Preparation and characterization of polymer-drug conjugate delivery system for efficient cancer therapy

THESIS PRESENTED

BY

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To

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Abstract:

The aim of this study is to prepare and characterize polymer drug-conjugate delivery system loaded with doxorubicin and melphalan for successful passive or active targeting of these anti-cancer agents to the tumor environment by the enhanced permeability and retention effect and targeted tumor drug delivery. The polymers will be investigated in \textit{in vitro} cell cultures of various cancers.

The preliminary study in this research focuses on preparation and characterization of polymer-drug conjugates. Doxorubicin and melphalan constitute the model chemotherapeutic agents for conjugation because doxorubicin exhibits maximum absorbance at 490nm whereas melphalan exhibits maximum absorbance at 260nm. This difference in absorbance allowed differential characterization of both drugs when conjugated to the polymers. In addition, the mechanisms of chemotherapeutic actions of doxorubicin and melphalan are different.

In-vitro studies using polymer drug-conjugate were also conducted to support the concept that tumor toxicity would be enhanced compared to equal concentrations of individual anti-cancer agents. The rationale is due to these reasons: 1) macromolecules such as polymer-drug conjugates will be concentrated in the tumor environment by enhanced permeability and retention effect, and 2) two different anti-cancer agents that have different cytotoxic activities delivered simultaneously to tumor cells should be more effective than individual drugs.

Successful completion of the above mentioned study confirm that the polymer-drug delivery system can be used for more efficient multi drug cancer therapy.
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A. Introduction

1.1 Objectives and Hypothesis:

**Objective:** Design and characterize a polymer drug-conjugate delivery system to which two different anti-cancer agents: doxorubicin and melphalan are covalently linked and to establish enhanced tumor toxicity of the polymer-drug conjugates compared to equal concentrations of free anti-cancer agents, to demonstrate the reduction in cardio-toxicity of doxorubicin and melphalan by polymer–conjugation. Comparative *in vitro* studies will be undertaken with human mammary adenocarcinoma (BT-20) cells, H9C2 embryonic cardiomyocytes.

**Hypothesis:** Delivery of multiple anti-cancer drugs loaded on a polymer into the tumor environment would be more efficient in killing tumor cells than with free conventional chemotherapeutic drugs.

1.2 Statement of Problem:

Current cancer therapies include surgical resection of tumor, chemo and radiation therapy. The primary drawback of conventional chemotherapy using cytotoxic drugs is the bystander toxicity on normal healthy cells. Various chemotherapeutic agents with anti-cancer properties such as doxorubicin, paclitaxel, camptothecin, melphalan etc. are limited by their narrow therapeutic index due to unwanted toxicity to normal healthy but rapidly dividing cells. These cytotoxic drugs utilize the same processes in the normal and cancerous cells for cell killing. In order to prevent unwanted effects to normal healthy dividing cells, it would be desirable to target these cytotoxic drugs to tumor cells. Additional benefits would be obtained if these drugs are delivered as prodrugs to malignant cells. Targeting to tumor cells can be achieved by passive targeting using macromolecular drug delivery systems or by active targeting using antibodies or ligand/receptor or oligo nucleotide interaction.
As tumor growth depends on the supply of nutrients through newly formed tumor vasculature, it is possible to treat certain cancer using anti-angiogenic agents. In addition some tumors are resistant to chemotherapy due to the efflux of the drugs initiated by P-glycoprotein transporters leading to multi drug resistance (MDR) (1). One approach to overcome MDR is to use modulators such as verapamil that inhibit the efflux of the therapeutic drugs (1). Therefore, it would be beneficial to deliver a combination of therapeutic reagents such as cytotoxic drugs, MDR modulators and anti-angiogenesis drugs through a single delivery system to the tumors. Such a combination therapy may be achieved by loading multiple drugs on polymers as polymer-multi-drug conjugates (PMDC).

1.3 Specific Aims:

**Aim – 1: Preparation of the multi layer Polymer-drug conjugates:**

- Cross linking doxorubicin to Poly-L-Glutamic acid (PGA) via covalent peptide linkage
- Covalent coupling of melphalan to Poly-L-Lysine (PLL) via peptide bond
- Covalent cross linking of PGA-doxorubicin and PLL-melphalan
- Cross linking doxorubicin and melphalan to Poly-L-Glutamic acid via peptide linkage

**Aim – 2: Characterization of the multi layer polymer-drug conjugate:**

- Characterization of the polymer drug conjugates using UV spectrophotometer, Sephadex G-50 size exclusion chromatographic column (85x1cm), Elisa, SDS-PAGE, Zeta potential.

**Aim – 3: Evaluation of tumor toxicity and cardiotoxicity:**

- *In vitro* studies on BT – 20 human mammary adenocarcinoma cell line to establish tumor toxicity of polymer-drug conjugates.
In vitro studies on H9C2 embryonic cardiomyocyte cell line to establish reduced cardio-toxicity of polymer-doxorubicin conjugate, polymer-melphalan conjugate and polymer-multi drug conjugates.

B. Background and Significance:

2.1 Cancer:

Various cancers occur due to the manifestation of six essential alterations in cell physiology that eventually lead to malignancy (2). The six alterations are: 1) autocrine-sufficiency in growth signals, 2) insensitivity to inhibitory signals, 3) evasion of apoptosis, 4) the ability to replicate indefinitely, 5) angiogenesis to support newly formed tumor mass and 6) the ability to invade other tissues (metastasis). These alterations are acquired by transformed cells during tumor genesis which eventually breach the anti-cancer defense mechanisms of the organism.

Normal cells require external mitogenic growth signals from other tissues or cells in order to proliferate. But cancerous cells are capable of generating their own growth factors for autocrine stimulation and thus liberate themselves from the dependency on external growth factors. Cells respond to most of the anti-proliferative signals through Retinoblastoma protein (pRb) and the other proteins such as p107 and p130. When in a hypophosphorylated state, pRb blocks proliferation by sequestering the E2F transcription factors that are essential for cell to proceed from G1 phase to S phase in the cell cycle. Phosphorylation of pRb is regulated by Transforming Growth Factor β (TGF β). TGF β prevents phosphorylation of pRb and subsequent inactivation of pRb (2). Cancer cells can regulate the effects of TGF β by down regulation of TGF β receptors or by producing mutant dysfunctional TGF β receptors (3). Additional resistance to apoptosis can be acquired by p53 tumor suppressor gene mutation. Normal cells undergo limited rounds of cell divisions and then withdraw from cell division. Telomeres in the
chromosomes are degraded with each division and after few cell divisions, the loss of telomeres leads to permanent cessation of cell division. Cancer cells produce an enzyme called telomerase that keeps the telomeres intact after each cell division (2). Thus, they can replicate indefinitely.

Tumors are also capable of angiogenesis by activating the angiogenic switch due to a change in the balance of angiogenesis inducers and inhibitors. Many tumors produce angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) in greater amounts than normal cells leading to tumor angiogenesis (2).

There are many other pathways involved in the development of malignant tumors. However, most of the human cancers exhibit the above described characteristics. So, cancer treatment is possible if one can treat the above conditions. Nevertheless, most of these processes described take place in cancer and normal cells as well. Therefore, cytotoxic drugs used for cancer therapy have collateral effects on normal cells leading to a necessity to develop targeted anti-cancer drugs for therapy.

2.2 Polymers:

Polymers are macromolecules containing repeating sub-units. When they are injected into the circulation, these macromolecules passively accumulate in the tumor environment due to the leaky tumor vasculature known as enhanced permeability and retention (EPR) effect (4).
Figure-1: Enhanced permeability and retention effect in the tumor environment (Ref: Ruth Duncan; The dawning era of polymer therapeutics; Nature reviews drug discovery (May - 2003) 347-360)

The leaky tumor vasculature facilitates extravasation of macromolecules from the circulation into the tumor environment. Additionally, retention of macromolecules in the tumor environment is enhanced by the lack of intra-tumoral lymphatic drainage.

In addition to the EPR effect, tumor cells also exhibit higher degree of endocytosis because of the enhanced metabolic activity (5,6,7). In opposition to these processes of drug delivery, the high intratumoral pressure that exists in solid tumors would promote exit of macromolecules from the center to the periphery of the tumors (5). Studies have shown that macromolecules localize preferentially in the periphery of tumors relative to normal tissues (6,7). Therefore, pharmaceuticals may be attached to carriers and delivered to the tumors by the carriers.

Small molecular drugs enter cells by diffusion, whereas macromolecules enter cells by endocytosis (8), and the endosomes fuse with lysosomes. The membrane of lysosomes is impermeable to macromolecules (9). Therefore, the macromolecular polymer-drug conjugates cannot escape the endo-lysosomes. The bond between the drug and the polymer must be dissolved in order for the drug to escape in to the cytoplasm. The acidic environment, lysozymes
and other proteolytic enzymes of the lysosomes promote release of the drug from the polymers and then into the cytoplasm.

In addition, certain drugs such as paclitaxel are hydrophobic. Conjugation of such drugs to polymers promotes solubility of such hydrophobic drugs (6,7). Polymers also offer steric protection to their conjugated moieties and reduce their interaction with proteolytic enzymes, antibodies etc in the circulation. Lastly, the endocytotic route of uptake of polymer-drug conjugates also reduce the efflux of the drugs because the drugs do not come in the vicinity of the membrane bound p-glycoproteins responsible for MDR (10).

2.2.1 Poly-L-glutamic acid(PGA) and Poly-L-lysine(PLL) as drug carriers: In general, natural polymers have higher polydispersity. Synthetic polymers however, exhibit lesser polydispersity. Hence, it is easier to characterize synthetic polymers. PGA is composed of natural glutamic acid residues. It is biodegradable and non-toxic. It contains free carboxyl groups in the side chain which can be modified for drug attachment and one N-terminal amino group. The negative charge of PGA polymer renders the polymer water soluble. PLL is also biodegradable and its positive charge helps in attachment to the cell surface by ionic interaction.

2.3 Doxorubicin:

Doxorubicin is the first line chemotherapeutic agent for treatment of breast cancer, sarcomas, leukemia, Hodgkin’s disease and many other tumors (28). The structure of doxorubicin is shown in Figure 2.
Mechanism of action: Topoisomerase II is the target of doxorubicin (11). Topoisomerase II enzyme plays a key role in relaxing the supercoil of the DNA during DNA replication. During this process, the supercoiled double strands are cut and covalently bound to Topoisomerase II. This intermediate complex helps the DNA supercoil to relax and facilitates DNA replication. Then, the DNA strands are released from topoisomerase II and are annealed. Doxorubicin binds to the topoisomerase II and stabilizes the above intermediate and inhibits DNA replication leading to cell cycle arrest. Double strand breaks thus created induce apoptosis in cancer cells (12).

Doxorubicin is also known to cause cardiotoxicity. The quinone moiety of doxorubicin undergoes redox cycling and generates reactive oxygen species (ROS). Normally, cells are protected from ROS injury by superoxide dismutase enzyme. Cardiac tissue is greatly affected by doxorubicin because: 1) anthracyclines are shown to be retained in cardiac myocytes (13), 2) cardiac tissue exhibit weak antioxidant activity (14), 3) cardiomyocytes are rich in mitochondria and are damaged by ROS generated by doxorubicin (15,16,17). This undesirable side effect can be overcome by conjugating doxorubicin to polymers such as PGA. It has been shown that the polymer-drug conjugates are less cardio-toxic (18) because free doxorubicin enters the cardiocytes through diffusion, whereas polymer-drug conjugate requires pinocytosis to enter the cells only when there is high extracellular concentration of the polymer-drug conjugates.
2.4 Melphalan:

Melphalan is an anti-cancer drug which belongs to the class of nitrogen mustards. It is the drug of choice for treatment of various human cancers which include Hodgkin’s disease, multiple myeloma, Burkitt’s lymphoma and in combination chemotherapies. The structure of melphalan is shown in Figure 3.

![Melphalan Structure](image)

**Figure-3**: Chemical structure of Melphalan (25)

**Mechanism of action**: The chloroethyl side chain present in melphalan cyclize spontaneously in aqueous solution to form an aziridinium ion which is capable of targeting the nucleophilic site in the DNA. This monoadduct can form a second aziridinium ion which can add to another nucleophilic site in the DNA resulting in crosslink of two DNA bases (19). This causes a halt in DNA replication and causes cell cycle arrest.

Melphalan is reported to have a maximum absorbance at 260nm (22). Also, it consists of both carboxylic group and amine group in its side chain which can be modified for conjugation. So, melphalan may be conjugated to polymers such as Poly-lysine via its carboxylic group and/or to Poly-Glutamic acid via its amine group.

2.5 Polyelectrolyte multilayers:

Polyelectrolyte multilayers (PEM) have wide variety of applications ranging from optical devices to biomaterials used in drug delivery. They are formed by alternate deposition of polyanions and polycations (20,21). The advantage of these PEM multilayers is that they can be precisely controlled up to nanometer scale to achieve desired thickness, targeting,
One of the main requirements to be fulfilled by PEMs is that they should be biodegradable by enzymes in the cells in order to release their contents into the cytoplasm. The growth of the multilayer is based on electrostatic interactions between the outermost layer on the film and the polyelectrolyte solution containing the polymer with opposite charge. After incubation for a certain time, there will be a complete reversal of the charge on the surface of the film due to the deposition of a new layer.

Polymers carrying multiple equal charges on their subunits allow surface charge reversal with two important consequences: 1) the surface charge repulses the equally charged molecules thus ensuring that adsorption of only a single layer takes place at each incubation step. 2) surface charge facilitates the deposition of oppositely charged polymer in the subsequent incubation step. Thus, multilayers are formed by cyclic repetition of alternate adsorption steps of the oppositely charged polymers. Polyelectrolytes are more suitable for multi-layer formation instead of small molecules because polyelectrolytes can form greater number of ionic bonds with the oppositely charged layers which leads to better adsorption of the layers (21).

Poly-L-Glutamic acid is an anionic polymer with the carboxylic groups in the side chain of the glutamic acid residues and Poly-L-lysine is a cationic polymer with amine groups in the side chain of the lysine residues. These two polymers can be loaded with two different anti-cancer drugs and subsequently, they can be alternately adsorbed and then covalently cross linked to form polyelectrolyte multilayer drug delivery system suitable for drug delivery in to the tumor environment.
C. Materials and Methods

Materials:

Poly-L-Glutamic acid (molecular weight 11,600 Da.), Poly-L-lysine (molecular wt: 13000 Da.) and bicyclic anhydride of Diethylene Triamine Pentaacetic acid (DTPA) are purchased from the Sigma Aldrich (st.Louis,MO). Goat anti-mouse antibody modified with horse radish peroxidase is purchased from MP biochemicals, LLC. K-Blue is purchased from Neogen corporation, KY. Doxorubicin is purchased from sigma Aldrich (st. Louis, MO). Melphalan is purchased from Fisher Scientific.

3.1 Preparation and characterization of (DTPA)-PGA-Doxorubicin conjugate:

3.1.1 Conjugation of DTPA to PGA: A solution of PGA (50mg/ml) in 0.1M NaHCO₃ (pH=8) is prepared. An aliquot of 3x moles excess (4.6 mg) of DTPA is dissolved in minimal amount of DMSO (200µl). DTPA solution in DMSO is added to the PGA solution drop by drop while vigorously stirring the PGA solution. This mixture is incubated at room temperature for two hours and then extensively dialyzed in 3 liters of 0.1M Phosphate Buffer Saline (PBS) (pH=7.4) overnight at 4°C. This ensures that mixture is free from unreacted DTPA. Reaction of PGA with bicyclic DTPA anhydride is shown in Figure – 4. Similarly, DTPA is conjugated to Bovine Serum Albumin (BSA) to be used as control to determine conjugation of DTPA to PGA. BSA has 62 lysine residues. Hence, 62 molecules of DTPA can be theoretically conjugated to one molecule of BSA. 3x moles excess (9.6 mg) of DTPA in 500ul of DMSO is mixed with 10mg/ml BSA in 0.1M NaHCO₃ (pH – 8.3). The mixture is incubated overnight at room temperature.
Figure – 4: Reaction of poly-L-glutamic acid with DTPA to form N-modified PGA with DTPA

Conjugation of DTPA to PGA and BSA was assessed by 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) assay. TNBS reacts with free amines to form a chromogenic derivative which can be quantitated by absorbance at 420nm. Bovine serum albumin (BSA) and unmodified PGA containing free amines are used to determine total free available amines for reaction.

3.1.2 Anti-DTPA ELISA for detection of PGA-DTPA: For qualitative analyses of conjugation of DTPA to PGA, anti-DTPA ELISA is performed. The 96-well microtiter plate (BD Falcon) is coated with 100µl of DTPA-Bovine Serum Albumin (1µg/ml) in each of 12 wells each of A and B rows and 100 µl of PGA-DTPA (1µg/ml) in the wells of the C, D, E and F rows of the plate. The antigens are incubated at 37°C for one hour and then washed with 0.1M PBS-T (0.1M Phosphate Buffer Saline-Tween) (pH=7.4) x 3-5 times. Then, the plate is blocked with 200µl of
5% spent fetal calf serum in each well, and incubated at 37°C for one hour and washed with 0.1M PBS-T (pH=7.4) as before. Serial dilutions of primary antibody (2C31E11 monoclonal anti-DTPA antibody) (1 µg/ml, 0.1 µg/ml, 0.01 µg/ml, 0.001 µg/ml and 0.0001 µg/ml) in 100 µl aliquots are added to the plate in quadruplicates (n=4) and incubated at 37°C for one hour. The wells are washed with 0.1M PBS-T (pH=7.4). Aliquots of 50 µl of the secondary antibody (Goat anti-mouse antibody modified with horse radish peroxidase) 2 µg/ml are added to each well and incubated at 37°C for one hour and washed with 0.1M PBS-T (pH=7.4). Then, 50 µl of K-Blue color reagent is added to each well, incubated in the dark for 30 minutes and the plate is read at 405nm.

**3.1.3 Coupling of doxorubicin to PGA-DTPA:** 10 mg/ml of PGA-DTPA in 0.1M PBS (pH=7.4) is mixed with 9 mg of doxorubicin (24x moles excess) in minimal amount of DMSO (200µl). 17.2mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is dissolved in minimal amount (300 µl) of DMSO. This is added to the previous mixture of PGA-DTPA and doxorubicin. EDC activates carboxylic group of PGA to form amide bond with the amino group of doxorubicin. The final solution is incubated in the dark for two hours at 4°C and then at room temperature overnight.
Figure – 5(i): Reaction of D-PGA complex with doxorubicin

Figure – 5(ii): General mechanism of activation of Carboxylic group by Carbodiimide(27)

In order to separate free doxorubicin from the D-PGA-dox, gel filtration is carried out using Sephadex G-25 column (1x10cm column). The fractionational range of this column is
between 1000-5000 Da. 0.1M PBS (pH=7.4) is used as the elution buffer. One ml (15 drops) fractions were collected and absorbance at 490nm was determined.

3.2 Stability studies of D-PGA-dox:

The stability of the D-PGA-dox complex is studied in various buffer systems at pH 4.0, 7.4 and 9.6 at 37°C. Results are shown in figure – 8.

3.2.1 Stability studies in 0.1M sodium acetate buffer pH-4.0:

An aliquot of 1ml of D-PGA-doxorubicin conjugate (OD=0.396 at 490nm) was placed in dialysis bag (MWCO: 3500Da.). This assembly was placed in 40ml of 0.1M sodium acetate buffer, pH-4.0 at 37°C and was stirred using a magnetic stirring bar at 100rpm. 1ml samples from the dialysate buffer (0.1M sodium acetate buffer, pH-4.0) were collected at the following time intervals: 5 min, 10min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 50 min, 1 hrs, 3 hrs, 6hrs, 12 hrs, 18 hrs, 24 hrs, 48 hrs. UV spectrophotometric absorbance at 490nm was obtained to determine the concentration of doxorubicin that is released from the dialysis membrane due to cleavage of the bond between the PGA and doxorubicin. Each aliquot was returned to the dialysate buffer after spectrometric reading. The % doxorubicin dissociated from D-PGA-doxorubicin complex was assessed in the dialysate from the OD of the initial sample of D-PGA-doxorubicin conjugate and the OD of the samples collected at different time intervals.

3.2.2 Stability studies in 0.1M phosphate buffer saline, pH-7.4:

An aliquot of 1ml of D-PGA-doxorubicin conjugate (OD=0.396 at 490nm) was placed in dialysis bag (MWCO: 3500Da.). This assembly was placed in 40ml of 0.1M phosphate buffer saline, pH-7.4 at 37°C and the release of doxorubicin was assessed as described above.
3.2.3 Stability studies in 0.1M sodium bicarbonate buffer, pH-9.6:
An aliquot of 1ml of D-PGA-doxorubicin conjugate (OD=1.969 at 490nm) was placed in
dialysis bag (MWCO: 3500Da.). This assembly was placed in 40ml of 0.1M sodium bicarbonate
buffer, pH-9.0 at 37°C and the release of doxorubicin was assessed as described above.

3.3 Coupling of melphalan to Poly-L-Lysine:
10 mg/ml of Poly-L-Lysine in 0.1M PBS (pH=7.4) is mixed with 5.6 mg of melphalan (24x
moles excess) in minimal amount of DMSO (200µl). 17.2mg of 1-ethyl-3-(3-
dimethylaminopropyl) carbodiimide (EDC) is dissolved in minimal amount (200 µl) of DMSO.
This is added to the previous mixture of Poly-L-Lysine and melphalan. The final solution is
incubated at room temperature overnight. Then, the PLL-melphalan solution is extensively
dialyzed in 0.1M Phosphate Buffer Saline, pH 7.4 in order to remove the free drug from the
solution. The absorbance of the PLL-mel solution before and after the dialysis was checked at
260nm. Using the equation for standard curve for melphalan (Figure-11), corresponding
concentrations were calculated. The concentration before dialysis was 5.6mg (melphalan
equivalent) and the concentration after dialysis was 5.5mg. The lack of major difference between
the initial and the final concentrations confirm that there was almost 100% conjugation of the
drug to the polymer.
3.4 Deposition of PLL-mel on the D-PGA-dox:

Since Poly-L-Lysine (PLL) has multiple lysine residues, it can be covalently cross-linked to the carboxylic groups of Poly-Glutamic acid. Carboxylic groups on PGA in 10 mg/ml D-PGA-dox solution are activated by 17.2mg of EDC in minimal amount of DMSO (200µl). The 1x moles (10 mg/ml) of PLL-mel is added to 1x moles of D-PGA-dox and incubated at room temperature overnight. Then, the solution is extensively dialyzed in 0.1M Phosphate Buffer Saline, pH 7.4.
3.5 Measurement of Zeta Potential of polymer-drug conjugates:

There exists an electrical double layer at the interface between the polymer surfaces and the surrounding liquid medium. The inner electrical layer is tightly bound to the surface whereas the outer layer where balanced charges due to electrostatic forces and Brownian motion develop are relatively loosely attached (23). The polymer moves in the liquid medium along with its associated charges as a unit. The ionic potential at the surface between this unit and the surrounding medium is called the zeta potential and it is measured in milli volts (23). Measurement of zeta potential is necessary to distinguish between the PGA-dox, PLL-melphalan and PLL-mel—D-PGA-dox complexes. PGA is negatively charged due to the carboxylic groups in the side chain, PLL is positively charged due to the amine groups in the side chain. So, these polymers are expected to show negative and positive zeta potential respectively. And when the PLL-mel is covalently cross linked to D-PGA-dox polymer, the zeta potential is expected to shift to zero as we are cross linking two oppositely charged polymers. For the measurement of zeta potential of the polymer drug conjugates, ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation) equipped with a palladium electrode with acrylic support was used. All the measurements were taken at 25°C. The results were as expected and shown in Table-3. These polymer-drug conjugates are used in in vitro cytotoxicity cell studies as described in section 3.7.

3.6 Conjugation of melphalan and doxorubicin to Poly-L-Glutamic acid:

10 mg/ml of Poly-L-Glutamic acid in 0.1M PBS (pH=7.4) is mixed with 4.2 mg of melphalan in minimal amount of DMSO (200µl). 17.2mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is dissolved in minimal amount (200 µl) of DMSO. This is added to the previous mixture of Poly-L-Glutamic acid and melphalan. The final solution is incubated at room temperature
overnight. Then, the PGA-melphalan solution is extensively dialyzed in against 0.1M Phosphate Buffer Saline, pH 7.4 in order to remove the free drug from the solution. Then, to this PGA-mel conjugate, 9 mg of doxorubicin in 200µl of DMSO and 17.2mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 300 µl are added. This solution is incubated in dark at 4°C for 2 hours and then at room temperature over night. Then, the solution is extensively dialysed in 0.1M Phosphate Buffer Saline, pH 7.4.

3.7 In vitro studies:

Doxorubicin, melphalan and the polymer-drug conjugates (PGA-dox, PLL-mel, PGA-mel, PGA-dox-mel, PLL-mel—PGA.dox) were used for in vitro experiments. The polymer-drug conjugates were prepared as mentioned above and were characterized using differences in zeta potential.

3.7.1 Cell culture:

Embryonic rat cardiocytes (H9C2 cell line) and human mammary adenocarcinoma (BT-20 cell line) are grown in high glucose concentration (4.5g/l) DMEM supplemented with 10% Fetal clone II (Hyclone) with 5 ml of penicillin-streptomycin (10,000 units/ml each) (Gibco, NY) and a fungicidal agent Amphotericin B (2.5µg/ml) (Sigma) at 37°C in 5% CO₂ in cell incubators. The cells are then dissociated using Trypsin-EDTA solution (Sigma) for passing the cells. A 10X solution of Trypsin-DTPA solution is diluted to 1X with 0.1M PBS using 2 ml of this solution. The digested cells are then seeded in new culture flasks (25 ml) at 10,000/ml cell concentrations. Cells on reaching confluency were then dissociated from the bottom of the flasks by trypsin digestion as described above. The cells are washed in F-DMEM and counted in a Hemocytometer. Aliquots of 40,000 H9C2 cells are delivered in F-DMEM to each well of a 12 well sterile culture plates. The culture media was removed when the cells reached 70% confluency. Fresh media containing 2.5ug/ml, 5ug/ml, 10ug/ml, 15ug/ml and 30ug/ml of
doxorubicin or melphalan or each of the polymer-drug conjugates mentioned above is added to
the wells of the culture plates. After 24 hrs of incubation at 37°C, the cells were collected from
each well and the number of dead cells in each well is determined by trypan blue exclusion test.

3.7.2 Cell viability test (Trypan blue exclusion test):

Trypan blue exclusion test is usually used to estimate viability as it is excluded from the
cytoplasm of viable cells; dead cells (even if they appear intact) are unable to exclude this dye
and appear blue when examined under a microscope. A 20µl aliquot of cell suspension was
removed from the microcentrifuge tube and gently mixed with an equal volume of 0.5% (w/v)
trypan blue solution; 10 µl of this mixture was then placed on a hemocytometer and viability was
counted under an inverted microscope (Olympus ck2; Optical, Inc. Hudson, MA) and viewed at
10x. Dead cells which were stained blue were counted and cytotoxicity was then estimated.

3.7.3 Statistical analysis:

N-way Anova analysis is performed using JMP-7 software to determine the statistical difference
between different groups of drugs and polymer-drug conjugates. The significance level is set at
0.05. The groups were found to be statistically different.
D. Results

4.1 TNBS Assay for PGA-DTPA and BSA-DTPA:

**Table – 1:** TNBS assay for PGA-DTPA and BSA-DTPA

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>µl</th>
<th>dH₂O(µl)</th>
<th>0.1M sodium tetraborate (µl)</th>
<th>TNBS(2mg/ml) (after this, the samples are incubated at 37°C for 30 minutes)</th>
<th>1.5mg/ml Na₂SO₃ in 50 ml of 0.2 M NaH₂PO₄</th>
<th>OD at 420 nm</th>
<th>Avg OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>375</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>375</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>-0.005</td>
<td>0.135</td>
</tr>
<tr>
<td>BSA</td>
<td>5.2</td>
<td>369.8</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.132</td>
<td>0.053</td>
</tr>
<tr>
<td>BSA</td>
<td>5.2</td>
<td>369.8</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.139</td>
<td>0.057</td>
</tr>
<tr>
<td>PGA</td>
<td>10.4</td>
<td>364.6</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.057</td>
<td>0.007</td>
</tr>
<tr>
<td>PGA</td>
<td>10.4</td>
<td>364.6</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.048</td>
<td>0.0085</td>
</tr>
<tr>
<td>PGA-DTPA</td>
<td>10.4</td>
<td>364.6</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.002</td>
<td>0.012</td>
</tr>
<tr>
<td>PGA-DTPA</td>
<td>10.4</td>
<td>364.6</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.012</td>
<td>0.0085</td>
</tr>
<tr>
<td>BSA-DTPA</td>
<td>6.25</td>
<td>368.8</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.007</td>
<td>0.012</td>
</tr>
<tr>
<td>BSA-DTPA</td>
<td>6.25</td>
<td>368.8</td>
<td>375</td>
<td>187.5</td>
<td>375</td>
<td>0.007</td>
<td>0.007</td>
</tr>
</tbody>
</table>

In this TNBS assay, the OD for PGA-DTPA is 0.007 and that of PGA is 0.053 \([(0.007/0.053)\times100=13.2\%]\). Therefore, 86.8\% of N-terminal amino groups of PGA were modified with DTPA. The OD for BSA-DTPA is 0.0085 and that of BSA is 0.135,\([(0.0085/0.135)\times100=6.3\%]\). Hence 93.6\% of the total amino groups in BSA were modified providing 62 moles of DTPA per mole of BSA.
4.2 Anti-DTPA ELISA for PGA-DTPA:

In Figure 7, 1µg/ml of DTPA-PGA shows equivalent binding OD$_{405}$ nm to that of 0.002µg/ml of DTPA-BSA. Since there are 62 lysine residues modified in DTPA-BSA, (62 DTPA moles/mole of BSA), 0.001µg/ml of BSA contains approximately 1.0x10$^{-12}$ moles of DTPA/ml and 0.002 µg/ml of DTPA-BSA contain 2 x 10$^{-12}$ moles of DTPA/ml. Binding of DTPA-PGA at 1 µg/ml is equivalent to 2 x 10$^{-12}$ moles of DTPA/ml on DTPA-BSA. Therefore 2x10$^{-12}$ moles of DTPA are detected in 1 µg of PGA-DTPA (8.5x10$^{-13}$ moles/ml). Hence, it can be inferred from the ELISA that there is almost 2:1 modification of DTPA/PGA, however since there is only one mole N-terminal amino group per mole of PGA, we assumed that every PGA is modified with 1 molecule of DTPA.
4.3 Gel filtration (Sephadex G-25 column) to separate free doxorubicin from D-PGA-dox:

**Figure - 8**: elution profile of D-PGA-dox (blue) and free dox (red) using sephadex G-25 column (1x10cm).

Polymer-drug conjugate (D-PGA-dox) is eluted in the void volume (starting from fraction number 5) whereas the free doxorubicin is eluted in the inclusion volume as can be seen in the figure-8.
4.4 Stability studies of D-PGA-Doxorubicin:

Table – 2: Stability of D-PGA-doxorubicin conjugate in 0.1M sodium carbonate buffer (pH-9.6); 0.1M sodium acetate buffer (pH-4.0); 0.1M phosphate buffer saline (pH-7.4)

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>% of drug dissociated in 0.1M sodium bicarbonate buffer, pH-9.6</th>
<th>% of drug dissociated in 0.1M sodium acetate buffer, pH-4.0</th>
<th>% of drug dissociated in 0.1M phosphate buffer saline, pH-7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.083</td>
<td>30.3</td>
<td>20.2</td>
<td>3.04</td>
</tr>
<tr>
<td>0.166</td>
<td>40.4</td>
<td>20.2</td>
<td>6.09</td>
</tr>
<tr>
<td>0.25</td>
<td>60.6</td>
<td>30.3</td>
<td>6.09</td>
</tr>
<tr>
<td>0.33</td>
<td>60.6</td>
<td>30.3</td>
<td>9.14</td>
</tr>
<tr>
<td>0.42</td>
<td>60.6</td>
<td>50.5</td>
<td>9.14</td>
</tr>
<tr>
<td>0.5</td>
<td>80.8</td>
<td>50.5</td>
<td>9.14</td>
</tr>
<tr>
<td>0.583333</td>
<td>80.8</td>
<td>50.5</td>
<td>9.14</td>
</tr>
<tr>
<td>0.666667</td>
<td>80.8</td>
<td>50.5</td>
<td>9.14</td>
</tr>
<tr>
<td>0.833333</td>
<td>80.8</td>
<td>60.6</td>
<td>9.14</td>
</tr>
<tr>
<td>1</td>
<td>80.8</td>
<td>60.6</td>
<td>9.14</td>
</tr>
<tr>
<td>3</td>
<td>80.8</td>
<td>60.6</td>
<td>9.14</td>
</tr>
<tr>
<td>6</td>
<td>80.8</td>
<td>60.6</td>
<td>9.14</td>
</tr>
<tr>
<td>12</td>
<td>80.8</td>
<td>60.6</td>
<td>9.14</td>
</tr>
<tr>
<td>18</td>
<td>90.9</td>
<td>60.6</td>
<td>9.14</td>
</tr>
<tr>
<td>24</td>
<td>90.9</td>
<td>60.6</td>
<td>9.14</td>
</tr>
</tbody>
</table>

The plot is shown below.
Figure – 9: Chart showing the % of doxorubicin dissociated from the D-PGA-doxorubicin conjugate in different buffer systems as indicated (Green line – in 0.1M PBS, pH – 7.4; Red line – in 0.1M sodium acetate buffer, pH – 4.0; Blue line – in 0.1M sodium carbonate buffer, pH – 9.6).

From the results of stability studies, it can be inferred that the D-PGA-dox conjugate is more stable in 0.1M phosphate buffer saline (pH-7.4) and is relatively less stable in both acidic and basic conditions.
4.5 Maximum absorbance of Melphalan:

As shown in the Figure – 10, maximum absorbance was observed at 260nm. The peak at 220nm might be due to noise interference.

4.6 Standard curve of Melphalan:

Table – 3: Standard curve of Melphalan

<table>
<thead>
<tr>
<th>conc. (ug/ml)</th>
<th>OD 260nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.046</td>
</tr>
<tr>
<td>1</td>
<td>0.079</td>
</tr>
<tr>
<td>5</td>
<td>0.33</td>
</tr>
<tr>
<td>10</td>
<td>0.652</td>
</tr>
<tr>
<td>25</td>
<td>1.62</td>
</tr>
<tr>
<td>50</td>
<td>2.91</td>
</tr>
</tbody>
</table>

The plot is as shown below:
4.7 Zeta potential analysis for the polymer-drug conjugates:

Table – 3: Zeta potential of polymer-drug conjugates measured at 25°C

<table>
<thead>
<tr>
<th>Polymer-drug conjugate</th>
<th>Zeta potential at 25°C(mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-PGA-dox</td>
<td>-18.49</td>
</tr>
<tr>
<td>PLL-mel</td>
<td>17.52</td>
</tr>
<tr>
<td>PLL-mel—D-PGA-dox</td>
<td>0</td>
</tr>
</tbody>
</table>

As seen in Table - 3, the D-PGA-dox and PLL-mel conjugates resulted in negative and positive zeta potential respectively. And, PLL-mel—D-PGA-dox resulted in zero potential.
4.8 *In-vitro* studies:

4.8.1 *Cytotoxicity studies on H9C2 and BT-20 cell lines:*

This section deals with the results of *in vitro* studies of PLL-mel—D-PGA-dox, PLL-melphalan, D-PGA-dox, doxorubicin and melphalan performed on H9C2 (embryonic cardiomyocyte) cell line and BT-20 (human adenocarcinoma) cell line.

![Cytotoxicity studies on H9C2 cell line](image)

**Figure – 12:** Cytotoxicity studies on H9C2 cell line; melphalan(green), PLL-mel(pink), doxorubicin(red), D-PGA-dox(brown), PLL-mel—D-PGA-dox(black) (n = 3)

**Figure – 12** shows the cytotoxic effects of doxorubicin, melphalan, D-PGA-dox, PLL-melphalan and PLL-mel—D-PGA-dox at increasing concentrations on H9C2 cell line (embryonic cardiomyocytes). PLL-mel—D-PGA-dox exhibited highest cell killing ability. PLL-
melphalan exhibited higher cytotoxicity when compared to that of melphalan and PGA-dox exhibited lower cytotoxicity when compared to that of doxorubicin at equal concentrations.

The same experiment is repeated on a different occasion to test the precision of the results. The results are as shown in Figure – 13

**Figure – 13:** Cytotoxicity studies on H9C2 cell line; melphalan(green), PLL-mel(pink), doxorubicin(red), D-PGA-dox(brown), PLL-mel—D-PGA-dox(black) (n = 3)

The cytotoxic pattern followed by the polymer-drug conjugates and the free drugs in Figure – 13 are similar to that shown in Figure – 12. So, the results from the in-vitro studies from both the occasions (Figure 12, 13) are combined in Figure - 14 with each data point corresponding to n=6.
Figure - 14: Combined results of Cytotoxicity studies on H9C2 cell line from Figure 12, 13; melphalan(green), PLL-mel(pink), doxorubicin(red), D-PGA-dox(brown), PLL-mel—D-PGA-dox(black) (n=6)
Figure – 15: Cytotoxicity studies on BT-20 cell line; melphalan(green), PLL-mel(pink), doxorubicin(red), D-PGA-dox(brown), PLL-mel—D-PGA-dox(black) (n=3)

Figure – 15 shows the cytotoxic effects of doxorubicin, melphalan, D-PGA-dox, PLL-melphalan and PLL-mel—D-PGA-dox at increasing concentrations on BT-20 cell line (human adenocarcinoma cells). PLL-mel—D-PGA-dox exhibited highest cell killing ability. PLL-melphalan exhibited higher cytotoxicity when compared to that of melphalan and PGA-dox exhibited lower cytotoxicity when compared to that of doxorubicin at equal concentrations.

The same experiment is repeated on a different occasion to test the precision of the results. The results are as shown in Figure – 16.
Figure – 16: Cytotoxicity studies on BT-20 cell line; melphalan(green), PLL-mel(pink), doxorubicin(red), D-PGA-dox(brown), PLL-mel—D-PGA-dox(black) (n=3)

The cytotoxic pattern followed by the polymer-drug conjugates and the free drugs in Figure – 16 are similar to that shown in Figure – 15. So, the results from the in-vitro studies from both the occasions (Figure 15, 16) are combined in Figure - 17 with each data point corresponding to n=6.
4.8.2 In vitro studies on H9C2 and BT-20 cell lines:

This section deals with the results of *in-vitro* studies of PGA-dox-mel, PGA-dox, PGA-mel, doxorubicin and melphalan on H9C2 (embryonic cardiomyocytes) cell line and BT-20 (human adenocarcinoma) cell line.

**Figure – 17:** Combined results of Cytotoxicity studies on BT-20 cell line from Figure 15, 16; melphalan(green), PLL-mel(pink), doxorubicin(red), D-PGA-dox(brown), PLL-mel—D-PGA-dox(black) (n=6)
Figure – 18: Cytotoxicity studies on H9C2 cell line; melphalan(green), PGA-melphalan(blue), doxorubicin(red), D-PGA-dox(brown), PGA-dox-mel(black) (n=3)

Figure – 18 shows the cytotoxic effects of doxorubicin, melphalan, D-PGA-dox, PGA-melphalan and PGA-dox-mel at increasing concentrations on H9C2 cell line (embryonic cardiomyocytes). Doxorubicin exhibited highest cell killing ability. PGA-dox-mel, D-PGA-dox and PGA-melphlan exhibited lower cytotoxicity when compared to that of the free drugs doxorubicin and melphalan.

The same experiment is repeated on a different occasion to test the precision of the results. The results are as shown in Figure – 19.
Figure – 19: Cytotoxicity studies on H9C2 cell line; melphalan(green), PGA-melphalan(blue), doxorubicin(red), D-PGA-dox(brown), PGA-dox-mel(black) (n=3)

The cytotoxic pattern followed by the polymer-drug conjugates and the free drugs in Figure – 18 are similar to that shown in Figure – 19. So, the results from the in-vitro studies from both the occasions (Figures 18, 19) are combined in Figure - 20 with each data point corresponding to n=6.
**Figure – 20:** Combined results of Cytotoxicity studies on H9C2 cell line from Figures 18, 19. melphalan(green), PGA-melphalan(blue), doxorubicin(red), D-PGA-dox(brown), PGA-dox-mel(black) (n=6)
Figure – 21: Cytotoxicity studies on BT20 cell line; melphalan (green), PGA-melphalan (blue), doxorubicin (red), D-PGA-dox (brown), PGA-dox-mel (black) (n=3)

Figure – 21 shows the cytotoxic effects of doxorubicin, melphalan, D-PGA-dox, PGA-melphalan and PGA-dox-mel at increasing concentrations on BT-20 cell line (human adenocarcinoma cells). Doxorubicin exhibited highest cell killing ability. PGA-dox-mel, D-PGA-dox and PGA-melphalan exhibited lower cytotoxicity when compared to that of the free drugs doxorubicin and melphalan.

The same experiment is repeated on a different occasion to test the precision of the results. The results are as shown in Figure – 22.
Figure – 22: Cytotoxicity studies on BT20 cell line; melphalan(green), PGA-melphalan(blue), doxorubicin(red), D-PGA-dox(brown), PGA-dox-mel(black) (n=3)

The cytotoxic pattern followed by the polymer-drug conjugates and the free drugs in Figure – 22 are similar to that shown in Figure – 21. So, the results from the in-vitro studies from both the occasions (Figures 21, 22) are combined in Figure - 23 with each data point corresponding to n=6.
Figure – 23: Combined results of Cytotoxicity studies on BT-20 cell line from Figures 21, 22. melphalan(green), PGA-melphalan(blue), doxorubicin(red), D-PGA-dox(brown), PGA-dox-mel(black) (n=6)

4.8.3: **IC50 values of the free drugs and polymer-drug conjugates in H9C2 and BT-20 cell lines:**

Table – 4: IC50 values of free drug and polymer-drug conjugates in H9C2 cell line

<table>
<thead>
<tr>
<th>Cytotoxic agent</th>
<th>IC50 (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL-mel–D-PGA-dox</td>
<td>1.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.6</td>
</tr>
<tr>
<td>PGA-dox</td>
<td>15.8</td>
</tr>
<tr>
<td>Melphalan</td>
<td>6.4</td>
</tr>
<tr>
<td>PLL-melphalan</td>
<td>3.6</td>
</tr>
<tr>
<td>PGA-dox-mel</td>
<td>12</td>
</tr>
<tr>
<td>PGA-mel</td>
<td>14.1</td>
</tr>
</tbody>
</table>
Table – 5: IC50 values of free drug and polymer-drug conjugates in BT-20 cell line

<table>
<thead>
<tr>
<th>Cytotoxic agent</th>
<th>IC50 (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL-mel--D-PGA-dox</td>
<td>1.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>2</td>
</tr>
<tr>
<td>PGA-dox</td>
<td>16.3</td>
</tr>
<tr>
<td>Melphalan</td>
<td>6.6</td>
</tr>
<tr>
<td>PLL-melphalan</td>
<td>3.7</td>
</tr>
<tr>
<td>PGA-dox-mel</td>
<td>10.6</td>
</tr>
<tr>
<td>PGA-mel</td>
<td>19.5</td>
</tr>
</tbody>
</table>

As seen in Tables 4 & 5, free drugs doxorubicin and melphalan have low IC50 values when compared to their respective PGA-drug conjugates. Whereas, the polymer-drug conjugates containing Poly-lysine have low IC50 values when compared to that of the free drugs.
**5. Discussion:**

The model drugs used in this study, doxorubicin and melphalan, have maximum absorbance at specific wavelengths (490nm for doxorubicin and 260nm for melphalan) which helped in quantification of the amount of drug conjugated to polymers after the extensive dialysis of the polymer-drug conjugate solutions.

Size exclusion chromatography using Sephadex G-25 column (1x10 cm) was performed for D-PGA-dox conjugate in order to see if there is any free doxorubicin left after conjugation. As can be seen in Figure – 8, the polymer-drug conjugate was eluted in the void volume. Free doxorubicin is shown to be eluted starting from fraction 25. No such peak was seen in the D-PGA-dox elution profile showing that the polymer-drug conjugation was efficient. Also, the concentrations of the polymer-drug conjugates did not vary before and after extensive dialysis of the polymer-drug conjugates.

As shown in Figure – 9, the polymer-drug conjugates are stable at neutral pH when compared to acidic and basic pH conditions. So, 0.1M Phosphate Buffer Saline, pH 7.4 was used for the dilution purpose of all the polymers and polymer-drug conjugates. This study also supports the fact that polymer-drug conjugates are unstable in lysosomes where the pH is acidic (24). Along with the reduction in pH in lysosomes, a proteolytic enzyme Cathepsin B also helps in degradation of the bond between the drug and the polymer leading to the release of the free drug (24). The polymers that are employed in this study (Poly-L-Glutamic acid and Poly-L-Lysine) are bio-degradable. These polymers are broken down into their respective amino acid residues in lysosomes (by proteolytic enzymes) which are then removed from the body in the process similar to that of other natural amino acids (24).
As shown in Table – 3, the differences in zeta potential of different polymer-drug conjugates were used in characterizing them. Poly-L-Glutamic acid consists of carboxylic acids in the side chain of glutamic acid residues because of which the polymer is negatively charged. So, it exhibits negative zeta potential. Similarly, Poly-L-lysine consists of amine groups in the side chains of the lysine residues which confer positive charge to the polymer. As a result, it exhibits positive zeta potential. In case of PLL-mel—D-PGA-dox, the positive charge of Poly-L-Lysine negates the negative charge on Poly-L-Glutamic acid. So, it exhibits zero zeta potential. As expected, PLL-mel—D-PGA-dox conjugate exhibited zero potential which is different from the individual zeta potentials of the constituting polymers (PLL-mel and D-PGA-dox).

In vitro studies were conducted using mammary adenocarcinoma (BT-20) cells, and embryonic cardiomyocytes (H9C2). PGA-dox exhibited lower cell killing ability when compared to that of free doxorubicin at equal concentrations (Figures 12 - 23). Doxorubicin is almost 10 times more cytotoxic than PGA-dox which is evident from the IC50 values shown in Table 4 & 5. This is due to the inherent negative charge on the polymer which causes the negatively charged cell membranes to repel the polymer due to repulsion by similar charges. As a result, very few polymer-drug conjugates would interact with the cell membranes that would lead to internalization. This reduced cytotoxicity would be beneficial for reducing the cardiotoxicity of doxorubicin if the polymer drug conjugate can be then targeted to cancer cells.

As seen in Figures 12 -23, PLL-melphalan exhibited higher cytotoxicity when compared to that of melphalan due to the inherent positive charge on PLL which causes the polymer to extensively stick to the negatively charged cell membranes leading to higher uptake of the polymer-drug conjugates. Whereas, PGA-melphalan followed the pattern similar to that of PGA-
doxorubicin in exhibiting lower cytotoxicity when compared to that of melphalan and for the same reason of carrying negative charge on its surface.

PLL-mel—D-PGA-dox exhibited highest cytotoxic ability (Figures - 12-17). This might be due to the presence of positive charge on Poly-L-lysine which makes the polymers to stick extensively to the negatively charged cell membranes. Also, two drugs (doxorubicin and melphalan) are simultaneously delivered into the cells when PLL-mel—D-PGA-dox conjugates are internalized. As a result, highest cytotoxicity is achieved. Even though PLL-mel—D-PGA-dox conjugate exhibited highest cytotoxic effect on BT-20 cells which is desirable, it exhibited the similar effect on H9C2 cells which is undesirable. In order to address this, we conjugated melphalan and doxorubicin to PGA. Through PGA-dox-mel conjugate, we are able to deliver two drugs simultaneously into the cells. As shown in Figures – 18-23, PGA-dox-mel conjugate exhibited lower cytotoxicity when compared to free drugs in both the cell lines. This reduced toxicity is due to the negative charge on PGA polymer. This showed that the side effects of the drugs like cardiotoxicity of doxorubicin can be mitigated by conjugating the drug to the polymer.

The cytotoxic effect of PGA-dox-mel can be enhanced in tumors by specifically targeting these polymer drug conjugates in to the tumor environment. Due to their higher molecular weight, polymer-drug conjugates are passively targeted in to the tumor environment due to the Enhanced Permeability and Retention effect (4) as explained in section 2.2. Specific targeting into tumor environment would lead to higher internalization of polymer-drug conjugates like PGA-dox-mel by tumor cells, leaving the other cells and organs to be safe. And by using a polymer-drug conjugate like PGA-dox-mel, two different anti-cancer drugs can be
simultaneously deployed into the tumor environment which can lead to a better anti-cancer therapy.

6. Conclusion:

Polymer-drug conjugates were prepared and characterized taking advantage of the optical properties of the two model drugs that we used in this study and also the significant surface charge differences between the polymers such as Poly-L-lysine and Poly-L-Glutamic acid. The in-vitro experiments proved that the polymers loaded with multiple drugs exert less side effects than free drugs at equal concentrations. The main drawback of side effects exerted by chemotherapeutic agents can be mitigated by conjugating them on to bio-degradable polymers. The efficacy of these polymer-drug conjugates on tumors can further be increased by targeting them to tumors. Although this thesis did not going into the targeting aspect of the polymer-drug conjugates, it stands to reason that if these multi-drug polymer conjugates are targeted to cancer cells, tumor toxicity should be enhanced with simultaneous reduction of non-targeted by-stander toxicity.
E. Bibliography


2. Douglas Hanahan and Robert A. Weinberg; The Hallmarks of Cancer; Cell, Vol. 100, 57–70, January 7, 2000


9. S. Foster, J.B. Lloyd, Solute transport across the mammalian lysosome membrane,
    Poupon MF; Doxorubicin-loaded nanospheres bypass tumor cell multidrug resistance;
    Animati F: Anthracyclines: selected new developments. Curr Med Chem Anticancer
    mediated up-regulation of CD95 gene expression upon genotoxic treatment in human
13. Johnson BA, Cheang MS, Goldenberg GJ: Comparison of adriamycin uptake in chick
    embryo heart and liver cells an murine L5178Y lymphoblasts in vitro: role of drug uptake
    Dystrophin-deficiency increases the susceptibility to doxorubicin-induced cardiotoxicity.
15. Berthiaume JM, Wallace KB: Adriamycin-induced oxidative mitochondrial
    insights into doxorubicin induced cardiotoxicity: the critical role of cellular energetics. J


24. R. Duncan, S. Gac-Breton, R. Keane, R. Musila, Y. N. Sat, R. Satchi, F. Searle; Polymer-drug conjugates, PDEPT and PELT: basic principles for design and transfer from the laboratory to clinic; Journal of Controlled Release 74 (2001) 135–146


Appendix 1: Laboratory Safety Training

Chemical Hygiene Training Part 1 - Right to Know

Northeastern University Office of Environmental Health & Safety

On May 5, 2010, Savitri Mandapati successfully completed 01: "Chemical Hygiene Training Part 1 - Right To Know" program on the web, answering 93% of the 15 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: To complete this training you must also take Part 2

Record Locator: tw0000000010022 (Session).

Chemical Hygiene Training Part 2 - Lab Safety and Hazardous Waste Management

Northeastern University Office of Environmental Health & Safety

On May 5, 2010, Savitri Mandapati successfully completed 02: "Chemical Hygiene Training Part 2 - Lab Safety and Hazardous Waste Management" program on the web, answering 90% of the 10 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: Record Locator: tw0000000010030(Session).