13 g) were added under magnetic stirring. The activation reaction of carboxylic acid end group of PLGA was continued by 12 hrs magnetic stirring at room temperature. Insoluble dicyclohexyl urea was filtered and the resultant solution was precipitated by dropping into ice cold anhydrous diethyl ether. The activated PLGA was completely dried under vacuum. The NHS ester of PLGA was conjugated with PEG diamine via amide linkage. The activated PLGA (100 mg) was dissolved in DCM (20 ml) and then excess of PEG-diamine (100 mg) was added. The coupling reaction was performed under magnetic stirring for 9 hrs at room temperature. An excess amount of PEG-diamine was used to prevent the formation of PLGA-PEG-PLGA triblock copolymers. The amine terminated PEG-PLGA diblock copolymer was precipitated with cold methanol and purified with excess of ethanol (to eliminate unconjugated PEG diamine) and finally dried under vacuum. The synthesized PLGA-PEG diblock copolymer structure was confirmed by FTIR and XRD.

4.2.3.3. FTIR of PLGA-PEG diblock co-polymer

Fourier transform infrared spectroscopic measurement of the PLGA-PEG co-polymer was carried out by depositing purified polymer on Si (111) wafers with simple dropcoating and subjecting them to analysis (6700 spectrum; Thermo Nicolet, Madison, WI) in a diffuse reflectance mode at a resolution of 4 cm$^{-1}$ (Chauhan et al., 2011).

4.2.3.4. XRD analysis of PLGA-PEG co-polymer

An X-ray diffractometer (Rigaku, Mini Flex II powder diffractometer), was used for diffraction studies. The samples were exposed to CuKα radiation (40 kv, 20 mA) and
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scanned from 0° to 85°, 2θ at a step size of 0.02°, and step time of 5 sec (Gajendiran et al., 2013; Derakhshandeh, 2010).

4.2.3.5. Preparation of tumor antigen encapsulated PLGA-PEG nanocomposite

The preparation of nanocomposites was conducted using minor modifications of a previously published water-in-oil-in-water (w/o/w) solvent evaporation method as standardized in our laboratory (Garinot et al., 2007). Briefly, a known quantity of tumor antigen (30 mg) was dissolved in 1 ml of PBS buffer, mixed with PLGA-PEG copolymer solution (300 mg of PLGA-PEG copolymer dissolved in 5.0 ml DCM), and sonicated in a bath-type sonicator to form the primary emulsion. The primary emulsion was mixed with 100 ml of 10% PVA (w/v) and homogenized using a Silverson L4RT homogenizer (Silverson Machines, East Longmeadow, MA). The resulting w/o/w emulsion was stirred at 25°C for 18 hrs to allow solvent evaporation and formation of antigen-entrapped PLGA-PEG nanoparticles. The nanocomposites were centrifuged at 20,000 g and thoroughly washed with phosphate buffered saline (PBS) (0.15 M NaCl containing 20 mM sodium phosphate, pH 7.4) to remove PVA. The nanoformulation was lyophilized and finally stored at 4°C until further use.

4.2.3.6. Preparation of PLGA-PEG immuno-nanocomposites

The immuno-nanocomposites were prepared by slight modifications in the method reported by Dhankar et al., 2011. The preparation of immuno-nanocomposites includes three steps:

1. Activation of NH₂ group of PLGA-PEG nanocomposites with MBS ester.
2. Thiolation of anti-DEC 205 monoclonal antibody.
3. Conjugation of thiolated anti-DEC 205 mAb on the surface of MBS activated nanocomposites.

In the first step, PLGA-PEG nanocomposites (5 mg) were dispersed in 1 PBS (pH7.2) and incubated with 100 µl crosslinker MBS solution (3.14 mg MBS dissolved in 1 ml of DMSO) for 30 min at RT in shaker incubator. The excess of MBS was separated from activated nanoparticles using sephadex G25. Secondly, for thiolation of anti-DEC 205 mAb, 5.7 mg of 2-iminothiolane (Traut’s Reagent) was dissolved in 5 ml PBS (pH 7.4) and 100 µl anti-DEC 205 mAb was incubated with 4.2 µl of 2-iminothiolane for 2 hrs at 20°C under constant shaking. Finally, 100 µl thiolated mAb
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was added in 100 µl MBS-activated nanoparticles for 30 min at RT under constant shaking for preparation of PLGA-PEG immuno-nanocomposites.

4.2.3.7. Characterization of PLGA-PEG nanocomposite size and surface morphology

The size and shape of as-synthesized antibody conjugated PLGA-PEG nanocomposites was determined by SEM (Farazuddin et al., 2012). For electron microscopic analysis, SEM (JEOL, Japan) was used. The lyophilized preparation of antigen loaded PLGA-PEG nanocomposites was mounted on clear glass stub and coated with gold-palladium alloy using a sputter coater. An accelerating voltage of 20 kV was used for imaging.

4.2.3.8. Antigen entrapment efficiency

Entrapment of tumor antigens in PLGA-PEG nanocomposites (not conjugated to antibody) was assessed by dissolving an aliquot of the NCs in 0.1 N NaOH followed by analysis of protein content by BCA Protein Assay kit (Sigma, India) following manufacturer's instructions. Entrapment of antigen was calculated with the help of standard curve plotted using BSA. The percentage entrapment efficiency (% EE) was calculated with the following formula:

\[
\% \text{ EE} = \frac{\text{Amount of protein entrapped}}{\text{Total amount of protein used in the beginning}} \times 100
\]

4.2.3.9. Determination of the conjugation of anti-DEC 205 mAb onto PLGA-PEG nanocomposite surface

The as-synthesized PLGA-PEG NCs were characterized for antibody conjugation employing protein estimation using BCA Protein Assay kit following manufacturer's instructions and detection by FITC labelled anti-mouse 2' antibody. To 50 µl of PLGA-PEG immuno-nanocomposite solution, FITC tagged anti-mouse 2'antibody was added in the ratio 1:2500 and incubated for 30 min. The incubated solution (10 µl) was dropped on a glass slide to be analysed under fluorescence microscope (magnification, 100X).

4.2.3.10. Mice immunization for prophylactic studies

In prophylaxis studies, female Swiss albino mice were immunized by subcutaneous injection in the abdominal flank with 50 µg free antigen/mouse, 50 µg antigen
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entrapped in PLGA-PEG NC/mouse, 50 μg antigen entrapped in antibody tagged PLGA-PEG NC/mouse or PBS alone at weeks 1, 2, and 4. One week after the last immunization, skin carcinogenesis was induced in the mice by following the protocol discussed above under the subtitle “tumor induction and isolation of tumor cell proteins”. The experiment was terminated at 21 weeks after DMBA exposure. Each group contained at least 10 mice. The various groups employed for the study are as detailed in Table 4.1.

Table 4.1. Various groups of animals used for prophylactic study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Formulations administered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1.</td>
<td>Unimmunized control animals</td>
</tr>
<tr>
<td>Group 2.</td>
<td>Animals immunized with free skin cancer antigen (not encapsulated in NPs); Free Ag group</td>
</tr>
<tr>
<td>Group 3.</td>
<td>Mice immunized with skin cancer antigens entrapped within PLGA-PEG NCs; PLGA-PEG NC [Ag] group</td>
</tr>
<tr>
<td>Group 4.</td>
<td>Animals immunized with anti-DEC mAb conjugated and skin cancer antigen entrapped PLGA-PEG NC formulation; Ab@PLGA-PEG NC [Ag] group</td>
</tr>
</tbody>
</table>

4.2.3.11. Tumor Measurement

The tumor mass was isolated aseptically. The diameter of the tumors was measured using a Digital Vernier Caliper and the tumor size was determined by the formula:

\[ V = D \times d^2 \times \frac{\pi}{6} \]

(where 'V' denotes tumor volume, 'D' is the biggest dimension and 'd' denotes smallest dimension).

4.2.3.12. Isolation of T lymphocytes from spleens of immunized mice

Single cell suspension of the spleen was prepared as described elsewhere (Ansari et al., 2011). Briefly, spleens isolated from animals belonging to various immunized groups were macerated and suspension was treated with ACK lysis buffer (0.15 mol/L ammonium chloride, 10 mmol/L potassium bicarbonate, and 88 mmol/L edetic acid) for lysis of the red blood cells. The cell suspension was centrifuged at 1500 g for 5
min and cell pellet was washed with HBSS solution 3 times and resuspended in RPMI 1640 medium containing 10% fetal bovine serum and 0.1% antimycotic cocktail.

4.2.3.13. Cytokine assay: Determination of IFN-γ, IL-6, IL-12 and TNF-α
Cytokines induced by splenocytes isolated from various groups upon their co-culture in the presence of skin cancer antigens were estimated using appropriate and specific biotinylated antibody pairs according to the manufacturer's protocols. Briefly, 50 μl of the purified capture antibodies were adsorbed overnight on polystyrene microtitre plates at 4°C in carbonate buffer of pH 9.5. Plates were washed five times with PBST and blocked with 5% skimmed milk. After the usual steps of washing, 50 μl of the supernatant (isolated from cultured splenocytes after 48 hrs) was dispensed in each well to determine its cytokine content. After stipulated incubation time, the plates were thoroughly washed and incubated with biotinylated polyclonal goat anti-mouse cytokine detection antibody. Afterward, the plates were washed three times with PBST. Subsequently, 100 μl of streptavidin-HRP conjugate was added to each well and plate was incubated for 30 min at RT. The plates were again washed three times with PBST and finally colored complex was developed with tetra methyl benzidine. The absorbance was read at 450 nm with a microtitre plate reader (Bio-Rad). A known specific recombinant cytokine was used as standard for calculating level of given cytokine in the samples tested and concentration was expressed as pg/mL.

4.2.4. Methods for therapeutic study

4.2.4.1. Preparation of dendrimer-siRNA complex
The dendrimer-siRNA complex was prepared by following the protocol as published elsewhere (Dutta et al., 2010). Briefly, 50 nM of siRNA was added to fourth-generation PAMAM dendrimers (N/P ratio 100) in phosphate buffer saline (PBS pH 7.4) and incubated at 25°C for 20 min to allow complex formation.

4.2.4.2. Preparation of dendrosome
The in-house prepared dendrimer-siRNA complex was used for the preparation of dendrosomes which were synthesized as described elsewhere (Dutta et al., 2010). Briefly, PC and Cholesterol were dissolved in diethyl ether, in varying molar ratios, to which was added the solution of dendrimer-siRNA complex in distilled water. The mixture was sonicated for 5 min at room temperature (25°C-27°C), followed by vortexing the thick emulsion to remove traces of organic solvent. Dendrosome
formation takes place by phase inversion. The dendosome so formed was size-reduced by passing through a 0.2 µm membrane (Whatman, Maidstone, United Kingdom) using a Lipex Extruder (Northern Lipids Inc., Burnaby, British Columbia, Canada). Multiple passes (10 times) were needed to obtain the desired particle size (approx. 200 nm). Purification of dendosomes was carried out by centrifugation followed by re-suspending the dendosomes in distilled water.

4.2.4.3. Characterization of dendosomes for size and morphology
TEMP samples were prepared following protocol as published elsewhere (Farazuddin et al., 2012) by placing a drop of synthesized nanoparticles over gold coated negative grid followed by evaporation of the solvent. TEM analysis was performed on JEOL model (1200 EX, JOEL Inc, Peabody, MA) which was operated at an accelerating voltage of 200 kv.

4.2.4.4. Entrapment efficiency of dendrimer-siRNA complex in dendosomes
The entrapment efficiency (ratio of the amount of dendrimer-siRNA complex entrapped to the amount of complex added expressed as a percentage) was determined by lysing dendosomes with n-propanol. A dispersion of dendosomes in distilled water was mixed with an equal volume of n-propanol and vortexed for 10 min. The resulting solution was centrifuged for 15 min at 2500 g. Supernatant was withdrawn and analyzed using UV spectrophotometry at 260 nm after appropriate dilutions against a dendrimer solution as reagent blank.

4.2.4.5. In vitro release kinetics of active siRNA from dendosomes
In order to assess the release kinetics of siRNA from dendosomes, dendrimer-siRNA complex bearing dendosomes were dispensed in microvials containing buffer (PB) of pH 7.4. To 1.5 ml vial, 50 µl of dendrimer-siRNA-dendosome stock formulation (in sterile normal saline) was added and volume was made up to 1 ml with buffer. The mixture was allowed to incubate at 37°C. Aliquots (100 µl) of supernatant were removed after centrifugation at 9,168 g for 10 min at different time intervals and were analyzed for siRNA content. Release runs were continued for 7 days. The aliquots were subjected to HPLC analysis to determine siRNA content. The calculated amount of the released siRNA (in %) in PB (pH 7.4) was plotted against time.
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4.2.4.6. Cytotoxicity studies
Epidermoid cancer cell line procured from NCCS PUNE (India) was maintained in RPMI 1640 media supplemented with 10% FCS, antimycotic and antibiotic solution (sigma) at 37°C in 5% CO₂ atmosphere. Cytotoxicity of dendrimer and dendrosome were assessed on epidermoid cell line following the protocol of MTT assay as published elsewhere (Farazuddin et al., 2012). Briefly, cells were plated at a density of 2.5 X 10⁴ cells per well in 96 well plate before 24 hrs of treatment. Cells were incubated with 50 µl dendrimer, 50 µl dendrimer-siRNA complexes containing 50 nM of siRNA and PC along with 100 µl complete medium for 4hr at 37°C. After 4 hrs incubation, medium was removed followed by washing with sterile PBS and fresh medium of 100 µl was added. After 24 hr addition of medium, cell viability was measured by adding 20 µl of MTT dye (5 mg/ml) in sterile PBS. Plate was incubated further for 3 hrs at 37°C. Formazan crystal formed due to reduction was dissolved in DMSO and absorbance was read at 570 nm. The absorption values were expressed as cell viability (%), considering untreated cells as a 100%.

4.2.4.7. In vitro study of PLK-1 depletion and analysis for the presence of apoptotic factors in epidermoid cell line
Epidermoid cells were incubated with different formulations of siRNA after 80% confluency. After 6 hrs of incubation with various siRNA formulations, fresh medium containing 20% FCS was added and cells were again incubated for 24 hrs at 37°C in 5% CO₂ atmosphere. After incubation, the cells were scraped, centrifuged, and washed with RPMI medium and lysed with TNN lysis buffer. The lysate was subjected to 12% SDS PAGE, and gels were electroblotted onto PVDF membrane. The membrane was probed for the presence of p53 wt, Bax and PLK-1 using specific antibodies and the bands were developed onto X-ray film by ECL using ECL kit (BioRad).

4.2.4.8. In vivo study; treatment schedule
Tumor induction was done as detailed in ‘methods for prophylactic study’ section. For assessment of anticancer efficacy of siRNA dendrosomes, tumor bearing animals were divided into five different groups each consisting of not less than 10 animals. The various groups employed for the therapeutic study are detailed in Table 4.2.
Table 4.2. Various groups of animals used for therapeutic study

<table>
<thead>
<tr>
<th>Groups</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Untreated control animals</td>
</tr>
<tr>
<td>Group 2</td>
<td>Mice exposed to mixture of dendrimer and PC; dendrimer+PC group</td>
</tr>
<tr>
<td>Group 3</td>
<td>Animals treated with free siRNA; Free siRNA group</td>
</tr>
<tr>
<td>Group 4</td>
<td>Animals belonging to the group treated with dendrimer conjugated siRNA; dendrimer-siRNA</td>
</tr>
<tr>
<td></td>
<td>mice</td>
</tr>
<tr>
<td>Group 5</td>
<td>Mice treated with siRNA-dendrimer complex encapsulated in dendosome; dendosome-siRNA</td>
</tr>
<tr>
<td></td>
<td>treated group</td>
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</table>

4.2.4.9. Topical delivery of siRNA

Various siRNA formulations (siRNA-dendrimer, siRNA-dendrimer-dendosome or free siRNA form) were applied onto the DMBA induced skin papillomas in Swiss albino mice according to the protocol approved by the Institutional Animal Ethics Committee. Mice were placed under anesthesia and 200 µl of 50 nM of siRNA in different formulations was topically applied over 2 cm² areas on the back of animal for consecutive 10 days.

4.2.4.10. Preparation of nuclear fraction

The skin tumor tissues were removed from experimental mice with sharp scalpel blades (Farazuddin et al., 2012). The tissue samples were placed on ice and fat was scraped off before further processing. Finally, the samples were homogenized to prepare nuclear fraction.

4.2.4.11. Western blot analysis

Skin papilloma homogenate (30 µg protein) was resolved on 10% SDS-PAGE gel and electroblotted onto PVDF membrane (Farazuddin et al., 2012). Employing an enhanced chemiluminescence kit, the membrane was probed for the presence of p53 wt, p53 mut, cyclin B1, Bax and PLK-1 using specific antibodies. Blots were re-probed with an antibody for β-actin and used as a control for equal protein loading and transfer.
4.2.4.12. Determination of caspase-9 level by confocal microscopy

Single cell suspension of skin papilloma cells was permeabilized with 0.1% Triton X100 in PBS at RT for 10 min followed by fixation in 4% paraformaldehyde for 2 hrs at room temperature. The fixed cells were blocked with 2% FCS followed by incubation with monoclonal antibody which detects only 35 Kda cleaved caspase 9. Finally, the cells were incubated with FITC tagged 2' antibody. Fluorescence microscopy was performed using Zeiss fluorescence microscope at 100X magnification.

4.2.4.13. TUNEL Assay

Single cell suspension of skin papilloma cells was prepared by pelleting cells at 300 g for 10 min at 4°C from various experimental groups. Cells were re-suspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4). Suspension was placed on ice for 30 min followed by centrifugation for 5 min at 300 g and supernatant was discarded. The cell pellet was resuspended in 70% (v/v) ice-cold ethanol followed by washing. After keeping for 30 min in 70% ethanol, cells were washed 3 times and pellet was resuspended in 50 μl of DNA labelling solution (Reaction buffer + TdT enzyme and Br-dUTP). Cells were incubated in the DNA labelling solution for 60 min at 37°C in a temperature controlled water bath. Cell pellet was incubated with the FITC-labelled anti-BrdU antibody staining solution in the dark for 30 min at room temperature. After labelling the tumor cells with FITC-tagged anti-BrdU antibody, PI/RNase (50 μg/ml) was added to the antibody labelled cells which were analyzed by flowcytometer (GUAVA, Millipore, MA, USA) followed by incubation for 30 min at 37°C.

4.2.5. Methods for combinatorial study

For combined skin papilloma antigen vaccination and siRNA therapy, the animals immunized with the formulation exhibiting the best prophylactic response (Ab@PLGA-PEG NC [Ag]) were selected and treated with the siRNA formulation showing the desirable therapeutic response (dendosome-siRNA). siRNA therapy was initiated in immunized animals after ten weeks of DMBA exposure. At the same time siRNA therapy was started in unimmunized animals as well. Various groups taken for combined study included in Table 4.3.
Chapter 4: Materials and methods

Table 4.3. Various groups of animals employed for combinatorial study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Formulations administered</th>
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<tbody>
<tr>
<td><strong>Group 1.</strong></td>
<td>Unimmunized and untreated control animals; unimmunized &amp; untreated group</td>
</tr>
<tr>
<td><strong>Group 2.</strong></td>
<td>Unimmunized but animals treated with siRNA-dendrostone; unimmunized &amp; treated group</td>
</tr>
<tr>
<td><strong>Group 3.</strong></td>
<td>Animals immunized with Ab@PLGA-PEG NC [Ag] but untreated; Immunized only group</td>
</tr>
<tr>
<td><strong>Group 4.</strong></td>
<td>Mice immunized with Ab@PLGA-PEG NC [Ag] and treated with dendrostone-siRNA as well; immunized and treated group</td>
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</table>

4.2.5.1. Histopathological studies

To examine the combined effect of immunization and siRNA therapy on the tumor treatment, three mice from each group were sacrificed by cervical dislocation and fixed in 10% formaldehyde solution. Tumor tissues were then excised and sections were prepared by using conventional paraffin sections and hemotoxylin-eosin staining.

4.2.6. Statistical analysis

Data were analyzed and two groups were compared with the Student $t$ test, and one way ANOVA (Holm-Sidak method) to assess the differences among various groups, using Sigma-Plot version 10 software. $P$ values $<$0.05 were considered to be significant.
4.3. Results

4.3.1. Results for nanocomposite characterization

4.3.1.1. Characterization of PLGA-PEG copolymer

In the present study, first PLGA-PEG copolymer was synthesized for further synthesis of PLGA-PEG nanocomposites. To confirm the success of PLGA-PEG conjugation, FTIR and XRD analyses were taken into consideration. Figure 4.1A shows the FTIR spectrum of our obtained product. The strong peak observed at 1767 cm\(^{-1}\) can be due to C=O double bond and the peak found at 3457 cm\(^{-1}\) can be attributed to N-H stretching vibration (Dao et al., 2014). These results confirmed the conjugation of PEG with PLGA since PLGA-PEG conjugation results in the formation of amide bond, therefore, FTIR vibrations for amide bond ascertain the synthesis of PLGA-PEG co-polymer which is however absent in PLGA and PEG only. The FTIR result was further supported by XRD analysis of PLGA-PEG co-polymer as shown in Figure 4.1B. The PLGA-PEG co-polymer exhibits a broad region from 10° to 30° and peaks at around 35°, 42°, 62° and 75°. This indicates crystallinity in the PLGA-PEG co-polymer, which is consistent with previous reports (Gajendiran et al., 2013).

![Graphs showing FTIR and XRD analysis of PLGA-PEG copolymer](image)

**Figure 4.1.** Characterization of PLGA-PEG copolymer. (A) FTIR spectrum of PLGA-PEG copolymer. (B) XRD analysis of PLGA-PEG copolymer. At least three independent experiments were performed for each sample and data are representative of at least two similar observations.
4.3.1.2. Size and shape analysis of in-house synthesized nanocomposites
As-synthesized PLGA-PEG nanocomposites and dendrosomes were analysed by SEM and TEM respectively. Figure 4.2A shows a representative SEM image of the tumor antigen-bearing PLGA-PEG NCs. Formation of spherical NCs with a size in the range of 100-200 nm is clearly revealed by SEM analysis. The shape and dimensions of siRNA-bearing dendrosomes were further established by TEM analysis. The dendrosomes appear to be spherical with 200-300 nm size. The TEM analysis of dendrosomes further reveals dendrimer core within the liposomal shell as shown by arrow heads (Figure 4.2B). Table 4.4 shows the dimensions of as-synthesized nanocomposites.

Figure 4.2. Characterization of nanocomposites and release kinetics of entrapped payload. (A) SEM analysis of antigen encapsulated PLGA-PEG nanocomposites. (B) TEM analysis of siRNA bearing dendrosome. At least three independent experiments were performed for each sample and data are representative of at least two similar observations. (C) and (D) Release kinetics of antigen and siRNA entrapped within
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PLGA nanoparticles and dendrosomes respectively. Data are mean ± standard deviation of three independent experiments.

4.3.1.3. Entrapment efficiency and release kinetics of payload from as-synthesized nanocomposites

PLGA NPs and PLGA-PEG NCs exhibited entrapment efficiency of 55.23 ± 1.19% and 62.37 ± 1.25% respectively (Table 4.4). The in vitro release profile of PLGA NPs and PLGA-PEG NCs is shown in Figure 4.2C. The studies were carried out in PBS (pH 7.4) at 37°C on a constant shaking mixer. The antigen release was studied over a period of 15 days. Both exhibit a biphasic release pattern that was characterized by an initial burst up to 24 hrs, followed by a slower sustained release that levelled off after about 12 days. The PLGA-PEG NCs showed higher release rate of antigen than PLGA NPs. The PLGA NPs exhibited 13.31 ± 1.2 % antigen release in the first 24 hrs while PLGA-PEG NCs showed 22.04 ± 0.9% release in the same period. At day 15, PLGA NPs and PLGA-PEG NCs had released 30.32 ± 1.6% and 47.05 ± 1.1% of antigen respectively.

Dendrimer-siRNA complex encapsulated dendosome formulation showed approximately 40.12 ± 2.8% entrapment efficiency of PLK-1-specific siRNA (Table 4.4). In vitro release kinetics of dendrosomal siRNA was studied at 37°C in PB, pH 7.4. Dendrosomes showed a sustained release, with approx. 23% of the entrapped siRNA leaking out in the initial 24 hrs. Dendrosomes were found releasing 35% of loaded siRNA in the initial 72 hrs with an overall total 40% release in 168 hrs (Figure 4.2D). Altogether, dendrosomes showed an initial burst release pattern followed by sustained release kinetics for extended time period.

Table 4.4. Size and entrapment efficiency of PLGA-PEG NCs and dendrosomes

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Size (nm)</th>
<th>Entrapment efficiency (%)</th>
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<tr>
<td>PLGA-PEG NC</td>
<td>100-200 nm</td>
<td>62.37±1.25</td>
</tr>
<tr>
<td>Dendosome</td>
<td>200-300 nm</td>
<td>40.12±2.8</td>
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Figure 4.3. Cytotoxicity assay. Cytotoxicity analysis of dendrimer, dendosome, PLGA NP and PLGA-PEG NC on PBMCs as revealed by MTT assay. Data are mean ± standard deviation of three independent experiments.

4.3.1.4. Cytotoxic effect of in-house synthesized nanocomposites on PBMCs
Cytotoxicity studies using MTT assay were carried out to ascertain the toxicity of various as-synthesized nanocomposites. As shown in Figure 4.3, dendrimers were found to be extremely toxic to PBMCs. However, the PLGA NPs, PLGA-PEG NCs and dendrosomes were found to be feebly toxic with PLGA-PEG NCs being the least toxic followed by PLGA NPs which were further trailed by dendrosomes. This clearly demonstrates the superiority of dendrosomes over the dendrimeric formulation. The data shows that PLGA-PEG NCs were toxic to a small 10% of the population followed by PLGA NPs which rendered killing of approx. 20% of PBMC population. The dendrimers resulted in killing of about 65% of the cell population. On the other hand, dendrosomes were found to obliterate only approx. 25% of the cells.

4.3.1.5. Determination of anti-DEC 205 antibody conjugation to PLGA-PEG nanocomposites and their targeting to dendritic cells
Fluorescence microscopy was employed to determine the conjugation of anti-DEC 205 antibody to PLGA-PEG NCs. Antibody tagged PLGA-PEG NCs were incubated
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with FITC labelled secondary antibody and then the incubated particles were washed to further visualize them under fluorescence microscope. The particles exhibiting green fluorescence in Figure 4.4A ascertain the conjugation of antibodies onto PLGA-PEG NCs. The fluorescence in Figure 4.4B shows dendritic cell targeting of the as-synthesized anti-DEC 205 antibody functionalized PLGA-NCs.

Figure 4.4. Fluorescence microscopic analysis anti-Dec 205 antibody conjugated PLGA-PEG NCs and their interaction with dendritic cells. (A) Green fluorescence exhibits conjugation of antibody onto PLGA-PEG NC surface. Antibody tagged PLGA-PEG NCs were incubated with FITC-tagged antimouse secondary antibody and the NCs were then analysed under fluorescence microscope. (B) Green fluorescence refers to the PLGA-PEG NCs targeting the dendritic cell surface. At least three independent experiments were performed for each sample and data are representative of at least two similar observations.

4.3.2. Results for prophylactic study

4.3.2.1. Prophylactic effect of tumor antigen loaded PLGA-PEG immuno-nanocomposites on skin papilloma induction and growth

Immuno-prophylactic potential of tumor antigen bearing PLGA-PEG immuno-nanocomposites was evaluated by assessing the first tumor incidence, percentage of tumor inhibition, average tumor volume and number. PLGA-PEG immuno-NCs were found to be successful in delaying onset of tumorogenesis. As depicted in Figure 4.5A, the onset of tumorogenesis was delayed for a considerable period in the mice.
Chapter 4: Results

immunized with tumor antigen bearing PLGA-PEG NCs in comparison to animals that were vaccinated with free form of tumor antigen ($p < 0.05$). The first tumor incidence in the mice immunized with antigen loaded PLGA-PEG immunonanocomposites was delayed by a period of more than a week as compared to animals immunized with PLGA-PEG NCs ($p < 0.05$). Besides delaying the onset of tumorigenesis, immunization with tumor antigen bearing PLGA-PEG immunonanocomposites resulted in significant growth inhibition (81%) as compared to vaccination with antigen loaded PLGA-PEG NCs (66%, $p < 0.05$) and free antigen (42%, $p < 0.01$) (Figure 4.5B).

Time bound growth in tumor volume and number was also used as a parameter to assess the prophylactic efficacy of PLGA-PEG NCs against DMBA-induced skin papilloma. As shown in Figures 4.5C and 4.5D, tumor antigen loaded PLGA-PEG immuno-NCs exhibited a significantly slower growth in tumor volume as well as number when compared to naked antigen bearing PLGA-PEG NCs and free tumor antigen (for tumor volume: Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], $p < 0.05$ and Ab@PLGA-PEG NC [Ag] vs Free Ag, $p < 0.01$; for tumor number: Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], $p < 0.05$ and Ab@PLGA-PEG NC [Ag] vs Free Ag, $p < 0.01$). Figure 4.6 shows gross images of mice vaccinated with various formulations. As shown in Figure 4.6, mice immunized with tumor antigen encapsulated PLGA-PEG immuno-NC exhibited a significantly lower number of tumors with significantly smaller size as compared to immunized control. The group immunized with naked PLGA-PEG NCs also showed lower number of tumors with smaller size than unimmunized control but the result was not as significant as observed for PLGA-PEG immuno-NC. The unimmunized group shows multiple large pedunculated and papillomatous growth with keratin deposition on the surface in contrast to antigen bearing PLGA-PEG immuno-NC group that exhibited scantily as well as significantly smaller papillomatous growth. Slightly smaller tumors were also observed in groups immunized with free tumor antigen in comparison to unimmunized control.
Figure 4.5. Effect PLGA-PEG immuno-nanocomposite formulation on tumor induction and prevention. (A) Immunoprophylactic effect of various formulations on the onset of mouse skin tumorigenesis. (B) Percent-inhibition of tumor growth by various formulations of tumor antigen. The animals were immunized with various antigen formulations prior to exposure to DMBA as described in Materials and Methods. The tumor growth inhibition was calculated by comparing the average size of the tumor induced in immunized animals with unimmunized control. (C) Immuno-preventive effects of various antigen formulations on average tumor volume. The immuno-preventive efficacy of various formulations was assessed by measuring the size of the tumors using a caliper. The tumor volume was determined by the formula as described in Materials and Methods. (D) Effect of PLGA-PEG immunonanocomposite based prophylaxis on the development of average number of
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tumors per mouse. Prophylactic efficacy of various formulations was assessed on the basis of their ability to prevent development of tumors. Data are mean ± standard deviation of three independent experiments. (For first tumor incidence, Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], p < 0.05; for percentage of tumor inhibition, Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], p < 0.05 and Ab@PLGA-PEG NC [Ag] vs free antigen, p < 0.01; for tumor volume: Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], p < 0.05 and Ab@PLGA-PEG NC [Ag] vs Free Ag, p < 0.01; for tumor number: Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], p < 0.05 and Ab@PLGA-PEG NC [Ag] vs Free Ag, p < 0.01).

Figure 4.6. Immunoprophylactic effect of PLGA-PEG immuno-nanocomposite formulation on the development of DMBA induced skin cancer in Swiss albino mice. Swiss albino mice pre-immunized with various PLGA-PEG NC based various tumor antigen formulations were exposed to DMBA to induce skin tumor following protocol as described in materials and methods section. Various groups include (A)
unimmunized control (B) animal vaccinated with free tumor antigen (C) PLGA-PEG NC entrapped antigen administered animal (D) and mouse immunized with anti-DEC 205 antibody functionalized PLGA-PEG NC encapsulated antigen. At least three independent experiments were performed for each sample and data are representative of at least two similar observations.

Figure 4.7. Anti-DEC 205 antibody conjugated PLGA-PEG NC induces pro-inflammatory cytokine expression in the immunized mice. PLGA-PEG NC mediated pro-inflammation was ascertained by determining cytokine response in splenocyte culture supernatant belonging to various immunized groups at different time points; (A) IFN-γ, (B) IL-12, (C) IL-6 and (D) TNF-α. To activate splenocytes belonging to various groups of immunized animals, free form of 20 mg of tumor antigen was used. Level of pro-inflammatory cytokines in splenocyte culture
supernatant was determined by sandwich ELISA as described in Materials and methods section. Data are mean ± standard deviation of three independent experiments. For IFN-γ (week eight PE): Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], p < 0.05; Ab@PLGA-PEG NC [Ag] vs Free Ag, p < 0.01. For IFN-γ (week four PE): Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], p < 0.05; Ab@PLGA-PEG NC [Ag] vs Free Ag, p < 0.01. For IFN-γ (week one PB): Ab@PLGA-PEG NC [Ag] vs PLGA-PEG NC [Ag], p < 0.05; Ab@PLGA-PEG NC [Ag] vs Free Ag, p < 0.01. For IL-12, IL-6 and TNF-α, p values were found to be same amongst various groups as those observed for IFN-γ at various time points. ‘PB’ and ‘PE’ denote ‘post booster’ and ‘post exposure’ time points respectively.

4.3.2.2. Immunization with tumor antigen bearing PLGA-PEG immuno-NCs induces the production of pro-inflammatory cytokines

In order to determine the kind of immune response evoked by tumor antigen bearing PLGA-PEG immuno-NCs, we measured the level of pro-inflammatory cytokines in the culture supernatant of splenocytes isolated from immunized animals in response to stimulation with free tumor antigen. IFN-γ, TNF-α, IL-6 and IL-12 act as a pro-inflammatory cytokines and are produced in response to infection or tissue injury. As depicted in Figure 4.7, pro-inflammatory cytokines were found to consistently increase with increasing time points, viz. post booster (PB) and post DMBA exposure (PE). The amount of pro-inflammatory cytokines released in the group of animals immunized with the formulation Ab@PLGA-PEG NC [Ag] was found to be significantly higher than those immunized with antigen bearing naked PLGA-PEG NCs (p < 0.05) as well as free antigen (p < 0.01) at various time points. A substantial level of pro-inflammatory cytokines was also found to be present in PLGA-PEG NC immunized animals even after eight weeks of DMBA exposure.
Figure 4.8. Effect of dendosome-siRNA formulations on the expression of pro-apoptotic molecules. (A) PLK-1 inhibition by siRNA-bearing dendrosomes causes upregulation of p53 wildtype and Bax in Epidermoid cell line. Lane 1: untreated control; lane 2: free siRNA; lane 3: dendrimer-siRNA complex and lane 4: dendrosome-siRNA formulation. (B) Skin cancer cells isolated from mice treated with various siRNA formulations were lysed and examined for the expression of various apoptotic molecules by immunoblotting. Lane 1: untreated control; lane 2: dendrimer+PC; lane 3: free siRNA; lane 4: dendrimer-siRNA complex and lane 5: dendrosome-siRNA. Three independent experiments were performed for each sample and data are representative of at least two similar observations.

4.3.3. Results for therapeutic study

4.3.3.1. Effect of siRNA encapsulated dendrosomes on apoptosis

The ability of various siRNA formulations in causing apoptosis of cancer cell line as well as skin tumor cells was evaluated. As shown in Figure 4.8A, dendosome-siRNA downregulated the expression of PLK-1 while enhanced the expression of p53 wild-type (p53wt) and bax in epidermoid cancer cell line (Figure 4.8A, lane 4). However, other formulations viz. free siRNA (Figure 4.8A, lane 2) and dendrimer-siRNA (Figure 4.8A, lane 3) could also modulate the apoptotic factors in comparison to untreated control (Figure 4A, lane 1) but not as effectively as dendrosome-siRNA formulation. The results clearly suggest that siRNA-dendrimer complex while encapsulated in dendrosomes is delivered efficiently to the cancer cells and can easily modulate various apoptotic factors and eventually result in apoptosis of the cancer cells.
Next, we proceeded to analyse the apoptosis of cancer cells in response to administration of various siRNA formulations in mouse model. Treatment with dendrimer-PC did not show significant effect on upregulation of p53 wild-type (p53wt) (Figure 4.8B, lane 2) with respect to untreated control (Figure 4.8B, lane 1). Moreover, free siRNA failed to upregulate the expression of p53wt (Figure 4.8B, lane 3) although dendrimer-siRNA complex treatment resulted in moderate increase in the expression of p53wt (Figure 4.8B, lane 4). Significant upregulation of p53wt was observed when cancerous mice were treated with dendrosomal siRNA formulation (Figure 4.8B, lane 5). Exposure of animals to DMBA ensued in upregulation of p53mut (Figure 4.8B, lane 1). Treatment with dendrosome-siRNA nanoparticle formulation resulted in its significant downregulation (Figure 4.8B, lane 5). The effect of dendrosome-siRNA nanoparticle formulations on the expression of Bax was also recorded. Immunoblot analysis showed downregulated expression of Bax in cancerous (untreated) mice, whereas its level was upregulated in the group treated with dendrosomal siRNA (Figure 4.8B, lanes 1 and 5, respectively). Exposure to DMBA resulted in overexpression of PLK-1 and cyclin B1 in experimental animals (Figure 4.8B). The level of PLK-1 as well as cyclin B1 were reduced to normal in the animals treated with dendrosome-siRNA nanoparticle formulation when compared to DMBA exposed but no siRNA treatment group.
Figure 4.9. Dendroosome-siRNA formulation induces caspase-9 mediated apoptosis in skin cancer cells and causes augmented DNA fragmentation in treated skin papilloma cells. (A) Involvement of caspase-9 in dendosomal siRNA mediated apoptosis of cancer cells as revealed by fluorescence microscopy. Upper panel; fluorescence micrographs, lower panel; phase contrast images. While group (a) served as control (untreated), the other treated groups are: (b) dendrimer+PC, (c) free siRNA, (d) dendrimer-siRNA complex, (e) dendroosome-siRNA. The isolated cells were washed and fixed with 1% paraformaldehyde, 0.19% picric acid in PBS (pH 7.4) for 1 hr at RT. Fixed cells were permeabilized with 0.1% SDS in PBS at RT for 10 min, blocked with 2% FCS, stained with a polyclonal antibody which detects only cleaved 35 kDa caspase-9 (BD, India) and revealed with a conjugated rabbit anti-mouse IgG-FITC probe (Sigma, India). The cells were then visualized under a fluorescence microscope (excitation at 488 nm, emission at 505-530 nm). (B) Dendroosomal nanocomposite induced DNA fragmentation as analysed by APOBrdUTM study employing flow cytometer (B). Histograms shows the data obtained after antibromodeoxyuridine staining. Dendroosome siRNA treated cells harbour an increased numbers of DNA breaks. The group (a) is untreated control, while the other treated groups are (b) dendrimer+PC, (c) free siRNA, (d) dendrimer-siRNA complex, (e) dendroosome-siRNA. At least three independent experiments were performed for each sample and data are representative of at least two similar observations.

4.3.3.2. Effect of dendroosome-encapsulated siRNA on the expression of caspase-9

Skin cancer cells were squeezed out from experimental animals and the efficacy of the siRNA treatment was evaluated by determining the expression of caspase-9 using confocal microscopy. Apoptosis induction was quite visible in free siRNA-treated cells in comparison with dendrimer+PC-treated and untreated groups, where caspase-9 expression was almost negligible (Figures 4.9Aa,b,c). However, expression of caspase-9 was significantly enhanced in cells isolated from siRNA-bearing dendroosome-treated mice (Figure 4.9A,e). The group treated with dendrimer-siRNA complex also exhibited a significantly higher expression of caspase-9 (Figure 4.9A,d) than free siRNA but the expression was not as significant as observed for dendroosome-siRNA formulation treated group.
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4.3.3.3. *Effect of dendosome-encapsulated siRNA on DNA fragmentation*

Activation of endonucleases during apoptotic events in the cell generally result in DNA fragmentation (Chauhan et al., 2014). Skin papilloma cells were isolated from mice belonging to various experimental groups (untreated control, free siRNA, dendrimer-siRNA complex and dendosome-siRNA) and fixed using paraformaldehyde. Efficacy of dendosome-entrapped siRNA was observed by 5-bromo-2'-deoxyuridine (BrdU) TUNEL analysis using FACS (Figure 4.9B). In a manner similar to fluorescence microscopy results, FACS analysis too revealed that dendosome-siRNA led to significantly higher apoptosis induction in cells isolated from dendosome-siRNA treated group in comparison to free siRNA, dendrimer-siRNA complex and untreated control mice (Figure 4.9B).

4.3.3.4. *Effect of dendosome-encapsulated siRNA formulation on tumor regression in mice with skin papilloma*

The in-house prepared dendosome-siRNA formulation in addition to inducing apoptosis in cancer cells could also regress tumors. As shown in Figure 4.10, mice treated with dendosome-siRNA formulation exhibited a significant regression in tumor as compared to control untreated animal. The untreated group shows multiple large pedunculated and papillomatous growth with keratin deposition on the surface in contrast to dendosome-siRNA treated group that exhibited scantily as well as significantly smaller papillomatous growth. A slight tumor regression was also observed in group administered dendrimer-siRNA complex. However, the groups treated with free siRNA exhibited negligible tumor regression (Figure 4.10).
Figure 4.10. Dendosome-siRNA nanocomposite formulation mediated regression of DMBA induced skin papilloma in Swiss albino mice. Swiss albino mice were exposed to DMBA to induce skin papilloma following method as described in methodology section. Group (A) represents untreated control animals while other groups are (B) free siRNA administered animal (C) mouse treated with dendrimer-siRNA complex and (D) animal treated with dendosome-siRNA formulation. At least three independent experiments were performed for each sample and data are representative of at least two similar observations.

4.3.4. Results for combinatorial study

4.3.4.1. Histopathological studies

One of the strategies to establish the efficacy of combined vaccination and PLK-1-specific siRNA therapy was histopathological studies. Skin papilloma tissue samples from mice belonging to various experimental groups were used for histopathological analysis. Figure 4.11 shows sections of skin cancer cells belonging to various experimental groups. Unimmunized and untreated group showed profuse
papillomatous growth, moderate acanthosis, and marked keratosis, complex fibrovascular core with congested vessels and loss of pilosebaceous units. Mice belonging to "immunized only" and "unimmunized and treated" groups exhibit moderate response to treatment with reduction in both acanthosis and keratosis while vaccinated as well as treated animals show good response to treatment as revealed by mild acanthosis and keratosis, and absence of the complexity of fibrovascular core.

Figure 4.11. Histopathological studies of animals given combined vaccination and siRNA treatment. Photomicrograph of mouse skin from unimmunized and untreated control (A) unimmunized and treated (B) immunized only (C) immunized and treated (D) groups. Unimmunized and untreated group showed profuse papillomatous growth, moderate acanthosis, and marked keratosis, complex fibrovascular core with congested vessels and loss of pilosebaceous units. Mice belonging to "immunized only" and "unimmunized and treated" groups exhibit moderate response to treatment with reduction in both acanthosis and keratosis while
vaccinated as well as treated animals show good response to treatment as revealed by mild acanthosis and keratosis, and absence of the complexity of fibrovascular core. At least three independent experiments were performed for each sample and data are representative of at least two similar observations.

4.3.4.2. Combined vaccination with tumor antigen and siRNA therapy inhibits the tumor growth and regresses the tumor volume as well as number
As shown in Figure 4.12, mice which were immunized as well as treated with dendroosome-siRNA formulation exhibited a significant regression in tumor as compared to control ‘unimmunized and untreated’ animals. The ‘unimmunized and untreated’ group shows papillomatous growth with heavy acanthosis and keratosis in contrast to animals given combinatorial treatment that negligibly smaller papillomatous growth. A marked tumor regression was also observed in ‘unimmunized and treated’ and ‘immunized only’ groups. (Figure 4.12). To evaluate the efficacy of combinatorial therapy, tumor volume and tumor number were measured since the day combinatorial therapy was begun. As shown in Figure 4.13 A, the ‘immunized and treated’ group of animals showed a significantly smaller tumor volume in contrast to ‘immunized only’ (p < 0.05) and ‘unimmunized and treated’ (p < 0.01) groups at week four. In case of animals belonging to ‘immunized only’ group, a slow increase in tumor growth is observed that reached approx. 300 mm³ at week four whereas animals in the ‘unimmunized and treated’ group exhibited a slow decrease in tumor volume resulting in downsizing the tumor volume from approx. 1000 mm³ at week one to approx. 800 mm³ at week four. Animals belonging to the ‘immunized and treated’ group exhibited a continous decrease in tumor growth so that it reached approx. 150 mm³ at week four from approx. 250 mm³ at week one (Figure 4.13A). A similar pattern of decrease and increase in tumor number for various groups was observed (Figure 4.13B). At week four, mice in the ‘immunized and treated’ group showed significantly lower number of tumor papillomas as compared to animals belonging to ‘immunized only’ (p < 0.05) and ‘unimmunized and treated’ group (p < 0.01) (Figure 4.13B).

4.3.4.3. Effect of combined vaccination and siRNA therapy on overall weight and survival of skin papilloma bearing mice
We also monitored the body weight and survival of animals for a period of 24 weeks. The bodyweights of mice belonging to various groups were examined at an interval of
10 days (Figure 4.13C). The 'immunized and treated' group showed mitigation of weight loss comparatively higher than 'unimmunized and treated' animals ($p < 0.05$) and 'immunized only' group ($p < 0.05$). Animals belonging to 'unimmunized and untreated' group demonstrated almost 50% reduction in bodyweight by day 120. Our results show that the mice which were immunized as well as treated with dendosome-siRNA formulation showed a significantly higher survival (70%) even after 24 weeks of DMBA exposure in contrast to 'unimmunized and treated' group and 'immunized only animals which exhibited 50% ($p < 0.01$) and 40% ($p < 0.01$) survival respectively. No animals survived in the 'unimmunized and untreated' group after week 18 (Figure 4.13D).

Figure 4.12. Combined vaccine and siRNA therapy induced highly effective anti-tumor activity. Swiss albino mice were immunized subcutaneously three times at intervals of 1, 2 and 4 weeks with anti-DEC 205 antibody tagged PLGA-PEG nanocomposite entrapped skin papilloma antigens obtained by maceration of excised tumor. After one week of last immunization, mice were exposed to DMBA. After 10
weeks of DMBA exposure, the immunized animals were treated with dendrosomal PLK-1 specific siRNA. The animals were monitored time to time till 24 weeks counted from the day of first immunization. The figures represent images of animals left at the end of the combinatorial study. While group (A) represents unimmunized and untreated control, other groups are (B) unimmunized and treated (C) immunized only and (D) immunized and treated. At least three independent experiments were performed for each sample and data are representative of at least two similar observations.

Figure 4.13. Effect of combinatorial vaccination and siRNA therapy on tumor volume, tumor number, overall weight and survival of mice with DMBA induced skin papilloma. (A) After siRNA therapy, tumor measurements were performed using calipers. Data represent tumor measurements performed at time points 1, 2, 3 and 4 weeks post siRNA treatment in pre-immunized animals. The data are presented as the average tumor volume per group and are reported as mm³. (B) Average tumor number/mouse was calculated at time points 1, 2, 3 and 4 weeks post siRNA treatment in pre-immunized animals. (C) Body weight of animals evaluated at an
interval of 10 days since first immunization. (D) Percent survival of animals monitored on a weekly basis from the day of first immunization. Data are mean ± standard deviation of three independent experiments. (For tumor volume, ‘immunized and treated’ group vs ‘immunized only’ group, p < 0.05; ‘immunized and treated’ group vs ‘unimmunized and treated’ group, p < 0.01. For tumor number, ‘immunized and treated’ group vs ‘immunized only’ group, p < 0.05; ‘immunized and treated’ group vs ‘unimmunized and treated’ group, p < 0.01. For body weight, ‘immunized and treated’ group vs ‘immunized only’ group, p < 0.05; ‘immunized and treated’ group vs ‘unimmunized and treated’ group, p < 0.05. For survival, ‘immunized and treated’ group vs ‘immunized only’ group, p < 0.01; ‘immunized and treated’ group vs ‘unimmunized and treated’ group, p < 0.01).
Chapter 4: Discussion

4.4. Discussion

Surgery, radiation, and chemotherapy are the mainstay of cancer management. Surgery and radiation therapy are relatively precise and used to achieve local control. In contrast, cytotoxic chemotherapy exerts a systemic effect and is used to cytocreduce established tumors and eradicate micrometastatic disease (Emens LA and Jaffee; 2005). When properly sequenced, these treatment modalities can cure a substantial number of hematologic malignancies and a smaller subset of various early-stage solid tumors. However, despite such success, a significant percentage of cancers remain incurable (Emens LA and Jaffee; 2005). Furthermore, the imprecise nature of chemotherapy results in collateral damage to normal tissues, resulting in significant side effects that adversely affect the patient’s quality of life. The associated toxicities and limited success of traditional treatments in maximizing cure rates is a clear mandate for developing innovative therapeutic strategies that capitalize on emerging insights about tumor biology and the host-tumor interaction. Immune-based approaches that recruit the host antitumor immune response are particularly attractive for improving clinical outcomes in malignant disease.

Progress in manipulating the cellular arm of the immune response has been slower, but viable cancer therapies based on the adoptive transfer of lymphocytes or the de novo induction of an effective antitumor immune response by active vaccination are under intense investigation (Tabi and Man, 2006; Moingeon, 2001; Emens LA and Jaffee; 2005). Immunization with tumor vaccines in particular offers advantages that other cancer therapies do not. First, it is highly specific and can target antigens integral to the process of transformation. Second, it is well tolerated, with minimal side effects limited primarily to injection site reactions and minor systemic toxicities (transient fever and flu-like symptoms). Third, vaccination offers the unique potential for a durable antitumor effect due to the phenomenon of immunologic memory, potentially obviating the need for prolonged, repetitive cycles of therapy. However, the clinical efficacy of immune based therapeutics can be severely curtailed by the burden of established cancer compared with the magnitude of immune effectors in play. Therefore, developing prophylactic vaccines given prior to tumor establishment can prove comparatively better being able to mount desired immune response in the absence of tumor burden (Zhao et al., 2013; Finn and Forni, 2002). Although prophylactic vaccines against cancers caused by viruses like cervical cancer (caused
by human papilloma virus) are intensely investigated (Palmer et al., 2009),
development of prophylactic vaccines against cancers progressed upon exposure of
non-viral anti-cancer agents remains in its infancy. Moreover, integrating cytotoxic
therapies with tumor vaccines using unique doses and schedules is another strategy to
break down the barriers exhibited by established tumors, releasing the full potential of
the antitumor immune response to eradicate disease. Anti-tumor effect of combining
therapeutic tumor vaccines with chemotherapy has been widely studied (Vanneman
M and Dranoff G, 2012; Foy et al., 2012; Andersen et al., 2010). However, to the
best of our knowledge integrating a prophylactic tumor vaccine with siRNA based
therapy has not yet been explored.

In the present study, first of all we performed anti-skin papilloma prophylactic study
wherein we evaluated the prophylactic potential of anti-DEC 205 antibody
functionalized and tumor antigen loaded PLGA-PEG immuno-nanocomposites in
prevention of skin papilloma. In a separate set of study, we evaluated the potential of
dendosome encapsulated PLK-1 specific siRNA in treatment of skin papilloma in
mouse models. Since the dendosome encapsulated siRNA was found to produce a
potent anti-tumor effect, we further administered the formulation to animals
immunized with tumor antigen loaded PLGA-PEG immuno-nanocomposites and
determined the anti-tumor effect of the combination. The significant outcomes of the
study are: For the prophylactic study i) DC targeting of tumor antigen significantly
inhibits tumor progression enabling marked reduction in tumor size and number ii)
Immunization with anti-DEC 205 antibody functionalized NCs strongly up-regulates
inflammatory cytokines in the host. For the therapeutic study i) The dendosome
encapsulated siRNA successfully reduces the cumulative numbers as well as sizes of
tumor nodules ii) The dendosome based nanocomposite formulation effectively
modulates pro-apoptotic factors. For the combinatorial study i) The combinatorial
vaccination and treatment with as-synthesized NCs display synergism ii) Integrating
siRNA therapy with anti-tumor vaccination may prove highly effective in reducing
tumor volume and number, significantly improving the body weight and survival of
‘immunized & treated’ animals.

In the prophylactic study, PLGA was employed for nanoparticle synthesis owing to
the potential of its nanoparticles to cross-present the antigen entrapped to MHC-I as
well as MHC-II, a pre-requisite for evoking a CTL response required for developing a
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potent vaccine against tumor cells. PLGA-PEG nanocomposites were synthesized from as-synthesized PLGA-PEG co-polymer. In this diblock copolymer, PLGA is a hydrophobic core that enables entrapping hydrophobic molecules to protect them from the surrounding environment while PEG chains orient themselves towards the external aqueous phase in micelles, thus surrounding the encapsulated species. This layer of PEG acts as a barrier and reduces the interactions with foreign molecules by steric and hydrated repulsion, giving enhanced shelf stability. Anti-DEC 205 antibody functionalization of the PLGA-PEG NC was done with the premise to facilitate targeted delivery of tumor antigens to dendritic cells. DCs may endocytose the antibody coated nanocomposites by receptor mediated endocytosis and process the tumor antigen entrapped in the particles into major histocompatibility complex (MHC) class I and MHC class II pathways, and display the resulting peptide epitopes in an immunogenic context to stimulate CD8+ T cells and CD4+ T cells, respectively as illustrated by Figure 4.14.

The data presented in Figures 4.5 and 4.6 indicate the successful functioning of the strategy owing to the higher efficacy of Ab@PLGA-PEG NC [Ag] which was assessed on the basis of its ability to delay, onset of tumor induction, significant inhibition of tumor growth decreasing the tumor volume and number dramatically as compared to naked PLGA-PEG NC ent Ag. As shown in Figure 4.7, Ab@PLGA-PEG NC [Ag] induces a significantly higher pro-inflammatory immune response in the immunized animals at various time points than naked PLGA-PEG NC as assessed by determining the level of proinflammatory cytokines IFN-γ, IL-12, IL-6 and TNF-α. The decreased tumor progression and increased tumor regression in the immunized animals can be owed to the generation of these inflammatory cytokines since systemic administration of IL-12 has been reported to induce production of IFN-γ that together activate CTLs and NK cells resulting in decreased tumor progression and increased tumor necrosis in administered animals (Puddu et al., 2005; Heo et al., 2014). Therefore, from the prophylactic study it can be concluded that anti-DEC antibody conjugated multifunctional polymer nanoparticles capable of delivering tumor antigens to DCs can induce an effective immune response against tumor and hence harbour the attributes of a potent anti-tumor prophylactic vaccine.
Figure 4.14. Schematic illustration of DC targeting of anti-DEC 205 antibody conjugated PLGA-PEG NCs and subsequent antigen processing by MHC-I and MHC-II to generate tumor specific CD4^+ and CD8^+ cells.

A separate study employing siRNA therapy against DMBA induced skin papilloma established higher efficacy of dendrosome-siRNA formulation that can be solely attributed to the effective delivery of siRNA in the leaky tumor vasculature by dendrosomes. The therapeutic efficacy of dendrosomal siRNA was indicated owing its modulation of apoptotic factors. The dendrosome-siRNA formulation was successful in downregulating the expression of target protein PLK-1 and therefore enhancing the expression of p53wt and bax in cell line as well as skin cancer cells (Figure 4.8). On the other hand, free siRNA or dendrimer-siRNA complex could feebly modulate the expression of these proteins. In fact, tumor cells overexpress PLK-1-mediated cyclin-dependent kinase 1 (Cdc2)/cyclin B1 that help in rapid proliferation of the cells. This ensued in less production of p53wt and upregulated production of p53mut. Earlier studies have demonstrated involvement of the suppressor p53 in PLK-1 depletion-induced apoptosis (Chauhan et al., 2014). In principle, the silencing of the PLK-1 gene should reinstate normal production of the
p53wt protein. As depicted in Figure 4.8A, comparatively higher expression of p53wt protein was recorded in the cells treated with siRNA-bearing dendrosomes compared with free siRNA and dendrimer-siRNA administered groups. The data clearly suggest that siRNA-bearing dendrosomes play a determining role in upregulation of p53wt expression. Similarly, treatment with various forms of siRNA was also successful in aborting upregulation of p53mut in cancer cells. In fact, PLK-1 has also been reported to have an ability to phosphorylate p53 and also binds physically with the latter.

Caspase-9, an apical caspase in the intrinsic pathway initiated by cytochrome c released from the mitochondrial membrane space into the cytosol, induces apoptosis formation during apoptosis (Chauhan et al., 2014). Caspase-9 activates the effector caspases-3 and -7, which cleave several cellular proteins, resulting in the characteristic biochemical and morphological features associated with apoptosis. Expression of caspase-9 was found to be enhanced upon treatment with dendrosome-siRNA nanocomposites compared with the free siRNA form (Figure 4.9A). An augmented apoptosis induction in the cancer cells by dendrosome-siRNA formulation in comparison to free siRNA and dendrimer-siRNA complex was further supported by APO-BrdT™ TUNEL assay employing FACS analysis (Figure 4.9B). Moreover, administering dendrosome-siRNA formulation resulted in an efficient regression in tumor growth as compared to the naked dendrimer-siRNA complex (Figures 4.10). This clearly suggests that putting the dendrimer-siRNA complex in a lipophilic shell offers an effective strategy to augment the protective efficacy of PLK-1 specific siRNA against skin papillomas.

Considering the experimental outcomes of prophylactic and therapeutic studies where prophylactic multifunctional PLGA-PEG immuno-nanocomposite and cytotoxic siRNA therapy employing dendrosome could successfully prevent and treat DMBA induced skin papilloma respectively, integrating PLGA-PEG immuno-nanocomposite based prophylactic tumor vaccine with dendrosome based siRNA therapy seems to be highly attractive. As shown in Figures 4.11, 4.12 and 4.13 vaccination followed by therapy resulted in highly successful anti-tumor effect resulting in significant improvement in anatomical features of cancer inflicted tissues, almost complete regression tumor size and number, marked mitigation of body weight and highly enhanced survival of 'immunized & treated' animals as compared to 'unimmunized & untreated' group. The data of combinatorial therapy suggest that integrating the two
approaches might have complementary roles in cancer treatment, and that combinatorial therapy could prove synergistic. Because siRNA mediated therapy can induce rapid tumor regressions, they may afford a favourable window for immunoprophylaxis to achieve more potent cytotoxicity. Potent vaccination might potentiate anti-tumor immune responses by breaking ‘oncogene addiction’, in turn triggering tumor cell senescence and facilitating tumor clearance by T cells. Moreover, the higher efficacy of the combined treatment as compared to individual therapies can be owed to the release of large amounts of antigenic debris on tumor cell death by siRNA therapy which may contribute to the success of vaccination in situ, particularly in the present study where concurrent DC activation would have been triggered. Thus, vaccination might consolidate the dramatic tumor responses that are achieved with siRNA into durable and long-lasting remissions. The interplay of vaccination and siRNA mediated therapy seems to be complex, however, parameters such as timing, dosage and sequence of administration are all likely to influence the overall anti-tumor effects and toxicity profiles of the combined treatments. A more complete understanding of the effect of combined therapies on specific molecular pathways in immune cells should help to advance the integration vaccination with siRNA therapy.


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