Abstract of the thesis entitled "Biopolymer Based Theranostic Agents For Anticancer Activity And Enzymatic Stability" to be submitted to the Savitribai Phule Pune University for the Degree of Doctor of Philosophy in Chemistry by Ms. Deepanjali Dattatray Gurav under the guidance of Dr. (Mrs.) Vaishali S Shinde, Department of Chemistry, Savitribai Phule Pune University, Pune.

The thesis is divided into three chapters:

**Chapter 1: Synthesis And Anticancer Activity Study Of Biopolymer Coated Gold Nanoparticles.**

Present chapter is divided in to three sections. Section-A includes introduction to role of biopolymer based theranostic agents in anticancer studies. Section-B describes synthesis of hemocompatible chondroitin sulfate coated gold nanoparticles for anticancer activity. Section-C comprises of synthesis of fucoidan-mimetic glycopolymer coated gold nanoparticles for anticancer therapy.

**Section A: Introduction to biopolymers coated theranostic agents for anticancer activity.**

Biopolymers are polymeric biomolecules produced by living organisms and classified according to the monomeric units: polymolecules (RNA and DNA), polypeptides and polysaccharides. Biopolymer based theranostic agents viz. nanoparticles represent a well-established option to formulate drug delivery systems. Since extracellular matrix (ECM) based biopolymers such as hyaluronic acid (HA), heparin (HP) and chondroitin sulfate (CS) are biocompatible, biodegradable and non-toxic, suitable modification of these polymers could make them ideal drug delivery system. Gold nanoparticles (AuNPs) have been exploited for various biomedical applications as a biocompatible carrier of biopharmaceuticals, a contrast agent for diagnostic imaging, and a photoabsorber for photothermal therapy. Therefore, chapter 1 focuses on utilizing biopolymers with AuNPs for designing an appropriate targeted drug delivery system.
Section B: Synthesis of hemocompatible chondroitin sulfate coated gold nanoparticle for targeted anticancer activity.

Thrombotic complications in cancer patients undergoing chemotherapy are believed to be due to acute bone marrow toxicity and procoagulant activity induced by chemotherapeutic agents. We hereby show that doxorubicin (DOX) cause acute platelet toxicity and trigger coagulation cascade in human whole blood model. To circumvent these side effects, we present here, chondroitin sulfate (CS) coated gold nanoparticles conjugated to DOX (CS-Au-DOX) as a safe platform for targeted delivery of chemotherapeutic agent. DOX was conjugated to the nanoparticle via pH-responsive hydrazone linkages, which yielded sustained drug release profile at acidic pH (Scheme 1)

Scheme 1: (A) Scheme for the synthesis of CS-DTPH; (B) Schematic representation of CS-Au-NP synthesis from DTPH modified CS; (Inset) Dynamic light scattering profile and SEM image of CS-Au-NP.

Cytotoxicity results of CS-Au-DOX exhibited higher toxicity towards CD44 overexpressing human colon cancer cells (HCT116 and GP5D) as compared to human breast cancer cells (MCF-7) that express less CD44 receptor as well as free DOX. This nanoparticle mitigated DOX mediated toxicity to human platelets and suppressed
thromboinflammation, demonstrating the significance of nanoparticle design for the delivery of toxic drugs (Figure 1).

Figure 1: (A) Dose dependent cytotoxicity profile of HCT116, GP5D and MCF-7 cell line and (B) caspase 3/7 activities of HCT116, GP5D and MCF-7 cell line.

Section C: Synthesis of fucoidan-mimetic glycopolymer coated gold nanoparticles for anticancer therapy

Fucoidan is a fucose-rich sulfated polysaccharide obtained primarily from brown seaweeds. Structurally it resembles heparin; therefore fucoidan elicits a variety of biological functions such as being anticoagulant, antithrombotic, antiangiogenic, antiproliferative and anticancer etc. Transforming fucoidan-mimetic glycopolymers (FM-glycopolymers) glycopolymers into a nanoparticle formulation would promote efficient cellular delivery and would display interesting biological properties. Considering these factors, we report the synthesis of FM-glycopolymers via free-radical chain transfer polymerization reaction with improved polydispersity and higher yield. Subsequently, this sulfated fucoside-grafted polymer to tailor FM-glycopolymer coated gold nanoparticles, and evaluated the anticancer properties (Scheme 2).
Scheme 2: (A) Scheme for the synthesis of FM-glycopolymer. (B) Synthesis of FMG-Au-NP with DLS profile and SEM image.

We performed the cytotoxicity study of FMG-Au-NP and FM-glycopolymer with human colon cancer cell line (HCT116) and compared it with a non-cancer mouse fibroblast cells (NIH3T3).

Figure 2. Dose dependent cytotoxicity profile of HCT116 and NIH3T3 cell line and Caspase activity

Interestingly, it showed a dose dependent cytotoxicity for FMG-Au-NP with the HCT116 cells. On the other hand, FM-glycopolymer was non-toxic to these cells in the concentrations we tested. Both FM-glycopolymer and FMG-Au-NP did not induce any
toxicity to NIH3T3 cells. This clearly indicates that FMG-Au-NP triggers apoptosis specifically to cancer cells and not fibroblast cells. Also, neither FM-glycopolymer nor FMG-Au-NP triggered any significant caspase activity in HCT116 and NIH3T3 cells, suggesting mechanism of apoptosis is not mitochondria mediated (Figure 2).

Chapter 2: Synthesis of Theranostic Glycosaminoglycan Derived Polymerosome for Targeted Anticancer Drug Delivery

This chapter is divided into three Sections as mentioned below.

Section A: Introduction of theranostic agents for targeted drug delivery systems

The term “theranostic” is defined as a material that combines the modalities of therapy and diagnostic imaging. The most promising aspects of utilizing nanoparticles as therapeutics and diagnostics are their potential to localize (or be targeted) in a specific manner to the site of disease and reduce or eliminate the possible numerous untoward side effects. In case of cancer treatment due to the altered anatomy of tumor vessels, nanosized particles can easily extravagate from the blood pool into tumor tissues and be retained due to poor lymphatic drainage using the enhanced permeability and retention (EPR) effect. Additionally, nanoparticles have high surface area-to-volume ratios, yielding high loading capacities. Thus, nanoparticles can be loaded with therapeutic drugs and imaging agents.

Section B: Synthesis of glycosaminoglycan derived polymersome for targeted delivery of doxorubicin

We present a simple and versatile strategy to design polysaccharide based multifunctional theranostic nanoparticles (NP) for delivering aromatic hydrophobic chemotherapeutic drugs. We used hyaluronic acid and chondroitin sulfate as polymer scaffold for tailoring these NP, as they possess diverse biological function with cancer targeting ability. The amphiphilicity was induced by conjugating planar fluorescein moiety, which provided theranostic property in addition to excellent drug stabilization by $\pi-\pi$ stacking interaction. The drug loaded NP showed superior toxicity than free drug in breast cancer cell (Scheme 3).
Scheme 3: HA-FTSC or CS-FTSC loaded DOX theranostic particles.

Cytotoxicity of synthesized HA-DOX-NP and CS-DOX-NP was evaluated using MTT and LDH assay. Both these assays, which measure either mitochondrial function or cell membrane integrity, provide a good assessment of cytotoxicity of NP. It was observed that CS based NP showed improved selectivity and toxicity as compared to free drug in breast cancer cell line (Table 1).

Table 1: In vitro cytotoxicity study of DOX, HA-DOX-NP and CS-DOX-NP by MTT and LDH assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Drug Loading (%)</th>
<th>Loading Efficiency (%)</th>
<th>IC₅₀ HCT 116 (μM)</th>
<th>IC₅₀ MCF-7 (μM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTT</td>
<td>LDH</td>
</tr>
<tr>
<td>DOX</td>
<td>100</td>
<td></td>
<td>1.319</td>
<td>1.77</td>
</tr>
<tr>
<td>HA-DOX-NP</td>
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<td>80</td>
<td>2.985 (2.25)[]</td>
<td>5.87 (3.31)[]</td>
</tr>
<tr>
<td>CS-DOX-NP</td>
<td>4.8</td>
<td>98</td>
<td>1.662 (1.25)[]</td>
<td>3.52 (1.98)[]</td>
</tr>
</tbody>
</table>
Section C: Synthesis of hyaluronic acid-pluronic nanogels and study its importance for delivery of doxorubicin

Hydrogels are a three dimensional (3D) structure class of materials which retain a significant amount of water. Hydrogels can be in the form of macroscopic networks or confined to smaller dimensions such as microgels or nanogels. Gels derived from natural polymers often undergo rapid degradation upon contact with body fluids or medium and hence this problem can be overcome by modifying these polymers with various synthetic polymers. Nanogels have tunable size of several nanometers and a large surface area for physical entrapment of bioactive molecules such as drugs, proteins, carbohydrates and DNA in the polymeric network, as well as their in vitro release behaviour. Thus, we propose the work involving the combination of pluronic thermosensitivity and biodegradability of hyaluronic acid into a molecular network by molecular design (Scheme 4).

Scheme 4: Synthesis of DOX loaded HA-Pluronic nanogels with TEM images of nanogels.

In order to investigate the in vitro cellular uptake, inherent fluorescence of DOX was utilized. Since DOX is known to be intercalated in DNA, maximum accumulation was observed in the nucleus as seen in Figure 3. The images taken after 48 h revealed higher toxicity of DOX loaded Plu-HA nanogels (Figure 3B) as compared to native DOX (Figure 3A), while only nanogels sample did not show any effect on the cancer cells (Figure 3C). The characteristic phenomenon of in vitro efficient drug release was achieved using the prepared polymeric NP system.
Figure 3: Confocal images of intracellular DOX release from (A) DOX; (B) Plu-HA-DOX nanogel and (C) Plu-HA nanogel after 48 h incubation.

The efficiency of DOX loaded nanogels were evaluated by quantification of cell death in HeLa cell line using the MTT assay. The data showed a dose dependent cytotoxicity for DOX and nanogel-DOX samples as seen in Figure 4. The % cell viability was higher for native DOX samples as compared to DOX loaded nanogels (96 h highest toxicity), thus exhibiting an efficient system for anticancer therapy.

Figure 4: In vitro cytotoxicity studies for DOX and DOX-nanogels.

Chapter 3: Synthesis And Enzymatic Stability Study Of 2'-N-Guanidino, 4'-C-Ethylene Bridged Thymidine (GENA-T) Modified Oligonucleotide.

This chapter is also divided into three sections.

Section A: Introduction to oligonucleotides and its modified derivatives

Chemically modified nucleotides have generated enormous interest in recent years as they improve the enzymatic stability and efficacy properties of oligonucleotides, transforming them into drug molecules toward therapeutic applications. Of particular
interest has been North-type (C3'-endo) conformationally locked nucleotides, namely, Locked/Bridged Nucleic Acid (LNA/BNA). Since the amino group (positive charge) near phosphates offers extra enzymatic stability (as observed in azetidine Vs oxetane comparison) we anticipated that converting amino residue of aza-ENA to guanidino group could impart interesting features to the existing properties of aza-ENA.

Section B: Synthesis of 2'-N-Guanidino,4'-C-Ethylene Bridged Thymidine (GENA-T) modified oligonucleotide.

Synthesis of GENA derivative was initiated from the previously synthesized aza-ENA intermediate. To prepare this intermediate we slightly modified the synthetic strategy for the incorporation of cyano intermediate. Trifluoroacetyl group was deprotected using aqueous CH₃NH₂ in methanol and the product obtained was directly used for the guanylation reaction (Scheme 5).

Reagents and conditions: (a) Tf₂O, pyridine, CH₂Cl₂, 0 °C, 3 h; (b) acetone cyanohydrin, MeLi, LiH, DMF, r.t., 3 days; (Ref) Oommen et al. JACS, 2009; (i) 40% aqueous CH₃NH₂, CH₃OH, 3 h, rt; (ii) dry CH₂Cl₂, 17, Et₃N, 4 days, rt, (iii) N,N-diisopropylphosphoramido chloridite, DIPEA, dry CH₂Cl₂, 2 h, rt.

Scheme 5: Synthesis of GENA-T modified Oligonucleotide

Stability study of GENA-T modified oligonucleotide in presence and absence of enzyme

Enzymatic stability of such modified ON in human serum and with snake venom phosphodiesterase (SVPDE) was studied. Human serum mainly comprises of 3'-
exonucleases along with some endonucleases and 5'-exonucleases whereas, the SVPDE is a 3'-exonuclease. Comparison of enzymatic stability studies with single aza-ENA and GENA modifications incorporated at second position from the 3'-end showed great improvements in stability as compared to the unmodified sequence. Interestingly, the guanidine-residue of GENA seemed to have higher impact on stability of the modified ON as compared to that of the amine residue of aza-ENA (Figure 5).

**Figure 5:** Footprints after enzymatic digestion of $^{32}$P labeled DNA. Autoradiograms of 20% denaturing PAGE, showing the degradation pattern of 5'-$^{32}$P-labeled ON sequence 3'-d(CTTCTTTTCTTC)-5' where T is either, GENA, aza-ENA or T (a) in SVPDE and (b) in human serum.