POLYETHYLENE GLYCOL (PEG) AS A KEY COMPONENT OF LONG-CIRCULATING DELIVERY SYSTEMS FOR THERAPY AND IMAGING

Doctoral Thesis Presented

By

Rishikesh Manohar Sawant

To,

The Graduate School of Bouvé College of Health Sciences

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Pharmaceutical Sciences with Specialization in Pharmaceutics & Drug Delivery Systems

NORTHEASTERN UNIVERSITY

BOSTON, MASSACHUSETTS

November, 2008
Thesis Approval Form

Thesis Title: Polyethylene glycol (PEG) as a key component of long circulating delivery systems for therapy and imaging

Author: Rishikesh Sawant

Program: Pharmaceutical Sciences with specialization in Pharmaceutics & Drug Delivery Systems

Approval for Thesis Requirements of the Doctor of Philosophy degree in Pharmaceutical Sciences

Thesis Committee (Chairman) __________________________ Date 11/13/08

Other committee members:

__________ Robert Schaff ______________ Date 11/13/05

__________ Kim Chen ______________ Date 11/13/08

__________ Robert B. Canary ______________ Date 11/13/08

__________ Nolan Weinberg ______________ Date 11/19/08

Director of the Bouvé College Graduate School:

_________________________ Suzanne B. Greenberg ______________ Date 11/20/2008
ABSTRACT

The undesired side-effects of many therapies and diagnostics result from their accumulation in the non-target tissues. There is a clear need to design pharmaceutical delivery systems capable of delivering drugs, DNA and diagnostics to the target tissue with minimal accumulation in the non-target tissues. Targeted delivery of pharmaceuticals will help in reducing accumulation and undesired side-effects in the non-target organs, and increase the amount of drug delivered and drug bioavailability at intended target organs. Targeting can be achieved passively by long circulation time of the pharmaceuticals in the blood, actively by using target-specific ligands or by combination of both. The major long term goal of this project is to develop optimal delivery systems capable of delivering drugs, DNA or imaging agents to the intended target site using polymeric carriers like dextran, or nanoparticulate carriers like liposomes and micelles.

In this study, polyethylene glycol (PEG), a biocompatible hydrophilic polymer was used as the key component of various delivery systems to make them long-circulating for passive targeting and/or actively targeted via the attachment of various ligands onto their surfaces. PEG conjugates have been used to modify existing delivery platforms like dextran, to increase its circulation time in blood. PEG-phosphatidylethanolamine (PEG-PE) conjugates spontaneously form micelles with a hydrophobic lipid core to entrap super-paramagnetic iron oxide nanoparticles (SPION) and form stable nano-sized suspensions of SPION-micelles. There was a significant improvement in MRI signal from the SPION-micelles compared to “plain” SPION. To prepare targeted contrast agents, para-nitrophenyl PEG-PE (pNP-PEG-PE) conjugates were used to surface-modify SPION-micelles with the anti-cancer nucleosome-specific
monoclonal antibody 2C5 (mAb2C5). mAb2C5-SPION-micelles demonstrated increased association with cancer cells and were able to bring more MRI contrast signal to cancer cells in vitro. Moreover, physical targeting of SPION-micelles into subcutaneous tumor models in mice in vivo was also possible by using an external magnet.

Stimuli (pH)-sensitive PEG-PE conjugates [with a pH-cleavable hydrazone bond between PEG and PE (PEG-Hz-PE) making PEG detachable from the conjugate at lowered pH] can be used to prepare multifunctional nanocarriers with “hidden” functions that will be developed only upon external stimuli (lowered-pH values in tumor interstitium). Cell-penetrating peptides (CPPs) have been shown to effectively deliver the cargoes into the cell. Using pH-sensitive PEG-Hz-PE, we have constructed multifunctional nanocarriers which, in addition to prolonged circulation (via the attached PEG) and target recognition (via the attached antibody), carry the temporarily hidden CPP function. The CPPs attached to the nanocarriers are “shielded” with PEG chains under normal pH values (as in blood), however upon the incubation at low-pH values, hydrazone bond hydrolyzes, PEG detaches, and the CPPs become exposed and help internalize the nanocarriers into the cells. This is a significant step on the way toward “smart” multifunctional pharmaceutical nanocarriers capable of both target accumulation and intracellular penetration in a controlled fashion.
ACKNOWLEDGEMENTS

I would like to acknowledge and express by sincere thanks to all who made it possible to complete my doctoral thesis dissertation:
First and foremost, I am extremely grateful to my Ph.D. advisor, Prof. Vladimir Torchilin, for providing me the opportunity to work in his lab, and the financial support throughout my Ph.D. research years. His understanding and patience helped instill confidence and courage to develop myself as a research scientist. His constant guidance and mentoring helped me overcome many hurdles throughout my Ph.D. research.

My sincere thanks to all my thesis committee members, Dr. Robert Campbell, Dr. Kim Lewis, Dr. Robert Schatz, and Dr. Volkmar Weissig, for their time, invaluable advice on my thesis project and moral support.

I would also like to acknowledge the efforts of Dr. James Hurley and Dr. Anatoly Lukyanov for help with chemical synthesis, Dr. Tatiana Levchenko for help with the radioactivity labeling, Dmitry Mongayt for in vitro cell-interaction studies, Dr. William Hartner for in vivo biodistribution studies and Dr. Srinivas Sridhar's lab for synthesis of SPION. My heartfelt thanks to all the lab members and graduate students from the Department of Pharmaceutical Sciences who helped me either directly or indirectly during my dissertation work.

I, humbly, thank my parents, who have sacrificed much to ensure that I have the best in life. I, certainly, am very fortunate to have such a wonderful family. And finally, I would like to thank my wonderful wife Rupa, who has been there for me through the toughest of times and completely supporting my dreams.
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td><strong>1  INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1  STATEMENT OF THE PROBLEM</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Need for site-specific drug delivery</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Need for long circulation of drug delivery systems</td>
<td>1</td>
</tr>
<tr>
<td>1.1.3 Systems for targeted drug delivery</td>
<td>3</td>
</tr>
<tr>
<td>1.1.4 Active targeting of drug carriers</td>
<td>4</td>
</tr>
<tr>
<td>1.1.5 Polyethylene glycol (PEG) in drug delivery carriers</td>
<td>4</td>
</tr>
<tr>
<td>1.2  REVIEW OF THE LITERATURE</td>
<td>5</td>
</tr>
<tr>
<td>1.2.1 Water-soluble polymers and prolonged circulation</td>
<td>5</td>
</tr>
<tr>
<td>1.2.2 Rationale for using PEG in drug delivery systems</td>
<td>6</td>
</tr>
<tr>
<td>1.2.3 PEGylation chemistry</td>
<td>8</td>
</tr>
<tr>
<td><strong>2  CONCEPTS AND DESIGN OF PHARMACEUTICAL DELIVERY SYSTEMS</strong></td>
<td>15</td>
</tr>
<tr>
<td>2.1  PEGylation of dextran to make long-circulating pharmaceutical carrier</td>
<td>17</td>
</tr>
<tr>
<td>2.2  PEG-lipid conjugates as the basis for micellar pharmaceutical nanocarriers and PEG-lipid micelles with magnetic sensitivity</td>
<td>19</td>
</tr>
<tr>
<td>2.2.1 PEG-lipid conjugates as micellar systems</td>
<td>19</td>
</tr>
<tr>
<td>2.2.2 Superparamagnetic iron oxide nanoparticles (SPION)</td>
<td>21</td>
</tr>
</tbody>
</table>
2.2.3 MRI and MRI Contrast agents ................................................................. 22
2.2.4 Antinuclear Autoantibodies: Monoclonal antibody 2C5 .............. 23

2.3 Detachable PEG to make stimuli-sensitive multifunctional liposomes and micelles ...................................................... 25
  2.3.1 Cell-penetrating peptides ................................................................. 25
  2.3.2 Developing multifunctional nanocarrier systems ....................... 26
  2.3.3 The new concept of multifunctionality ........................................ 28

3 Objectives and Specific Aims ................................................................. 30

4 Experimental Design and Methods ..................................................... 31

4.1 PEGylation of dextran to make long-circulating pharmaceutical carriers ................................................................. 31
  4.1.1 Materials .......................................................................................... 31
  4.1.2 Synthesis of aminodextran .............................................................. 31
  4.1.3 Introduction of DTPA residues into aminodextran .................... 32
  4.1.4 PEGylation of aminodextran .......................................................... 32
  4.1.5 Amino group concentration ............................................................ 33
  4.1.6 Radiolabeling of PEGylated dextrans by transchelation ............ 33
  4.1.7 Biodistribution studies ................................................................. 34
  4.1.8 Statistics ........................................................................................ 34

4.2 PEG-lipid micelles with magnetic sensitivity .................................. 35
  4.2.1 Materials .......................................................................................... 35
  4.2.2 Synthesis of SPION ................................................................. 35
  4.2.3 Formulation of SPION-micelles .................................................. 36
  4.2.4 Size distribution and zeta potential measurements of SPION-micelles ................................................................. 36
  4.2.5 Freeze-fracture electron microscopy ........................................... 37
  4.2.6 Relaxation rate of SPION-micelles ............................................. 37
  4.2.7 Preparation of SPION-immunomicelles ....................................... 38
4.2.8 ELISA of immunomicelles. ............................................................. 39
4.2.9 Cell culture. ..................................................................................... 39
4.2.10 Interaction of SPION-loaded mAb 2C5-immunomicelles with cancer cells in vitro ................................................................. 40
4.2.11 Fluorescence microscopy. ........................................................... 40
4.2.12 Subcutaneous tumor models in mice for studying magnetic targeting of SPION-micelles............................................................ 41
4.2.13 Radiolabeling of SPION-micelles with 111-Indium.................. 41
4.2.14 Magnetic targeting of SPION-micelles in tumor of tumor-bearing mice in vivo under influence of external magnet. ......................... 42

4.3 DETACHABLE PEG TO MAKE STIMULI-SENSITIVE MULTIFUNCTIONAL LIPOSOMES AND MICELLES. ......................................................... 43
4.3.1 Materials. ....................................................................................... 43
4.3.2 Synthesis of pH-cleavable mPEG2000-hydrazone-phosphatidyl ethanolamine (mPEG2000-Hz-PE). ......................................................... 44
4.3.3 Acidic pH cleavability of mPEG2000-Hz-PE. ............................... 46
4.3.4 Kinetics of the pH-dependent degradation of mPEG2000-Hz-PE. 46
4.3.5 Preparation of pH-sensitive drug delivery systems. ................. 47
4.3.6 Preparation of pH-sensitive immunocarriers. ......................... 48
4.3.7 ELISA. ........................................................................................... 48
4.3.8 Biotin-avidin binding. ................................................................... 49
4.3.9 Interaction of TATp-containing pH-sensitive drug delivery systems with cells. ................................................................................. 49
5 RESULTS AND DISCUSSION .................................................................................................................. 51

5.1 PEGYLATION OF DEXTRAN TO MAKE LONG-CIRCULATING PHARMACEUTICAL CARRIERS .................................................................................................................. 51

5.1.1 Synthesis of aminodextran and PEGylation of aminodextran .......................................................... 51

5.1.2 PEGylation profile of aminodextran .................................................................................................. 52

5.1.3 Biodistribution profile of PEGylated aminodextran ......................................................................... 53

5.2 PEG-LIPID MICELLES WITH MAGNETIC SENSITIVITY .................................................................. 56

5.2.1 Particle size and surface potential of SPION micelles ..................................................................... 56

5.2.2 Freeze-fracture electron microscopy ............................................................................................... 56

5.2.3 Relaxation rate of SPION-micelles ................................................................................................... 57

5.2.4 SPION-immunomicelles and their characterization ......................................................................... 58

5.2.5 In vitro cell interaction of mAb 2C5 SPION-immunomicelles ............................................................ 60

5.2.6 T2 relaxation rates of mAb2C5 SPION-immunomicelles on cells in vitro ......................................... 62

5.2.7 Magnetic targeting of SPION-micelles in tumor of tumor-bearing mice in vivo under the influence of external magnet ................................................................................................... 63

5.3 DETACHABLE PEG TO MAKE STIMULI-SENSITIVE MULTIFUNCTIONAL LIPOSOMES AND MICELLES ............................................................................................................. 65

5.3.1 Synthesis of pH-cleavable PEG-PE conjugate .................................................................................. 66

5.3.2 Immunoreactivity of multifunctional drug delivery systems .............................................................. 70

5.3.3 Avidin binding of multifunctional biotin-containing drug delivery systems ...................................... 71

5.3.4 TATp-mediated interaction of multifunctional drug delivery systems with cells in vitro .................. 73

6 CONCLUSIONS .................................................................................................................................. 77

7 REFERENCES ...................................................................................................................................... 79

APPENDIX 1: LIST OF PUBLICATIONS ............................................................................................. 92
LIST OF TABLES

Table I. Some of the currently marketed PEGylated products .................. 7

Table II. Aminodextran PEGylation efficiency and blood circulation half-life in female C57BL/6J mice ................................................................. 53

Table III. Relaxation rate (1/T2) of the MCF-7 cells after the incubation with different concentrations of micellar preparations......................... 62

Table IV. PEG2000-Hz-PE micelle stability at different pH values (as per cent of remaining micelles)................................................................. 70
LIST OF FIGURES

Figure 1. Schematic representation of the enhanced permeability and retention (EPR) effect................................................................. 2

Figure 2. Chemistry for amino-reactive PEG conjugation .................... 11

Figure 3. Chemistry for thiol-reactive PEG conjugation ....................... 11

Figure 4. Example of detachable “double ester” PEG.......................... 12

Figure 5. Attachment and detachment of PEG maleic anhydride with proteins ..................................................................................... 13

Figure 6. Example of detachable PEG-lipid conjugates using disulphide linkages ................................................................. 13

Figure 7. Chemical structure of PEG-PE............................................. 20

Figure 8. Micelle formation from amphiphilic unimers and drug incorporation into micelle.......................................................... 21

Figure 9. Schematic of interaction of the multifunctional pH-responsive pharmaceutical nanocarrier with the target cell. pH-dependent removal of protecting PEG chains or mAb-PEG moieties allows for the direct interaction of the CPP moiety with the cell membrane....................... 27

Figure 10. Synthesis of PEGylated dextran................................. 52

Figure 11. Biodistribution of Dextran(PEG)$_{21}$ in comparison with Dextran: A) Liver; B) Spleen; C) Kidney; D) Lung .................. 54
Figure 12. Particle size for SPION-micelles measured using dynamic light scattering in ZetaPlus particle size analyzer................................. 56

Figure 13. Freeze-fracture micrographs of (A) ‘plain’ PEG-PE micelles and (B) SPION-loaded PEG-PE micelles .................................................. 57

Figure 14. T2 relaxation rate (1/T2) of SPION-loaded micelles compared to the relaxation rate of “plain” SPION in HBS pH 7.4 at room temperature................................................................. 58

Figure 15. Binding of mAb 2C5 SPION- immunomicelles and control ‘plain’ SPION-micelles or bovine IgG SPION-micelles to a monolayer of nucleosomes................................................................. 59

Figure 16. Epifluorescence microscopy of human breast cancer MCF-7 cells after incubation with SPION-micelles for 2 h................................. 60

Figure 17. Cell-associated rhodamine fluorescence (by fluorescence spectrometer) using micelles at concentration of A) 0.35 mM PEG-PE or B) 0.70 mM PEG-PE . ................................................................. 61

Figure 18. Accumulation of SPION-micelles in tumor tissue of tumor-bearing Balb/C mice under influence of external magnets................. 64

Figure 19. Schematic for the design of the multifunctional DDS used in this study that includes pH-cleavable PEG-Hz-PE (a), and TATp (b), and monoclonal antibody (c) attached to the surface of DDS via pH-non-cleavable spacer................................................................. 65

Figure 20. Schematic description of the conjugation reaction for preparing pH-sensitive PEG-Hz-PE ......................................................... 68
Figure 21. HPLC analysis of the pH-sensitive mPEG2000-Hz-PE micelles after incubation in pH 8.0 (A) and after incubation in pH 5 (B) at room temperature.......................................................... 69

Figure 22. Binding of antimyosin mAb 2G4-PEG2000-Hz-PE-immunomicelles to a monolayer of dog cardiac myosin in comparison to the native mAb 2G4 at corresponding pH values ........................................... 71

Figure 23. Binding of pH-sensitive biotin-micelles to NeutrAvidin columns after 15 min incubation at room temperature at pH 8.0 and at pH 5.0...... 72

Figure 24. Fluorescence microscopy showing internalization of Rh-PE-labeled-TATp-containing micelles by NIH 3T3 fibroblast cells after incubating micelles at pH 8.0 and pH 5.0 for 30 min................................. 73

Figure 25. Fluorescence microscopy showing internalization of Rh-PE-labeled-TATp containing pH-sensitive liposomes by U-87 MG astrocytoma cells .............................................................. 75
1 INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

1.1.1 Need for site-specific drug delivery.

The most important requirement during treatment or diagnosis of a disease condition is to get the maximum effect with minimum dose of pharmaceutical agents. In most of the cases, the administered pharmaceuticals more or less evenly distribute throughout the body with the blood, before providing the desired therapeutic or diagnostic effect at the disease site, for example tumors, or infarcts. This type of distribution leads to unnecessary exposure of non-target tissue to the drugs which is the main reason for drug-related toxicities and side-effects (1). Also because of this, the amount of the pharmaceuticals administered has to be in “excess” to obtain an effective or therapeutically desirable concentration at the disease site.

An obvious way to address this problem would be to formulate or design drug delivery systems (drug carriers) that are capable of accumulating in desired pathological sites and have minimal accumulation in non-target tissues (1-3). Such targeted drug carriers are the solution to reduce effective drug dose, and also drug-related toxicities.

1.1.2 Need for long circulation of drug delivery systems.

In order to design targeted delivery systems, it is important to understand and exploit the characteristics / conditions unique to the pathological tissue to enhance / facilitate therapy. For example, in the case of solid tumors, uncontrolled cell proliferation demands angiogenesis (i.e. new blood vessel development for supporting this growth). New vessel formation is often accompanied by discontinuity or “leakiness” within the vascular endothelial layer (4). Studies indicate the pore size of most peripheral human tumors to
range from 200 nm to 600 nm in diameter (5). This “leakiness” makes the tumor vasculature hyperpermeable for high molecular weight (≤ 40 kDa) long-circulating macromolecules and nanoparticles like liposomes (50 -to- 400 nm) or polymeric micelles (10 -to- 50 nm). In addition to this, defective or underdeveloped lymphatic drainage system causes the permeabilized macromolecules to be retained. Thus, macromolecules entering tumor tissues are retained with higher efficiency and for a prolonged time compared to normal tissues. Maeda and coworkers observed this passive targeting phenomenon while studying styrene maleic anhydride–neocarzinostatin (SMANCS) and described it as the Enhanced Permeability and Retention Effect (EPR effect) (1, 6-8) (Figure 1).

**Figure 1.** Schematic representation of the enhanced permeability and retention (EPR) effect (9).
Thus, pharmaceuticals or pharmaceutical carriers with increased residence time in blood, (i.e. long-circulating) will result in repeated passages of pharmaceuticals over the discontinuous tumor vascular bed. Long-circulating pharmaceuticals will thus be able to extravasate more efficiently through the vasculature and accumulate in the diseased tissue areas. Thus, long circulation of pharmaceuticals could be viewed as the mantra to make them accumulate more in the compromised vasculature areas in diseased tissue.

1.1.3 Systems for targeted drug delivery.

For parenteral administration, drug delivery systems to be safe and effective they require the following properties: they should be small in particle size (nm range), biocompatible, bio-degradable, devoid of immunogenicity, have high drug-loading capacity, exhibit long blood circulation, extravasate into the required pathological sites and be able to release the drug contents at the target site for therapeutic effect (1, 10).

Particulate delivery systems like liposomes, micelles and polymeric nanoparticles have been extensively studied with a great deal of success and provide most of the properties desired in delivery systems for parenteral administration. Because of their small size and ability to functionalize their surfaces with various polymers or ligands, these particles can be used for target specific delivery. Various other constructs or assemblies exist which can serve the same function of targeted delivery include drug- polymer conjugates, cyclodextrins, niosomes, solid lipid particles, lipoproteins, microemulsions, dendrimers, metal nanoparticles, protein cages, polyplexes, and cochleates (11). The choice of delivery system used is mostly influenced by the physico-chemical characteristics of the drug to be delivered.
1.1.4 Active targeting of drug carriers.

In addition to long circulation and passive targeting, target-specific ligands attached to the surface of drug delivery systems can provide even better accumulation or even allow for delivery at the cellular level. Various ligands like monoclonal antibodies, their fragments, peptides, sugar moieties, etc having specific recognition for their target receptors have been identified and well characterized, and can be used to achieve targeted active delivery of pharmaceuticals and pharmaceutical carriers.

1.1.5 Polyethylene glycol (PEG) in drug delivery carriers

Among many materials used to make or modify pharmaceutical carriers (lipids, natural and synthetic polymers, emulsions, or dendrimers) special attention was paid to PEG, which was used both for chemical modification of various drugs (peptide and protein, first of all) to make them more stable and long-circulating and for the decoration of pharmaceutical carriers to improve their pharmacokinetic properties (12-15). FDA approved PEG is a highly hydrophilic, flexible polymer which has an inherent long circulating property. The array of already available versatile PEG chemistries make it an attractive polymer to be used in modifying pharmaceuticals or surfaces of pharmaceutical carriers to achieve the desired long-circulating property or add convenient functional groups to conjugate ligands for active targeting.

The goal of this study was to design various platforms for the efficient delivery of therapeutic and diagnostic agents using PEG to screen its application in engineering various delivery systems for both passive and/or active targeted delivery of pharmaceuticals for the potential treatment and diagnosis of various disease states.
1.2 REVIEW OF THE LITERATURE.

1.2.1 Water-soluble polymers and prolonged circulation.

Water-soluble polymers are popular as carriers for various therapeutic and diagnostic agents (14-16). Conjugation with polymers increases the circulation lifetime of many low-molecular weight drugs, by preventing their renal filtration when the total molecular weight of the polymer-drug conjugate exceeds approximately 40 kDa and by preventing drug degradation by the action of various body enzymes (17). In addition, polymer conjugation prevents the passive diffusion into cells typical for low-molecular-weight therapeutics, thereby increasing circulation time and decreasing the accumulation of the drug in non-targeted tissues (12, 18). This latter effect allows for a substantial decrease in side-effects for certain drugs (19). Conjugation with polymers also allows for an increase in therapeutic potential of biologically active proteins and peptides (18). The use of potential therapeutic agents of this nature is frequently hampered by their rapid proteolytic degradation in the blood and uptake by the cells of the reticuloendothelial system (RES). The attachment of polymer molecules to polypeptides creates steric hindrances for their interaction with degrading enzymes of the host and receptors of RES cells (14). The hydrophilic polymer chains exposed to the aqueous surroundings interfere with the inter-particle attraction caused by van der Waals forces. This prevents adsorption of blood proteins (including opsonins) in vivo onto the surface of biologically active proteins, peptides or any pharmaceutical carrier surface in general. Preventing protein adsorption onto carrier surfaces not only prevents their RES mediated clearance, but can also influence important characteristics such as biodistribution, stability, or drug release profile. The efficacy of protection depends both on the surface density of hydrophilic polymer blocks and the
thickness of the protective layer, i.e. the molecular size of the hydrophilic polymer block (16, 20, 21).

1.2.2 Rationale for using PEG in drug delivery systems.

A number of water-soluble polymers like PEG (15, 21), M-(2-hydroxypropylmethacrylamide) (HPMA) copolymers (22), poly(acryl amide) and poly(vinyl pyrrolidone), poly(acryloyl morpholine), poly(2-methyl-2-oxazoline), poly(2-ethyl-2-oxazoline), phosphatidyl polyglycerols, polyvinyl alcohol and poly(glutamic acid) (PGA) (23-28) have been tested as steric protectors in various nanoparticulate systems with various degrees of success. However, PEG has emerged as one of the most popular polymers for drug delivery, in particular for the development of protein- or peptide-based long-circulating therapeutics (16, 18, 29). PEGylated agents have been approved by the Food and Drug Administration (FDA) for parenteral or topical administration and as a component of various foods, cosmetics, and drug-delivery systems, such as liposomes, and nasal sprays. Table I shows some of the marketed PEGylated products.
Table I. Some of the currently marketed PEGylated products (16).

<table>
<thead>
<tr>
<th>PEG Conjugate</th>
<th>Generic Name (Trade Name)</th>
<th>Biactivity of Native Agent</th>
<th>Main Effect of Pegylation</th>
<th>Reason for Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA</td>
<td>Pegademase (Adagen) / Enzon (March 1999)</td>
<td>Enzyme replacement, reverses symptoms of ADA deficiency</td>
<td>Longer half-life, reduced immune response&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SCID as a result of ADA deficiency</td>
</tr>
<tr>
<td>Asparaginase</td>
<td>Pegaspargase (Concasparg) / Enzon (February 1994)</td>
<td>Hydrolyzes asparagine, on which leukemic cells are dependent</td>
<td>Longer half-life, reduced immune response&lt;sup&gt;b&lt;/sup&gt;</td>
<td>In combination chemotherapy, for treatment of acute lymphoblastic leukemia in patients hypersensitive to L-asparaginase</td>
</tr>
<tr>
<td>Granulocyte colony-stimulating factor</td>
<td>Pegfilgrastim (Neulasta) / Amgen (January 2002)</td>
<td>Stimulation of neutrophil production</td>
<td>Longer half-life, self-regulating clearance&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Prophylaxis against severe neutropenia and its complications during myelosuppressive chemotherapy</td>
</tr>
<tr>
<td>Interferon α2b</td>
<td>Peginterferon α2b (PEG-intron) / Schering (January 2001)</td>
<td>Antiviral cytokine</td>
<td>Slower clearance, sustained serum concentration&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Hepatitis C in patients with normal liver function</td>
</tr>
<tr>
<td>Interferon α2a</td>
<td>Peginterferon α2a (Pegasys) / Roche (October 2002)</td>
<td>Antiviral cytokine</td>
<td>Slower clearance, sustained serum concentration&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Hepatitis C in patients with compensated liver disease</td>
</tr>
<tr>
<td>Methotrexate liposomes for delivery of doxorubicin</td>
<td>Pegylated liposomal doxorubicin (Caelyx, Doxil) / Alza (June 2000)</td>
<td>Antitumor, anticyclines</td>
<td>Slower clearance, greater distribution into tumors&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Kaposi's sarcoma, refractory ovarian cancer</td>
</tr>
</tbody>
</table>

PEG = polyethylene glycol; FDA = Food and Drug Administration; ADA = adenosine deaminase; SCID = severe combined immunodeficiency disease.

PEG offers dramatic protection to the molecules to which it is conjugated. Chapman and colleagues demonstrated that by attaching a single 60 kDa branched molecule of PEG onto antibody fragments, sufficient steric protection was achieved (30). In this case, PEGylation resulted in the modification of only one protein reactive group, which increased the chances to obtain a modified protein with preservation of its specific activity.

In the area of nanoparticulate delivery systems, “conventional” first-generation liposomes (phospholipid bilayer vesicles) were beneficial for the transport of small drug molecules by reducing the rate of drug clearance and increasing the accumulation of drug molecules into the target organ/tissue. In spite of the advantages offered, liposomal delivery had a number of limitations including sequestration into organs like liver, spleen, kidney and recognition and removal by the RES. When the liposomes were additionally surface-modified by either
glycolipids like monosialoganglioside or hydrophilic-polymers, such as PEG, the liposomes were able to evade recognition by the immune system resulting in long-circulation in the blood pool along with improved distribution to the tumors (20, 21, 23, 31-34). The success of clinically approved Doxil® is an superb example of such a PEGylated liposome system (33). Overall PEG provides an excellent combination of properties with a highly flexible hydrophilic polymer chain to act as a protecting polymer; it has very low toxicity, devoid of any antigenicity, does not accumulate in the RES organs and also has minimum change in biological properties of the modified pharmaceuticals (14, 29, 35).

1.2.3 PEGylation chemistry.

PEGylation can be defined as conjugation of one or more PEG chains to proteins, peptide, non-peptide molecules or any particle surface (29). PEGylation technology was first developed by Davis et al. in the 1970s (36). PEG polymers are repeating units of ethylene oxide with molecular weights ranging from 500 Da – 30 kDa in linear chains or branched chains linked together by chemical linkers (16) having the general structure:

\[ \text{HO}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2\text{-OH} \]

PEG is synthesized by ring opening polymerization of ethylene oxide initiated by nucleophilic attack of hydroxide ions on the epoxide ring. However PEGs having hydroxy groups on both sides generally have higher polydispersity and high molecular weights since polymerization can occur at both ends of the polymer. To address this, monomethoxy PEG or mPEG are useful having the general structure:

\[ \text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2\text{-OH} \]
In order to couple the PEG chains onto different molecules such as proteins, peptides or particle surfaces it is necessary to have PEG activated with a functional group at one or both of the termini. The choice of the functional group is influenced by the functional groups available on the molecule of interest. In proteins or peptides the side chain amino groups (lysine, arginine), sulfhydryl (cysteine), hydroxyl (serine, threonine), carboxy (aspartic acid, glutamic acid) or N-terminal amino and C-terminal carboxy can be considered; whereas in the case of glycoproteins, the hydroxyl groups can be utilized. The majority of the cases for PEGylation of proteins or peptides make use of available primary amine groups from lysine, arginine or the N-terminal amino group. PEG chemistries for amine conjugating involve acylation and include derivatives like PEG-dichlorotriazine, PEG-tresylate, PEG-succinimidylichloroformate, PEG-benzotriazole carbonate, PEG\(p\)-nitrophenyl carbonate, PEG-trichlorophenyl carbonate, PEG-carbonylimidazole, PEG-succinimidyl succinate, PEG tresylate. In general, active esters of PEG carboxylic acids are commonly used as acylating agents for proteins that react with primary amines to form stable amides (37). Some of the amino group reactions are as shown in Figure 2.
**Figure 2.** Chemistry for amino-reactive PEG conjugation (37).
Conjugating to thiol groups (Figure 3) usually involves the formation of disulfide, thioether or thioester bonds using PEG derivatives such as PEG-maleimide, vinylsulfone, iodoacetamide and orthopyridyl disulfide (37).

![Chemistry for thiol-reactive PEG conjugation](image)

In most cases, PEGylation chemistry involves formation of a stable irreversible conjugate linkage because of the obvious advantage of long-term storage, as well as effective steric protection from opsonins. However, the steric protection property of PEG can also block the active site of the PEGylated protein and decrease its biological activity. The best solution to prevent such a problem requires a chemistry that allows for the removal of PEG chains from the conjugate to expose the active or binding site to generate the native protein.
or peptide. For example, in the case of PEG-intron, PEG-succinimidyl carbonate was conjugated to interferon alpha-2b histidine residue (His$^{34}$) to form a carbamate bond that was released over time (38). PEG “double esters” have also been developed for this purpose wherein hydroxy acids are attached to carboxylic acids of PEG to create a PEG acid that has an ester linkage between hydroxy acid and PEG acid. The terminal acid is then activated and attached to $\alpha$- and $\varepsilon$-amino groups. The problem however with PEG “double esters” chemistry is that they release the protein with residual chemical groups still attached to the protein which could lead to protein immunogenicity (Figure 4).

Figure 4. Example of detachable “double ester” PEG (37).
To circumvent this problem, chemistries like PEG maleic anhydride by Garman et al. (39) or mPEG phenyl ether succinimidyl carbonates and mPEG benzamide succinimidyl carbonates developed by Zhao et al. have been used (Figure 5).

\[ \text{Figure 5. Attachment and detachment of PEG maleic anhydride with proteins (37).} \]

Zalipsky et al. have also described the synthesis of detachable PEG-lipid polymers cleaved by cysteine (40). The linkage employs a \( p \)- or \( o \)-disulphide of a benzyl urethane which when subjected to mild reducing conditions present in endosomal compartments of cell releases PEG from the conjugate. The removal of PEG chains proceeds as shown in Figure 6.

\[ \text{Figure 6. Example of detachable PEG-lipid conjugates using disulphide linkages (40).} \]
Thus there is an array of available PEGylation chemistry to tailor the requirements for drug molecules, proteins, peptides or any particulate drug delivery systems where long-circulation is desired. FDA-approved PEGylated products clearly indicate the improved therapeutic efficacy of the drugs using this technology. Even though many studies have been conducted demonstrating the theoretical and commercial usefulness of PEGylation technology there are many more untapped applications that are still to be explored.
Thus it is clear that PEG offers the versatile properties of flexibility, hydrophilicity, and biocompatibility. PEG-conjugation modifies the physico-chemical properties of the conjugated molecule thereby changing its pharmacokinetic and biodistribution profile.

A detailed systematic investigation was carried out to explore the applications of PEG in developing various drug carrier systems.

As a first example, PEG with a molecular mass of 5 kDa was used to modify dextran (molecular mass 73 kDa). Dextran was initially modified to introduce amino groups. Partial modification of these amino groups with PEG residues resulted in a construct with a nearly doubled circulation half-life in mice of up to 7 h (41). Residual dextran amino groups can be used for attachment of drugs, diagnostic agents or even stimuli-responsive polymer blocks, thus providing a convenient platform for long-circulating multifunctional polymer-drug pharmaceutical preparations. We have prepared PEG-modified dextran to make it long-circulating. Dextran, a polysaccharide, has hydroxyl groups which can be used to conjugate small drug molecules proteins/peptides or diagnostic moieties and be developed to be used as a drug carrier system.

Another application of PEG is in combination with lipids. When PEG is conjugated to lipid molecules as in the case of PEG-phosphatidylethanolamine (PEG-PE), the conjugates can form micelles with a central hydrophobic lipid core and outer PEG corona. These micellar structures can be loaded with poorly water-soluble drugs like, paclitaxel and camptothecin (10, 42, 43). PEG
forms the external hydrophilic corona that can offer long-circulation as well as the convenience of attaching various ligands at the distal PEG-terminus to make the micelles actively target the specific diseased tissue.

To develop magnetically sensitive micelles, superparamagnetic iron oxide nanoparticles (SPION) were incorporated into PEG-PE based micelles to form stable SPION-micelles. SPION have excellent MRI contrast properties, however, they are not stable in physiological systems and show aggregation \((44, 45)\). PEG-lipid based micellar formulation not only prevented the SPION from aggregation but also improved its MRI signal. Because of the small size and long-circulating property, SPION-micelles can be targeted passively by EPR effect. SPION-micelles can also be targeted to the disease site under influence of external magnets. Moreover to prepare actively targeted MRI contrast agents, SPION-micelles can be easily surface-modified by active targeting ligands.

An interesting application of PEG-lipid conjugates can be achieved when the bond between PEG and lipid is stimuli-sensitive and degradable. This stimulus can either be the locally decreased pH, locally increased temperature, or reducing conditions as the found in inflammation areas or tumor interstitium \((46-49)\). Because of its steric protection function PEG is used in targeted long-circulating PEGylated liposomes and PEG-PE-based micelles \((10, 43, 50, 51)\). Cell-penetrating peptides (CPPs) like TAT can be incorporated onto the surface of liposomes or micelles and these ligands could be easily “shielded” or “exposed” by using stimuli (pH)-sensitive PEG-PE. PEG-PE used in this work for liposome surface modification or for micelle preparation was made degradable by inserting the pH-sensitive hydrazone bond between PEG and PE (PEG-Hz-PE). Under normal pH values, TATp functions on the surface of nanocarriers were “shielded” by long protective PEG chains. At pH 7.4-8.0,
TATp-containing both liposomes and micelles demonstrated very limited internalization by NIH/3T3 or U-87 cells. However, upon brief incubation at lowered pH values (pH 5.0-6.0), the nanocarriers lost their protective PEG shell because of acidic hydrolysis of PEG-Hz-PE and acquired the ability to become effectively internalized by cells via TATp moieties. This result could be considered as the first step in the development of multifunctional stimuli-sensitive pharmaceutical nanocarriers.

2.1 PEGylation of dextran to make long-circulating pharmaceutical carrier.

Dextran, a natural polymer of glucose residues linked mainly with 1,6-bonds has also been used as a drug carrier for a long time. This polymer is widely available from natural sources and can be prepared in a variety of molecular weights (52). For example, as early as 1978, it was shown that conjugation of daunomycin with dextran increased the therapeutic efficacy and decreased the toxicity of the drug (53). However, there are certain problems associated with dextran. The degree of dextran branching (the property associated with dextran immunogenicity) depends on the source of dextran and may vary over a broad range (54). The high-molecular-weight dextrans used as drug carriers may have a rather broad molecular size distribution and can cause anaphylactic reactions (53). Although dextran-bound drugs demonstrated somewhat increased circulation times, dextrans still undergo biodegradation via various dextranases found in many tissues (55, 56).

On the other hand, synthesis of monofunctional (containing an activating group at only one terminus) forms of high molecular weight PEG with narrow polydispersity is relatively difficult to achieve (57). The disadvantage of PEGs with molecular weights exceeding 30 kDa is their reduced excretion and
increased kidney accumulation. Although PEG has a very low toxicity and has been approved for a variety of products for human use (14), this polymer is not metabolized. While PEG molecules with the molecular weight of 30 kDa or lower are easily cleared by renal filtration, PEGs with higher molecular weights are cleared much more slowly (57). Such biologically uncleavable polymers with high molecular weight can accumulate in the kidneys, decrease the efficiency of renal filtration and cause various undesirable side effects (17).

Thus, the idea comes to mind of designing long-circulating but still biodegradable delivery systems built from a biodegradable core polymer, dextran and grafted with relatively short PEG residues as a good alternative to using either high molecular PEGs or pure dextrans. Such co-polymers synthesized using monofunctional PEG derivatives with a low-to-moderate molecular weight will slowly degrade to fragments small enough to be eliminated by the renal filtration. The dextran core, after the attachment of multiple PEG residues, might still bear free reactive groups suitable for the attachment or single-point modification of various drugs including proteins and peptides to obtain their long-circulating forms (58).
2.2 PEG-lipid conjugates as the basis for micellar pharmaceutical nanocarriers and PEG-lipid micelles with magnetic sensitivity.

2.2.1 PEG-lipid conjugates as micellar systems

PEG-PE-based lipid-core polymeric micelles have been the delivery system of choice for various hydrophobic drugs/diagnostic agents (42, 50, 59-63). They have prolonged blood circulation and can be easily functionalized with various target-specific ligands like nucleosome-specific monoclonal antibody 2C5 (mAb2C5) on the water-exposed PEG termini (10, 43). Polymeric micelles, in general, have been studied extensively for encapsulating various cancer chemo therapeutics. In addition to long blood-circulating time and EPR effect in tumors, they have also been shown to reduce toxicity and side-effects of the drugs (64).

Diacyl lipid-PEG or PEG-PE conjugates have been successfully used to surface-modify liposomes to make them long circulating (65). The structure of PEG-PE polymer is similar to that of amphiphilic co-polymers of A-B type with acyl chains of lipids forming the hydrophobic core of the micelles that can be used as a cargo space for encapsulating a variety of sparingly water-soluble therapeutic and diagnostic agents. PEG-PE conjugates are commercially available or can be easily synthesized (65).
Figure 7. Chemical structure of PEG-PE (50).

The chemical structure of PEG-PE is shown in Figure 7. A single molecule of PEG-PE consists of flexible, hydrophilic PEG conjugated with a relatively short but very hydrophobic diacyl phospholipid. The lipid allows the anchoring of the molecule in the lipid bilayer in case of liposomes, leading to its initial use of modifying the surface of liposomes with PEG chains.

The presence of PEG protects liposomes from the interaction with opsonins in the blood plasma and prevents rapid uptake of liposomes by the RES. This results in a dramatic increase in the liposome circulation time. One peculiar observation is that the PEG-PE molecule, if used in liposomes above certain critical limit forms micelles (66). Thus, the proportion of PEG-PE used in a liposomal formulation is important. Later it was realized that PEG–PE micelles have a substantial potential as particulate carriers for the delivery of therapeutic and diagnostic agents (Figure 8). The use of lipid moieties as hydrophobic blocks provides a very high stability of the micelles because of very strong hydrophobic interactions between the double acyl chains of the phospholipids residues. As with long-circulating liposomes, the exposure of PEG residues on the surface of micelles prevents rapid uptake of the micelles by the RES, making them long circulating. PEG-PE has very low toxicity and
it is currently used in the clinic as a component of Doxil® — doxorubicin-loaded long-circulating liposomes.

2.2.2 Superparamagnetic iron oxide nanoparticles (SPION).

SPION have received increased attention during the last decade due to their characteristic inducible magnetic moments in the presence of an external magnetic field, their small size (ca. 4 - 10 nm) and most importantly T2 magnetic resonance imaging (MRI) contrast properties. The most important property of the SPION is their paramagnetic nature. SPION behave like magnets only in presence of an external magnetic field and do not retain any

![Diagram of micelle formation](image.png)

**Figure 8.** Micelle formation from amphiphilic unimers and drug incorporation into micelle (50).

In this work PEG-PE was used to prepare magnetically sensitive micelles. Hydrophobic SPION particles were loaded into the PEG-PE based micellar system to form SPION-micelles.
residual magnetism upon removal of the external magnetic field. This property makes it possible for their application as MRI contrast agent or their targetability under the influence of external magnets. However, “plain” SPION are not stable at normal physiological conditions and show a tendency to aggregate because of the hydrophobic nature of these particles. These magnetic nanoparticles can be dispersed to form homogenous suspensions into suitable solvents by using proper surface coating. Attempts have been made to stabilize SPION by their incorporation into polymeric micelles (44).

2.2.3 MRI and MRI Contrast agents.

Currently, MRI is the most routinely used imaging modality in both clinical and research settings. It offers outstanding resolution down to 1-2 mm in plane at magnet field strengths as low as 1.5 Tesla. The signal of MRI depends on the longitudinal and transverse (T2) proton relaxation times of mainly water. The difference in proton relaxation times (because of differences in water content of tissues) provides MRI contrast in the tissues. The tissue water content, however, is dependent on the physiological microenvironment and may be slightly altered depending on the pathology state. However, this slight change in water environment is not evident until the late phases of the disease. In order to make the differences visible at early stages and reveal the pathologic condition at early stages, certain MRI contrast agents can be used. MRI contrast agents, because of their intrinsic paramagnetic nature, produce local micro-magnetic fields, and thus decrease the T1 or T2 relaxation times of tissue water protons that results in enhanced contrast in imaging of the pathological tissue.

The capability of a contrast agent to change T1 and T2 is defined as the relaxivity, r1 or r2, expressed as mM⁻¹ s⁻¹. Depending on the r2/r1 ratios, the MRI contrast agents are classified as positive contrast agents or negative
contrast agents. Positive contrast agents have low r2/r1 ratios and thus generate hyper-intense (bright) T1-weighted images (e.g. gadolinium-based paramagnetic chelates), while the negative contrast agents have high r2/r1 ratio and thus generate hypo-intense (dark) T2-weighted images (e.g. iron-oxide based superparamagnetic chelates). MRI applications are becoming more and more dependent on contrast agents. MRI in combination with the contrast agent has been an effective tool to get a perspective of inflammation, infarct, tumor, atherosclerotic plaques, live stem-cell tracking, brain perfusion and many other applications (45, 67).

2.2.4 Antinuclear Autoantibodies: Monoclonal antibody 2C5 (mAb2C5).

mAb2C5 belongs to a subset of anti-nuclear antibodies of the IgG2a isotype (68). mAb2C5 is capable of inhibiting tumor growth of various murine and human tumors both prophylactically and therapeutically (69). However at sub-therapeutic levels, mAb2C5 is capable of specifically recognizing a wide variety of tumors with no affinity for normal cells through the surface-bound nucleosomes and thus serves as a targeting moiety to deliver drug carriers such as liposomes and micelles into different murine and human tumors (70, 71). Nucleosomes are an assembly of DNA and four pairs of histones, which are released from dying cells in the center of the tumor. As these nucleosomes migrate from the center to the periphery of the tumor, they get attached and expressed on the surface of the neighboring live tumor cells. The uniqueness of using mAb2C5 as the targeting ligand is that its target antigen is not tumor-type specific, but rather a universal target that is typical of almost all tumor types (71, 72). Thus it can be expected that the nanocarriers decorated with mAb2C5 will recognize a broad variety of murine and human cancer cell types. Previously we have demonstrated efficient tumor targeting capability of mAb2C5 with several drug carriers like paclitaxel-loaded mAb2C5 surface-
modified immunomicelles (73) or doxorubicin-loaded mAb2C5 surface-modified liposomes (36, 74-77).

Taking into account the benefits of using lipid-core based PEG-PE polymeric micelles and the tumor-targetability of mAb2C5, we formulated SPION-loaded PEG-PE micelles (SPION-micelles) and additionally surface modified SPION-micelles with mAb2C5 to form the SPION-immunomicelles. We hypothesized that these immunomicelles would bring an increased quantity of SPION to cancer cells thus increasing the T2 MRI signal from these cancer cells compared to either “plain” SPION-micelles or “non-specific” SPION-bovine-IgG immunomicelles.

Because of the magnetic properties of SPION, we also hypothesized that we would be able to target SPION-micelles to the tumor tissue in subcutaneous tumor models in mice using external magnets.
2.3 Detachable PEG to make stimuli-sensitive multifunctional liposomes and micelles.

Another important application of PEG is to engineer multifunctional pharmaceutical nanoparticles by using PEG conjugates with special properties such as pH-sensitivity. The concept of synthesizing cleavable PEG-lipid polymers was described earlier by Zalipsky et al. The cleavable bond was based on \( \text{p-} \) or \( \text{o-} \)-disulphide of a benzyl urethane which, when subjected to mild reducing conditions such as in endosomal compartments of cells releases PEG from the conjugate (40).

Ideally, a nanoparticle drug delivery system (DDS) should be able: (a) to specifically accumulate in the required organ or tissue, and then (b) penetrate inside target cells delivering its load (drug or DNA) intracellularly. Organ or tissue (tumor, infarct) accumulation could be achieved by passive targeting via the EPR effect (8, 78); or by antibody-mediated active targeting (10, 79), while the intracellular delivery could be mediated by certain internalizable ligands (folate, transferrin) (80, 81) or by cell-penetrating peptides (82, 83).

2.3.1 Cell-penetrating peptides

CPPs such as HIV trans-activating transcriptional activator peptide (TATp), polyarginine (polyArg), homeodomain of Antennapedia (Antp), or Herpes simplex virus type 1 (HSV-1) protein VP22 all contain short sequences of less than 30 amino acids that are able to penetrate cell membranes and translocate different cargoes into cells. For example, the minimal protein transduction domains or the cell penetrating function domains of TAT-peptide have the sequence Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg while the domains in Antp have the sequence Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-
Trp-Lys-Lys. As is evident, the common feature of these peptides is that they are amphipathic and net positively charged.

The exact mechanism of cell translocation is not yet clearly understood but in general it is accepted that energy-dependent macropinocytosis is responsible for TAT-mediated intracellular delivery of large molecules and nanoparticles with subsequent enhanced escape from endosomes into the cell cytoplasm (84, 85). Energy-independent electrostatic interactions and hydrogen bonding seem to be responsible for cell penetration of individual CPPs or CPP-conjugated small molecules (86, 87).

### 2.3.2 Developing multifunctional nanocarrier systems.

The “ideal” drug delivery system (DDS) should simultaneously carry on its surface various active moieties, i.e. be multifunctional and possess the ability to “switch on” and “switch off” certain functions when necessary, for example under the action of local stimuli typical of the target pathological zone (increased temperature or lowered pH values characteristic for inflamed, ischemic, and neoplastic tissues). Another important requirement is that different properties of the multifunctional DDS should be coordinated in a certain orchestrated manner. For example, if a system is to be constructed that can provide the combination of the longevity allowing for target accumulation via the EPR effect and specific cell surface binding allowing for its internalization by target cells, two requirements have to be met. First, the half-life of the carrier in the circulation should be long enough (hours) to fit EPR effect requirements, and second, the internalization of the DDS by the target cells should proceed fast enough (minutes) to prevent carrier degradation and drug loss in the interstitial space. However, systems like this still represent a challenge (88).
Intracellular transport of different biologically active molecules is one of the key problems in drug delivery in general. Nanoparticulate DDS, such as liposomes and micelles, are frequently used to increase the efficacy of drug and DNA delivery and targeting \((50, 51)\). So far, multiple and not always successful attempts have been made to deliver various drug carriers directly into the cell cytoplasm, bypassing the endocytic pathway, to protect drugs and DNA from the lysosomal degradation, thus enhancing drug efficiency and DNA incorporation into the cell genome \((89-92)\).

Figure 9. Schematic of interaction of the multifunctional pH-responsive pharmaceutical nanocarrier with the target cell. pH-dependent removal of protecting PEG chains or mAb-PEG moieties allows for the direct interaction of the CPP moiety with the cell membrane.
2.3.3 *The new concept of multifunctionality.*

Keeping this in mind we came up with an interesting idea to combine both the longevity of the PEG and the cell-penetrating capability of TAT-peptide by designing liposomes and micelles such that during the first phase of delivery, a non-specific cell-penetrating function is shielded providing organ/tissue-specific delivery (sterically-protecting polymer or antibody). Upon the accumulation in the target, protecting polymer or antibody attached to the surface of the DDS via the stimuli-sensitive bond should detach under the action of local pathological conditions (abnormal pH or temperature) and expose the previously hidden second function allowing for the subsequent delivery of the carrier and its cargo inside cells. Such a DDS should be stable in the blood for a long time (hours) to allow for an efficient target accumulation. It has to lose the protective coat inside the target almost instantly to allow for fast internalization (minutes). Such a delivery system can prove beneficial as it will carry the drug directly into the target cell (88). The schematic pattern of such system is shown in Figure 9. Intracellular trafficking, distribution, and fate of the carrier and its cargo can be additionally controlled by its charge and composition, which can drive it to the nuclear compartment or toward other cell organelles.

In this study, we have prepared and tested *in vitro* stable targeted PEGylated DDS (liposomes and micelles) containing the second specific function (responsible for the intracellular internalization of the DDS), which, under normal conditions, is shielded by the protecting polymer or polymer-antibody conjugate, however can be “exposed” upon brief incubation at lowered pH values characteristic of inflamed or neoplastic areas. As the first step on the way to multifunctional drug delivery systems, we have prepared liposomes and micelles sterically protected by the low pH-cleavable PEG chains and
additionally containing a monoclonal antibody attached to the surface of the DDS via a noncleavable longer PEG spacer and an additional function (biotin or TATp) attached to the surface of the DDS via the noncleavable anchor shorter than the cleavable PEG chain.
3 OBJECTIVES AND SPECIFIC AIMS

The overall objective of this thesis project is to test applications of PEG in constructing platforms for delivery of therapeutics and diagnostics.

Polymers like dextran and drug delivery nanocarriers like liposomes and micelles have been used and improved by either modifying them by using PEG to prepare more effective drug carrier formulations and thus to improve the delivery of pharmaceuticals therapeutic outcome.

I. To develop and characterize a biodegradable drug carrier platform using dextran and test if PEGylation of dextran improves their circulation time \textit{in vivo}.

II. To develop and characterize “targeted” SPION as an imaging agent loaded in PEG-lipid polymeric micelles, and to use either external magnets or nucleosome-specific monoclonal antibody 2C5 on the micelle surface for target-specific delivery, and to test the targetability of such micelles to cancer cells \textit{in vitro} and \textit{in vivo}.

III. To develop and characterize multifunctional liposomes and micelles simultaneously carrying a number of ligands, which are expressed only upon the action of external stimuli like local pH, using pH-degradable PEG-lipid conjugates and test their functionality \textit{in vitro}.
4 EXPERIMENTAL DESIGN AND METHODS

4.1 PEGylation of dextran to make long-circulating pharmaceutical carriers.

4.1.1 Materials

Dextran with average molecular weight of 73 kDa, carbonyldiimidazole (CDI), ethylenediamine (EDA), diethylene triamine pentaacetic acid anhydride (DTPA) and fluorescamine were purchased from Sigma Chemical, Inc. (St. Louis, MO). PEG-succinimidyl propionate (PEG-SPA) with a molecular weight of 5 kDa was from Nektar Therapeutics (San Carlos, CA). All other chemicals and organic solvents were of analytical grade. Distilled, deionized water was used.

4.1.2 Synthesis of aminodextran

Aminodextran was synthesized from CDI-activated dextran by conjugating EDA using a previously described procedure for attaching diamines to cross-linked agarose (93). In order to determine the rate of CDI activation, 17 µmol of dextran dissolved in 30 ml of formamide was incubated with 360 µmol of CDI (21 molar excess over dextran) for 15 min, 30 min, 1 h and 3 h at room temperature. After overnight incubation at room temperature the reaction was stopped by the addition of at least 100-fold molar excess (over CDI) of EDA. The resultant aminodextran was precipitated by adding 2-fold volume excess of acetone. The white precipitate was washed with acetone and collected by centrifuging at 1500xg for 1 h, dissolved in water, dialyzed against at least a 3000-fold excess of water for 2-4 days with several buffer changes, and freeze
dried. The number of EDA residues attached per dextran molecules was estimated by determining the concentration of amino groups in the samples using the fluorescamine method as described below.

As a typical preparative procedure, 68 µmol of dextran was dissolved in 125 ml of formamide and 1.44 mmol of CDI (21 molar excess over dextran) dissolved in 10 ml of formamide was added. After an hour of incubation, 25 ml of EDA was added to the mixture, and stirred at room temperature overnight. Purification was carried out as described above.

4.1.3 Introduction of DTPA residues into aminodextran.

Aminodextran (28.5 µmol) was dissolved in 25 ml of formamide containing 0.8 ml of pyridine and mixed with of DTPA anhydride dissolved in 0.5 ml of formamide (2-fold molar excess over aminodextran). The mixture was incubated for 1-5 h at room temperature, dialyzed against water and freeze dried.

4.1.4 PEGylation of aminodextran.

Aminodextran (20.5 µmol) was dissolved in 50 ml of formamide containing 2 ml of pyridine. To this solution, 103 µmol of PEG-SPA dissolved in 5 ml of formamide was added. To study the kinetics of PEG attachment, the mixture was incubated for 30 min, 2 h, 5 h and overnight at room temperature with stirring. The reaction was stopped by adding 5 ml of water per 10 ml of the reaction mixture. The sample was purified by dialysis and freeze-dried as described above. The number of PEG residues attached per each aminodextran molecule was estimated by amino group loss determined by the fluorescamine method (see below).
For preparative purposes, 20 µmol of aminodextran was dissolved in 50 ml of formamide containing 1.2 ml of pyridine. To this solution, 300 µmol or 600 µmol of PEG-SPA dissolved in formamide was added. The mixture was incubated at room temperature with stirring overnight. Organic solvents and unbound PEG were removed by dialysis against water using dialysis bags with a cutoff size of 50 kDa (Spectrum Medical Industries, Rancho Dominguez, CA) and freeze-dried. For biodistribution studies, PEGylated aminodextran samples were acetylated in formamide containing pyridine by addition of a 10-fold molar excess of acetic anhydride and purified as described above.

4.1.5 Amino group concentration.

The number of primary amino groups was estimated using fluorescamine according to a procedure adapted from a previous publication (94). The material of interest was dissolved at about 0.2 mg/ml in 10 mM phosphate buffered saline, pH 7.4. A 1.5 ml aliquot of the sample was mixed with 0.5 ml of 3 mg/ml fluorescamine solution in acetone and incubated in the dark at room temperature for 30 min. The number of amino groups was determined by fluorescence at an excitation wavelength of 400 nm and an emission wavelength of 460 nm on a F-2000 spectrofluorimeter (Hitachi, Japan) using a sample of commercially available aminodextran with a known number of amino groups per dextran molecule as a standard (Molecular Probes, OR).

4.1.6 Radiolabeling of PEGylated dextrans by transchelation.

DTPA-modified aminodextran or PEGylated aminodextran was dissolved in 10 mM HEPES buffered saline, pH 7.4 (HBS) at a concentration of 10 mg/ml. To 1 ml of this solution, 10-20 µCi of $^{111}$In in 0.1 ml of 0.1 M sodium citrate, pH 3.7 (a weak chelator for $^{111}$In) was added and incubated for 1 h at room
temperature. Any unbound $^{111}$In was removed by dialysis using 25 kDa MWCO dialysis tubing against HBS at 4°C overnight.

**4.1.7 Biodistribution studies.**

In vivo biodistribution studies were carried out using $^{111}$In-labeled dextran or PEG-dextran in female C57BL/6J mice (Charles River Laboratories, Wilmington, MA). The mice were injected with 100 µl of 10 mg/ml of $^{111}$In-labeled dextran or PEG-dextran via the tail vein. The animals were allowed free access to food and water. Mice were sacrificed by cervical dislocation at time points between 0.5 and 17 h post injection with 4 animals in a group for each time point. Blood, liver, spleen, renal and lung samples were collected and analyzed by $\gamma$-counting for the presence of the dextran-associated $^{111}$In radioactivity as CPM using a Beckman 5500B gamma-counter. Dextran circulation half-lives were determined assuming a first order elimination process from the Log [% of injected dose in blood] vs. Time plots using a computer program, Origin (Microcal Software, Inc, Northampton, MA).

**4.1.8 Statistics.**

Data is represented as mean ± SE, unless specified otherwise. Student’s t-test was applied for test of significance. $P \leq 0.05$ was considered statistically significant.
4.2 PEG-lipid micelles with magnetic sensitivity.

4.2.1 Materials.

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol) 2000] (PEG2000-PE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl] [ammonium salt] (Rhodamine-PE or Rh-PE) and 1,2-Dimyristoyl-sn-Glycero-3-Phosphoethanolamine-N-DTPA (DTPA-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Trimethylamine oxide [(CH3)3NO], Iron(0) pentacarbonyl [Fe(CO)5], hexane, ethanol, octyl ether and all other chemicals were purchased from Sigma Chem., Inc. (St. Louis, MO, USA). HOECHST 33342 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Dulbecco’s Modified Eagle’s Medium, Hanks’ Balanced Salt Solution (HBSS), and penicillin/streptomycin stock solutions were purchased from CellGro (Kansas City, MO, USA). Tissue culture grade fetal bovine serum and trypsin was obtained from ICN Biomedicals (Costa Nesa, CA, USA). The production and purification of the anti-nucleosomal cancer-specific mAb 2C5 were carried out by Harlan Bioproducts (Indianapolis, IL, USA) using the hybridoma cell line from our laboratory. Control bovine IgG antibody was purchased from Millipore Corp. (Bedford, MA, USA). p-Nitrophenylcarbonyl-polyethyleneglycol-3400-phosphatidyl-ethanolamine (pNP-PEG3400-PE) was synthesized in our lab following the procedure described in (95). Cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Distilled and deionized water was used in all experiments.

4.2.2 Synthesis of superparamagnetic iron oxide nanoparticles (SPION).

The nanoparticles were synthesized using the thermal decomposition method developed by Hyeon et. al. with some slight modifications (96). Briefly, 1.28 g
oleic acid was dissolved in 10 mL octyl ether, heated to about 100°C. To this solution 200 µl of Fe(CO)₅ was added. The mixture was refluxed at 280°C for 1 hour and then cooled to room temperature. The solution changed color from pale-yellow green to dark black during the heating process. To this mixture 0.34 g of (CH₃)₃NO was added along with 500µl of octyl ether to facilitate complete transfer of the salt. The mixture was then heated to 130°C for 2 hours under an argon atmosphere. The temperature was slowly increased to reflux temperature of 280°C and maintained for an additional 1 hour. The iron oxide nanoparticles were precipitated from this solution using ethanol, separated from the mother liquor by centrifugation, dried under argon, weighed and re-dispersed in hexane to get a 1.5 mg/mL SPION solution.

4.2.3 Formulation of SPION-micelles.

SPION-micelles were prepared by the rehydration method of dry lipid film and SPION. Briefly, 10 mg of mPEG₂₀₀₀-PE in chloroform was mixed with 0.25 mg of SPIONs in hexane. The organic liquids were removed using a rotary evaporator to dryness. To remove any residual organic liquid the film was freeze-dried overnight. Next day, the film was rehydrated using 1 mL HBS, pH 7.4. The film was vortexed vigorously for 5 min followed by bath sonication for 5 min to get SPION-loaded PEG-PE micelles. Unincorporated SPION were removed by applying an external magnet and the supernatant was removed to a fresh vial.

4.2.4 Size distribution and zeta potential measurements of SPION-micelles.

The size distribution and surface potential of pure micelles and the SPION-micelles were determined by dynamic light scattering (DLS) measurement with a Brookhaven Instruments 90Plus particle size analyzer. The
measurements were performed five times. For the zeta potential measurement, a phase analysis light scattering (PALS) method was used. For each sample 5 runs with 10 cycles each were performed and an average value was obtained from the five measurements. For DLS size measurements the samples were prepared in HBS pH 7.4 whereas for the zeta potential measurements the samples were prepared in 1 mM KCl.

4.2.5 Freeze-fracture electron microscopy.

For determining the structure of the micelles after the incorporation of SPION, the freeze fracture electron microscopy was carried out for ‘plain’ PEG-PE micelles and SPION-micelles (10 mg/mL in HBS pH 7.4). The sample was quenched by using the sandwich technique and liquid nitrogen-cooled propane. A cooling rate of 10,000 Kelvin per sec avoids ice crystal formation and artifacts possibly caused by the cryofixation process. The cryo-fixed samples were stored in liquid nitrogen for less than 2 hours before processing. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment, and the exposed fracture planes were shadowed with platinum for 30 sec at an angle of 25–35° and with carbon for 35 sec [2 kV, 60–70 mA, 1 x 10^-5 torr]. The replicas were cleaned with fuming HNO₃ for 24 h followed by repeated agitation with fresh chloroform/methanol [1:1 by vol] at least five times and examined with a JEOL 100 CX electron microscope.

4.2.6 Relaxation rate of SPION-micelles.

When used as a contrast agent for MRI, SPION increases the relaxation rate of water and has the greatest effect on the T2 (spin-spin) relaxation rate. However, this change in the relaxation rate depends on the size of the SPION, degree of aggregation, surface coating thickness, etc. In vitro relaxation parameters of both, ‘plain’ SPION and SPION-micelle samples were measured
using a benchtop 5 mHz RADX NMR Proton Spin Analyzer at room temperature in HBS, pH 7.4, at different SPION concentrations ranging from 0.002-to-0.125 mg/mL.

4.2.7 Preparation of SPION-immunomicelles.

First, mAb 2C5 or nonspecific control bovine IgG was conjugated to pNP-PEG_{3400}-PE as previously with some modifications (36, 75-77, 97). Briefly, 14 mg PEG_{2000}-PE (97 mol %), 0.45 mg pNP-PEG_{3400}-PE (2 mol %), 0.064 mg Rh-PE (1 mol %) in chloroform and 0.35 mg SPION in hexane was dried in a rotary evaporator and freeze-dryer to form a thin film. The film was hydrated with 1 mL of 5mM citrate buffered saline, pH 5.0, by vortexing for 5 min. followed by bath sonication for 5 min to form the micelle solution. Separately, 1 mL solution (1.56 mg/mL) of the mAb 2C5 or non-specific bovine gamma immunoglobulin antibody (IgG) was prepared in 50 mM phosphate-buffered saline, pH 8.7, and incubated with the above micelle solution with stirring overnight at 4°C to allow the attachment of the antibody to the activated PEG terminus with the simultaneous hydrolysis of non-reacted pNP groups, thus forming the SPION-2C5-immunomicelles. The formulations were then purified using dialysis bags MWCO 250 kDa against HBS, pH 7.4, with 4 changes within 6 hours. After purification, the antibody concentration in the micelle preparation was estimated by the BCA protein assay kit (Pierce Biotechnology, Inc. Rockford, IL, USA) according to manufacturer’s recommendations. The micelle size was confirmed by the dynamic light scattering in ZetaPlus particle size analyzer. (Brookhaven Instruments Corporation Holtsville, NY, USA).
4.2.8 ELISA of immunomicelles.

The specific activity of the immunomicelles was determined by ELISA as described below. An ELISA assay (indirect, using an enzyme-tagged secondary Ab) was performed to show the ability of the SPION-immunomicelles to recognize the target antigen, nucleosomes (NS). The 96-well plates were coated with 50 µL of 40 µg/mL poly-L-lysine (MW 30-70 kDa). After discarding the poly-L-lysine solution, the wells were blocked with 200 µL of TBS containing 0.05% w/v Tween 20 and 2 mg/mL casein (TBST-casein) for 1h at RT, and then incubated with 50 µL of 40 µg/mL NS in TBST-casein for 1 h at RT and washed three times with TBST. The wells were incubated with different concentrations of the native mAb 2C5 and mAb2C5- and IgG-modified SPION immunomicelles for 1 h at RT. After incubation, the wells were washed as before and incubated with 50 µL/well of 1:5000 dilution of goat anti-mouse IgG peroxidase conjugate (ICN Biomedicals, Inc., Aurora, OH) in TBST-casein for 1 h at RT. The wells were again washed as before, and each well was incubated with 100 µL of enhanced Kblue TMB peroxidase substrate (Neogen Corporation, Lexington, KY) for15 min. The microplate was read at a dual wavelength of 620 nm with the reference filter at 492 nm using a Labsystems Multiskan MCC/340 microplate reader installed with GENESISLITE windows based microplate software.

4.2.9 Cell culture.

Human breast carcinoma MCF-7 and murine breast carcinoma 4T1 cells (American Type Culture Collection, Manassas, VA, USA) were propagated using DMEM cell culture medium at 37 °C, 5% CO₂. DMEM media were supplemented with 10% FBS, 1 mM Na-pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin.
4.2.10 Interaction of SPION-loaded mAb 2C5-immunomicelles with cancer cells in vitro.

After the initial passage in tissue culture flasks, MCF-7 cells were grown on coverslips in 6-well tissue culture plates (100,000 cells per well) in DMEM with 10% BSA. After the cells reached a confluence of 70–80%, the plates were washed and blocked with 1% BSA in DMEM. Rhodamine-PE (Rh-PE)-labeled micelle samples in a serum-free medium were added to the cells at a final PEG–PE concentration of 0.35-0.70 mM and incubated for 2 h at 37°C, 5% CO₂. After the incubation, the cells were washed with HBSS. The cells were then digested using trypsin and collected in HBSS. T2 relaxation times for the cell samples were measured using a Varian INOVA 500MHz NMR Spectrometer at room temperature in HBSS. The rhodamine fluorescence associated with the cells was also measured using the Hitachi L2000 fluorospectrometer at the excitation wavelength of 550 nm and emission wavelength of 590 nm.

4.2.11 Fluorescence microscopy.

After the initial passage in tissue culture flasks, MCF-7 cells were grown on coverslips in 6-well tissue culture plates (50,000 cells per well) in DMEM with 10% BSA. After the cells reached a confluence of 40-50% the plates were washed and blocked with 1% BSA in DMEM. Rh-PE-labeled micelle samples in serum-free medium were added to the cells at a final PEG–PE concentration of 0.35-0.70 mM and incubated for 2 h at 37°C, 5% CO₂. After the incubation, the cells were washed with HBSS. The cells were stained with HOECHST 33342 at 5 µg/mL for 3 min followed by washing with HBSS. Individual coverslips were mounted cell-side down onto fresh glass slides with PBS. Cells were viewed and pictures were taken with a Nikon Eclipse E400 microscope using the epifluorescence microscopy.
4.2.12 Subcutaneous tumor models in mice for studying magnetic targeting of SPION-micelles.

All the animal work was performed at either in our lab facility or the animal care facility at Northeastern University. The studies were in compliance with the institutional guidelines. Balb/C female mice (Charles River Laboratories, Wilmington, MA) 6-8 weeks of age were injected subcutaneously with murine mammary epithelial tumor 4T1 cell suspension at $1 \times 10^5$ cells in 100 µL media into the right flank of the mice. Mice were allowed free access to food and water and were monitored regularly for tumor volume and body weight changes. The tumor dimensions were measured with vernier caliper and the tumor volume was calculated using the formula:

$$\text{Tumor volume} = \frac{[\text{Length} \times (\text{Width})^2]}{2} ; \text{with length being the longer axis (76).}$$

4.2.13 Radiolabeling of SPION-micelles with $^{111}$Indium.

To radiolabel with $^{111}$-Indium ($^{111}$In), SPION-micelles formulations were prepared as described above in section 4.2.4 along with addition of 0.5 mol% of DTPA-PE to the lipid film. DTPA-PE-containing SPION-micelles were incubated for 1 hour with $^{111}$In at room temperature, to allow for the trans-chelation of $^{111}$In from a weak citrate complex into a strong DTPA complex. The unbound $^{111}$In was removed by dialysis overnight against 4 L HBS pH 7.4 at 4°C using MWCO 2000 dialysis tubing.
4.2.14 Magnetic targeting of SPION-micelles in tumor of tumor-bearing mice in vivo under influence of external magnet.

Subcutaneous 4T1 tumors were grown in BALB/C mice as described above. After the tumor volume reached approx. 0.5 – 0.6 mm³, the mice were injected with 100 µL of Indium-¹¹¹In-labeled SPION-micelles at PEG-PE concentration of 33.6 mg/mL via tail vein. Test group consist of animals with magnets attached externally onto the tumors using cyanoacrylate glue and the other control group will be non-magnet bearing mice. Mice under anesthesia were sacrificed by cervical dislocation at time points 10 min, 30 min, 1 hour, and 4 hours post injection with 6 animals in a group for each time point. Tumor tissue was collected, weighed and analyzed by γ-counting for the presence of the SPION-micelle-associated ¹¹¹In radioactivity as CPM using a Beckman 5500B gamma-counter. From this data, the amount of the accumulated radioactivity per gram of tissue was calculated as percent of injected dose of radioactivity.
4.3 Detachable PEG to make stimuli-sensitive multifunctional liposomes and micelles.

4.3.1 Materials

Cell lines, mouse fibroblast NIH 3T3 and astrocytoma U-87 MG, were purchased from the American Type Culture Collection (Manassas, VA). All cell culture media, DMEM, and RPMI 1640, heat-inactivated fetal bovine serum (FBS), and concentrated solutions of sodium pyruvate and penicillin/streptomycin stock solutions were purchased from Cellgro® (Herndon, VA). TAT-peptide (11-mer: TyrGlyArgLysLysArgGlnArgArgArg; molecular mass, 1,560 Da; three reactive amino groups) was prepared by Research Genetics (Huntsville, AL). Monoclonal antibody (mAb) 2G4 was produced and purified in our laboratory. Also, pNP-PEG$_{3400}$-PE was synthesized and purified according to an established method in our laboratory (95). Egg phosphatidylcholine (PC), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-750] (ammonium salt) (mPEG$_{750}$-PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium Salt) (mPEG$_{2000}$-PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt) (biotin-PE) 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (sodium salt) (PE-SH) and rhodamine-PE (Rh-PE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). mPEG$_{2000}$-butyraldehyde (mPEG$_{2000}$-CHO) and mPEG$_{5000}$-butyraldehyde (mPEG$_{5000}$-CHO) was obtained from Nektar™ (Huntsville, AL). Control bovine antibody IgG was obtained from Serologicals Proteins, Inc. (Kankakee, IL). 3-(2-pyridyldithio) propionyl hydrazide (PDPH) and Immobilized NeutrAvidin™ Protein was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Bovine
serum albumin and all other chemicals and buffer solution components were from Sigma (St. Louis, MO) and were of analytical grade.

4.3.2 Synthesis of pH-cleavable mPEG2000-hydrazone-phosphatidyl ethanolamine (mPEG2000-Hz-PE).

Developing the method of coupling oxidized antibody to the PEG terminus through a hydrazone bond as suggested by Hansen et al (98), we devised our own scheme of conjugate reaction for synthesis of pH-cleavable PEG-PE. The reaction was performed in two steps: first, activation of mPEG2000-CHO with PDPH, and second, conjugation of 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (Na salt) (PE-SH) to activated mPEG-CHO.

For step I of the synthesis, 150 mg (64 µmole) of mPEG2000-CHO was dissolved in dry chloroform containing 3.5 molar excess of 3-(2-pyridyldithio) propionyl hydrazide (PDPH) to obtain 50 mg/ml solution of mPEG2000-CHO. The mixture was incubated for 48 h at room temperature with stirring under argon. TLC (CHCl₃ : CH₃OH : H₂O - 80:20:2) revealed that the reaction was complete. The starting material mPEG2000-CHO did not absorb UV and was positive to Dragendorff spray, while PDPH absorbed UV and was negative for Dragendorff spray. The product, mPEG2000-Hz-PDP, absorbed UV and was positive to Dragendorff spray. Organic solvents were then removed using a rotary evaporator. mPEG2000-Hz-PDP was then dissolved in deionized water (adjusted to pH 10-11 using 1 M NaOH) and purified from unreacted PDPH using a Sepharose G25 column and deionized water (adjusted to pH 10.5 using 1 M NaOH). Pooled fractions containing mPEG2000-Hz-PDP (Dragendorff and UV positive) were freeze-dried.
For step II of the reaction, 20 mg (26 µmole) of 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (sodium salt) (PE-SH) was dissolved in dry chloroform containing 1.5 molar excess of mPEG2000-Hz-PDP, to get a 10 mg/mL solution of PE-SH. The solution was supplemented with 15 µL (approx. 3-fold molar excess over PEG) of triethylamine (TEA). The sample was incubated overnight at room temperature with stirring under argon. TLC (CHCl₃:CH₃OH:H₂O - 80:20:2) revealed that the reaction was complete. The starting material mPEG2000-Hz-PDP was positive to Dragendorff spray and negative for molybdenum blue, while PE-SH was positive to molybdenum blue and negative for Dragendorff spray. The product, mPEG2000-Hz-PE, was positive to both Dragendorff spray and molybdenum blue. The organic solvents were then removed using a rotary evaporator. The mPEG2000-Hz-PE micelles were formed in deionized water (adjusted to pH 10.5 using 1 M NaOH) by vortexing. The micelles were separated from the unbound PEG and released pyridine-2-thione on CL-4B column using deionized water (adjusted to pH 10.5 using 1 M NaOH) as an eluent.

Pooled fractions containing mPEG2000-Hz-PE were freeze-dried, and extracted with chloroform. mPEG2000-Hz-PE was stored as 10 mg/mL in chloroform at -80° C under argon until further use. mPEG5000-Hz-PE used in some experiments was synthesized in the same way starting with mPEG5000-CHO. ¹H NMR (500 MHz, CDCl₃) δ (ppm) for mPEG2000-Hz-PE: 0.87 (t, CH₃ of lipid, 6H), 1.27 (b,s, CH₂, ≈ 56H), 2.29 (t, OCOCH₂, 4H), 2.40 (t, COCH₂CH₂S, 2H), 2.45 (t, SCH₂CH₂O, 2H), 2.5 (t, COCH₂CH₂S, 2H), 2.59 (m, CH₂CH=N, 2H), 3.1 (t, CH₂CH, 2H), 3.39 (s, OCH₃ of PEG, 3H) and 3.5 (bm, PEG, ≈ 184H). Thus, there was a clear indication for the presence of the expected conjugate.
4.3.3 *Acidic pH cleavability of mPEG2000-Hz-PE.*

**TLC analysis.** TLC-verified degradation of the polymer conjugates after pH treatment and spots corresponding to plain PEG and plain PE were observed after incubation of the polymers at pH 5.0 for 15 minutes at 37° C.

**HPLC analysis.** Micelles of mPEG$_{2000}$-Hz-PE were prepared containing 1 mol % of rhodamine-PE as fluorescent marker as follows. Lipid film was prepared by mixing chloroform solutions of both the lipids in a round bottom flask and then removing chloroform using a rotary evaporator. To ensure complete removal of any traces of chloroform further drying was done using a lyophilizer. The appropriate volume of pH 8.5 phosphate buffer (100 mM phosphate, 150 mM sodium chloride) was added and vortexed for 2 min to form a 0.5 mM solution of mPEG$_{2000}$-Hz-PE micelles. The sample was then divided into equal volumes and incubated at different pH values. For pH 7.4 treatment, a 50 µL aliquot of the first half of the micelle formulation was applied, as is, to Shodex KW-804 size exclusion column at regular intervals using pH 7.4 phosphate buffer (100 mM phosphate, 150 mM sodium sulfate) as eluent and run at 1.0 ml/min. Both UV (from 200 to 400 nm) and fluorescence (excitation 550 nm and emission 590 nm) was used to monitor the micelles. To the second half of the micelle formulation an appropriate volume of 1N HCl was added to a final pH of ~ 5.0, aliquots of which were then analyzed as above at different intervals. As a control, micelles of mPEG$_{2000}$-PE (non-pH-sensitive micelles) were prepared and analyzed after treatment at both the pH values as above.

4.3.4 *Kinetics of the pH-dependent degradation of mPEG2000-Hz-PE.*

The degradation of the micelles spontaneously formed by mPEG$_{2000}$-Hz-PE under the action of the acidic pH was studied by following the presence or absence of micelles over the period of time in buffer solutions at different pHs
(i.e. pH values 6.0, 7.0, 8.0 and 10). Rh-PE-labeled micelles of mPEG_{2000}-Hz-PE conjugate were prepared in phosphate buffer (10mM phosphate, 150mM NaCl) solutions of different pH values. The pH of the solution was adjusted using appropriate amounts of either 1N HCl or NaOH. Aliquots (50 µl) were sampled at different time intervals for size exclusion chromatographic analysis in 100mM phosphate buffer (pH 7.0) containing 150mM sodium sulphate using a fluorescence detector (excitation 550 nm and emission 590 nm). The area under the micelle peak (mean retention time: 9.35 min) was determined for each chromatogram.

4.3.5 Preparation of pH-sensitive drug delivery systems.

**Micelles.** For micelle preparations, a mixture of mPEG_{750}-PE, pH-sensitive mPEG_{2000}-Hz-PE, biotin-PE (or TATp-PE), and Rh-PE at a molar ratio of 40:54:5:1 was prepared in chloroform. Chloroform was removed on a rotary evaporator followed by freeze-drying on a Freezone 4.5 (Labconco, Kansas City, MO). The film was hydrated with PBS, pH 8.0 (10 mM phosphate, 150 mM sodium chloride) at room temperature and vortexed for 5 min. Micelle size was determined by using a Coulter N4 Plus submicron particle analyzer.

**Liposomes.** For liposome preparations, a mixture of phosphatidylcholine and cholesterol in 7:3 molar ratio and with the addition of various quantities (up to 18% mol) of PEG_{5000}-Hz-PE or mPEG_{5000}-PE was prepared in chloroform. When required, the composition for liposome preparation was supplemented with 0.5 to 1 % mol of TATp-PEG_{2000}-PE [prepared as described earlier in (92)] and with 0.5 % mol of Rh-PE (for the fluorescent labeling). Chloroform was removed on a rotary evaporator followed by freeze-drying on a Freezone 4.5 (Labconco, Kansas City, MO). The film obtained was hydrated with HBS buffer (pH 8.0) at room temperature for 5 min. The lipid dispersion was
extruded 20 times through polycarbonate filters (pore size 200 nm) by using a Micro extruder (Avanti). Vesicle size was determined by using a Coulter N4 Plus submicron particle analyzer.

4.3.6 Preparation of pH-sensitive immunocarriers.

First, mAb 2G4 or nonspecific control bovine IgG was conjugated to pNP-PEG₃₄₀₀-PE as in (95) with some modifications. Briefly, pNP-PEG₃₄₀₀-PE and mPEG₇₅₀-PE was dried in a rotary evaporator and freeze-dryer to form a thin film. The film was hydrated with 5mM citrate buffered saline, pH 5.0, and vortexed. Antibody solution was prepared in 50mM tris-buffered saline, pH 8.7 and incubated with a 10-fold molar excess of pNP-PEG₃₄₀₀-PE for 24 h at 4°C to allow the attachment of the antibody to the activated PEG terminus with the simultaneous hydrolysis of non-reacted pNP groups, thus forming the antibody-micelle solution. Then, the required aliquot of this solution was added to liposomes or micelles prepared as described above and incubated for an hour to allow for the quantitative incorporation of the modified antibody into the appropriate drug delivery systems (DDS) (73).

4.3.7 ELISA.

An ELISA assay (indirect, using an enzyme-tagged secondary Ab) was performed to show the ability of the pH-sensitive immunocarriers to recognize the target antigen at different pH values (pH 8.0 and 5.0). First, ELISA plates were coated with 50 µl of 10 µg/ml cardiac myosin and incubated overnight at 4°C. Then, each well was washed three times with 200 µl of TBST (TBS containing 0.05% w/v Tween-20), and incubated with 50 µl of serial dilutions of 2G4 antibody (or non-specific IgG) in TBST-Casein (TBST with 2 mg/mL casein) for 1 h at RT. After incubation, the wells were washed as before and incubated with 50 µl/well of 1:5000 dilution of goat anti-
mouse IgG peroxidase conjugate (ICN Biomedicals, Inc., Aurora, OH) in TBST-Casein for 1 h at RT. The wells were again washed as before, and each well was incubated with 100 µl of enhanced Kblue\textsuperscript{®} TMB peroxidase substrate (Neogen Corporation, Lexington, KY) for 15 min. The microplate was read at a dual wavelength of 620nm with the reference filter at 492nm using a Labsystems Multiskan MCC/340 microplate reader installed with GENESIS-LITE windows based microplate software.

4.3.8 **Biotin-avidin binding.**

To test in a simple test-system, the binding of biotin-bearing Rh-PE-labeled DDS before and after incubation at lowered pH values, the corresponding samples were kept for 15 min at pH 8.0 or pH 5.0 and then applied onto the Immobilized NeutrAvidin\textsuperscript{TM} protein column. The retention of the corresponding preparation on the column was estimated following the decrease in the sample rhodamine fluorescence at 550/590 nm after passing through the NeutrAvidin\textsuperscript{TM} column.

4.3.9 **Interaction of TATp-containing pH-sensitive drug delivery systems with cells.**

For experiments with micelles, NIH 3T3 cells (fibroblasts) have been chosen. After the initial passage in tissue culture flasks, NIH 3T3 cells were grown on coverslips in 6-well tissue culture plates (100,000 cells per well) in DMEM with 10% BSA. After 48 h the plates were washed twice with PBS, pH 7.4, and then treated with various Rh-PE-labeled micelle samples (without and with pre-incubation for 15 min at pH 5.0) in serum-free medium (2 ml/well, 30µg total PEG-PE/ml). After a 1 h incubation period, the media were removed and the plates washed with serum-free medium three times. Individual coverslips were mounted cell-side down onto fresh glass slides with PBS. Cells were
viewed with a Nikon Eclipse E400 microscope under bright light, or under epifluorescence with rhodamine/TRITC.

For experiments with the liposomes, U-87 MG cells (astrocytoma) have been chosen. After the initial passage in tissue culture flasks, U-87 MG cells were grown on coverslips in 6-well tissue culture plates (20,000 cells per well) in DMEM with 10% BSA. After 48 h the plates were washed twice with PBS, pH 7.4, and then treated with various Rh-PE-labeled liposome samples (with and without pre-incubation for 20 min at pH 5.0) in serum-free medium (2 ml/well, 30 µg total lipid/ml). After 1 hour incubation, the media were removed and the plates washed with serum-free medium three times. Individual coverslips were mounted cell-side down onto fresh glass slides with PBS. Cells were viewed with a Nikon Eclipse E400 microscope under bright light, or under epifluorescence with rhodamine/TRITC.
5 RESULTS AND DISCUSSION

5.1 PEGylation of dextran to make long-circulating pharmaceutical carriers.

5.1.1 Synthesis of aminodextran and PEGylation of aminodextran.

The synthesis is outlined in Figure 10. First, hydroxyl groups of dextran were activated with carbonyldiimidazole (CDI). CDI forms imidazole carbamate complexes with dextran hydroxyl groups. These complexes react with amino groups forming urethane bonds. In our design, ethylenediamine (EDA) was added to the activated dextran without purification of the latter. To avoid the formation of cross-linked dextrans, EDA was used in at least 100-fold molar excess over CDI. The quantification of amino groups in the sample obtained by the fluorescamine method has shown that, on average, 21 ± 5 amino groups were introduced into each dextran molecule with a molecular weight of 73 kDa. This corresponds to a quantitative yield of CDI hydroxy group activation. The incubation of dextran with CDI for longer than 30 min before the addition of EDA did not result in a reaction yield increase. Incubation for 15 min, however, resulted in the introduction of substantially fewer amino groups. These data suggested an incubation of 30 min to 5 h for the CDI activation of dextran. The aminodextran preparation was further modified with PEG-SPA (Figure 10). To characterize the kinetics of this step, the number of PEG residues attached per each aminodextran molecule was quantified by amino group loss after incubation of aminodextran in the presence of 5 molar (over aminodextran) excess of PEG-SPA for 0.5 to 17 h (data not shown). The results indicated that the reaction's completion requires at least overnight incubation.
**Figure 10.** Synthesis of PEGylated dextran. a. General scheme of aminodextran conversion into Dextran-PEG. b. Chemical specifics of (a).

### 5.1.2 PEGylation profile of aminodextran.

For the biodistribution study, aminodextran was modified with PEG-SPA at 15/1 mol/mol and 30/1 mol/mol ratios. The degree of PEGylation of the samples obtained is shown in Table II. Modification at initial PEG-SPA/aminodextran ratio of 15/1 mol/mol resulted in introduction of 12±2 PEG residues per aminodextran molecule indicating that the reaction yield of PEG attachment was close to 80% (12 of possible 15 PEG residues were attached). The addition of 30 molar excess of PEG-SPA over aminodextran resulted in the PEGylation of almost all of the available amino groups.
<table>
<thead>
<tr>
<th>Initial PEG-SPA -to-</th>
<th>PEG/Aminodextran</th>
<th>Circulation half-Life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminodextran ratio</td>
<td>attached</td>
<td>(hours)</td>
</tr>
<tr>
<td>(mol/mol)</td>
<td>(mol/mol,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean±S.D.)</td>
<td></td>
</tr>
<tr>
<td>0/1 (Non modified</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>Aminodextran)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15/1</td>
<td>12±2</td>
<td>5.3</td>
</tr>
<tr>
<td>30/1</td>
<td>21±2</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*Table II.* Aminodextran PEGylation efficiency and blood circulation half-life in female C57BL/6J mice (n=3).

5.1.3 **Biodistribution profile of PEGylated aminodextran.**

Blood clearance and biodistribution of dextran with 12 or 21 PEG molecules attached [Dextran(PEG)$_{12}$ or Dextran(PEG)$_{21}$] in comparison with an appropriate aminodextran with its amino groups acylated (non-PEGylated aminodextran) was studied in female C57BL/6J mice. The blood concentration profiles for both samples showed a good linear fit in Log [% of dose] vs. time coordinates ($R^2 \geq 97$) indicating that the samples followed a first order elimination process. The PEGylation of dextran caused a substantial increase in circulation time. The circulation half-life of Dextran(PEG)$_{21}$ was 7.0 h as opposed to 4.0 h for Dextran. The circulation half-life of Dextran(PEG)$_{12}$ was intermediate at 5.3 h (Table II) The results indicate that the modification of
dextran with PEG residues increased dextran circulation time and that the increase depends on the amount of PEG molecules attached.

The results of the comparative biodistribution study with Dextran and Dextran(PEG)$_{21}$ are shown in Figure 11. Uptake by the main RES organ, liver, primarily responsible for dextran elimination from the systemic circulation was substantially decreased in the case of PEGylated dextran. At the same time, the uptake by spleen, lung and kidney is comparable for both Dextran and Dextran(PEG)$_{21}$.

![Graphs A, B, C, D showing biodistribution of Dextran and Dextran(PEG)$_{21}$](image)

**Figure 11.** Biodistribution of Dextran(PEG)$_{21}$ in comparison with Dextran: A) Liver; B) Spleen; C) Kidney; D) Lung (mean± std. error of mean, n=6; * P ≤ 0.05 compared to non-PEGylated dextran).
In conclusion, the activation of dextran with CDI provides a convenient method for the preparation of aminodextran by the attachment of EDA residues. Introduced amino groups can be used for the further attachment of PEG residues using PEG-SPA with a nearly quantitative reaction yield. The modification with PEG decreases the uptake of dextran by RES organs and substantially increases the dextran circulation time. The increase in circulation time depends on the number of PEG residues introduced. Thus, PEGylated dextrans (with residual reactive groups, such as amino groups) can be used as convenient long-circulating carriers for therapeutic and diagnostic agents.
5.2 PEG-lipid micelles with magnetic sensitivity.

5.2.1 Particle size and surface potential of SPION micelles.

The SPION-micelle formulation was stable and did not show signs of aggregation of SPION for approx 2 weeks at 4°C. The size of the micelles was generally in the range of 30 – 50 nm (Figure 12). The surface potential for the SPION-micelles was $-26.7 \pm 3.1$ mV and was not different than pure mPEG$_{2000}$-PE micelles of $-29.7 \pm 2.4$ mV.

![Figure 12. Particle size for SPION-micelles measured using dynamic light scattering in ZetaPlus particle size analyzer.](image)

5.2.2 Freeze-fracture electron microscopy.

Freeze fracture electron microscopy was used to study the morphology of the SPION-micelles and confirm whether there is a difference from the morphology of ‘plain’ PEG-PE micelles as well as for determining whether the SPION were loaded inside the lipid core of the PEG-PE micelles or were present as aggregates externally. As can be clearly seen from the electron micrographs taken from several freeze-fracture preparations of either ‘plain’ PEG-PE micelles or SPION-loaded PEG-PE micelles, both samples contain
medium concentration of small (ca. 30-to-50 nm), overall spherical particles, which indicated that the normal morphology of the micelles was maintained. They display their shadows behind the structure, which is typical for ‘tight-core’ particles such as micelles. The only clear difference in electron micrographs of the two samples was that in case of SPION-loaded PEG-PE micelles, the structures show an additional darker inner core, a clear indication of encapsulated SPION (typical micrographs in Figure 13).

Figure 13. Freeze-fracture micrographs of (A) ‘plain’ PEG-PE micelles and (B) SPION-loaded PEG-PE micelles (red arrows pointing at darker inner core).

5.2.3 Relaxation rate of SPION-micelles.

For any MRI contrast the relaxation rate depends on the size of the contrast agent, degree of aggregation and concentration. PEG-PE based polymeric micelles were able to prevent the SPION form aggregation and hence the T2 relaxation rate was much greater in case of SPION-micelles when compared to ‘plain’ SPION at the same concentration (Figure 14). For example, at 0.0625 mg/mL of SPION, the relaxation rate of SPION-micelles was more than 30 times the relaxation rate of ‘plain’ SPION. This is a very important and
desirable property for developing MRI contrast agents as more intense MRI signals will be obtained at low concentrations of SPION, thus preventing unnecessary high doses of the contrast agents to obtain clinically relevant MRI images of the pathological organs.

![Graph showing T2 relaxation rate (1/T2) of SPION-loaded micelles compared to "plain" SPION in HBS pH 7.4 at room temperature (mean± S.D., n=6).](image)

**Figure 14.** $T_2$ relaxation rate ($1/T_2$) of SPION-loaded micelles compared to the relaxation rate of "plain" SPION in HBS pH 7.4 at room temperature (mean± S.D., n=6).

Thus, the incorporation of SPIONs into mPEG-PE based polymeric micelles prevented the SPION aggregation and also improved the MRI signal at low concentration.

### 5.2.4 SPION-immunomicelles and their characterization.

In order to further improve the SPION-micelles we modified their outer PEG corona with tumor cell-targeting nucleosome-specific mAb 2C5, using a
simple and reproducible chemistry using pNP-PEG$_{3400}$-PE. (95). As was seen previously, the attachment of mAb 2C5 did not change the size of the SPION-immunomicelles (10, 36, 44, 74-77, 99). These SPION-immunomicelles were also fluorescently-labeled using Rh-PE to follow their interaction with cells in vitro. The conjugation of the antibody to the Rh-PE-labeled SPION-micelles was gentle enough, since the ELISA assay indicated that the activity of the mAb 2C5-SPION-immunomicelles towards the specific antigen, nucleosomes, was maintained almost at the level of native unmodified mAb 2C5, while the ‘plain’ SPION-micelles or non-specific bovine IgG-modified SPION-micelles did not show any binding to the monolayer of the nucleosomal antigen (Figure 15).

![Graph](image)

**Figure 15.** Binding of mAb 2C5 SPION-immunomicelles and control ‘plain’ SPION-micelles or bovine IgG SPION-micelles to a monolayer of nucleosomes (mean ± S.D., n=3).
5.2.5 *In vitro* cell interaction of mAb 2C5 SPION-immunomicelles.

After ensuring that the mAb 2C5 maintained its specific activity upon the conjugation to SPION-micelles, their interaction with MCF-7 human breast tumor cell *in vitro* was carried out using both epifluorescence microscopy and fluorescence spectrometry.

The specificity of the conjugated mAb 2C5 resulted in the increased association of these micelles with the MCF-7 cells, when cells were incubated with mAb 2C5-SPION-immunomicelles in comparison to the ‘plain’ and ‘non-specific’ bovine IgG-modified SPION-micelles (following the micelle-associated Rh fluorescence) (Figure 16).

![Figure 16](image)

**Figure 16.** Increased fluorescence can be observed from epifluorescence microscopy of human breast cancer MCF-7 cells after incubation with SPION-micelles for 2 h. (Left panel shows nuclear HOECHST 33342 staining of the cell nuclei and the Right panel shows associated micellar rhodamine fluorescence from the micelles. Magnification of images at 40x objective).
In order to quantify the amount of fluorescence associated with the cells, the cells were digested/lysed using trypsin and the cell lysate fluorescence was measured using fluorescence spectrometer. In agreement with the epifluorescence microscopy results, the fluorescence associated with MCF-7 cells was significantly higher in case of cells treated with mAb 2C5-SPION-micelles in comparison to the ‘plain’ and ‘non-specific’ bovine IgG-modified SPION-micelles (Figure 17).

Figure 17. Cell-associated rhodamine fluorescence (by fluorescence spectrometer) using micelles at concentration of A) 0.35 mM PEG-PE or B) 0.70 mM PEG-PE (mean ± S.D., n=6), * P ≤ 0.05 compared to “plain” SPION-micelles or bovine IgG-SPION-micelles. Also there is a dose-dependant increase in association observed for mAb 2C5-SPION-micelles whereas the amount of association remains unchanged for “plain” SPION-micelles or bovine IgG-SPION-micelle.
5.2.6 *T2 relaxation rates of mAb2C5 SPION-immunomicelles on cells in vitro.*

The goal of developing cancer cell-targeted SPION-micelles was to ensure that the higher amount of SPION is carried to the cancer cells for their more efficient MR imaging. We determined the T2 relaxation time of the lysed cells after their co-incubation with plain, non-specific IgG-modified or mAb 2C5-modified SPION-micelles. The signal associated with the cells was determined using 500 MHz NMR. We observed a significant increase in cell-associated T2 relaxation rate (1/T2) in case of cells treated with mAb 2C5 SPION-immunomicelles (compared to non-targeted SPION micelles) (Table III). This increased signal is the direct result of target cell recognition by mAb 2C5-SPION-immunomicelles.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Concentration of PEG-PE applied to MCF-7 cells.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.35 mM</td>
<td>0.70 mM</td>
</tr>
<tr>
<td>‘Plain’ SPION-micelles</td>
<td>4.3 ± 0.02 s⁻¹</td>
<td>5.2 ± 0.05 s⁻¹</td>
</tr>
<tr>
<td>mAb 2C5 SPION-micelles</td>
<td>5.6 ± 0.01 s⁻¹               *</td>
<td>7.1 ± 0.02 s⁻¹   *</td>
</tr>
<tr>
<td>Bovine IgG SPION- micelles</td>
<td>4.3 ± 0.00 s⁻¹</td>
<td>6.0 ± 0.02 s⁻¹</td>
</tr>
</tbody>
</table>

*Table III.* Relaxation rate (1/T2) of the MCF-7 cells after the incubation with different concentrations of micellar preparations. (The relaxation rate of the sample increases when larger quantity of SPION is associated with the cells). *(n=3, mean±std. error of mean, *p*<0.05 when compared to ‘Plain’ SPION-micelles and Bovine IgG SPION-micelles)*
PEG-PE-based polymeric micelles offer a convenient carrier system for suspending SPION in aqueous medium. The formulations were stable with no apparent aggregation compared to ‘plain’ SPION. SPION-loaded PEG-PE micelles provided dramatically improved T2 relaxation rates. These SPION-micelles can be easily modified with anti-cancer nucleosome-specific mAb 2C5 with the preservation of the antibody specific activity to make them cancer cell-specific. mAb 2C5-SPION-immunomicelles specifically recognize and bind cancer cells in vitro, bring more SPION to these cells, and have the potential to serve as an MRI contrast agent with improved T2 for better tumor imaging. In addition, the specific accumulation of mAb 2C5-SPION-immunomicelles into the cancer cells make these micelles good candidates for use in conjunction with AC-field-assisted hyperthermic cancer therapy.

5.2.7 Magnetic targeting of SPION-micelles in tumor of tumor-bearing mice in vivo under the influence of external magnet.

Since SPION have magnetic properties in presence of external magnetic fields, we hypothesized that SPION-micelles could be targeted to the intended target area using external magnets.

There is significantly more tumor accumulation of SPION-micelles in presence of external magnet than compared to control non-magnet bearing BALB/c mice at all time points (Figure 18). For example, at time 4 hr (240 min) the amount of SPION-micelles accumulated in external magnet-bearing mice is twice that of control non-magnet bearing mice. Thus targeting can be achieved using SPION-micelles and external magnets. The SPION-micelles could moreover be co-loaded with poorly soluble anti-cancer drugs to direct them specifically to the tumor area. Magnetically targeted SPION-micelles may effectively reduce the dose of anti-cancer drug required to get therapeutic effects.
The only limitation to magnetic-targeting remaining, is that the tumor must be easily accessible for focusing magnetic fields. Solid magnets as used in this study or electro-magnets could be used as the source for focusing magnetic fields in the intended target area. Also, depending on the field strength used there is scope for improvement of the accumulation of the SPION-micelles.

**Figure 18.** Accumulation of SPION-micelles in tumor tissue of tumor-bearing Balb/C mice under influence of external magnets (n=6, mean±std. error of mean).
5.3 Detachable PEG to make stimuli-sensitive multifunctional liposomes and micelles.

The particular design of the multifunctional DDS we have used in this study is presented in Figure 19. The suggested DDS (liposome or micelle) bears on its surface a “hidden” function (we have used biotin and TATp moieties for this purpose) inserted into the liposome membrane or micelle core via its modification with PE moiety; protecting PEG chains (PEG\textsubscript{2000}) attached to the surface via the pH-cleavable bond; and specific antibody attached to the surface via non-cleavable long PEG spacers (PEG\textsubscript{3400}). In some experiments with liposomes, cleavable PEG\textsubscript{5000}-Hz-PE and non-cleavable TATp-PEG\textsubscript{2000}-PE conjugates were used (88).

\[ \text{Figure 19. Schematic for the design of the multifunctional DDS used in this study that includes pH-cleavable PEG-Hz-PE (a), and TATp (b), and monoclonal antibody (c) attached to the surface of DDS via pH-non-cleavable spacer.} \]
Such DDS (Figure 19) are expected to demonstrate specific targeted properties (via antibody-mediated recognition) at both normal (7.5-8.0) and acidic (5.0-6.0) pH values, however, the incubation of the DDS at lowered pH should eliminate (detach) protecting PEG chains and un-shield the second function. After exposure to the lowered pH, in addition to immune recognition, DDS should be able to bind with the avidin column if the second, “hidden” function is biotin, or demonstrate internalization by cells if the second, “hidden” function is TATp. As an antibody, we have used the cardiac myosin-specific monoclonal 2G4 antibody (mAb 2G4) (100). The coupling of mAb 2G4 and biotin or TATp to the DDS surface was performed using the reactive derivative of PEG-PE conjugate activated at the free PEG terminus with a p-nitrophenylcarbonyl (pNP) group (pNP-PEG-PE) (95).

5.3.1 Synthesis of pH-cleavable PEG-PE conjugate.

The scheme of the synthesis of PEG-PE conjugated via the pH-cleavable hydrazone group (PEG-Hz-PE) is shown in the Figure 20. The synthesis was carried out in 2 steps. The first step involves the conjugation of 3-(2-pyridyldithio) propionyl hydrazide (PDPH) to mPEG$_{2000}$-CHO. The hydrazide group in PDPH reacts with the aldehyde group of mPEG$_{2000}$-CHO to form the acidic-pH-labile hydrazone bond. Since this bond is vulnerable to hydrolysis, efforts were taken to carry out the reaction in anhydrous conditions. Use of PDPH as the cross linker not only offers the advantage of forming the hydrazone bond but also introducing pyridyldisulfanyl groups for subsequent conjugation of PEG to the thiol component of 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (PE-SH) in the second step of the synthesis. The PE-SH in the conjugate serves as a hydrophobic anchor to assure its association with the lipid bilayer of liposomes or the hydrophobic core of micelles. All steps of the
reaction were followed on TLC to confirm the progress of the reaction. The structure of the final conjugate, mPEG_{2000}-phosphatidyl ethanolamine hydrazone (mPEG_{2000}-Hz-PE) was confirmed by proton NMR characterization.
Step 1.

\[
\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{O} + \text{H}_2\text{N} \cdot \text{N} \cdot \text{O} \cdot \text{N} \cdot \text{N} \cdot \text{H}_2\text{O} \cdot \text{C}_15\text{H}_31 \cdot \text{O} \cdot \text{C}_15\text{H}_31 \cdot \text{PDPH} \\
\downarrow \text{dry CHCl}_3 \quad \text{stirring, room temperature} \quad 48\text{h} \\
\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{N} \cdot \text{H} \cdot \text{C}_15\text{H}_31 \cdot \text{O} \cdot \text{C}_15\text{H}_31 \\
\text{mPEG-Hz-PDP}
\]

Step 2.

\[
\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{N} \cdot \text{H} \cdot \text{C}_15\text{H}_31 \cdot \text{O} \cdot \text{C}_15\text{H}_31 \cdot \text{PE-SH} \\
\downarrow \text{dry CHCl}_3, \text{TEA} \quad \text{stirring, room temperature} \quad \text{overnight} \\
\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{N} \cdot \text{H} \cdot \text{C}_15\text{H}_31 \cdot \text{O} \cdot \text{C}_15\text{H}_31 \\
\text{mPEG-Hz-PE}
\]

Figure 20. Schematic description of the conjugation reaction for preparing pH-sensitive PEG-Hz-PE.
The stability of the conjugate and the kinetics of its degradation were analyzed by size-exclusion HPLC following the area under the micelle peak on the chromatogram after conjugate incubation for different time intervals at different pH values. The conjugate spontaneously forms micelles in aqueous solutions similar to “normal” PEG-PE conjugate. Rhodamine-PE was incorporated into the micelles as a fluorescent tag, and the sample was monitored using a fluorescence detector with excitation at 550 nm and emission at 590 nm. As a typical example, HPLC for mPEG\textsubscript{2000}-Hz-PE is shown in Figure 21. After appropriate pH treatment the peak at retention time 9.7 min (peak for micelle) observed in case of intact micelles (pH 8.0) disappears after incubation at pH 5.0. This disappearance is indicative of the destruction of the micelle structure due to the loss of the PEG corona, while non-pH-sensitive micelles produce the peak at both pH values (data not shown).

**Figure 21.** HPLC analysis of the pH-sensitive mPEG2000-Hz-PE micelles after incubation in pH 8.0 (A) and after incubation in pH 5 (B) at room temperature.
Table IV shows the representative data for the degradation kinetics of PEG$_{2000}$-Hz-DPPE micelles at different pH values. It is clearly seen that such micelles are quite stable at high pH value (8 and above) but they disintegrate within a few minutes at pH 5.0. This result was also confirmed by TLC, where two separate spots for PEG and PE were observed after a few minutes of incubation at pH 5.0 with no TLC spot corresponding to intact PEG-PE (data not shown).

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>pH value</th>
<th>5.0</th>
<th>7.0</th>
<th>8.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td>5.0</td>
<td>3</td>
<td>56</td>
<td>94</td>
<td>99</td>
</tr>
<tr>
<td>40 min</td>
<td>5.0</td>
<td>2.5</td>
<td>28</td>
<td>62</td>
<td>99</td>
</tr>
<tr>
<td>60 min</td>
<td>5.0</td>
<td>2</td>
<td>10</td>
<td>53</td>
<td>99</td>
</tr>
</tbody>
</table>

Table IV. PEG$_{2000}$-Hz-PE micelle stability at different pH values (as per cent of remaining micelles)

5.3.2 Immunoreactivity of multifunctional drug delivery systems.

Fully assembled 2G4 antibody-bearing DDS demonstrated clear immunoreactivity towards the antigen, the monolayer of dog cardiac myosin in the standard ELISA test at both pH 8.0 and 5.0 in PEG-PE-based 2G4-immunomicelles (Figure 22). There is a decreased affinity for the antibodies modified with the pNP-PEG-PE anchor and incorporated into the micelle structure. The same pattern is observed for immunoliposomes. This decrease is more apparent than real, since not all DDS-attached antibodies, even remaining active, can interact with the substrate because of their steric...
orientation to the DDS surface, and as was shown earlier, is compensated by the multipoint interaction of antibody-modified drug delivery systems with the target \((74, \ 101)\). Thus, the systems prepared are immunologically active at both pH values. Control preparations bearing a non-specific IgG did not show any binding with myosin at any pH.

![Graph showing binding of antimyosin mAb 2G4-PEG2000-Hz-PE-immunomicelles to a monolayer of dog cardiac myosin in comparison to the native mAb 2G4 at corresponding pH values (mean ± S.D.; n =3).](image)

**Figure 22.** Binding of antimyosin mAb 2G4-PEG2000-Hz-PE-immunomicelles to a monolayer of dog cardiac myosin in comparison to the native mAb 2G4 at corresponding pH values (mean ± S.D.; n =3).

### 5.3.3 Avidin binding of multifunctional biotin-containing drug delivery systems.

Avidin-biotin complexation was used initially as an easy-to-use test system to follow the shielding and un-shielding of the second hidden function in pH-sensitive drug delivery systems. Liposomes and micelles containing 5 % mol of the biotin-PE in addition to 2G4-PEG_{3400}-PE and pH-sensitive PEG_{2000}-Hz-PE, were prepared and labeled with rhodamine-PE, and their ability to interact
with avidin (NeutrAvidin affinity column) was investigated at pH 8.0 and after the brief (15 min) exposure at the lowered pH of 5.0. Although biotin-containing 2G4-DDS demonstrated identical immunoreactivity at both pH values, their ability to bind to avidin was minimal at pH 8.0 and it increased after 15 min incubation at pH 5.0. This is an indication that a substantial portion of the shielding PEG<sub>2000</sub> micelle corona (or liposome coating) was cleaved away at pH 5.0. The data in Figure 23 (for micellar drug delivery systems) clearly show that at pH 8.0 only about 15% of micelles were retained by the avidin column, whereas after 15 min incubation at pH 5.0 nearly 75% of micelles were retained (the degree of the binding was estimated following the decrease in the sample rhodamine fluorescence at 550/590 nm after passing through the avidin column). The elimination of the pH-cleavable PEG coat unshields the hidden biotin function and allows for more biotin moieties to interact with avidin on the column.

**Figure 23.** Binding of pH-sensitive biotin-micelles to NeutrAvidin columns after 15 min incubation at room temperature at pH 8.0 (a) and at pH 5.0 (b) (mean ± std. error of mean, n=4).
5.3.4 TATp-mediated interaction of multifunctional drug delivery systems with cells in vitro.

For *in vitro* cell culture experiments, we have used rhodamine-labeled DDS similar to those described above, but containing TATp moieties attached to their surface instead of biotin groups. In this case, we have investigated DDS internalization by various cells (non-targets for the 2G4 antibody) at pH 8.0 and after a brief (20-30 min) exposure at pH 5.0. TATp-containing DDS interact differently with cells at pH 8.0 than after incubation at pH 5.0, which indicated that at pH 5.0 a substantial portion of the shielding PEG is lost from the micelle corona (or liposome coating). Figure 24 clearly shows that while cleavable PEG-PE-based TATp-containing micelles kept at pH 8.0 show only marginal association with NIH-3T3 murine fibroblasts, the same micelles pre-incubated for 30 min at pH 5.0 demonstrated dramatically enhanced association with the cells (higher fluorescence), i.e. better accessibility of TATp moieties for cell interaction.

![Fluorescence microscopy showing internalization of Rh-PE-labeled-TATp-containing micelles by NIH 3T3 fibroblast cells after incubating micelles at pH 8.0 (A) and pH 5.0 (B) for 30 min.](image)

**Figure 24.** Fluorescence microscopy showing internalization of Rh-PE-labeled-TATp-containing micelles by NIH 3T3 fibroblast cells after incubating micelles at pH 8.0 (A) and pH 5.0 (B) for 30 min.
In the case of TAT-bearing liposomes (Figure 25), we have shown that the incorporation of 9 % mol of PEG-PE strongly diminished TATp-uptake of liposomes (Figure 25B), and the incorporation of 18 % mol of PEG-PE completely eliminated it (Figure 25C). However when pH-degradable PEG-PE was used, 20 min preincubation of both preparations at pH 5.0 significantly increased the association of both preparations with cells, bringing the cell binding of the liposomes with 9 % mol PEG (Figure 25D) almost back to the level of PEG-free TATp-liposomes (Figure 25A), and significantly improving the cell binding of the TATp-liposomes with 18 % mol of the initial PEG (Figure 25E). These results clearly confirm that the elimination of the pH-cleavable PEG coat de-shield the hidden TATp function and allows for better association of DDS with cells.
Figure 25. Fluorescence microscopy showing internalization of Rh-PE-labeled-TATp containing liposomes by U-87 MG astrocytoma cells using: PEG-free TATp-liposomes (A); 9 % mol pH-non-cleavable PEG-PE at pH 7.4 (B); 18 % mol pH-non-cleavable PEG-PE at pH 7.4 (C); 9 % mol pH-cleavable PEG-Hz-PE after incubation at pH 5.0 for 20 min (D)*, 18 % mol pH-cleavable PEG-Hz-PE after incubation at pH 5.0 for 20 min (E)*.

* The pH of these formulations was raised back to pH 7.4 after their incubation at pH 5.0 and prior to incubation with cells.
Using pH-sensitive PEG-PE conjugates double-targeted drug carriers have been developed, which, in addition to prolonged circulation (via the attached PEG) and target recognition (via the attached antibody) can carry a temporarily hidden function which can be developed under the action of certain local stimuli (such as lowered pH). This represents a significant step on the way towards “smart” multifunctional pharmaceutical nanocarriers capable of both target accumulation and intra-cellular penetration in a controlled fashion.
6 CONCLUSIONS

PEG is an important biomedical polymer and has proved to be backbone for successful design and application of many therapeutic or diagnostic delivery systems.

A. PEG modification of biocompatible and biodegradable polymer dextran results in dextran-based degradable polymeric drug carrier which has improved and longer blood-circulating properties.

B. Polymeric micelles based on PEG-PE conjugates can be successfully used for various purposes including the preparation of actively and passively targeted tumor-targeted magnetically sensitive micelles loaded with SPION.

C. PEG-based conjugate with lowered pH-sensitive bond between PEG and PE allow for the preparation of multifunctional pharmaceutical nanocarriers where the cell-penetrating function is sterically shielded by PEG chains under normal conditions in the circulation, but becomes exposed and functional after PEG detaches in the acidic environment in the tumor.
P.S. Some of the data in this thesis work has already been published in (58, 88) and other parts of the data have been submitted for publication.
7 REFERENCES


85


(93) Bethell, G. S., Ayers, J. S., Hancock, W. S., and Hearn, M. T. (1979) A novel method of activation of cross-linked agaroses with 1,1'-carbonyldiimidazole which gives a matrix for affinity chromatography devoid of additional charged groups. *Journal of Biological Chemistry* 254, 2572-4.


(101) Klibanov, A. L., Muzykantov, V. R., Ivanov, N. N., and Torchilin, V. P.
APPENDIX 1: List of Publications

Below is the list of articles that were published during the course of my Ph.D. program at Northeastern University.


(9) **Sawant, R. M.,** Sawant, R. R., Gultepe, E., Nagesha, D., Papahadjopoulos-Sternberg, B., Sridhar, S., and Torchilin, V.P. Nanosized Cancer Cell-Targeted Polymeric Immunomicelles Loaded with Superparamagnetic Iron Oxide Nanoparticles. (Submitted)