CYTOTOXICITY EVALUATION OF TEMPERATURE SENSITIVE POLY(*N*-ISOPROPYLACRYLAMIDE) NANOPARTICLES FOR DRUG DELIVERY

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS AT ARLINGTON

August 2008

ACKNOWLEDGEMENTS

I would like to thank Dr. Kytai Nguyen for opening the door for me to work in her laboratory and being my advisor. Her continuous support and belief in my abilities encouraged me to complete this thesis successfully. She always motivated me to reach my goal. She was also positive and optimistic about my results and lab work. I appreciate her suggestion, guidance, and patience throughout this work.

I would also like to thank Dr. Jian Yang and Dr. Young-tae Kim for serving on my thesis committee. I would like to extend my thanks to Dr. Thuy Chastek and Dr. Thomas Chastek, my collaborators at NIST, who greatly contributed to complete this work. Thanks to Dr. Pranesh Aswath who improved my critical thinking and analytical skills. I would like to take opportunity to thank Maham Rahimi and Abhimanyu Sabnis who trained and guided me during my first steps in the world of nanomedicine and tissue engineering. I would like to express my deep gratitude to all my lab mates, in particular Soujanya Kona, Bhanuprasanth Koppolu, Khaushik Subramanian, Hao Xu, Danyel Specht, and Ghida Sleiman for their continuous support in the lab.

Lastly and most importantly, I thank my parents, Mr. Sharad Wadajkar and Mrs. Smita Wadajkar, for their unlimited love and support in all my endeavors. I thank my brothers, Shailesh Wadajkar and Vishal Wadajkar, for improving my personality and making me strong. Without their best wishes, I would not have succeeded in my life.

July 21, 2008

ABSTRACT

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The objective of this research project was to develop temperature sensitive poly(*N*-isopropylacrylamide) (PNIPA) nanoparticles for drug delivery applications. These nanoparticles were formulated by a radical polymerization method, and their toxicity was studied *in vitro* using MTS assays and various cell types such as fibroblasts, smooth muscle cells, and endothelial cells. Cytotoxicity results did not show a significant difference in cell survival when cells were exposed to different particle sizes (100, 300, and 500 nm). In addition, dose studies of 100 nm nanoparticles showed that the cells exposed to PNIPA nanoparticles at concentrations less than or equal to 5 mg/ml were biocompatible. Photomicrographs also showed that cells exposed to PNIPA nanoparticles maintained their normal morphology. Additionally,

we improved the biocompatibility of PNIPA nanoparticles using various Pluronic surfactants (L64, P65, P85, and F127) instead of the common surfactant, sodium dodecyl sulfate (SDS). The MTS assay results of free surfactant toxicity studies showed that Pluronic surfactants, especially F127 and P85, were more biocompatible than SDS over the studied range of concentrations. We also found that PNIPA nanoparticles synthesized using Pluronic were more biocompatible than those of SDS. Finally, drug release studies were done using Doxorubicin as a drug model. PNIPA nanoparticles synthesized using Pluronic surfactants produced higher amount of Doxorubicin release than PNIPA nanoparticles synthesized using SDS at 37^{o}C over the same period of time. In general, Pluronic P85 and F127 used as surfactants to formulate temperature sensitive PNIPA nanoparticles increase the biocompatibility drug release of PNIPA nanoparticles.

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CHAPTER 1

INTRODUCTION

1.1 Nanoscopic Drug Carriers

Most systemically administered drugs exert their biological effects not only at their target sites but also at non-target sites, which often results in undesired side effects and hampers their therapeutic potential [1]. In addition, drugs are administered several times over a time course for an effective treatment. These issues emphasizes the importance of drug delivery systems which could control the release of drugs over a time course and deliver biologically active compounds selectively to the pathological area. Thus, controlled and targeted drug delivery is of particular importance for the treatment of various diseases, leading to an increased interest to use synthetic polymers in the development of drug carriers for controlled and targeted drug delivery [2]. Polymers used as drug carriers usually show an improved pharmacokinetics with longer circulation time compared to drugs only. These carriers also provide the potential for cell/tissue targeting. Recently, drug carriers in a nanometer size is characterized by the term 'nanomedicine', which deals with polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, polymeric micelles, and polyplexes [2].

Extensive research for the ideal drug carrier system in last few decades has resulted in a great variety of nanoscopic drug carriers [3]. Nanoscopic drug carriers have, as their name implies, dimensions in nanometer and can be categorized into

particulate systems and water-soluble macromolecular systems. The first category includes liposomes [4], emulsions [5], nanoparticles [6], polymeric micelles [7] and polymeric vesicles [8]. The second category of nanoscopic drug carrier systems encompasses polymer-drug conjugates [9] and dendrimers [10]. Nanoscopic drug carriers can be administered intravenously, providing possibilities to reach the pathological sites while avoiding many biological barriers in the human body, such as limited gastrointestinal absorption and high hepatic first-pass effect of orally administered drugs. The size of nanoscopic drug carriers is an important factor which determines their biological fate [11]. In general, the size of the particles needs to be below the renal threshold (size $\leq$ 200 nm) ensuring that it is not accumulated in the body [12]. Also, generally nanoscopic drug carriers cannot be detected easily by reticular endothelial system as compared to microscopic drug carriers. Therefore, they remain in circulation for longer duration before clearing out by liver and kidneys [12]. Many drug carriers adopt a more active role such as releasing drugs and bioactive molecules including hormones, growth factors, peptides or oligo/poly(nucleic acid) upon an external stimulus using stimuli-responsive polymers in the formation process.

1.2 Stimuli-Responsive Polymers

Over the last decade, there has been much research activity in the development and use of pulsed and self-regulated drug delivery systems [13, 14]. In particular, stimuli-responsive polymer (SRP) systems that have potential applications in controlled drug delivery have been studied extensively [15]. The SRPs are also coined with

different names such as, smart polymers [16], intelligent polymers [17], and environmental-sensitive polymers [18]. The characteristic feature that makes them 'smart' is their ability to respond to very slight changes in the surrounding environment. The uniqueness of these materials lies not only in the fast macroscopic changes occurring in their structure but also the reversibility of these transitions [19]. As shown in Figure 1.1, the responses are manifested as changes in one or more of the following: dissolution/precipitation, degradation, drug release, hydration state, swelling/collapsing, shape, surface characteristics, solubility, and others [20]. The environmental trigger behind these transitions can be either changes in temperature [21], pH shift [22], an increase in ionic strength [22], a presence of certain metabolic chemicals [23], an addition of an oppositely charged polymer [24], polycation-polyanion complex formation [25], changes in an electric field [26], changes in a magnetic field [27], and light or radiation forces [28]. The physical stimuli, such as temperature, electric or magnetic fields, and mechanical stress alter molecular interactions inside the polymers at critical onset points. These polymers then undergo fast, reversible changes in microstructure from a hydrophilic to a hydrophobic state [29]. An appropriate proportion of hydrophobicity and hydrophilicity in the molecular structure of the polymer is required for the phase transition to occur [20].

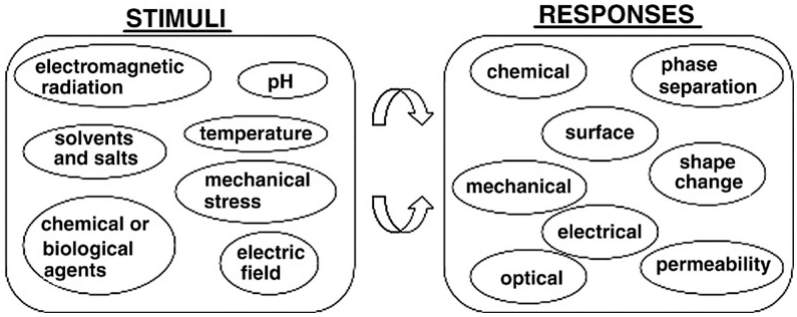


Figure 1.1 Potential stimuli and responses of synthetic stimuli-responsive polymers.

SRPs in solution can be conjugated with biomolecules or can be used as a carrier shell for drugs and bioactive molecules. These biological molecules are DNA plasmids, simple lipids/phospholipids, proteins/oligopeptides, sugars/polysaccharides, single-/double-stranded oligonucleotides, other recognition ligands, and synthetic drug molecules. The polymer-biomolecule hybrid system is capable of responding to biological, physical and chemical stimuli [20]. SRPs have been used in a large variety of bio-related applications such as drug delivery [18], bioseparation [19], chromatography [17], and cell culture [30]. Even though, there are systems, which show a linear response to an external stimulus, it is more interesting to study polymers with a non-linear behavior because biological systems also accomplish specific settings of environmental conditions in different parts of the body [2]. The lower critical solution temperature behavior of temperature sensitive polymers is a classic example of a non-linear behavior.

1.3 Temperature Sensitive Polymers

Temperature sensitive polymers have been investigated widely for biomedical applications, especially for controlled drug delivery, due to their phase transition behavior at physiological temperatures [12]. The temperature at which these polymers undergo a reversible phase transition is called as the cloud point or lower critical solution temperature (LCST) [31]. Polymers of this type are soluble in a solvent (water) at low temperatures and become insoluble as the temperature rises above the LCST. Phase transition of these polymers is controlled by a delicate balance between hydrophobic-hydrophilic conditions [32]. Below the LCST, water is bound to the hydrophilic moieties of the polymer and the presence of hydrated water prevents the interaction between different polymer chains as well as intra-polymer association. This means that below the LCST the polymer exists in a water-soluble form. Once temperature of the polymer solution is above the LCST, the hydrogen bonds between water molecules and the hydrophilic moieties in the polymer chain are disrupted and water is expelled from the polymer chains. As a result, interactions between the hydrophobic moieties of the polymer chain increase, which is associated with the collapse of the polymer, resulting in the aggregation/precipitation of the polymer. This conversion from soluble to insoluble form can be achieved by either reducing the number of hydrogen bonds which the polymer forms with water or by neutralizing the electric charges present on the polymeric network [12, 20, 31, 32].

Temperature sensitive polymers can be classified into different groups depending on the mechanism and chemistry of the groups. These are (a) poly(*N*-alkyl

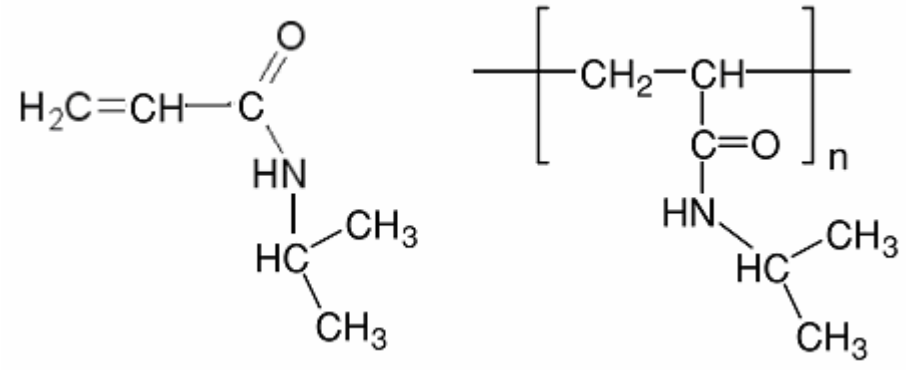
substituted acrylamides) e.g. poly(*N*-isopropylacrylamide) [2, 33] and (b) poly(*N*-vinylalkylamides) e.g. poly(*N*-vinylcaprolactam) [34]. Other types of temperature-responsive polymers include poly(ethylene oxide)$_{106}$-poly(propylene oxide)$_{70}$-poly(ethylene oxide)$_{106}$ co-polymer [35], which has the trade name Pluronic F127 (discussed in detail in chapter 3), and poly lactic acid-co-poly ethylene glycol-poly lactic acid (PLLA-PEG-PLLA) triblock copolymers [36]. Another interesting class of temperature sensitive polymers, which involves elastin like polymers (ELPs) e.g. oligo- and polypeptides [2, 37] have recently emerged. The specific LCST of all these different polymeric systems show potential applications in bioengineering and biotechnology. Yet only poly(*N*-isopropylacrylamide), among all temperature sensitive polymers, will be discussed in the next section.

1.4 Poly(*N*-isopropylacrylamide)

1.4.1. Introduction

Poly(*N*-isopropylacrylamide) (PNIPA) is the most extensively investigated and popular polymer among a variety of temperature sensitive polymers [38-40]. It is synthesized by a free radical polymerization of *N*-isopropylacrylamide (NIPA) monomers using potassium persulfate (KPS) as initiator. Cross-linkers and surfactants can be added to the polymerization reaction to form nanoparticles of PNIPA [41]. The PNIPA nanoparticle synthesis method uses various surfactants to obtain the particle stability, and the most common surfactant is sodium dodecyl sulfate (SDS) [41]. The chemical structures of NIPA monomer and PNIPA repeat unit are shown in Figure 1.2.

PNIPA has negative thermo-sensitivity, i.e. it shrinks with increasing temperature [27]. In contrast, polymers with positive thermo-sensitivity have upper critical solution temperature (UCST). UCST polymers are not in the scope of this report.



(a) (b)

Figure 1.2 Chemical structure of (a) NIPA monomer and (b) PNIPA

PNIPA undergoes a reversible phase transition at around 32°C (LCST) which is close to the physiological temperature [27]. Below the LCST, PNIPA is hydrophilic and swells by absorbing large quantities of water and water-soluble drugs, whereas above the LCST, PNIPA becomes hydrophobic, forms a cloudy solution, shrinks and expels the encapsulated materials [43, 44]. A molecular level explanation of the LCST phenomena involves a balance between hydrogen bonding and the hydrophobic effect. PNIPA has hydrophilic amide groups, and hydrophobic isopropyl pendant groups. At temperatures well below the LCST, the solution is stabilized by the presence of hydrogen bonds between the amide groups and the water, and by ice-like structures that water molecules form around the hydrophobic groups. As the temperature increases, the hydrogen bonding weakens, the pendant groups become mobile by increasing

attraction between the hydrophobic isopropyl groups, causing phase separation at the LCST [45, 46]. LCST of PNIPA can be easily varied by copolymerization with hydrophilic or hydrophobic monomers [47] and by addition of salts or surfactants to the polymer solution [48]. Since the LCST of PNIPA in water is slightly below body temperature and can be changed to or above physiological temperature, PNIPA has become a good candidate for pharmaceutical use and is widely used for designing temperature sensitive controlled drug delivery systems [43, 44, 47-49].

1.4.2. Applications of Temperature Sensitive PNIPA

1.4.2.1 Cell Culture and Cell Sheet Engineering

Because cells often attach to the hydrophobic surfaces and detach from the hydrophilic surfaces, PNIPA can be used to coat surfaces for cell culture and growth. The hydrophobic phase of PNIPA acts as an adhesive surface for cells and the hydrophilic phase acts as a releasing surface [50, 51]. As the temperature drops below 32^{o}C, the PNIPA chains rapidly hydrate causing the cells to detach from the surface [52]. This behavior is advantageous because it eliminates the need for enzymatic or mechanical detachment of cells allowing them to retain their morphology and function as shown in Figure 1.3. It has been found that enzymatic and mechanical detachment can disrupt the cell membrane and cause a change in cellular activity [53].

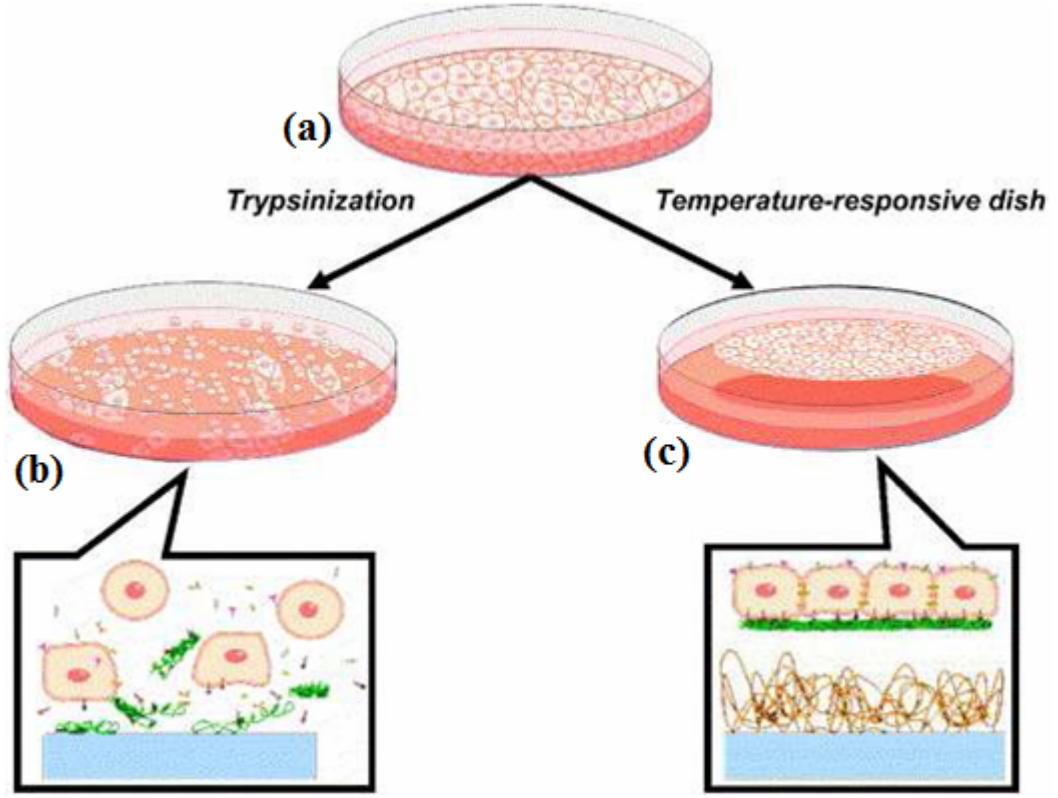


Figure 1.3 Temperature-responsive culture dishes [55]. (a) During culture, cells deposit extracellular matrix (ECM) molecules and form cell-to-cell junctions. (b) With typical proteolytic harvest by trypsinization, both ECM and cell-to-cell junction proteins are degraded for cell recovery. (c) Cells harvested from temperature-responsive dishes are recovered as intact sheet along with deposited ECM.

As mentioned above, PNIPA grafted surfaces have already been used for cell culturing, especially for tissue engineering and cell sheet engineering use. This type of engineering is advantageous because it yields cell sheets that retain their native extracellular matrix, which is responsible for the intrinsic adhesion [54]. Tissue reconstruction can be performed with single or multilayer cell sheets. Unlike biodegradable scaffolds used for tissue generation, cell sheets do not induce the foreign body response and result in more uniform cell growth along the entire length of the

construct [55]. Cell sheets detached from PNIPA grafted surfaces can be stacked in vitro and adhere quickly in vivo. PNIPA coated surfaces in cell sheet engineering can be modified with peptide sequences and can be used to grow co-culture systems and to form micropatterns [54, 55]. PNIPA can also be used for microencapsulation of islet cells [56].

1.4.2.2 Hyperthermia

Cancer cells are very sensitive to elevated levels of heat as cancer cells die and tumor sizes reduce upon exposure to prolonged heat, temperatures between 40-46^{o}C [57, 58]. The prolonged heating of the tissue in the range of 40-46^{o}C is known as hyperthermia, and it has been used for treatments of various cancer diseases. Different mechanisms such as protein coagulation membrane fluidity and nucleic acid modifications might be responsible for the cancer killing seen in hyperthermia [59, 60]. Temperature sensitive PNIPA micro/nanoparticles are a potential candidate for inducing drug release in combination with hyperthermia therapy. After intravenous injection, these particles can be targeted to the cancerous tissue. Heat provided in hyperthermia therapy would in turn act as a stimulus to the particles to release the drugs to the surrounding tissue. Hyperthermia also makes cancer cells more sensitive to anticancer therapies including anticancer drugs [61]. PNIPA particles would release anticancer drugs at these temperatures causing a significant reduction in the tumor size due to the combination of hyperthermia and drug activity.

1.4.2.3 Drug Delivery

For drug delivery applications, PNIPA has been used to make drug or protein loaded micro/nanoparticles, micelles, and hydrogels [62-64]. PNIPA nanoparticles offer several advantages in temperature dependent controlled drug delivery compared to conventional drug delivery methods. PNIPA nanoparticles can be used to treat various diseases. One such disease is cancer as these targeted carriers could reduce the side effects caused by the chemotherapeutic agents on the healthy tissue. This makes treatment more effective and less painful for the patient. After intravenous administration, drug carriers should accumulate at the tumor sites and deliver the drugs to the cancer cells due to the so-called enhanced permeability and retention (EPR) effect [65, 66]. The EPR effect is attributed to two factors. Firstly, the angiogenic tumor vasculature, as well as blood vessels in other pathological tissues, has a higher permeability compared to normal ones due to its discontinuous endothelium. Secondly, it has been shown that in tumors lymphatic drainage is not fully developed. These features lead to the fact that colloidal particles (e.g. PNIPA nanoparticles) extravasate through the "leaky" endothelial layer in tumor tissues and are subsequently retained there. After accumulating to the tumor site, PNIPA nanoparticles would release anticancer drugs in response to changes in the local temperature.

Another interesting application of PNIPA is in wound healing. PNIPA hydrogels are water based dressings which maintain a moist micro-environment at the wound site. These types of dressings are simple to apply and remove. They allow greater comfort and promote the cell migration. In addition, drug loaded PNIPA

nanoparticles can be incorporated in to the hydrogels to make nanoparticle composite hydrogels. A controlled and sustained release of drugs, in response to the temperature, can be achieved from such composite hydrogels on wound site [67, 68].

1.5 Objective of the Research Project

1.5.1. Specific Aims

As mentioned above, PNIPA nanoparticles have several attractive applications, especially in controlled drug delivery. The **goal** of this research project is to develop temperature sensitive nanoparticles and evaluate their biocompatibility for controlled drug delivery applications. **Two specific aims** were pursued to achieve this goal.

- Aim 1: Evaluate the cytotoxicity of the temperature sensitive poly(*N*-isopropylacrylamide) nanoparticles using various cell types.
- Aim 2: Improve the biocompatibility of the temperature sensitive poly(*N*-isopropylacrylamide) nanoparticles using Pluronic surfactants.

1.5.2. Successful Outcome of This Research Project

The successful **outcome** of this research project would provide an in depth understanding of the cytotoxicity of the *N*-isopropylacrylamide monomers and temperature sensitive poly(*N*-isopropylacrylamide) nanoparticles as a function of dialysis, dose, and incubation period. It is also expected that this study will help in designing poly(*N*-isopropylacrylamide) nanoparticles with controlled drug release profiles while maintaining their biocompatibility.

CHAPTER 2

CYTOTOXICITY EVALUATION OF THE TEMPERATURE SENSITIVE PNIPA NANOPARTICLES USING VARIOUS CELL TYPES

2.1 Introduction

Although PNIPA shows advantages in the controlled drug release in response to changes in the temperature, biocompatibility is still the major concern in the use of PNIPA nanoparticles as drug carriers. Some investigators have reported the cytotoxicity of PNIPA polymers in comparison with the cytotoxicity of corresponding monomers. For instance, Vihola et al. [44] have tested PNIPA polymers and NIPA monomers on two human carcinoma cell lines, intestinal Caco-2 and bronchial Calu-3. Cytotoxicity of various copolymers of PNIPA has also been studied in mice and on human cervix epithelioid cell (HeLa) lines [69-71]. In addition, Tanii and Hashimoto have studied the neurotoxicity of acrylamide and several related compounds using mice and rat models [72, 73]. Results from these studies indicate that PNIPA polymers are biocompatible, but NIPA monomers may have some toxic effects. However, these studies are concentrated on certain cell lines and animals for particular applications, and have not explored the effects of NIPA and PNIPA incubation periods on cell survival. In addition, they provide no data on the biocompatibility of PNIPA nanoparticles on cell types such as fibroblast cells, smooth muscle cells, and endothelial cells.

In general, it is important to evaluate the potential toxicity of PNIPA nanoparticles and NIPA monomers on different cell types before venturing into the public health area for various applications. Many studies have proposed the use of PNIPA nanoparticles in controlled drug delivery to treat various diseases such as restenosis, wound healing, and cancer [41, 74]. For *in vivo* applications, these PNIPA nanoparticles will be delivered via intravenous injection, and for wound healing applications, PNIPA nanoparticles will be applied on wound sites. Therefore, it is important to study the effects of PNIPA nanoparticles on cell types such as fibroblasts, smooth muscle cells and endothelial cells as they are predominately in contact with nanoparticles. The aim of this study is to extensively investigate the cytotoxicity of PNIPA nanoparticles in comparison with the NIPA monomers using three different cell types including fibroblast cells, smooth muscle cells, and endothelial cells. In this study, PNIPA nanoparticles were synthesized by a radical polymerization method using the most popular anionic surfactant, sodium dodecyl sulfate. We investigated the effects of nanoparticle sizes, concentrations of NIPA monomers and PNIPA nanoparticles, and incubation periods on the cell viability using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assays. The amount of NIPA monomers remaining on PNIPA nanoparticles after synthesis and dialysis was also determined using bromination and spectrophotometry methods.

2.2 Materials and Methods

2.2.1. Materials

N-isopropylacrylamide (NIPA, 97% Sigma-Aldrich), sodium dodecyl sulfate (SDS, 99% Sigma-Aldrich), *N,N′*-Methylenebisacrylamide (BIS, Sigma-Aldrich), potassium persulfate (KPS, 99+% Sigma-Aldrich), bromine (99.99+% Sigma-Aldrich), and carbon tetrachloride (CCl_4, 99% Sigma-Aldrich) were purchased and used without further purification. Cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA), whereas serum was purchased from HyClone (Logan, UT).

2.2.2. Synthesis of PNIPA Nanoparticles

PNIPA nanoparticles of three different sizes (100, 300, and 500 nm) were synthesized by a free radical polymerization reaction as reported previously by Ramanan et al. [41]. Briefly, for 100 nm particle size, 1.54 g of NIPA monomers was dissolved in 100 ml de-ionized (DI) water. 26.2 mg BIS and 43.8 mg SDS were added to the reaction flask while continuously stirring under argon gas for 30 minutes at room temperature. Initiator, KPS (62.4 mg), was then added to the solution. The reaction was heated to 70°C and stirred for 4 hours under the presence of argon gas. After 4 hours of reaction, the formed nanoparticle solution was dialyzed (molecular weight cutoff of 6000-8000 Da) for 3 days to remove free surfactants and un-reacted monomers. Dialyzed nanoparticle solution was lyophilized, and PNIPA nanoparticles of 100 nm diameter were collected. PNIPA nanoparticles of 300 nm and 500 nm sizes

were synthesized following the same method as described above by varying concentrations of the reagents as specified in Table 2.1.

Table 2.1 Reagents and Their Respective Amounts for Synthesizing Different Sizes of PNIPA Nanoparticles

Reagents	**PNIPA Nanoparticle Size**		
	100 nm	**300 nm**	**500 nm**
BIS (mg)	26.20	26.10	30.00
SDS (mg)	43.90	30.80	19.00
KPS (mg)	62.40	60.00	30.00
NIPA (g)	1.54	1.50	1.50

2.2.3. Characterization of PNIPA Nanoparticles

Transmission electron microscope (TEM, JEOL 1200 EX Model, JEOL) was used to determine the size and shape of the synthesized PNIPA nanoparticles. In general, samples were prepared by drop casting an aqueous dispersion of nanoparticles onto a carbon coated copper grid. The nanoparticles were stained with phosphotungstic acid (PTA) at a concentration of 0.01 wt% before observation. Measurements of nanoparticle sizes and size distributions were also performed in de-ionized water using dynamic light scattering (DLS) technology (Nanotrac 150, Microtrac. Inc.).

2.2.4. Fibroblast Cell and Human Aortic Smooth Muscle Cell Culture

Fibroblast cells (FCs, NIH/3T3) and human aortic smooth muscle cells (HASMCs, Cascade Biologics) were cultured in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% serum and 1% penicillin-streptomycin. Cells were incubated in a humid environment at 37°C and 5% CO_2. Upon 80-90% confluency, the cells were passaged or used for experiments. FCs up to passage 12 and HASMCs up to passage 9 were used in the experiments. For all the experiments, the cells were seeded in 48-well plates at a density of 5000 cells/well. After seeding, the cells were incubated at 37^{o}C in the 5% CO_2 humid environment for 2 days to allow cellular attachment and growth before further experiments.

2.2.5. Human Micro-Vascular Endothelial Cell Culture

Human micro-vascular endothelial cells (HMVECs, Cascade Biologics) were cultured in complete medium consisting of Medium 199 (M199) supplemented with 20% serum and 1% penicillin-streptomycin. Cells were incubated in a humid environment at 37°C and 5% CO_2. Upon 80-90% confluency, the cells were passaged or used for experiments. Cells up to passage 9 were used in the experiments. For all the experiments, the cells were seeded following the same method as for FCs and HASMCs.

2.2.6. Effects of PNIPA Nanoparticle Sizes on Cell Viability

The first cytotoxicity study was done to evaluate the effect of the PNIPA nanoparticle size on the cell survival. Cells were seeded and cultured as described above. PNIPA nanoparticles of 100, 300, and 500 nm sizes were sterilized under ultraviolet (UV) light for 30 minutes and then dissolved separately in respective cell complete media to obtain a final nanoparticle concentration of 2 mg/ml. Media containing PNIPA nanoparticles were added to the wells consisting of FCs, HASMCs, and HMVECs. Cells cultured in media without nanoparticles were used as controls. Cells were incubated with nanoparticle solutions for 6 hours and then cell survival was determined using colorimetric MTS assays (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega) and UV-Vis spectrophotometer (Infinite M200 plate reader, Tecan), following the manufacturer's instructions.

2.2.7. Effects of NIPA Monomer and PNIPA Nanoparticle Concentrations on Cell Viability

A dose dependent cytotoxicity study was done to evaluate the effects of varying concentrations of PNIPA nanoparticles and NIPA monomers on cell survival. Following the results of the nanoparticle size dependent cytotoxicity study, PNIPA nanoparticles of 100 nm size were used for the rest of the studies, after sterilizing, as described earlier. Nanoparticles and monomers were dissolved separately in respective complete media to obtain final concentrations of 0.1, 1, 5, and 10 mg/ml. Cells were incubated in these solutions for 6 hours and then cell survival was determined using

MTS assays. The cells incubated with nanoparticle- and monomer-free complete media served as controls.

2.2.8. Effects of NIPA Monomer and PNIPA Nanoparticles Exposure Duration on Cell Viability

To evaluate the effects of the exposure time, cells were exposed to PNIPA nanoparticles and NIPA monomers at various time points (6, 24, 48, and 96 hours). From the dose dependent cytotoxicity studies of PNIPA nanoparticles and NIPA monomers, a concentration of 1 mg/ml was chosen for this study. Cell survival was determined using MTS assays. The control wells consisted of cells incubated with nanoparticle- and monomer-free complete media.

2.2.9. Effects of NIPA Monomers and PNIPA Nanoparticles on Morphology of Cultured Cells

To visually observe the effects of the PNIPA nanoparticles and NIPA monomers on cell morphology, HMVECs were incubated with the respective media containing final concentrations of 5 mg/ml at 37°C for 12 hours. We selected HMVECs for this study since this cell type was the most sensitive one based on the results of dose and incubation time studies. Cells were then observed and photographed under an inverted microscope (Axiovert 40 CFL, ZEISS).

2.2.10. Determination of Un-reacted NIPA Monomers on PNIPA Nanoparticles

The un-reacted NIPA monomers remaining on the PNIPA nanoparticles after nanoparticle formation was assessed using spectrophotometry, as previously described [75]. In brief, serial dilutions of NIPA monomers were prepared in 1% (v/v) bromine in carbon tetrachloride (CCl_4), in order to determine the standard curve. Similar to standard NIPA monomer samples, PNIPA nanoparticle solution samples were mixed with bromine-CCl_4 solution. The absorbance was recorded at 540 nm wavelength and the amount of the NIPA monomers on PNIPA nanoparticles after the nanoparticle formation was determined against the standard curve.

2.2.11. Statistical Analysis

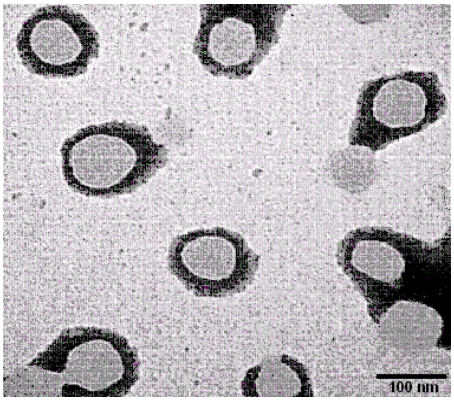
Analysis of the results was performed using t-tests with $p < 0.05$. For each study, the sample size was four (n=4), and all the results are presented as mean ± standard deviation (SD). Percentage cell survival was determined by dividing the absorbance reading of a cell sample by the representative control.

2.3 Results and Discussion

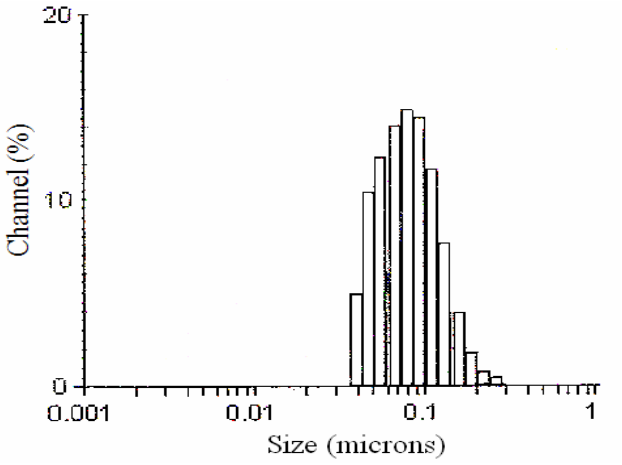
2.3.1. Characterization of PNIPA Nanoparticles

The average size of the PNIPA nanoparticles was analyzed using TEM. As an example, the 100 nm nanoparticles are illustrated in Figure 2.1 (a). The result indicates that the nanoparticles are spherical in shape. The black staining around the nanoparticles is the negative stain that was used to define the outer edge of the

nanoparticles due to the properties of hydrogel nanoparticles. Furthermore, the average size of the synthesized PNIPA was analyzed using DLS. The nanoparticle size distribution of 100 nm particles is shown in Figure 2.1 (b). The size analyzed by DLS was in the size range measured by TEM. Similar to previous studies [41, 76], we also observed that the nanoparticle size is inversely related to the amount of surfactants used.



(a)



(b)

Figure 2.1 Characterization of PNIPA nanoparticles: (a) TEM image of 100 nm diameter sized PNIPA nanoparticles and (b) PNIPA nanoparticle size distribution.

2.3.2. Effects of PNIPA Nanoparticle Sizes on Cell Viability

In our study, cells were exposed to PNIPA nanoparticles of different sizes (100, 300, and 500 nm) to determine the effects of nanoparticle sizes on cell survival. The effects of PNIPA nanoparticle sizes on the FC, HASMC, and HMVEC survival are shown in Figure 2.2. Exposure of these cell types to different sized PNIPA nanoparticles at 2 mg/ml concentration did not show a statistically significant difference in cell survival. This might be due to the size range of PNIPA nanoparticles was not very large (100 nm to 500 nm). Previous studies found that the uptake of nanoparticles by cells inversely depends upon the nanoparticle size [77]. Since smaller nanoparticles are better for cellular uptake [77], and there was no significant difference in cell survival over the studied range of PNIPA nanoparticle size, the PNIPA nanoparticles of 100 nm diameter size were chosen for further studies.

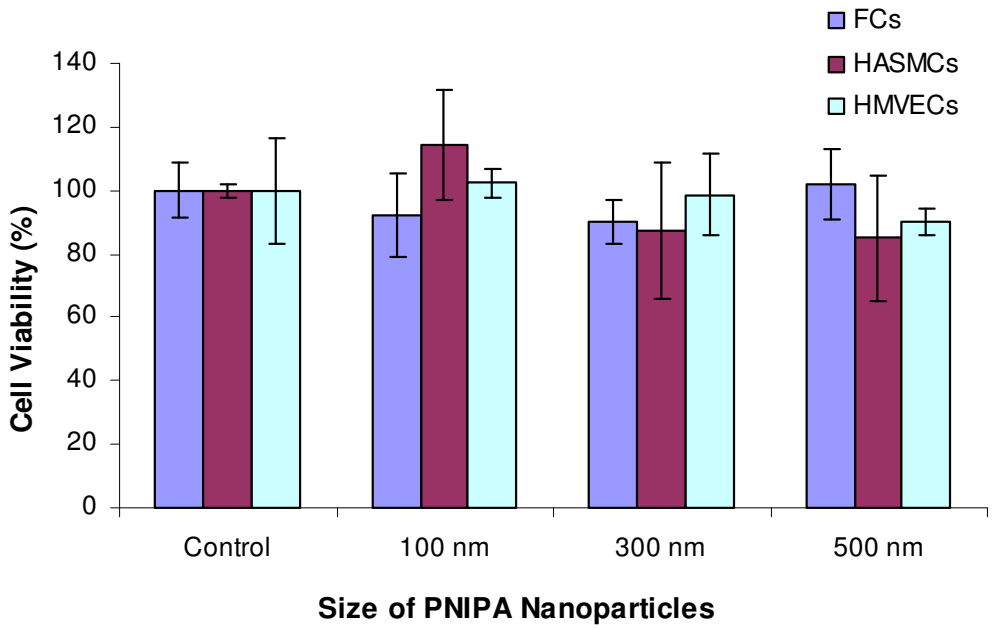


Figure 2.2 Effects of PNIPA nanoparticle sizes on cell survival.

2.3.3. Effects of NIPA Monomer and PNIPA Nanoparticle Concentrations on Cell Viability

FCs, HASMCs, and HMVECs were incubated with various NIPA monomer and PNIPA nanoparticle concentrations (0.1, 1, 5, and 10 mg/ml) to evaluate the effects of their concentrations on cell viability. The cytotoxic effects of the NIPA monomer and PNIPA nanoparticle concentrations on FCs, HASMCs, and HMVECs are shown in Figures 2.3, 2.4, and 2.5, respectively. PNIPA nanoparticles did not show a significant decrease in cell survival when FCs were exposed to all studied concentration solutions (Figure 2.3). NIPA monomers did not show a significant decrease in cell survival when FCs were exposed to 0.1, 1, and 5 mg/ml concentration solution. However, upon increasing monomer concentration above 5 mg/ml, a statistically significant decrease was noticed in the relative cell survival.

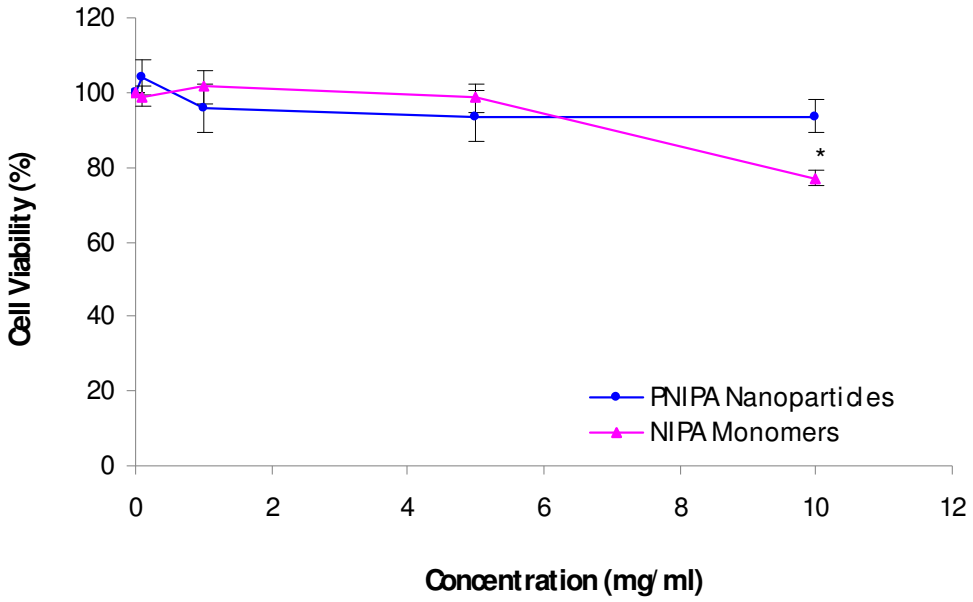


Figure 2.3 Effects of NIPA monomer and PNIPA nanoparticle doses on FC survival. * $p < 0.05$ as compared to control.

In the case of HASMCs, NIPA monomers and PNIPA nanoparticles showed a significant decrease in cell survival at concentrations above 5 mg/ml (Figure 2.4). Similar to HASMCs, there was no significant difference in cell survival when the HMVECs were exposed to NIPA monomer and PNIPA nanoparticle solutions of concentrations below 5 mg/ml compared to those of controls. However, monomer and nanoparticle concentrations above 5 mg/ml showed a statistically significant decrease in cell survival (Figure 2.5). We also observed that cell survival was cell type-dependent. HMVECs showed the highest cell mortality compared to HASMCs and FCs when cells were exposed to NIPA monomers and PNIPA nanoparticles. Finally, the important observation in all three cell types was that PNIPA nanoparticles were more biocompatible compared to NIPA monomers.

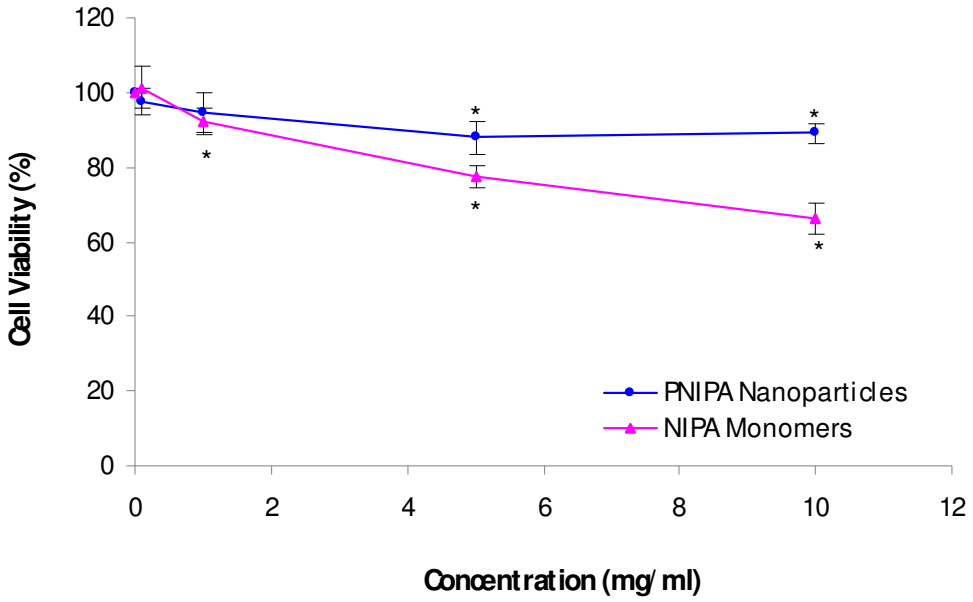


Figure 2.4 Effects of NIPA monomer and PNIPA nanoparticle doses on HASMC survival. * $p < 0.05$ as compared to control.

Similar to our observation with PNIPA nanoparticles, Vihola et al. [44] found that the survival of human carcinoma cell lines (intestinal Caco-2 and bronchial Calu-3) was higher with cells exposed to PNIPA polymers (concentration range from 0.01 to 10 mg/ml) than those with NIPA monomers at the same concentration range. Cross-linked surface structure of PNIPA polymers might have reduced the cytotoxicity as suggested by this study [44].

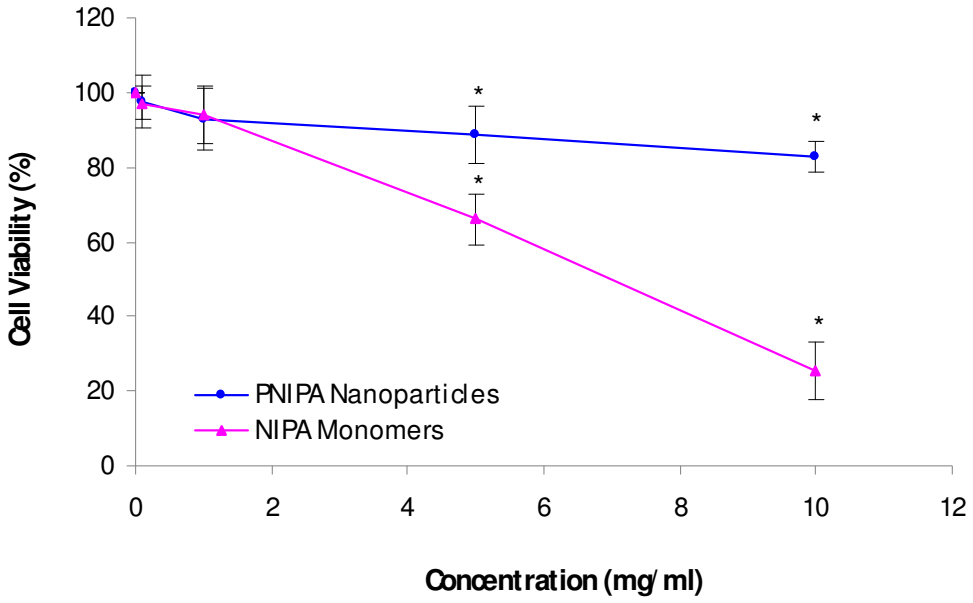


Figure 2.5 Effects of NIPA monomer and PNIPA nanoparticle doses on HMVEC survival. * $p < 0.05$ as compared to control.

2.3.4. Effects of NIPA Monomer and PNIPA Nanoparticle Exposure Duration on Cell Viability

The cytotoxic effects due to different incubation periods of NIPA monomer and PNIPA nanoparticle solutions are shown in Figure 2.6. For FCs and HASMCs, the viability of cells exposed to longer time periods (24, 48, and 96 hours) was similar to those of 6 hours for concentration of 1 mg/ml (results not shown). For these cell types,

the incubation period does not affect the cell behavior. In contrast, the incubation period does affect HMVECs. Longer exposure (48 and 96 hours) to NIPA monomers reduced the cell survival, whereas exposure to PNIPA nanoparticles did not show a significant decrease in cell survival until 96 hours. Weng et al. [76] found that PNIPA nanoparticles triggered lesser inflammatory and fibrotic responses among tested nanoparticles (e.g. poly-L-lactic acid, polystyrene, and hydroxypropyl cellulose) in mice models after 96 hours. Our observation and results from previous studies reveal the potential of the PNIPA nanoparticles for controlled drug delivery systems due to their biocompatibility.

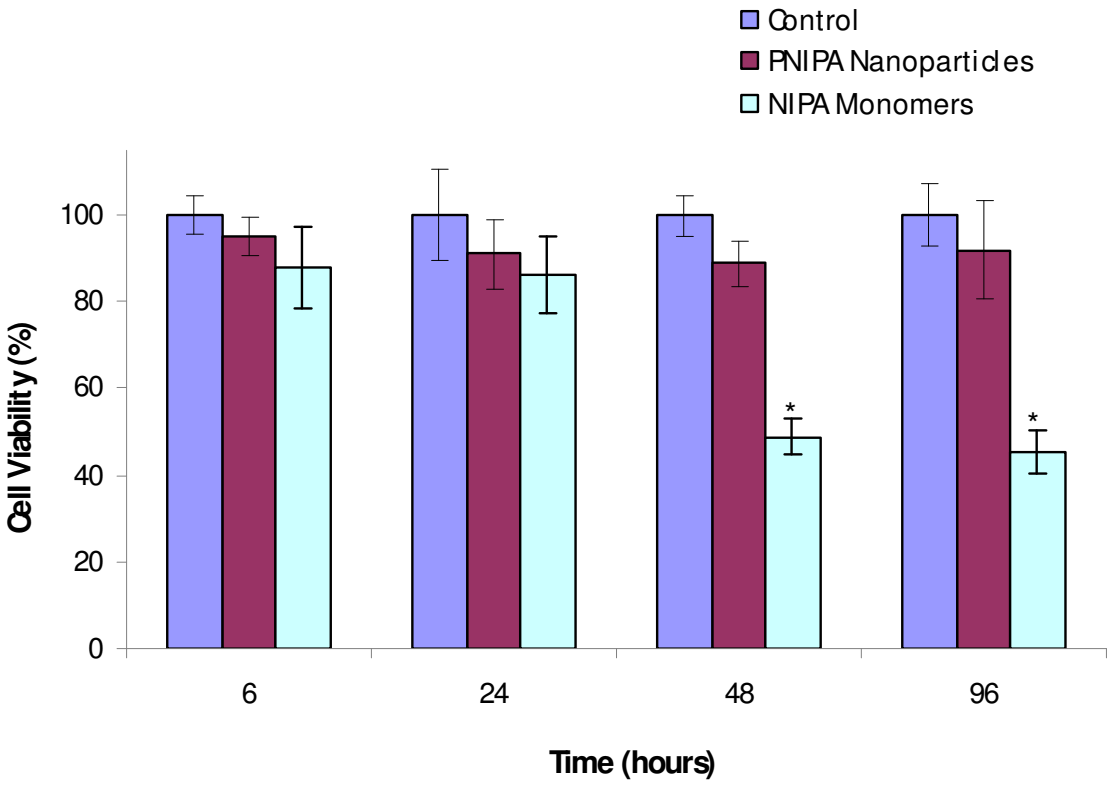


Figure 2.6 Effects of exposure duration toward NIPA monomers and PNIPA nanoparticles on HMVEC survival. * $p < 0.05$ as compared to control.

2.3.5. Effects NIPA Monomers and PNIPA Nanoparticles on Morphology of Cultured Cells

After exposure to either NIPA monomers or PNIPA nanoparticles, cells were observed for change in their morphology. Figure 2.7 shows the phase contrast photographs of HMVECs after exposure. The control cells and the cells exposed to media containing PNIPA nanoparticles did not show apparent differences in cell morphology (Figures 2.7 (a) and (c)). However, cells exhibited spherical morphology when they were exposed to media containing NIPA monomers (Figure 2.7 (b)). Takezawa et al. [78] found the same cell behavior when dermal FCs were exposed to NIPA monomer and PNIPA solutions. These results confirm that the NIPA monomers are cytotoxic, whereas PNIPA nanoparticles exhibited cytocompatibility similar to that of the controls at the studied ranges.

Figure 2.7 HMVECs morphology after exposure to (a) media only, (b) NIPA monomers, and (c) PNIPA nanoparticles.

2.3.6. Determination of Un-reacted NIPA Monomers on PNIPA Nanoparticles

The amount of NIPA monomers left on PNIPA nanoparticles was determined by bromination. A very small amount of NIPA monomers was left on nanoparticles after the formation process. Even at the highest concentration (10 mg/ml) in our studies,

PNIPA nanoparticles showed only 0.44 ± 0.01 mg/ml NIPA monomers left over after the nanoparticle synthesis and dialysis. Amount of NIPA monomers detected was negligible since dialysis removes most of the un-reacted monomers from the PNIPA nanoparticle solution.

2.4 Conclusion

In this study, the cytotoxicity of PNIPA nanoparticles was compared with NIPA monomers on different cell types. Our results showed that the studied PNIPA nanoparticle size range did not affect the cell survival. In addition, PNIPA nanoparticles were cytocompatible, whereas NIPA monomers were cytotoxic even at low concentrations. Cells exposed to nanoparticles for longer durations survived well similar to those of the controls. In contrast, cells exposed to the same concentrations of monomers showed a decrease in cell survival with exposure time. Finally, the un-reacted NIPA monomer concentration on the nanoparticles after the formation process was found to be negligible. These results are indicative of biocompatibility of PNIPA nanoparticles for controlled drug delivery applications.

CHAPTER 3

IMPROVEMENT OF THE BIOCOMPATIBILITY OF PNIPA NANOPARTICLES USING PLURONIC SURFACTANTS

3.1 Introduction

PNIPA nanogel is synthesized by a radical polymerization reaction [41]. The necessary reagents for this reaction include monomers, cross-linkers, initiators and surfactants. Among them, the surfactant stabilizes nanoparticles and controls their size [41, 79]. Sodium dodecyl sulfate (SDS), an anionic surfactant, is a widely used surfactant for PNIPA as well as other types of nanoparticles. However, this surfactant is the most toxic reagent in the reaction. The nanoparticle size decreases as SDS concentration increases [41]. However, the induced SDS amount causes the increase in the toxicity of the nanoparticles. Thus there is a need to find a replacement for SDS with biocompatible surfactants.

Pluronic, a non-ionic surfactant, has been of great interest nowadays. Pluronic is relatively non-toxic and has been approved by Food and Drug Administration (FDA). It is a block copolymer terminating in primary hydroxyl groups and can be used as a surfactant. Pluronic block copolymers, also termed as 'Poloxamer' or 'Synperonic', consist of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a triblock structure: EOx–POy–EOx. This arrangement makes it an amphiphilic copolymer. The number of hydrophilic EO (x) and hydrophobic PO (y) units can be altered to vary the size, hydrophilicity and lipophilicity [80-84]. Figure 3.1 shows the structure formula of

Pluronic block copolymers. Copolymers with various *x* and *y* values are characterized by distinct hydrophilic–lipophilic balance (HLB). As EO contain decreases, HLB decreases and hydrophobicity increases [80].

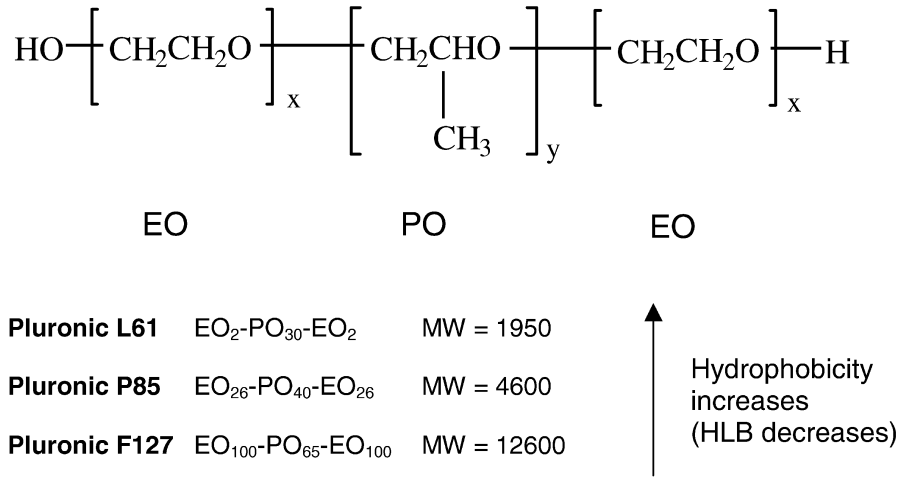


Figure 3.1 Chemical structure of Pluronic block copolymers contain two hydrophilic EO blocks and a hydrophobic PO block.

Pluronic block copolymers have a unique nomenclature. It includes one letter, 'F', 'P', or 'L', followed by a 2 or 3 digit numeric code. The letters stand for solid (F), paste (P) or liquid (L). It defines physical state of the Pluronic. The numeric code defines the structural parameters of the block copolymer. The last digit of the code represents weight content of EO block in tens of weight percent (for example, if the digit is 4, 40% wt). The remaining first one or two digits stand for molecular mass of the central PO block. The digit should be multiplied by 300 to obtain the molecular mass in Da. For example, the code 'F127' defines the block copolymer, which is a solid, has a PO block of 3600 Da (12×300) and 70% wt of EO [80].

A defining property of Pluronic is the ability of individual block copolymer molecules to self-assemble into micelles in aqueous solutions [83]. Pluronic micelles containing hydrophilic low molecular mass drugs and polypeptides are being actively investigated as potential drug delivery systems. They are also useful as surfactants [80-84]. Aim of this study was to investigate the biocompatibility of SDS and various Pluronic surfactants and to synthesize the PNIPA nanoparticles using these surfactants. We also studied the effects of surfactants, and compared the effects of non-dialyzed and dialyzed PNIPA nanoparticles synthesized using various surfactants on fibroblast cell viability using MTS assays. In addition, we compared drug release profiles of Doxorubicin from dialyzed PNIPA nanoparticles at 37^{o}C.

3.2 Materials and Methods

3.2.1. Materials

The chemicals, if not specified, were purchased from Sigma-Aldrich (St. Louis, MO) including *N*-isopropylacrylamide (NIPA), sodium dodecyl sulfate (SDS), *N,N′*-Methylenebisacrylamide (BIS), and potassium persulfate (KPS). Pluronic L64 (PL64), Pluronic P65 (PP65), Pluronic P85 (PP85), and Pluronic F127 (PF127) were kindly provided by National Institute of Standards and Technology (NIST).

3.2.2. *Synthesis of PNIPA Nanoparticles*

PNIPA nanoparticles of approximately 250 nm size were synthesized using SDS and various Pluronic surfactants by a free radical polymerization reaction as described

elsewhere [41]. Briefly, for PNIPA nanoparticles using SDS surfactant (PNIPA-SDS), 1.5 g of NIPA was dissolved in 100 ml de-ionized (DI) water. 26.1 mg of BIS and 30.8 mg SDS were added to the reaction flask while continuously stirring under argon gas for 30 minutes at room temperature. Initiator, KPS (60 mg), was then added to the solution. The reaction was stirred for 4 hours at 70°C under the presence of argon gas. After 4 hours of reaction, the formed nanoparticle solution was dialyzed (molecular weight cutoff of 6000-8000 Da) for 3 days to remove free surfactants and un-reacted monomers. Dialyzed nanoparticle solution was lyophilized, and PNIPA-SDS nanoparticles were collected. PNIPA nanoparticles using Pluronic surfactants (PNIPA-PL64, PNIPA-PP65, PNIPA-PP85, and PNIPA-PF127) were synthesized following the same method as described here by varying the concentration of the reagents as specified in Table 3.1.

Table 3.1 Reagents and Their Respective Amounts for Synthesizing Different PNIPA Nanoparticles

Reagents	**PNIPA Nanoparticles Using**				
	SDS	**PL64**	**PP65**	**PP85**	**PF127**
BIS (g)	0.026	0.037	0.034	0.039	0.049
Surfactant (g)	0.031	0.196	0.197	0.111	0.300
KPS (g)	0.060	0.102	0.122	0.112	0.112
NIPA (g)	1.500	1.564	1.665	1.632	1.632

3.2.3. Characterization of PNIPA Nanoparticles

Transmission electron microscope (TEM) was used to determine the size and shape of the synthesized PNIPA nanoparticles. In general, samples were prepared by drop casting an aqueous dispersion of nanoparticles onto a carbon coated copper grid. The nanoparticles were negatively stained with 1% uranyl acetate before observation. Measurements of nanoparticle sizes and size distributions were also performed using dynamic light scattering (DLS) technology.

3.2.4. Effects of Free Surfactants on Cell Viability

To evaluate the effects of free surfactants on fibroblast cell survival, cells were cultured and seeded in a 48-well plate. SDS, PL64, PP65, PP85, and PF127 were sterilized under ultraviolet (UV) light for 30 minutes and then dissolved in cell complete media to obtain final concentrations of 0.54, 1.62, 4.87, 14.63, and 43.9 mg/ml. Cells were incubated with surfactant solutions for 6 hours. Cells cultured in media without surfactants were used as control. Cell survival was determined using colorimetric MTS assays following the manufacturer's instructions.

3.2.5. Effects of Non-dialyzed and Dialyzed PNIPA Nanoparticles on Cell Viability

To determine the necessity of particle dialysis, non-dialyzed and dialyzed (3 days) PNIPA-SDS nanoparticles were chosen for the study. Nanoparticles were sterilized under UV light for 30 minutes and then dissolved in cell complete media to obtain final concentrations of 5 and 20 mg/ml. The nanoparticle solutions were added

to the wells containing cells. Cells were incubated for 6 hours and then cell survival was determined using MTS assays. The cells incubated with complete media only served as controls.

3.2.6. Biocompatibility Studies of Various PNIPA Nanoparticles

Upon analyzing the results of effect of dialysis study, all types of PNIPA nanoparticles (synthesized using SDS and Pluronic surfactants) were dialyzed for 3 days to get rid of free surfactants and un-reacted monomers. A dose dependent cytotoxicity study was done to evaluate the effect of nanoparticles on cell survival. Nanoparticles, sterilized as described earlier, were dissolved in cell complete media to obtain final concentrations of 1 and 5 mg/ml. Cells were incubated in nanoparticle solutions for 6 hours and then cell survival was determined using MTS assays.

3.2.7. LCST Determination

3.2.7.1 UV-Vis Spectrophotometry

The LCST of PNIPA nanoparticles was determined using an UV-Vis spectrophotometer coupled with a temperature controller as described elsewhere [85]. 200 µl of PNIPA nanoparticle solution samples (n=4) were used for the experiment. The samples were heated from 22^{o}C to 41^{o}C. Absorbance was recorded at 500 nm wavelength at several temperatures. The absorbance values were then converted into percentage of transmittance and a graph of percentage of transmittance versus temperature was plotted.

3.2.7.2 Differential Scanning Calorimetry

The LCST of PNIPA nanoparticles was also determined by differential scanning calorimetry (DSC). The DSC measurements were carried out using a differential scanning calorimeter (Perkin-Elmer 7 series, DSC 7). The PNIPA nanoparticle solution was placed in aluminium hermetic sealed pans and the nanoparticle solution was heated at a rate of 1°C/min. A graph, heat flow versus temperature, was generated by DSC. The peak in the curve represents the LCST of the PNIPA nanoparticles.

3.2.7.3 Visual Observation

The LCST and the turbidity (change in the color of solution) was observed by naked eyes. PNIPA nanoparticle solution was taken in 2 different glass tubes. One tube was kept at room temperature and the other was heated above the LCST of PNIPA. Change in the color (cloudiness or turbidity) was observed by naked eyes. The temperature at which solution became turbid was noted as LCST of the PNIPA nanoparticles.

3.2.8. Drug Loading

Doxorubicin, trade name Adriamycin or Hydroxydaunorubicin, an anticancer drug, was used to load into the PNIPA nanoparticles. Doxorubicin is known to interact with DNA by intercalation. This inhibits the progression of the enzyme topoisomerase II, which unwinds DNA for transcription. Doxorubicin stabilizes the topoisomerase II

complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication [86].

From the results of nanoparticle biocompatibility study, only PNIPA-PP85 and PNIPA-PF127 nanoparticles were chosen for drug release study as they were found to be the most biocompatible nanoparticles among all tested nanoparticles. Drug release profiles of these nanoparticles were compared with drug release profile of PNIPA-SDS nanoparticles. To load the Doxorubicin into the PNIPA nanoparticles, 1.5 mg of Doxorubicin and 7.5 mg of PNIPA nanoparticles were dissolved in phosphate buffer solution (PBS). The solution was incubated at 4^{o}C on a shaker for 3 days. The Doxorubicin loaded nanoparticle solution was then transferred to dialysis bag (molecular weight cut-off of 10 kDa) and dialyzed against PBS for 3 hours at 4^{o}C to separate un-loaded Doxorubicin from the solution. The dialysate was then read and analyzed using UV-Vis spectrofluorometer (λ_{ex} 470 nm and λ_{em} 585 nm) to determine the amount of un-loaded Doxorubicin. This value was then compared with the initial amount of Doxorubicin to determine the loading efficiency of the nanoparticles. Loading efficiency was calculated using following formula:

$$\text{Loading Efficiency} = \frac{\text{Initial amount of doxorubicin used - Doxorubicin present in dialysate}}{\text{Initial amount of doxorubicin used}} \times 100\%$$

3.2.9. Drug Release Study

To study the Doxorubicin release over time, Doxorubicin loaded PNIPA nanoparticles were dialyzed against PBS at 37^{o}C. At predetermined time intervals, 1 ml of dialysate was removed and stored at -20^{o}C for later analysis. 1 ml of fresh PBS was added to reconstitute the dialysate volume. After all samples were collected, the dialysate samples were read at excitation wavelength of 470 nm and emission wavelength of 585 nm using an UV-Vis spectrofluorometer. The concentration of Doxorubicin in the dialysate samples were calculated against the Doxorubicin standard samples. Percentage cumulative release kinetics of Doxorubicin was plotted as a function of time.

3.2.10. Statistical Analysis

Analysis of the results was performed using t-tests with $p < 0.05$. For each study, the sample size was four (n=4), and all the results are presented as mean ± standard deviation (SD). Percentage cell survival was determined by dividing the absorbance reading of a cell sample by the representative control.

3.3 Results and Discussion

3.3.1. Characterization of PNIPA Nanoparticles

The size and morphology of the PNIPA nanoparticles was analyzed using TEM. From Figure 3.2, it was observed that the nanoparticles were spherical in shape. Their average size was measured to be 150-200 nm. The black color on the nanoparticles is

the negative stain that was used to emphasize the PNIPA nanoparticles. Furthermore, the average size and distribution of the PNIPA was analyzed using DLS. The size distribution of the particles is shown in Figure 3.3.

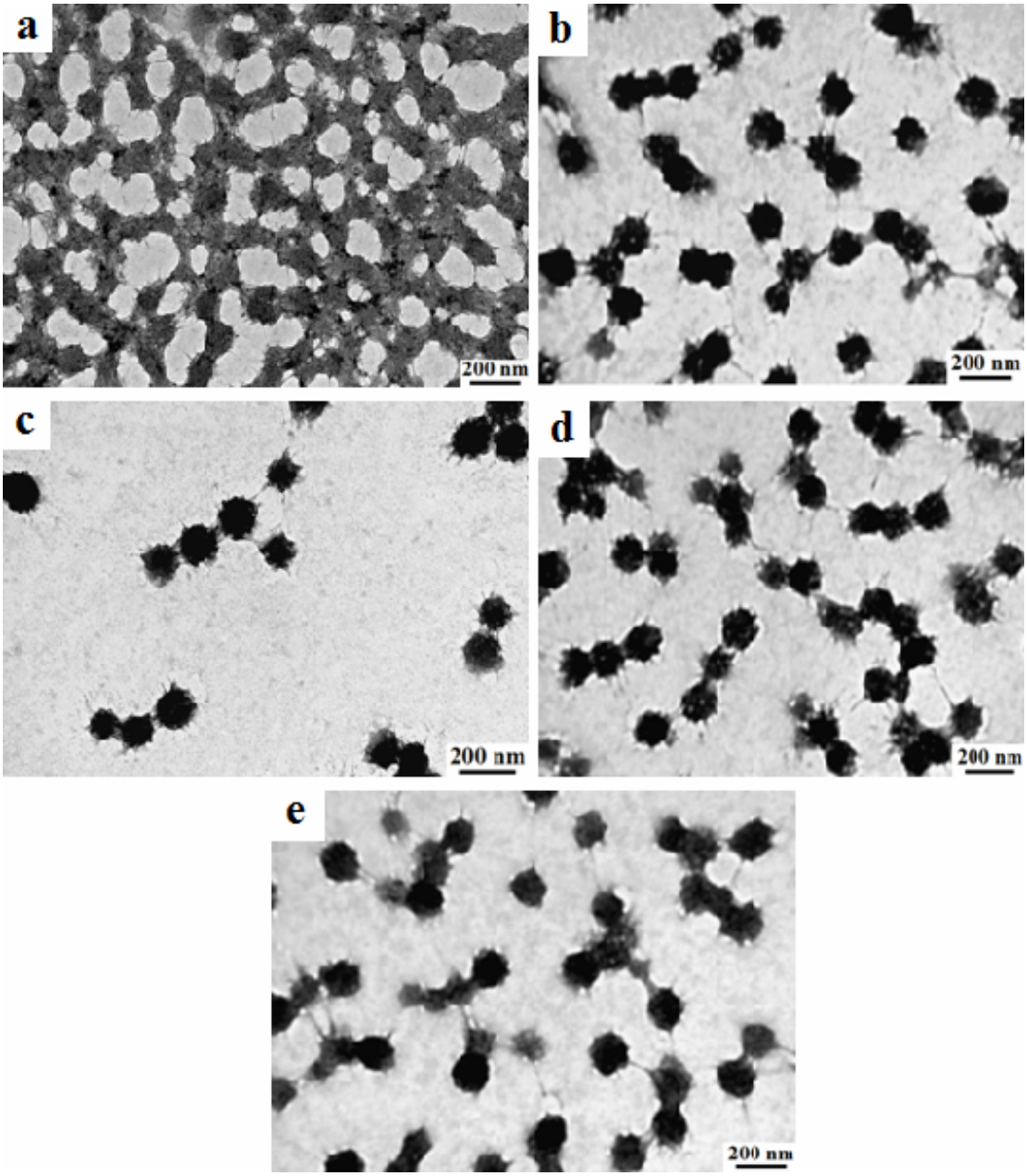


Figure 3.2 TEM image of (a) PNIPA-SDS, (b) PNIPA-PL64, (c) PNIPA-PP65, (d) PNIPA-PP85, and (e) PNIPA-PF127 nanoparticles.

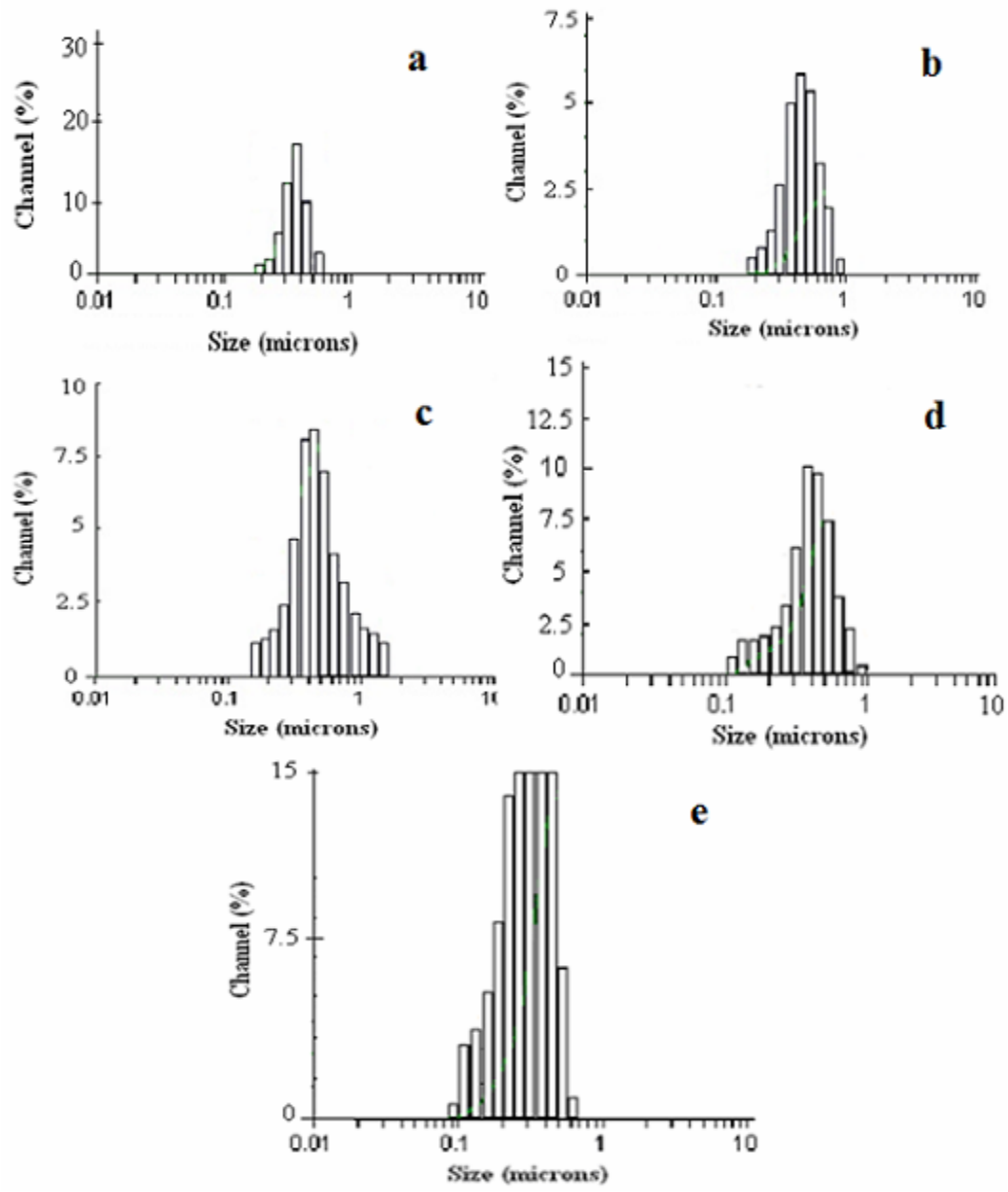


Figure 3.3 Size distribution of (a) PNIPA-SDS, (b) PNIPA-PL64, (c) PNIPA-PP65, (d) PNIPA-PP85, and (e) PNIPA-PF127 nanoparticles.

The PNIPA nanoparticle sizes obtained from TEM and DLS were compared in Table 3.2. The nanoparticle sizes measured by DLS were found to be slightly greater

than sizes measured by TEM. However, DLS measurements have large standard deviation which overlaps the results obtained from TEM measurements.

Table 3.2 PNIPA Nanoparticle Size Measured by DLS and TEM

Nanoparticle Type	**Nanoparticle Size (nm)**	
	DLS	**TEM**
PNIPA-SDS	266.7 ± 119	179.3 ± 12
PNIPA-PL64	289.3 ± 137	168.0 ± 15
PNIPA-PP65	263.3 ± 113	168.8 ± 13
PNIPA-PP85	261.7 ± 110	172.0 ± 07
PNIPA-PF127	258.3 ± 112	183.5 ± 11

3.3.2. Effect of Free Surfactants on Cell Viability

Cells were incubated with various concentrations (0.54, 1.62, 4.87, 14.63, and 43.9 mg/ml) of free surfactants to evaluate their effects on cell survival. The results of the fibroblast cell survival are shown in Figure 3.4. SDS was found highly toxic while all Pluronic surfactants were more biocompatible than SDS at all of the studied concentrations. In addition, PL64 and PP65 were found to be least biocompatible among Pluronic surfactants by exhibiting cytotoxicity even at low concentrations of 1.62 and 4.87 mg/ml, respectively. PP85 was more biocompatible than PL64 and PP65 (at high concentrations). PF127 showed the highest percentage of cell survival, thereby being the most biocompatible surfactant among all.

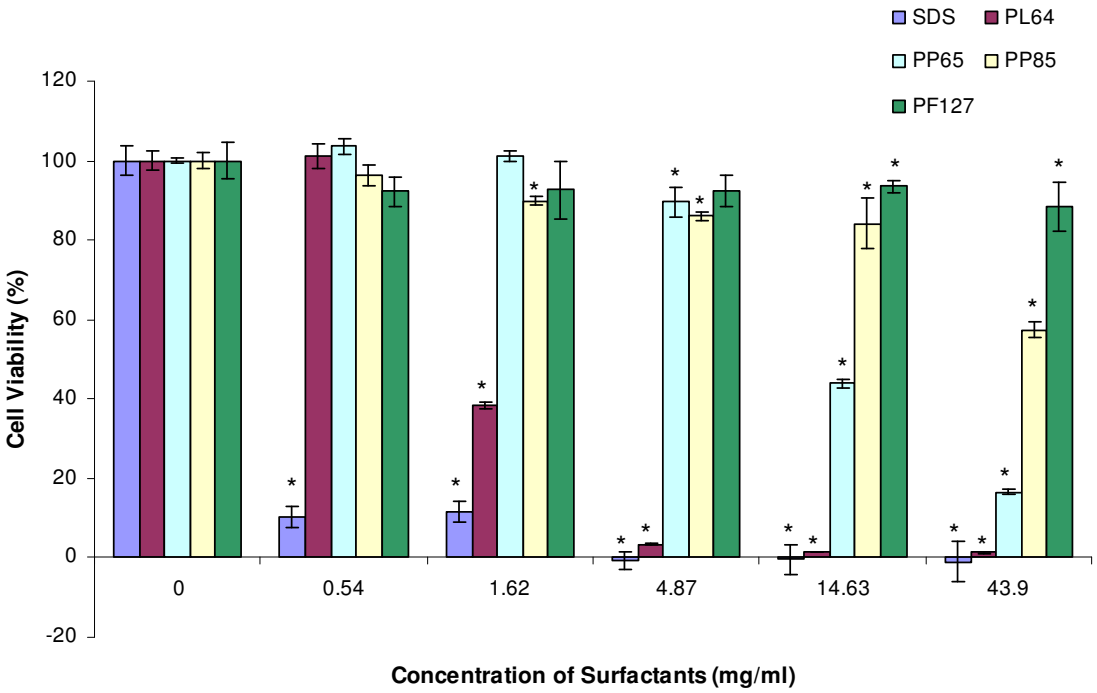


Figure 3.4 Effects of free surfactant dose on cell viability. * $p < 0.05$ as compared to control.

A graph of percentage of EO versus percentage of cell viability was also plotted to understand the effect of EO weight content on the cell survival (Figure 3.5). It was observed that the cell survival was increased upon increasing EO content. As described earlier, PL64 contains 40%, PP65 and PP85 contains 50%, and PF127 contains 70% wt of EO. Upon exposure to 70% wt of EO, percentage of cell survival was highest indicating that PF127 was the most biocompatible surfactant. Cell survival decreased consecutively in 50% and 40% wt of EO, indicating PL64 as the most cytotoxic surfactant among tested Pluronic surfactants. EO is the hydrophilic component of the Pluronic block copolymer and is used as food sterilizing agent and a chemical

intermediate in the production of ethylene glycol [87, 88]. Kier et. al. [89] tested 8 types of EO/PO copolymers and similar to our observation, they found PL64 as the most cytotoxic compound having the least percentage of EO weight content.

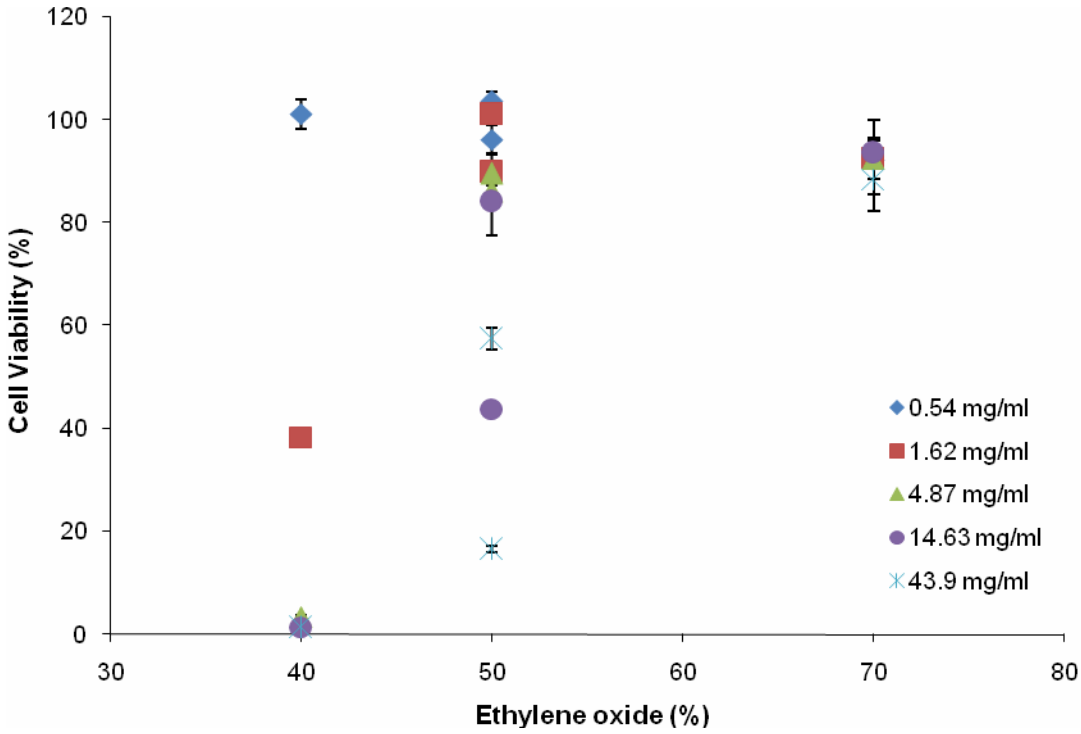


Figure 3.5 Effects of EO weight content on cell viability.

3.3.3. Effects of Non-dialyzed and Dialyzed PNIPA Nanoparticles on Cell Viability

To study the effect of dialysis of nanoparticles on cell survival, cells were incubated with 5 and 20 mg/ml concentration of non-dialyzed and dialyzed PNIPA-SDS nanoparticles. Cell survival is shown in Figure 3.6. Non-dialyzed nanoparticles decreased cell survival when compared to both controls and dialyzed nanoparticles at both the concentrations. Cell survival when cells were exposed to non-dialyzed

nanoparticles was lesser than that of dialyzed nanoparticles at a low concentration of 5 mg/ml. At a higher concentration (20 mg/ml) of non-dialyzed nanoparticles, the cell survival was extremely reduced. Dialysis removes free surfactants and un-reacted monomers from the solution [41]. These reagents might be present in non-dialyzed nanoparticles solutions exhibiting more cytotoxicity compared to dialyzed nanoparticles and controls. Thus, dialysis is an important step to reduce the toxicity of PNIPA nanoparticles.

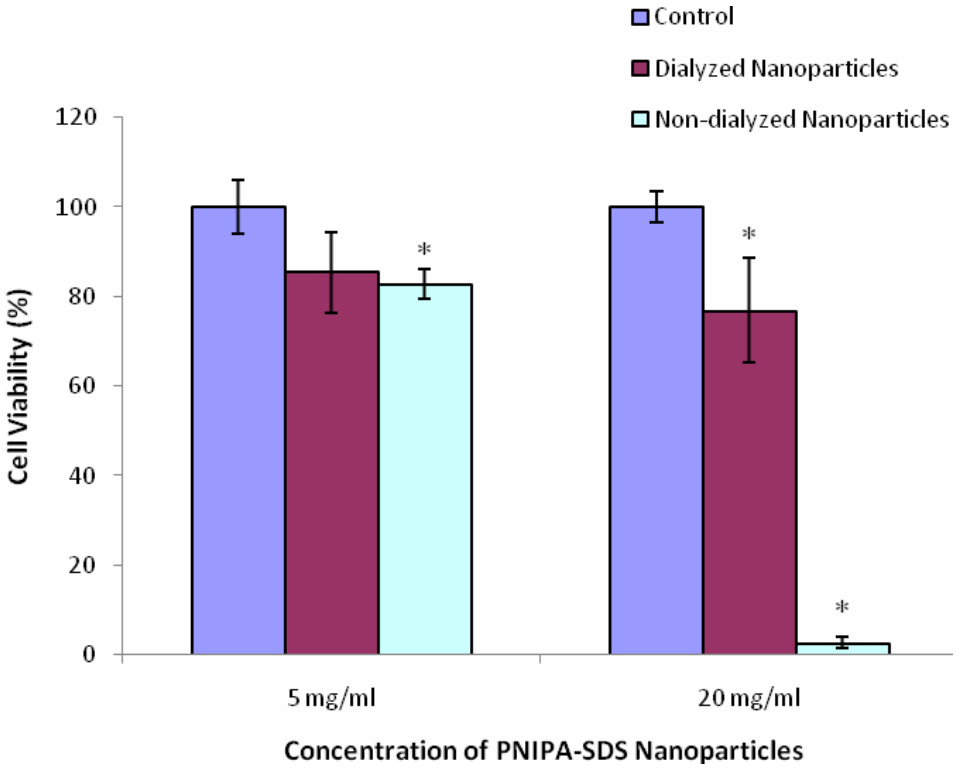


Figure 3.6 Effect of PNIPA-SDS nanoparticle dialysis on cell viability. * $p < 0.05$ as compared to control.

3.3.4. Biocompatibility Studies of Various PNIPA Nanoparticles

To compare the biocompatibility of various PNIPA nanoparticles, cells were incubated with 1 and 5 mg/ml concentration of dialyzed PNIPA nanoparticles. Cell survival in PNIPA nanoparticles, synthesized using Pluronic surfactants, was compared with cell survival in PNIPA-SDS nanoparticles for the statistical significance. PNIPA-PF127 nanoparticles were the most biocompatible nanoparticles followed by PNIPA-PP85 at both the concentrations (Figure 3.8).

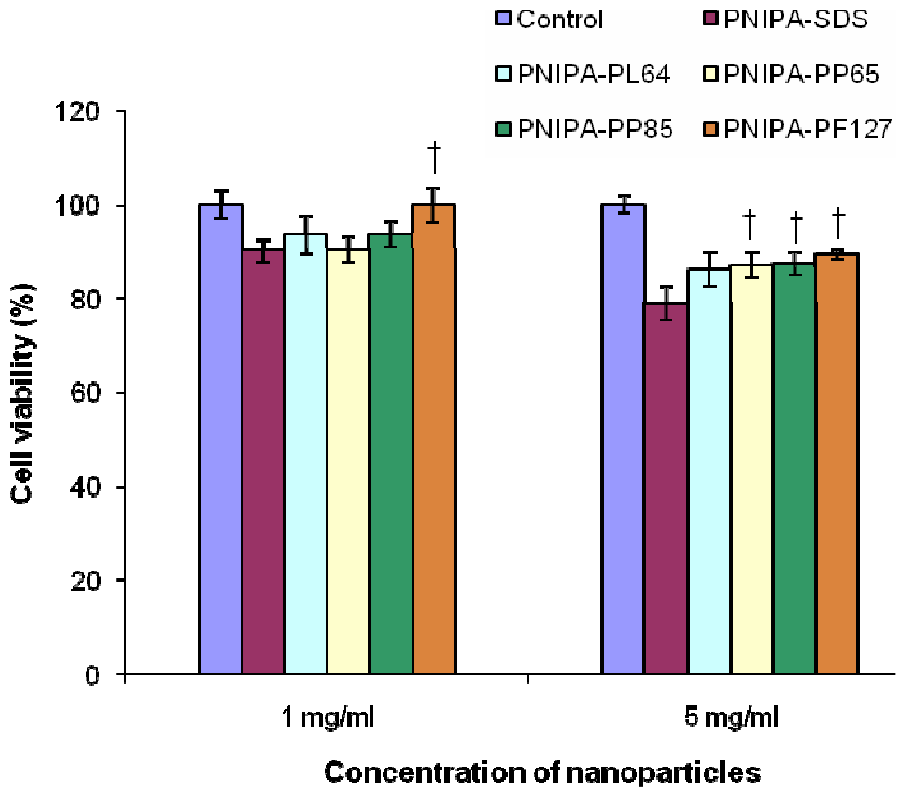


Figure 3.7 Effects of dialyzed PNIPA nanoparticles on cell viability. † $p < 0.05$ as compared to PNIPA-SDS.

Cell survival in PNIPA-PF127 nanoparticles was significantly higher than in PNIPA-SDS nanoparticles at both the concentrations. In addition, cell survival in PNIPA-PP85 nanoparticles was significantly higher than in PNIPA-SDS nanoparticles at 5 mg/ml concentration. On the other hand, cell survival in PNIPA-PL64 and PNIPA-PP65 nanoparticles was not significantly different than in PNIPA-SDS nanoparticles at both the concentrations; however, percentage cell survival was greater in PNIPA-PL64 and PNIPA-PP65 nanoparticles than in PNIPA-SDS.

Again, this can be explained by taking the results of free surfactant cytotoxicity study into the consideration. Those results were already discussed earlier. PP85 and PF127 were found to be biocompatible among all tested surfactants. More importantly, PF127 was the most biocompatible surfactant. Consequently, PNIPA nanoparticles synthesized using PF127 would show the highest cell survival compared to all other nanoparticles followed by PNIPA-PP85. Moreover, EO weight content is more in PNIPA-PF127 compared to other nanoparticles.

3.3.5. LCST Determination

3.3.5.1 UV-Vis Spectrophotometry

Percentage of transmittance was plotted as a function of temperature in Figure 3.9. The LCST is the temperature at the inflection point in the plotted curve. The inflection point was observed around 32^{o}C indicating the LCST of PNIPA nanoparticles. At temperatures below LCST, nanoparticle solution is clearer; hence transmits more light and absorbs less. However, at temperatures above LCST,

nanoparticle solution becomes cloudy; absorbs more light and transmits less. Inflection point is the stage where this transition occurs.

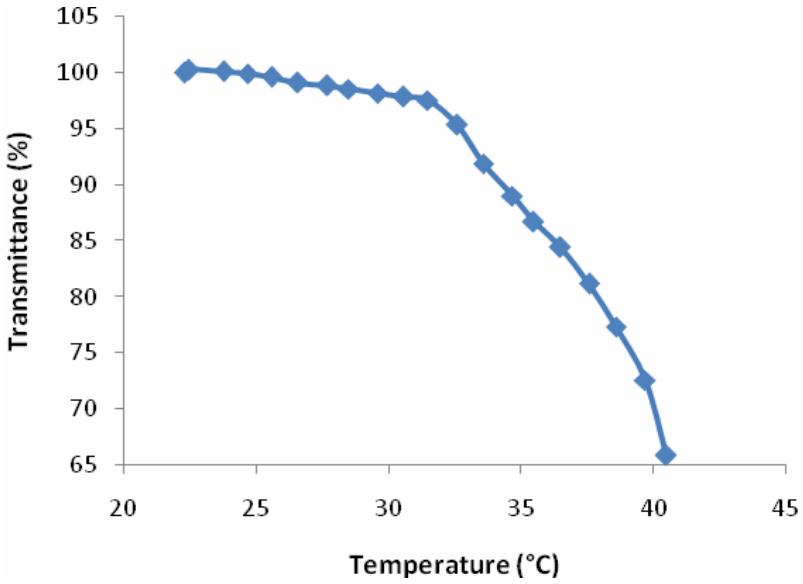


Figure 3.8 LCST of PNIPA nanoparticles determined by UV-Vis spectrophotometry.

3.3.5.2 Differential Scanning Calorimetry

DSC generated a graph of heat flow versus temperature which was represented in Figure 3.10. DSC analysis showed a detectable endothermal peak at 34°C which is close to the LCST of PNIPA nanoparticles. Many literatures showed that LCST of PNIPA nanoparticles lie in between 32-34^{o}C.

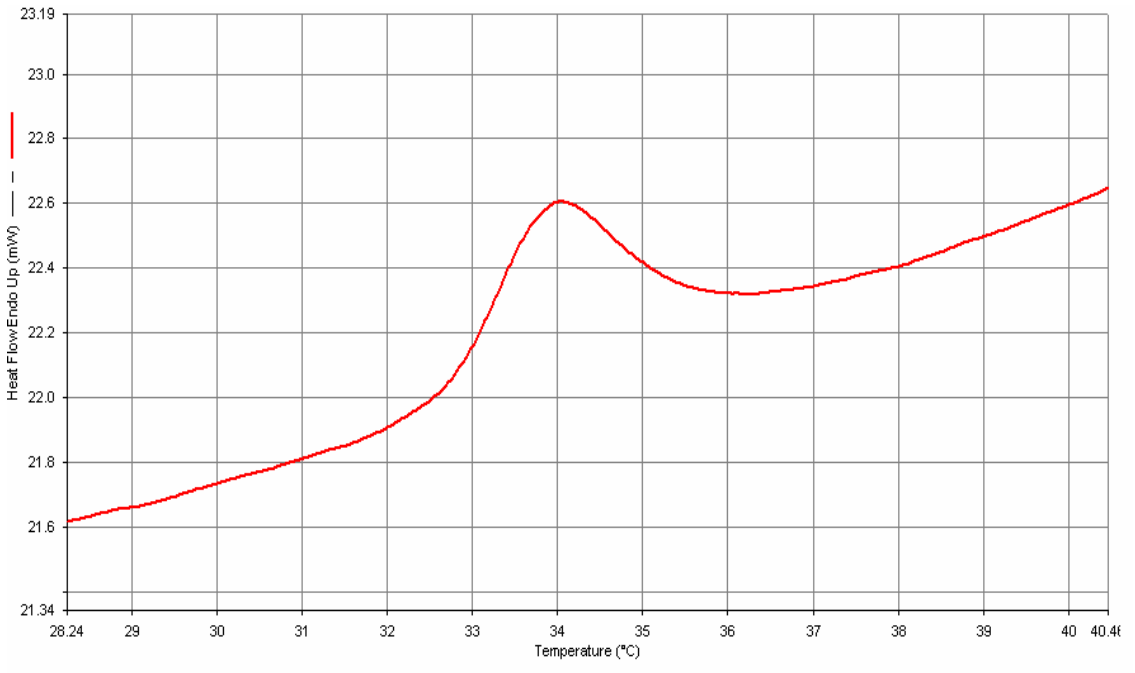


Figure 3.9 LCST of PNIPA nanoparticles determined by DSC.

3.3.5.3 Visual Observation

The change in the color or turbidity of the PNIPA nanoparticle solution was observed by naked eyes. The nanoparticle solution became turbid at 32°C. The turbidity in the solution was compared with clear solution maintained at room temperature in Figure 3.11. The LCST behavior is due to the dehydration of the polymer chain. Coil-to-globule transition occurs when PNIPA nanoparticle structure collapses and particles shrink. This transition is composed of four thermodynamically stable states: coil, crumpled coil, molten globule, and globule [90]. At temperatures above LCST, PNIPA nanoparticle solution becomes cloudy due to fluctuations in the refractive index which is caused by the partitioning into a polymer rich phase and a solvent rich phase.

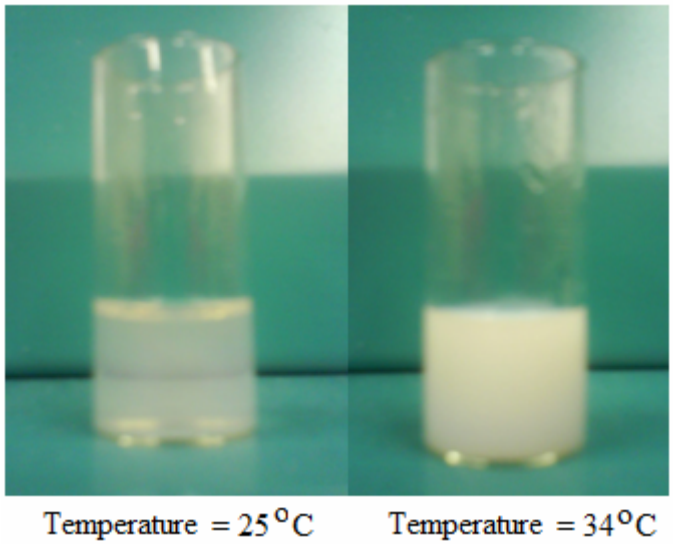


Figure 3.10 LCST of PNIPA nanoparticles determined by visual observation.

3.3.6. Drug Loading Efficiency

The loading efficiency of Doxorubicin into the PNIPA nanoparticles was determined according to the formula described earlier in the methods section. Results indicated that approximately 70.56%, 72.98%, and 72.12% of the incubated Doxorubicin was loaded into the PNIPA-SDS, PNIPA-PP85, and PNIPA-PF127 nanoparticles, respectively. Loading efficiency of PNIPA-PP85 and PNIPA-PF127 was found to be approximately same; however loading efficiency of PNIPA-SDS was lesser than other two types of the nanoparticles.

3.3.7. Drug Release Kinetics

Doxorubicin release profiles for PNIPA-SDS, PNIPA-PP85, and PNIPA-PF127 nanoparticles at 37^{o}C were plotted as percentage of cumulative release versus time in Figure 3.12. Drug release profiles showed that the PNIPA-PF127 nanoparticles released highest amount of Doxorubicin over the studied time period; while the PNIPA-SDS nanoparticles release the lowest amount of Doxorubicin. From the drug release profiles, it can be observed that all types of nanoparticles showed a biphasic drug release, with an initial burst release (up to 24 hours) followed by a plateau release (up to 103 hours). At temperatures above the LCST, nanoparticles collapse, shrink, and squeeze the drug out. Most of the loaded Doxorubicin was released within the first 24 hours and remaining Doxorubicin was released slowly till 103 hours. In case of PNIPA-PF127, approximately 77% of the encapsulated Doxorubicin was released within 24 hours. This percentage of Doxorubicin release was decreased to 71% and 68% in case of PNIPA-PP85 and PNIPA-SDS, respectively. Over the time period, Doxorubicin released from PNIPA-PP85 and PNIPA-PF127 was higher compared to that of PNIPA-SDS.

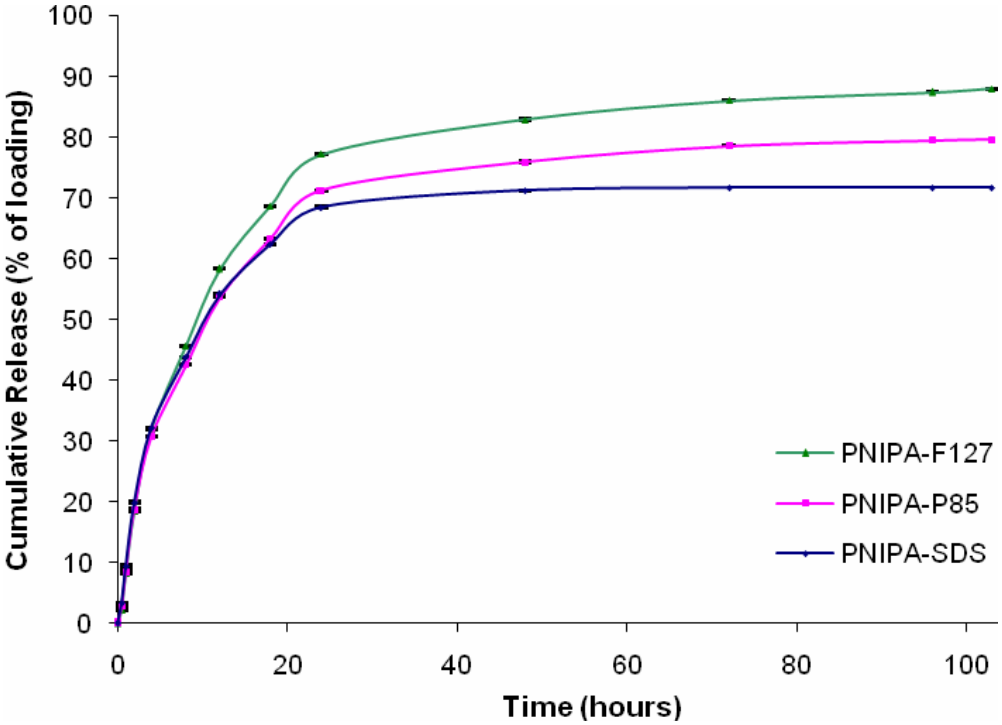


Figure 3.11 Biphasic release profiles of Doxorubicin from PNIPA nanoparticles at 37°C.

PF127 contains more EO (70% wt) and less PO than in PP85. PF127 is more hydrophilic than PP85 as EO is the hydrophilic block in the Pluronic block copolymer. Therefore, PNIPA-PF127 nanoparticles are more hydrophilic than PNIPA-PP85 [80-84]. Some Pluronic surfactants might be at the nanoparticle surface even after the dialysis as dialysis cannot remove 100% of the surfactant. At temperatures above LCST, PNIPA becomes hydrophobic and squeezes drug out. However, due to more hydrophilic surfactant content on the nanoparticles, hydrophilic Doxorubicin molecules come out of PNIPA-PF127 more easily as compared to PNIPA-PP85 and PNIPA-SDS. Thus, PNIPA-PF127 nanoparticles become the best choice for drug delivery systems.

This type of drug release is useful for diseases like cancer, where an aggressive release of drugs is necessary to kill the tumor.

3.4 Conclusion

In this study, the biocompatibility of PNIPA-Pluronic nanoparticles was tested and compared with PNIPA-SDS nanoparticles. Our results showed that the Pluronic surfactants were more biocompatible than SDS surfactants. Dialysis of nanoparticles is necessary to improve the biocompatibility of PNIPA-SDS nanoparticles, especially when used at high concentrations. In addition, PNIPA-PF127 and PNIPA-PP85 nanoparticles were most biocompatible nanoparticles even at high concentrations. PNIPA nanoparticles showed a phase transition at LCST in all the LCST experiments. Finally, PNIPA-PF127 nanoparticles showed highest amount of Doxorubicin release at 37^{o}C. These results indicate that PNIPA-Pluronic, especially PNIPA-PF127 and PNIPA-PP85, are biocompatible nanoparticles with potential applications in controlled drug delivery.

CHAPTER 4

LIMITATIONS AND FUTURE WORK

A major disadvantage of PNIPA nanoparticles is that, thermal treatment (hyperthermia or hypothermia) is required for the controlled destabilization of the micelles and concurrent drug release, which is not always feasible in clinical practice. Previous work in our lab, by Rahimi, had made this system target specific and solved the problem of thermal treatment by covalently coating PNIPA on magnetic nanoparticles. PNIPA coated magnetic nanoparticles can be guided to the target site by applying alternating magnetic field externally. These particles would vibrate (Brownian motion) due to alternating magnetic field, and heat generated from magnetic core would serve as a temperature stimulus to the PNIPA coating shell.

Another disadvantage of the system is that the drug has to be hydrophilic. Drug loading into the PNIPA nanoparticles is done below its LCST where PNIPA is hydrophilic. Therefore, the drugs chosen must be hydrophilic in nature.

Since PNIPA is in its precipitated form at body temperature, this system is not suitable for *in vivo* application without modification. LCST of PNIPA needs to be increased above body temperature. The LCST of PNIPA can be modulated by copolymerizing with hydrophobic or hydrophilic monomers. Hydrophobic monomers decrease the LCST whereas hydrophilic monomers have the opposite effect [91]. We increased LCST of PNIPA nanoparticles to 39^{o}C by copolymerizing it with hydrophilic

acrylamide (AAm) monomers. Synthesis and LCST results of copolymer of PNIPA and AAm (PNIPA-AAm) are discussed below.

4.1 Synthesis of PNIPA-AAm Nanoparticles

PNIPA-AAm nanoparticles were prepared as described elsewhere [41, 91]. 1.3644 g of NIPA, 0.1756 g of AAm, 0.0262 g of BIS, and 0.0439 g of SDS was added to 100 ml of DI water. The solution was stirred for 30 minutes under argon gas. Initiator, KPS (62.4 mg), was added to the solution and free radical polymerization was carried out in an oil bath at 70°C for 4 hours under argon. The resulting PNIPA-AAm nanoparticles were cooled to room temperature and dialyzed (molecular weight cut-off of 6000-8000 Da) against DI water for 3 days to remove un-reacted monomers and surfactants.

4.2 LCST Determination

LCST of PNIPA-AAm nanoparticles was determined by UV-Vis spectrophotometry and visual observation as described earlier in chapter 3 in methods section. Results were analyzed in the same way as for LCST of PNIPA nanoparticles alone.

4.2.1. UV-Vis Spectrophotometry

Percentage of transmittance was plotted as a function of temperature in Figure 4.1. The LCST is the temperature at the inflection point in the plotted curve. The

inflection point was observed around 39°C indicating the LCST of PNIPA-AAm nanoparticles. Phase transition of PNIPA-AAm nanoparticles at temperatures above LCST is sharper than PNIPA alone. This was confirmed by turbidity determination by visual observation.

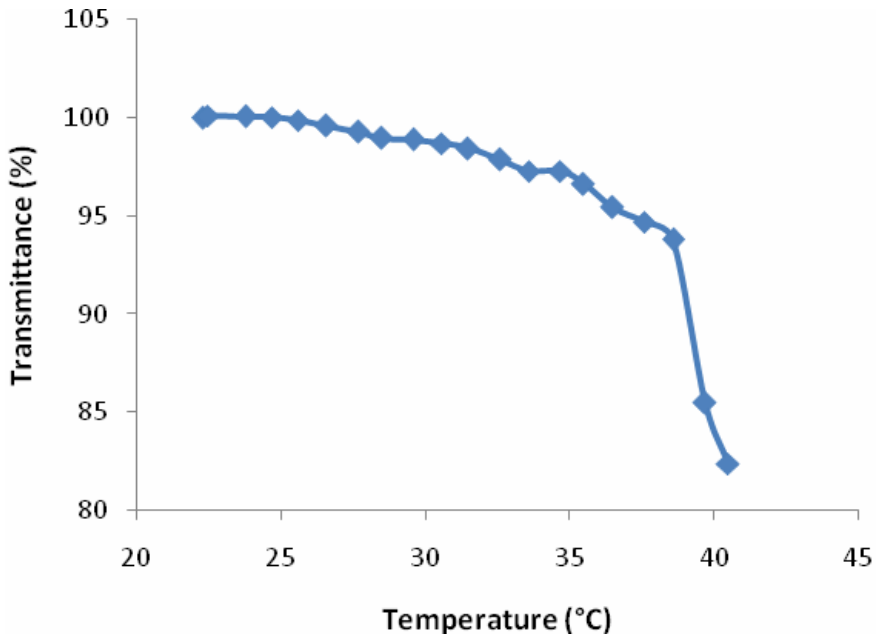


Figure 4.1 LCST of PNIPA-AAm nanoparticles determined by UV-Vis spectrophotometry.

4.2.2. Visual Observation

The change in the color or turbidity of the PNIPA-AAm nanoparticle solution was observed by naked eyes at 39°C. The turbidity in the solution was compared with clear solution maintained at room temperature in Figure 4.2.

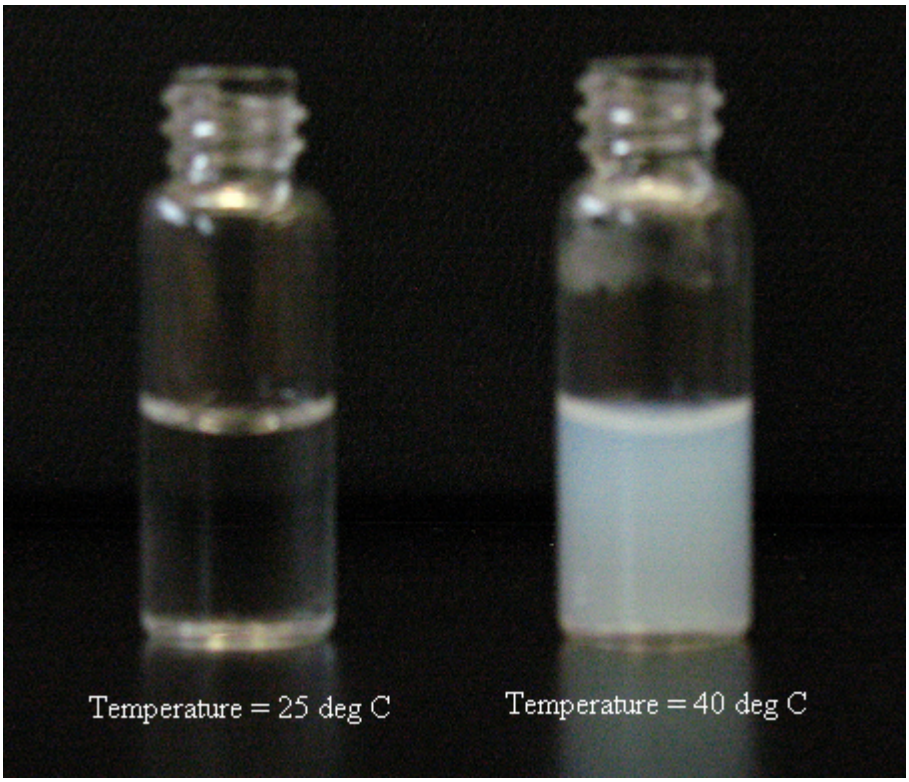


Figure 4.3 LCST of PNIPA-AAm nanoparticles determined by visual observation.

CHAPTER 5

CONCLUSION

The overall goal of our project was to develop temperature sensitive PNIPA nanoparticles for drug delivery applications. The specific aims were to evaluate the cytotoxicity of the temperature sensitive PNIPA nanoparticles using various cell types and improve the biocompatibility of these nanoparticles using Pluronic surfactants. Cytotoxicity studies on FCs, HASMCs and HMVECs revealed that the PNIPA nanoparticles were biocompatible, while NIPA monomers were cytotoxic even at low concentrations. Size of the PNIPA nanoparticles, in the range of 100-500 nm, had no significant effect on cell survival. Further, the cytotoxicity of the NIPA monomers and PNIPA nanoparticles at same concentrations was found to be different for all three cell types. Additionally, dialysis removed most of the un-reacted monomers and surfactants from the PNIPA nanoparticle solution as a negligible amount of NIPA monomers remained on PNIPA nanoparticles was detected.

In addition to the cytotoxic comparison between monomers and polymeric nanoparticles, the biocompatibility of PNIPA nanoparticles was improved using Pluronic surfactants and drug release study was performed. Importance of dialysis was also confirmed as a higher cell survival was observed in dialyzed PNIPA-SDS nanoparticles compared to that of non-dialyzed nanoparticles. Furthermore, Pluronic surfactants were found to be more biocompatible than SDS, and hence the PNIPA-

Pluronic nanoparticles. Moreover, LCST of PNIPA nanoparticles was detected around 32^{o}C using three different methods. Finally, the most biocompatible PNIPA-Pluronic nanoparticles, PNIPA-PF127 and PNIPA-PP85, release higher amounts of doxorubicin than PNIPA-SDS nanoparticles. This study has improved our understanding about the effects of monomers and surfactants on biocompatibility and drug release behavior of PNIPA nanoparticles. Following these results, PNIPA nanoparticles can be modified to have specific LCST and drug release profiles while maintaining their biocompatibility.

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BIOGRAPHICAL INFORMATION

Aniket Wadajkar was born in Nanded, India in September 1982. He graduated with a Bachelor of Engineering degree in Instrumentation and Control Engineering from the Pravara Rural Engineering College, University of Pune, India in June 2005. He joined a nanotechnology research group in University of Pune, India in February 2006 and worked on nanoparticles that are useful for biomedical applications. He moved to the United States in August 2006 to pursue his dream of becoming a nanotechnology scientist. He found it interesting how integrating different disciplines in science and engineering contribute to the improvement of human life. After enrolling in the Biomedical Engineering program at the University of Texas at Arlington, he began conducting research under the guidance of Dr. Kytai Nguyen. His undergraduate knowledge and work experience gave him a better understanding of different disciplines in science and engineering. His research interests include drug delivery, biomaterials and tissue engineering. After completing the Master of Science degree in Biomedical Engineering, he plans to pursue a Ph.D. degree in the same field.