DESIGN OF INTELLIGENT NANOPARTICLES FOR USE IN CONTROLLED RELEASE

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ABSTRACT

DESIGN OF INTELLIGENT NANOPARTICLES FOR USE IN CONTROLLED RELEASE

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The aim of this project was to design an intelligent controlled release system based on thermoresponsive nanoparticles for cancer therapy and to evaluate the efficiencies of these systems with *in vitro* cell culture. Poly(N-isopropylacrylamide), an important thermoresponsive polymer, was selected for this study to prepare the responsive nanoparticles. This polymer has an lower critical solution temperature (LCST) of 32 ^{o}C, below which it is hydrophilic and above this temperature, it shows hydrophobic behavior. Controlling drug release with this property was the objective of this study.

Nanoparticles were prepared by nanoprecipitation method. By using different solvent:non-solvent ratios and polymer concentrations, different samples were prepared. The particle size was decreased when solvent:non-solvent ratio was increased and polymer concentration was decreased. This was found to be related with the solution viscosity.

Nanoparticles prepared from polymers prepared with different initiator-accelarator amounts had significantly different sizes and release rates, and additionally the size of particles prepared from polymers with various crosslinker amounts were decreased with increased croslinker amount.

In situ release experiments were performed both below and above polymer's LCST degree. Uncrosslinked nanoparticles demonstrated higher release rate of Celecoxib above LCST. However, there was no significant difference with the crosslinked nanoparticles.

Crosslinked and uncrosslinked nanoparticles were tested on Saos-2 cells to assess their toxicity. Both Celecoxib loaded and free crosslinked particles were found to be cytotoxic. Uncrosslinked nanoparticles showed an increased toxicity upon loading with the bioactive agent, Celecoxib. In conclusion, uncrosslinked particles would be a proper drug carrier for cancer therapy with enhanced drug loading.

Key words: Cancer Therapy, Nanoparticle, Thermoresponsive Release

ÖZ

KONTROLLÜ SALIM AMACIYLA AKILLI NANOPARTİKÜLLERİN TASARIMI

Bayyurt, Banu

Yüksek Lisans, Biyoteknoloji ABD

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Bu çalışma kanser tedavisinde kullanılmak üzere, sıcaklığa duyarlı, akıllı nanopartiküllerin tasarlanmasını ve oluşan kontrollü salım sisteminin etkinliğinin *in vitro* koşullarda test edilmesini amaçlamaktadır. Sıcaklığa duyarlı bir polimer olan poli(N-izopropilakrilamit), bu projede kullanmak için seçilmiştir. LCST derecesi 32 olan bu polimer, LCST'nin altında hidrofilik, üstünde hidrofobik davranışlar göstermektedir. Bu özelliği kullanılarak, nanopartiküllerin ilaç salımını kontrol etmek amaçlanmıştır.

Nanopartiküller nanoçöktürme yöntemi kullanılarak hazırlanmıştır. Çözüldüğü solüsyonun çöktüğü solüsyona oranı ve bu karışımdaki polimer miktarı değiştirilerek farklı örnekler elde edilmişitir. Bu nanopartiküller karşılaştırıldığında, solüsyon oranı arttığında ve polimer miktarı azaltıldığında partikül boyutlarının anlamlı bir şekilde küçüldüğü gözlenmiştir. Bunun nedeni solüsyon akışkanlığına bağlanmıştır.

Farklı başlatıcı ve hızlandırıcı miktarları kullanılarak elde edilen polimerlerden hazırlanan nanopartiküllerin boyutlarının ve salım hızlarının belirgin bir şekilde farklı olduğu belirlenmiş ve ayrıca farklı oranlarda çapraz bağlanmış polimerlerden elde edilen partiküllerin de partikül boyutunun çapraz bağlama oranı arttıkça küçüldüğü gözlenmiştir.

Polimerin LCST derecesinin üstünde ve altında *in situ* salım deneyleri yapılmış ve çağraz bağlanmamış partiküllerin LCST'nin üstünde salım hızının daha fazla olduğu gözlemlenmiştir. Bunun yanında çağraz bağlı partiküllerde belirgin bir fark bulunmamıştır.

Çapraz bağlanmış ve bağlanmamış nanopartiküller, toksik etkilerini araştırmak için Saos-2 hücre hattında test edilmiştir. Çapraz bağlı partiküller göreceli olarak sitotoksik bulunmuştur. Biyoaktif ajan olan Celecoxib yüklü çapraz bağlanmamış nanopartiküllerin artan bir toksisiteleri olduğu gösterilmiştir. Sonuç olarak, çapraz bağlanmamış nanopartiküllerin, daha fazla ilaç yüklenmesi sağlanarak, kanser terapi için uygun bir taşıyıcı olabileceği düşünülmüştür.

Anahtar kelimeler: Kanser Terapi, Nanopartikül, Sıcaklığa Duyarlı Salım

Dedicated to my parents

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LIST OF ABBREVIATIONS

APS	Ammonium persulphate
ATCC	American Type Cell Collection
DMEM	Dulbecco's Modified Eagle Medium
DMSO	N,N-Dimethylformamide
FBS	Fetal bovine serum
FT-IR	Fourier Transform Infrared
MBA	N,N-methylene bisacrylamide
MTS	CellTiter 96 AQueous One Solution Cell Proliferation Assay
NIPAM	N-Isopropylacrylamide
PB	Phosphate Buffer
Saos-2	Sarcoma osteogenic cell line
SEM	Scanning electron microscopy
TCPS	Tissue Culture Polystyrene
TEMED	N,N,N,N-tetramethyl ethylenediamine

CHAPTER 1

INTRODUCTION

1.1 Controlled Drug Delivery Systems and Therapeutic Use of Controlled Release Systems (CRS)

One of the basic goals of chemical therapeutics is to deliver the drug efficiently and specifically to the site of disease in order to reduce disease morbidity and increase life expectancy (Pierigè et al., 2008). In order to achieve this, drugs have administered through different paths such as oral, intramuscular, transdermal, transmucosal, ocular, pulmonary, or implantation (Hughes, 2005). Among these paths, oral and injection routes are the most encountered drug delivery routes, however, they are not efficient for recent drugs such as proteins, cancer drugs and nucleic acids and for some drugs which are rapidly cleared from the area of introduction or the circulation or obstructed by biological barriers which they cannot permeate (Pierigè et al., 2008, Hughes, 2005).

In conventional drug delivery, drug level in the blood increases soon after injected or orally taken, and then the level is reduced to an ineffective level by blood components, metabolism or excretion so the frequency of drug administration should be increased to maintain a stable drug level in the blood (Zhang et al., 2002). To overcome the inadequacy of drug delivery, higher drug concentrations can be applied, however, this increases the cost of the treatment and toxic side effects of the drug (Pierigè et al., 2008). In order to

eliminate or decrease the above disadvantages, drug delivery systems (DDS) have been developed aiming to provide drug with the predetermined dose in the blood or targeted organ (Zhang et al., 2002) (Figure 1).

Drug delivery systems provide drug to have some additional advantageous in comparison to free drug. Firstly, even if the drug is insoluble in blood and other fluids, the intraveneously or intraperitoneally administered drug-loaded carrier can carry them into central circulation or the intraperitoneum to enable drug transport within the body. Drug will have a protection from serum protein binding, enzymatic attack, macrophages and other scavenging processes, while in the carrier. As a result, the circulation time in reticuloendothelial system of the drug will be longer and renal clearance will be minimized. Also while protecting the drug from the clearance; it will protect body from possible toxic side effects due to excessive doses. Additionally, incorporating some linkers or spaces to the carrier enables targeting and endocytotic cell entry (Neuse, 2008). In addition to mentioned benefits such as providing safety, targeting, minimal side effects and biodistribution of the drugs, the administration of higher drug quantities reaching the therapeutic concentration is another crucial advantageous (Pierigè et al., 2008).

An ideal drug carrier should be biocompatibile in intact form and after the degradation, as well. It should carry an effective dosage and be stable during treatment, should be suitable for regular clinical administration and economically feasible (Kuo, 2005). Also, it should have a desired release rate providing a local concentration until the end of the treatment. Considering this expectation, controlled release systems (CRS), which deliver a drug at a predetermined rate for a definite time period, are being sought. The aim of controlled drug release systems is to achieve a drug level within a range above which is toxic and below is ineffective in a single administration (Langer, 1990) (Figure 1). If the design and process of the CRS can be properly controlled, the release of the drug can be in a desired kinetic profile.

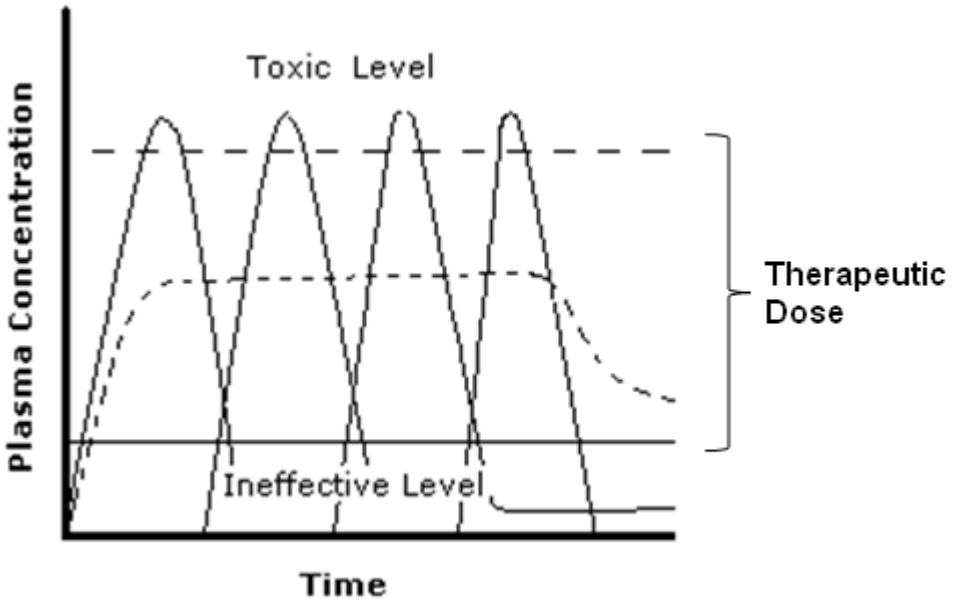

Figure 1 Plasma concentration of drug in conventional (straight line) and controlled drug delivery (dashed-line).

Diffusion of the drug through the system, chemical or enzymatic degradation of the system, solvent activation either through osmosis or swelling of the system are the main release mechanisms observed in controlled drug release systems. Consequently these mechanisms obey Zero order, First order, and Higuchi kinetics, which are the most common release profiles. The conventional delivery through oral administration or by injection is by First order kinetic and generally, the ideal release profile is a steady release rate (Zero order) which maintains constant drug levels in the system (Hughes, 2005).

1.1.1 Types of Drug Delivery Systems

A number of drug delivery systems exist. They can be classified as cellular, liposomal and polymer based system.

1.1.1.1 Cellular Drug Delivery

In the construction of CRS, a carrier is needed. This carrier needs to be accepted by the body and has to be eventually eliminated. Sometimes drug delivery systems fail to target their drugs due to being recognized as a foreign by the immune system. Consequently, it is advantageous to use various types of cells, such as bacterial cells, macrophages, red blood cells, as drug delivery carriers.

Bacterial cells are used as ghost cells, due to the absence of cytoplasm and nucleus, with surface structures for specific attachment to specific cells (Dou et al., 2006). Bacterial ghosts from *Mannheimia haemolytica* for delivery of doxorubicin to human colorectal adenocarcinoma cells was used and improved results related with targeting and efficiency of doxorubicin on Caco-2 cells were obtained when compared with the free doxorubicin administration (Paukner et al., 2004). The same bacterium was also used as a delivery system for DNA vaccines (Ebensen et al., 2004).

Macrophages could phagocytose nanoparticles and upon administration they could accumulate in the spleen and liver and this target the drug especially to bacteria or parasites or viruses, which have special receptors for macrophages. In a study, a novel bone marrow–derived macrophage (BMM) pharmacologic nanoparticle (NP) Indinavir delivery system was designed to enhance the stability of the carrier, to achieve controlled release and bioavailability of the drug (Dou et al., 2006).

Red blood cells (RBC) are used as ghost cells with large carrier volumes. Differently, they have another important property: they shrink in hypertonic solutions or swell when in hypotonic solutions and this could be employed as a release trigger. In a study, Dexamethasone-21-phosphate encapsulated into autologous RBCs was slowly dephosphorylated by RBC resident enzymes and slowly released in circulation as Dexamethasone (Pierigè et al., 2008).

Embryonic stem cells can be used as release vehicles for the regulated delivery of drugs or therapeutic proteins, as well. Insulin-producing cells

derived from stem cells were reported encouraging results in mouse diabetes model (Gorba et al., 2003).

Besides the fascinating results obtained from cell-based drug delivery, there are several difficulties with isolation, stability and half-life of cell carriers.

1.1.1.2 Liposomes

A widely studied drug delivery system is liposomes. They are spherical vesicles with a membrane composed of a mostly phospholipid and a cholesterol bilayer and are nontoxic, degradable, and nonimmunogenic due to the proper selection of their ingredients (Langer, 1990; Edwards et al., 2006).

Liposomes can carry the drug for long periods, and release it slowly via diffusion. Protecting the drug from the degradation, loss of their efficiency, protecting the body from overdose or side effects, and targeting are the some important properties seeked in a good carrier and liposome has all these properties (Crommelin et al., 2003). A wide variety of hydrophilic molecules can be encapsulated within the inner cavity; enzymes (Petrikovics et al., 2000), DNA (Liu et al., 1995), vaccines (Tiwari et al., 2009), fluorescent dyes, electrochemical and chemiluminescent markers are among those compounds that can be carried by liposomes (Edwards et al., 2006). Also the drug can be carried within the lipid bilayers if it is lipophilic or amphiphilic (Frézard et al., 2006). Some of these drugs are cyclosporine, progesterone, paclitaxel, and doxorubicin.

In a clinical study employing liposomes as the drug carrier, an 86% reduction of cardiovascular toxicity of Doxorubicin was observed (Crommelin et al., 2003). Other drugs, daunorubicin, amphotericin B, morphine, and cytarabine are also approved by Food and Drug Administration (FDA) for pharmaceutical uses (Langer, 1990).

Some advantageous characteristics of liposomes are the ability to incorporate not only water soluble but also lipid soluble agents, specific targeting to the required site in the body and versatility in terms of fluidity, size, charge and

number of lamellae. For delivery applications liposomal formulations should preferably have high entrapment efficiencies, narrow size distributions, long-term stabilities and ideal release properties (Mozafari, 2005). Targeting options with liposomes (through incorporation of molecules in the membrane) change the distribution of the drug through the body thereby enhancing therapeutic response and reducing the dose of the drug associated toxicity. Since liposomes undergo endocytosis and phagocytosis, they can deliver the drugs intracellularly. They can be used to deliver DNA inside the cells, hence they are very important as non-viral transfection systems (Sharma et al., 2006).

PEG-modified liposomes (PEG-liposomes), which are also called "long-circulating liposomes" or "stealth liposomes", have been widely used as drug carriers for systemic injection. Formulations such as Doxil® (Alza Co.) and Visudyne® (Novartis Co.) have already been approved for clinical use (Nishiyama et al., 2006).

Despite the enormous research and development works on liposomes, only a small number of liposomal products have been approved for human use so far. This may be due to many reasons including: toxicity of some liposomal formulations, low entrapment of molecules and compounds into liposomes, instability of the liposomal carriers, and high cost of liposome production especially on large scales (Hans et al., 2002).

1.1.1.3 Polymer Based Systems

Polymers have been the most commonly used vehicles in controlled release systems due to wide selection with a great variability in properties, easy synthesis and characterization methods, increased stability and lower cost.

Many polymeric drug-delivery systems have been developed over the years and have been extensively reviewed. Wide variety of delivery systems has been constructed with polymers like polyvinyl acetate, polypropylene, polyurethanes, polymethacrylate. Researchers have also used specific organic

polymers such as poly(D,L-lactide-co-glycolide) (PLGA), polylactide (PLA), polycaprolactone (PCL), poly(alkyl cyano-acrylate) (PACA) and its copolymers due to their biocompatibility and biodegradability (Yang et al., 2008).

Modifications on these polymeric carriers can enhance targeting and controlled release of loaded agent. Diversity of polymeric structure can be achieved either by changing the bulk or surface properties of polymeric products to design CRS with desired chemical, mechanical and biological features providing proper delivery properties. Surface properties (hydrophilicity, smoothness and surface energy) control and determine the biocompatibility of the polymeric release systems, as well about physical properties such as stability, permeability and degradability. Surface properties can be further improved by chemical, physical and biological approaches. Molecular weight, solubility and crystallinity are bulk properties that should be considered when designing polymeric controlled release systems (Pillai et al., 2001).

Polymeric carriers can also be designed or modified to carry some groups such as RGD that facilitate cell entry acting as a homing device capable of directing the polymer-drug conjugate selectively to target tissue. Functional groups could also help reversible drug binding to the polymer (Neuse, 2008).

All forms of polymeric products have different kinetic release profiles. The selection of the proper polymeric products provides adequate drug release without exposing any side effects. Polymeric devices generally release drugs by diffusion, solvent activation (swelling or osmotic effects) or chemical reaction (degradation or chemical cleavage) (Langer, 1990). Thus, the application areas of these products should be decided considering their release rates.

Generally, polymer based biomaterials are classified in terms of chemical nature, biodegradability and vehicle form of products.

1.1.1.3.1 Synthetic and Natural Polymers

In the construction of the polymeric CRS, synthetic or biological origin polymers are used. Natural polymers are usually biodegradable and biocompatible, whereas, synthetic polymers have adjustable properties since found in a wide variety of compositions.

Natural polymers, such as collagen, chitosan, hyaluronic acid, chondroitin sulfate, gelatin, starch, are usually biodegradable and have excellent biocompatibility, but the properties differ from batch to batch due to difficulties or conditions used in purification and also with the source (Pillai et al., 2001).

Collagen is widely involved in various tissues, thus it has excellent biocompatibility (Cascone et al., 1995). Hence, the use of collagen in biomedical fields attracts great attention. Systems for drug delivery applications such as ophthalmology (Gebhardt et al., 1995), wound and burn dressing (Marks et al., 1991), tumor treatment (Davidson et al., 1995), and tissue engineering (Wallace et al., 2003) have been designed (Friess, 1998) with collagen in a various forms such as shields, sponges, mini-pellets and tablets, as well as gel formations, micro and nanoparticles. Also, collagen can be used in gene delivery systems (Sanoet al., 2003; Dang et al., 2006). Some disadvantages of collagen-based systems are hard and inadequate isolation procedures, poor mechanical strength, and ineffectiveness in the management of infected sites. To achieve better collagen delivery systems, the structure of the collagen matrix can be adjusted with proteins, such as elastin, fibronectin or glycosaminoglycans and it can be combined with liposome or silicone or can be crosslinked to achieve more stable structure (Lee et al., 2001).

Chitosan is a promising natural polymer that shows good adhesion-enhancing properties with mucoadhesive feature and positive charge (Pillai et al., 2001, Agnihotri et al., 2004). Also it is nontoxic, inexpensive and biodegradable. By controlling its properties such as degree of acetylation and molecular weight, chitosan would have some advantageous such as controlled release of an agent and increase residual time in the application place. Thus, it is

extensively used in drug delivery systems (Shiraishi et al., 1993), in the preparation of mucoadhesive formulations (Lehr et al., 1992), improving the dissolution rate of the poorly soluble drugs (Gentaet al., 1994), drug targeting (Hassanet al., 1992) and enhancement of peptide absorption (Luehen et al., 1994). Additionally, chitosan is one of the most reported natural polymer used for gene delivery due to the high affinity to DNA (Leong et al., 1998; Agnihotri et al., 2004). Disadvantages of chitosan is limited solubility in water and some organic solvents, however, there are some studies using hydrophilic compounds to enhance solubility. Also, efficient transport of gene is only observed in acidic environment other than normal or alkaline pH. This can be explained by the protonation of chitosan below normal pH (Tiyaboonchai, 2003).

Hyaluronic acid (HA) is a polyanionic natural polymer that is mostly present in connective tissues. Due to non-immunogenic, biodegradable and viscoeleastic properties, it has been ideal for medical applications. Commonly, it is used in scaffold design and wound healing due to its supportive feature. HA has also been extensively studied in ophthalmic (Bucolo et al., 1999; Langer et al., 1997), nasal (Lim et al., 2002), pulmonary (Surendrakumar et al., 2003), gene delivery (Yun et al., 2004; Kim et al., 2004) and parenteral drug delivery (Peer et al., 2003). Generally, HA is also considered as a mucoadhesive and it retains the drug at application area same as chitosan (Brown et al., 2005).

Natural polymers could be immunogenic, poorly stable, vary in purity, often require crosslinking that could denature the embedded drug and inadequate mechanical properties (Hans et al., 2002), thus, synthetic polymers was mostly preferred due to the versatility, reproducibility, improved control of physical and chemical behaviors by altering the chemical composition, molecular weight, and addition or removal of functional groups, as well as minimization of systemic toxicity and immunogenic properties (Neuse, 2008).

The synthetic polymers used in various areas of biomedicine include Dacron and Teflon in the cardiovascular system; polyethylene, polypropylene in orthopaedics and ophthalmic devices; silicone polymers in dentistry, soft implants and ophthalmology; cellulose and polyacrylonitrile in artificial organs

and drug delivery systems. The selection of these polymers is mostly according to the required mechanical properties of the specific application (Cascone et al., 1995).

Poly(lactic-co-glycolic acid) (PLGA) is currently the most frequently used synthetic, biodegradable and biocompatible polymer. Porous Doxorubicin loaded particles (Yang et al., 2009), recombinant human bone morphogenetic protein-2 (Kenley et al., 1995), Dopamine and Norepinephrine loaded microparticles (McRae et al., 1994), Paclitaxel (Fonseca et al., 2002), Cisplatin (Avgoustakis et al., 2002), Doxorubicin (Yoo et al., 2000) loaded nanoparticles (Fonseca et al., 2002) are manufactured for local sustained inhalational therapy of pulmonary diseases, tissue engineering, neural degenerative diseases and cancer therapy. Several clinically approved products are also prepared such as PLGA-based microparticles loaded with Leuprolide (Lupron Depot) or Triptorelin (Trelstar) (Klose et al., 2008).

Poly(L-lactic acid) (PLLA) is another extensively studied biodegradable synthetic polymer in tissue engineering as a cell carrier with controlled release of various growth factors (Lo et al., 1996; Kim et al., 2004) and implantable depot for sustained release drug delivery such as Methotrexate for reducing inflammation in rheumatoid arthritis (Liang et al., 2004), Paclitaxel in the prevention of tumor growth in the peritoneal cavity (Liggins et al., 2001).

Poly(methyl methacrylate) (PMMA) is an acrylic hydrophobic biostable polymer that is widely used in the biomedical field as bone cement in orthopedics, and as implant carrier for sustained local delivery of anti-inflammatory or antibiotics drugs such as Cisplatin (Yan et al., 2005), Vancomycin (Minelli et al., 2004) and Gentamicin (Neut et al., 2001; Elviraet al., 2004). It has an ability to slowly release high concentrations of an antibiotic, but, it may cause undesired tissue reactions due to its synthetic structure. (Greco et al., 1991).

Poly(N-isopropylacrylamide) (pNIPAM) is one of the most widely studied thermoresponsive, synthetic polymer mainly due to the sharpness of its phase transition, the closeness of its lower critical solution temperature (LCST) to the physiological temperature, and of the easiness to vary its LCST by copolymerisation. It has a LCST of 32 ºC, above which the polymers are

insoluble in water and below which the solubility is enhanced. So far, intestinal drug delivery of some hormones such as calcitonin and insulin (Nolan et al., 2006) were studied. Additionally, in some studies thermoresponsive properties of the pNIPAM is utilised for an on-off release mechanism by grafting carriers (Park et al., 2007; Ohya et al., 2005).

1.1.1.3.2 Biodegradable and Non-biodegradable Polymers

When permanent applications in the body or external use such as cell culture matrix, surface modification and sensors are considered, the nondegradable polymers are advantageous. By contrast, for internal temporary uses such as drug delivery and tissue engineering applications, biodegradable polymers should be considered (Jeong et al., 2002).

Biodegradability enables the removal of polymers from the body in the form of molecules in a same way other biological waste products are removed (Neuse, 2008).

Biodegradable carriers release their drug contents upon degradation along with diffusion and other kinetics (Mao et al., 1999). This degradation proceeds first by diffusion of water into the material, followed by random hydrolysis fragmentation of the material, and finally more extensive hydrolysis accompanied by phagocytosis, diffusion, and metabolism. The hydrolysis is affected by the size, hydrophilicity, and crystallinity of the polymer and the pH and temperature of the environment (Kenawy et al., 2009).

Degradation is a chemical process; whereas, erosion is physical and depends on dissolution and diffusion. Erosion mostly on the surface but could also happen in the bulk on the chemical structure of the polymer backbone. The kinetics of erosion and release rate of drug might be highly correlated. Generally, biodegradable polymers used in drug delivery undergo bulk erosion. In micro and nanoparticles due to the large surface to volume ratio, erosion appears both on the surface and in the bulk.

A polymer is biodegradable if it carries certain groups in its backbone. The degradation rate can be manipulated if groups such as ester, orthoester, anhydride, carbonate, amide and urea are included in their backbone.

Polyester-based polymers are among the most widely investigated polymers for drug delivery. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers poly(lactic acid-co-glycolic acid) (PLGA) are some of the most well knowns (Pillai et al., 2001). Biodegradable polymeric products prepared from this group such as PLA, polyglycolic acid (PGA), or a copolymer of PLA and PGA, are being investigated for the delivery of proteins and genes (Panyam, 2003), vaccines (Katare et al., 2003), anticancer drugs (Lee et al., 2002; Brannon-Peppas et al., 2004), ocular drugs (Kompella, 2003), and cytokines (Sanchez et al., 2003; Hughes, 2005).

Polyorthoesters are the unique biodegradable polymers due to varying mechanical properties by choosing appropriate diols during sysnthesis. A number of applications have been found for polyorthoesters such as Indomethacin (Solheim et al., 2004), 5-fluorouracil release (Guerra et al., 2001) and Tetracycline (Schwach-Abdellaoui et al., 2002).

Polyanhydrides have higher degradation rates due to rapid erosion of the material, while this property can be manipulated by suitable choice of monomers to be able to release drugs such as methotrexate, 5-fluorouracil agents for days to weeks (Bai et al., 2001).

Polycaprolactone (PCL) is another synthetic biodegradable polymer attracted due to its low cost, sustained biodegradability, availability at low molecular weight, and excellent drug permeability (Prabu et al., 2006). PCL is a soft and hard tissue-compatible material that is used for the controlled delivery of proteins, vaccines (Singh et al., 2006), and gene (Zhao et al., 2008; Elvira et al., 2004).

Formulating biodegradable systems are more complicated than nondegradable systems. The constant degradation kinetics and geometrical shaped to achieve stable surface area even during the erosion are the properties that should be considered while designing biodegradable drug delivery systems. Another problem that occurs with bioerodable systems is the slow diffusion of

the drug from the polymer matrix. This becomes a major challenge to overcome when developing bioerodable systems (Dash et al., 1998).

The development of nondegradable systems is less expensive and complex than biodegradable system. However, these systems need minor surgery when implanted. Nonbiodegradable polymers can not benefit (or suffer) from degradation (Mao et al., 1999).

A variety of non-degradable polymers are used in drug delivery which include polysaccharides, dendrimers and polyacrylates (Uekama et al., 1999).

Dextrans were employed in the sustained delivery of therapeutic agents, particularly for injectables and colon-specific drug delivery systems (Mehavar, 2000).

Polyethylene oxide (PEO) and polyoxypropylene (POP) copolymers are used for nanoparticle preparations which could mimick biological molecules such as lipoproteins or viruses.

Dendrimers are highly branched three dimensional polymers (Liu et al., 1999) and have a large number of controllable peripheral functionalities making them targetable, macrophage avoiding, fluorescing.

Polycationic polymers such as polyaminodiamine (PMAM) can form complexes like chitosan with the negatively charged nucleic acids (Pillai et al., 2001).

1.1.1.3.3 Form of Drug Delivery Vehicles

The mobility of a drug in delivery systems does not only depend on its own physico-chemical properties and the type of used polymer, but also to a large extent on the size and shape of the device (Klose et al., 2008).

Various vehicle types can be produced by several methods when the place, target or needed drug amount are taken into consideration. Controlled release systems exist in many forms such as fibers, films and membranes, sponges and particulate forms (capsules, spheres or irregular particles and a combination of those).

Fibers as drug carriers have a promising future in biomedical applications, especially postoperative local chemotherapy and tissue engineering (Xie et al., 2006), however today fiber carriers for drug delivery is very limited (Zeng et al., 2003; Peng et al., 2008). Many studies have demonstrated the low efficiency of drug delivery and complete drug (such as Tetracycline (Kenawy et al., 2002), Mefoxin (Zong et al., 2002)) releases in short periods with burst release of drugs at the beginning from the fibers. These problems are mostly due to having drugs on or near the fiber surface. Recently, encapsulation of the drugs inside the fibers is studied. Fibers containing various percents of Rifampin were prepared and the drug was capsulated inside the fibers (Zeng et al., 2003). Along with degradation of the fibers, the drug was released constantly. No burst release was observed. The main advantages of the fibrous carriers are ability to encapsulate more than one drug, having high surface area and porous structure, offer site-specific delivery of any number of drugs from the scaffold into the body (Peng et al., 2008)

Hydrogel is three-dimensional network formed by hydrophilic polymers that can swell in water and hold a large amount of water while maintaining the structure. Hydrogels can protect the drug from hostile environments such as the presence of enzymes and low pH in the stomach. Hydrogels can also control drug release by changing the gel structure in response to environmental stimuli. They have been used extensively in the development of the smart drug delivery systems (Fujii et al., 2009). Hydrogels containing such 'sensor' properties can undergo reversible volume phase transitions or gel–sol phase transitions upon only minute changes in the environmental condition (Qiu et al., 2001).

Polymeric particles represent very promising drug delivery systems. The capsule corresponds to a polymeric wall enveloping an aqueous core, whereas the sphere consists of a polymeric matrix throughout (Blouza et al., 2006). In spheres, the drug can be either adsorbed at the surface of the particle or incorporated inside the matrix core by dispersion between the hydrocarbon chains (Lemos-Senna et al., 1998). Capsules are more advantageous than spheres in some reasons. One of them is their low polymer content and a high loading capacity for hyrophilic drugs. Other advantages of confining the drug

within a central cavity is that a burst effect may be avoided, the drug is not in direct contact with the tissues, and therefore, irritation at the site of administration will be reduced and the drug may be protected from degradation both during storage and after administration (Blouza et al., 2006).

In a large number of applications, nano and microspheres were used. Among the recent carrier systems, nanotubes have become highly attractive due to their structural attributes, such as the distinctive inner and outer surfaces. Inner voids can be used for capturing, concentrating, and releasing species ranging in size from small molecules to large proteins because tube dimensions can be controlled. Outer surfaces can be functionalized with environment-friendly and/or targeting molecules (Son et al., 2005). Recently, *in vivo* study of PEG-modified gold nanorods, which have been used as agents for controlled release of DNA into cells, have demonstrated promising results with long lasting circulation (Niidome et al., 2006).

1.2 Nano and Microparticles in Drug Delivery

The delivery of the drug by particulate CRS efficiently to various parts of the body can be affected by particle size (Hughes, 2005). By altering their size, shape, and composition loading of various drugs with various pharmacological properties can be controlled (Dou et al., 2006).

1.2.1 Microparticles

The microparticle is defined as a particulate system larger than 1 mm. Injectable microparticles have been successfully used in therapies that deliver various drugs including anti-inflammatory agents (Duarte et al., 2006), proteins such as insulin (Kim et al., 2005), immunoglobulin (Bot et al., 2000), hormones such as recombinant human growth hormone (Kim et al., 2006; Lassalle et al., 2007).

1.2.1.1 Preparation and Characterization Methods

The production of uniform polymer particles in the 1–5 mm range has received a great deal of attention in the recent years because of its many applications in new technical fields. Micro size polymer particles have been generally prepared by emulsion polymerization (Kriwet et al., 1998). They were also prepared by other methods such as dispersion polymerization (Wang et al., 2002); spray drying methods (He et al., 1999). The selected method determines the characteristics of spheres, including the size, the most important property. As a consequence, knowledge of the experimental parameters (solvents, temperature, stabilizer, agitation rate, etc.) involved in each method is crucial (Lassalle et al., 2007).

Emulsion-based methods include the elimination of the solvents. Aqueous and oily phases can be present. In one report, PLA microspheres were prepared through the emulsion-solvent evaporation method as a biodegradable polymeric carrier for the non-steroidal anti-inflammatory drug, Nimesulide. Recently, 5-fluorouracil loaded PLGA-based microparticles are produced to be able to use for the treatment of brain tumours (Lassalle et al., 2007). In addition, various drugs such as bovine serum albumin (BSA) (Benoit et al., 1999), Nifedipine and propranol HCl (Perez et al., 2000) have been encapsulated with polycaprolactone (PCL) using the emulsion method (Sinha et al., 2004).

The spray drying method, in which the drug is dissolved or suspended in an organic phase and then solution is sprayed in a hot air flow, is another method for preparation micro and nanoparticles. Contrary to the conventional solvent evaporation methods, spray drying is a very rapid procedure especially useful at the industrial scale as a mild method. In spite of its advantages, it has limitations on particle shape uniformity and homogenity. PLGA microspheres were formed through this method and used in the controlled release of cytochrome C. Also, the spray drying technique has been also used in the preparation of PCL microspheres loaded with Ketoprofen.

Polymers with low melting points have been fabricated into microspheres by the hot melt technique. The molten polymer is dispersed in a suitable dispersion medium and slowly cooled to form the microspheres. This method is found to be particularly successful in microspheres which are susceptible to hydrolysis in the presence of moisture (Sinha et al., 2004).

1.2.2 Nanoparticles

Nanoparticles with size ranging between 10 and 1000 nm are colloidal drug delivery systems, which act as transport carrier compartments for drugs or other active molecules encapsulated, dispersed or absorbed in the nanoparticles (Sinha et al., 2004). In situations where the drug is administered via systemic circulation or have to cross the mucosal membrane, particles less than 500 nm are required (Lassalle et al., 2007). Therefore, nanoparticles have an advantage over larger microparticles, because they are better suited for intravenous delivery. Also, the smallest capillaries in the body are 5–6 μm in diameter. The size of particles being distributed into the blood stream must be significantly smaller than 5 μm, without forming aggregates, to ensure that the particles do not form an embolism (Hans et al., 2002).

Nanostructured delivery architectures are promising candidates that enable efficient and targeted delivery of novel drug compounds. They have the potential to enhance drug bioavailability, improve drug release duration, and enable targeting. This system can be used in pulmonary therapies (Courrier et al., 2002), as gene delivery vectors (Senior, 1998), and in entrapment of drugs with fast degradation (LaVan et al., 2003). Some biologic nanostructures used in drug delivery include lipid nanoparticles (Seki, 2004), peptides (Djalali et al., 2004), chitosan (Park et al., 2004), viral nanoparticles (Yamada et al., 2004) and nucleic acid nanostructures (Niemeyer, 2001).

Nanostructured drug carriers help overcome the problems such as the penetration of the drug through the blood brain barrier, the branching pathways of the pulmonary system, and the tight epithelial junctions of the

skin. Also, they help to the delivery drug into the cell and to target tumors. The discontinuous nature of the tumor microvasculature containing pores ranging from 100 to 1000 nm makes this penetration possible. The microvasculature of healthy tissue such as heart, brain or lung are less than 10 nm, and targeting to tumors within these tissue types is achieved with nanostructured delivery systems, which are smaller than the pores of the tumor vasculature, but larger than pores within the healthy tissue (Hughes, 2005).

Nanoparticles can be used for selective targeting via reticuloendothelial system to liver and to cells that are phagocytically active. The size of nanoparticles allows them to be administered intravenously unlike many other colloidal systems which occlude both the needles and the capillaries. However, they have the disadvantage of fast elimination from the body by the reticuloendothelial system. A solution for this problem was seeked by developing monodisperse biodegradable nanoparticles from amphiphilic copolymers. These demonstrated an increase of half-life in blood circulation and reduced accumulation in liver (Sinha et al., 2004).

The effect of size on NP biodistribution has been shown to be organ specific and non-linear due to organ-specific physical and physiological barriers. Thus, controlling the NP size is very important for their use in various clinical applications, allowing for optimization of NP delivery vehicles for systemic administration (Cheng et al., 2007).

There are several different methods for preparing nanoparticles. Additionally, numerous methods exist for incorporating drugs into the particles. For example, drugs can be entrapped in the polymer matrix, encapsulated in a nanoparticle core, surrounded by a shell-like polymer membrane, chemically conjugated to the polymer, or bound to the particle’s surface by adsorption.(Hans et al., 2002)

1.2.2.1 Preparation and Characterization Methods

Different methods have been reported in the literature for the preparation of nanoparticles including emulsion polymerization in continuous aqueous phase, interfacial polymerization, interfacial disposition (nanoprecipitaion), solvent evaporation, desolvation of macromolecules, dialysis method, etc. (Sinha et al., 2004).

Nanopreciptation (or solvent displacement method), was first described by Fessi et al. and is based on the interfacial deposition of polymers. It is a straight forward technique, rapid and easy to perform. Ideally, both the polymer and the drug must dissolve in solvent, but not in the non-solvent. Nanoprecipitation occurs by a rapid desolvation of the polymer when the polymer solution is added to the non-solvent. The rapid nanoparticle formation is governed by the so-called Marangoni effect, which is due to interfacial turbulences that take place at the interface of the solvent and the non-solvent and result from complex and cumulated phenomena such as flow, diffusion and surface tension variation (Bilati et al., 2005; Blouza et al., 2006). Consequently, reduced or even no drug leakage toward the outer medium leads to approximately 100% entrapment efficiency (Bilati et al., 2005). A nanoparticle system with high entrapment efficiency is expected to decrease the quantity of carrier and the drug waste during manufacturing (Govender et al., 1999). Additionally, this method is free from use of large amounts of toxic solvents and external energy sources. The general limitation is the solubility of the drug in organic solvents (Lassalle et al., 2007). This technique is mostly suitable for compounds having a hydrophobic nature such as Indomethacin, which is soluble in ethanol or acetone, but displays very limited solubility in water (Bilati et al., 2005). Nanoprecipitation is mostly used for the entrapment of hydrophobic drugs (Lassalle et al., 2007). Mainly water insoluble drugs have been incorporated into nanoparticles using the nanoprecipitation technique with typical drug content values being: Indomethacin, 2.0% w/w or 5.8% w/w; Dexamethasone, 0.9% w/w and Itraconazole, 4.1% w/w (Govender et al., 1999). The main problem with the nanoprecipitation method is the frequent agglomeration of particles due to the

lack of a stabilizer. This can be solved ensuring rapid and efficient stirring, by slow addition of the organic phase to the aqueous phase, and by the selection of an adequate solvent system (Lassalle et al., 2007).

1.2.3 Surface Modification and Targeting Methods

Generally, intravenously injected carriers are quickly removed from the circulation by macrophages. As a result they decrease the site specific delivery of drugs. In an attempt to reduce or minimize particle interaction with opsonins, which facilitate this phagocytic process, the concept of steric stabilization of particulates was introduced (Govender et al., 2000). Particles should be designed with a defense system to avoid being cleared from the body. They are called as stealth or long circulating particles. Particles can gain stealth properties by adding polyethylene glycol (PEG) (Dong et al., 2004). PEG is a hydrophilic and biocompatible polymer that has long time circulation through prevention of protein adhesion and the enhanced permeation and retention (EPR) effect if nano in size (Yang et al., 2008).

PEG chain binds at one end to particles and the other is free to form hydrophilic shell, which becomes an effective protective layer to inhibit opsonization (Dong et al., 2004). Particulates, surface modified with PEG, are being increasingly investigated. The *in vivo* behaviour of particulate systems prepared from diblock polymers with PEG as the hydrophilic component, such as PLA–PEG, has been widely examined (Govender et al., 2000).

CRS vesicles may be targeted either passively or actively. Passive targeting involves the natural uptake by cells that scavenge foreign particulates such as reticuloendothelial cells, concentration in tissues such as the liver or spleen, or circulating monocytes. Active targeting generally involves placing a charge or recognition sequence onto the vesicle such that it is more rapidly taken up by certain cell types (such as cancer cells) than others (Blouza et al., 2006). So, the drug targeting could be further increased by binding pilot molecules such as antibodies or sugars or by introducing a polymer sensitive to variation in temperature or pH (Jones et al., 1999).

In recent years, ligands which are able to identify cancer cells greater viewed with interest. Receptors have site-specific properties and increase cellular uptake within target cells in natural metabolism. These properties may allow for the selective targeting of imaging and therapeutic agents to tumor tissue, reducing the side effects of drugs. However, they are not specific to tumor types. In order to overcome this problem, tissue specific ligands are being used to increase specific targeting. Monoclonal antibodies, peptide, biotin, and folic acid (FA) are some of the examples of agents used in anticancer drug delivery (Yadav et al., 2007). Folic aid (FA) is well known receptor which has a high affinity to tumor cells (Yang et al., 2008).

Magnetic microparticles have also been used to target drugs to specific locations in animal models via external magnetic fields (Langer, 1990). Additionally, similar studies investigating stimuli responsive drug delivery systems, which can be targeted by local temperature, pH or ionic changes are being viewed with great interest.

1.3 Intelligent (Responsive) Systems

It would be highly beneficial if the active agents were delivered by a system that sensed the signal caused by disease, judged the magnitude of signal, and then acted to release the right amount of drug in response. Such a system would require coupling of the drug delivery rate with the physiological need by means of some feedback mechanism (Qiu et al., 2001).

Compared to the conventional drug delivery systems, the smart drug delivery systems are more advantageous due to control of release behavior by external changes, such as temperature (Qiao et al., 2005; Shin et al., 2006), ionic strength (Gelfuso et al., 2008; Zhang et al., 2005), electric fields (Zaharoff et al., 2002) and pH (Hruby et al., 2005; Zhang et al., 2002). External stimuli are produced with the help of stimuli generating devices, whereas internal stimuli are produced within the body to control the structural changes in the polymer network and to exhibit the desired drug release (Gupta et al., 2002). pH and/or thermoresponsive systems have been the

most promising, since these factors are the most available parameters within the body (Shin et al., 2006).

1.3.1 pH Sensitive Systems

Polymers containing ionizable functional groups that respond to changes in pH are called pH-sensitive polymers. As a result of the generation the charge along the polymer backbone, the electrostatic repulsion causes an increase in the hydrodynamic volume of the polymer (Jeong et al., 2002). Swelling and shrinking of pH dependent materials due to the acidic or basic components are reversible changes.

Variations in the pH are known to occur at several body sites, such as the gastrointestinal tract, vagina and blood vessels, and these can provide a suitable base for pH-responsive drug release. In addition, local pH changes in response to specific substrates can be used for modulating drug release. The pH-responsive drug delivery systems have been targeted for peroral controlled drug delivery, taste-masking of bitter drugs and intravascular drug release during elevated blood pH in certain cardiovascular defects. Among others, the most commonly studied ionic polymers with potential for pH-responsive behavior include poly(acrylamide) (PAAm), poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), poly(diethylaminoethyl methacrylate) (PDEAEMA) and poly(dimethylaminoethyl methacrylate) (PDMAEMA) [30] Apart from the use of synthetic polymers, various natural polymers, such as albumin (Mao et al., 1999) and gelatin (Uekama et al., 1999), have also shown pH-responsive behaviour (Gupta et al., 2002).

Recently, pH-sensitive polymers have been regaining attention for applications in gene delivery and gene therapy research. Transport of naked DNA into a cell is a difficult process because of its negative charge and the large size under physiological conditions. Positively charged polymers are used to balance the charge and condense the DNA in nanoparticles of ca. 100 nm. Poly(L-lysine), poly(ethyleneimine), amine-containing dendrimers, and amine-containing fractured dendrimers have been investigated for this

purpose. Additionally, a slight difference of pH in cancer tissue in comparison with normal cells is important in the localization of pharmaceutical agents in cancer cells (Jeong et al., 2002).

pH/ temperature dual responsive materials are polymer networks which contain both pH- and temperature-sensitive components in their structure and are used for Methotrexate delivery. Also, hydrogels from poly(NIPAM-co-butyl methacrylate-co-dimethylaminoethylmethacrylate) have been investigated for releasing human calcitonin (Shin et al., 2006).

Semi-IPN of crosslinked chitosan and PEO showed more swelling under acidic conditions as in the stomach. This type of hydrogels would be ideal for localized delivery of antibiotics, such as Amoxicillin and Metronidazole, in the stomach for the treatment of *Helicobacter pylori* (Qiu et al., 2001).

1.3.2 Thermoresponsive Systems

Most polymers increase their water-solubility as the temperature increases. Polymers with LCST, however, decrease their water-solubility as the temperature increases.

Polymers shrink as the temperature increases above the LCST. This type of swelling behavior is known as negative temperature-dependence. These polymers are generally hydrophobic or contain a mixture of hydrophilic and hydrophobic segments. At lower temperatures, hydrogen bonding between hydrophilic segments of the polymer chain and water molecules are stronger causing to be insoluble in water. When the temperature increases, hydrophobic interactions become strengthened, while hydrogen bonding becomes weaker. Thus shrinking of the polymers occurs due to inter-polymer chain association through hydrophobic interactions. Generally, if the polymer chain contains more hydrophobic constituent, LCST becomes lower. The LCST can be changed by adjusting the ratio of hydrophilic and hydrophobic segment of the polymer. One way is to make copolymers of hydrophobic and hydrophilic monomers (Qiu et al., 2001).

Thermosensitive drug carriers are a promising design for targeted delivery of particularly cancer therapeutics. They release the encapsulated drug at the targeted site caused by the temperature difference between tumor site and normal tissues (Li et al., 2008).

Thermoresponsive hydrogel is the most extensively studied, polymeric responsive material. They can be constructed of various polymers, such as the N-substituted polyacrylamide, polymethylacrylamide and poly(ethylene oxide). A number of thermo-sensitive hydrogels show a negative response to temperature. A positive controlled release pattern which means high drug release at increased temperature and slow drug release at decreased temperature, is needed especially in inflammation or in cancer therapies where the local temperature is increased. (Zhang et al., 2002).

1.3.2.1 p(N-Isopropylacrylamide) Based Systems

Poly(N-isopropylacrylamide) (pNIPAM) is one of the most widely studied thermoresponsive, synthetic polymer (Figure 2). It is soluble below 32 °C and precipitates above 32 °C in water (Figure 3). The phase transition temperature is referred to as the lower critical solution temperature (LCST). Below the LCST, favorable interactions via hydrogen bonding between amide groups of the polymer and the water molecules lead to the dissolution of the polymer. Above the LCST, the hydrogen bonds are broken and water molecules are expelled from the polymer, resulting in precipitation. By controlling the polymer composition and topology, the coil-to-globule transition could be kinetically and thermodynamically controlled. Copolymerization of NIPAM with hydrophobic butylmethacrylate decreases the LCST of aqueous copolymer solution and copolymerization with hydrophilic comonomers, such as acrylic acid or hydroxyethyl methacrylate, results in an increase in LCST. Polymer systems with more than one transition can be obtained by controlling the polymer architecture. Recent studies of NIPAM copolymers focused on biological applications such as separations, enzyme immobilization, gene delivery, cell culture and cell sheet engineering. In a

related application, the pNIPAM polymers were investigated for on–off control of avidin–biotin binding. Hoffman et al. reviewed progress in smart bioconjugates that use stimuli-responsive properties of pNIPAM (Jeong et al., 2002).

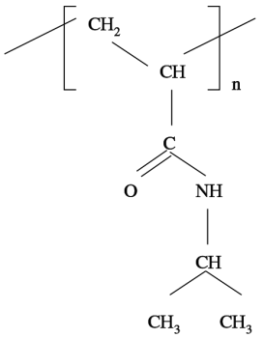

Figure 2 Structural formula for pNIPAM

Hydrogels of pNIPAM and PAA exhibited dual sensitivities (Mehavar, 2000) that PAA is pH-sensitive. These hydrogels were able to respond rapidly to both temperature and pH changes, and were used in the delivery of insulin and calcitonin. Such systems were also evaluated for prolonged mucosal delivery of bioactive agents, specifically peptide drugs and release of streptokinase and heparin. The system provided control over the drug release in a pulsatile pattern with a high swelling ratio of hydrogel in the presence of both stimuli (Gupta et al., 2002).

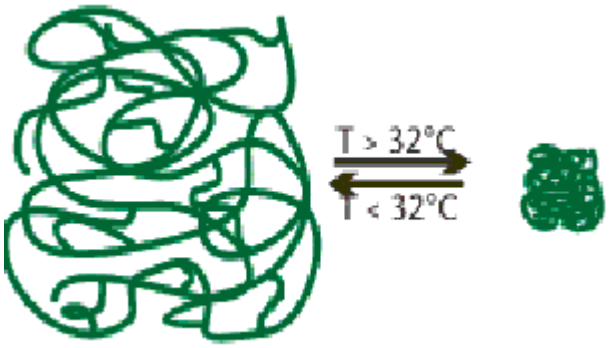

Figure 3 Schematic sketch of swelling-deswelling of thermoresponsive products

Many studies have reported on the applications of pNIPAM-based hydrogels as versatile tools in biology for processes such as separation and purification of proteins, drug delivery, control of enzyme activity, carriers for biomolecules, therapeutics and diagnostics. These applications were also found to be applicable to be microgel colloidal particles prepared by precipitation polymerization of N-alkyl acrylamides (especially NIPAM) in the presence of a crosslinker, methylene bisacrylamide (MBA), with or without a surfactant.

Clinical applications of thermosensitive hydrogels based on NIPAM and its derivatives have limitations. The monomers and crosslinkers used in the synthesis of the hydrogels are not known to be biocompatible, i.e. they may be toxic or carcinogenic (Qiu et al., 2001).

1.4 Therapeutic Use of Controlled Release Systems

In order to achieve therapeutic levels of drug at the tumor site without causing any damage to healthy organs and tissues, it is important to consider the physiological differences between diseased and normal sites. An exciting potential solution in cancer treatments is to encapsulate the drug in a biocompatible material that can be injected into the blood stream expecting to

deliver the drug to a tumor site as a response to a thermal stimulus (Li et al., 2008).

1.4.1 CRS in Cancer Therapy

Chemotherapy is a major treatment for cancers. However, anticancer drugs are not specific to cancer cells. Thus, the selective uptake of anticancer agents would be a great interest in chemotherapy. The properties of this agents such as administration route, biodistribution and elimination from the body, can be modified by controlled drug delivery systems. Most current anticancer agents can not differentiate between cancer and normal cells. This leads to systemic toxicity and adverse effects. Therefore, severe side effects in other tissues such as bone marrow suppression, cardiomyopathy, and neurotoxicity, limit the administration of maximum dose of the drug. Additionally, large drug quantities are needed to be used clinically due to rapid elimination and widespread distribution of the drug in healthy tissues. Thus, it is not an economical approach.

The diameter of a human cell is in the range 10 to 20 μm. Nanoscale devices can interact with biomolecules on the cell surface and within the cells (Yadav et al., 2007). Also, it was reported that smaller particles tend to accumulate in the tumor sites due to the facilitated extravasation and a greater internalization was also observed. Addtionally, spleen filtering can be prevented by using less than 200 nm particles (Dong et al., 2004).

Carriers tend to accumulate in solid tumors due to enhanced vascular permeability. Lymphatic system covers all macromolecules in interstitial space in normal tissues; however, lymphatic clearance is retarded in tumor tissues. Passive targeting, lower systemic toxicity and effective polymer-drug conjugate administration can be achived by this enhanced permeability and retention (EPR) effect of tumor sides (Neuse, 2008).

In several studies, it was demonstared that cell distribution of anticancer drugs can be controlled by entrapment in colloidal carrier. As an example, for the solubilization of paclitaxel (PTX), alternative drug delivery systems such

as liposomes, microspheres, nanoparticles and polymeric micelles are investigated. Also, a Cremophor-free formulation of PTX nanoparticles, Abraxane®, has recently been approved by the FDA for breast cancer (Danhier et al., 2009).

1.4.1.1 Celecoxib

The potential use of nonsteroidal anti-inflammatory drugs (NSAIDs) in cancer chemoprevention has been suggested. Epidemiological studies indicate that the use of aspirin and other NSAIDs reduces the risk of colorectal cancer by 40-50% and the risk of breast cancer. All NSAIDs in clinical use are cyclooxygenase (COX) inhibitors. There is two isoforms of COX: COX-1 is constitutively expressed in almost all tissues, whereas COX-2 is upregulated during pathological conditions such as inflammation and cancers. The COX-2 gene is rapidly induced in response to tumor promoters, cytokines, and growth factors. Moreover, oncogenes and p53 regulates the COX-2 expression positively and negatively, by turns. It is suggested that COX-2 is involved in the pathologic processes of cancer growth and progression, which is further supported by animal studies showing that tumorigenesis is inhibited in COX-2 knockout mice.

Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in many cancer cells such as colon, stomach, prostate, and breast (Hsu et al., 2000).

Celecoxib belongs to the new generation of NSAIDs. In clinical trials in familial adenomatous polyposis patients, celecoxib efficacy reduced the formation of colonic polyps without toxicity. Also, effect of Celecoxib on tumor growth inhibition and enhanced survival in the mouse model of urinary bladder cancer was demonstrated and this is suggesting that it might have a chemopreventive property. However, due to the cardiovascular risks of celecoxib, it is suggested to be used as a last choice. Also, it remains unclear how celecoxib displays its preventive effect on tumor proliferation and also how it triggers apoptosis in cancer cells (Arico et al., 2002).

1.5 Scope of The Study

The aim of this study is to design a thermoresponsive system that can deliver an anticancer agent, Celecoxib, by controlling the local temperature. This kind of a system is especially useful when pain relieve, insulin and the like are administered upon need by the patient rather than continuously.

In this study, Celecoxib carrying nanoparticles of pNIPAM were prepared and several parameters such as particle size, loading efficiency and release profiles known to be effective in the design of controlled release systems were investigated.

Nanoparticles prepared from polymers with different compositions produced by changing the initiator and crosslinker amounts were also compared in order to select the system with optimum loading, size and drug release kinetics.

Particles were prepared by using nanoprecipitation method variation of particle size was attempted by changing solvent:nonsolvent volume ratio and polymer concentration in the preparation medium.

In situ release behavior was studied at different temperatures in order to determine influence of size and preparation conditions on release profiles.

In vitro cytotoxicity and efficiency tests of selected nanoparticle types were performed on cell culture with Saos-2 cells.

The most suitable nanoparticle Celecoxib delivery system to use in cancer therapy by local hyperthermia was determined.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

N-Isopropylacrylamide was purchased from Sigma-Aldrich Co. (USA). Celecoxib was gift of Dr. Ibrahim Yilmaz (Fako Ilac San., Ist, Turkey). Ammonium persulphate (APS) was purchased from Fluka Biochemica (Switzerland).

N,N,N,N-tetramethyl ethylenediamine (TEMED) and N,N-methylene bisacrylamide (MBA) were purchased from Merck (Germany). Cyclohexane and acetonitrile were purchased from Riedel de Haën (Germany). Acetone and ethanol were purchased from J. T. Baker (Netherlands).

The human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection (ATCC). RPMI-1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from HyClone® (USA). NucleoCounter reagents were purchased from Chemometec (Denmark). CellTiter 96* AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Germany).

2.2 Methods

2.2.1 Preparation of pNIPAM Based Nanoparticles

2.2.1.1 Synthesis of Crosslinked & Uncrosslinked N-Isopropylacrylamide Homopolymers

Uncrosslinked pNIPAM polymers were prepared via free radical polymerization. N-Isopropylacrylamide (1 g, 10% w/v) was dissolved in distilled water (10 mL). The polymerization was carried out at 65 ^{o}C for 16 h in the oven by using ammonium persulfate (APS, 0.3 mL from 5% APS stock solution) as the initiator and N,N,N,N-tetramethyl ethylenediamine (TEMED, 0.3 mL from 10% TEMED stock solution) as the accelerator under nitrogen atmosphere. Before polymerization, N_2 gas was bubbled through the solution for a few minutes and then the system was sealed. After the completion of the reaction, polymer was washed several times with ethanol and distilled water.

To synthesize crosslinked polymers, N,N-methylene bisacrylamide (MBA, 0.44% w/v) was added to the monomer solution and the above procedure was repeated. In order to vary the release behavior or response rate of the polymeric product, different initiator, accelerator and crosslinker amounts were used (Table 1).

Polymers were freeze-dried (FreeZone® 6 Liter Freeze Dry System, Labconco Co., USA) for ease of handling before using in nanoparticle preparation.

Table 1 Monomer (NIPAM), initiator (APS)/accelarator (TEMED) and crosslinker (MBA) content of the polymerization medium

NIPAM (%, w/v)	APS and TEMED* (%, v/v)	MBA (%, w/v)
10	1.5	-
10	3.0	-
10	6.0	-
10	3.0	0.22
10	3.0	0.44
10	3.0	0.88

* APS and TEMED were used in equal quantities.

2.2.1.2 Preparation of Nanoparticles

Nanoparticles were prepared by nanoprecipitation method (Fessi et al., 1989). Briefly, polymer was dissolved in organic solvent (S), acetone, to form the diffusing phase. Then, this phase added to dispersing phase which was cyclohexane, in which polymer is insoluble, non-solvent (NS), by micropipette without using any surfactant. An anticancer agent, Celecoxib, was loaded into the nanoparticles by dissolving the drug in acetone together with polymer. As soon as polymer solution diffused into the non-solvent medium, a turbid nanoparticle suspension was formed and it was left overnight in the hood to evaporate the solution (Damiani et al.,2003).

Parameters controlling the formation of NPs were systematically varied in order to obtain NPs with different particle sizes and release rates. The solvent-nonsolvent (S:NS) volume ratios and polymer concentrations in the solvent are presented in Table 2.

Table 2 Solvent-nonsolvent ratios and polymer concentrations used in nanoparticle preparation

Polymer content* (mg)	S content (mL)	NS content (mL)	Polymer Concentration (% w/v)	S:NS Volume Ratio
50	25.00	225.00	0.02	1:9
50	12.50	112.50	0.04	1:9
50	6.25	56.25	0.08	1:9
50	93.75	31.25	0.04	3:1
50	62.50	62.50	0.04	1:1
50	31.25	93.75	0.04	1:3
50	12.50	125.00	0.04	1:10

* Uncrosslinked pNIPAM prepared by using 3.0% initiator and accelerator amount and crosslinked pNIPAM prepared with 3.0% APS and TEMED and 0.44% amount of MBA was used in nanoparticle preparation for this experiment.

S: Solvent, NS: nonsolvent

To assess the effect of initiator, accelerator and crosslinker amount on nanoparticle size, loading efficiency and release behavior, polymers with

different structures mentioned in Table 1 was used in NP preparetion. For this experiment, S:NS volume ratio and polymer concentration in mixture was kept constant at 1:10 and 0.04%.

All nanoparticles and preparation parameters are presented in Table 3.

Table 3 Polymer and nanoparticle preparation conditions

Sample*	Polymer		NP	
	APS and TEMED* (%, v/v)	MBA (%, w/v)	S:NS volume ratio	Polymer Conc. (%, w/v)
$NP_{1.5,0,1:10,\ 0.04}$	1.5	-	1:10	0.04
$NP_{3,0,1:10,\ 0.04}$	3.0	-	1:10	0.04
$NP_{6,0,1:10,\ 0.04}$	6.0	-	1:10	0.04
$NP_{3,0,1:9,\ 0.02}$	3.0	-	1:9	0.02
$NP_{3,0,1:9,\ 0.04}$	3.0	-	1:9	0.04
$NP_{3,0,1:9,\ 0.08}$	3.0	-	1:9	0.08
$NP_{3,0,3:1,\ 0.04}$	3.0	-	3:1	0.04
$NP_{3,0,1:1,\ 0.04}$	3.0	-	1:1	0.04
$NP_{3,0,1:3,\ 0.04}$	3.0	-	1:3	0.04
$NP_{3,0,1:10,\ 0.04}$	3.0	-	1:10	0.04
$NP_{3,0.22,1:10,\ 0.04}$	3.0	0.22	1:10	0.04
$NP_{3,0.44,1:10,\ 0.04}$	3.0	0.44	1:10	0.04
$NP_{3,0.88,1:10,\ 0.04}$	3.0	0.88	1:10	0.04
$NP_{3,0.44,1:9,\ 0.02}$	3.0	0.44	1:9	0.02
$NP_{3,0.44,1:9,\ 0.04}$	3.0	0.44	1:9	0.04
$NP_{3,0.44,1:9,\ 0.08}$	3.0	0.44	1:9	0.08
$NP_{3,0.44,3:1,\ 0.04}$	3.0	0.44	3:1	0.04
$NP_{3,0.44,1:1,\ 0.04}$	3.0	0.44	1:1	0.04
$NP_{3,0.44,1:3,\ 0.04}$	3.0	0.44	1:3	0.04
$NP_{3,0.44,1:10,\ 0.04}$	3.0	0.44	1:10	0.04

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture

2.2.2 Characterization of Stimuli Responsive Systems

2.2.2.1 Infrared Spectroscopy of pNIPAM Nanoparticles

Infrared spectra of nanoparticles were obtained with Jasco FT/IR 4200 infrared spectrophotometer (Germany). The samples were dried in vacuum oven in order to remove the moisture, before grinding and mixing with KBr (ratio of 10:1 w/w KBr:sample).

2.2.2.2 Nanoparticle Topography by Scanning Electron Microscopy

Samples were prepared by spreading concentrated nanoparticle dispersions over the stubs. NPs were coated with gold under vacuum and were observed by using a QUANTA 400F Field Emission scanning electron microscope (FE-SEM).

2.2.2.3 Drug Loading

Drug loading in various nanoparticles (NPs) was determined by extraction of the content with appropriate solvent. Nanoparticles with encapsulated drug, Celecoxib (5 mg) were dissolved in acetone (1 mL) and then added dropwise into diethyl ether (9 mL). The supernatant was removed by Pasteur pipettes and filtered with 0.02 μm pore size Anodisc filters (Whatman International Ltd., England). The filtered solution was completely evaporated at room temperature under the hood. Then, by dissolving Celecoxib in acetonitrile:PB solution (70:30, v/v), the drug quantity was determined by UV–VIS spectrophotometry (UV 2100*;* Shimadzu, Japan) at 254 nm (Damiani et al.,2003; Jadhav et al, 2005). The Celecoxib amount was calculated from the

calibration curve which was prepared by measuring the absorbances of various Celecoxib concentrations (1-30 µg/mL) dissolved in acetonitrile:PB (70:30) solution by UV–VIS spectrophotometer at 254 nm and plotting a concentration vs. absorbance graph (Appendix A).

The encapsulation efficiency (EE) was expressed as the fraction of the drug in the produced NP with respect to the input drug amount.

The loading was expressed as the fraction of the drug in the produced NP with respect to the total NP weight.

This procedure was also performed with unloaded nanoparticles as a control and each experiment was performed in triplicate.

2.2.2.4 *In situ* Release Studies

For the *in situ* release, the thermoresponsive nanoparticles (5 mg) containing anticancer agent were added to phosphate buffer solution (1 mL, 0.01 M, pH 7.4) in Eppendorf tubes. At predetermined time points, Eppendorfs were centrifuged at 13500 rpm for 5 min, the supernatant was removed and diluted with acetonitrile to obtain acetonitrile:PB solution (70:30, v/v). The pellets were resuspended in fresh PB (1 mL) and the process was repeated. Celecoxib in the treated supernatants was determined by using UV–VIS spectrophotometer at 254 nm. Celecoxib amount was calculated from the calibration curve (Appendix A).

In situ release was carried out at two temperatures; 30 ^{o}C and 37 ^{o}C in triplicate.

For evaluation of release kinetics, the obtained release data were fitted to first order, zero order and Higuchi equations. Determination of the best fit was based on the comparisons of the relevant correlation coefficients (Jadhav et al., 2005).

2.2.3 *In vitro* Cell Viability Studies

2.2.3.1 Trypsinization and Cell Seeding

Human osteosarcoma cell line, Saos-2, was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit) at 37 °C in a humidified 5% carbon dioxide incubator (Sanyo MCO-17AIC, Japan). Cells were incubated in T-75 tissue culture polystyrene (TCPS) flasks until confluency.

For passaging or cell seeding, cells were detached from the flask by incubation at 37 °C for 5 min with trypsin-EDTA solution (3 mL of 0.05%). Before the addition of trypsin, the medium in the flasks was removed and cells were washed twice with sterile PBS solution. Then, RPMI-1640 medium (6 mL, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit)) was added to the flask to inhibit trypsin activity. The detached cells were centrifuged at 3000 rpm for 5 min, the supernatant was removed and the cells were resuspended in RPMI-1640 medium (2 mL, 1% penicillin/streptomycin (100 unit), 10% FCS).

For passaging, these cells were replated to the T-75 tissue culture flasks. For cell seeding, the number of viable cells was determined with the NucleoCounter (Chemometec A/S Nucleo Counter, Denmark) before and after the treatment with a lysis buffer, giving an estimate of nonviable and total cells. The cell suspension was then replated to 24 well plates at needed density. For each parameter, all the experiments were conducted in triplicate and cell-free medium was used as a blank.

2.2.3.2 MTS Assay for Quantification of Saos-2 Cell Line

MTS assay was used to determine cell viability and proliferation. Calibration curve for MTS was prepared. Briefly, after trypsinization process and cell counting as described in Section 2.2.3.1, the collected cells were seeded on 24 well plates in different amounts $5x10^3$ to $2.5x10^5$. As a control, cell free medium was added into the TCPS. The 24 well plates was incubated at 37 °C in a humidified 5% carbon dioxide incubator for 3 h in order to maintain cell attachment onto TCPS. Then, the medium was removed and the wells were washed three times with sterile PB solution. MTS solution (0.5 mL) containing 10% MTS, 90% Low glucose DMEM medium (1% penicilin/streptomicin, 10% FCS) was added into each well and the cells were incubated at 37 °C in a humidified 5% carbon dioxide incubator for a further 2 h.

After incubation, 200 µL of solution was removed from each well and added into a 96 well plate and the absorbances were measured at 490 nm by using Elisa Plate Reader (Maxline Vmax®, Molecular Devices, USA).

The obtained absorbance values were plotted against the related cell numbers to establish a standard calibration curve (Appendix B). Viable cell numbers in the experiments were then determined from this curve by measuring their absorbances.

2.2.3.3 Effect of Free Celecoxib on Human Osteosarcoma Saos-2

In order to investigate the effect of Celecoxib on cell viability, the cells were plated in 24 well plates at a density of $2x10^4$ cells/well. Trypsinization and cell seeding procedures were performed as described in Section 2.2.3.1. After 24 h incubation to allow reattachment, the medium in the wells was replaced with the fresh RPMI-1640 medium (supplemented with 1% penicillin/streptomycin (100 unit), 10% FCS) containing varying concentrations of Celecoxib (1-200 µM). Cell free medium was used as a blank and Celecoxib free cell culture was used as control group.

To determine the effect of Celecoxib dose on Saos-2 cell line, at predetermined time points, cell viability and proliferation was determined with MTS assay as previously described in Section 2.2.3.2.

For each time point, both the experimental and control group were independently studied. The results were evaluated by comparing the experimental group with the control group at identical time points.

2.2.3.4 Effect of Nanoparticles on Saos-2 Cell Line

The effect of Celecoxib loaded and Celecoxib free nanoparticles on *in vitro* cell proliferation was determined by seeding cells at a density of $5x10^4$ cell/well in 24 well plates as described previously (Section 2.2.3.1). Cells were allowed to adhere by incubating for 24 h and nanoparticles (5 mg) sterilized by 30 min exposure to UV were introduced to the medium. Cell free medium was used as a blank and NP free cell culture was used as control group.

In predetermined time points, cell viability and proliferation was determined with MTS assay as previously described in Section 2.2.3.2.

For each time point, both the experimental and control group were independently studied. The results were evaluated by comparing the experimental group with the control group at identical time points.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Characterization

pNIPAM was characterized by FTIR spectrophotometry to ascertain complete polymerization. The formation of nanoparticles and drug encapsulation was assessed by SEM and spectrophotometric assays.

3.1.1 FTIR Spectrophotometry

N-Isopropylacrylamide monomer has a number of distinctive bonds that can be used for identification such as C=C (917, 965, and 992 cm^{-1}), C=O (1655 cm^{-1}), N-H (3268 and 1542 cm^{-1}) and CH_3 (2970 cm^{-1}) peaks (Figure 4a).

In the FTIR spectra of the free and drug loaded pNIPAM NPs, the absence of the C=C bond of the monomer, indicates that NPs had no trace of the monomer and the polymerization of NIPAM is achieved. A representative FTIR spectrum of pNIPAM-based NPs is presented in Figure 4b.

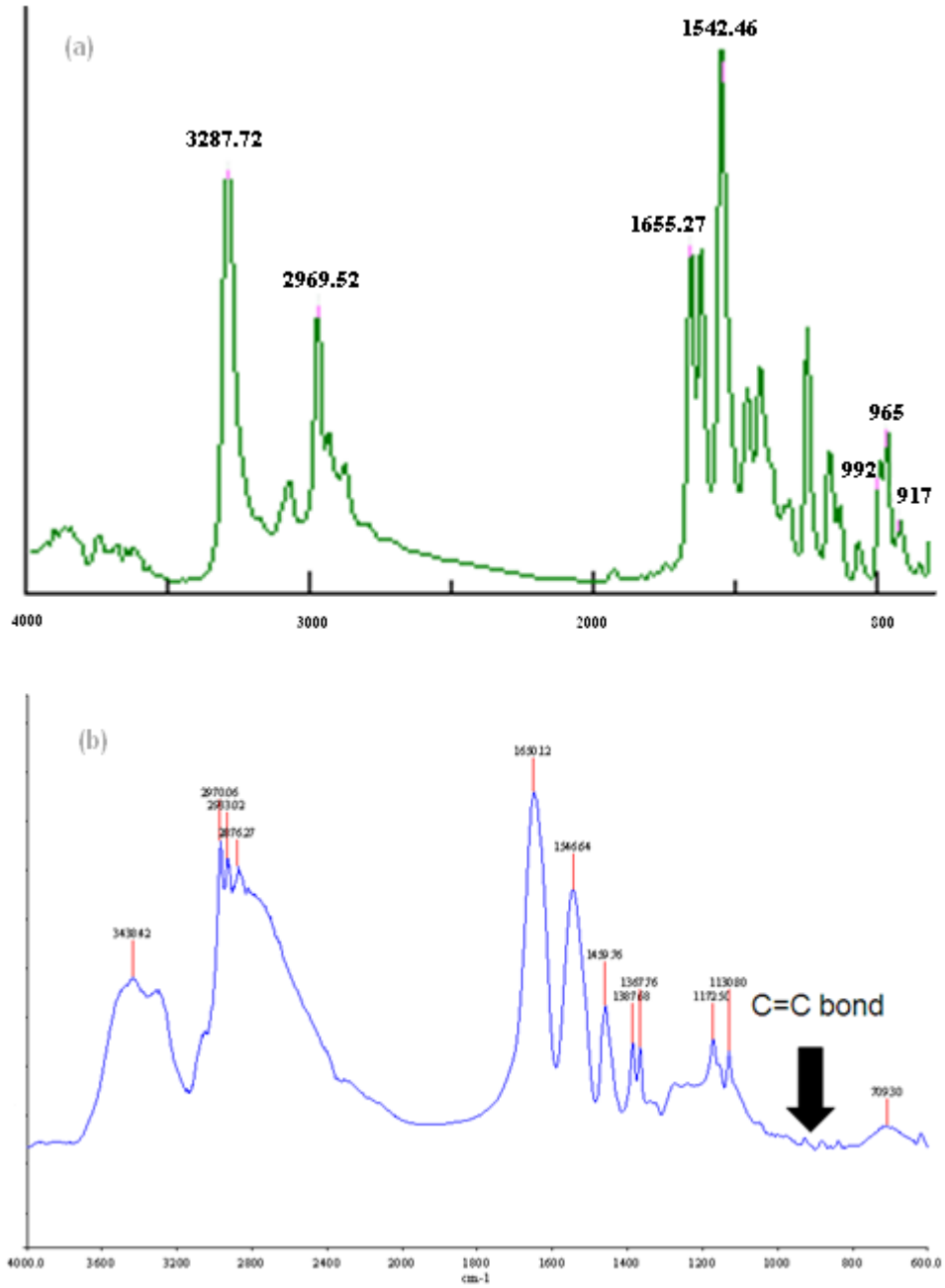

Figure 4 Representative FTIR spectra of NIPAM (a) monomer and (b) polymer

3.1.2 Particle Size and Shape of the Nanoparticles

Physical appearance of the particles (roundness, smoothness, and formation of aggregates) was studied by scanning electron microscopy (SEM). The size distribution of particles was investigated by using image analysis software Image J (NIH, U.S.A.) on these SEM micrographs.

Statistical analyses were carried out using one way ANOVA for multiple sample comparisons, Tukey's test for multiple pairwise comparisons when ANOVA produced significant differences. P values less than 0.05 were considered as statistically significant.

3.1.2.1 Influence of Crosslinker Concentration

Polymerization and NP preparation conditions were kept constant while investigating the influence of the crosslinker amount on particle properties. The initiator amount, polymerization duration and temperature of the polymerization solution were maintained as 3%, 16 h and 65 ^{o}C, respectively. NPs were produced by keeping solvent to nonsolvent (S:NS) ratio and the polymer concentration in the S:NS mixture constant at 1:10, 0.04%, respectively. Influence of the crosslinker amount (varied between 0.22-0.88%) on the size and shape of pNIPAM NPs was evaluated.

The mean size of particles was determined by measuring the diameters of ten particles selected from SEM micrographs via Image J Software. The size of pNIPAM NPs prepared by using 0.22, 0.44 and 0.88% (w/v) crosslinker were found to be 234±71, 225±17 and 96±25 nm, respectively (Figure 5). This demonstrated that an increase in the crosslinker amount lead to a decrease in particle size. Even though the effect of crosslinker concentration on size distribution was found to be statistically significant ($p=0.001$, ANOVA), the difference between the size distribution of pNIPAM NPs prepared with 0.22 and 0.44% crosslinker was not ($p=0.892$, Tukey's test).

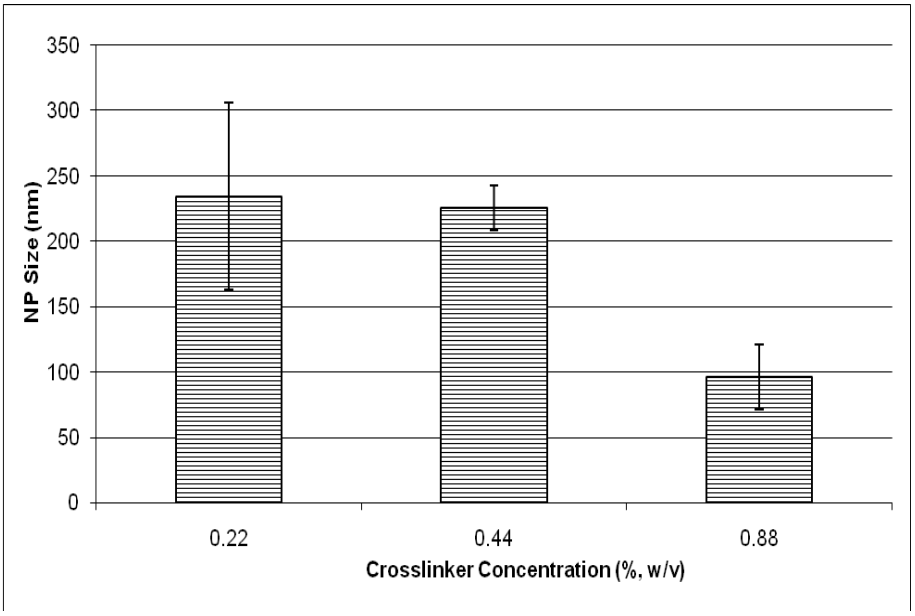

Figure 5 Effect of crosslinker amount on NP size

In a similar study investigating influence of crosslinker concentration on particle size, it was reported that addition of crosslinker may reduce the particle sizes (Cao et al., 2000) supporting the results of this study.

SEM images obtained from pNIPAM NPs prepared by using various crosslinker concentrations are presented in Figure 6. The shape of the particles was mostly spherical in the samples prepared with 0.022 and 0.44% crosslinker. The angular shaped NPs were more abundant in the samples prepared with 0.88% crosslinker. It might be suggested that increasing the crosslinker amount decreases particle size and causes the formation of stiffer and less spherical particles with sharp, angular edges.

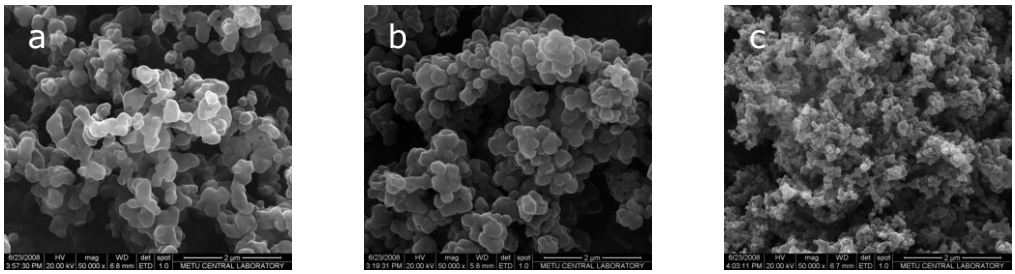

Figure 6 SEM of NPs based on pNIPAM, which was prepared by using various concentrations of crosslinker: (a) 0.22% (b) 0.44% (c) 0.88% (x50 000)

3.1.2.2 Influence of Initiator and Accelerator Concentration

Influence of initiator and accelerator concentration on size and shape of uncrosslinked pNIPAM NPs were evaluated by analyzing the SEM micrographs. The S:NS ratio and polymer concentration in the S:NS mixture were kept constant at 1:10 and 0.04%, respectively, while the initiator and accelerator concentration varied between 1.5-6.0% (v/v).

The sizes of pNIPAM NPs prepared with 1.5, 3.0 and 6.0% (v/v) initiator were 197±50, 67±14 and 196±36 nm, respectively (Figure 7). Increase of initiator concentration leads to higher number of free radicals, thus the number of chains increase and chain sizes become smaller. As a result, molecular weight of polymer decreases. However, the molecular weight of chains in a droplet influences the solution viscosity and thus the final droplet dimension. Less viscous solutions would probably lead to a looser, and thus smaller, particle so one would expect an increase of initiator concentration to lead to a NP size decrease. This is partially observed in Figure 7. However, there is no distinct trend in this figure and the literature is also not clear on this point.

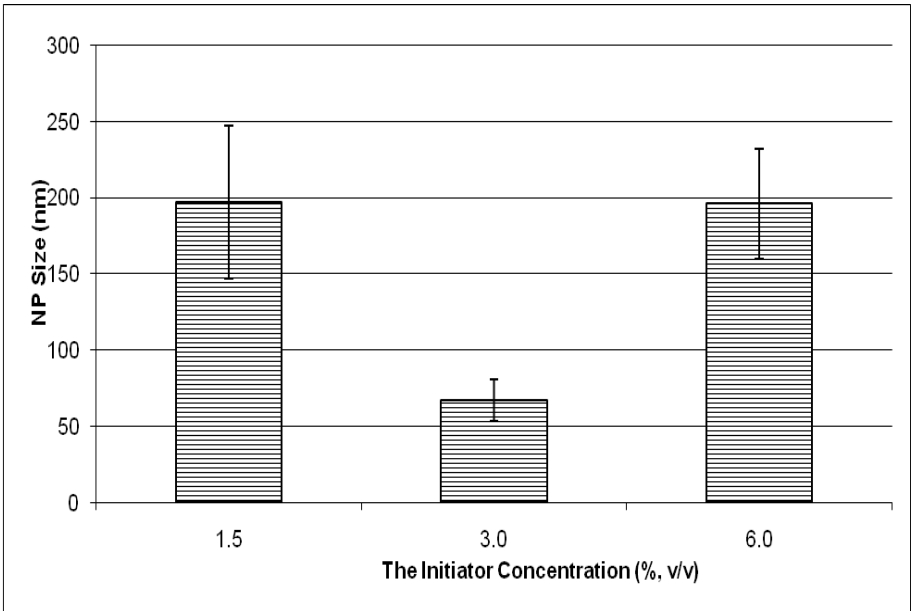

Figure 7 Effect of initiator concentration on NP size

In earlier studies, it was observed that an increase in initiator concentration causes larger particles (Cao et al., 2000; Jeong et al., 2004). In another study with PLA–PE NPs, increasing the molecular weight (MW) of PLA block from 3 to 110 kDa increased particle size from 27.6 to 152.4 nm. In the mentioned studies, particle preparation methods, polymer types and initiators are different, thus these disagreements are not unexpected. In the present study, there was a statistically significant influence of initiator concentration on the particle size ($p=0.001$, ANOVA). No statistically significant difference was observed between pNIPAM NPs prepared with 1.5 and 6.0% APS and TEMED in terms of particle size ($p=0.998$, Tukey's test).

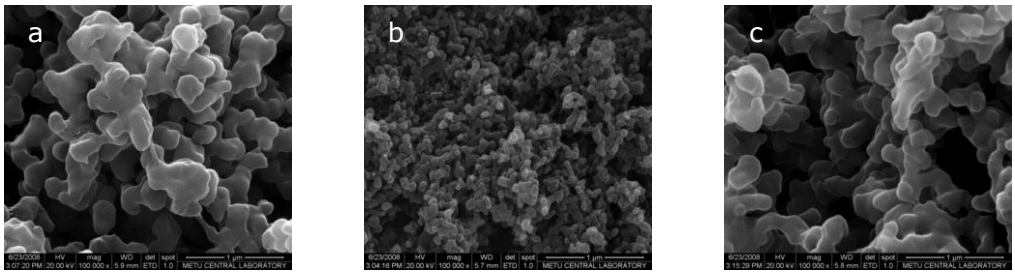

Figure 8 SEM of NPs based on pNIPAM which was prepared by using various amounts of initiator and accelerator amount: (a) 1.5% (b) 3.0% (c) 6.0% (x100 000)

Round, smooth and smaller particles were observed only with NPs prepared with 3.0% APS and TEMED concentration. In other samples, more aggregates were present (Figure 8).

Additionally, when the NPs were compared in terms of crosslinking, generally the uncrosslinked particles were found to be more spherical; however, more clusters were observed. The interesting observation was that the NPs formed using crosslinked polymers had larger sizes than the uncrosslinked ones. It appears that this study should be carried out with the use of surfactants to maintain droplet shape and dimension.

3.1.2.3 Influence of Polymer Concentration

The influence of the polymer concentration in S:NS mixture on particle size and shape was investigated both for crosslinked and uncrosslinked pNIPAM particles using SEM micrographs. The S:NS ratio was kept constant at 1:9.

The sizes of uncrosslinked pNIPAM NPs prepared by using 0.02, 0.04 and 0.08% (w/v) polymer in the S:NS mixture were found to be 51±6, 53±12 and 87±17 nm, respectively (Figure 9).

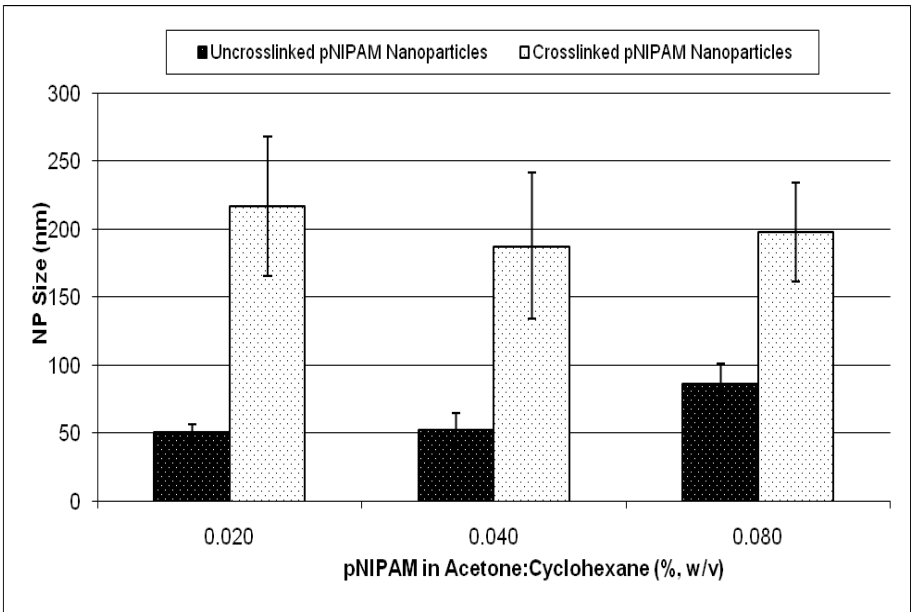

Figure 9 Effect of polymer concentration in the mixture on NP size

The effect of polymer concentration on the size distribution of uncrosslinked pNIPAM NPs was found to be statistically significant (p=0.001, ANOVA); however, no difference between NPs prepared by using 0.020 and 0.040% polymer concentration in the mixture was observed (p=0.934, Tukey). These

showed that polymer concentration increase leads to NP size increase. This indicates that an increase in droplet viscosity due to higher polymer concentration, leads to an increase in droplet size showing a higher ability retain droplet size. The less viscous ones shrank upon exposure to non-solvents.

The mean size of crosslinked pNIPAM NPs prepared by using 0.020, 0.040 and 0.080% (w/v) polymer concentration in S:NS mixture was found to be 217±51, 188±54 and 198±36 nm, respectively. No statistically significant effect of polymer concentration in S:NS mixture on the size distribution of crosslinked pNIPAM NPs was observed (p=0.381, ANOVA).

The micrographs of uncrosslinked NPs prepared by using various polymer concentrations are illustrated in Figure 10. The shape of the particles is spherical with some aggregation which was most probably caused by drying, sample preparation for SEM imaging and also due to the increase in the polymer concentration.

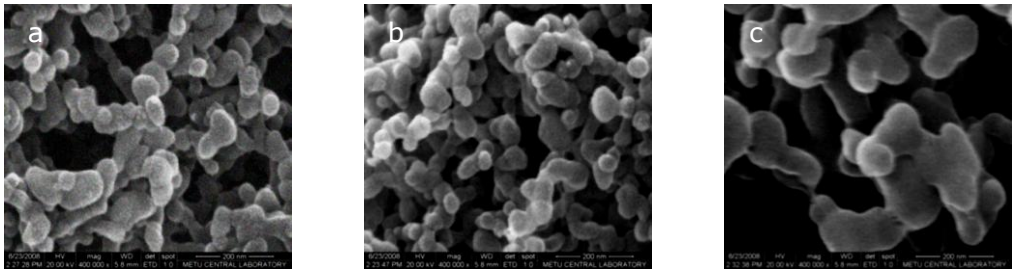

Figure 10 SEM micrographs of uncrosslinked pNIPAM NPs prepared by using various polymer concentrations: (a) 0.020% (b) 0.040% (c) 0.080% (x400 000)

The micrographs of crosslinked pNIPAM NPs prepared by using various polymer concentrations are presented in Figure 11. The shape of the

crosslinked particles was observed to be more spherical and less aggregated than the uncrosslinked NPs. Nevertheless, no influence of polymer concentration either on particle shape or size was observed in the samples of crosslinked NPs.

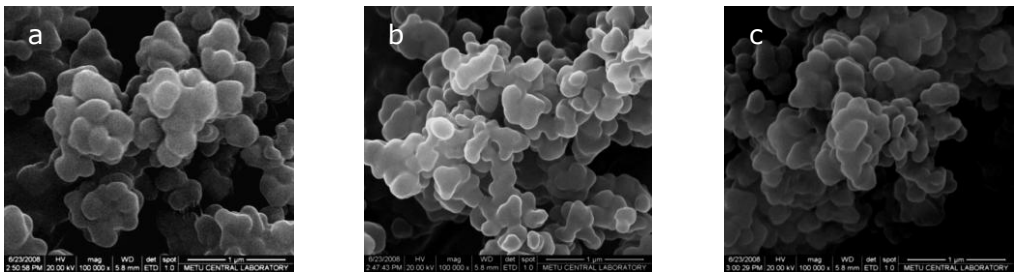

Figure 11 SEM micrographs of crosslinked pNIPAM NPs prepared by using various polymer concentrations in the S:NS mixture: (a) 0.020% (b) 0.040% (c) 0.080% (x100 000)

In a study by Chorny et al. (2002), who investigated PLA NP preparation by nanoprecipitation, the particle size has been found to depend on the PLA concentration that increasing the PLA concentration increased the particle size. In another study, it was reported that using high polymer concentrations prevented NP preparation (Bilati et al., 2005). Additionally, other studies with similar observations have suggested that the diffusion rate of solvent decreases with the viscosity increase and this prevents the complete dispersion of the solvent through nonsolvent leading to larger NPs (Bilati et al., 2005; Zhang et al., 2006).

In the current study, similar results were obtained when uncrosslinked polymer concentration was increased, even though particle preparation was not prevented. The particle size was found to be approximately two-fold larger when the uncrosslinked pNIPAM amount was increased four times. In the

present study, unlike the uncrosslinked NPs, crosslinked polymer chains might not separate from each other completely, thus no influence of polymer concentration was observed.

Also, it is suggested that polymer chains which are in independent and separated form in the diffusing phase, fuse and isolate smaller sizes when they penetrate into the dispersing phase as a result of which particles form. When the viscosity increases by increasing polymer concentration, particle size also increase due to the decrease in the solvent ratio to polymer concentration and leads to chains overlap each other until reaching semi-dilute regime (Legrand et al., 2007). It is well known that NP size is dependent on the rate of diffusion of solvent to non-solvent environment. When the diffusion rate is faster, smaller particles are formed (Yadav et al., 2007). This correlation may lead to nanoparticle preparation with desired size and shape (Cheng et al., 2007).

3.1.2.4 Influence of S:NS Ratio Used in Precipitation Method

In the production of particulates a solvent (single or mixture) and a nonsolvent one selected. The solvent should dissolve the polymer, and also it must be miscible in the nonsolvent. The solvent should also have a low boiling point to facilitate evaporation of solvent (Legrand et al., 2007). Acetone, which was used as a solvent in this study, fulfills all these criteria.

The influence of S:NS ratio (v/v) on particle size and shape was investigated for both crosslinked and uncrosslinked pNIPAM NPs prepared with polymer concentration fixed at 0.04% (w/v).

No nanoparticle formation was observed when 1:1 and 3:1 S:NS (v/v) were used. The polymer formed a gel upon contacting the nonsolvent.

However NP could be prepared with lower S:NS ratios. The mean size of uncrosslinked pNIPAM NPs prepared by using 1:3, 1:9 and 1:10 S:NS ratio was found to be 632±154, 47±7 and 60±12 nm, respectively, indicating a decrease in particle size upon increase of NS fraction.

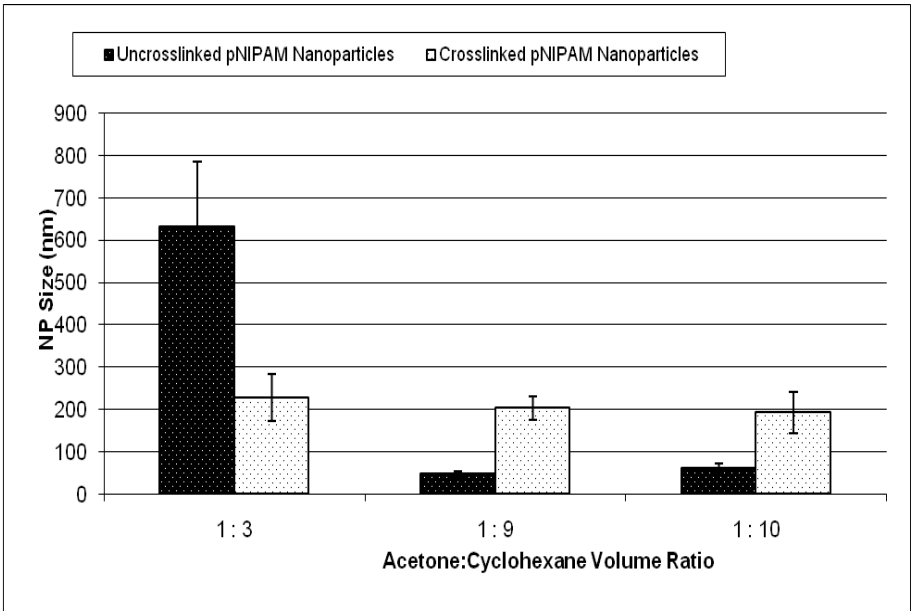

Figure 12 Effect of solvent to nonsolvent volume ratio (S:NS) on the size of crosslinked and uncrosslinked pNIPAM nanoparticles

This effect of S:NS volume ratio on the size distribution of uncrosslinked pNIPAM NPs was statistically significant (p=0.001, ANOVA); as can be expected from the closeness of the S:NS ratio, no difference was found between the size and the size distribution of NPs prepared by using 1:9 and 1:10 S:NS volume ratio (p=0.935, Tukey) (Figure 12).

The influence of S:NS ratio was much less when crosslinked polymer was used. The mean size of crosslinked pNIPAM NPs prepared by using 1:3, 1:9 and 1:10 S:NS ratio was found to be 228±56, 203±56 and 192±49 nm, respectively. This also shows a trend similar to that of the uncrosslinked but to a much lower level. As a result, no statistically significant effect of S:NS ratio on the size distribution of crosslinked pNIPAM NPs was observed (p=0.212, ANOVA). There is an increasing relation between the uncrosslinked and crosslinked samples. When the S:NS ratio is the least the particle size of the uncrosslinked is much larger than that of crosslinked. Upon increase of

the S:NS ratio, the decrease in the particle size of the uncrosslinked is much more significant and these NP are smaller than the crosslinked ones.

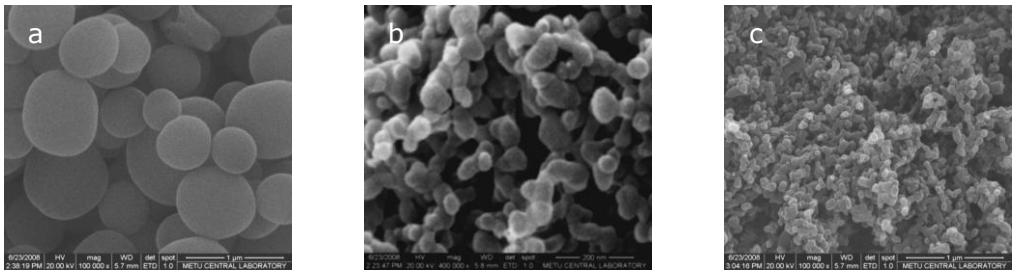

Figure 13 SEM micrographs of uncrosslinked pNIPAM NPs prepared by varying S:NS volume ratios: (a) 1:3 (x100 000) (b) 1:9 (x400 000) (c) 1:10 (x100 000)

In Figure 13, the micrographs of uncrosslinked NPs prepared by using various S:NS volume ratios are presented. The shapes of the particles are spherical. The most regular shaped particles were obtained by using 1:3 S:NS ratio, and their particle size of this group was the highest among all. Aggregation was observed more frequently when the S:NS ratio increased. Smoothness of the surface was certainly related with the size as was also observed in Figure 13. Regardless, the influence of S:NS ratio on particle shape was obvious.

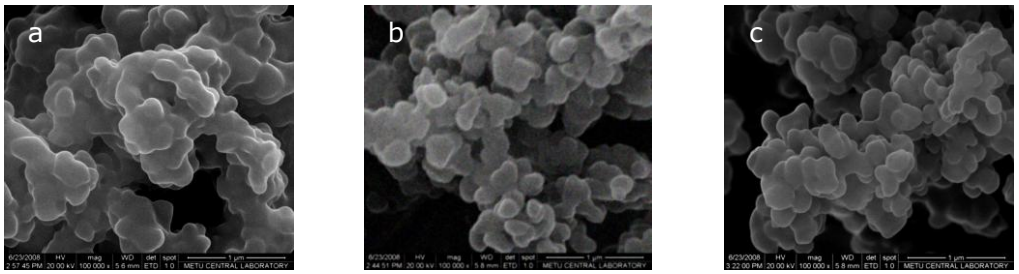

Figure 14 SEM micrographs of crosslinked pNIPAM NPs prepared by using different S:NS volume ratios: (a) 1:3 (b) 1:9 (c) 1:10 (x100 000)

The micrographs of crosslinked pNIPAM NPs prepared by using various S:NS volume ratio are presented in Figure 14. The shape of the crosslinked particles was observed to be spherical and less aggregated when compared with uncrosslinked NPs. Besides, no influence of S:NS ratio on particle shape was observed.

However, in a study by Zhang et al. (Zhang et al., 2006), the volume ratios of S:NS lower than 1:13 were found to cause gel formation in addition to particle formation. In the same study, an increase of volume ratio from 1:13 to 1:20 resulted in a decrease of particle size from 700 to 400 nm. In our study, similar results were found with both the crosslinked and uncrosslinked particles. When the volume ratio for the uncrosslinked polymer was increased from 1:3 to 1:10, the particle size was decreased approximately ten-fold.

In a study by Bilati et al., the choice and interaction of the S-NS has been considered to be more important than the solvent characteristics such as dielectric constant or solubility in terms of influence on particle size. The S:NS volume ratio has been found not effective in particle size even increased in 12-fold, and they concluded that the polymer concentration was the more crucial parameter (Bilati et al., 2005). In another study, no clear correlation has been found between particle size and volume ratio, as well (Cheng et al., 2007).

3.1.3 Encapsulation Efficiency

The influence of parameters on encapsulation efficiency was investigated by determining the drug content of the nanoparticles using an extraction method. This data was used to calculate the encapsulation efficiency and loading. The encapsulation efficiency was expressed as the amount of the drug in the produced NP with respect to the input drug amount and the loading was the weight fraction of the drug in the produced NP with respect to the nanoparticle weight.

3.1.3.1 Influence of Crosslinker Concentration

When the particle size and the influence of crosslinker were studied, it was observed that an increase in crosslinker concentration led to a decrease in NP size. However, when the influence of the same variable of encapsulation efficiency was studied no significant correlation could be found (Table 4). The highest encapsulation efficiency was obtained when 0.44% crosslinker was used (80±7) Encapsulation efficiency showed small maxima with the crosslinker concentration. A similar result was obtained with loading, as long as the yield is similar.

Table 4 Effect of crosslinker amount on Celecoxib loading and encapsulation efficiency of NPs

Samples	Crosslinker Amount (%, w/v)	Particle Size (nm)	Encapsulation Efficiency* (%, mean±SD)	Loading* (%, mean±SD)
$NP_{3,0.22,1:10,0.04}$	0.22	234±71	73.9±2.6	6.7±0.2
$NP_{3,0.44,1:10,0.04}$	0.44	225±17	80.7±7.3	7.3±0.7
$NP_{3,0.88,1:10,0.04}$	0.88	96±25	57.5±11.9	5.2±1.1

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture (%)

*Encapsulation efficiency and loading values were obtained from three independent NP preparation.

*Batches in each preparation calculations were done on triplicate samples.

3.1.3.2 Influence of Initiator and Accelerator Concentration

It was observed that similar to crosslinker amount, initiator and accelerator content had no significant effect on encapsulation efficiency (Table 5).

Table 5 Effect of initiator/accelerator concentration on Celecoxib loading and encapsulation efficiency of uncrosslinked NPs

Samples	Initiator Amount (%, v/v)	Particle Size (nm)	Encapsulation Efficiency* (%, mean±SD)	Loading* (%, mean±SD)
$NP_{1.5,0,1:10,0.04}$	1.5	197±50	71.9±0.6	6.5±0.1
$NP_{3,0,1:10,0.04}$	3.0	67±14	68.5±5.6	6.2±0.5
$NP_{6,0,1:10,0.04}$	6.0	196±36	72.1±0.1	6.6±0

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture

*Encapsulation efficiency and loading values were obtained from three independent NP preparations.

*Batches in each preparation calculations were done on triplicate samples.

Higher entrapment efficiency was expected with NPs prepared from polymers with lower amounts of initiator and accelerator due to higher MW (longer polymer) chains and the resultant larger NPs. This, however, was not observed. In a study, it has been indicated that when the number of polymer chains increase, the interaction between polymer and hydrophobic drug would be higher, which causes higher drug entrapment (Jeong et al., 2004). This would indicate higher encapsulation and loading with higher initiator concentration; this, however, was not either. In another study, similar drug content and loading was found for NPs prepared with various PLA–PEG copolymers, even though the authors expected higher loading with higher particle size (Govender et al., 2000). It appears that loading is a result of a number of parameters and polymer chain length does not appear to be the most influential.

3.1.3.3 Influence of Polymer Concentration in the S:NS Mixture

Table 6 presents the encapsulation and loading of both uncrosslinked and crosslinked pNIPAM NPs prepared by using different polymer concentrations. No change was observed in the encapsulation efficiencies or loading of crosslinked NPs upon increase of polymer concentration varied. With the uncrosslinked NP a maximum was observed with the 0.04% polymer concentration.

Table 6 Effect of polymer concentration on Celecoxib loading and encapsulation efficiency of NPs

Samples	Polymer Conc. (%, w/v)	Particle Size (nm)	Encapsulation Efficiency* (%, mean±SD)	Loading* (%, mean±SD)
$NP_{3,0,1:9,0.02}$	0.02	51±6	56.7±2.8	5.2±0.3
$NP_{3,0,1:9,0.04}$	0.04	53±12	86.6±1.7	7.9±0.2
$NP_{3,0,1:9,0.08}$	0.08	87±17	44.9±0.4	4.1±0
$NP_{3,0.44,1:9,0.02}$	0.02	217±51	73.8±5.8	6.7±0.5
$NP_{3,0.44,1:9,0.04}$	0.04	188±54	77.6±3.6	7.1±0.3
$NP_{3,0.44,1:9,0.08}$	0.08	198±36	78.8±3.4	7.2±0.3

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture

*Encapsulation efficiency and loading values were obtained from three independent NP preparations.

*Batches in each preparation calculations were done on triplicate samples.

3.1.3.4 Influence of S:NS Ratio Used in Precipitation Method

Table 7 presents the Celecoxib encapsulation and loading of both uncrosslinked and crosslinked pNIPAM NPs prepared by using various volume ratios of S:NS. The uncrosslinked NP showed a maximum with the 1:9 S:NS ratio whereas the crosslinked NPs had a small decrease in the efficiency upon decrease of S:NS.

Table 7 Effect of S:NS ratio on Celecoxib loading and encapsulation efficiency of NPs

Samples	**S:NS Volume Ratio**	**Particle Size (nm)**	**Encapsulation Efficiency*** **(%, mean±SD)**	**Loading*** **(%, mean±SD)**
$NP_{3,0,1:3,0.04}$	1:3	632±154	68.1±2.4	6.2±0.2
$NP_{3,0,1:9,0.04}$	1:9	47±7	86.6±1.7	7.9±0.2
$NP_{3,0,1:10,0.04}$	1:10	60±12	68.5±5.6	6.2±0.5
$NP_{3,0.44,1:3,0.04}$	1:3	228±56	87.5±6.5	8.0±0.6
$NP_{3,0.44,1:9,0.04}$	1:9	203±56	77.6±3.6	7.1±0.3
$NP_{3,0.44,1:10,0.04}$	1:10	192±49	80.7±7.3	7.3±0.7

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture

*Encapsulation efficiency and loading values were obtained from three independent NP preparations.

*Batches in each preparation calculations were done on triplicate samples.

When crosslinked NPs were compared with uncrosslinked ones, generally the encapsulation efficiency was found to be higher in crosslinked NPs. This might be due to the higher particle size which induces higher NP volume and also due to the higher polymer density reducing loss of drug during the preparation stage. Still the differences are too small in comparison to the large changes in the particle sizes.

3.1.4 *In situ* Release Profiles

The ultimate aim of this study was to design responsive drug delivery systems with different release profiles to achieve sequential or responsive release by optimizing the NP preparation conditions and evaluating various polymer structures. The obtained NPs were therefore, investigated in terms of Celecoxib release profiles and kinetics.

The release kinetics was assessed by measuring the released Celecoxib by UV-VIS spectrophotometry and the obtained data was fitted to the release relation of Higuchi, Zero and First Order Release kinetics.

Zero Order release model was assessed by plotting Mt vs t according to equation of $M_t/M = k_0 t$. First Order release model plotting $\ln M_t$ vs t according to equation of $M_t = M\, e^{k1t}$ and Higuchi release model requires plotting M_t/M_∞ vs $t^{1/2}$ according to the equation $M_t/M_\infty = k_H t^{1/2}$. M_t and M_∞ are the amounts of drug released at time t (h) and at time infinity (h), respectively, where t is time (h) and k_0, k_1 and k_H are rate constants for Zero and First Order kinetics and Higuchi release models, respectively.

3.1.4.1 Influence of Crosslinker Amount on Celecoxib Release Kinetics

The samples tested had different crosslinker concentration during preparation. An initial burst effect was observed for all the NPs (Figure 15). Then, the rate with which the drug is released decreased substantially. NPs prepared with

different crosslinker concentrations released 8.7, 9.3 and 14.4% of entrapped drug at the end of 96 h.

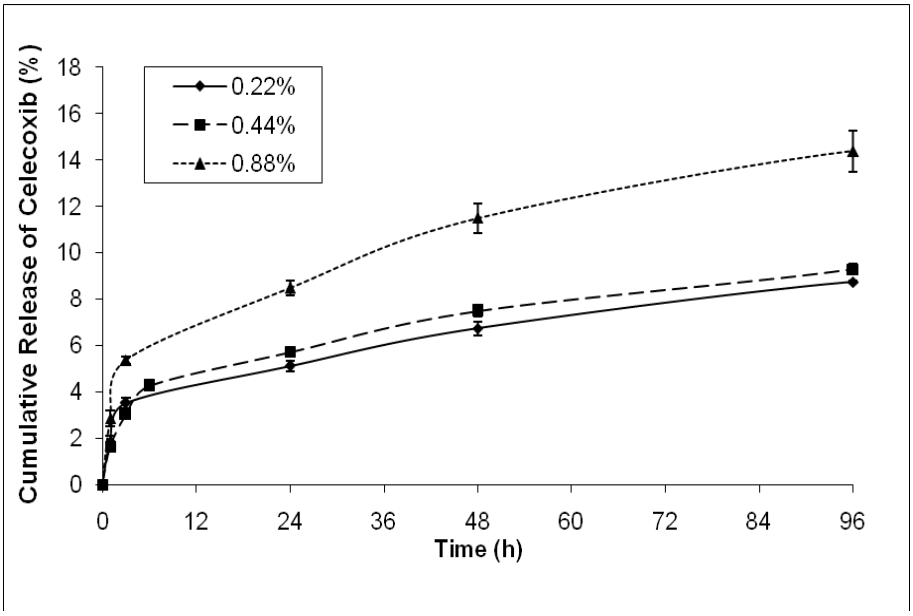

Figure 15 Time course, cumulative Celecoxib release from NPs prepared with pNIPAM crosslinked with various MBA amounts at 37 ^{o}C in phosphate buffer solution (n=3)

Higuchi equation, which defines the release from a monolithic structure via diffusion, was found to be the best fitting release profile for all the pNIPAM NP types (Table 8). The release rates slightly increased with increasing crosslinker concentration. This might be due to the fact that the lower diffusion path length in the smaller particles causes this higher release rate (Chen et al., 2007). NPs prepared with 0.88% crosslinker amount was the smallest among the crosslinked pNIPAM NPs and had the highest release rate. Thus, the higher release is probably not due to the crosslinker concentration but rather as a result of it; smaller size, faster rate.

Table 8 Kinetic analysis of Celecoxib release from NPs prepared from polymers obtained by using various crosslinker amounts

Samples	Release Models, Rate Constant (k) and (r^2)					
	Zero Order		First Order		Higuchi	
	k_o	r^2	k_1	r^2	k_H	r^2
$NP_{3,0.22,1:10,0.04}$	0.259	0.829	0.022	0.467	0.008	0.954
$NP_{3,0.44,1:10,0.04}$	0.301	0.802	0.022	0.423	0.009	0.956
$NP_{3,0.88,1:10,0.04}$	0.34	0.835	0.024	0.462	0.014	0.965

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture (%)

3.1.4.2 Influence of Initiator and Accelerator Concentration on Celecoxib Release

The release of Celecoxib from NPs prepared by using pNIPAM prepared with 1.5, 3.0 and 6.0% initiator and accelerator concentration is presented in Figure 16. A rapid release was observed for the first 6 h and this rate decreased later and at the end of 96 h they released 9.7, 8.8 and 10.2% of loaded Celecoxib, respectively. Thus there were no significant differences between them.

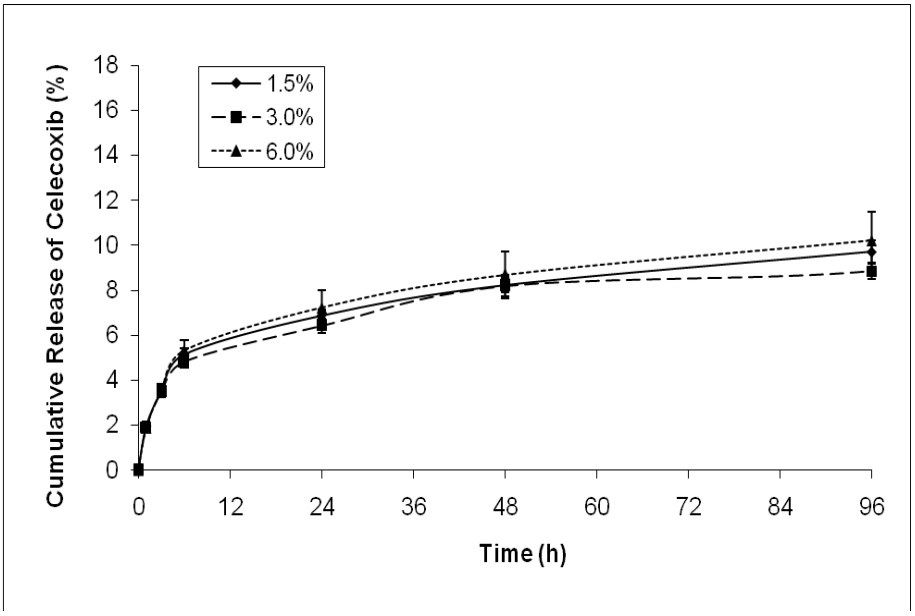

Figure 16 Cumulative Celecoxib release from NPs based on pNIPAM prepared with various initiator and accelarator amounts at 37 ^{o}C in phosphate buffer solution

The release kinetics of all 3 samples was found to fit Higuchi kinetics (Table 9). In a study the release rates were found to be higher in NPs made from polymers with higher MW (Jeong et al., 2004). But in this study initiator concentration did not have any effect on the release profiles.

Table 9 Kinetic analysis of Celecoxib release from NPs prepared from uncrosslinked pNIPAM by using various initiator and accelarator amounts

Samples	Release Models, Rate Constant (k) and r^2 values					
	Zero Order		First Order		Higuchi	
	k_o	r^2	k_1	r^2	k_H	r^2
$NP_{1.5,0,1:10,0.04}$	0.272	0.727	0.021	0.382	0.009	0.915
$NP_{3,0,1:10,0.04}$	0.237	0.698	0.020	0.379	0.009	0.900
$NP_{6,0,1:10,0.04}$	0.292	0.734	0.022	0.390	0.009	0.916

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture (%)

When uncrosslinked NPs were compared with the crosslinked NPs, even though crosslinked ones had higher entrapment efficiency and larger particle size, no difference was observed in terms of release profiles. This demonstrates that crosslinking is not influential on the release systems.

3.1.4.3 Influence of S:NS Ratio Used in Precipitation Method on Celecoxib Release Behavior

Among the S:NS volume ratios used in the assessment of both average particle size and entrapment efficiency, 1:3 and 1:10 samples were selected for use in release kinetics studies knowing that the results that NPs prepared with 1:9 volume ratio were quite similar to the NPs prepared with 1:10.

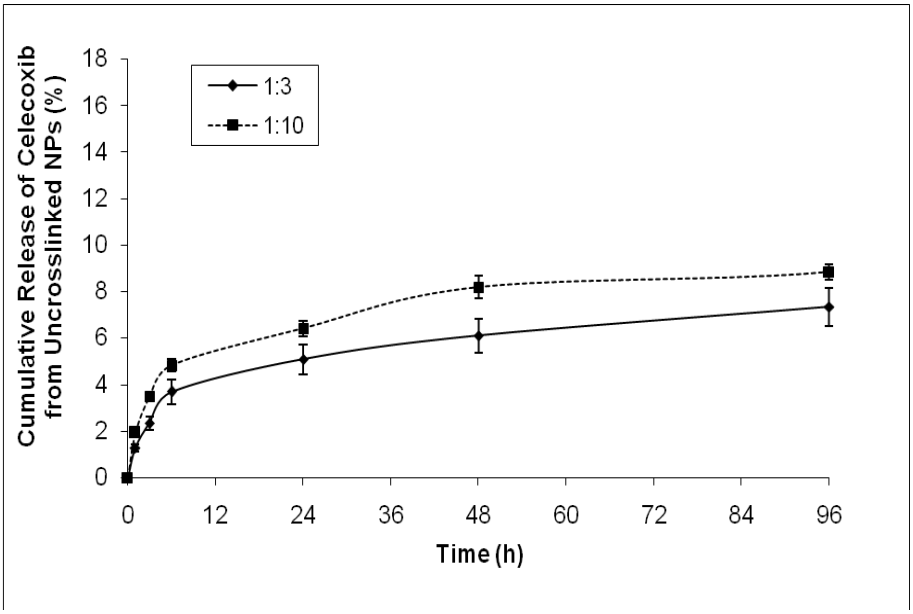

Figure 17 Influence of S:NS volume ratio on Celecoxib release profiles of uncrosslinked NPs at 37 ^{o}C in phosphate buffer solution

With both 1:3 and 1:10 S:NS volume ratio, samples of uncrosslinked NPs have similar release profiles were obtained but the 1:10 samples released more (Figure 17). Higuchi equation was found to be the best fitting release profile for these NPs (Table 10). They had similar entrapment efficiencies, but 1:3 samples were approximately 10 fold larger. Thus, larger samples released at a lower rate as expected from their lower surface area-to-volume ratios (Jeong et al., 2004). Besides larger particles have a longer diffusion path, thus, a slower release behavior is to be expected (Chen et al., 2007).

Table 10 Kinetic analysis of Celecoxib release from uncrosslinked NPs prepared by using various S:NS volume ratios

Samples	Release Models, Rate Constant (k) and r^2 values					
	Zero Order		First Order		Higuchi	
	k_o	r^2	k_1	r^2	k_H	r^2
$NP_{3,0,1:3,0.04}$	0.201	0.755	0.021	0.450	0.007	0.933
$NP_{3,0,1:10,0.04}$	0.237	0.698	0.020	0.379	0.009	0.900

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture (%)

When the crosslinked NPs are studied, it was observed that a higher burst of drug than the uncrosslinked ones was obtained. While crosslinked NPs released 6.7 and 4.2% (in 1:3 and 1:10 S:NS volume ratio, respectively) at the 6th hour, uncrosslinked ones released 3.6 and 4.8% (in 1:3 and 1:10 S:NS volume ratio, respectively) of their drug content at the same time. At the end of the 96 h, crosslinked NPs prepared by using 1:3 and 1:10 S:NS volume ratio had released 13.8 and 9.3%, respectively. During the same period uncrosslinked NPs prepared by using 1:3 and 1:10 S:NS volume ratio released lesser, 7.3 and 8.8% of their contents (Figures 17 and 18).

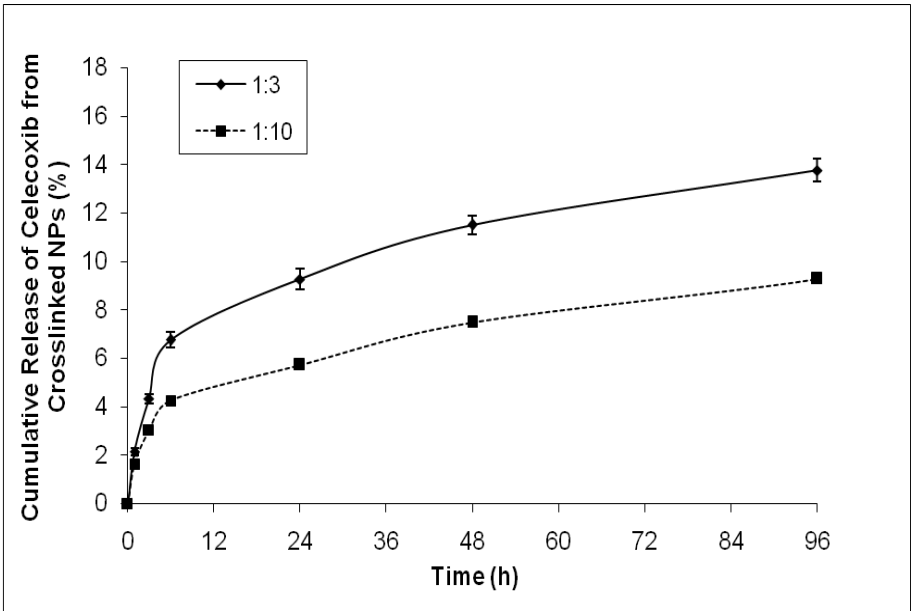

Figure 18 Influence of S:NS volume ratio on release profiles of crosslinked NPs at 37 ^{o}C in phosphate buffer solution

In addition, a negative correlation was observed in crosslinked particles prepared with S:NS volume ratios. Increasing the volume ratio decreased the release rate (Table 11). This was the opposite for the uncrosslinked NPs. It was considered that these contradictory results were due to the difference of particle size distribution. Although, no significant difference was observed in crosslinked particles in terms of size distribution, uncrosslinked particles statistically significantly varied when prepared by using different S:NS volume ratios.

Table 11 Kinetic analysis of Celecoxib release from crosslinked NPs prepared by using various S:NS volume ratios

Samples	Release Models, Rate Constant (k) and r^2 values					
	Zero Order		First Order		Higuchi	
	k_o	r^2	k_1	r^2	k_H	r^2
$NP_{3,0.44,1:3,0.04}$	0.491	0.765	0.024	0.379	0.014	0.938
$NP_{3,0.44,1:10,0.04}$	0.301	0.802	0.022	0.423	0.009	0.956

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture (%)

3.1.4.4 Influence of Temperature

In order to investigate the effect of environmental temperature on drug release behaviors of NPs, the *in situ* release study was performed at two different temperatures: 30 ^oC (below pNIPAM LCST) and 37 ^oC (above pNIPAM LCST) (Choi, 2006).

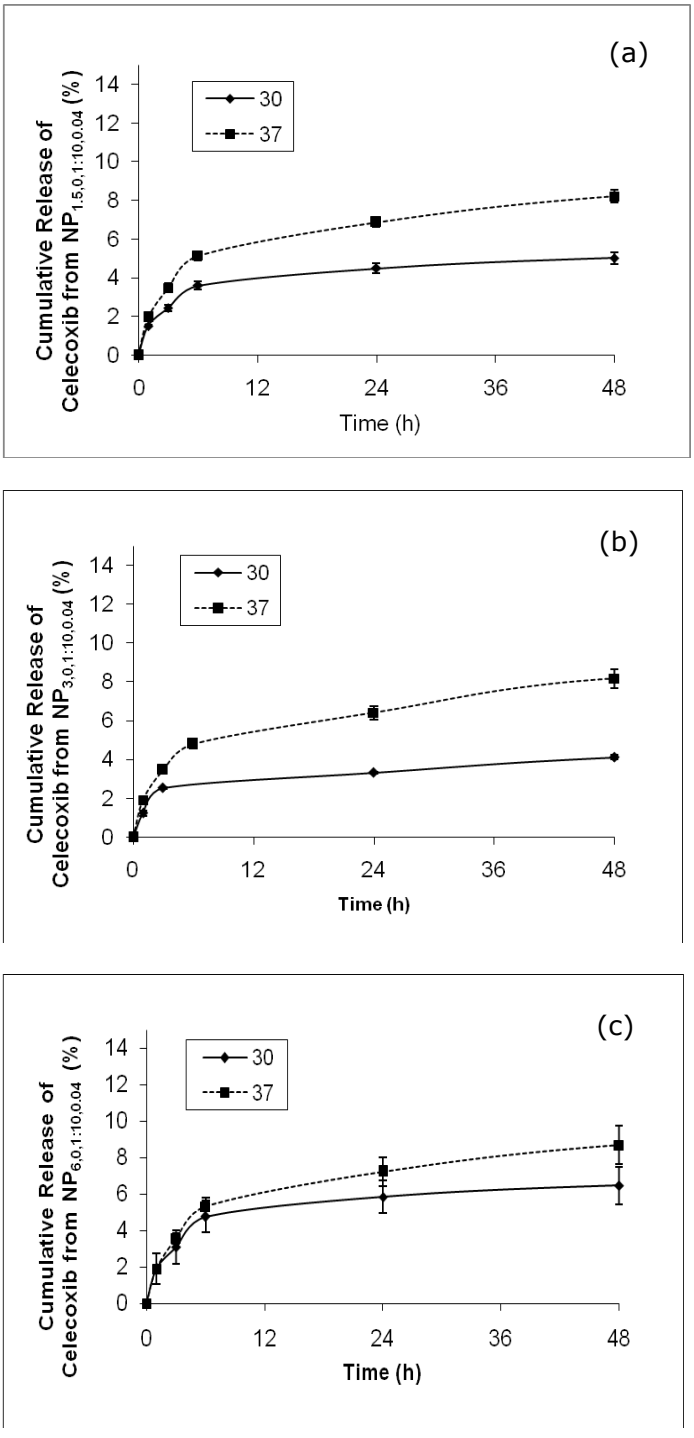

Figure 19 Influence of temperature on release profiles of uncrosslinked pNIPAM based NPs: (a) 1.5% (b) 3% (c) 6% initiator (v/v) in phosphate buffer solution (n=3)

Figure 19 illustrates the release profiles of uncrosslinked NPs at 30 °C and 37 °C (below and above LCST). All uncrosslinked NPs were found to be responsive to the environmental temperature change, as expected. Drug release rates were found to be higher at 37 °C than at 30 °C (Table 12) for all samples with different initiator concentrations. This result can be explained using the scheme shown in the Figure 20. At lower temperature, release rate was slower due to swelling of the particles, which provides encapsulation of the drug. At the higher temperature, particles collapsed and released higher amounts of drug to the media.

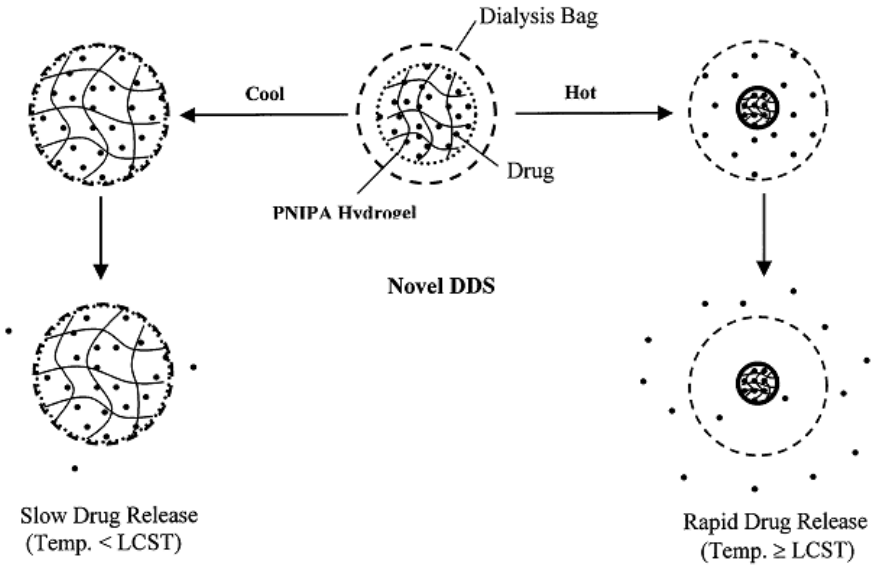

Figure 20 Schematic illustration of thermo-responsive drug delivery systems (Zhang et al. 2002)

It was demonstrated that drug release rate of NPs increases by increasing temperature due to the shrinking of the material in response to temperature changes (from 25 to 45 °C) (Shin et al., 2006).

Table 12 Kinetic analysis of Celecoxib release from uncrosslinked NPs at different temperatures

Samples	Release Models, Rate Constant (k) and r^2 values					
	Zero Order		First Order		Higuchi	
	k_o	r^2	k_1	r^2	k_H	r^2
$NP_{1.5,0,1:10,0.04}$						
at 30 °C	0.147	0.622	0.017	0.359	0.005	0.841
at 37 °C	0.272	0.727	0.021	0.382	0.009	0.915
$NP_{3,0,1:10,0.04}$						
at 30 °C	0.131	0.739	0.019	0.468	0.005	0.901
at 37 °C	0.237	0.698	0.020	0.379	0.009	0.900
$NP_{6,0,1:10,0.04}$						
at 30 °C	0.186	0.599	0.018	0.332	0.007	0.824
at 37 °C	0.292	0.734	0.022	0.390	0.009	0.916

Figure 21 illustrates the release profiles of NPs at 30 and 37 °C prepared with polymers with various crosslinker amounts. No significant difference was observed in terms of release profiles upon temperature change, which was considered to be due to lower swelling behavior of crosslinked particles. Therefore, the scheme represented in Figure 20 did not apply to crosslinked NPS. As can be seen from the earlier tests the crosslinked polymers lead to NPs did not respond to parameter changes probably due to the restricting effect o crosslinkage.

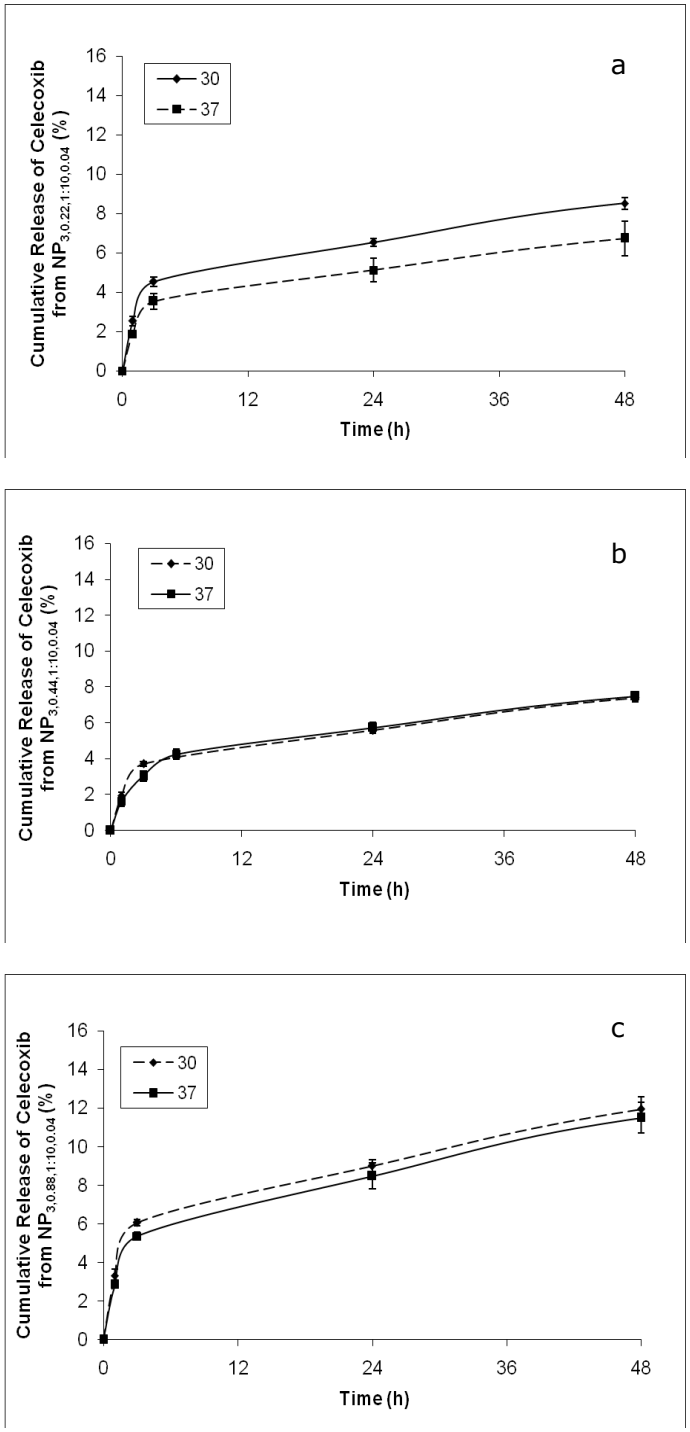

Figure 21 Influence of temperature on release profiles of different crosslinked pNIPAM based NPs: (a) 0.22% (b) 0.44% (c) 0.88% crosslinker (w/v) in phosphate buffer solution

On the whole the release rates of crosslinked NPs were higher than those of the uncrosslinked ones (Table 13).

Table 13 Kinetic analysis of Celecoxib release from crosslinked NPs produced from pNIPAM at different environment temperature

Samples	Release Models, Rate Constant (k) and r^2 values					
	Zero Order		First Order		Higuchi	
	k_o	r^2	k_1	r^2	k_H	r^2
$NP_{3,0.22,1:10,0.04}$						
at 30 °C	0.306	0.793	0.023	0.419	0.010	0.939
at 37 °C	0.259	0.829	0.022	0.467	0.008	0.954
$NP_{3,0.44,1:10,0.04}$						
at 30 °C	0.305	0.823	0.023	0.454	0.009	0.956
at 37 °C	0.301	0.802	0.022	0.423	0.009	0.956
$NP_{3,0.88,1:10,0.04}$						
at 30 °C	0.356	0.833	0.024	0.439	0.015	0.958
at 37 °C	0.34	0.835	0.024	0.462	0.014	0.965

NPxyzw: x:APS and TEMED amount; y:crosslinker amount; z: S:NS volume ratio; w: polymer concentration in mixture (%)

3.1.5 *In vitro* Effectiveness of the Thermoresponsive CRS

3.1.5.1 Effect of Free Celecoxib on Cell Proliferation

Celecoxib can be used in the treatment of both solid tumors and hematologic cancer indications. Celecoxib was demonstrated to have the greatest *in vitro* anti-proliferative potency when compared with other non-steroidal anti-inflammatory drugs (NSAID) such as Refocoxib (Waskewich et al., 2002).

Celecoxib induced apoptosis and inhibited cell growth in human colon cancer HT-29 Cell Line and human prostate cancer cells (Arico, 2002; Hsu et al., 2000). The dose and time dependent inhibitory effect of Celecoxib on a human osteosarcoma Saos-2 cell line was not determined. As a matter of control, effect of Celecoxib on Saos-2 cells was examined. Stock Celecoxib concentrations were prepared by using DMSO at a final concentration of 0.01% (Lai et al., 2003) due to insoluble characteristic of the agent in the medium. 0.01% DMSO was also used in control groups and no cell death was observed due to the solvent.

Celecoxib doses of 50 μM and below were found to be ineffective on Saos-2 cell growth. Celecoxib at concentrations higher than 50 μM inhibited the growth of cells (Figure 22). The suppressive effect was observed with different dosages reported in other studies using various cell types. In one study, the minimal effective dose was found as 20 μM for colon cancer cell line, and in another as 35 μM (Lai et al., 2003). In another study, 50 μM Celecoxib inhibited all seeded human prostate cancer cells in 3-4 h (Hsu et al., 2000). In another study, Celecoxib concentrations of <10 μM were used and it has been found to have no effect on cell viability or growth, while treatment with >20 μM celecoxib decreased drammatically the cell viability (Williams et al., 2000). Treatment with 50 μM celecoxib caused significant apoptosis in three different cell lines (LLC, HCA-7, and HCT-15 cells, 88%, 37%, and 15%, viability, respectively) demonstrated that Celecoxib at concentrations more than 20 μM induces apoptosis *in vitro* (Williams et al., 2000). In another

study, with an incubation of 16 h, 35-65 μM has been found to be effective for both epithelial and hematopoietic cell lines (Waskewich, 2002). In conclusion, there is no consensus on effective dose of Celecoxib in *in vitro* studies but the general view is that Celecoxib is effective with doses around 50 μM or higher.

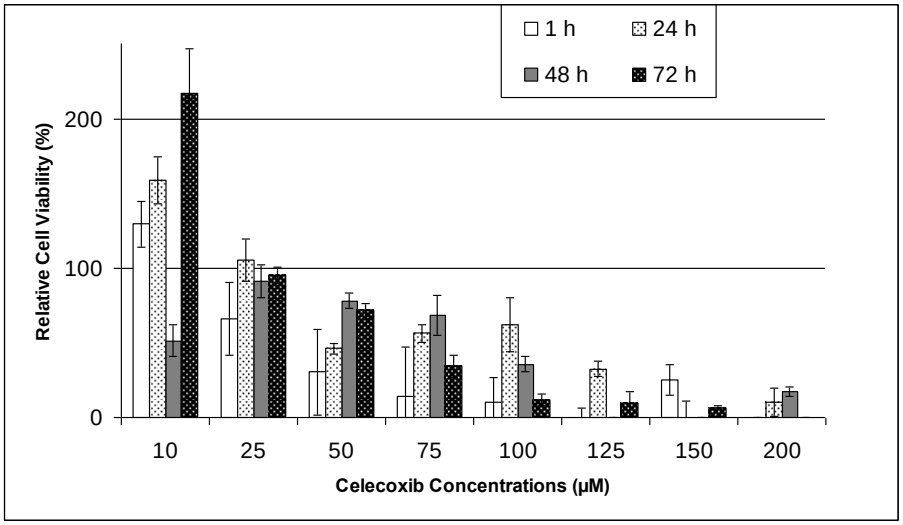

Figure 22 The dose and time dependent effect of free Celecoxib on relative cell viability of Saos-2 cells (n=3)

In this study, Celecoxib significantly induced cell death in a time and dose dependent manner (Figure 22). The treatment with 50 μM celecoxib for 72 h caused a loss of viability up to 29% and with 75 μM up to 66%. In the literature, the similar effect has been reported for various types of cell lines including prostate cancer cells (Hsu et al., 2000), lung carcinoma cells and colon cancer (Williamset al., 2000).

In the light microscopy examination, morphological changes of cells such as shrunken, round shaped cells that detached from the well surface were observed (data not shown) which might suggest apoptosis (Hsu et al., 2000).

3.1.5.2 Effect of Free and Celecoxib Carrying pNIPAM NPs on Cell Proliferation

In biomedical applications, thermosensitive pNIPAM was considered to have great a potential as a drug carrier, which can be targeted to solid tumors and the drug released with local hyperthermia. Therefore, the cytotoxicity of this polymer and its monomer has been investigated.

In the present study, 3% initiator used uncrosslinked NPs and 3% initiator and 0.44% crosslinker used crosslinked NPs, which were prepared using 1:10 S:NS volume ratio and 0.04% polymer concentration ($NP_{3,0,1:10,0.04}$ and $NP_{3,0.44,1:10,0.04}$), were tested *in vitro*. Both free and loaded pNIPAM NPs were applied to Saos-2 cells at 5 mg/mL concentration. NP free cell culture was used as the control group. Separate experimental and control groups were used for each time point. Relative cell viability was expressed as the ratio of the mean cell number of experimental group determined at a time to the mean cell number of control group belonging to same time point. Student t-test was used for the comparisons of cell numbers for each cell culture.

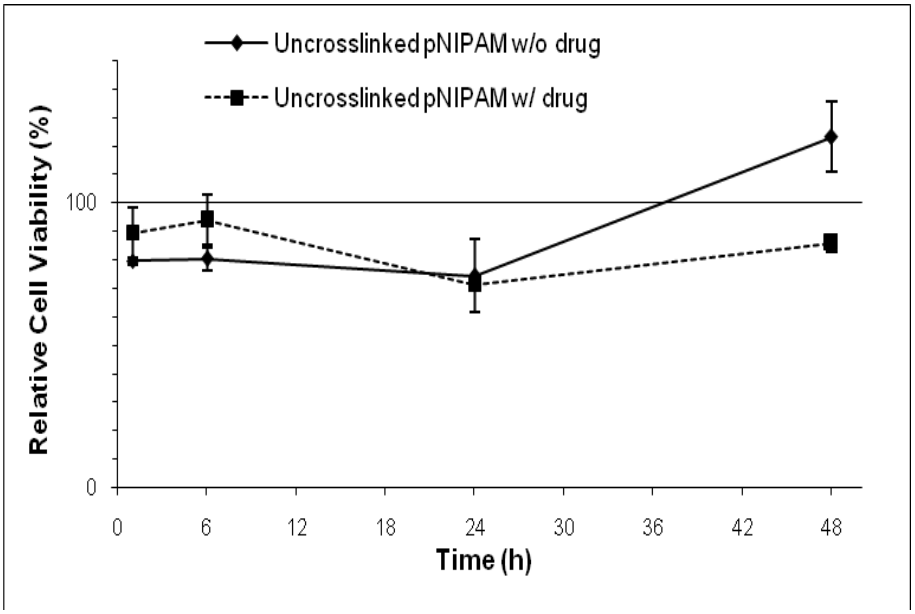

Figure 23 Relative cell viability of Saos-2 cell line after incubation with uncrosslinked pNIPAM NPs carrying Celecoxib (n=3)

During 24 h incubation, no significant difference was observed between drug free and loaded uncrosslinked NPs in terms of cell growth; the proliferation decreased in time for both. After 24 h, the unloaded NPs had a statistically significantly higher cell number than the control group in the 48 h incubation ($p=0.044$, Figure 23). Although no significant difference was observed between the control group and cell culture with Celecoxib loaded NPs in terms of cell number ($p=0.230$), loaded Celecoxib loaded NPs were reduced the relative cell proliferation to 86% after 48 h. Additionally, there was a statistically significant difference between cell cultures with free and Celecoxib loaded uncrosslinked NPs ($p=0.000$). These results demonstrated that the uncrosslinked NPs was not harmful for Saos-2 cell line after 48 h incubation and, it was observed that the loaded NPs did not release enough drugs for total growth suppression.

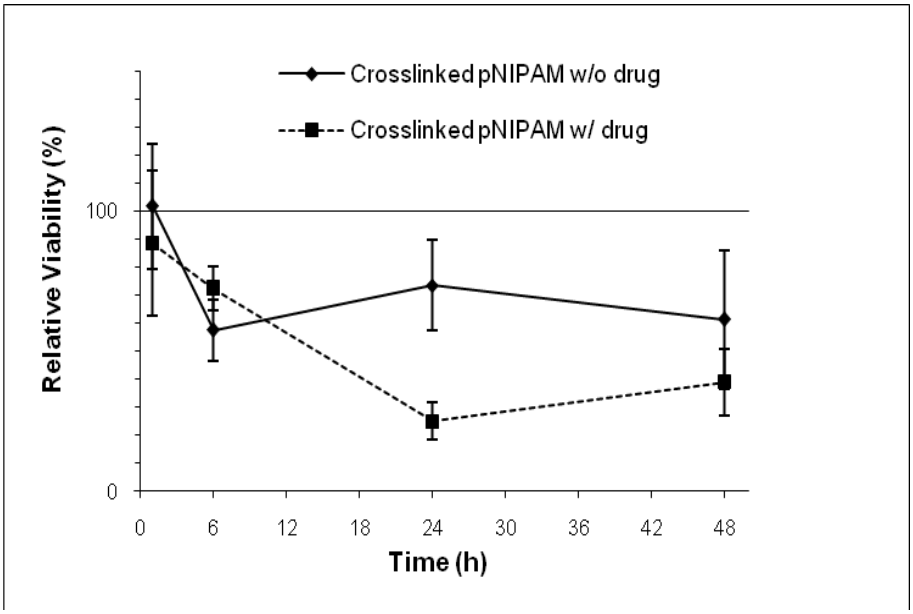

Figure 24 Relative cell viability of Saos-2 cell line after incubation with crosslinked pNIPAM NPs carrying Celecoxib

Similarly, the decrease in cell proliferation in both free and loaded crosslinked NPs treated cell cultures were similar by 6 h post-treatment. After this time, cell cultures with drug free NPs had higher proliferation rate than cell cultures treated with Celecoxib loaded NPs. When the cell proliferation of control group was compared with the cells with drug free crosslinked pNIPAM NPs, lower relative cell viability was determined and it was statistically significant ($p= 0.033$), in addition, cell growth was found to be significantly inhibited with loaded crosslinked NPs ($p= 0.021$). No statistically significant difference was found between cell proliferations of free and Celecoxib loaded crosslinked NPs ($p= 0.219$). Relative cell viability was reduced to 39% after 48 h incubation of Celecoxib loaded crosslinked NPs (Figure 24).

In a study conducted by Vihola, pNIPAM polymers in a varied concentration (0.01-10 mg/mL) were found to be not harmful for Caco-2 cells at 37 ^{o}C after 12 h, additionally, the cell viability was observed at or above 100% when

compared to the control; while NIPAM monomer had some cytotoxicity (Vihola et al., 2005).

In another study investigating the pNIPAM-co-AAc based microgels, slight toxic effect on cells were observed (Teng et al., 2008). NIPAM copolymers without drug have demonstrated no significant cytotoxicity by using various concentrations (Dufresne et al., 2004). Thus the observed cell proliferation decreases in a result of the result of the Celecoxib release from the NP.

CHAPTER 4

CONCLUSION

Different nanoparticles were prepared by changing some parameters involved in polymerization medium and nanoprecipitation method, and produced NPs were compared in terms of particle size, drug loading, release profiles and thermoresponsiveness.

NPs prepared from polymers with higher crosslinker amount had lower particle size; however, their shape was more angular and they were stiffer. Smaller sized particles had a higher release rate than the other croslinked NPs probably due to the shorter path the drug followed. Except this parameter, no difference was found between the crosslinked NPs. The particle size range of crosslinked NPs was found to be 100-250 nm. Thus, it was concluded that crosslinker amount used in the preparation of the polymer is not a crucial parameter in designing controlled drug release systems. Their encapsulation efficiency was exceptionally high, near 100%, in some crosslinked NPs. In addition, they released around 15% of their drug content in the end of the 96 h, which means drug can be delivered for a much larger period. Consequently, they can be used as a long term drug delivery system.

Nanoparticles produced from polymers prepared with different initiator/accelerator amount were smaller and spherical. However, different sized particles were prepared when the initiator and accelarator amounts were varied; there was no linear correlation was determined. This situation was same in encapsulation efficiency and release profiles. Uncrosslinked particles demonstrated different release rates when temperature of the release

medium was varied due to the response of the NPs to the temperature changes. Increasing the temperature provided a faster release. This was considered that this NPs can be used in the cancer therapy by local hyperthermia.

Changing parameters involved in nanoprecipitation method only affected the uncrosslinked nanoparticles. Solvent:nonsolvent ratio and polymer concentration had an effect on solution viscosity. Increasing S:NS ratio and decreasing the polymer concentration in this mixture led to smaller particles. Crosslinked polymers were stiffer and could not separated easily.

Additionally, crosslinked NPs was found to be somewhat cytotoxic when compared to the uncrosslinked NPs. Uncrosslinked NPs was observed to be not toxic even higher cell proliferation was determined than the control. Drug loaded uncrosslinked NPs were effective and thermoresponsive but not able to suppress the growth, however, by increasing the drug loading, this material could become a proper thermoresponsive carrier.

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APPENDIX A

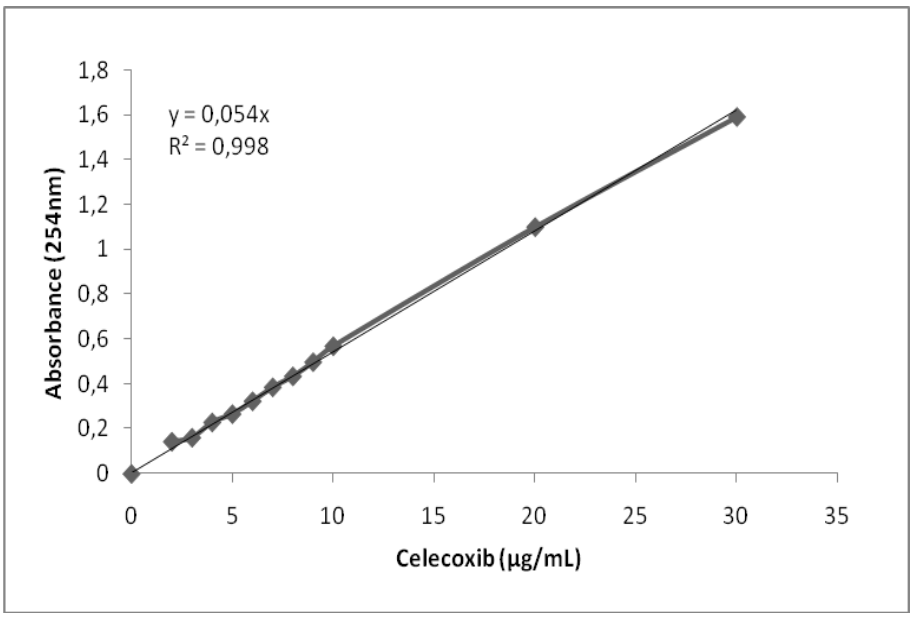

Figure A Celecoxib calibration curve prepared for encapsulation efficiency and release studies

APPENDIX B

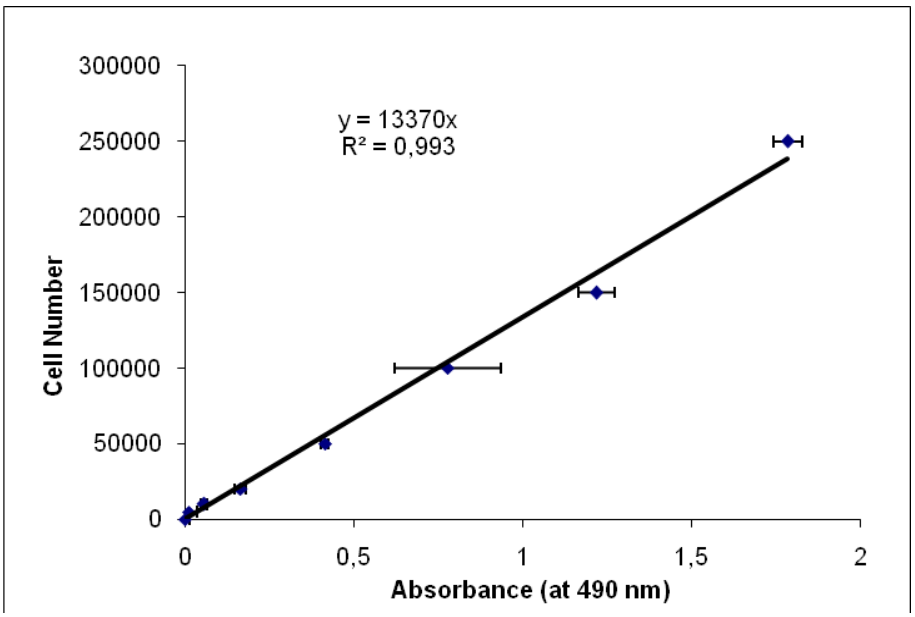

Figure B Calibration curve for Saos-2 prepared by MTS Assay