DESIGNED MULTIPURPOSE NANOPARTICLES FOR TARGETED DELIVERY OF ANTICANCER THERAPEUTICS FOR MALIGNANT MESOTHELIOMA

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A DISSERTATION

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PHYSICS

ABSTRACT

Malignant mesothelioma is an aggressive deadly pleural cancer overexpressing Ephrin A2 receptors which are biomarkers for malignant mesothelioma. The Ephrin A1 ligand binding to the Ephrin A2 receptor downregulates overexpression of the Ephrin A2 in malignant mesothelioma cells (MMC). Similarly, the YSA peptide can mimic the Ephrin A1 ligand and both could be suitable as targeting agents and tumor suppressing agents for MMC.

In this work, we conjugated the surface of nanoparticles with the YSA peptide and Ephrin A1 ligand to deliver paclitaxel with low solubility by targeting the MMC.

We have obtained nanoparticles ranging 188-278 nm with the Ephrin A1 surface conjugated nanoparticles having 57-90 μm smaller size compared to any the other four nanoparticle formulations we studied. Nanoparticles released 32% of the drug encapsulated by day 5 and showed slow sustained release afterwards with very minimal variation in drug release amount. This stable and sustained release profile of paclitaxel loaded nanoparticles provides the desired amount of drug supplied to cells.

Highest uptake (100%) was achieved by the Ephrin A1 conjugated nanoparticles (EPH NP) at 125 μg/ml concentration in 5 hours. The highest killing (71%) was achieved by the YSA peptide conjugated and paclitaxel loaded nanoparticles (YSA-PTX NP) at 1.5 μg/ml over 48 hours. This is 32% more cytotoxicity with 90% less of the drug used compared to free paclitaxel.

Based on combined results of efficient targeting ability and improved cytotoxicity, surface conjugated and paclitaxel loaded nanoparticle formulations stands out with YSA-PTX NP as being one step further. This is ideal and promising in that it has significant potential for *in vivo* targeted delivery.

In this study, we successfully prepared particles in an ideal nano-range with successful targeting agent conjugation and sufficient anticancer drug encapsulation. They are suitable and ideal for cancer diagnoses and treatments. The targeting ability of the Ephrin A1 ligand and YSA peptide is already proven. This work has introduced successful formulation of PLGA nanoparticles with high loading efficiency as a great potential for diagnosis and treatment of many cancer types overexpressing the Ephrin A2 receptors. This work further will be carried by *in vitro*/ *in vivo* studies of the Ephrin A2 overexpressing other cancer cells.

Keywords: ephrin A1 ligand, YSA peptide, paclitaxel, malignant mesothelioma, nanoparticles, targeted drug delivery

DEDICATION

I would like to dedicate this thesis to my dear wife Fatma and my beloved children Afra, Taha, and Bera.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DLS	Dynamic light scattering (also known as photon correlation spectroscopy
DMSO	Dimethyl sulfoxide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)- carbodiimide
EPH NP	Ephrin A1 ligand surface conjugated nanoparticles without PTX
EPH-PTX NP	PTX loaded nanoparticles surface conjugated with Ephrin A1 ligand
FITC	Fluorescein isothiocyanate. A derivative of fluorescein
IC50	Half Maximal Inhibitory Concentration
MMC	Malignant Mesothelioma Cells
MTT	A yellow tetrazole, is reduced to purple formazan in living cells
NP	Placebo nanoparticles
PBS	Phosphate-buffered saline
PLGA	poly (lactic-co-glycolic acid
PTX	Free paclitaxel
PTX NP	Paclitaxel loaded nanoparticles
PVA	poly (vinyl alcohol)
SEM	Scanning electron microscope

YSA NP	YSA peptide surface conjugated nanoparticles without PTX
YSA-PTX NP	PTX loaded nanoparticles surface conjugated with YSA peptide
YSA	YSAYPDSVPMMS (Tyr-Ser-Ala-Tyr-Pro-Asp-Ser-Val-Pro-Met-Met-Ser)

CHAPTER 1

INTRODUCTION

Cancer is a malignant disease caused by uncontrolled growth of aberrant cells. Cancer is the second highest mortality rank with one out of every four death in U.S.A. [1]. Cancer is a malignant disease caused by uncontrolled growth of aberrant cells. Cancer is the second highest mortality rank with one out of every four death in U.S.A. [2].

1.1 Malignant Mesothelioma

Malignant mesothelioma is an aggressive deadly pleural cancer disease that occurs as a result of inhalation of asbestos fibers which is naturally occurring silicate minerals used commercially, mainly in construction [3]. The pleural space is a target for malignant involvements in primary tumors of the pleura such as mesothelioma and in metastatic tumors [4-8]. There are over 200,000 patients with malignant pleural effusions each year [4, 9]. Diagnosis and therapy for these patients remain palliative at best and have not changed for several decades [9-13]. Approximately 3000 cases are diagnosed annually in U.S. It is disappointing that most of the cases result in death after 12 months [14, 15]. Health care and compensation costs for this disease in the U.S. over the next several years are expected to reach 200 billion dollars [5].

In Europe, where the incidence of malignant mesothelioma has not yet peaked, over 250,000 deaths are predicted in the next ten years. Because asbestos use is still prevalent in third-world countries, the morbidity and mortality with this disease are expected

to increase globally. Over 90% of patients with malignant mesothelioma have an undiagnosed pleural effusion [16]. Patients with Stage 1A disease, where the tumor is limited to an isolated nodule on the surface of the pleura, have the best prognosis. However, a small isolated nodule cannot be easily visualized on chest radiographs or CT scans, particularly in the presence of an effusion [4].

1.2 Tumor

Knowing the structure and mechanism of tumors is essential to developing targeting and treatment techniques for cancer. Impaired lymphatic drainage, angiogenesis, defective vascular architecture and increased permeability factors are major characteristics of solid tumors. The architecture of tumors allows small particles, typically less than 100 nm, such as liposomes, nanoparticles, and macromolecular drugs to accumulate in tumor sites. This phenomenon is called the enhanced permeability retention (EPR) effect. This property of tumors could be further explored to improve the drug delivery to tumor sites. The EPR effect by itself may be an insufficient targeting mechanism, however better-targeting strategies could be developed by incorporating ligands such as peptides, monoclonal antibodies (mAbs), sugar moieties etc. to the drug therapeutics [17].

1.3 Defining the Problem

Some of the major problems with cancer treatments can be summarized as; (1) early detection of tumor formation, (2) targeting the tumor site and (3) delivery challenges of anticancer therapeutics. Insufficiency of current therapeutic techniques leads to interest and exploring the molecular markers to identify the tumors and to the need to de-

velop designed nanocarriers with targeting signals attached on the surface that will selectively target tumors of interest and deliver proteins/drugs/genes to the area of interest to stop/slow tumor growth.

1.4 Nanocarriers in Drug Delivery

Nanotechnology in drug delivery offers a variety of tools to deliver micro or macromolecules such as genes, proteins, peptides, chemical compounds etc. to the interest of tissue by selectively targeted techniques or localized administration [18].

The cellular uptake, safety, and biodistribution of nanoparticles depend on the surface charge, hydrophobicity and size. Nanoparticles with negative surface charge clear faster *in vivo* [19]. Since cell membranes are usually negatively charged, it reduces the cellular uptake of nanoparticles [20-22]. Hydrophilic nanoparticles can avoid the uptake by mononuclear phagocyte system (MPS), which primarily contains monocytes and macrophages [20]. Hydrophilic nanoparticles can also provide longer circulation time *in vivo* [20], whereas hydrophobic nanoparticles aim to be better taken up by lungs, liver and spleen [23, 24]. Nanoparticles larger than 100 nm in diameter are more likely to be absorbed by MPS in the liver, spleen, lungs and bone marrow [19].

The use of nanoparticles in anti-cancer therapeutics has many advantages, such as: (i) preventing degradation of therapeutics *in vivo* and protects them, (ii) increasing the amount of payload delivered to tumor tissue, (iii) specifically targeting the tumor tissue when incorporated by targeted ligands, and (iv) providing sustained controlled release of therapeutics [19].

Moreover, nanoparticles having comparable sizes to pathogens trigger internalization by antigen-presenting cells [2, 25].

1.4.1 Poly-lactic-co-glycolic Acid

Poly-lactic-co-glycolic acid (PLGA) (Figure 1) is widely used in nanotechnology and has been widely facilitated in many FDA approved therapeutics in drug delivery, vaccines, tissue engineering applications, and diagnostics and treatment of cancer, cardio-vascular diseases and in many other clinical applications and etc. [26, 27].

PLGA types are distinguished by their monomer ratios. PLGA 50:50 is one of the most commonly used polymers in nanotechnology, specifically in drug delivery and where 50:50 represents the composition of the copolymer ratio of 50% lactic acid to 50% glycolic acid [27, 28]. Alteration of the amount of lactic acid in polymer composition lengthens or shortens the degradation time [29].

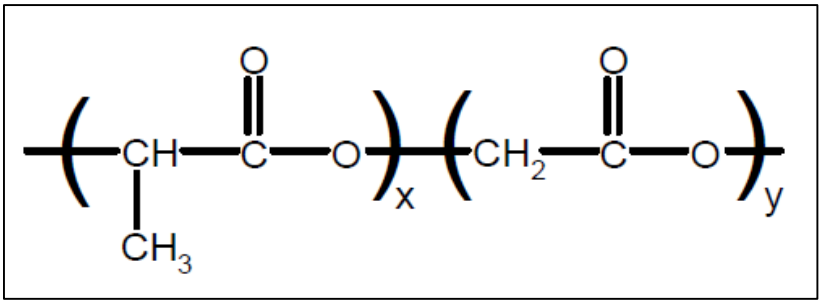

Figure 1. Chemical structure of poly (lactic-co-glycolic acid) (PLGA)

1.4.2 Effect of PLGA Nanoparticles

Much controversy still exists regarding the potential adverse effects of nanoparti-cles. However, concerns over the effects of the physicochemical properties of nanoparti-cles such as size, surface area, and molecular weight of the material warrants further in-vestigation. It has been speculated that the specific surface area of nanoparticles is re-sponsible for the potential adverse effects that nanoparticles could have in drug delivery

applications. Therefore, the specific surface area is an important parameter to consider when studying the possible adverse effects of a nanomaterial. However, such adverse effects and toxicity of PLGA particles are very minimal compared to advantages of such drug delivery systems [30].

However, delivery of anticancer therapeutics via nanoparticles is not sufficient enough by itself. More effective cellular uptake and specific targeting may be achieved by incorporating the targeting signals onto nanoparticles [20].

1.5 Targeting

Peptides are great for tissue penetration and can be easily synthesized and conjugated and it makes them great candidates for targeting purposes in drug delivery [20, 31].

Peptide/ligand conjugated nanocarriers loaded with anticancer drugs act more effectively than anticancer drugs themselves when administrated to cancer tissues [32].

1.5.1 Ephrin A2 Receptor and its Ligand Ephrin A1

Ephrin receptors are great candidates for cancer treatments since they are transmembrane proteins with the ability to recognize signals and have a role on the cell to cell interaction and migration. The Ephrin A2 receptor and its ligand Ephrin A1 are part of the family receptor tyrosine kinases and have an important role in embryonic development [33, 34]. The Ephrin A2 receptor is overexpressed in malignancy. The Ephrin A2 receptor activated by it ligand mediates the downstream of signals and regulates malignant behavior [35].

Nasreen et al. have recently demonstrated [35-37] that a biomarker for mesothelioma is the Ephrin A2 receptor on the surface of mesothelioma cells which is not expressed on adjacent, normal, pleural mesothelial cells. The Ephrin A1 ligand activating its receptor Ephrin A2 downregulates total Ephrin A2 expression in malignant mesothelioma cells (MMC). Resulting ERK1/2 signaling by activation of the receptor Ephrin A2 via its ligand Ephrin A1 suppresses the growth of MMC. It proves that the surface membrane receptor Ephrin A2 can act as a good target as well as a tumor-suppressing agent [15]. Please see Figure 2 describing the mechanism of the conjugated nanoparticles with the Ephrin ligand.

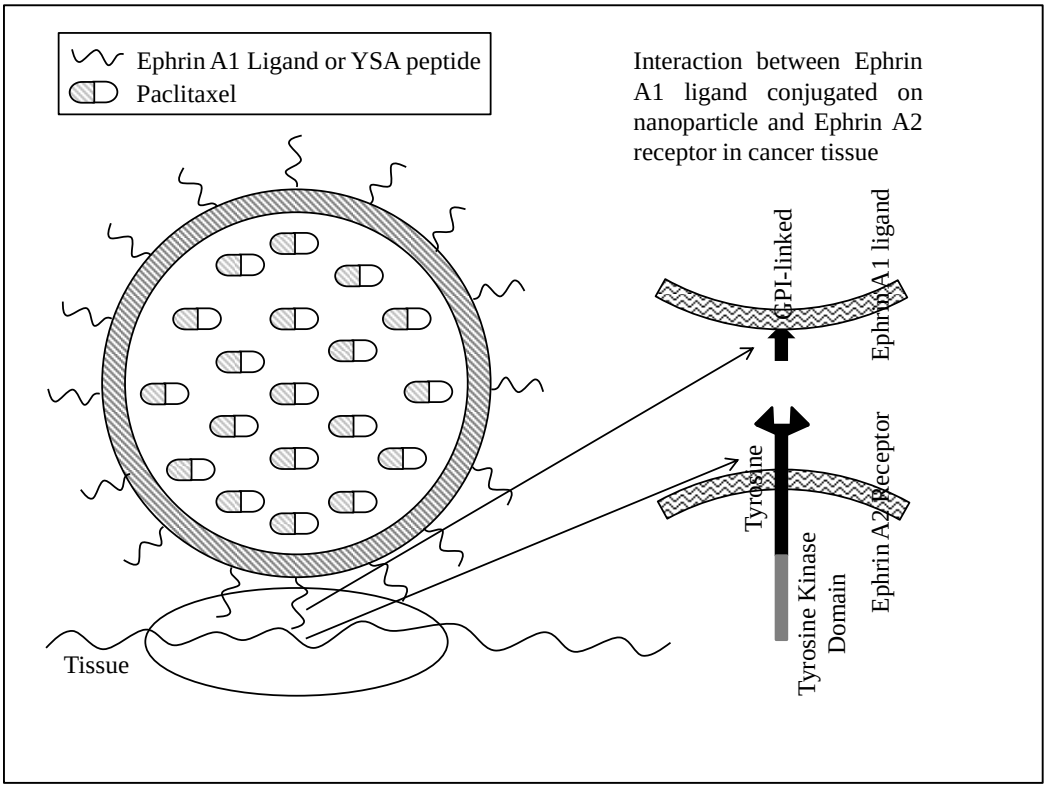

Figure 2. A schematic diagram depicting the reaction of the Ephrin A2 receptor targeted nanoparticle formulations with the Ephrin A2 receptor. The Ehprin A1 ligand and YSA peptide with their high affinity to the Ephrin A2 receptor are conjugated onto the paclitaxel loaded nanoparticle formulations. In the scheme an Ephrin A1 ligand (top) in contact with an Eph A2-expressing cell (bottom) is shown.

1.5.2 YSA (YSAYPDSVPMMS or YSA) Peptide

YSA, YSAYPDSVPMMS (Tyr-Ser-Ala-Tyr-Pro-Asp-Ser-Val-Pro-Met-Met-Ser) is a 12 amino acid peptide (Figure 3) that mimics the Ephrin A1 ligand binding the Ephrin A2 receptor [38].

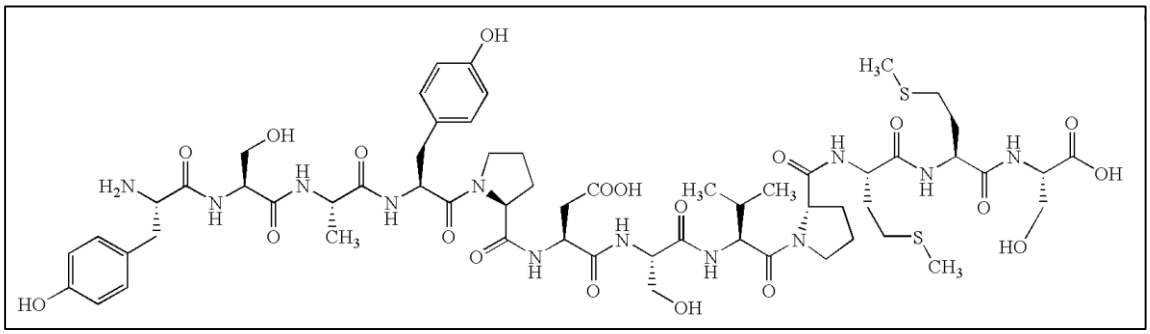

Figure 3. Structure of YSA Peptide. (Sequence: Tyr-Ser-Ala-Tyr-Pro-Asp-Ser-Val-Pro-Met-Met-Ser, MW: 1347.53, Formula: $C_{59}H_{86}N_{12}O_{20}S_2$)

Since protein based therapeutics have some drawbacks such as allergic reactions, immunogenic and homogeneity issues, incorporating proteins into drugs may not be very ideal as targeting signals for drug delivery. Therefore, peptides or small molecule ligands are more desirable for this purpose [39]. Wang et al. reported that they have identified YSA (amino acid sequence YSAYPDSVPMMS) and SWL (amino acid sequence SWLAYPGAVSYR) short peptides that selectively target Ephrin A2 receptors. They claim that the YSA peptide showed higher affinity for the Ephrin A2 compared to the SWA peptide and YSA inhibited the binding of Ephrin-A ligands to immobilized the Ephrin A2 receptor with low micromolar range values of IC50 (The half maximal inhibitory concentration). They also reported that the YSA peptides can initiate the active internalization of receptors into cells [32].

Scarberry et al. also reported that the YSA-coated magnetic cobalt ferrite nanoparticles can selectively target Ephrin A2 receptors in ovarian carcinoma cells. The YSA peptides can activate Ephrin A2 receptors and downstream signaling in addition to decreasing receptor levels on the cell surface [32, 40].

YSA is a short peptide sequence and it is single and small molecule whose size would not trigger an immune response. One should note the significant lower cost to synthesize a homogeneous short peptide than to prepare proteins and humanized antibodies [32].

1.5.3 Paclitaxel as Anticancer Therapeutics

Most anticancer drug treatments have not been very effective due to poor cell penetration. The paclitaxel (Figure 4) is a mitotic inhibitor that is most commonly used in chemotherapy. Due to its low therapeutic index and low solubility in water or in many pharmaceutical solvents, its clinical success for intravascular administration is limited [41]. Nanoparticles loaded with paclitaxel shows significant increase in antitumor efficiency of the drug compared to the free drug (Taxol®) [42].

Van Vlerken LE, et al. 2008, administrated nanoparticles loaded with paclitaxel intravenously to MCF7 and $MCF7_{TR}$ tumor-bearing mice. They observed a higher concentration of paclitaxel in the blood due to longer retention time and improved accumulation in the tumor area compared to the free drug [27, 43].

Multi-purpose nanoparticle formulations are great candidates as a drug carrier to overcome challenges with drug delivery to cancer or any other type of tissues. The YSA peptide and the Ephrin A1 ligand with their high affinity to malignant mesothelioma cells

provides targeting ability to nanoparticles. Fluorescent labeled targeting agents conjugated onto nanoparticles are great tools to visualize the location of the cancer tissue and diagnosed with. Paclitaxel with low solubility requires a carrier with high cellular uptake to deliver it into the cell nucleus for more efficient cancer treatment. The sustained release profile of nanoparticles is also an additional advantage over free paclitaxel. Such designed multipurpose nanoparticles are very promising for the diagnoses and treatment of malignant mesothelioma cancer.

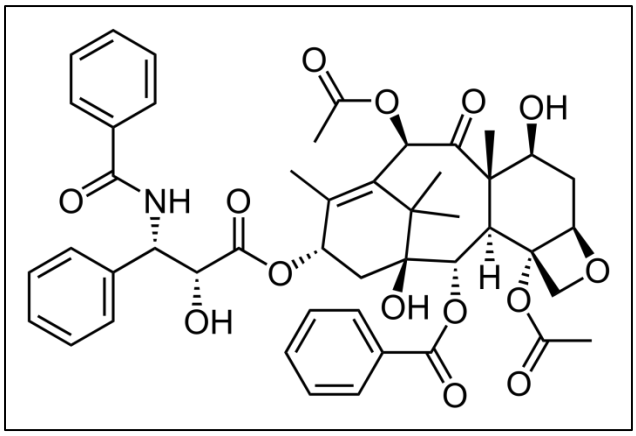

Figure 4. Chemical Structure of paclitaxel, Formula: C47H51NO14, Molecular mass: 853.906 g/mol

1.6 Objectives

Most of the literature focuses on either only targeting or delivery techniques of anticancer therapeutics for nanoparticles, and there is very little work of nanoparticles specifically for mesothelioma cancer treatment. Our aim was to combine current targeting and delivery techniques to improve the delivery of anticancer therapeutics for MM via designed multipurpose PLGA nanoparticles. This study has three main objectives.

1. Synthesis and optimization of placebo nanoparticles suitable for anti-cancer therapeutics
    - Characterization and *in vitro* studies of placebo nanoparticles (NP) (Section 3.1)
2. Encapsulating paclitaxel into designed nanoparticles achieved in objective 1
    - Characterization of paclitaxel loaded nanoparticles (PTX NP) and *in vitro* studies in comparison with free paclitaxel (PTX) (Section 3.2)
3. Conjugating the surface of nanoparticles achieved in objectives1 and 2 with Targeting Signals such as the Ephrin A1 ligand and the YSA peptide

    Characterization of surface conjugated nanoparticles with targeting agents (The Ephrin A1 ligand and YSA peptide) and *in vitro* studies (EPH NP, EPH-PTX NP, YSA NP, YSA-PTX NP) (Section 3.3)

CHAPTER 2

METHODOLOGY

This thesis includes seven studies with six nanoparticle formulations and free paclitaxel drug as follows:

- Placebo nanoparticles (NP) (Section 3.1)
- Free paclitaxel (PTX) (Section 3.2)
- Paclitaxel Loaded nanoparticles (PTX NP) (Section 3.2)
- YSA peptide surface conjugated nanoparticles with no paclitaxel (YSA NP) (Section 3.3)
- Ephrin A1 ligand surface conjugated nanoparticles with no paclitaxel (EPH NP) (Section 3.3)
- Paclitaxel loaded nanoparticles surface conjugated with YSA peptide (YSA-PTX NP) (Section 3.3)
- Paclitaxel loaded nanoparticles surface conjugated with Ephrin A1 ligand (EPH-PTX NP) (Section 3.3)

We have studied them three basic categories as;

1. Synthesis of nanoparticles
2. Characterization of nanoparticles
3. *in vitro* Studies

Please see Table 1 for the outline of the thesis.

Table 1. Outline of experiments

	Placebo-NP (3.1)	**PTX (3.2)**		**Surface Conjugated NPs (3.3)**			
	No Conjugation No PTX	**Free Drug**	**PTX Loaded NP**			**Surface Conjugated-NPs w/o PTX**	
	NP	**PTX**	**PTX NP**	**YSA-PTX NP**	**EPH-PTX NP**	**YSA NP**	**EPH NP**
Synthesis (2.1)							
Water-in-oil-in-water method and Fluorescent Encapsulation (2.1.1)	2.1. 1		2.1. 1				
Water-in-oil-in-water method w/ drug (2.1.1)			2.1.1				
Protein labeling and conjugation (2.1.2)				2.1.2			
Characterization (2.2)							
SEM, Microscope, Size, and Charge (2.2.1,2)	3.1.1		3.2.1	3.3.1			
Determination of Protein Amount (2.2.3)				3.3.2			
Determination of Drug Content (2.2.4)			3.2.2	3.3.3			
Drug Release (2.2.5)			3.2.3				
***in vitro* Studies (2.3)**							
Cellular Uptake (2.3.2)	3.1.2					3.3.4	
Visualization of NPs in Cells (2.3.3)	3.1.2						
Cytotoxicity (2.3.4)	3.1.2	3.2.4		3.3.4			
Determination of The half maximal inhibitory concentration (IC50) (2.3.5)		3.2.4		3.3.4			

2.1 Synthesis of Designed Multipurpose Nanoparticles

Materials: Paclitaxel MW: 83.92, PLGA with a lactide/glycolide molar ratio of 50:50 (MW: 38000–54000), polyvinyl alcohol (PVA, MW: 88000), and 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC or EDAC) MW: 191.70 purchased from Fisher Scientific (USA). Recombinant Ephrin A1/Fc Chimera (MW: 47500) is purchased from Biotang inc. (USA). Ephrin A2 Selective YSA-Peptide (MW: 1348.5) from Abgent, Inc. RPMI 1640 cell culture medium and phosphate-buffered saline (PBS, pH 7.4) were purchased from life technologies (USA). Coumarin-6 (MW: 350.43), Dichloromethane and DCM-D2 were purchased from Sigma–Aldrich (St. Louis, MO). A malignant mesothelioma cancer cell line, CRL-2081, was purchased from ATCC (American Type Culture Collection) (USA). All other chemicals were the highest grade possible and obtained from commercial sources.

2.1.1 Water-in-oil-in-water (w/o/w) Method

The most commonly used method for PLGA NP formation is the single or double-emulsion-solvent evaporation (Figure 5). Single-emulsion process involves oil-in-water (o/w) emulsification while the double-emulsion process is a water-in-oil-in-water (w/o/w) method. The w/o/w method is best suited to encapsulate water-soluble drugs, such as peptides, proteins, and vaccines while the o/w method is ideal for water-insoluble drugs, such as steroids [44].

Surfactants are commonly used to stabilize the emulsion during nanoparticle preparation by emulsion techniques. We used poly (vinyl alcohol) (PVA) as a surfactant, which is the most commonly used as the stabilizer. Upon removal of the organic solvent, the hydrophilic side chains of PVA remain exposed on the surface of the newly formed

nanoparticles suggesting that the stabilizer molecules are incorporated directly into the nanoparticle surface during production (Figure 6) [45, 46].

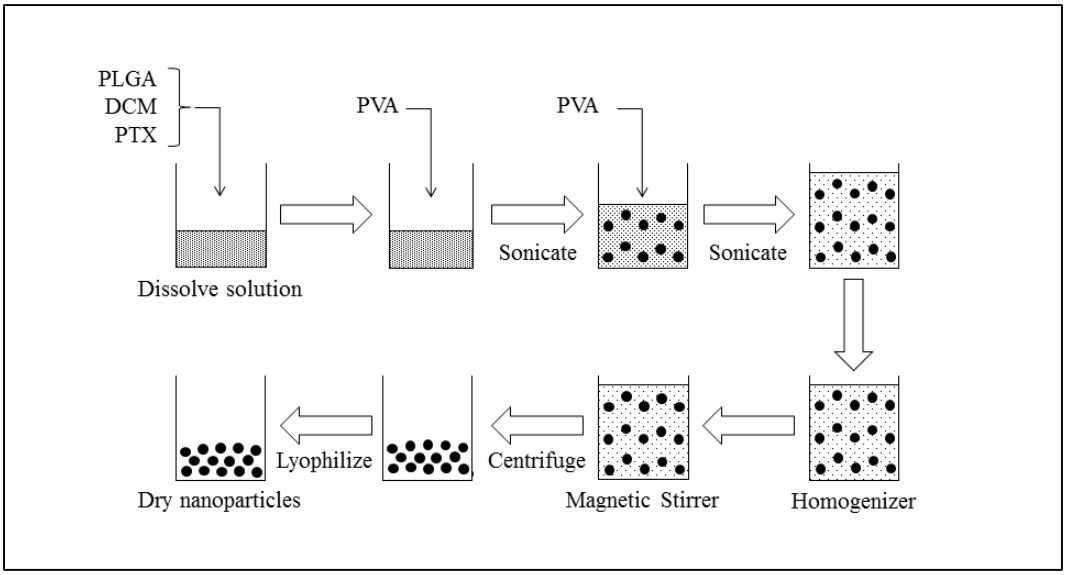

Figure 5. Preparation of nanoparticles via Double Emulsion technique

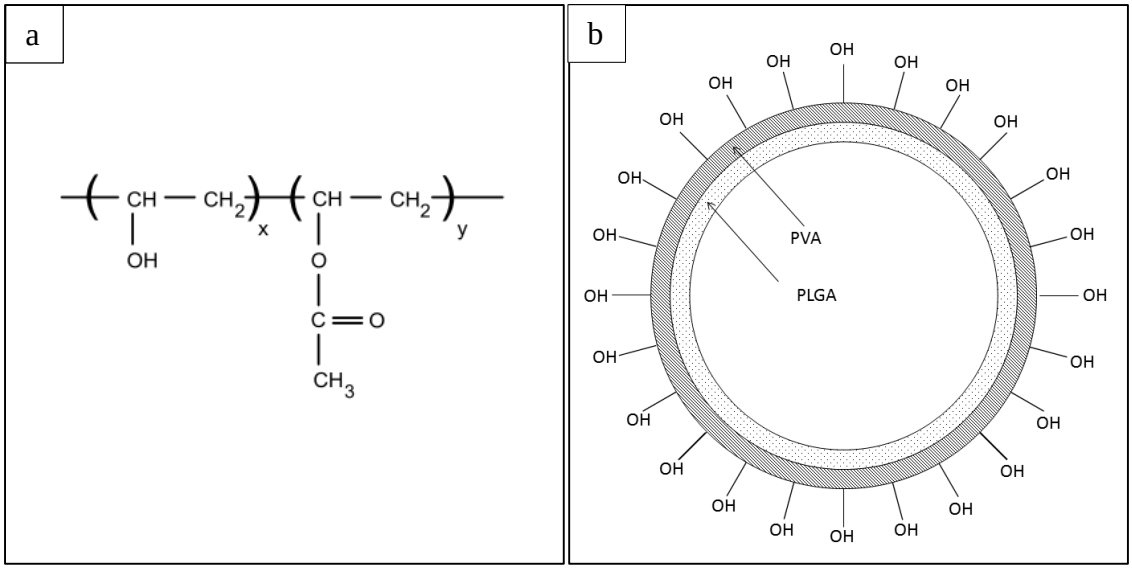

Figure 6. Role of PVA in nanoparticle preparation. Structure of PVA molecule (a), PVA stabilizes the nanoparticle emulsion and hydroxyl groups on nanoparticle surface are fromed (b).

Preparation of Nanoparticles: PLGA nanoparticles containing paclitaxel were prepared using a solvent evaporation method. Briefly, 100 mg PLGA was dissolved in 4 ml dichloromethane solution (DCM). Paclitaxel (5 mg) was added into 1 ml DCM. This step is skipped for placebo nanoparticles. And 20 ml 4% (w/v) water solution of PVA poured into vial of DCM solu-tion with PLGA and paclitaxel, vortexed 10 seconds and the mixture was sonicated on ice for 2 minute with pauses every 45 seconds. The mixture was then poured into a 100 ml glass beaker containing a 10 ml of 4% (w/v) water solution of PVA and 30 ml of Milli-Q water and sonicated for 4 min with 20-30 second pauses every 1 minute or so. And it has been transferred to 250 ml beaker and homogenized with IKA T25 homogenizer for 20 minutes and then it was moved to a magnetic stirrer and stirred for 4 hours to let the DCM evaporated. After stirring, the resulting PLGA dispersion was transferred to ultracentrifuge tubes (Beckman) and centrifuged at 1000 g for 10 minutes to separate the aggregated nanoparticles. The resulting supernatant was transferred to ultracentrifuge tubes (Beckmann Instruments, Fullerton, CA) and further centrifuged at 3000g for 10 minutes. The sediment obtained was resuspended in 50 ml millipore water with the aid of a sonicator and centrifuged at 15000 g for 15 min. This process (i.e., resuspension of sediment and centrifugation) was repeated twice to wash out the PVA, which is not allowed for use as an intravenous excipient. The sediment was centrifuged at 100000 g (29400 RPM) for 45 minutes for the last time to collect nanoparticles. The resulting dispersion was then kept at -80 °C overnight and lyophilized for 48 hours. The nanoparticles obtained were weighted stored in Eppendorf tubes at -20 °C for further experiments [47].

Protocol steps to prepare PLGA nanoparticles via w/o/w technique are as follows:

1. Prepare PVA solution;

i. Sprinkle 4 g of PVA slowly over 100 mL of Millipore water while stirring on a magnetic stirrer. This gives you 4% PVA solution.

ii. It takes 30 min to dissolve the PVA.

iii. Filter through a sterile 0.22 µm filter to remove any undissolved PVA from the solution.

2. Prepare PLGA solution;

- Dissolve 100 mg of PLGA 50:50 in 4 mL of DCM in glass vial with magnetic stirring.

3. Organic Solution;

- Mix 5 mg of paclitaxel (PTX) with 1 ml of DCM. (Skip this step for placebo nanoparticles.)
- Magnetic stir until it is fully dissolved (~30 minutes).
- And make it 5 ml DCM by adding 4 ml into each vial right before sonicating. This makes organic solution 20 mg/ml.

4. Nano-encapsulation;

- Add 20 ml 4% PVA into 5 ml PLGA/PTX solution in 40 ml vial, (This makes it ¼ Organic/PVA).
- Sonicate the emulsification as soon as PVA added as follows:

i. Clean and rinse the sonicator probe with isopropyl alcohol.

ii. Using a microtip probe sonicator (1/8") sonicate the emulsion for 2 min over an ice bath. During sonication, place the probe approximately in the center of the emulsion and avoid contact with the wall of the tube.

5. Add 30 ml water, 10 ml 4% PVA in 100 ml beaker.
    - Sonicate with 3/16" tip probe this solution at 85% amplitude, for 4 minutes pausing for 20 sec at 1.5-2 min intervals.
6. And disperse at the highest level for 20 min.
7. Stir the resulting emulsion overnight (18 hours) in the same tube at room temperature inside a chemical hood with gentle magnetic stirring to allow the chloroform to evaporate.
8. Ultra centrifugation;
    - Centrifuge at 1000 g for 10 minutes to separate the aggregated nanoparticles
    - The resulting supernatant was transferred to ultracentrifuge tubes and further centrifuged at 3000 g for 10 minutes. This will separate particles at the size of 1-3 µm.
    - The sediment obtained was resuspended in 50 ml Millipore water with the aid of a sonicator and centrifuged at 15000 g for 15 minutes.
    - This process (i.e., resuspension of sediment and centrifugation) was repeated twice to wash out PVA, which is not allowed for use as an intravenous excipient.
    - Recover nanoparticles by ultracentrifugation at 100,000 g for 45 minutes at 4 °C. Remove and collect the supernatant.
9. Resuspend the pellet in MPQW and vortex the suspension for 30 seconds over an ice bath.

10. Repeat Steps 8 and 9 to remove unencapsulated anticancer therapeutics and excessive PVA.
11. Keep at -80 °C overnight and lyophilize for 48 hours.
12. The nanoparticles obtained were weighted stored in Ependorf tubes at -20 °C for further experiments.

2.1.1.1 Encapsulating fluorescent dyes into nanoparticles. One of the major limitations of optical imaging using antibody conjugated to fluorescent dyes is the limited ability to detect the signal coming from few targeted molecules. It has been shown that the autofluorescence in the pleural tissues (diseased and normal tissues [9]) falls in the red to orange region of the electromagnetic spectrum, thus we would add coumarin-6 to the organic solution for fluorescent particles that is needed for cellular uptake experiment and visual imaging.

Briefly, 1 mg of coumarin-6 was mixed into nanoparticles to PLGA solution to obtain fluorescent nanoparticles. Same nanoparticle preparation protocol in section 2.1.1 is followed with modification at step 3, adding coumarin-6.

2.1.2 Preparation of Surface Modified Nanoparticles for Targeted Anti-Cancer Treatment

2.1.2.1 Labeling Ephrin A1 Ligand/YSA Peptide with FITC. The Ephrin A1 ligand and YSA peptide were labeled with FITC dye before conjugating them onto nanoparticles so that we can confirm the successful conjugation under optical microscope afterward.

We used commercial FluoReporter fluorescent labeling kit and followed manufacturer's protocol with slight modifications. The said protocol steps are as follows:

1. Prepare 1 M Bicarbonate solution of 10 ml at pH 9.

2. Prepare 100 µl of protein solution. Make it 1 mg/ml, 100 µg YSA/100 µl PBS.
3. Put 100 µl of protein solution into reaction tubes.
4. Add 20 µl of 1 M sodium bicarbonate solution to the tube.
5. Prepare 10 mg/ml dye stock solution by adding 37 µl of DMSO to the 370 µg of reactive dye. Pipet it up and down to dissolve it.
6. Calculate the appropriate amount of dye to be used by;

   For YSA peptide (MW: 1348.5);

   (1 mg/ml protein) x (0.1 ml protein solution) x (9389 MW of FITC) x 100 x (25 MR)/ (1348.5 MW of YSA peptide) = 72.1 µl of reactive dye to be used.

   For the Ephrin A1 ligand (MW: 46000);

   Calculate the appropriate amount of dye to be used by;

   (1 mg/ml protein) x (0.1 ml protein solution) x (389 MW of FITC) x 100 x (40 MR) / (46000 MW of YSA peptide) = 3.38 µl of reactive dye to be used.
7. Add this to the protein solution while stirring.
8. Stir the reaction for 1 hour at the room temperature protected from light.
9. Use HPLC to purify the protein from the dye.

   Purification:

1. Prepare a spin column; Remove the top, add the resin until full, after the resin is settled, remove the bottom to allow the column buffer to drain and discard the flowthrough.
2. Place the spin column in 2 ml collection tubes. Centrifuge for 3 min at 1100 g. Discard the buffer but keep the collection tube.

Inspect the labeling reaction, if any precipitate formed, centrifuge it for 5minutes in a microcentrifuge.

3. Load the sample to the center of spin column dropwise. Allow the solution to absorb into the gel.
4. Place the spin column into the collection tube and centrifuge for 5 minutes at 1100 g.

   The labeled proteins will be in the collection tube.

   Use HPLC to purify the YSA peptide since it has a very low MW.

2.1.2.2 Purification of fluorescent labeled targeting agents from fluorescent residues. For HPLC analysis, a reverse-phase Spirit Peptide C18 column (22.4 mm i.d., pore size 5 µm) was used. The column temperature was maintained at 40 °C. The mobile phase, a mixture of acetonitrile: 2 mM phosphoric acid (50:50, v/v), was delivered at a flow rate of 1.2 ml/min. 20 µl of samples is injected [48].

An HPLC experiment was done to purify FITC labeled the Ephrin A1 ligand and YSA peptide by separating FITC dye molecules from them. We ran a control sample of only the YSA peptide and its peak appeared at 19.8 retention time. In Figure 7, the first peak shows the YSA peptide with no dye. The rest of the peaks show the YSA peptide with a different amount of FITC molecule attached. The latter peaks having more molecular weight are due to more FITC molecule being attached to amino groups in the YSA peptide. HPLC analysis confirms the purification of the YSA peptide from non-labeled FITC dye and further confirms the successful labeling of FITC dye on the YSA peptide (Figure 7).

The molecular weight (MW) of the YSA peptide and FITC are 1348.5 g/mol and 389.4 g/mol respectively. Since MW of FITC 3.5 times less than MW of the YSA, it is supposed to appear before the YSA peptide. Since no peaks appeared before the YSA peak at 19.8 minutes, this means no free FITC is available and we successfully purified the YSA peptide from free FITC residues. This would mean we would not get fluorescent reading of free FITC but only YSA in the proceeding procedure.

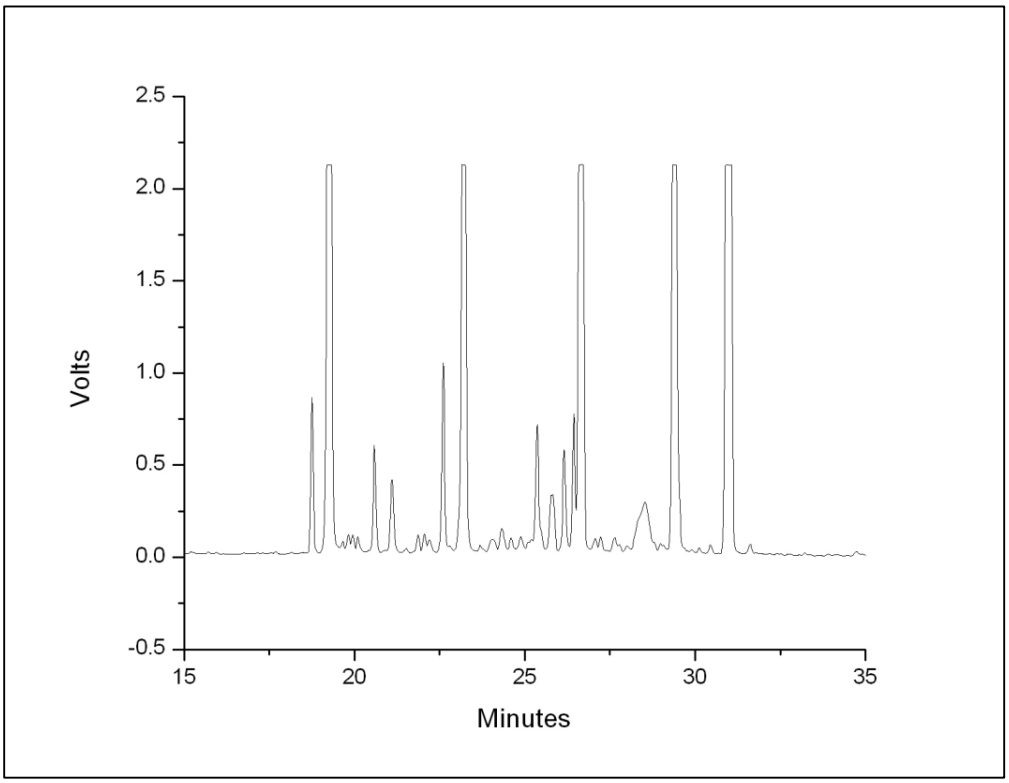

Figure 7. HPLC spectrum of purification of FITC labeled YSA peptide from free-excessive FITC dye

2.1.3 Conjugation of BSA, YSA, and Ephrin A1 onto Nanoparticle Surfaces

Conjugation of the Ephrin A1 ligand and YSA peptide onto the surface of the PLGA nanoparticles was done using the well-established carbodiimide chemistry [47].

Carbodiimide method: Carboxyl (COOH) nanoparticles can be used for covalent coupling of proteins by activating the carboxyl groups with the water-soluble carbodiimide (Figure 8). The carbodiimide reacts with the carboxyl groups to create an active ester that is reactive toward primary amines on the protein of interest [47]

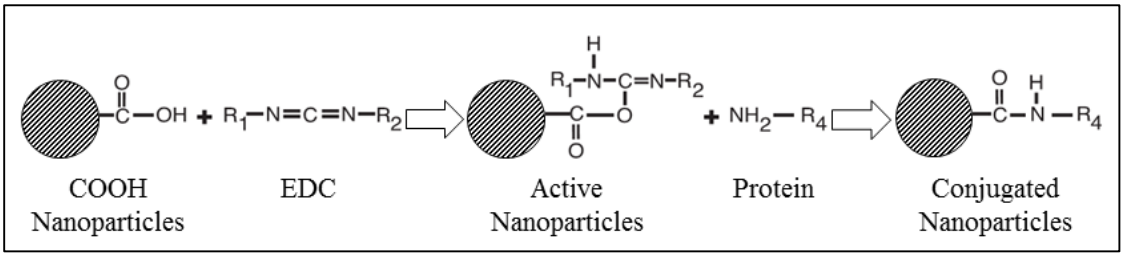

Figure 8. Conjugation process of peptides with nanoparticles

Methodology: The protocol for conjugation process below is slightly modified version of a manufacturer's protocol of Polysciences Technical (Data Sheet 238C) [46].

Protocol steps to conjugate the Ephrin A1 ligand and YSA peptide to the PLGA nanoparticles using the well-established carbodiimide chemistry are as follows:

Preparations:

Carbonate buffer; 0.1 M pH: 9.6

Dissolve 0.084 g of $NaHCO_3$ in 9 ml of DI water. Adjust pH to 9.6 by adding NaOH via pipette. Fill it up to 10 ml once pH is adjusted.

MES Buffer; 0.1 M, pH: 6

Dissolve 19.2g of MES free acid (MW 195.2) in 900ml of pure water. Titrate to desired pH (5.2-6.0) with 1N NaOH. Make up volume to 1,000 ml with pure water

Borate Buffer; 0.2 M pH: 8.5

Ethanolamine; 0.25 M

Add 20 µl of ethanolamine to 1.3 ml of borate buffer.

Carbodiimide; 2%

Add 20 mg of EDC in 1 ml MES buffer. Use in 15 min of preparation.

Procedure:

1. Nanoparticles are washed twice with 0.1 M of Carbonate buffer, pH=9
    a. 12.5 mg of nanoparticles is placed in 1.5 ml centrifuge tubes and fill it half way with carbonate buffer
    b. Vortex and fill it up with carbonate buffer
    c. Centrifuge at 20000 g for 20 min
    d. Remove supernatant with a pipette and discard
    e. Repeat these steps
2. Nanoparticles are washed three times with 0.1 M MES Buffer.
    a. Resuspend pellet to one half the tube volume in 0.1 M MES Buffer.
    b. Vortex and fill it up with MES buffer
    c. Centrifuge at 20000 g for 20 min.
    d. Remove supernatant using a pipette and discard.
    e. Repeat steps two times.
3. Carbodiimide
    a. Resuspend nanoparticles in 0.625 ml of 0.1 M MES Buffer
    b. Add 0.626 ml of 2% EDC in MES Buffer
    c. Incubate for 3 hours at room temperature on an end-to-end shaker.

    This step activates the carboxylic acid groups on the nanoparticle surface.
4. After incubation, nanoparticles are washed 3 times with MES Buffer

a. Centrifuge for 20 minutes at 20000 g, remove and discard the supernatant.
b. Resuspend pellet to one half the tube volume in 0.1 M MES Buffer.
c. Vortex and fill it up with MES buffer
d. Centrifuge at 20000 g for 20 min.
e. Remove supernatant using a pipette and discard.
f. Repeat steps two times.

   This is to get rid of the unreacted carbodiimide.

5. Add peptide
   a. Resuspend nanoparticles in 1.2 ml of 0.2 M borate buffer, pH=8.5.
   b. Add 200 µg of peptide to couple (100 µl of 2mg/ml FITC-BSA solution)
   c. Incubate overnight at room temperature on an end-to-end shaker.
6. After coupling,
   a. Centrifuge at 20000 g for 20 minutes and remove the supernatant and keep it. Record the volume
   b. Resuspend in 1.2 ml of borate buffer
   c. Add 50 µl of 0.25 M Ethanolamine
   d. Incubate for 30 minutes at room temperature.

      This is to block unreacted sites on the nanoparticle surfaces.
7. Add BSA
   a. Centrifuge at 20000g for 20 min. Remove and discard supernatant.
   b. Resuspend pellet in 1ml of 10mg/ml BSA solution in 0.2 M Borate Buffer. Cap and vortex.
   c. Mix gently for 30 minutes at room temperature and Repeat steps a-c.

This step will block any remaining non-specific protein binding sites.

8. Lyophilize;
    a. Centrifuge for 20 min at 20000 g, remove and discard supernatant.
    b. Nanoparticles are washed twice with Milli-Q water twice
    c. Resuspended in 4 ml MQW water and lyophilized

The fluorescent labeled YSA peptide was conjugated onto PLGA particles and optical image was taken to confirm the successful conjugation visually.

2.2 Physicochemical Characteristics of Placebo Nanoparticles (NP)

2.2.1 Scanning Electron Microscopy (SEM) and Optical Microscopy

SEM images were acquired with a Quanta FEG 650 Scanning Electron Microscope under high-vacuum. Prior to imaging, the samples were prepared with an Au-Pd sputter coating to promote surface conductivity and reduce charging artifacts. The beam causes deterioration of the samples. Thus, a low accelerating voltage 10kV or 5kV was used for imaging.

Loading of comarin-6 (fluorescent) on placebo nanoparticles with no surface conjugation or paclitaxel content was confirmed using an optical microscope. Briefly, lyophilized nanoparticles diluted with deionized water to an appropriate concentration and pipetted onto a microscope slide, then droplets were carefully spread onto slide with the tip of the pipette and let dry.

2.2.2 Size and Surface Charge Measurements of Nanoparticles

The mean particle diameter (Z-average), polydispersity index (PDI), and size distributions of nanoparticles were determined by dynamic light scattering (DLS). Lyophilized nanoparticles were re-dispersed in deionized Millipore water (0.2mg/ml) by a short period of sonication (<1 min). The measurements were performed on samples (n=3) in deionized Millipore water at 25 °C using a DynaPro DLS (Wyatt Technology), equipped with a 633 nm laser and 173° detection optics. Dynamics software (v.6.10) by Wyatt Technology was used for data acquisition and analysis.

Surface charge measurements of nanoparticles were done using Zetasizer Nano (Malvern). Nanoparticle suspension in water was prepared and 1 mL of it was pipetted into a plastic four-sided cuvette. The intensity of laser light (620 nm) is back-scattered at 172°. For surface charge measurements, electrophoretic mobility of the nanoparticles in aqueous suspension was measured. Zeta-potential values were obtained by inputting the dispersant viscosity and applying the Smolu-chowski equation. The surface-charge value of nanoparticles was obtained by averaging measurements of 40 sub-runs.

2.2.3 Determination of the Degree of Conjugation

Since Ephrin A1 ligand/YSA peptide contain amino groups, bicinchoninic acid (BCA) protein assay was used to determine their amount. 200 µg of each of the BSA protein, YSA peptide and Ephrin A1 ligand was used for conjugation in the final volume of 1.2 borate buffer. This makes a final concentration of proteins 16.7 µg/ml.

BCA protein assay is a detergent-compatible formulation. It is based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of the total protein. This method combines the well-known reduction of Cu+2 to Cu+1 by protein in an alkaline

medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu+1) using a unique reagent containing bicinchoninic acid [49].

Supernatant after carbodiimide will be collected to determine the concentration of non-conjugated the Ephrin A1/YSA peptide. This amount will be subtracted from the initial peptide/ligand amount used to determine the amount of ligand/peptide conjugated. Pierce® BCA Protein Assay Reagent Kit Protocol is used as follows:

Protocol for BCA Protein Assay:

Materials and Reagents;

- Bovine serum albumin (BSA) (Sigma)
- BCA protein assay reagents (Pierce, catalog number: 23235)
- BCA working reagent (WR)

Equipment;

Plate reader (Biotek)

Procedure;

- Prepare bovine serum albumin (BSA) standards. Prepare 1 ml of BSA stock 2 mg/ml dissolved in H_2O) and then make serial (5-8) dilutions with a range of 30-1000μg /ml.30-1000
- Prepare BCA working reagent (WR). Calculate the total volume of WR needed. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50: 1, Reagent A: B) (the mixture appears to be clear and green solution).
- For microplate measurement, 200 μl of WR reagent is required. Sample to WR ratio is 1:20

- Pipette 25 µl of each standard or protein sample replicate into a microplate well. Protein sample preparation buffer (Borate buffer) is used as blank solutions for the standard curve and protein samples, respectively.
- Add 200µl of the WR to each well.
- Cover and incubate tubes at 37 °C for 30 min.
- Keep all tubes at room temperature for 10 min before measurement.
- Take absorbance readings at 562 nm on a plate reader

The plate reading values of each standards (x axis) are compared against their corresponding concentration (y axis) via linear graph. Obtained the linear equation y=mx+b is used to calculate corresponding amount of the YSA peptide and Ephrin A1 ligand in the supernatant. This result is divided by the initial protein amount used 16.7 µg/ml. This is the percentage of the protein in supernatant against the initial amount used.

2.2.4 Determination of the Paclitaxel Content in Nanoparticles

Paclitaxel concentration in nanoparticles was measured by nuclear magnetic resonance technique (NMR). NMR samples were prepared in d2-dichloromethane (d2-DCM). Each sample contained a known mass of the salicylic acid. The 1D 1H NMR experiments were performed at 298 K on a Bruker Avance 700 equipped with a cryoprobe. Each FID consisted of 256 scans which were collected with a 10 second recycle time. Internal TMS was used as a shift reference.

The drug loading efficiency will be determined in triplicate by NMR. The encapsulation efficiency will be defined by the ratio of measured and initial amount of PTX encapsulated in nanoparticles [48, 50].

2.2.5 Determination of the Amount of the Drug Released

Paclitaxel loaded nanoparticles (20 mg) were diluted with 20 mL of phosphate-buffered saline (PBS, pH 7.4, 0.01 M) in a 50 ml falcon tube. 9 of 1.5 ml of the nanoparticle solution were allocated into 4 different 2 ml Eppendorf tubes for each time points (6hrs to day 8) and gently shaken at 120 rpm in an orbital shaker in a water bath (37 °C). At specified time points, the tube was taken out of the shaker and centrifuged at 20000g for 20 minutes. 1 ml of supernatant was taken, lyophilized and kept at -80 °C. The amount of paclitaxel in supernatants was determined by NMR as described before.

2.3 *in vitro* Studies

2.3.1 Cell Culture

CRL-2081 was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in T75 flasks in RPMI 1640 (Life Technologies) containing 10% fetal bovine serum and 1% of 100X Antibiotic-Antimycotic (Gibco®). In brief, cells were incubated at 37 °C in 5% CO2 and 95% air and were cultured to 80% confluence for 3–4 days [51].

2.3.2 Cellular Uptake of Nanoparticles

To quantify the uptake of Coumarin-6 EPHNP, YSA NP and placebo nanoparticles by the CRL-2081 cell line, cells were seeded onto 24-well plates at a density of 1.0×10^5 cells/well. Confluent cells were incubated for 5 hours at 37 °C with 200 µl of the respective nanoparticle dispersion (250, 125, 62.5 and 31.25 µg/ml in media). Uptake was terminated by washing the cell monolayers twice with PBS and solubilizing the cells

with 200 µl of 5% SDS in 0.1 M NaOH. Cell-associated nanoparticles were quantified by analyzing the fluorescence intensity of the cell lysates in the plate reader [52].

Cell Lysis:

1. Make a 10% Triton X100 stock solution in water and dilute to 0.5% to make lysis buffer.

2. Spin cells to be lysed down in 15 or 50 mL centrifuge tubes. Aspirate off media, leaving around 1 mL. Transfer that 1 mL to a microcentrifuge tube and spin the cells to pellet (spin for 5-10 seconds at max speed in the microcentrifuge).

3. Resuspend cells in lysis buffer. Usually for cell lines, add around 20 µl of lysis buffer per million cells.

4. Let cells sit in lysis buffer on ice for 10-15 minutes to ensure complete lysis.

6. Spin cells at max speed for 10 minutes at 2-4 °C to pellet nuclei.

7. Harvest supernatants into fresh tubes.

2.3.3 Visualization of Cell-Associated Nanoparticles

CRL-2081 cell lines were cultured on 12-well tissue culture slides in their supplemented RPMI-1640 media as described. Cells were plated at 10^5 cells/well and incubated with fluorescent nanoparticles at 1 mg/ml for 4 hours *in vitro* and then cells were collected and centrifuged at 850 rpm for 5minutes and reconstituted in 200 µl of media. Cells were cytospun onto glass slides at 850 rpm for 4 minutes. Spun cells were fixed with 3.7% formaldehyde at 37 °C for 15 minutes. Cells were rinsed with PBS three times and incubated with DAPI at 300 ng/ml for 30 minutes at room temperature. Following

mounting, cells were imaged on an optical microscope (Carl Zeiss inc., Thornwood, NY). Images were taken without software enhancement [53].

Cytospin Protocol:

Protocol is followed per Thermo Shandon, Cytospin. Manufacturer's manual:

1. Wash 10^5 cells in cold 2% FCS-PBS twice and dilute in 100 µl of cold 1% BSA-PBS. Be sure to keep all samples on ice.

2. Place slides and filters into appropriate slots in the cytospin with the cardboard filters facing the center of the cytospin. In the event that there are few cells available, aliquot about 100 µl of cold 1% BSA-PBS into each of the wells and spin for 1-2 minutes. This will serve to wet the filter and allow more cells to reach the slide. Also, be sure that each filter and slide pair is flush with each other and that the hole in the filter is in proper position so that cells will be able to reach the slide.

3. Quickly aliquot 100 µl of each sample into the appropriate wells of the cytospin. Be careful not to confuse the slides so that the samples are not aliquoted into the wrong wells.

4. Carefully place the lid of the cytospin over the samples and spin at maximum speed for 1-3 minutes.

5. Remove the filters from their slides without contacting the smears on the slides.

6. Examine each slide under the microscope to be sure that the cells have annealed properly. The cells should appear to have normal morphology and should be lying flat on the slide. For staining purposes, the cells should also be in a flat layer on the slide.

7. Dry the slides in a desiccation chamber overnight.

2.3.4 Anti-Proliferative Activity of Designed Nanoparticle Formulation

Cells were seeded in 96-well plates at the density of 8000 cells per well and incubated 24 hours to allow cell attachment. The cells were then incubated, YSA NP, EPH NP, YSA-PTX NP, and EPH-PTX NP at nanoparticle concentrations of 1.48, 0.3, and 07 µg/ml 24, 48, and 72 hours. At the determined times, the formulations were replaced with media containing MTT (5mg/ml) and cells were then incubated for additional 4 hours. MTT was aspirated off and 100 µl of DMSO was added into each well to dissolve the formazan crystals and cells were incubated for 15 minutes. Absorbance was measured at 570 nm using a BioRad microplate reader. Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance [54].

2.3.5 Determination of the Half Maximal Inhibitory Concentration (IC50) IC50

The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function [55]. Cytotoxicity of free paclitaxel drug was measured at ranging concentrations (nM) in 24, 48, and 72 hours via MTT assay. Concentration of paclitaxel that kills 50% of the cells (IC50) is calculated with the use of the trendline equations the IC50 graphs. In logarithmical trend line equations, y is set to 50 to calculate the x value which is the concentration that gives 50% cytotoxicity (IC50 in a certain time period).

2.4 Statistical Analyses

Data are presented as mean ± standard deviation, and analyzed by one-way ANOVA with the Tukey's test applied post hoc for paired comparisons of means (SPSS

10, SPSS Inc. Chicago, IL, USA). Values of $p<0.05$ were indicative of significant differences, and $p<0.01$were indicative of a very significant difference [41].

CHAPTER 3

RESULTS

In order to synthesize nanoparticles for targeting and anti-cancer abilities, studies were done in three main sections.

1. Characterization and *in vitro* studies of placebo nanoparticles (NP)

2. Characterization of paclitaxel loaded nanoparticles (PTX NP) and *in vitro* studies in comparison with free paclitaxel (PTX)

3. Characterization and *in vitro* studies of designed multipurpose nanoparticles for targeted delivery of anticancer therapeutics for Malignant Mesothelioma (EPH NP, EPH-PTX NP, YSA NP, YSA-PTX NP).

Experiments in this section were done in two main sections;

a. Characterization,

b. And *in vitro* experiments of nanoparticles

3.1 Characterization and *in vitro* Studies of Placebo Nanoparticles (NP)

3.1.1 Physicochemical Characteristics of Placebo Nanoparticles (NP)

The double-emulsion technique was successfully used with some modifications to prepare mono-dispersed nanoparticles. SEM image in Figure 9 shows nicely formed spherical nanoparticles obtained without any defects on the surface.

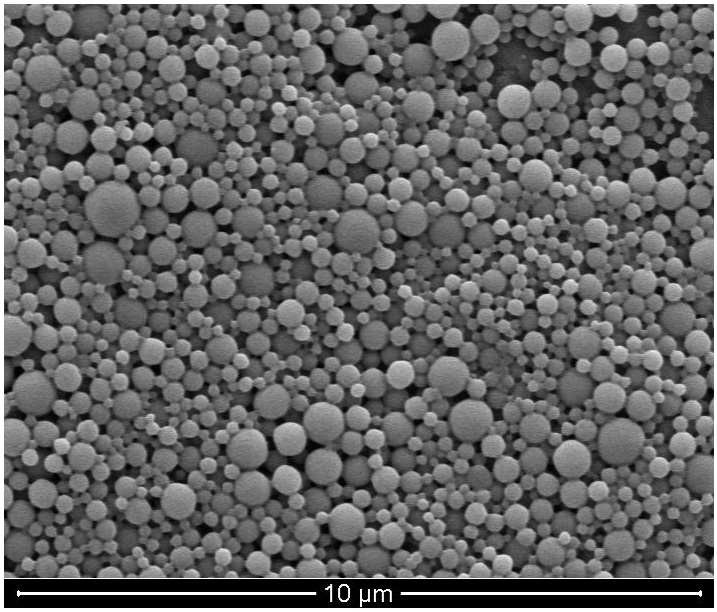

Figure 9. SEM image of placebo nanoparticles

It was found that the mean diameter of the PLGA nanoparticle was 254 nm with polydispersity of 0.14 (Table 2).

Table 2. Physical characteristics of placebo nanoparticles (NP). Size was measured by DLS. Surface charge was determined by a nanosizer.

Formulation[1]	Size (nm)	PD[2]	Zeta Potential (mv)
NP	253.6±3.4	0.14	-29.1±4.7

[1. NP: Placebo nanoparticles with no surface modification or paclitaxel. 2. PD: Poly-dispersity]

A DLS analysis of nanoparticles shows the particle size distribution (Figure 10a). The particle size distribution is relatively narrow which is considered as an ideal characteristic for the nanoparticle formulation for anticancer therapeutics, as demonstrated in the size distribution diagram. Prepared PLGA particles were within an acceptable PD

(<0.2) and were in the nanometer range. Surface charge of placebo nanoparticles (NP) was measured with a nanosizer and came out to be -29 mV (Table 2) (Figure 10b).

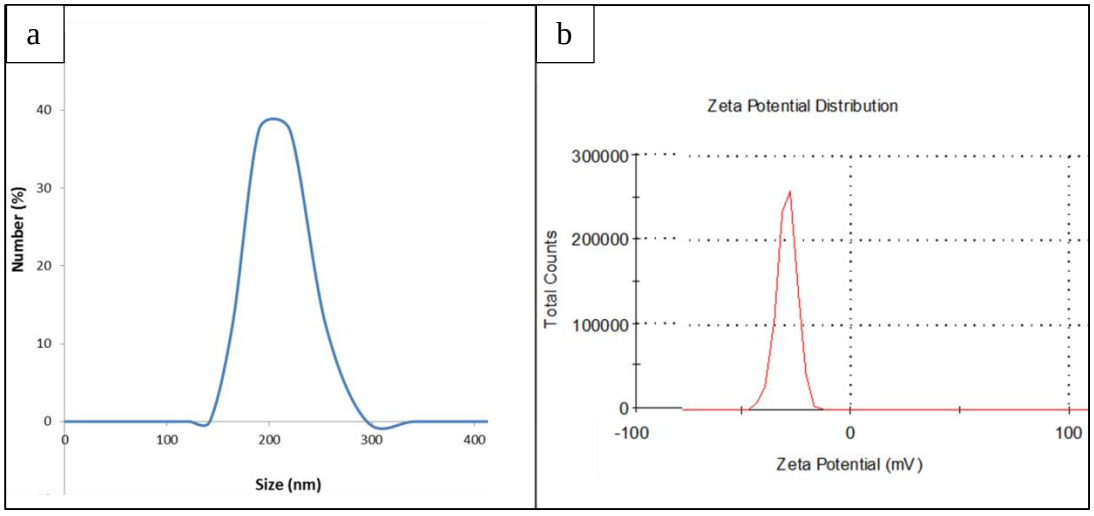

Figure 10. DLS Size distribution (a) and surface charge of placebo nanoparticles (NP) (b)

Monodispersed nanoparticles were successfully prepared that encapsulates fluorescent dye as it was discussed in section 2.1.1. The encapsulation of FITC in PLGA nanoparticles was determined and confirmed using optical imaging in Figure 11.

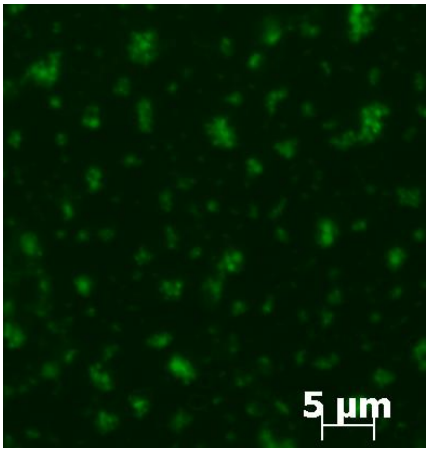

Figure 11. Placebo nanoparticles (NP) loaded with FITC dye is observed under optical microscope

3.1.2 in vitro Studies of Placebo Nanoparticles (NP)

3.1.2.1 Cellular uptake. Nanoparticles were seeded in cells and uptake readings were taken after 5 and 24 hours of incubation. The uptake experiment was done in concentrations of 32.5 to 250 µg/ml. The percentage of the cellular uptake was calculated by comparing fluorescent nanoparticle concentration uptake to the fluorescent nanoparticle concentration in the well that started with 100% of fluorescent nanoparticles.

It is seen that more than half (62%) of fluorescent nanoparticles were taken within 5 hours at 250 µg/ml.

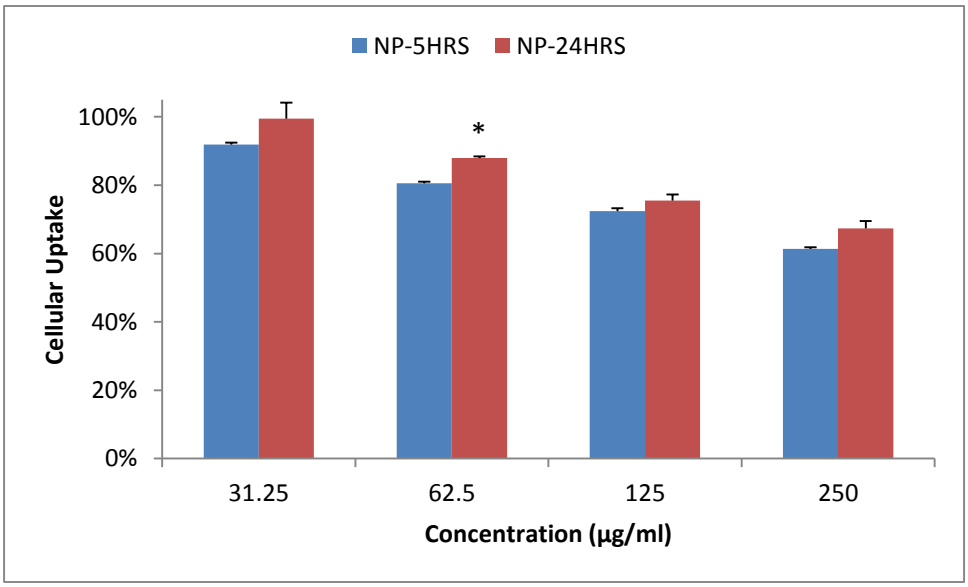

Figure 12. Comparing cellular uptake of placebo nanoparticles in 5 hours (NP-5HRS) and 24 hours (NP-24HRS) at concentrations from 32.5 µg/ml to 250 µg/ml. [*: $p<0.05$; Significance of NP-24HRS to NP-5HRS].

As the concentration decreases in the 5 hour experiment, the uptake increases up to 92% at the 32.5 µg/ml. It is worth to note that the uptake of NP-5HRS drops logarithmically with the R^2 value of 0.9964 as concentration increases (Figure 13a). This means

that the uptake decreases as concentration drops and becomes stable with very little decrease afterwards. This leads to the conclusion that increasing amount of concentration does not help the uptake to increase.

Similarly, the uptake of nanoparticles was also measured after 24 hours of incubation (Figure 13b). The uptake at 250 µg/ml increases up to 67.35% within 24 hours compared to the 61% uptake in 5 hours at 250 µg/ml concentration. We see that the uptake in 24 hours increases up to almost 100% (99.51%) at 32.5 µg/ml of concentration. Please note that the uptake reaches above 90% at 32.5 µg/ml concentration in both 5 hours and 24 hours (Figure 12). It is clearly seen that cellular uptake in 24 hours logarithmically decreases with R^2 value of 0.9929 as the concentration of fluorescent nanoparticles increases. Error bars are negligible in all concentration except the 24 hours uptake at 32.5 5 µg/ml concentration (Figure 12).

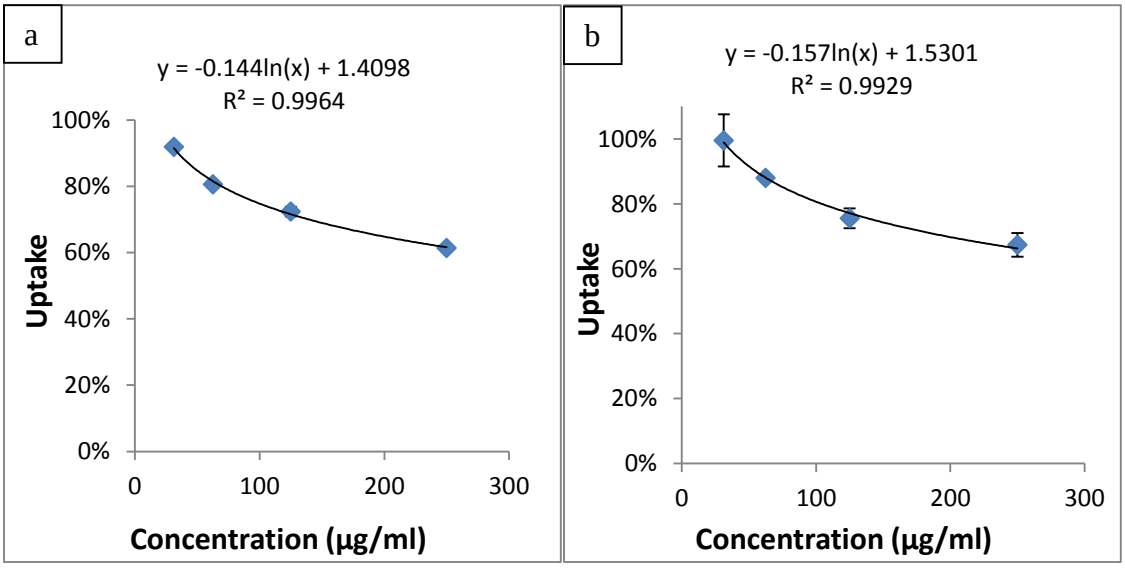

Figure 13. Regression graph with logarithmical trend line of cellular uptake of placebo nanoparticles (NP) over concentration after 5 hours (a) and 24 hours of incubation (b)

Comparison of the bar graph of the uptake of nanoparticles in 5 hours and in 24 hours is shown in Figure 12. We see that the uptake is not much higher at 24 hours in all concentrations compared to it is in 5 hours. We can safely conclude that the ideal uptake is observed at the concentration of 32.5 µg/ml in 5 hours for NP. This could also be interpreted as extending incubation time from 5 hours to 24 hours does not make much difference.

3.1.2.2 Visualization of cell-associated nanoparticles. In Figure 14, Fluorescent image of uptaken fluorescent nanoparticles in the cells is shown where fluorescent nanoparticles (in green) (Figure 14a), cell nucleus (in blue) (Figure 14b), fluorescent nanoparticles and cell nucleus all together (Figure 14c) are shown. We can clearly see that the uptaken fluorescent nanoparticles are surrounding the cell nucleus and accumulating around it. Nanosize of nanoparticles helps them easily seize into cells and arrive at the cell nucleus. After 5 hours, the majority of fluorescent nanoparticles was uptaken and accumulated around the cell nucleus (Figure 14).

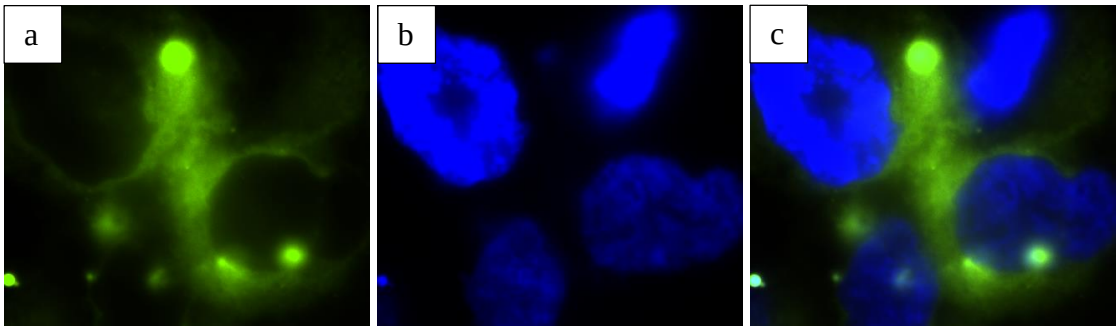

Figure 14. Visualization of cellular uptake of fluorescent nanoparticles via fluorescent microscopy. Fluorescent nanoparticles (Green) uptaken in the cells (a), cell nucleus (Blue) stained with DAPI (b), and combined image of a and b (c).

3.1.2.3 Anti-proliferative activity of placebo nanoparticle formulation (NP). The cytotoxicity of placebo nanoparticles were checked with MTT assay at concentration ranges of 0.075-1.5 µg/ml for 24 hours. Nevertheless we see some cytotoxicity at 0.03 µg/ml, it is negligible considering the variation of viability value in normal cells with 100% viability. So we can say that we did not observe any cytotoxicity at any of the four nanoparticle concentrations we picked (Figure 15).

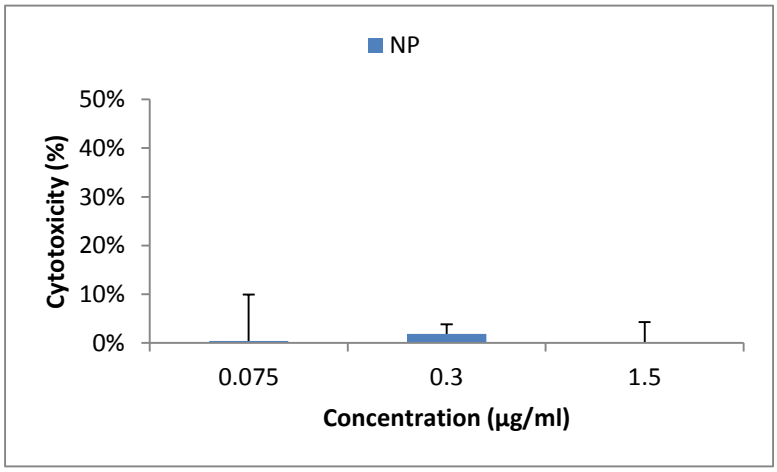

Figure 15. Cytotoxicity of placebo nanoparticles (NP) in 24 hours

3.2 Characterization of Paclitaxel loaded nanoparticles (PTX NP) and *in vitro* studies in comparison with Free Paclitaxel (PTX)

3.2.1 Physicochemical Characteristics of Paclitaxel loaded Nanoparticles (PTX NP)

As it is discussed in section 2.1, using the same technique, we incorporated paclitaxel into nanoparticles by adding the drug content in the polymer solution. As it is seen in SEM image in Figure 16, similar to placebo nanoparticles, we obtained monodispersed nanoparticles without any defects on the surface (Figure 16).

Size of PTX NP was measured with DLS is 268.6 nm with PD value of 0.12. The sizes of placebo nanoparticles and paclitaxel loaded nanoparticles are within the same

size range considering the standard deviation of sizes of the two. The surface charge of PTX NP came out to be -30.1 mV, which is the surface charge similar to the one of placebo nanoparticles (-29.1 mV) (Table 3). We can conclude that the inclusion of paclitaxel into NP nanoparticle formulation does not change the characteristics of nanoparticles.

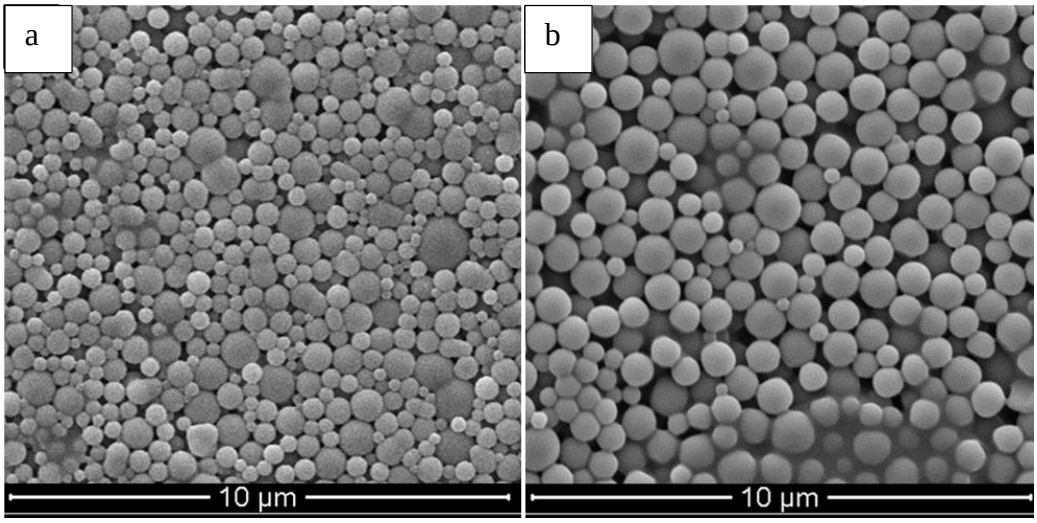

Figure 16. SEM image of placebo nanoparticles (NP) (a) and paclitaxel loaded PTX NP (b)

Table 3. Physical characteristics of NP and PTX NP. Size of nanoparticle formulations was measured by DLS and surface charge was determined by a nanosizer.

Formulation[1]	Size (nm)	PD[2]	Zeta Potential (mv)
NP	253.6±3.4	0.14	-29.1±4.7
PTX NP	268.6±14.4	0.12	-30.1±5.7

[1. NP: Placebo nanoparticles, 2. PTX NP: nanoparticles loaded with paclitaxel. 2. Polydispersity)]

3.2.2 Determination of Paclitaxel Amount in PLGA Nanoparticles

Paclitaxel amount in nanoparticles was determined by the nuclear magnetic resonance (NMR). Paclitaxel (PTX) concentrations were calculated from relative peak areas

of salicylic acid (one proton each) comparing to the peak areas of paclitaxel in the spectrum. Each paclitaxel aromatic peak represents one proton, except for the peaks at 8.2 ppm and 7.8 ppm which are two protons (Figure 17). The mass of paclitaxel would be calculated from the salicylic acid peak at 6.95 PPM as;

$$M_{SA} \times \frac{MW_{PTX}}{MW_{SA}} \times \frac{p \times I_{PTX}}{I_{SA}}$$

(M=Mass, MW: Molecular Weight, I=Integrated area under each peak, PTX: Paclitaxel, and SA: Salicylic Acid. p=Number of protons in the molecule that paclitaxel peaks represent)

Based on the results of NMR analyzes and such calculations, paclitaxel amount was determined. The amount of paclitaxel is calculated from NMR spectrum which is compared against the amount of paclitaxel included into the polymer during the preparation process. This ratio is called the encapsulation efficiency (EE). It is determined that nanoparticles successfully encapsulated 66.2±3.1 % paclitaxel used (Table 4).

The amount of drug recovered compared to the amount of nanoparticles recovered is called the loading efficiency. This ratio gives the amount of paclitaxel in 1 mg of nanoparticles. Corresponding loading efficiency (LE) is 50.0 ±2.3 µg (58.8 ±2.7 µM) paclitaxel in 1 mg of PLGA nanoparticles recovered (Table 4).

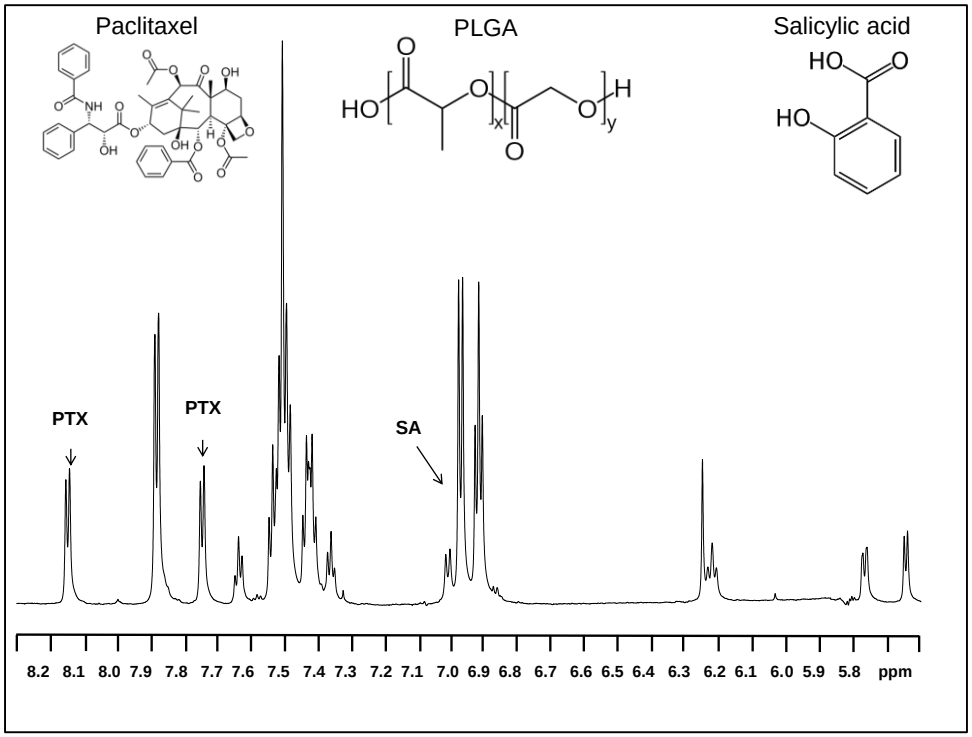

Figure 17. NMR spectrum of paclitaxel loaded nanoparticles (PTX NP). PTX is seen at 7.8 and 8.2 PPM with 2H. Control, salicylic acid shows up at 6.95 PPM. Areas under peaks are calculated and compared against known amount of salicylic acid via equation mentioned above. (PTX: Paclitaxel, SA: Salicylic Acid)

Table 4. Determined amount of paclitaxel content in PTX NP

Formulation[1]	EE (%)[2]	LE (µg drug/mg NP)[3]
PTX NP	66.2±3.1	50.0±2.3

[1. PTX NP: Nanoparticles loaded with paclitaxel. 2. Paclitaxel encapsulation efficiency (EE) (%) =drug recovered in PTX NP/drug fed 3. Paclitaxel loading efficiency (LE): drug recovered in nanoparticles/nanoparticles recovered]

3.2.3 Drug Release Profile of Paclitaxel Loaded Nanoparticles

As it is discussed in section 2.2.4 and in 3.2.2, the amount of the drug released in the supernatant is determined at each time point determined. In the first 6 hours, 14% of the paclitaxel released out of nanoparticles. We see an exponential increase in the release

of nanoparticles in the first 5 days which goes up to 32% (Figure 18). The drug release after day 5 is only 1%.

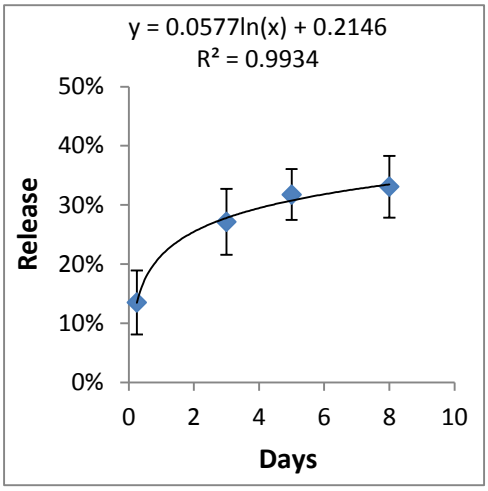

Figure 18. Release profile of paclitaxel loaded nanoparticles (PTX NP) over 8 days. PTX NP samples were kept in PBS and incubated on a shaker. Supernatants at determined time points were collected and amount of PTX released was measured by NMR.

3.2.4 in vitro Studies of Paclitaxel Loaded Nanoparticles (PTX NP) in Comparison with Free Paclitaxel

3.2.4.1 Anti-proliferative activity of free PTX and PTX NP. At concentrations of 3.13, 25, and 200 nM, cytotoxicity levels of free paclitaxel and paclitaxel loaded nanoparticles in CRL2081 cell lines was determined. It is determined by comparing the viability of cells with free PTX or PTX NP respect to the viability of cells without any treatment in percentage over different concentration points. At the end of 24 hours of incubation, cytotoxicity of free PTX ranges between 1% to 32% and it increases as the concentration increases (Figure 19a).

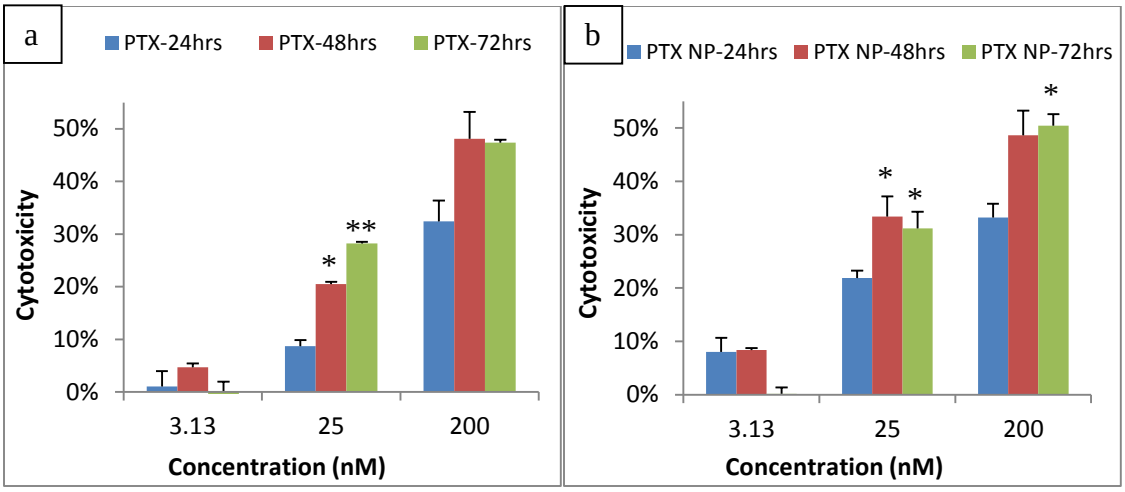

Figure 19. Cytotoxicity in 24 hours, 48 hours, and 72 hours for free PTX (a) and PTX NP (b). (3.4 µg PTX NP contains 200 nM PTX). [*: $p<0.05$, **: $p<0.01$; significance of PTX-24hrs compared to PTX-48hrs or PTX-72hrs (a). *: $p<0.05$, **: ($p<0.01$); significance of PTX NP-24hrs compared to PTX NP-48hrs or PTX NP-72hrs (b).]

When incubation time goes up to 48 hours, cytotoxicity level of free PTX at concentration of 200 nM increases almost up to the IC50 value. Free PTX gets ~50% cytotoxicity at 200 nM in 48 hours which is the highest cytotoxicity value for it, and its cytotoxicity drops down to below 30% at 25 nM in the 24, 48 and 72 hour experiments and the highest cytotoxicity at 25 nM point is observed in 72 hour experiment with a value of 28% highest cytotoxicity in 72 hour for PTXs. Nevertheless, we do not observe a significant change in cytotoxicity levels between 48 and 72 hour cytotoxicity of PTX at 200 nM (Figure 19a). Cytotoxicity of PTX NP stays above 21% at 25 nM concentration point for all three days (Figure 19b).

In all three days, no significant difference of cytotoxicity percentage at 200 nM is observed between free PTX and PTX NP (Figure 20). Moreover, both PTX and PTX NP show no significant cytotoxicity at 3.13 nM PTX concentration in the 72 hour experiment (Figure 20c). Cytotoxicity values of both PTX and PTX NP at 200 nM concentration are

above ~30% in all three days (Figure 20a). At 24 and 48 hours, cytotoxicity of PTX NP shows higher values with $p<0.01$ and $p<0.05$ respectively compared to free paclitaxel at 25 nM (Figure 20a and b). Cytotoxicity value of PTX NP (33%) is 1.5 times the value of free PTX (21%) at 25 nM in 48 hours (Figure 20b). Cytotoxicity levels of free PTX and PTX NP become similar for all three concentration values in 72 hours (Figure 20c).

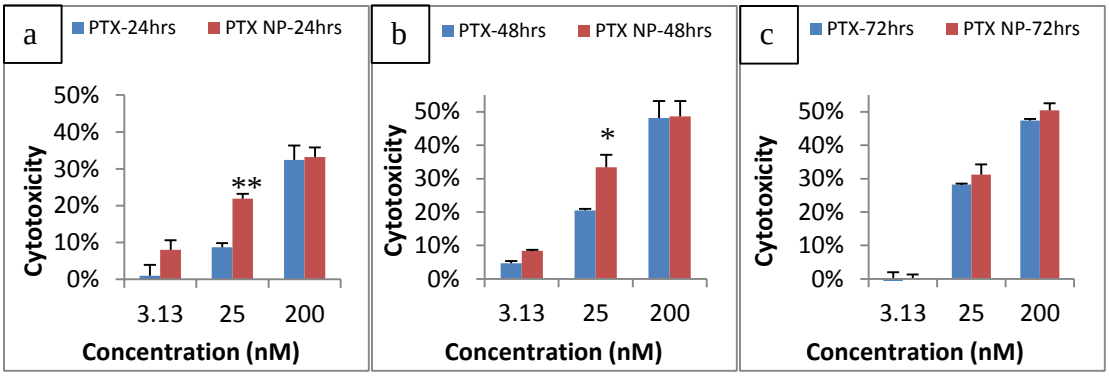

Figure 20. Cytotoxicity of free PTX and PTX NP is compared in 24 hours (a), 48 hours (b), and 72 hours (c). (3.4 µg PTX NP contains 200 nM PTX) [*: $p<0.05$, **: ($p<0.01$); significance of PTX NP against PTX]

3.2.4.2 IC50 Calculations of free PTX and PTX loaded nanoparticles (PTX NP).

The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function [55]. IC50 shows the concentration needed for the 50% of the cells being killed.

Cytotoxicity of free paclitaxel drug was measured at ranging concentrations (nM) and at 24, 48, and 72 hours via MTT assay. Concentration of paclitaxel that kills 50% of the cells (IC50) is calculated using the trendline equations in Figure 21.

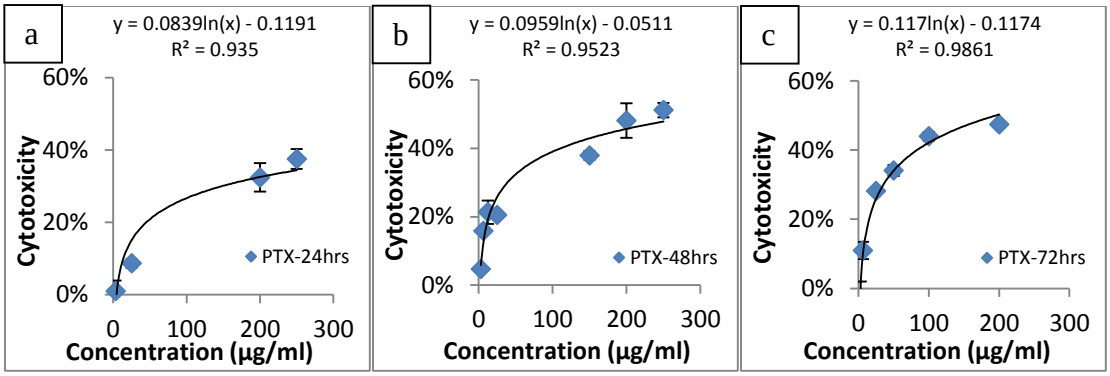

Figure 21. Cytotoxicity graphs of free PTX over concentrations at 24 hours (a), 48 hours (b), and 72 hours (c). [Logarithmical trendlines are added to calculate IC50 values; R^2 is the coefficient of determination that indicates how well trendline fits to graph.]

Table 5. Calculated IC50 values of free PTX in 24, 48, and 72 hour experiments (1. IC50 of free PTX in nM).

Time	PTX (nM)[1]
24HRS	1602.0
48HRS	313.1
72HRS	195.8

In logarithmical trendline equations in Figure 21, y is set to 50 to calculate the x value which is the concentration that gives 50% cytotoxicity (IC50 in a certain time period). By such calculation, using the trendline equation in Figure 21a, the IC50 value in 24 hours came up to be 1602 nM (Table 5).

We are getting more ideal IC50 at 48 hours. Calculating it in the similar way, IC50 in 48 hours came out to be 313 nM (Table 5 and Figure 21b). The reading results of MTT assay after 72 hours give a lower IC50 of 196 nM (Figure 21a, b, and c). Negligible error bars and R^2 values above 0.93 (R^2=1 means perfect fit.) in Figure 21 assure the reliability of our results.

We have measured the IC50 values of paclitaxel loaded nanoparticles (PTX NP) in the similar way. The IC50 value of 24 hour cytotoxicity experiment is above 2000 nM (Table 6). However, IC50 value of PTX NP at 48 hours (366 nM) is similar to IC50 value of free paclitaxel (313 nM) in 24 hours. This corresponds to 6.3 µg/ml of PTX NP (Table 6). Free PTX and PTX NP show their ideal IC50 in 72 hours.

Table 6. Calculated IC50 values of PTX and PTX NP. (1. Corresponding amount of PTX in PTX NP in nM unit. 2. Total weight of nanoparticles including the PTX inside.)

Time	PTX(nM)	PTX NP(nM)[1]	PTX NP (µg)[2]
24HRS	1602.0	2318.3	39.6
48HRS	313.1	366.2	6.3
72HRS	195.8	303.9	5.2

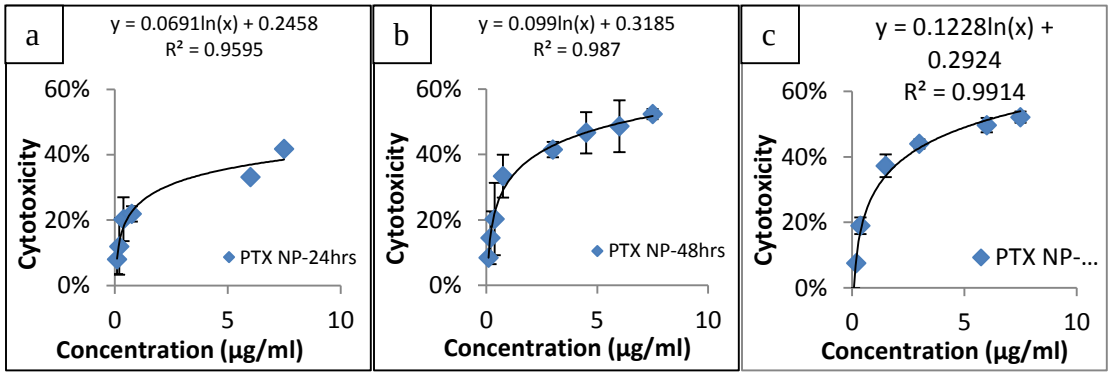

Figure 22. Cytotoxicity graphs of PTX NP over concentrations at 24 hours (a), 48 hours (b), and 72 hours (c). [Logarithmical trendlines are added to calculate IC50 values, R^2 is the coefficient of determination that indicates how well trendline fits to graph.]

The logarithmic trend lines of IC50 graphs of PTX NP in Figure 22 have R^2 values that are above 0.095 in all three graphs. This means that PTX NP has much consistent cytotoxicity values and much reliable IC50 values.

Please see the comparison of IC50 values of PTX and PTX NP at 24, 48, and 72 hours in the Figure 23 below. Free PTX reaches its lowest cytotoxicity value in 72 hours which is 196 nM. Keeping in mind that 1 µg of PTX NP contains 50 µg PTX drug which corresponds to ~60 nM (Figure 23).

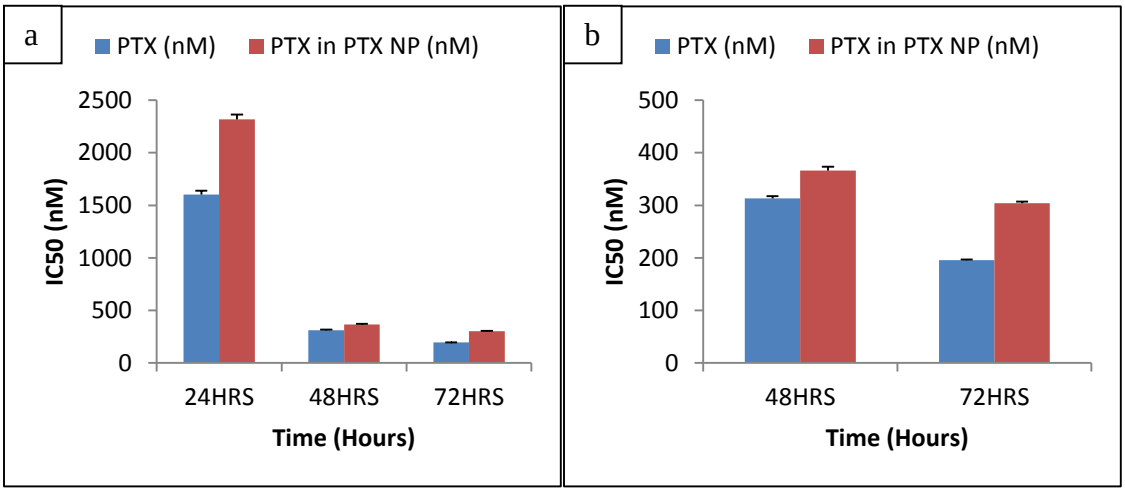

Figure 23. Comparing IC50 values of PTX and PTX NP on each day (a), Rescaled graph for 48 and 72 hours only (b) Characterization and *in vitro* studies of Nanoparticles Surface Conjugated with Targeting Agents (Ephrin A1 ligand and YSA peptide)

3.3 Characterization and *in vitro* studies of Nanoparticles Surface Conjugated with Targeting Agents (Ephrin A1 ligand and YSA peptide)

3.3.1 Physicochemical Characteristics of Surface Conjugated Nanoparticles

The YSA peptide or Ephrin A1 ligand was conjugated onto surfaces of NP and the YSA peptide conjugated nanoparticles (YSA NP) and the Ephrin A1 ligand conjugated nanoparticles (EPH NP) were obtained. Similarly, the YSA peptide or Ephrin A1 ligand was conjugated onto surfaces of PTX NP and the YSA peptide conjugated and paclitaxel loaded nanoparticles (YSA-PTX NP) and the Ephrin A1 ligand conjugated and

paclitaxel loaded nanoparticles (EPH-PTX NP) were obtained. Figure 24 shows nicely formed spherical surface conjugated nanoparticles EPH NP and YSA NP.

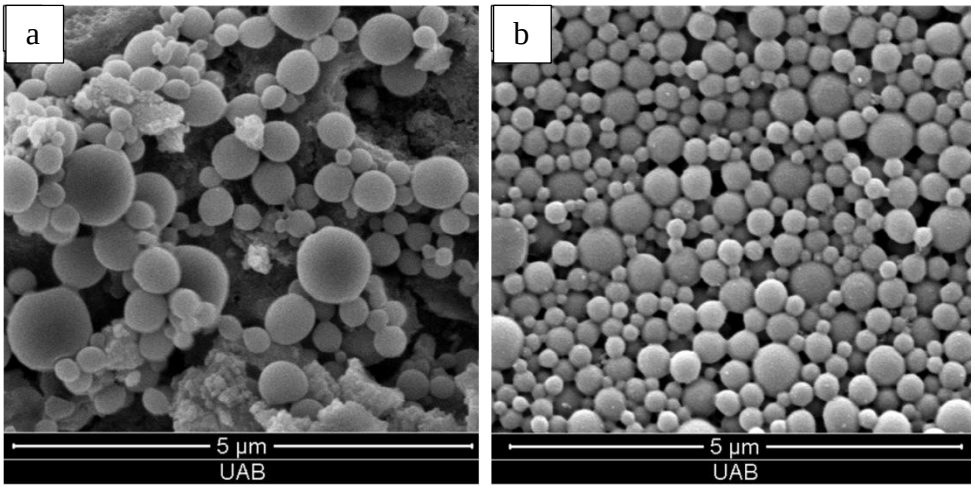

Figure 24. SEM image of EPH NP (a) and YSA NP (b)

The particle size distribution was determined with DLS showed that the mean diameters of conjugated nanoparticle formulations ranges between 187 and 278 nm where EPH-PTX NP is having the lowest and YSA NP is having the highest diameter over all six nanoparticle formulations we have studied (Table 7).

The Ephrin A1 ligand conjugated nanoparticles are having 188 nm (EPH-PTX NP) and 197.2 nm (EPH NP) of sizes whereas the YSA peptide conjugated nanoparticles are 247.7 nm (YSA-PTX NP) and 287.7 nm (YSA NP) (Table 7).

Please note that sizes of only surface conjugated nanoparticles (YSA NP and EPH NP) are higher compared to their corresponding surface conjugated and paclitaxel loaded nanoparticles (YSA-PTX ENP and EPH-PTX NP) and we do not see a significant difference in polydispersity of all.

Table 7. Physical characteristics of Ephrin A1 or YSA conjugated nanoparticles with or without paclitaxel content. Size of nanoparticle formulations was measured by DLS and surface charge was determined by a nanosizer.

Formulation[1]	Size (nm)	PD[2]	Zeta Potential (mv)
YSA NP	278.7±2.2	0.10	-22.0±4.9
EPH NP	197.2±2.6	0.13	-37.8±5.5
YSA-PTX NP	247.7±9.1	0.14	-16.1±4.0
EPH-PTX NP	188.0±5.6	0.14	-40.6±4.8

[1. YSA NP: nanoparticles with YSA conjugation, EPH NP: nanoparticles with Ephrin A1 conjugation, YSA-PTX NP: nanoparticles with YSA conjugation and paclitaxel loaded, and EPH-PTX NP: nanoparticles with Ephrin A1 conjugation and paclitaxel loaded. 2. PD: Poly-dispersity.]

The surface charges of the Ephrin A1 conjugated nanoparticles (EPH NP and EPH-PTX NP) are around -40 mV. It should be noted that the YSA conjugated nanoparticles (YSA NP and YSA-PTX NP) show the lowest surface charge of approximately -20mV (Table 7).

3.3.2 Determination of the Degree of Conjugation

FITC labeled YSA peptide was conjugated onto nanoparticles as it is discussed in section 2.1.2. Optical image of nanoparticles that were conjugated with fluorescent labeled YSA peptide was taken via microscopy to confirm the successful conjugation. Since excessive FITC and the non-conjugated peptide were removed, the fluorescent emission would only indicate the conjugated YSA peptide (Figure 25). To quantify the amount of the protein conjugated, BCA assay was done. Please keep in mind that placebo nanoparticles were conjugated with BSA and they will be used as a control.

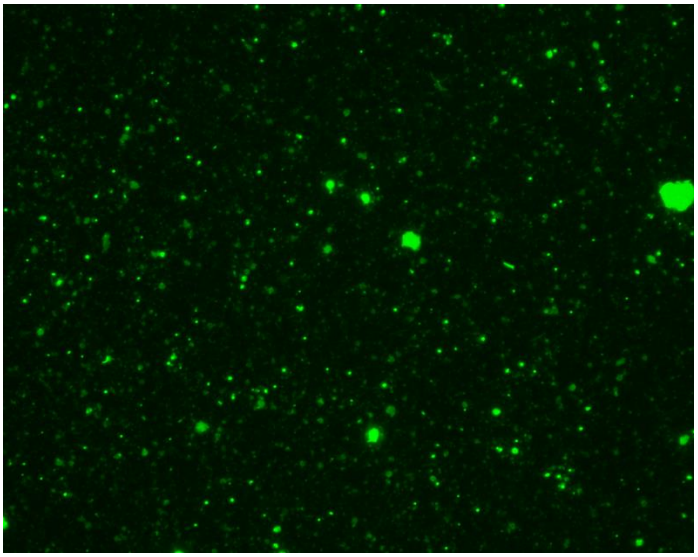

Figure 25. Optical image of fluorescent labeled YSA peptide that is conjugated onto nanoparticles

Table 8. Protein (BSA, Ephrin A1 ligand, or YSA peptide) recovery on the surface of nanoparticles after conjugation. Protein amount was calculated with BCA protein assay.

Formulation[1]	Protein recovery[2] (%)	Protein Amount[3] (µg /mg)	Protein Amount[3] (nM/mg)	MW (g/mol)
BSA NP	65.5±1.5	10.9±0.2	165.3±2.5	66000.0
EPH NP	57.2±0.3	9.7±0.1	203.4±1.4	47500.0
YSA NP	36.2±1.6	6.1±0.61	4534.3±449.5	1348.5
BSA-PTX NP	65.0±0.9	10.8±0.1	164.1±1.4	66000.0
EPH-PTX NP	56.4±0.4	9.5±0.1	200.5±1.9	47500.0
YSA-PTX NP	38.6±1.3	6.5±0.5	4821.4±349.6	1348.5

[BSA NP: nanoparticles with BSA conjugation. YSA NP: nanoparticles with YSA conjugation. EPH NP: nanoparticles with Ephrin A1 conjugation. BSA PTX NP: paclitaxel loaded nanoparticles with BSA conjugation. EPH-PTX NP: paclitaxel loaded nanoparticles with Ephrin A1 conjugation. YSA-PTX NP: paclitaxel loaded nanoparticles with YSA conjugation. 2. Amount of protein recovered compared to the amount of protein used. 3. Amount of protein recovered in 1 mg of nanoparticles used.]

Protein recovery after conjugation process for the Ephrin A1 NP with or without paclitaxel is around 57 % whereas protein recovery of the YSA conjugated NP with or

without paclitaxel is around 37%. Protein recovery of the YSA conjugation is much lower than the Ephrin A1 conjugation. Please note that YSA has the lowest molecular weight of all. BSA NP or BSA-PTX NP has the highest protein conjugation percentage with approximately 65% and the BSA has the highest molecular weight (Table 8).

3.3.3 Determination of Paclitaxel Content in YSA-PTX NP and EPH-PTX NP

Same procedure as discussed in section 2.2.4 was followed, and the amount of PTX content in surface conjugated nanoparticles was determined via NMR (Table 9). PTX peaks are appeared at 8.2 in Figure 26a and b.

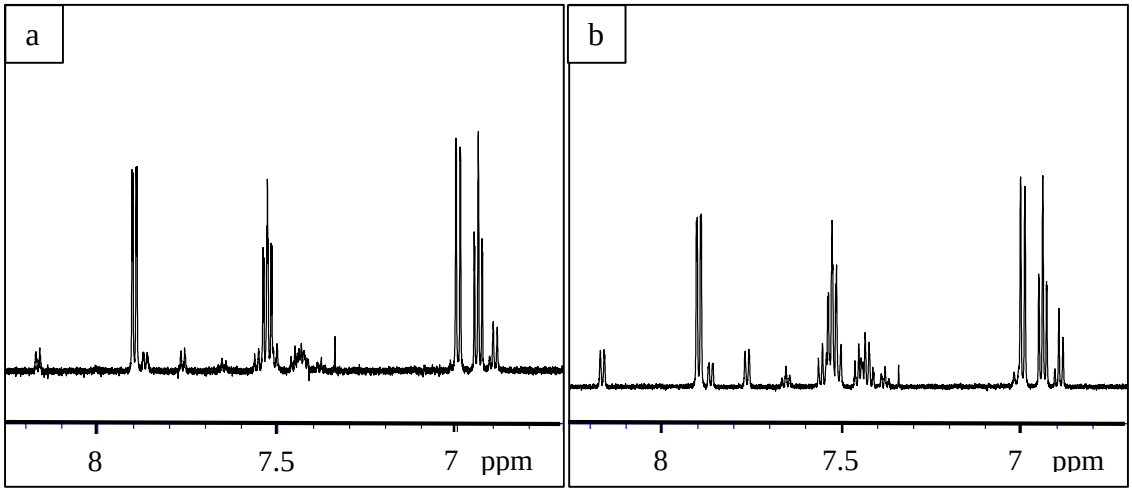

Figure 26. NMR spectrum of EPH-PTX NP (a) and NMR spectrum of YSA-PTX NP (b).

Surface modified nanoparticles YSA-PTX NP and EPH-PTX NP contain much lower paclitaxel encapsulation efficiency compared to PTX NP. Paclitaxel encapsulation efficiency of YSA-PTX NP is 29.5±1.1% and EPH-PTX NP is 32.5 ±1.3 %.

It should be noted that YSA-PTX NP has the lowest paclitaxel loading efficiency of all paclitaxel formulations (Table 9). Loading efficiency of surface modified nanoparticles YSA-PTX NP and EPH-PTX NP came out to be 22.2±0.8± µg/mg and 24.5 µg/mg respectively (Table 9).

Table 9. Determined amount of paclitaxel content in YSA-PTX NP and EPH-PTX NP

Formulation[1]	EE (%)[2]	LE (µg drug/mg NP)[3]
YSA-PTX NP	29.5±1.1	22.2±0.8
EPH-PTX NP	32.5±1.3	24.5±1.0

[1. YSA-PTX NP: nanoparticles with YSA conjugation and paclitaxel loaded. EPH-PTX NP: nanoparticles with the Ephrin A1 conjugation and paclitaxel loaded. 2. Paclitaxel encapsulation efficiency (EE) (%) =drug recovered in NP/drug fed 3. Paclitaxel loading efficiency (LE): drug recovered in nanoparticles/nanoparticles recovered]

3.3.4 in vitro Studies of Surface Conjugated Nanoparticles (EPH NP, EPH-PTX NP, YSA NP and YSA-PTX NP

3.3.4.1 Cellular uptake. At concentration 250 µg/ml, we see no significant difference among the cellular uptake of three nanoparticle formulations. Cellular uptake of EPH NP becomes ~100% at 125 µg/ml concentration and stays around 100% as concentration drops down. Cellular uptake of YSA NP and NP is around 72% at 125 µg/ml concentration with no significant difference between the two, however EPH NP has significantly higher uptake ($p<0.01$) compared to the other two formulations. The uptake of YSA NP and NP becomes ~90% at 32.5 µg/ml concentration. Please note that the uptake of YSA NP is not much different from placebo NP for all concentration amounts (Figure 27).

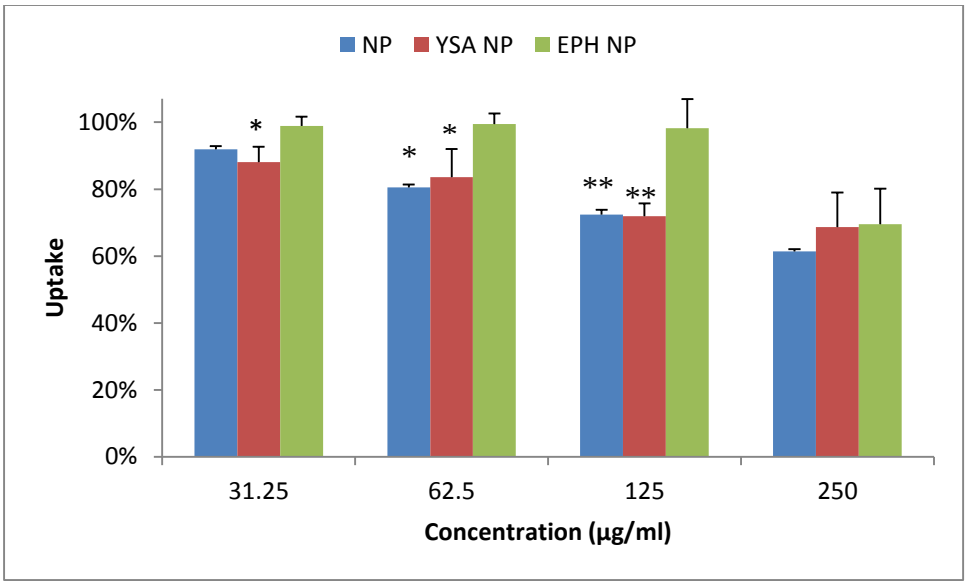

Figure 27. Cellular uptake of fluorescent (Coumarin-6) nanoparticles with YSA (YSA NP) or Ephrin A1 (EPH NP) or without any surface modification (NP). Cells were treated with the determined amounts of nanoparticle formulation with fluorescent and uptaken amount of nanoparticles was measured via a plate reader. [*: $p<0.05$, **: $p<0.01$; significance of any formulation to EPH NP within each concentration]

3.3.4.2 Anti-proliferative activity of surface conjugated nanoparticles. Cytotoxicity of conjugated nanoparticles in 24 hours is shown in Figure 28. The highest cytotoxicity is achieved at 1.5 µg/ml by the surface conjugated and paclitaxel loaded nanoparticles (YSA-PTX NP and EPH-PTX NP) with no significant difference between the two (40% and 44% respectively) (Figure 28b). Only surface conjugated nanoparticles at 0.075 µg/ml show negligible killing and it is around average of 10% for both. At the lowest concentration, 0.075 µg/ml, only 16% (YSA-PTX NP) and 29% (EPH-PTX NP) cytotoxicity is seen for the surface conjugated and paclitaxel loaded nanoparticles (Figure 28b). Cytotoxicity of EPH-PTX NP at 0.075 µg/ml and 0.3 µg/ml is not much different (Figure 28b).

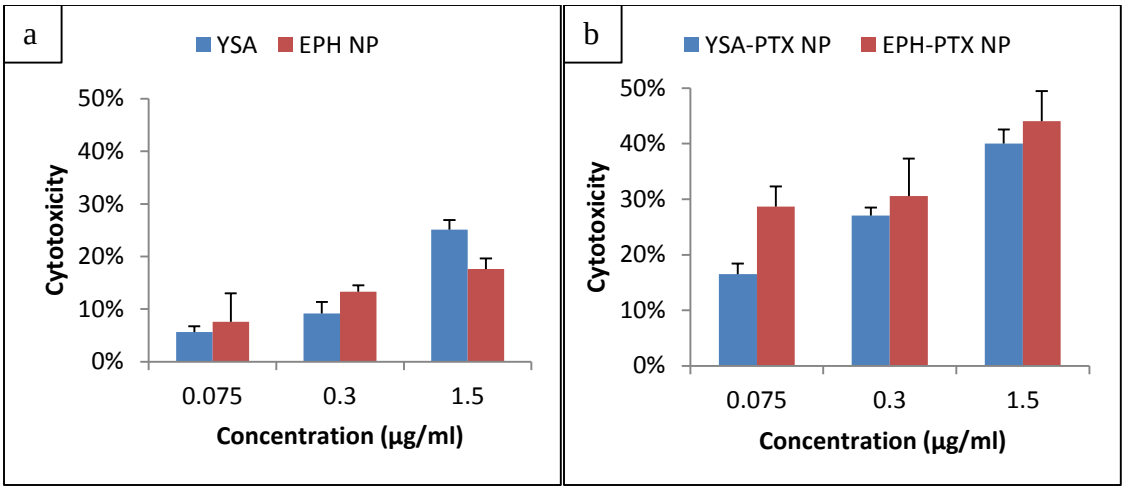

Figure 28. 24 hour cytotoxicity of surface conjugated nanoparticles with no paclitaxel (YSA NP and EPH NP) (a) and surface conjugated and paclitaxel loaded nanoparticle formulations (YSA-PTX NP and EPH-PTX NP) (b) over concentrations. Cytotoxicity was determined with MTT assay on cells treated with different amount of nanoparticle formulations over 24 hours

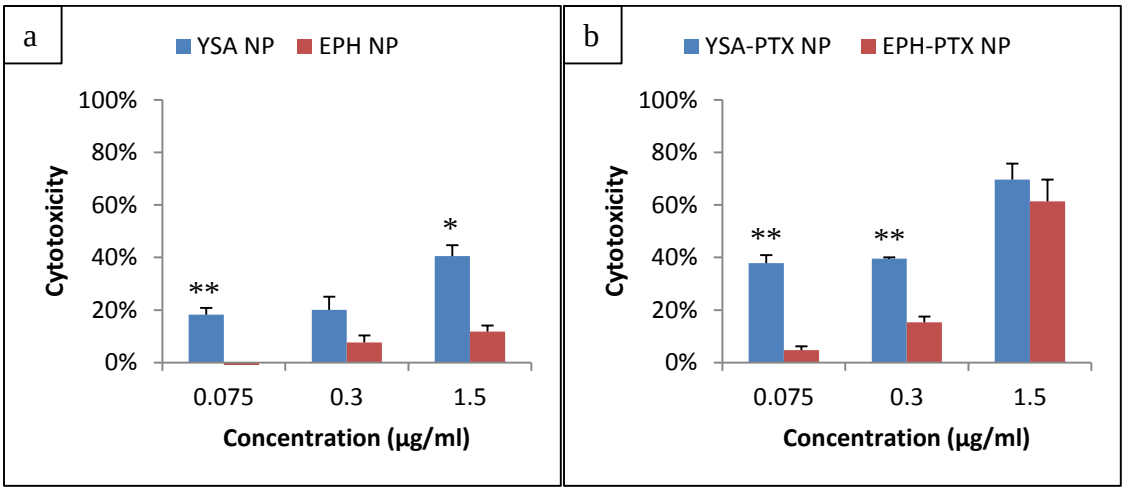

Figure 29. 48 hour cytotoxicity of surface conjugated nanoparticles with no paclitaxel (YSA NP and EPH NP) (a) and surface conjugated and paclitaxel loaded nanoparticle formulations (YSA-PTX NP and EPH-PTX NP) (b) over concentrations. Cytotoxicity was determined with MTT assay on cells treated with different amount of nanoparticle formulations over 48 hours. [*: $p<0.05$, **: $p<0.01$, significance of EPH-PTX NP compared to YSA-PTX NP within each concentration]

The cytotoxicity of only surface conjugated nanoparticles (YSA NP and EPH NP) are almost half way lower respect to their corresponding surface conjugated and paclitaxel loaded nanoparticles (YSA-PTX-NP and EPH-PTX NP) at 1.5 µg/ml (Figure 28). The cytotoxicity of EPH-PTX NP ranges around 30% for the two lower concentrations.

In the 48 hour experiment, the highest cytotoxicity for all nanoparticle formulations is seen at 1.5 µg/ml (Figure 29).

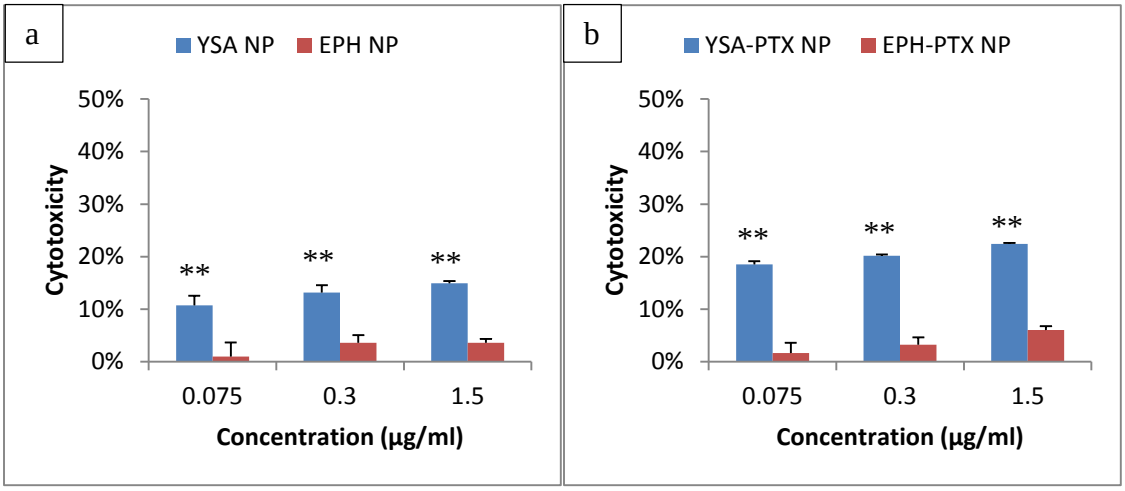

Figure 30. 72 hour cytotoxicity of surface conjugated nanoparticles with no paclitaxel (YSA NP and EPH NP) (a) and of surface conjugated and paclitaxel loaded nanoparticles (YSA-PTX NP and EPH-PTX NP) (b) over concentrations. Cytotoxicity was determined with MTT assay on cells treated with different amount of nanoparticle formulations over 72 hours. [**: $p<0.01$; significance of EPH-PTX NP to YSA-PTX NP within each concentration]

The cytotoxicity of YSA-PTX NP is around 20% throughout all three concentrations in the 72 hour experiment where the cytotoxicity of YSA NP is around 15% in all concentrations (Figure 30). All other formulations in the 72 hour experiment do not show much cytotoxicity.

The highest cytotoxicity of YSA NP among all three days is achieved in 48 hours which 41% (Figure 31a). The cytotoxicity of EPH NP at 1.5 µg/ml drops about 7% at the end of each 24 hours (Figure 31b). Quite noticeable increase (15%) is observed in the cytotoxicity of YSA-PTX NP at 1.5 µg/ml when the incubation time is extended from 24 to 48 hours (Figure 31a). The cytotoxicity of EPH-PTX NP at 1.5 µg/ml increases by ~50% from 44% (24 hours) to 61% (48 hours) within 48 hours (Figure 31d).

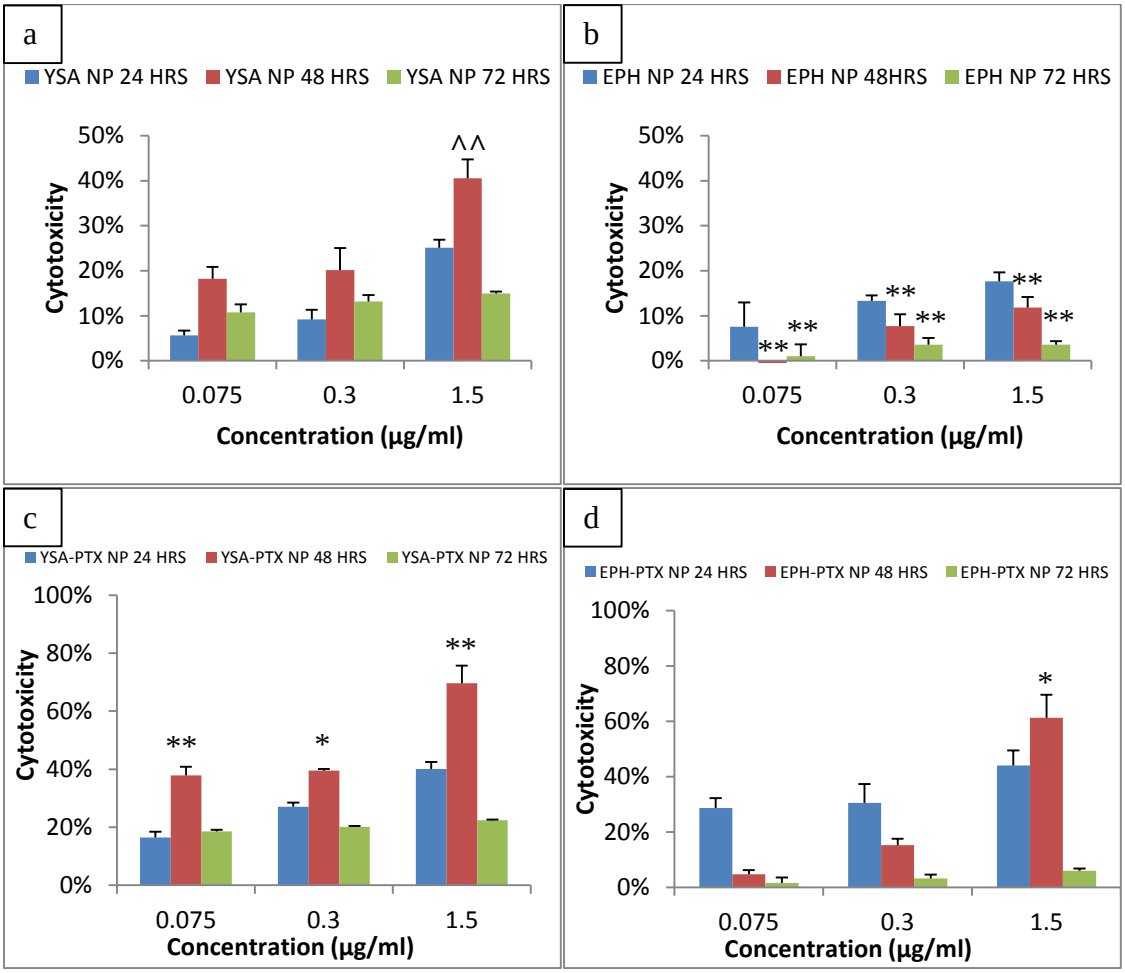

Figure 31. Comparing cytotoxicity in 24, 48, and 72 hours for YSA NP (a), EPH NP (b), YSA-PTX NP (c), and EPH-PTX NP (d). [*: $p<0.05$, **: $p<0.01$, significance of any nanoparticles in 24 hours against to any nanoparticles in 48 72 hours, ^^: $p<0.01$, significance of YSA NP 48 HRS to YSA NP 72 HRS]

3.2.4.3 IC50 calculation of surface conjugated nanoparticles. IC50 values of surface conjugated nanoparticles are calculated by trendline equations in Figure 32, Figure 33 and Figure 34.

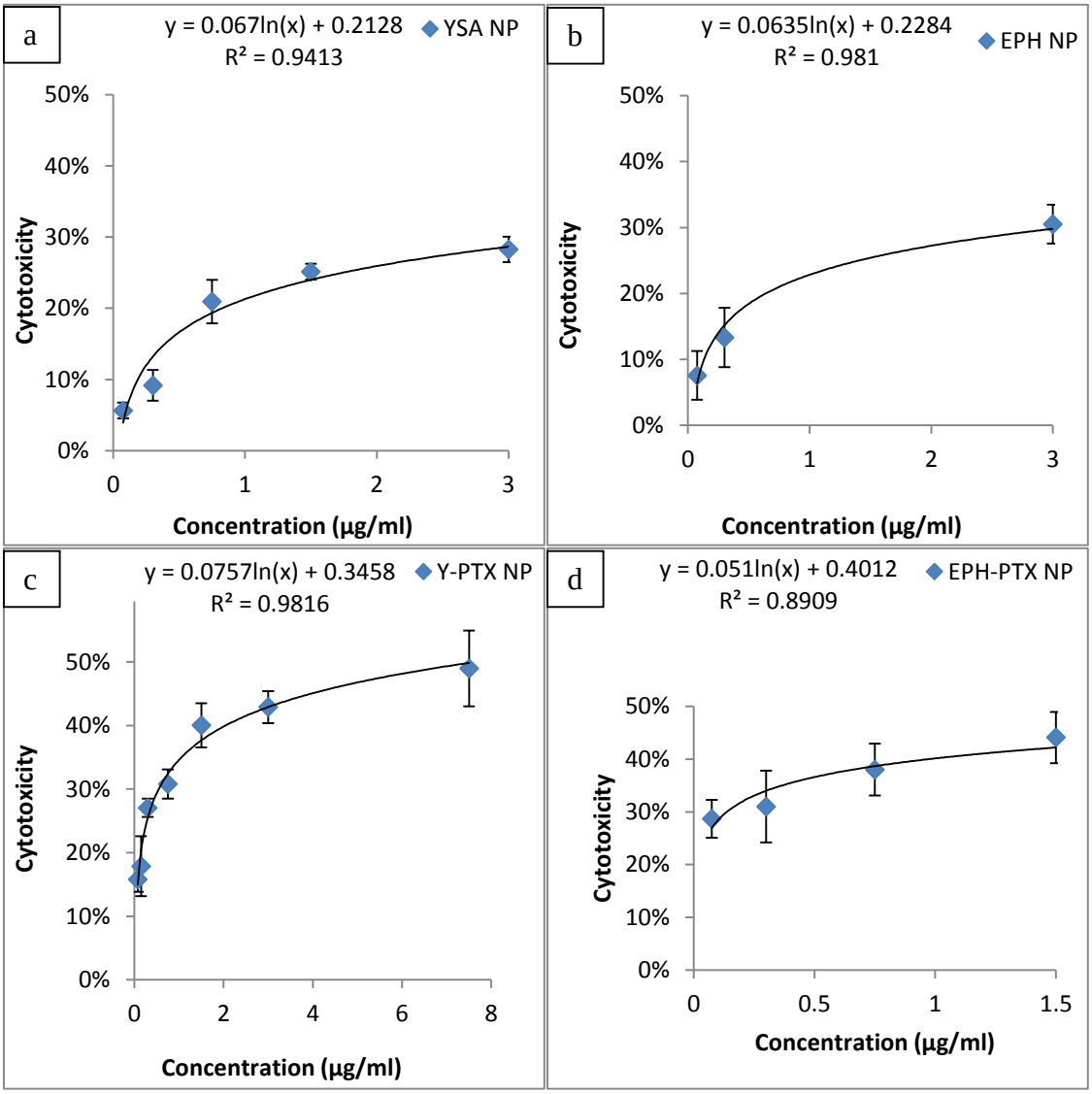

Figure 32. 24 hour cytotoxicity graphs of surface conjugated nanoparticles, YSA NP (Nanoparticles with YSA conjugation) (a), EPH NP (Nanoparticles with Ephrin A1 conjugation) (b), YSA-PTX NP (Nanoparticles with YSA conjugation and paclitaxel loaded) (c), and EPH-PTX NP (Nanoparticles with Ephrin A1 conjugation and paclitaxel loaded) (d) over concentrations. [Logarithmical trendlines are added to calculate IC50 values; R^2 is the coefficient of determination that indicates how well trendline fits to graph.]

IC50 values of only surface conjugated nanoparticles (YSA NP and EPH NP) in 24 hours are very similar to each other with the value of 72 µg/ml (Table 10).

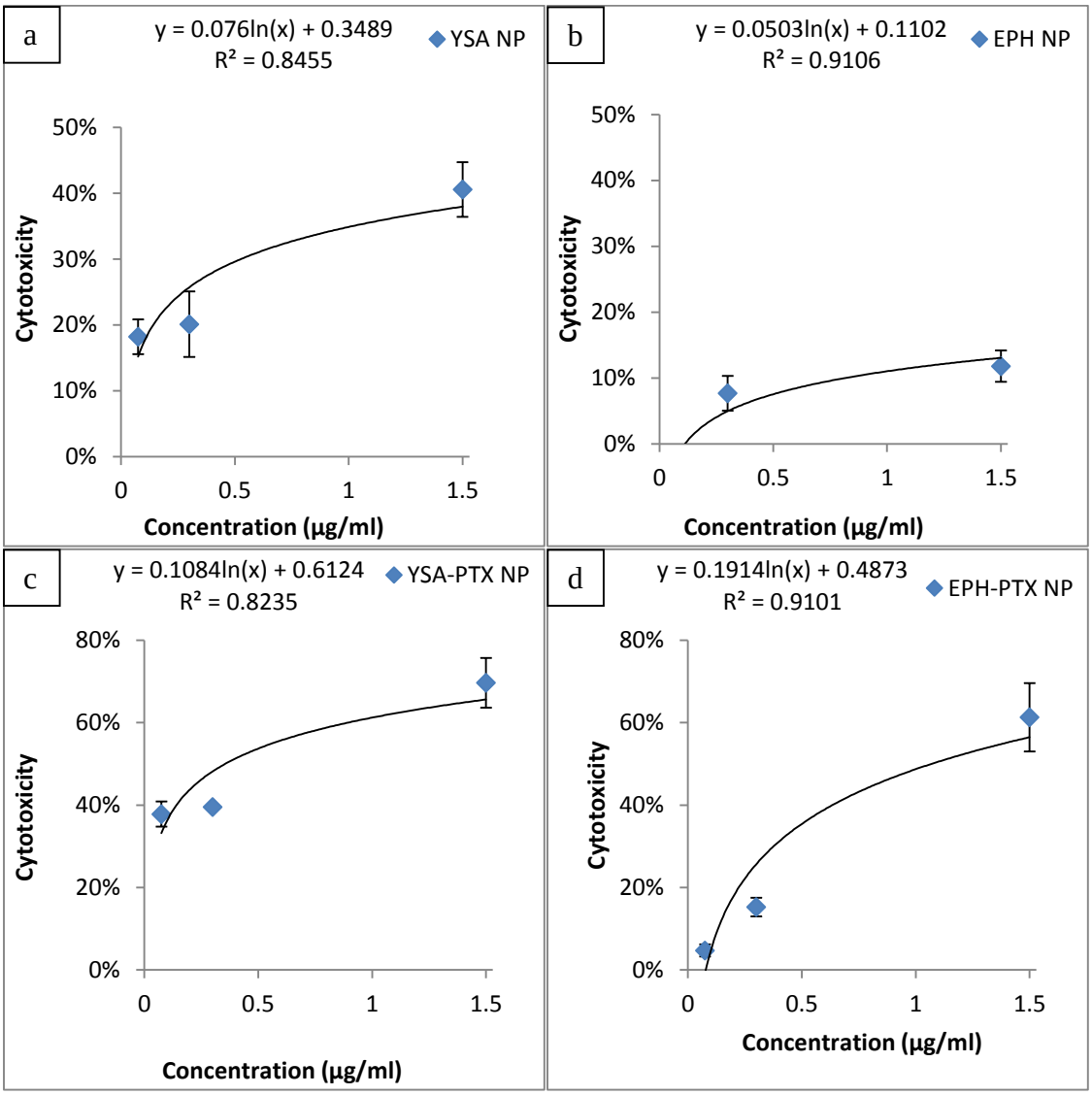

Figure 33. 48 hour cytotoxicity graphs of surface conjugated nanoparticles, YSA NP (Nanoparticles with YSA conjugation) (a), EPH NP (Nanoparticles with Ephrin A1 conjugation) (b), YSA-PTX NP (Nanoparticles with YSA conjugation and paclitaxel loaded) (c), and EPH-PTX NP (Nanoparticles with Ephrin A1 conjugation and paclitaxel loaded) (d) over concentrations. [Logarithmical trendlines are added to calculate IC50 values; R^2 is the coefficient of determination that indicates how well trendline fits to graph.]

Based on calculations, surface conjugated and paclitaxel loaded nanoparticles (YSA-PTX NP and EPH-PTX-NP) have very ideal IC50 values of 7.7 and 7 µg/ml in the 24 hour experiment respectively (Table 10).

Table 10. Calculated IC50 values of surface conjugated nanoparticles in 24 hours. (1. IC50 value in terms of weight of nanoparticles in µg/ml)

Time	YSA NP (µg/ml)[1]	EPH NP (µg/ml)[1]	YSA-PTX NP (µg/ml)[1]	EPH-PTX NP (µg/ml)[1]
24HRS	72.72	72.04	767	6.94

When incubation time was extended to 48 hours, all nanoparticle formulations but EPH NP had the ideal IC50 values of 7.3 µg/ml for YSA NP, 0.35 µg/ml for YSA-PTX NP, and 1.07 µg/ml for EPH-PTX NP (Figure 33 and Table 11).

Table 11. Calculated IC50 values of surface conjugated nanoparticles in 48 hours. (1. IC50 value in terms weight of nanoparticles in µg/ml)

Time	YSA NP (µg/ml)[1]	EPH NP (µg/ml)[1]	YSA-PTX NP (µg/ml)[1]	EPH-PTX NP (µg/ml)[1]
48HRS	7.30	2320.42	0.35	1.07

Table 12. Calculated IC50 values of surface conjugated nanoparticles in 72 hours. (1. IC50 value in terms weight of nanoparticles in µg/ml)

Time	YSA NP (µg/ml)[1]	EPH NP (µg/ml)[1]	YSA-PTX NP (µg/ml)[1]	EPH-PTX NP (µg/ml)[1]
72HRS	$1x10^{11}$	$8.77x10^{23}$	$2.56x10^{09}$	$1.59x10^{13}$

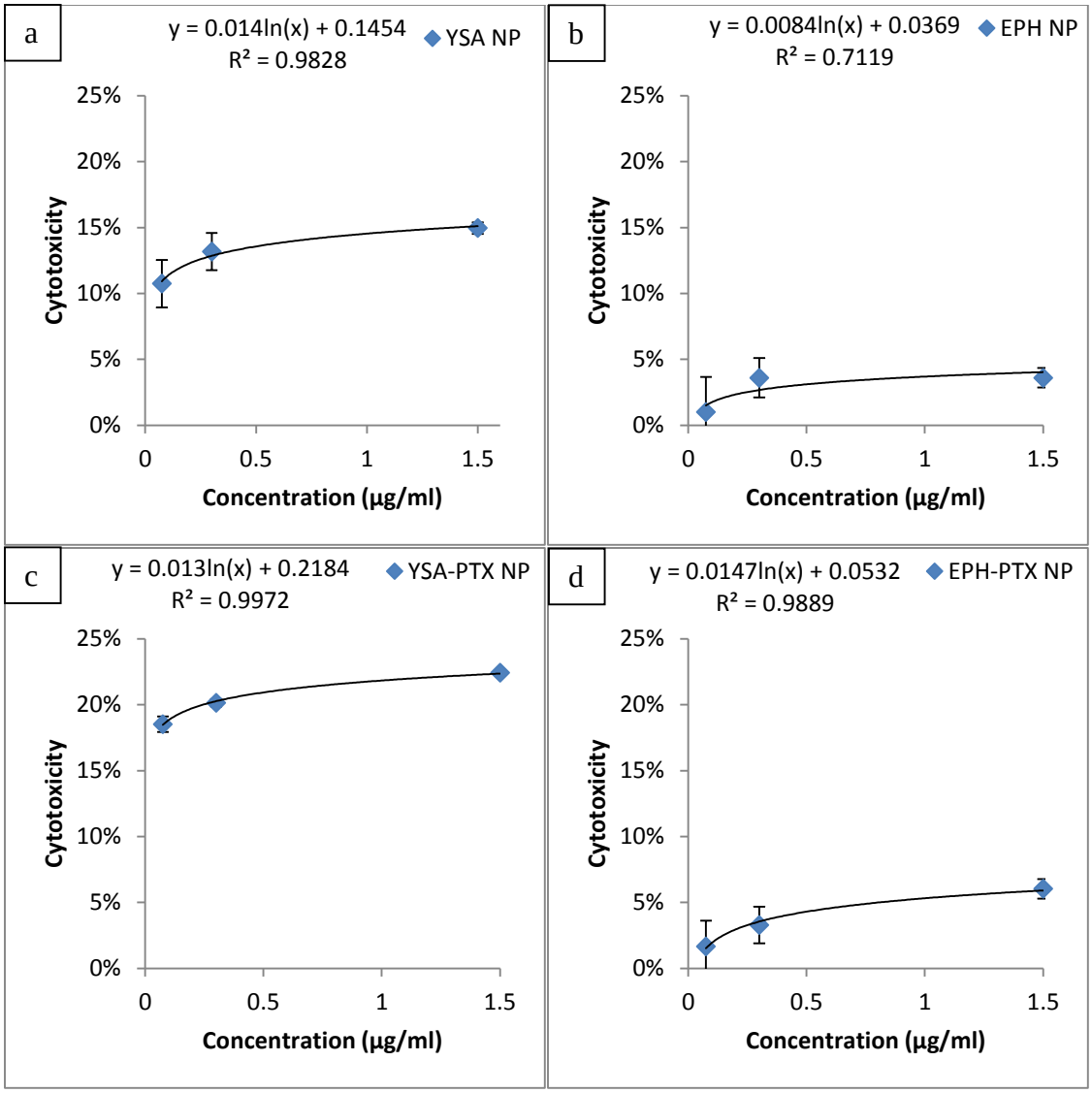

Figure 34. 72 hour cytotoxicity graphs of surface conjugated nanoparticles, YSA NP (Nanoparticles with YSA conjugation) (a), EPH NP (Nanoparticles with Ephrin A1 conjugation) (b), YSA-PTX NP (Nanoparticles with YSA conjugation and paclitaxel loaded) (c), and EPH-PTX NP (Nanoparticles with Ephrin A1 conjugation and paclitaxel loaded) (d) over concentrations. [Logarithmical trendlines are added to calculate IC50 values; R^2 is the coefficient of determination that indicates how well trendline fits to graph.]

After 72 hours incubation, very high IC50 values were obtained (Table 12).

In Figure 35, it is clearly seen that the surface conjugated and paclitaxel loaded nanoparticle formulations have very low IC50 values as opposed to free PTX or PTX NP.

Moreover, YSA-PTX NP shows much lower IC50 compared to EPH-PTX NP in 49 hours (Figure 35).

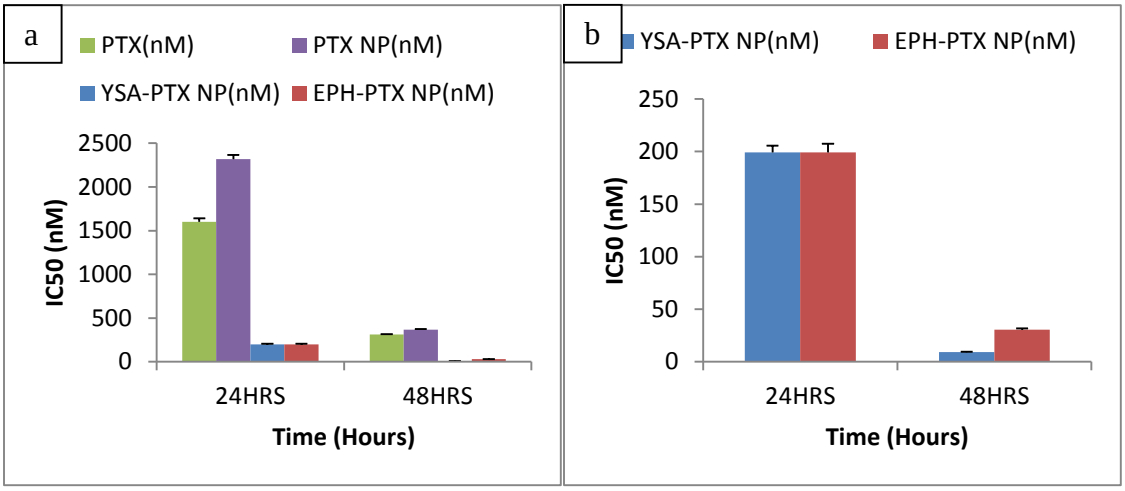

Figure 35. Comparing IC50 values of free PTX against paclitaxel loaded nanoparticles (PTX NP, YSA-PTX NP, and EPH-PTX NP) over 24 and 48 hours (a), Rescaled graph for only for only YSA-PTX NP and EPH-PTX NP (b).

CHAPTER 4

DISCUSSION

-Malignant pleural mesothelioma is still a frustrating clinical challenge with no effective treatment. Various therapeutic approaches, including surgery, radiotherapy, chemotherapy, and traditional gene therapy, showed limited effectiveness on improving the survival rate of patients [56].

Targeted delivery represents a potential approach to further enhance anti-tumoral efficacy and minimize toxicity. Targeted delivery to the tumor and tumor vasculature is considered a powerful strategy for cancer treatment since angiogenesis is essential for tumor growth [57].

The Ephrin A1 ligand was selected be-cause of its natural origin and demonstrated receptor-mediated cellular uptake as well as the YSA peptide which is known for its high affinity to the Ephrin A2 receptor and mimics the Ephrin A1 ligand.

Since MMC over expresses the Ephrin A2 receptor, in this study, we demonstrated the ability of multi-functional nanoparticles which were surface conjugated with the Ephrin A1 ligand or YSA peptide to target the tumor and tumor endothelium (via the binding to the Ephrin A2 receptor) and improve the anti-tumoral efficacy of paclitaxel. Paclitaxel is a potent chemotherapeutic drug. Its clinical applications are limited by poor water solubility.

The Ephrin A1 and YSA peptide can inhibit the proliferation and migration of the lung cancer cells via binding to overexpressed the EphA2 receptors. [15, 58, 59].

Thus, the Ephrin-A1 or YSA peptide on the nanoparticles serves not only as a specifically targeting molecule to lung cancer cells but also as a therapeutic reagent for cancer treatments.

We obtained particles in nano range which is very suitable for drug delivery into cancer cells. All nanoparticles, except the Ephrin A1 conjugated nanoparticles, are in the size range of 247 to 278 nm. The Ephrin A1 conjugated nanoparticles have the smallest size among any other nanoparticle formulations we studied in this work. The Ephrin A1 conjugated nanoparticles also have the highest charge which may be resulting from smaller nanoparticle size (Table 13).

Table 13. Physical characteristics of all nanoparticles. EE and LE determined by NMR

Formulation[1]	Size (nm)	PD[2]	Zeta Potential (mv)	EE (%)[3]	LE (μg drug/mg NP)[4]
NP	253.6±3.4	0.14	-29.1±4.7	N/A	N/A
PTX NP	268.6±14.4	0.12	-30.1±5.7	66.2±3.1	50.0±2.3
YSA NP	278.7±2.2	0.10	-22.0±4.9	N/A	N/A
EPH NP	197.2±2.6	0.13	-37.8±5.5	N/A	N/A
YSA-PTX NP	247.7±9.1	0.14	-16.1±4.0	29.5±1.1	22.2±0.8
EPH-PTX NP	188.0±5.6	0.14	-40.6±4.8	32.5±1.3	24.5±1.0

[1. NP: Placebo nanoparticles, 2. PTX NP: nanoparticles loaded with paclitaxel. YSA NP: nanoparticles with YSA conjugation, EPH NP: nanoparticles Ephrin A1 conjugation, YSA-PTX NP: nanoparticles with YSA conjugation and paclitaxel loaded, and EPH-PTX NP: nanoparticles with Ephrin A1 conjugation and paclitaxel loaded, 2. PD: Poly-dispersity, 3. Paclitaxel encapsulation efficiency (EE) (%) =drug recovered in NP/drug fed, and 4. Paclitaxel loading efficiency (LE): drug recovered in NP/NP recovered]

Lower surface charge causes the formation of aggregated nanoparticles versus high surface charge which would help to disperse and single out nanoparticles further, which affects DLS measurement readings despite sufficient vortex process (Table 13).

One noteworthy challenge is electrostatic repulsion that inhibits the cellular uptake of nanoparticles, or drugs with a high negative charge *in vivo* in contrast to *in vitro* [21, 22]. The YSA conjugated nanoparticles with the lowest charge are 15.7 to 33.3 mV lower compared to any other four nanoparticle formulations we studied (NP, PTX NP, EPH NP, and EPH-PTX NP) (Table 13).

The lowest surface charge of (YSA NP and YSA-PTX NP) may be due to more molecules attaching on the carboxylic groups on the surface of polymer nanoparticles since the YSA peptide has very low molecular weight (136 g/mol) (Table 13). This means that less negative carboxylic groups are available on the nanoparticle surface meaning negative charge.

We understand that conjugation of the Ephrin A1 ligand onto nanoparticles may cause the surface charge of nanoparticles to increase (Table 13). This may be one of the reasons the Ephrin A1 conjugated nanoparticles (EPH NP and EPH-PTX NP) have 57-90 µm less size compared to any other four nanoparticle formulations we have.

Sizes of surface conjugated nanoparticles (YSA NP and EPH NP) are higher compared to corresponding nanoparticles with surface modification and paclitaxel (YSA-PNP NP and EPH-PTX NP respectively) (Table 13). Despite not much difference in surface charges of YSA NP and YSA-PTX NP or EPH NP and EPH-PTX NP, incorporation of PTX along surface conjugation lowers the particle size. This difference may occur during

the conjugation process where we have more free PTX available in the preparation solution due to the release over 24 hours that the conjugation process takes. This may allow more PTX to attach on the surface of nanoparticles, even though paclitaxel residues were washed at the end of the conjugation process. This may lower the surface charge by paclitaxel neutralizing the negative charge of some carboxylic groups on the polymer surface.

The size difference between the YSA surface conjugated nanoparticles ranges between 20 to 42 μm and the Ephrin A1 conjugated nanoparticles ranges between 1-7 μm (Table 13). Nevertheless, this difference in sizes via incorporation of PTX is not big for the Ephrin A1 surface conjugated nanoparticles; it is a significant difference for the YSA conjugated nanoparticles. Since YSA with the lowest molecular weight will have a number of molecules available on the nanoparticle surface, we would expect more interaction between the YSA molecules and PTX residues than the Ephrin A1 molecules.

Loading efficiency of PTX NP is comparable to results that R. Yang, et al. and Y. Mo, et al. obtained [60, 61].

EE and LE of conjugated nanoparticles are lower due to loss of paclitaxel during the conjugation process taking 24 hours with overnight incubation (Table 13). Perhaps the conjugation process needs to be optimized, shortening the conjugation process time and resulting in better loading and encapsulation efficiency of PTX, provided that conjugation efficiency is not compromised.

56-65% of protein recovery was observed in all formulations except the YSA conjugated nanoparticles (YSA NP (36%) and YSA-PTX NP (39%)). Please note that the YSA peptide has a very low molecular weight of 1348.5 g/mol and it is more than 30

times lower than the molecular weight of any other nanoparticle formulation. It gets the highest protein recovery in terms of molarity with 4500 nM/mg compared to the other nanoparticle formulations (Table 8).

Nanoparticles showed sustained release profiles. 32% of the encapsulated drug is released on day 5 (Figure 18 and Table 16). Using the logarithmic equation in Figure 18, we calculate that it will take $8.2x10^5$ days for the entire drug to be released. Please note that only 33% of the drug was released by the day eight. After 22 more days, based on the calculation, setting x to 30 days and solving the logarithmic equation in Figure 18 for y, 41% of the drug will be released at the end of 30 days. The release will be very minimal after a few months (Figure 18).

We can assume that roughly 1% of the drug will be released every three days after day five. IC50 for PTX in three days is 196 nM (Table 6). 1 mg PTX NP has 58.6 nM PTX content and 200 nM corresponds to 3.4 µg PTX NP (Table 13). This is only 1% of the amount of encapsulated the drug PTX NP contains. So we need 340 µg PTX NP for 30 days. This is the amount needed to supply 200 nM PTX every three days. We need 3.4 mg for 30 more days of treatment that PTX NP can supply 200 nM PTX that is required to reach IC50 in three days.

This stable sustained release profile of PTX NP provides the desired amount of the drug to cells. Please note that this is a very rough calculation and the determination of dosage needs further procedures and tests.

Referring back to Table 13, 1 µg of YSA-PTX NP has 22.2 ng of PTX (26 nM), and 1 µg of EPH-PTX NP has 24.5 ng of PTX (28.69 nM). Here we found, as free PTX shows IC50 at 313 nM, that YSA-PTX NP shows much better IC50 at 9.2 nM with EPH-

PTX NP at 30.7 nM at the end of 48 hours of incubation (Table 14). Please note that all surface conjugated nanoparticles with PTX show their ideal IC50 in 48 hours, so we can safely conclude that 48 hours is the ideal time frame for the drug become effective (Table 14).

One should consider that only one-fourth of the drug is released out of the nanoparticles within the 48 hours, versus all free PTX being already available in the cell from the beginning of the 48 hours (Table 16 and Figure 18).

Table 14. Calculated IC50 values of free PTX, PTX NP and surface conjugated and PTX loaded nanoparticles (YSA-PTX NP and EPH-PTX NP) in nM. (1. IC50 value of free PTX. 2. IC50 value of PTX that nanoparticles containing)

Time	PTX(nM)[1]	PTX NP(nM)[2]	YSA-PTX NP(nM)[2]	EPH-PTX NP(nM)[2]
24 HRS	1602	2318	199.3	199.1
48 HRS	313	366	9.22	30.7
72 HRS	196	304	$7x10^{10}$	$5x10^{14}$

However, YSA-PTX NP and EPH-PTX NP show ideal values of IC50 in 24 hours, as well as in 48 hours, as opposed to free PTX or PTX NP (Figure 34). Nevertheless, we observe that PTX NP shows similar IC50 value to free PTX. Surface conjugated nanoparticles with paclitaxel, YSA-PTX NP (9.2 nM) and EPH-PTX (30.7 nM) show much lower IC50 values from PTX NP and free PTX. We also see that IC50 of YSA-PTX NP at 48 hours is more than three times more efficient compared to EPH-PTX NP (Table 14).

YSA NP arrives at 7.30 µg/ml, its ideal IC50, in 48 hours, whereas YSA PTX has a similar IC50 (7.67 µg/ml) in 24 hours (Table 15). Please remember that we cannot compare surface conjugated nanoparticles without PTX in nM, but in µg/ml.

I must remind you one more time that, ~20% of the drug is released out of nanoparticles in 24 hours, and ~25% is released at the end of 48 hours from PTX NP and is available in the cell environment. The release increases to 30% and it stays around 35% with a very slow increase by the 3rd day (Figure 18 and Table 16).

Table 15. Calculated IC50 values of surface conjugated nanoparticles (YSA NP, EPH NP, YSA-PTX NP, and EPH-PTX NP) in µg/ml

Time	YSA NP (µg/ml)	EPH NP (µg/ml)	YSA-PTX NP (µg/ml)	EPH-PTX NP (µg/ml)
24 HRS	72.72	72.04	7.67	6.94
48 HRS	7.30	2320.42	0.35	1.07
72 HRS	$1x10^{11}$	$8.77x10^{23}$	$2.6x10^{9}$	$1.6x10^{13}$

The reason I bring this up is that the amount of available drug in the cell environment varies, increasing over the first 72 hours, but staying stable with a very little increase after 72 hours.

Table 16. Amount of PTX released on each day in nM out of PTX loaded nanoparticles. (1. PTX released at 0.075 µg/ml concentration, 2. PTX released at 0.3 µg/ml concentration, 3. PTX released at 1.5 µg/ml concentration)

	PTX NP Concentrations			YSA-PTX NP Concentrations			EPH-PTX NP Concentrations		
Time	At 0.075^{1}	At 0.3^{2}	At 1.5^{3}	At 0.075^{1}	At 0.3^{2}	At 1.5^{3}	At 0.075^{1}	At 0.3^{2}	At 1.5^{3}
24 HR	0.9	3.5	17.6	0.4	1.6	7.8	0.4	1.7	8.6
48 HR	1.1	4.4	22.0	0.5	1.9	9.7	0.5	2.2	10.8
72 HR	1.2	4.9	24.6	0.5	2.2	10.9	0.6	2.4	12.0

Calculating corresponding drug availability in the cell over each day, we came up with the figures seen in Table 16. 1.5 µg/ml PTX NP contains ~90 nM PTX and releases

only ~17 nM PTX in 24 hours (Table 16). Referring back to the graphs in Figure 21 and after some calculations, both 90 nM of free PTX and PTX NP containing 90 nM of the drug kills ~26% of cells. PTX NP uses 80% less of the drug (17.6 nM) than free PTX in 24 hours.

It is definitely important to consider the surface conjugated nanoparticles to measure cytotoxicity efficiency and the amount of the drug used against free PTX. At 1.5 µg/ml, YSA PTX NP (consumes only 8 nM PTX) kills 40% and EPH-PTX NP (using only 9 nM PTX) kills 44% of cells both performing ~30-40% better than free PTX, and they kill with 90% less PTX used than free PTX in 24 hours (Table 16). Similarly, both YSA-PTX NP and EPH-PTX NP also show approximately 30-40% better cytotoxicity from only PTX loaded particles with no surface conjugation (PTX NP), and they both use less than twice the amount of the drug in the 24 hour experiment (Figure 30b and Table 16).

Using similar approach in the 48 hour experiments, we came up with a 38% killing for free PTX with 90 nM of PTX drug versus the killing of 36% of cells with only 22 nM drug delivered by PTX NP, therefore saving 77% of the drug with similar cytotoxicity (Table 16).

Similarly, using 1.5 µg/ml concentration over a 48 hour timeframe, only 9.7 nM and 10.8 nM PTX are used out of YSA-PTX NP and EPH-PTX NP, respectively, versus 90 nM of free PTX being used (Table 16). Per Figure 30b, we were able to kill 70% and 61% with YSA-PTX NP and EPH-PTX NP, respectively, in 48 hours. This corresponds to 32% more killing by YSA-PTX NP using 90% less of the drug and 25% more killing by EPH-PTX NP using 88% less of the drug than free PTX (Figure 30b, Table 16).

We should compare killing of surface conjugated nanoparticles with no drug against paclitaxel loaded nanoparticles (PTX NP) rather than free PTX since there is no PTX in them. Since we compare the nanoparticle formulations against paclitaxel, I am going to take PTX NP as our base. It is not possible to compare free PTX in nM to YSA NP and EPH NP with no drug in units of µg/ml referring to the amount of nanoparticles rather than PTX in nM. So we will compare cytotoxicity and IC50 within nanoparticles in terms of total amount of nanoparticles in µg/ml, regardless of anti-cancer therapeutics that are carried against PTX NP.

We are going to use results for PTX NP and other PTX loaded formulations, as seen in Table 17 and Table 16. In 24 hours, YSA NP performs similarly to PTX NP with ~26% killing at 1.5 µg/ml concentration point and EPH NP is 8% below PTX NP's cytotoxicity (Table 17). This is the performance of YSA NP or EPH NP only by themselves without any paclitaxel content against the anti-cancer drug PTX. This is a proof of the YSA peptide and EPH ligand being an efficient anti-cancer therapeutics, in addition to their great targeting skills with high affinity (Figure 26) to the Ephrin A2 receptor, which is overexpressed in some cancer cells, including malignant mesothelioma.

In the 48 hour experiment, YSA NP performs 3% better than cytotoxicity of free PTX, which corresponds to the amount of PTX in PTX NP, whose nanoparticle concentration is equal to YSANP and EPH NP.

Please note that we expect a natural loss of cells in 72 hours, which is a long time for cell lines to survive without change of their media that has nutrition necessary for them to live, and this may affect our results.

Table 17. Cytotoxicity at 1.5 µg/ml concentration of free PTX and PTX loaded nanoparticles (PTX NP, YSA-PTX NP, and EPH-PTX NP) in 24 and 48 hours.

Formulations[1]	24 HRS PTX released (nM)[2]	24 HRS CYT[3] (%)	48 HRS PTX released (nM)[2]	48 HRS CYT[3] (%)
PTX	87.8*	26%	87.8	38%
PTX NP	17.6	27%	22	36%
YSA-PTX NP	7.8	40%	9.7	70%
EPH-PTX NP	8.6	44%	10.8	61%
YSA NP	N/A	25%	N/A	41%
EPH NP	N/A	18%	N/A	12%

[1. Nanoparticle formulations. 2. PTX amount released at 24 hours or 48 hours out of nanoparticles.* This is the total PTX amount that PTX NP contatining. This concentration amount is used for free PTX for comparision purposes. 3. Cytotoxicity at 1.5 µg/ml for 24 or 48 hours]

The purpose of using targeting nanoparticles to deliver paclitaxel is to enhance the cytotoxicity efficiency on MMC by the cell surface receptor the Ephrin A2 targeted delivery. Introducing PTX to EPH NP or YSA-PTX NP formulation has even further enhanced the killing ability of the nanoparticle formulations. These results prove that the engineered multifunctional EPH–PTX-NP and YSA-PTX NP formulation was highly effective in cell cytotoxicity.

On the basis of combined results of higher cellular uptake ability and high cytotoxicity, the surface conjugated and paclitaxel-loaded nanoparticles formulation (YSA-PTX NP and EPH-PTX NP) stands out with YSA-PTX NP one step further as an ideal and promising solution in that it has significant potential for *in vivo* targeted delivery applications that aim to provide an enhanced therapeutic effectiveness for malignancies that overly express the Ephrin A2.

The paclitaxel nanoparticle formulations (PTX NP, YSA NP, and EPH NP) were more effective than Taxol® at delaying tumor growth. Concerning surface conjugated

and paclitaxel-loaded nanoparticle formulations, the combined effect of passive accumulation and specific tumor targeting contributed to significantly improve the therapeutic efficacy of PTX.

CHAPTER 5

CONCLUSION

In this work, we conjugated the surface of nanoparticles with the YSA peptide or Ephrin A1 ligand to deliver paclitaxel with low solubility by targeting the MMC. These nanoparticle formulations with the inclusion of a fluorescent dye could also be used as a tool to diagnose malignant mesothelioma cancer.

Most of the current literature focuses on either only targeting or delivery techniques of anti-cancer therapeutics for nanoparticles. There is very little work about using nanoparticles for specifically mesothelioma cancer treatment. Our aim was to combine current targeting and delivery techniques to improve the delivery of anticancer therapeutics for MM via designed multipurpose PLGA nanoparticles.

The Ephrin A2 receptor expression on the surface of malignant mesothelioma is a biomarker for malignancy [35-37]. Fluorescent biodegradable nanoparticles with such targeting agents conjugated and loaded with the anticancer drug, paclitaxel can be utilized as a probe to interrogate, diagnose, and initiate apoptosis of malignant mesothelioma cells *in vitro*.

Overall, the aim was increasing the cytotoxicity of free paclitaxel by the combination of ideal size and characteristics for malignant mesothelioma, a deadly disease, currently without any available treatment.

Intention of this study was to produce such nanoparticles with:

- Improved cellular uptake of MMC.
- Improved the delivery of paclitaxel into MMC with sustained release ability
- Higher cytotoxicity to kill MMC

In order to synthesize nanoparticles with targeting and anti-cancer abilities, studies were done in three main sections:

- Synthesis
- Characterization
- *in vitro* experiments

Specific aims of this study could be summarized as follows:

Aim 1, synthesis and optimization of placebo nanoparticles suitable for anti-cancer therapeutics

The aim was to prepare nanoparticles in nano range to be used as targeting drug carriers.

- For the purpose, double emulsion technique was optimized to achieve nanosize.
- Nanoparticles were characterized by SEM, DLS, and Nanosizer, etc.
- *in vitro* studies were done to confirm the cellular uptake ability of nanoparticles with obtained size, charge and characteristics.
- Cytotoxicity of placebo nanoparticles was also measured.

These placebo nanoparticles were used as a control and targeting agents were conjugated on the surface. The first nanoparticle formulation obtained was:

- NP: Placebo nanoparticles with no surface modification or paclitaxel.

Aim 2, Encapsulating paclitaxel into nanoparticles with the technique achieved in aim 1.

The aim was to produce nanoparticles with high encapsulation efficiency and ideal *in vitro* activity.

- Such nanoparticles were produced, the amount of drug loaded was determined, and a drug release profile of paclitaxel nanoparticles was examined.
- *in vitro* studies were done to determine cytotoxicity and IC50 of paclitaxel nanoparticles.

In this section, free paclitaxel was also studied for comparison purposes in cytotoxicity and IC50. The nanoparticle formulation obtained is:

PTX NP: Paclitaxel loaded nanoparticles without YSA or Ephrin A1.

Aim 3, Conjugating surface of nanoparticles achieved in aim 1 and 2 with targeting signals such as the Ephrin A1 ligand and YSA peptide.

The aim here was to decorate nanoparticles with targeting agents. The Ephrin A1 ligand and YSA peptides were chosen as targeting agents since they show high affinity to the Ephrin A2 receptor which is overexpressed in MMC.

- These targeting agents were conjugated onto NP.
- *in vitro* studies were done to determine the cytotoxicity and IC50 of surface conjugated nanoparticles.

The purpose was to obtain nanoparticles loaded with paclitaxel as well as the Ephrin A1 or YSA conjugated nanoparticles.

We obtained nanoparticle formulations with improved cellular uptake and cytotoxicity.

Four nanoparticle formulations obtained are as follows:

1. YSA NP: Nanoparticles with YSA conjugation.

2. EPH NP: Nanoparticles with Ephrin A1 conjugation.

3. YSA-PTX NP: Nanoparticles with YSA conjugation and paclitaxel loaded

4. EPH-PTX NP: Nanoparticles with Ephrin A1 conjugation and paclitaxel loaded

Poly (lactic-co-glycolic acid) (PLGA) 50:50 nanoparticles were prepared with a double emulsion solvent evaporation method. The Ephrin A1 or YSA peptide conjugated onto surfaces of nanoparticles via well-established carbodiimide chemistry.

Relatively narrow particle size distribution and low surface charge is considered an ideal characteristic for the nanoparticle formulations for anticancer therapeutics. We have obtained nanoparticles ranging from 188-278 nm, with the Ephrin A1 surface conjugated nanoparticles having 57-90 μm less size compared to any other four nanoparticle formulations we studied.

in vitro studies with CRL2081 cell lines showed an enhanced cellular uptake of the targeted nanoparticles when compared to non-targeted nanoparticles, mediated by the binding to the Ephrin A2 receptor.

Highest uptake (~100%) was achieved by the Ephrin A1 conjugated nanoparticles (EPH NP) at 125 μg/ml concentration in 5 hours.

1.5 μg/ml PTX NP contains ~90 nM PTX. 90 nM of free PTX kills ~26% of cells in 24 hours, as well as 1.5 μg/ml PTX NP does. 1.5 μg/ml PTX NP releases only ~17 nM PTX out of 90 nM PTX that it contains in 24 hours. We were able to produce PTX NP using 80% less of the drug (17.6 nM) from free PTX in 24 hours and found that PTX NP performs a similar killing of cancer cells as free PTX.

1.5 µg/ml YSA PTX NP (consumes only 8 nM PTX) kills 40% and EPH-PTX NP (using only 9 nM PTX) kills 44% of cells, both performing ~30-40% better than free PTX, and they kill with 90% less PTX used than free PTX in 24 hours.

Similarly, using 1.5 µg/ml concentration over a duration of 48 hours, only 9.7 nM and 10.8 nM PTX was used out of YSA-PTX NP and EPH-PTX NP, respectively, versus 90 nM of free PTX being used. They were able to kill 70% and 61% with YSA-PTX NP and EPH-PTX NP, respectively, in 48 hours. This corresponds to 32% more killing by YSA-PTX NP using 90% less of the drug and 25% more killing by EPH-PTX NP using 88% less of the drug than free PTX.

Nanoparticles without PTX but with surface conjugation showed that 1.5 µg/ml YSA NP performs ~26%, killing similar to 1.5 µg/ml PTX NP. And EPH NP is 8% below PTX NP's cytotoxicity of 1.5 µg/ml. This is the performance of YSA NP or EPH NP only by themselves without any paclitaxel content against the anti-cancer drug PTX.

This is a proof of the YSA peptide and EPH ligand being an efficient anti-cancer therapeutic, on top of their great targeting skills with high affinity to the Ephrin A2 receptor, which is overexpressed in some cancer cells, including malignant mesothelioma

The highest killing (71%) was achieved by the YSA peptide conjugated and paclitaxel loaded nanoparticles (YSA-PTX NP) at 1.5 µg/ml over 48 hours. This is 32% more cytotoxicity with 90% less of the drug used compared to killing of free paclitaxel.

YSA-PTX NP (9.2 nM) and EPH-PTX (30.7 nM) show much lower IC50 values compared to any other nanoparticle formulations. And we see the ideal IC50 of both YSA-PTX NP and EPH-PTX in 48 hours.

YSA NP is without paclitaxel and shows similar IC50 in 48 hours to YSA PTX NP that contains PTX in 24 hours.

On the basis of combined results of higher cellular uptake cytotoxicity, the surface conjugated and paclitaxel-loaded nanoparticles formulations come forward with YSA-PTX NP being one step further as ideal and promising in that it has significant potential for *in vivo* targeted delivery.

Our proof of concept *in vitro* shows that EPH-PTX NP could have great advantages increasing their anticancer efficacy by targeting the anti-cancer drug to the tumor as compared to non-targeted nanoparticles. However, optimizing nanoparticle size to near 100 nm will increase the loading of anti-cancer drug as well as the uptake of nanoparticles. We can conclude that the YSA peptide and Ephrin A1 conjugated nanoparticles loaded with paclitaxel on the surface will give the best result for targeting and killing the tumor cells per the results.

We intend to study YSA-PTX NP and EPH-PTX NP in different cancer cells such as breast cancer and lung cancer that are overexpressing the Ephrin A2 receptor.

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