Université de Montréal

Synthesis and Characterization of Cationic Polymers Derived from Cholic Acid

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Synthesis and Characterization of Cationic Polymers Derived from Cholic Acid

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SOMMAIRE

L'idée de cette recherche est inspirée par les interactions entre espèces chargées dans la nature. Les molécules d'ADN ainsi que la surface des cellules sont normalement chargées négativement et par conséquent des polymères cationiques ont le potentiel d'interagir avec celles-ci. Des interactions de ce genre ont déjà été étudiées pour des applications bio-médicales, incluant le relargage de médicaments, la thérapie génique, et en génie tissulaire. Les polymères contenant des composants endogènes tels que les acides biliaires, peuvent être utilisés pour ce genre d'applications.

Cette étude se concentre sur la synthèse de polymères chargés positivement, dérivés de l'acide cholique (un des acides biliaires). [(2'-Diméthyl amino)éthylène]-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoaminde (mo nomère A) et [(2'-*tert*-butyloxycarboxamido)éthylnè]-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide (monomère B), ont été synthétisés et serviront pour préparer des polymères cationiques. Cependant comme de nombreux problèmes de synthèse ont été rencontrés avec le monomère A, seulement monomère B a été utilisé. Des homopolymères et copolymères ont été synthétisés par polymérisation radicalaire. L'homopolymère est sensible au pH et a été copolymérisé avec le N-isopropylacrylamide qui est connu pour générer des polymères thermosensibles. Les différents ratios de ces deux composants (monomère B et le N-isopropylacrylamide) affectent la sensibilité au pH et à la température. Les propriétés physiques et chimiques de tous les homopolymères et copolymères ont été caractérisées par les techniques physico-chimiques telles que la chromatographie d'exclusion stérique, la spectroscopie de résonance nucléaire magnétique, la spectroscopie infra-rouge à transformée de Fourier, la spectrométrie de masse, l'analyse élémentaire, le titrage potentiométrique, l'analyse thermogravimétrique, et la spectrophotometrie UV-visible.

Mots clés : acide cholique; vecteur non-viral; charge positive; polymère thermosensible; polymère sensible au pH

ABSTRACT

The idea of this research stems from interactions observed between charged species in nature. DNA molecules and the surfaces of cells are normally negatively charged. Positively charged polymers can potentially interact with cells and DNA molecules, and have been considered for bio-medical applications including drug delivery, gene therapy and tissue scaffolds. Polymers containing endogenous compounds such as bile acids may be used for such applications.

The focus of this study relates to the synthesis of positively charged polymers derived from cholic acid (a bile acid). [(2'-Dimethylamino)ethylene]-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide (monomer A) and [(2'-*tert*-butyloxycarboxamido)ethylene]-3α-methacryloyl-7α,12α-dihydroxy-5β- chol anoamide (monomer B) were synthesized from cholic acid and served as monomers for preparing cationic polymers. However, because there are lots of synthetic problems with monomer A, only monomer B was used for polymerization. Homopolymers and copolymers were synthesized from monomer B by free radical polymerization. The homopolymer displayed pH sensitivity. Monomer B derived from cholic acid was copolymerized with N-isopropylacrylaminde, which is known to generate thermosensitive polymers. The different ratios of these two components affect the thermo- and pH-sensitivities of the copolymers. The chemical and physical properties of all the homopolymers and copolymers were characterized by the use of various physical-chemical techniques such as size exclusion chromatography, nuclear magnetic resonance, Fourier transform infrared spectroscopy, mass spectrometry, elemental analysis, potentiometric titration, thermogravimetry and UV-visible spectrophotometry.

Keywords: cholic acid; non-viral vector; positive charge; thermo sensitive polymer; pH sensitive polymer;

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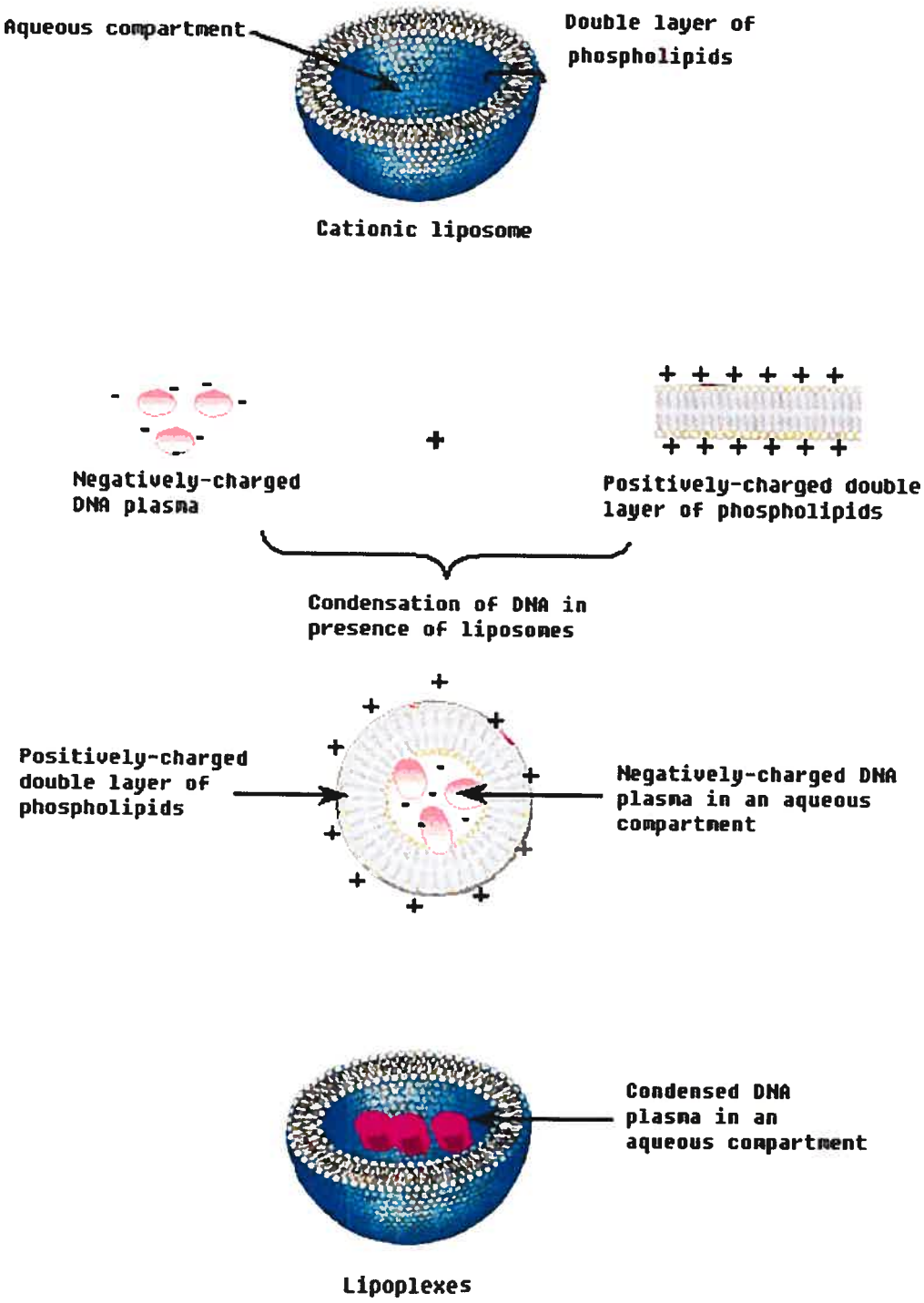

Figure 1.3 Formation of lipoplexes. (This figure is posted on web site *http://www.unilim.fr/theses/ 2003/sante/2003limo0100c/these_body.html*).

In vivo, an overall net positive charge of the lipoplexes helps to associate the complex with the negatively charged cell membrane. Entry of the lipoplexes into the cell may occur by the process of endocytosis via the lipid moieties of the liposome. [23] Following cellular internalization, the complexes appear in the endosomes and later in the nucleus.

(2) Cationic polymers

In general, the structures of cationic polymers (polycations) for non-viral vectors are divided into two parts: cationic branched polymer and hydrophilic linear polymers with ligands. Cationic polymers (i.e., proton-sponge polymers) can easily complex with anionic DNA molecules. The complexes formed between DNA and cationic polymers are called polyplexes as shown in Figure 1.4.

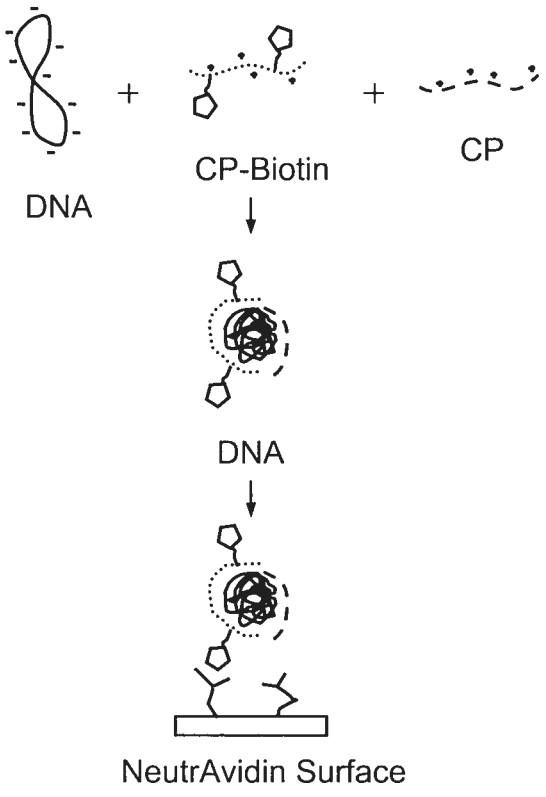

Figure 1.4 Schematic of the substrate-mediated delivery strategy, CP represents the cationic polymer used for condensation.[26]

Polyplexes are commonly used as non-viral vectors.[24] The general mechanism of action of polyplexes is based on the generation of positively charged complexes which can interact with the negatively charged cell surface. When cationic polymers are mixed with DNA, they readily self-assemble together. The cationic branched polymers trap DNAs by electrostatic interaction, and then the complex is surrounded by a hydrophilic linear polymer which may possess a terminal ligand (such as biotin). The result is the formation of small toroidal or spherical structures. When polyplexes are added to cells in culture, the polyplexes are taken up by the cells after the ligand is detected by the targeting cell. These complexes often take advantage of the pH change in the endosome to trigger an endosomal rupture and improve DNA uptake.[25] Similar to that of cationic lipids, transfection activity of cationic polymers varies with cell type, structure, size of the polymer, and polymer to DNA ratio.[26]

Compared to cationic lipids, the major drawback of cationic polymers is their relatively high toxicity. Also, the transfection efficiency of these systems is often below effective levels.[25] The properties of cationic polymers can be easily controlled to improve biocompatibility (toxicity or immunogenicity), biodegradability, and efficiency. The design of new polymer materials has turned out to be one of the most promising strategies for developing gene delivery vectors with improved properties.

Non-viral gene delivery is typically much safer but suffers from generally unsatisfactory delivery efficiency. The earliest synthetic vehicles reported in the literature were not originally designed for gene delivery (i.e. poly(*L*-lysine) (PLL) and poly(ethyleneimine) (PEI)).[27,28] The gene delivery efficacy of such materials is haphazard and typically insufficient for clinical application. In recent years, a variety of polymers have been designed specifically for gene delivery. Based on the large number of studies of these basic polymers, investigators learned a lot about the structure-function relationships existing for polymer vectors. With growing understanding of polymer gene delivery mechanisms it is likely that polymer-based gene delivery systems will become an important tool for human gene therapy.

1.3 Current research in cationic polymers

Cationic polymers are polymers that contain positively-charged groups covalently linked to the polymer molecule, such as phosphonium, sulphonium, and ammonium cations. They are used in different territories such as cosmetic and drug delivery. [29,30]

In cosmetics, the cationic polymer changes its behaviour when surfactants are included in the formulation. Because anionic surfactants bind to cationic polymers, they form a complex phase known as coacervate. Therefore, the polymer can deposit onto the anionic surface of hair and skin.[31] For instance, cationic polymer complexes formed between polymers like polyquaternium-6 and retinoic acid (Vitamin D) are a powerful skin exfoliating agent and help to stabilize the labile vitamin by prolonging its chemical life and potentially increasing its potency.[31] However, cationic polymers and their complexes are neither water-soluble nor dispersible in water, and have found less practical applications in personal care.

In drug delivery, cationic polymers contain pendant basic groups (e.g. amines or ammonium salts) which either accept or release protons in response to changes in the environmental pH.[32] Figure 1.5 shows a generalized structure of a cationic polyelectrolyte and its pH-dependent ionization. These characteristics are used for drug release and drug delivery, and also for their activity against a number of bacteria and fungi.[33] The positively charged sites are able to form polymeric cationic bridges in specific sites in the body including cell surfaces or neutralization of the negative charges of DNA. Such ionization causes cationic polyelectrolytes to exhibit greater solubility in aqueous solution at low pHs, or if cross-linked, to swell to a greater extent. These great qualities have been studied for gene therapy (for non-viral vectors) and drug applications (drug release).[34,35] The following sections will describe in further details of those applications.

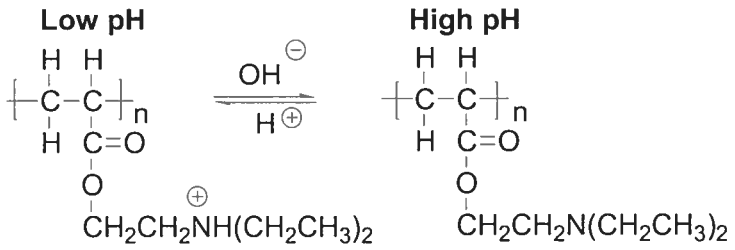

Figure 1.5 pH-dependent ionization of polyelectrolytes

1.3.1 Cationic polymer in gene therapy

Cationic polymers have been widely studied. They seem to be the most promising vehicle for gene therapy. Self-assembling complexes of nucleic acids and synthetic cationic polymers are formed as the result of electrostatic interactions between the negatively charged phosphate groups of the DNA and the positively charged groups of the polycations. Wide arrays of polycations are available for the studies of gene therapy and include those with linear, branched, dendritic and block or graft copolymer architectures. These polycations vary greatly in chemical composition as well as the number of repeating units, providing for a wide range of different polyplexes that can be easily assembled. Much of the research of polymer-based gene delivery indicates that polycations serve as potential reagents in the field.

The major mechanism of gene delivery for this non-viral vector is endocytosis of polyplexes (DNAs, cationic polymers), followed by disruption of the endosomal membrane as shown in Figure 1.6. However, in general, cationic polymers alone do not appear to be ideal candidates for bio-adhesion to cell surfaces because of toxicity. Some of the studies have indicated that cationic polymers tend to cause openings or holes in the cell membrane[22, 36] possibly due to neutralization of negative charges on the cell surface and formation of polymeric cationic bridges which crosslink opposite membrane surfaces. [22] Cationic polymers are inherently toxic to animal species because of their destructive interaction with cell membranes. The toxicity of such polymers is related to the charge density of the cationic

polymer.[37] By modifying the cationic polymer structures, these shortcomings have been improved in many studies.[34,38] Polycations have been modified with a number of groups and modalities, including chemical groups for shielding of the cationic charge, targeting groups for specific cells, and biodegradable linkers for increased biocompatibility.

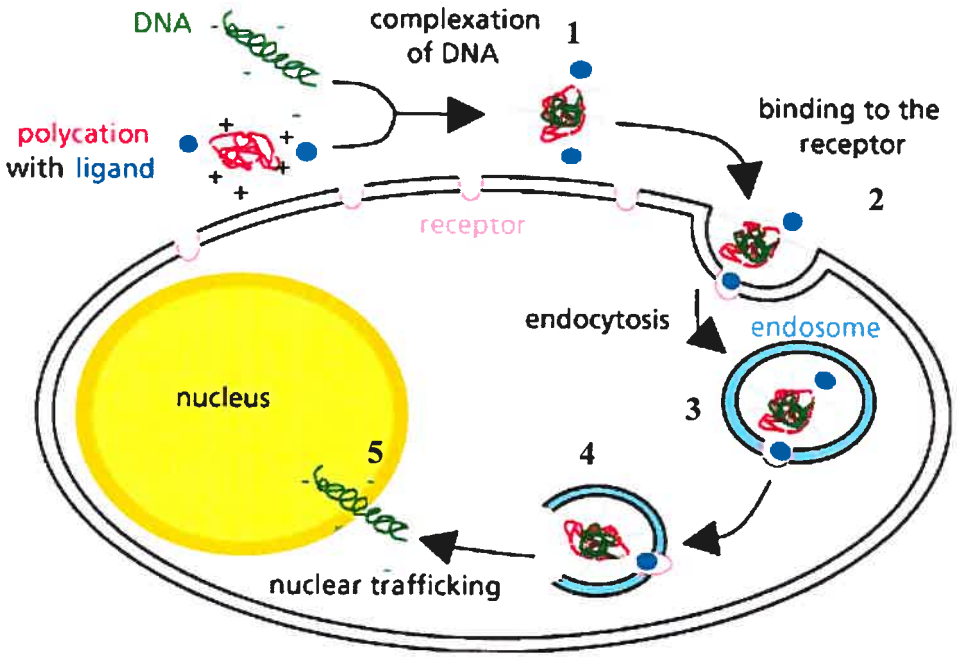

Figure 1.6 Schematic generalized representation of the delivery of a DNA-based therapeutic using a non-viral vector (cationic polymers) (this figure is post on *http://www.nano-lifescience.com/research/genedelivery.html*): (1) Cationic polymers which promote particle (complexation) act as a scaffold for DNA condensation and coupling of bioactive ligands (binding with a high specificity and affinity to the recognition sites). (2) The complexation binding to the cell surface. (3) The complexation crosses the plasma membrane. (4) DNA escapes form the endosome into the cytoplasm. (5) Transport the DNA into the nucleus.

Although the safety of the cationic polymers (in oncogenicity and immunogenicity) as non-viral vectors has been established in many studies,[39,40] non-viral vectors have much lower transfection efficiency than viral vectors. The presence of positive charges at the surface of polyplexes can promote unwanted non-specific interactions with serum proteins and cell membranes.[41] Therefore, *in vitro* (without plasma issue), the efficiency of polyplexes is good, but *in vivo,* it is

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Absorbance
A	Collision frequency factor
A_p	Frequency factor for propagation
AIBN	2, 2'-Azobis(isobutylonitrile)
amu	Atomic mass unit
atm	Atmosphere
aq	Aqueous
Boc	*tert*-butyloxycarbonyl
Boc_2O	Di-*tert*-butyl dicarbonate
CA	Cholic acid
CAME	Methyl ester of cholic acid
cm	Centimeters
CP	Cationic polymer
DMEDA	*N,N*-Dimethyl ethylenediamine
DCM	Dichloromethane
DMF	*N,N*-Dimethylformamide
DNA	Deoxyribonucleic acid
E	Arrhenius activation energy
E_p	Activation energy for propagation
EDA	Ethylenediamine
f	Initiator efficiency
FTIR	Fourier transform infrared spectroscopy

g	Gram
h	Hour(s)
Hz	Hertz
$[I]$	Concentration of the initiator
k_d	Rate constants of initiation
k_p	Rate constants of propagation
k_t	Rate constants of termination
LCST	Lower critical solution temperature
M	Molar per litre
$[M]$	Concentration of the monomer
mg	Milligram
mL	Millilitre
mmol	Millimole
mM	Millimolar per litre
M_n	Number-average molecular weights
MS	Mass spectrometry
M_w	Wight-average molecular weights
NIPAM	*N*-Isopropylacrylaminde
nm	Nanometer
NMR	Nuclear magnetic resonance
DMSO	Dimethyl sulfoxide
PAAM	Polyamidoamine
PDEAM	Poly(N,N-diethylacrylaminde)
PEI	Polyethyleneimine
PLL	Poly-*L*-lysine

PNIPAM	Poly(*N*-isopropylacrylamide)
ppm	Part per million
R	Rate of polymerization
R_f	Retention factor
RNA	Ribonucleic Acid
SEC	Size exclusion chromatography
*t*Bu	*tert*-butyl
T	Kelvin temperature
TEA	Triethylamine
TLC	Thin layer chromatography
T_m	Melting temperature
TGA	Thermogravimetric
THF	Tetrahydrofuran
UV	Ultraviolet
v	Kinetic chain length
$\overline{X}_n$	Number-average degree of polymerization
%T	Percent transmittance
μL	Microlitre
μm	Micrometer

CHAPTER 1
INTRODUCTION

1.1 Background

One of the most promising areas of therapeutic research is gene therapy. Therapeutic genes are used to "reprogram" cells into producing their own therapeutic agents and turn off unwanted or detrimental cell growth or activity. Many diseases are due to cellular processes which do not function properly at the molecular level. These cellular processes are regulated by proteins which are synthesized in the cell according to the genetic information stored in DNA. By adding external DNA or RNA into the cells to induce or suppress a specific function, gene therapy can be used to combat a wide range of diseases.

1.2 Vectors for gene therapy - Concept

Gene therapy faces numerous challenges, most notably getting the genes to, and then into, the targeted cells in the body. Currently, there are two main approaches: viral and non-viral vectors.

Viral and non-viral vectors have been widely studied for the past decade. Viral vectors include retroviruses, adenoviruses, and adeno-associated viruses. Non-viral vectors include cationic lipids/liposomes, and cationic polymers. The fundamental principles by which these vectors access the target cell are similar: the cells uptake the therapeutic gene carriers, transfect, and then replace the defective gene (Figure 1.1). However, the properties of such systems differ greatly.

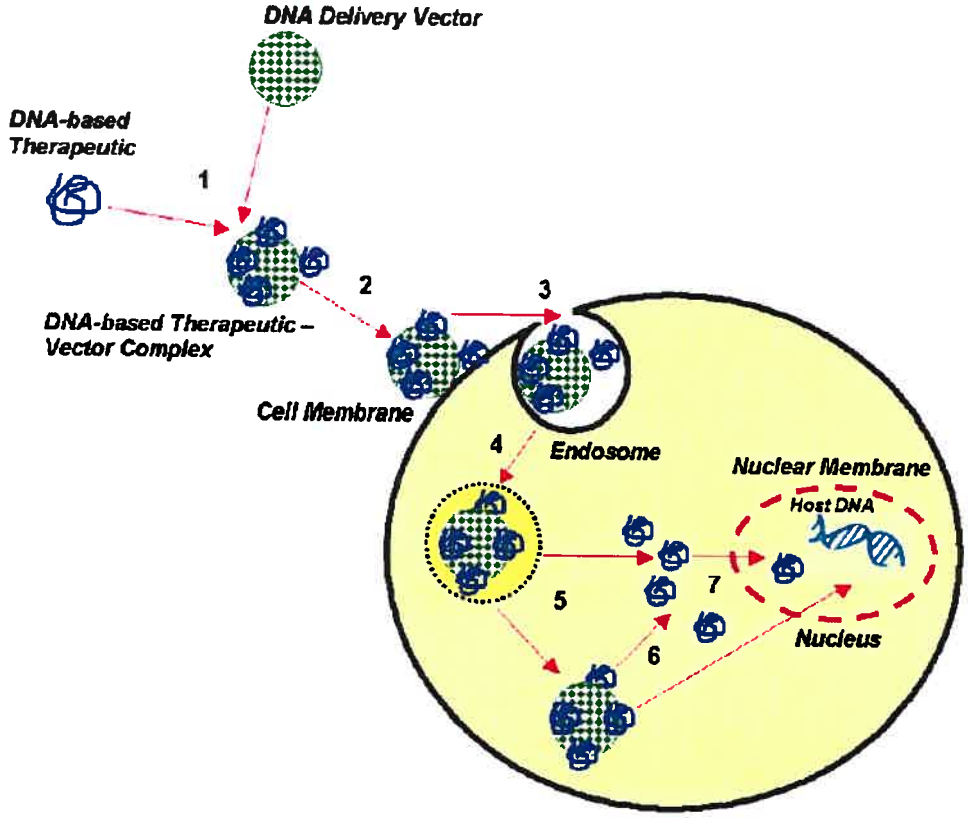


Figure 1.1 Generalized representation of the delivery of a DNA-based therapeutic using a viral or non-viral DNA delivery vector: (1) complexation and/or entrapment of DNA-based therapeutic with DNA delivery vector; (2) interaction of DNA-based therapeutic-vector complex with cell membrane; (3) cellular internalization via receptor- or non-receptor-mediated endocytotic pathways; (4) endosomal breakdown ; (5) cytoplasmic release of DNA-based therapeutic-vector complex or DNA-based therapeutic alone; (6) dissociation of DNA-based therapeutic from vector; (7) nuclear translocation of viral vectors or DNA-based therapeutics.[1]

1.2.1 Viral Vectors

Viruses are very efficient for transfecting into cells and producing new viral particles. A foreign gene can be placed into a virus, and these recombinant viral vectors are then used to enter the host cell. The main idea of gene therapy using viral vectors is that vectors infect the cell and change the cell's defective genes with the new substituted ones. Viral vectors can be sub-divided into three categories: [2]

Retroviruses are a large family of RNA viruses which include spumavirus (foamy viruses), lentivirus and moloney-murine-lentivirus-related viruses. The diameter of retroviral virions range from 80 to 130 nm, and their genomes consist of two identically positive-sense single-stranded RNA molecules. The genomes are encased inside a capsid (envelop) along with the integrase and reverse transcriptase enzymes. Retroviral vectors are capable of transfecting high populations of primary human endothelial and smooth muscle cells, a class of cells that is generally extremely difficult to transfect.[3] Retroviral vectors are the most widely used viral vectors in clinical trials at present. The most important advantages of retroviral vectors are their ability to transform and stably integrate into the target cell genome. However, they have been demonstrated to lose long-term expression capability which limits their potential for clinical application. [4] Furthermore, they are extremely difficult to produce on a large-scale because they are inherently unstable.

Adenoviruses are non-enveloped, icosahedral, double-stranded DNA viruses with a capsid diameter of 70–100 nm, and comprised of 252 capsomeres (240 hexons and 12 pentons). They are not incorporated into the genome of the target cell (non-integrating), but remain as an extrachromosomal entity in the nucleus of the host cell. Consequently there is no risk of insertional mutagenesis.[5] Replication-defective recombinant adenoviruses are the second most commonly used viral vectors in clinical trials today. Adenoviral vectors are known to generate inflammatory responses in tissues with short-lived gene expression. There is concern over loss of transgenic expression upon tissue maturation when cells have been transfected using viruses. [6] However, it has been demonstrated that

adenoviruses in formulations may lose their potency after storage in commonly used pharmaceutical vials.[7]

Adeno-associated viruses (AAV) are non-pathogenic human parvoviruses (parvoviruses are among the smallest, simplest eukaryotic viruses) which depend on a helper virus, usually an adenovirus, to proliferate. Compared to the other vectors, adeno-associated viruses (AVV) mediate stable transgenic expression in terminally differentiated cells without inducing significant inflammatory toxicity.[8] Therefore, AAV's only slightly damage the target cells.[9, 10] However, there is evidence to suggest that AAVs are significantly less efficient than retroviral vectors at transducing primary-cell cultures.[11,12] In primary-cell transductions, most of the DNA does not integrate into the host genome but remains extrachromosomal, and this inefficiency might limit its use for *in vivo* application.

Viral vectors have shown excellent transfection efficiencies. However, the limitations restrict their clinic applications. Viral vectors can cause immune responses, creating a number of serious safety risks such as potential oncogenicity, toxicity and the development of high immunogenicity after repeated administration.[13,14] Furthermore large scale production may be difficult to achieve. Because of these drawbacks, non-viral strategies are becoming more attractive.

1.2.2 Non-viral vectors

Although the number of clinical trials with viral carriers overshadows that of non-viral carriers due to their inherently high degree of transfection and their increased persistence of gene expression, these carriers are prone to trigger host immunogenic responses and are limited in the transgene size.[15] Non-viral vectors can overcome many of the problems encountered with viral vectors. Non-viral methods of DNA transfer require only a small number of proteins; have a virtually infinite capacity; have little or no infectious or mutagenic capability and large scale production is possible using pharmaceutical techniques.[16,17] DNA with cationic polymers are considered to be one of the most promising candidates for non-viral

gene delivery systems.[18,19,20] Cationic polymers, a non-viral system may, in the near future, overcome some of the problems inherent to currently employed viral gene delivery systems. Current studies of non-viral vectors are focused on cationic lipids/liposomes and cationic polymers.

(1) Cationic lipids/liposomes

The preparation of liposomes requires molecules with three specific parts: a hydrophobic anchor, a linker, and a head group (Figure 1.2).[21] The hydrophobic anchor and positively charged head group offer the important characteristics of self-assembly in aqueous solution. The cationic head group of the lipid compound associates with negatively charged phosphates on the nucleic acid, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex.

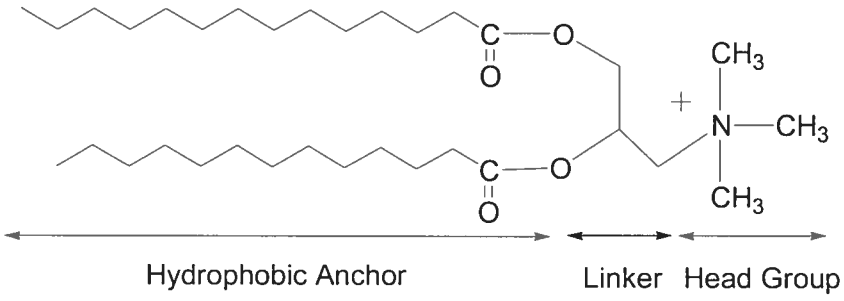
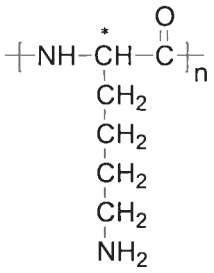


Figure 1.2 The general structure of a cationic lipid.

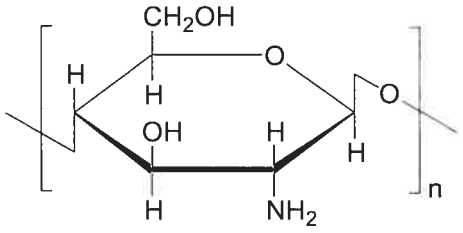
As shown Figure 1.3, the hydrophobic end of the molecule avoids contact with water, while the positively charged head group seeks contact with water. In order to protect their hydrophobic end from water and expose the hydrophilic end, the molecules self-assemble into positively charged bilayers of phospholipids structures. When DNAs are introduced into the aqueous center of the liposomes, they are referred to as lipoplexes. Lipoplexes, a negatively charged DNA plasma in an aqueous compartment, is centered into a micelle-type structure. Lipoplexes mostly or totally protect the DNA from the outside environment and have a high affinity towards cell membranes.[22]

significantly lower, because of the non-specific binding in the blood plasma.[42,43] To overcome these problems, the most attractive strategy is to replace non-specific electrostatic interactions between the transfection complexes and cells with cell-specific interactions that trigger receptor-mediated endocytosis of the DNA complexes. Such an active targeting requires the use of ligands such as sugar residues, peptides, proteins and antibodies[34] that bind with a high specificity and affinity to the recognition site (Figure 1.6).

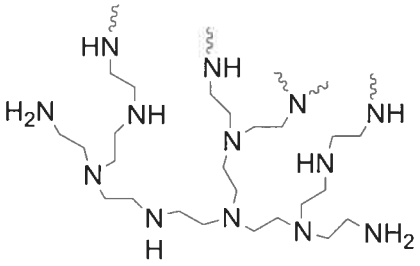
Cationic polymers are the first suitable candidate of gene carriers, as stated before. Currently, several cationic polymers are being studied and include PLL, PEI, chitosan and dendrimers (Figure 1.7). Polymeric transfection systems are advantageous in that they are safer than viral systems and are non-oncogenic. However, each polymer used for this application has its own specific advantages and disadvantages.



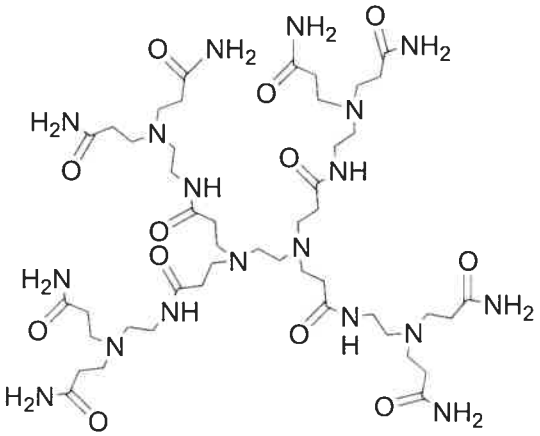
Poly(*L*-lysine)



Chitosan



Branched Poly(ethyleneimine)



Poly(amidoamine) (Dendrimer)

Figure 1.7 Structures of the commonly used cationic polymers for gene therapy

(1) PLL

The biodegradable cationic polymer PLL has been widely used as a non-viral vector because of its excellent DNA condensation properties.[44] PLL protects DNA from the attack of nucleases efficiently and the polyplexes can be rapidly internalized to the cell.[45,46] However, it has the highest toxicity among the polycations, because the cations on its amino acid backbone interact with the cell's surface, and reduces the recycling capacity of selected membrane components as well as membrane fluidity.[47] Moreover, PLL-DNA complexes show a low level of transfection since the complexes lack of normal communication with lysosomes and are therefore unable to be rapidly released from endosomes.[46] Many copolymers have been studied to reduce the toxicity and to enhance the transfection.[44] Many studies on copolymers with poly(ethylene glycol) (PEG) blocks bearing targeting ligands have shown that PEG shields the surface charges of PLL and can help in the steric stabilization due to its charge neutrality and water solubility.[44,48,49] The presence of the ligands can prevent non-specific interactions of the polymer with cellular components.[50,51] PLL-PEG complexes with ligands-grafted micelles had high transfection efficiencies and low cytotoxicities compared to PLL alone.[34]

(2) PEI

Today, PEI is the most commonly studied non-viral gene delivery polymer. Typically, the synthesis of PEI is through acid-catalyzed, ring-opening cationic polymerization of aziridine, resulting in ($NHCH_2CH_2$) monomer units with branched or linear conformations. Protonation of these amines confers PEI with the highest cationic density of all synthetic polymers for the purpose of DNA condensation. Simultaneously, PEI offers better protection against nuclease degradation and escape from the lysosomal compartment than other polycations, e.g. PLL, while also showing greater transfection.[52,53] However, a highly cationic PEI gene carrier may also attract anionic components in the blood stream such as erythrocytes.[54] Moreover, the unfavorable interactions may result in aggregation and removal from the body though the reticuloendothelial system.[55] Consequently,

there is an intricate balance for obtaining optimal cationic condensation of plasmid DNA without excessive cationic charge.[56]

PEI architecture has two forms: branched or linear. Both have advantages and disadvantages,[57] but the majority of PEI research reported in the litterature has been conducted with branched PEI, possibly due to its increased number of primary amines that are widely available for conjugation to other modalities. In order to control toxicity while maintaining high transfection, research is currently focused on lowering the molecular weight of PEI in combination with other molecules.[58, 59] Furthermore, PEI does not contain active groups for tissue or cell targeting; therefore, researchers have attached a number of targeting modalities such as cholesterol and antibodies.[60,61]

(3) Chitosan

Chitosan, a neutral polysaccharide having structural characteristics similar to glycosaminoglycans, is non-toxic and biodegradable.[62] It has therefore been widely employed in pharmaceutical and biomedical fields.[63] In recent years, biocompatible chitosan microspheres and beads have been investigated as non-viral vectors because positively charged chitosan can be complexed with negatively charge DNA.[64] Also, chitosan can effectively bind DNA and protect it from nuclease degradation.[65] In the manufacturing process of DNA-chitosan complexes, chitosan has many advantages during preparation and storage process. However, the levels of gene expression are very low, and response time is very long.[66,67] Furthermore, at high doses, chitosan causes hypercholesterolemia and therefore has limited applications.[68]

(4) Dendrimers

Dendrimers represent a promising class of molecules for use as vehicles for drug delivery.[69,70,71] The principle advantages that dendrimers offer include i) a well-defined composition, ii) multiple sites for manipulation and iii) a globular shape offering a protected hydrophobic interior that can be employed to solubilize

guests. The most commonly used dendrimer for gene delivery is polyamidoamine (PAAM) (Figure 1.7). PAAM can be synthesized from the core, and outspread generation by generation with very low polydispersity. The 3-dimensional spherical structure of the resulting polymer can be used for gene therapy.[72,73] Because of its low polydispersity, it can lead to reproducible gene delivery and a clinically reliable formulation.[74] The capability of dendrimers to transfect cells is dependent upon the size, structure and number of primary amino groups on the surface of the dendrimer. However an increased flexibility of the dendrimer with a better ability to complex DNA may be difficult to achieve.[75]

In truth, each platform (linear or dendritic polymer) has its advantages and disadvantages. Efforts to understand the appropriate roles for each strategy will require investigations of all systems at all levels. The first generation of polymer-bound therapeutics are in and emerging from clinical trials. These polymers convey three principal advantages to the drug: (1) they increase solubility; (2) they increase vascular circulation time; (3) they attenuate toxicity of the drug through selective targeting as a result of ligand/receptor interactions or enhanced permeability and retention that tumors show for large molecules. The success of these systems offers compelling motivation and proof of concept for the examination of the next-generation of polymer therapeutics.

1.3.2 pH sensitivity of cationic polymers in drug release

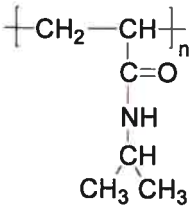
Cationic polymers were originally investigated for pH-sensitive controlled drug delivery in oral administration before the gene delivery. pH-sensitive polymers synthesized with either acidic or basic components demonstrate reversible swelling/deswelling in acidic or basic media. Because of the number of ionizable groups on its backbone, cationic polymers can respond to changes in pH (Figure 1.5). During the passage of the drug delivery systems from the esophagus through the stomach to intestine, the product is exposed to a variable pH ranging from 2-4 in the stomach and 6-8 in the intestines. Utilizing this physiological property, many drugs have been developed for controlled release.

Hydrophobicity and degree of ionization play important roles in pH-controlled drug systems. Traditionally, sustained delivery systems use the stomach as an area for drugs to dissociate from their host. In the intestines, the unique environmental conditions and the presence of high concentrations of bacteria can substantially alter the rate of drug release for the delivery system. Hydrogels made of cross-linked polyelectrolytes display large differences in swelling properties depending on the pH of the environment. For polycationic hydrogels, the swelling is minimal at neutral pH, thus minimizing drug release from the hydrogels. This property has been used to prevent release of foul-tasting drugs into the neutral pH environment of the mouth. Polycationic hydrogels would be ideal for localized delivery of antibiotics in the stomach.[76]

1.4 Thermo sensitive polymers

Thermo sensitive polymers are hydrogels which are a group of stimuli-sensitive polymers that show a phase transition in response to a temperature change.[77, 78] They have been extensively studied in the past decade because of their potential applications in many fields such as membranes, [79] drug delivery systems, [80] cell culture, [81] the isolation of bio-molecules[82] and enzyme activity control[83]. Many thermo sensitive polymers exhibit a lower critical solution temperature (LCST), which is defined as the temperature of the phase transition from a soluble to an insoluble state in an aqueous solution. It is presumed that the main mechanism of such a phase transition induced by a temperature change may be a drastic change in the hydrogen bonding between the polymer and surrounding water molecules. These polymers have attracted interest of many investigators because of their intelligent ability to deliver the drug they contain to the desired places under optimal conditions. Such polymers are physically characterized by their LCST or cloud point. The LCST of the polymers usually varies with the presence of additives such as electrolytes, organic solvents and surfactants.

Thermo sensitive drug release polymers are designed to possess sensitivity towards small changes of temperature (physiological temperatures), which leads either to an expansion or a contraction of their structure. Such systems exhibit dimensional changes leading to the release of their drug contents. Polymers such as poly(*N*-isopropylacrylamide (PNIPAM) (shown in Figure 1.8) or their copolymers are often used to immobilize enzymes or as carriers of certain functional groups important for biochemical or bio-medical applications. [84, 85] There are many researchers who have worked on PNIPAM which is one of the most frequently studied temperature-responsive hydrogels. PNIPAM exhibits a remarkable shrinkage with increasing temperature (LCST takes place around 32 °C). Copolymerization of NIPAM with other monomers can bring the LCST close to the body temperature.[86]



poly(*N*-isopropylacrylamide (PNIPAM)

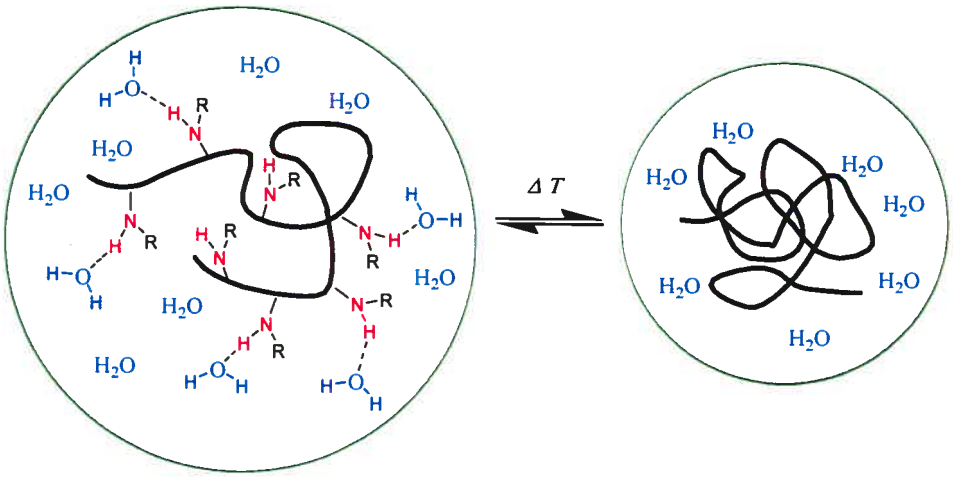


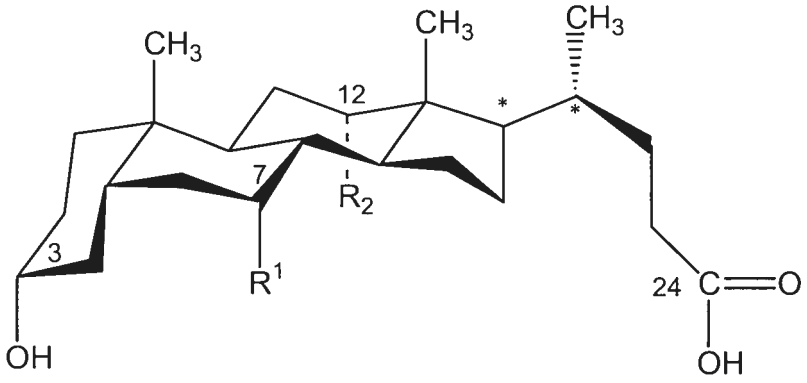
Figure 1.8 The chemical structure of PNIPAM and the dehydration of PNIPAM above its LCST

Figure 1.8 also shows that PNIPAM chain changes its shape at the LCST from a random coil (left) to spherical globule (right). This phenomenon has been reported widely.[87,88] As the temperature increases, inter-molecular hydrogen-bonding with water begins to break and the polymer changes conformation so as to favor intra-molecular hydrogen-bonding among the amide groups along its backbone. Because the changes induced by temperature are strictly due to conformation, the response of PNIPAM to temperature stimuli is reversible and fast.

Hydrogels which are responsive to both temperature and pH can be made by simply incorporating ionizable and hydrophobic (inverse thermo sensitive) functional groups to the same hydrogels. When a small amount of cationic monomers bearing cationic groups (such as amine) is incorporated into a thermo sensitive polymer, its LCST becomes dependent on the pH due to their ionization properties. As the pH of the medium decreases below the pK_a of the amine groups, the LCST shifts to higher temperatures due to the increased hydrophilicity and charge repulsion. These copolymers could be customized to be doubly sensitive to external temperature and pH stimuli.

1.5 Background on bile acids and their polymers

Bile acids are natural compounds which are in the family of steroids. Synthesized in the liver, they are generally conjugated with glycine and taurine (e.g., glycocholic and taurocholic acids). Different groups on positions 7 and 12 define different types of bile acids (shown in Figure 1.9). The bile acids in the salt form are detergent-like substances secreted from the gallbladder and aid in the digestion and absorption of lipids. Like a detergent, bile acids contain hydrophobic and hydrophilic components. Because of their idiosyncratic structures, bile acids have facial amphiphilicity which causes them to form micelles and supramolecular structures, especially cholic acid (shown in Figure 1.10). These surfactant properties are used to promote the absorption of water-insoluble compounds.



	R_1	R_2
Cholic acid	OH	OH
Deoxycholic acid	H	OH
Chemodeoxycholic acid	OH	H
Lithocholic acide	H	H

Figure 1.9 The chemical structures of selected bile acids. Bile acids have a rigid planar structure with a hydrophobic face and a hydrophilic face, making them excellent detergents.

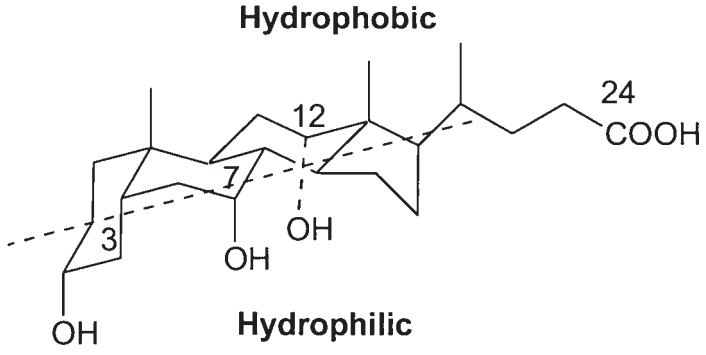


Figure 1.10 The structure of cholic acid.[89] Cholic acid is an amphiphilic molecule. It may be also important in disrupting membranes and activating an inflammatory response by macrophages.

Cholic acid is one of the most abundant bile acids in humans, being present for the most part conjugated in amide linkage with the amino acids glycine or taurine, yielding glycocholate and taurocholate, respectively. Chemically, cholic acid is a cholan-24-oic (cholanic) acid (the terminal C24 of cholane becoming a –COOH group); biologically, cholic acid is derived from cholesterol (a cholestane derivative) and displays oxidation (OH groups) and orientation at positions 3, 7 and 12. Cholic acid is soluble in acetic acid and most organic solvents, and sparingly soluble in water. Quaternary ammonium bile salts were synthesized by some researchers, with very interesting pharmacological applications (such as antiviral and antifungal agents).[90] Furthermore, cationic bile salt conjugates with polyamines were shown to dramatically increase the cellular uptake of DNA.[91]

Cholesterol-modified short interfering RNA also known as "siRNA" (the siRNA interferes with the expression of a specific gene in the RNA interference pathway) showed increased stability and gene silencing activity in the investigation.[92] As disease processes also depend on the activity of multiple genes, it is expected that in some situations turning off the activity of a gene with a siRNA could produce a therapeutic benefit. Because bile acids derivatives of cholesterol, it may also increase the tissue bioavailability in a variety of organs, just like cholesterol. The modified bile acids may also enhance the cellular penetration of the DNA or siRNA complex and overcome some of the problems of the transfection and toxicity in gene delivery systems.

Polymers made from the bile acids are extremely adaptable and present many properties which could be applied to both internal and external wrapping of pharmaceutical products. In certain cases they have been already used for such applications.[93, 94] Additional functionality can then be incorporated by using responsive polymers which can be triggered by a change in pH and temperature.[95, 96, 97] Amino derivatives of bile acids obtained by the modification of carboxylic side chain or replacement of OH groups with amino groups were found useful for binding inorganic ions and DNA receptors.[94,98]

Some of the polymers based on bile acids are hydrogels which can swell in water without dissolving. Hydrogels are usually made of hydrophilic polymers which are cross-linked via chemical bonds, hydrogen bonding, ionic interactions, and hydrophobic interactions.[99] The polymers of bile acids can preserve some of the properties of bile acids such as facial amphiphilicity, chirality and capacity to self-assemble. Our group has developed many bile acid-derived polymers. These polymers possess good biocompatibility and some of them are biodegradable, which present a potential for biomedical applications.[94]

1.6 Design of cationic polymers containing bile acid derivatives

Currently, non-viral vectors show rather lower transfection efficiency compared to viral vectors. In addition, many of the liposomes and polymers investigated thus far display considerable toxicity. Therefore, there is a great interest in improving the biocompatibility of polymeric gene vectors.

In the past, our group has studied polymers containing derivatives of bile acids, which maintain some of the characteristics of bile acids and show interesting biocompatibility. Up to now, neutral and negatively charged polymers of bile acids have been studied in our group.[94] To our knowledge, this work describes the first attempt to prepare cationic polymers containing derivatives of cholic acid.

The hypothesis of this work is that the incorporation of derivatives of bile acids in cationic polymers will help alleviate certain issues related to their use. Since bile acids are endogenous compounds, polymers made with such materials may be more biocompatible compared to the currently used polymers for non-viral vectors. Furthermore, the facial amphiphilicity of bile acids may promote complexation with DNA as well as transfection. The use of bile acids for preparing cationic polymers is considered justified given that polymers containing bile acids are adaptable and versatile.

In this research, the primary interest is to synthesize positively charged polymers containing derivatives of bile acids for use as a gene vector. Furthermore, since all the charged polymers are sensitive to pH, this aspect will also be explored. Its copolymer with NIPAM should add the interesting feature of thermo sensitivity.

For this purpose, we plan to modify the carboxylic acid group on cholic acid to a quaternary amine. The alcohol on position 3 will be modified to a polymerizable group. Alternatively, the quaternary amine group on these monomers can be left as a free primary amine, thus conferring pH sensitivity to the polymer. Finally, should solubility become an issue, these monomers could be copolymerized with other monomers such as NIPAM. The incorporation of NIPAM may also induce thermo sensitivity in the resulting copolymers, thus allowing for another mechanism for stimulated release.

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CHAPTER 2
EXPERIMENTAL

2.1 Materials

Cholic acid (CA, 98%), *N,N*-dimethyl ethylenediamine (DMEDA, 95%), *N,N*-ethylenediamine (EDA, 98%), di-*tert*-butyl dicarbonate (Boc_2O, 97%), and *N*-isopropylacrylamide (NIPAM, 97%) were purchased from Aldrich and were used as received without further purification unless specified in the text. 2,2-Azobis(isobutylonitrile) (AIBN) was purchased from Aldrich and recrystallized from methanol before use. Methacryloyl chloride 80% was purchased from Aldrich and distilled immediately prior to use. All organic solvents were purchased from Aldrich. Methanol, chloroform, tetrahydrofuran (THF, dried with sodium), and triethylamine (TEA, dried with sodium), *N,N*-dimethylformamide (DMF, dried with potassium hydroxide) were dried using a column solvent purification system unless otherwise specified.

2.2 Synthesis of monomers

2.2.1 [(2'-Dimethylamino)ethylene]-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide 4

(1) Cholic acid methyl ester (**2**)[1]

In a 150 mL round-bottom flask equipped with magnetic stirrer and condenser, 70 mL of methanol and 14 g of 98% CA **1** (32.8 mmol) were acidified with 0.5 mL HCl and heated to reflux for 1 h. The solution was allowed to cool to ambient temperature and the crude crystals of the methyl ester were separated and recrystallized in methanol. The purified crystals were collected and dried in a

vacuum oven at 40 °C overnight to yield 12.6 g (91%) of compound **2** (white powder ; T_m = 120-121 °C).

^{1}H NMR ($CDCl_3$) δ shift: 0.69 (s, 3H, C18-C**H$_3$**), 0.89 (s, 3H, C19-C**H$_3$**), 0.98 (d, 3H, C21-C**H$_3$**), 1.0-2.5 (various ring, aliphatic protons), 3.46 (m, 1H, C3-C**H**), 3.65 (s, 3H, OC**H$_3$**), 3.85 (s, 1H, C7-C**H**), 3.98 (s, 1H, C12-C**H**).

(2) [(2'-Dimethylamino)ethylene]-3α,7α,12α-trihydroxy-5β-cholanoamide (**3**)

In a 25 mL flask equipped with magnetic stirrer and condenser, 15 mL of 95% DMEDA and 6 g of cholic acid methyl ester **2** (14.4 mmol) were heated to reflux for 14 h. After the reaction, 30 mL of a water and ice mixture were added to the solution. The resulting mixture was extracted three times with CH_3Cl (3 × 20 mL). The combined organic extracts were washed with saturated brine, and then dried over anhydrous Na_2SO_4. The solvent was removed by rotary evaporation and the crude product was purified by column chromatography on silica gel with methanol as eluent (R_f = 0.12). 4.6 g (66%) of a colorless compound **3** was obtained (T_m = 192-193 °C).

^{1}H NMR ($CDCl_3$) δ shift: 0.73 (s, 3H, C18-C**H$_3$**), 0.94 (s, 3H, C19-C**H$_3$**), 1.05 (d, 3H, C21-C**H$_3$**), 1.0-2.5 (various ring, aliphatic protons), 2.58 (s, 6H, N-(C**H$_3$**)$_2$), 2.84 (s, 2H, $(CH_3)_2$-N-C**H$_2$**), 3.46(s, 2H, CONH-C**H$_2$**), 3.52(m, 1H, C3-C**H**OH), 3.86 (s, 1H, C7-C**H**OH), 4.01 (s, 1H, C12-C**H**OH).

(3) [(2'-Dimethylamino)ethylene]-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide (**4**) [1]

Method 1

In a 150 mL three-necked flask equipped a magnetic stirrer, 0.06 mL of distilled methacryloyl chloride (0.57 mmol) was dissolved in 3 mL anhydrous THF was added dropwise under nitrogen to a solution made of 0.19 mL dried triethylamine (2.6 mmol), 250 mg compound **3** (0.52 mmol), and 15 mL anhydrous THF at 0 °C (ice bath). After the addition of the methacryloyl chloride, the

temperature was slowly raised to room temperature and the solution left to react for a further 24 h. Under these conditions no reaction was evident by TLC.

Method 2

In a 150 mL three-neck flask equipped with a magnetic stirrer, 0.06 mL of distilled methacryloyl chloride (0.57 mmol) was dissolved in 3 mL anhydrous THF and added to a mixture of 0.16 mL pyridine (2.09 mmol), 250 mg of compound **3** (0.52 mmol), and 15 mL anhydrous THF at 0 °C (ice bath) under nitrogen purge. After the addition of methacryloyl chloride, the temperature was slowly raised to room temperature and mixture was left to react for a further 24 h. Under these conditions no reaction was evident by TLC

Method 3

In a 50 mL three-neck flask equipped with a magnetic stirrer, 0.06 mL of distilled methacryloyl chloride (0.57 mmol) was dissolved in 1 mL anhydrous DMF and added to a mixture of 0.19 mL triethylamine (2.6 mmol), 250 mg of compound **3** (0.52 mmol), and 7 mL anhydrous DMF at 0 °C (ice bath) under nitrogen purge. After the addition of methacryloyl chloride, the temperature was slowly raised to room temperature and the mixture was left to react for a further 24 h. Hydroquinone (inhibitor) (0.1 mg) was added, then DMF was removed by rotary evaporation at 50 °C. A drop of a NaOH solution (~3.12 mmol) and 8 mL methanol were added to the crude product. The mixture was stirred for 1 h, the salt removed by filtration, and the solvent evaporated. The crude product was purified by column chromatography on silica gel with methanol/THF (v/v = 1:1) as eluent (R_f = 0.3). The yield of **4** was 25.6 mg (9%) (T_m = 115 °C).

^{1}H NMR ($CDCl_3$) δ shift: 0.73 (s, 3H, C18-C**H**$_3$), 0.94 (s, 3H, C19-C**H**$_3$), 1.02 (d, 3H, C21-C**H**$_3$), 1.0-2.5 (various ring, aliphatic protons), 1.94 (s, 3H CH_2=C-C**H**$_3$), 2.37 (s, 6H, N-(C**H**$_3$)$_2$), 2.57 (m, 2H, (CH_3)$_2$-N-C**H**$_2$), 3.4 (m, 2H, CONH-

CH_2), 3.87 (s, 1H, C7-CHOH), 4.01 (s, 1H, C12-CHOH), 4.62 (m, 1H, C3-CH), 5.52 and 6.08 (d, 2H, CH_2=C).

2.2.2 [(2'-*tert*-butyloxycarboxamido)ethylene]-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide 7

(1) [(2'-Amino)ethylene]-3α,7α,12α-trihydroxy-5β-cholanoamide (**5**)[2]

In a 150 mL flask equipped with a magnetic stirrer and condenser, 50 mL of 98% EDA and 6 g cholic acid methyl ester **2** (14.4 mmol) were heated to reflux for ~16 h. The mixture was cooled to room temperature and 40 mL of a water and ice mixture were added and stirred for a further 5 h. The precipitate was filtered then washed with water. The crude product was dried in a vacuum oven overnight at 40°C until a constant weight was obtained. 5.5 g (85%) of compound **5** was obtained (T_m = 217 °C).

^{1}H NMR (MeOD) δ shift: 0.73 (s, 3H, C18-CH_3), 0.94 (s, 3H, C19-CH_3), 1.06 (d, 3H, C21-CH_3), 1.0-2.5 (various ring, aliphatic protons), 2.75 (t, 2H, NH-CH_2), 3.26 (d, 2H, CONH-CH_2), 3.47 (m, 1H, C3-CH), 3.81 (s, 1H, C7-CHOH), 3.97 (s, 1H, C12-CHOH).

(2) [(2'-*tert*-butyloxycarboxamido)ethylene]-3α,7α,12α-trihydroxy-5β cholanoamide (**6**)

In a 250 mL flask equipped with a magnetic stirrer and condenser, a solution of 97% Boc_2O, 20.7 g (73.5 mmol) in 30 mL MeOH was added dropwise into 70 mL of MeOH containing 31.5 g (70 mmol) of compound **5**. The reaction was left at room temperature for 5 h after the addition of Boc_2O. The solvent was removed by evaporation under reduced pressure and then ethyl acetate (120 mL) was added to dissolve the residue. The organic phase was washed with saturated brine twice, dried with Na_2SO_4, and then concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel with methanol and

ethyl acetate (v/v = 1:1) as eluent (R_f = 0.21). Product was dried in a vacuum oven overnight to yield 34.7 g (90%) of compound **6** (T_m = 112-113 °C).

^{1}H NMR ($CDCl_3$) δ shift: 0.7 (s, 3H, C18-C**H**$_3$), 0.91 (s, 3H, C19-C**H**$_3$), 1.02 (d, 3H, C21-C**H**$_3$), 1.0-2.5 (various ring, aliphatic protons), 1.46 (s, 9H, COO-C(C**H**$_3$)$_3$), 3.27 (s, 2H, CONH-C**H**$_2$), 3.37 (s, 2H, C**H**$_2$-NH-Boc), 3.48(m, 1H, C3-C**H**), 3.87 (s, 1H, C7-C**H**OH), 3.99 (s, 1H, C12-C**H**OH).

(3) [(2'-*tert*-butyloxycarboxamido)ethylene]-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide (**7**)[2]

To a 150 mL three-neck flask equipped with a magnetic stirrer, 0.67 mL of distilled methacryloyl chloride (6 mmol) dissolved in 10 mL dried THF was added dropwise to a solution containing 2.8 g of compound **4** (5.01 mmol), 7.3 mL of TEA (10 mmol), and 45 mL of anhydrous THF at 0 °C under a nitrogen purge. After the addition of methacryloyl chloride, the temperature was slowly raised to room temperature and the mixture was left to react for 24 h. The reaction mixture was filtered, and the solvent removed by rotary evaporation. Chloroform was added to dissolve the residue and was then washed twice with saturated brine. The mixture was dried with Na_2SO_4, and then the solvent was removed under vacuum. The crude product was purified by column chromatography on silica gel with methanol and ethyl acetate (v/v = 1:10) as eluent (R_f = 0.3). 1.8 g (57%) of **7** was obtained (T_m = 105-106°C).

Elemental analysis: Calc.: N: 4.53%, C: 67.96%, H: 9.39%; Found: N: 4.28%, C: 67.18% H: 9.66%.

^{1}H NMR ($CDCl_3$) δ shift: 0.73 (s, 3H, C18-C**H**$_3$), 0.94 (s, 3H, C19-C**H**$_3$), 1.02 (d, 3H, C21-C**H**$_3$), 1.0-2.5 (various ring, aliphatic protons),1.46 (s, 9H, COO-C(C**H**$_3$)$_3$), 1.94 (s, 3H, CH_2=C-C**H**$_3$), 3.29 (s, 2H, CONH-C**H**$_2$), 3.36 (s, 2H, C**H**$_2$-NH-Boc), 3.88 (s, 1H, C7-C**H**OH), 4.07 (s, 1H, C12-C**H**OH), 4.65 (m, 1H, C3-C**H**), 5.53, 6.09 (d, 2H, C**H**$_2$=C).

^{13}C NMR ($CDCl_3$) δ shift: 12.7 (1C, C18-**C**H_3), 17.98 (1C, C19-**C**H_3), 18.76(1C, C21-**C**H_3), 28.91 (3C, COO-C(**C**H_3)$_3$), 46.88 (2C, CONH-**C**H_2), 68.75 (1C, COO-**C**(CH_3)$_3$), 73.64 (1C, C7-**C**H), 75.20 (1C, C12-**C**H), 79.89 (1C, C3-**C**H), 125.4 (1C, **C**H_2=C), 137.5 (1C, CH_2=**C**), 157.42 (1C, NH-**C**O-O, Boc), 167.97 (1C,NH-**C**O-CH_2), 175.39 (CH_2=C-**C**O-O), 21.5-42.19 (various ring **C**H_2)

2.3 Syntheses of polymers

2.3.1 Homopolymers

All polymers were synthesized by free radical polymerization in anhydrous THF using AIBN as initiator. AIBN was purified by recrystallization in chloroform[3] or ethanol[4] and dried in a vacuum oven prior to use.

(1) Poly[(2'-*tert*-butyloxycarboxamido)ethylene-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide] (PCAMA-Boc; **8**)

The polymerization was carried out by the use of a previously reported procedure with some modifications.[5]

In a 25 mL flask equipped with a magnetic stirrer and condenser, 700 mg (1.13 mmol) of monomer **7** and 4.51 mg (0.03 mmol) AIBN were introduced and the system purged with a stream of nitrogen for 30 minutes. 10 mL anhydrous and degassed THF was then added and the temperature was raised from room temperature to 70 °C over a period of 2 h. The temperature was maintained at 70 °C for about 48 h (until the polymerization to complete). After verification that the reaction was complete by ^1H NMR spectroscopy, the solution was cooled to room temperature and THF was removed by rotary evaporation. The polymer was dissolved in a minimal amount of THF and precipitated in petroleum ether. The volume ratio of THF to petroleum ether was 1:3. The precipitate was collected by centrifugation of the reaction mixture. The white homopolymer was dried at 50 °C for 24 h in a vacuum oven. 0.61 g (87%) of compound **8** was obtained. The

molecular weight determined by SEC was M_w = 53,700, M_n = 37,900, M_w/M_n = 1.42.

(2) Poly[(2'-ammonium chloride-amino)ethylene-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide] (PCAMA-$NH_3^+Cl^-$; **9**)

In a 25 mL flask equipped with a magnetic stirrer and condenser, 250 mg of compound **8** (0.41 mmol) was dissolved in 5 mL THF. After addition of 0.82 mmol of 39% hydrochloride acid, the reaction was stirred at room temperature overnight. ^{1}H NMR spectroscopy was used to monitor the disappearance of the *tert*-butyloxycarbonyl group. THF was partially removed in vacuo and washed with deionized water. The deprotected and protonated polymer was dried at 50 °C for 24 h in a vacuum oven, yielding 0.23 g (93%) of compound **9**.

(3) Poly[(2'-aminoethylene)-3α-methylacryloyl-7α,12α-dihydroxy-5β-cholanoamide] (PCAMA-NH_2; **10**)

100 mg of compound **9** was dissolved into a small quantity of methanol. The solution was passed through a column containing (Dowex Z-X8, OH^- form), using methanol as the eluent. The methanol solution was collected and evaporated to dryness. Compound **10** was further dried at 50 °C for 24 h in a vacuum oven to give **10** (100% yield).

2.3.2 Copolymers

All copolymers were synthesized by free radical polymerization in anhydrous THF using AIBN as initiator. A series of Boc protected copolymers with different molar ratios of NIPAM and monomer **7** (namely 3, 6, 10, and 20 mol% of monomer **7**) were prepared. An example of a typical procedure for copolymers which contain 10 mol% of monomer **7** is given below.

(1) Poly{NIPAM-*co*-[(2'-*tert*-butyloxycarboxamido)ethylene-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide]} [poly(NIPAM-*co*-CAMA-Boc); **12**][5]

Copolymer **12** was prepared by combining 0.5 g (0.81 mmol) of monomer **7**, 0.82 g (7.29 mmol) of NIPAM **11**, and 32.2 mg (0.24 mmol) AIBN in a 25 mL flask equipped with magnetic stirrer and condenser. The system was purged with a stream of nitrogen gas for 30 minutes. Anhydrous THF (10 mL) was added and the system was heated from room temperature to 70 °C for 2 h (at a rate of ~0.5 °C/min). The temperature was maintained at 70 °C for 24 h. Once the copolymerization was complete, as verified by ^{1}H NMR spectroscopy, the solution was cooled to room temperature and THF was removed by evaporation. The copolymer was dissolved in a small amount of THF and precipitated by the addition of petroleum ether (volume ratio of THF to petroleum ether 1:1). The copolymer **12** precipitate was filtered and dried at 50 °C for 24 h in a vacuum oven to give a white solid 1.2 g (88%).

The copolymers poly(NIPAM-*co*-CAMA-Boc) **12** containing 3, 6 and 20 mol% of monomer **7** were prepared in the same fashion with yields of 0.95 g (92%), 1.02 g (88%) and 1.49 g (86%), respectively, by adding 0.15, 0.3 and 1 g of monomer **7** to 0.88, 0.86 and 0.73 g of NIPAM **11**.

Poly(NIPAM-*co*-CAMA-Boc) (containing 10 mol% of monomer **7**) ^{1}H NMR ($CDCl_3$) δ shift: 0.73 (s, 3H, C18-C**H$_3$**), 0.94 (s, 3H, C19-C**H$_3$**), 1.02 (d, 3H, C21-C**H$_3$**), 1.0-2.5 (various ring, aliphatic protons),1.46 (s, 9H, COO-C(C**H$_3$**)$_3$), 1.94 (s, 3H, CH_2=C-C**H$_3$**), 3.29 (s, 2H, CONH-C**H$_2$**), 3.36 (s, 2H, C**H$_2$**-NH-Boc), 3.88 (s, 1H, C7-C**H**OH), 4.0 (s, 1H, C12-C**H**OH), 4.65 (m, 1H, C3-C**H**), 5.53, 6.09 (d, 2H, C**H$_2$**=C).

(2) Poly{NIPAM-*co*-[(2'-ammonium chloride-amino)ethylene-3α-methacryloyl-7α,12α-dihydroxy-5β-cholanoamide]} [poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$); **13**]

In a 25 mL flask equipped with a magnetic stirrer and condenser, 250 mg (1.53 mmol) of 10% copolymer poly(NIPAM-*co*-CAMA-Boc) **12** was dissolved in 5 mL THF. 39% hydrochloride acid (3.1 mmol) was added and the reaction left at room temperature for 5 h. ^{1}H NMR was used to monitor the disappearance of the

tert-butyloxycarbonyl group. The solvent was evaporated and copolymer **13** dried at 50 °C for 24 h in a vacuum oven, to give a beige solid 0.24 g (98%).

The copolymers poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** containing 3, 6 and 20 mol% of monomer **7** were prepared in the same fashion with yields of 0.24 g (96%), 0.24 g (97%) and 0.24 g (95%), respectively, by adding 0.92, 1.84 and 6.2 mL of 39% hydrochloride acid to 250 mg of copolymer **13** containing 3, 6 and 20 mol% of monomer **7**.

To simplify the names and distinguish the copolymers, they are named by using mol% content of monomer **7**. For instance, poly(NIPAM-*co*-CAMA-Boc) contained 10 mol% of monomer **7** is named as poly(NIPAM-*co*-10%CAMA-Boc); poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) contained 3 mol% of monomer **7** is named as poly(NIPAM-*co*-3%CAMA-$NH_3^+Cl^-$).

2.4 Methods of physical characterization

2.4.1 Nuclear magnetic resonance

^{1}H NMR spectra were recorded on a Bruker AMX-400 spectrometer operating 400 MHz for protons and 100 MHz for carbon-13. The samples were dissolved in deuterated chloroform or deuterated methanol, as indicated in the text.

2.4.2 Elemental analysis

Elemental analyses of the monomers were done using an EA 1108 CHN analyzer by Fisons instruments

2.4.3 Mass spectrometry

The low resolution mass spectrometry of the monomer was done on a MS 50 TC TA instrument. The monomer was bombarded by a cluster of high-energy electrons so that some of the molecules converted into ions.

2.4.4 Thermogravimetric Analysis

The thermogravimetric behaviour of all polymers were recorded using a Hi-Res TGA 2950 thermogravimetric analyzer from TA instruments at a heating rate of 15 °C/min to a final temperature of 600 °C and under a flow of nitrogen.

2.4.5 Size exclusion chromatography

The molecular weight of the polymer samples was determined by size exclusion chromatography (SEC), using a Waters 1525 pump, a Waters 717 Plus autosampler, a Waters 2410 differential refractometer detector, and a column heater at 35 °C. Three 30 cm Waters columns (Waters Styragel HR3, HR4 and HR6) with range of molecular weights of 500-10^7 were used with THF as the mobile phase at a flow rate of 1 mL/min. The polymer samples were dissolved in the mobile phase and filtered through a 0.2 μm teflon filter. A volume of 100 μL of polymer solution was injected for each sample. Polystyrene Shodex SM-105 samples were used as standards to construct a calibration curve. Data collection and analysis were done using Waters Breeze software.

2.4.6 Ultraviolet-Visible spectrophotometry

The cloud points were measured optically, by the use of a CARY 100 BIO UV-visible spectrophotometer, coupled to a temperature controller. A quartz sample cell with a 10 mm optical path containing ~3 mL of the polymer solution was used. The polymer concentration was maintained at 1.5 wt/v%, and distilled water was used as a reference. The pH values (measured with a pH meter) were adjusted by the addition of NaOH solutions and ranged from ~2 to ~7 (see 2.5.1 pH sensitivity of the homopolymer). The polymer solutions were heated from 25 to 50 °C at a rate of 1 °C/min. The absorption was measured at a wavelength of 500 nm.

2.4.7 Fourier transform infrared spectroscopy

All the homopolymers were characterized on the Bomem (MB-series) Hartmann & Braun FTIR spectrometer. All the samples were ground and pressed with dry KBr to form thin transparent discs.

2.4.8 pH meter

pH was measured using a pH meter (VWR scientific model 2000) equipped with a Ag/AgCl pH, Gel and triode electrodes probe.

2.5 Characterization of pH or thermo-pH sensitivities

2.5.1 pH sensitivity of the homopolymer

PCAMA-$NH_3^+Cl^-$ **9** was dissolved in methanol at a concentration of 1.5 wt/v%. NaOH solutions of varying concentrations (namely, 1 M, 200 mM, 50 mM and 10 mM) were added in very small quantities to increase the pH in a controlled manner; pHs of 2.61, 3.1, 3.64, 4.06, 5.2, 5.58, 6.09 and 6.55 (pH was measured with the pH meter) were obtained. pH values higher than 7.35 could not be obtained because of the precipitation of the polymer. UV-visible spectrophotometry was performed in quartz cells with 10 mm optical path lengths. The pH-sensitivity of PCAMA-$NH_3^+Cl^-$ **9** was evaluated at 25 °C by measuring the absorbance of the homopolymer solutions at a wavelength of 500 nm.

2.5.2 pH sensitivities of the copolymer

(1) Concentration effect

Aqueous solutions with different concentrations of copolymer **13** were prepared (namely 0.25, 05, 0.75, 1, 1.25, 1.5, 1.75 and 2 wt/v% by dissolving 2.5, 5,

7.5, 10, 12.5, 15, 17.5 and 20 mg of **13** in 10 mL of water). The absorption at a wavelength of 500 nm was measured for all solutions in a 1 cm sample cell, containing approximately 3 mL of the copolymer solution, against distilled water (reference). The solutions were heated between 25 and 50 °C at a rate of 1 °C/min.

(2) Salt effect

Aqueous solutions with different concentrations of NaCl and containing the copolymer series **13** were prepared (Table 2.1). The absorptions at 500 nm of these solutions were measured as described above.

Table 2.1 Concentrations of NaCl in the copolymers solutions.

Copolymers **13**	[NaCl] (wt/v%)				
poly(NIPAM-*co*-3%CAMA-$NH_3^+Cl^-$)	0	1	5	10	15
poly(NIPAM-*co*-6%CAMA-$NH_3^+Cl^-$)	0	0.5	2	5	10
poly(NIPAM-*co*-10%CAMA-$NH_3^+Cl^-$)	0	2	6	10	---

(3) Thermo- and pH-sensitivity

180 mg of poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** were dissolved into 12 mL deionized water (concentration of 1.5 wt/v%). NaOH aqueous solutions of varying concentrations (namely, 1 M, 200 mM, 50 mM and 10 mM) were added in very small quantities to increase the pH. The volume and concentration of the NaOH solution which was added to the copolymer solution was noted so as to calculate the final concentration of NaCl. Absorptions at a wavelength of 500 nm of the copolymer solutions was measured as described above. The pH of the solutions ranged from approximately 2 to 7 (pH measured by pH meter) and the solutions of copolymer **13** were heated between 25 and 50 °C at a rate of 1 °C/min.

The copolymers poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) precipitated at certain pH values as follows: poly(NIPAM-*co*-3%CAMA-$NH_3^+Cl^-$) at pH 8.2; poly(NIPAM-

co-6%CAMA-$NH_3^+Cl^-$) at pH 8.32; poly(NIPAM-*co*-10%CAMA-$NH_3^+Cl^-$) at pH 8.06.

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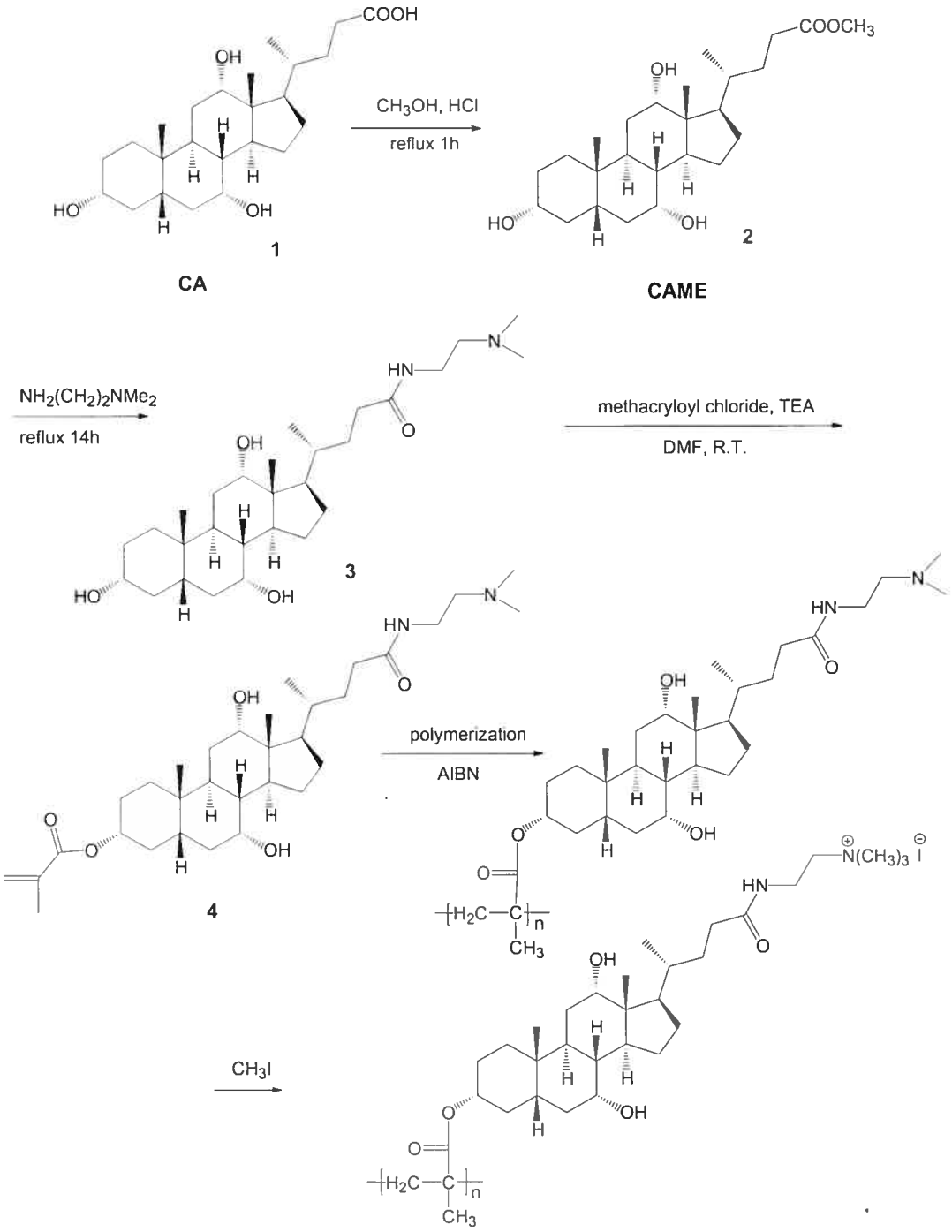
CHAPTER 3
RESULTS AND DISCUSSION

3.1 Synthesis of a monomer bearing a tertiary amine group

3.1.1 Synthetic procedures

The strategy employed to prepare polymers with positive charges involved the synthesis of monomers possessing a tertiary amine group, which can be quaternized once polymerized (monomers bearing positive charges are incompatible with most forms of polymerization). A methacrylate derivative of cholic acid bearing a tertiary amine group (such as monomer **4**) can be synthesized in three steps as illustrated in Figure 3.1.

In the first step towards the preparation of compound **4**, CA **1** was esterified to its methyl ester (**2**) under conditions previously established in our group.[1,2] This reaction proceeded well, with a yield of 91%. This was followed by trans-esterification with *N,N*-dimethyl ethylenediamine (DMEDA) to obtain **3** with a 66% yield after 24 h. Despite the moderate conversion of this reaction, isolation and purification by column chromatography are simple. The third step towards preparing monomer **4** was the acylation of the alcohol group on position 3 of compound **3** by reaction with methacryloyl chloride in the presence of a tertiary amine. Unfortunately, the optimization of reaction conditions for the successful synthesis of monomer **4** was complicated due to the poor solubility and ionization of compound **3** (yield ~9%). This will be further discussed in the following section.



CA: cholic acid and CAME: cholic acid methyl ester

Figure 3.1 Scheme for the synthesis of polymers containing cholic acid derivatives bearing quaternary amino groups.

3.1.2 NMR spectroscopy and mass spectrometry

The ^{1}H NMR spectrum of CA (compound **1**) in CD_3OD is shown in Figure 3.2. CD_3OD has two peaks at 3.3 and 4.8 ppm (due to traces of water in CD_3OD). Most of the peaks lie in a region between 1 and 2.6 ppm, except for those at 3.78 and 3.89 ppm, which are attributed to the methine protons deshielded by the OH groups at C7 and C12 positions of cholic acid. The peak at 3.35 ppm can be attributed to the C**H**OH group at C3. This peak splits into a quintet by the four hydrogen atoms on the neighboring carbons.

When CA is converted into CAME (compound **2**) changes in the ^{1}H NMR spectrum are observed (Figure 3.2). The additional sharp singlet at 3.65 ppm is the signal of methyl ester group at position 24. This is a distinctive feature of the methyl group of CAME.

The structures of **3** and **4** were confirmed by ^{1}H NMR spectroscopy as shown in Figure 3.3. The NMR spectrum of compound **3** confirms the successful isolation of the intermediate. The chemical peaks a, b, c and d at 3.43 (a), 2.84 (b) and 2.6 (c, d) ppm are attributed respectively to the protons on DMEDA which are attached to position 24 of cholic acid. Moreover, the peak at 2.6 ppm is due to the dimethyl groups on the DMEDA. The integrations of these peaks are also consistent.

Figure 3.3 shows the ^{1}H NMR spectrum of monomer **4**. We note the presence of two peaks at 5.6 and 6.1 ppm which confirms the presence of a double bond on monomer **4**. The sharp singlet at 1.9 ppm is attributed to the methyl group of the methacrylate. The peak for position 3 shifted from 3.52 to 4.62 ppm because the hydroxyl group was replaced by an ester. Thus, it is established that the methacrylate double bond was introduced into the molecular structure. The NMR integration of monomer **4** is in agreement with its structure. It is concluded from the experiments that the synthesized monomer **4** has the expected structure.

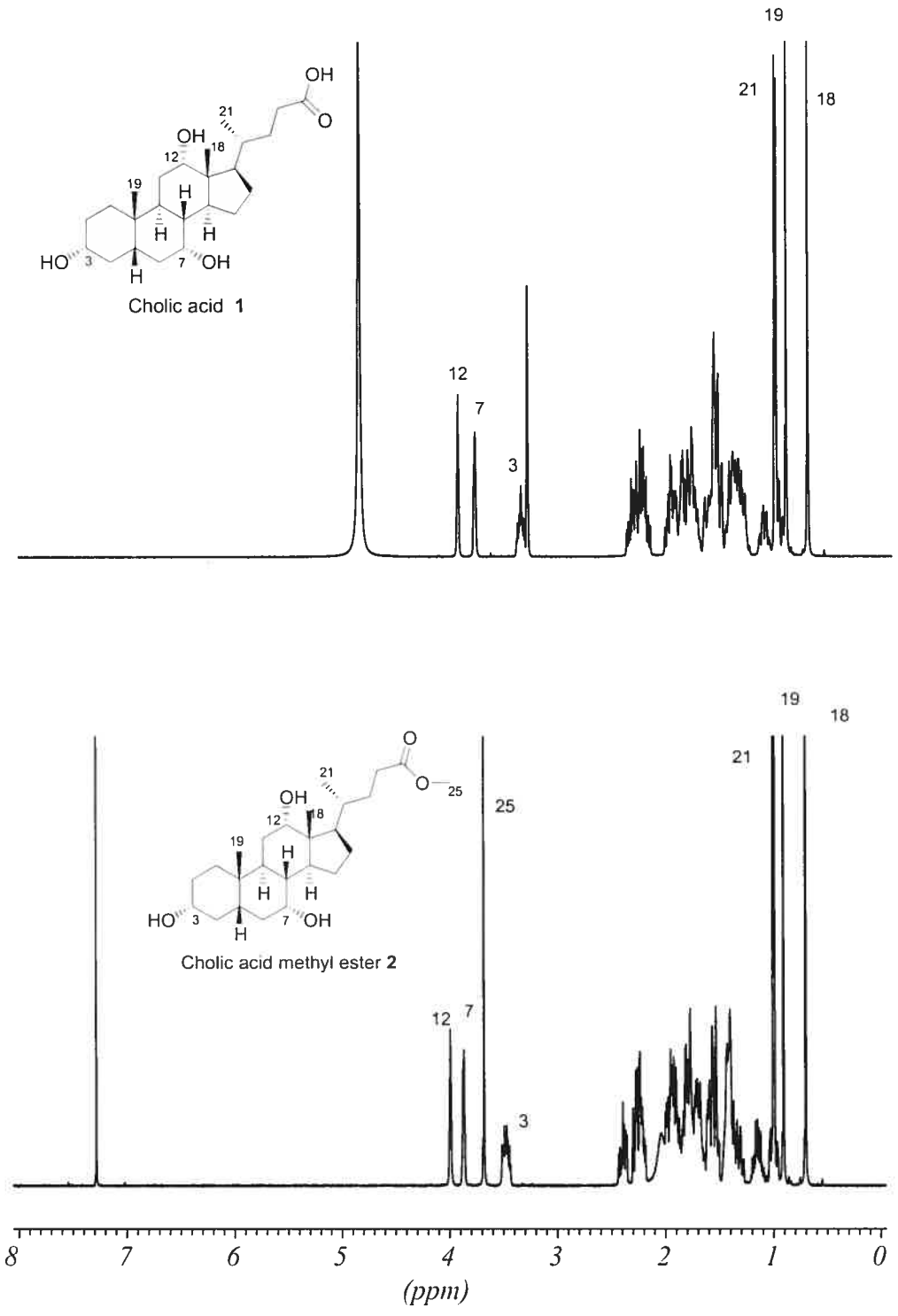


Figure 3.2 ^{1}H NMR spectra of compound **1** in CD_3OD and **2** in $CDCl_3$. These spectra show the appearance of a singlet at 3.65 ppm, characteristic of a methyl ester.

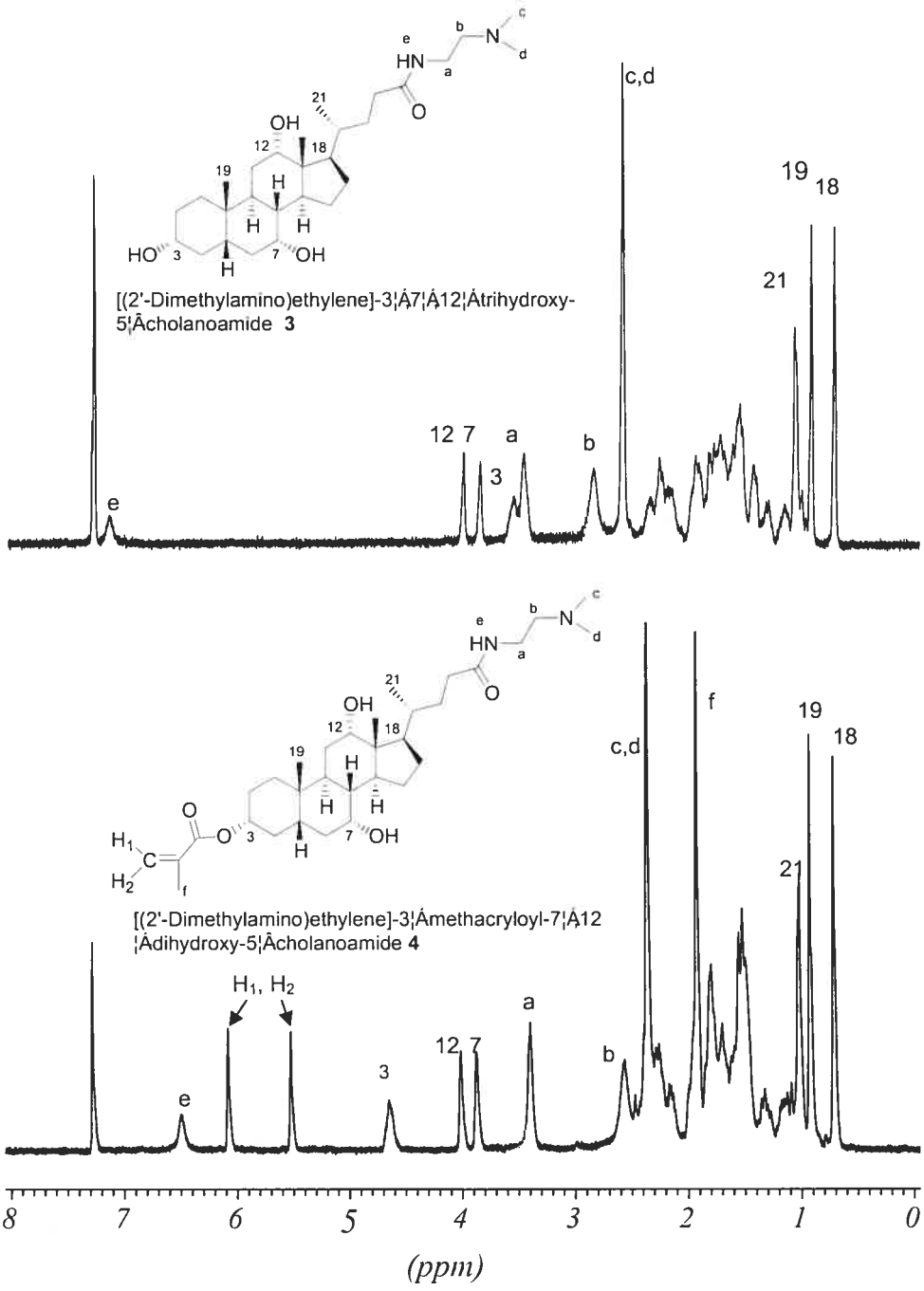


Figure 3.3 ^{1}H NMR spectra of compound **3** in $CDCl_3$ and **4** in $CDCl_3$. These spectra clearly show the presence of the DMEDA on position 24 of compounds **3** and **4** (peaks a-d) and a methacrylate group on monomer **4** (peaks H_1, H_2, f).

The structure of **4** also was confirmed by mass spectrometry (Figure 3.4). The mass spectrum of monomer **4** shows a peak at 547.4 amu corresponding to monomer **4** with the molecular ion +H [546 + 1 = 547]. Another peak at 569.4 amu, corresponding to monomer **4** with a sodium ion [546 + 23 = 569] is also observed.

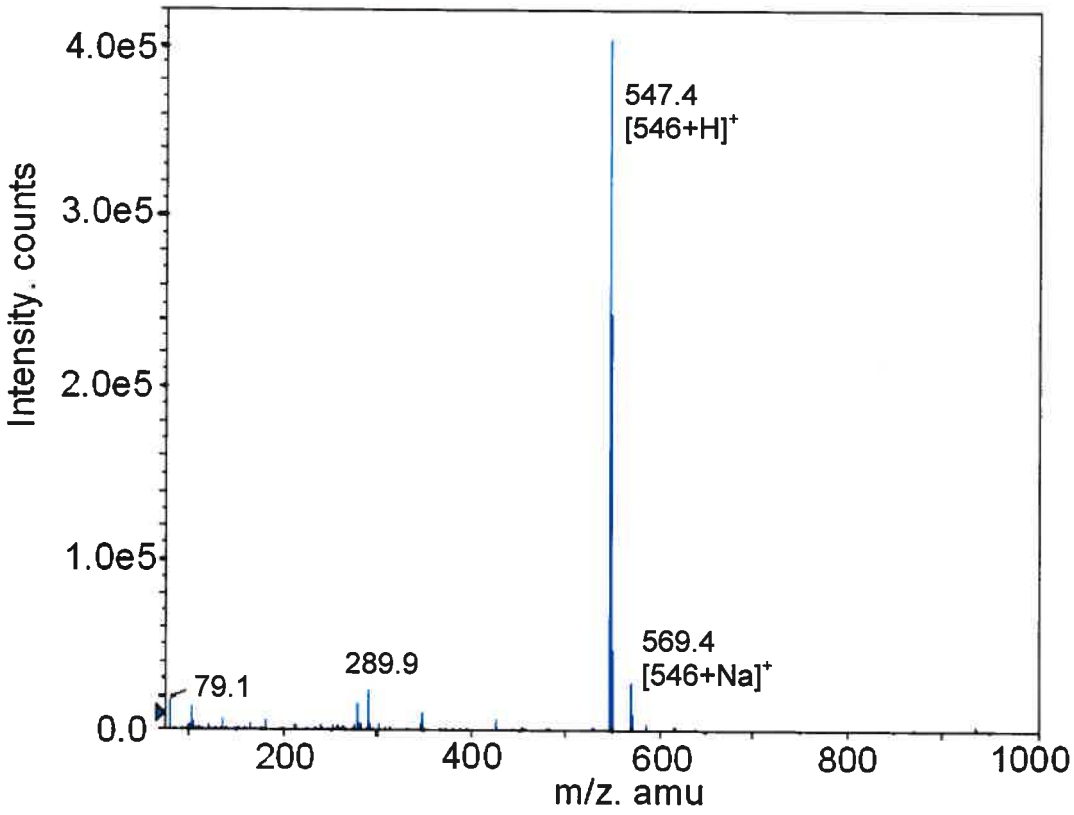


Figure 3.4 Mass spectrum of monomer **4**.

3.1.3 Aspects of the synthesis

The reaction of CAME with EDA served as a reference for the reaction of CAME with DMEDA[2]. DMEDA served two purposes in this reaction: reagent and solvent. The reaction had a yield of 66%. However, the reference reaction with EDA yields 92%. Comparing these two reactions, production of DMEDA occurs in much lower yield. The reaction was analyzed by thin layer chromatography (TLC) after 5, 16 and 24 h. On the TLC, two clear spots were observed: the starting material CAME with R_f = 0.92, and the product compound **2** with R_f= 0.12 using methanol as eluent. After 24 h the starting material CAME was present in the same

proportion as after 16 h. The main difference between these reactions is that the reaction with DMEDA was conducted in a very concentrated solution and the incomplete reaction caused a lower yield. However, due to the absence of by-products as well as marked difference in R_f of the starting material and the product, the purification by column chromatography was very simple.

As stated in the previous section, the acylation of compound **3** to the methacrylate **4** was greatly complicated by three factors: solubility, ionization, and purification.

(1) Solubility

Compound **3** was quite polar and only soluble in certain solvents. Compound **3** was insoluble in dichloromethane and toluene, and only poorly soluble in heated THF (~10 mg/mL). The reaction in THF was attempted, but, due to poor solubility, no reaction occurred. Solubility in DMF and in dimethylsulfoxide was adequate, but removal of the solvent was difficult given their high boiling points (153 and 189 °C at 1 atm, respectively). Generally, removal of the solvent under vacuum required that the reaction mixture be heated to a point where polymerization of the excess methacryloyl chloride (and by-products) and the monomer **4** occurred (~50 °C). To minimize this problem, small amounts of hydroquinone, a free radical polymerization inhibitor, were added to the reaction mixture.

(2) Ionization

Aside from the poor solubility of **3** in organic media, its acylation was further complicated by ionization of the ternary amino group on either **3** or **4** which caused immediate precipitation to occur. Typically, acylation with acid chlorides required the presence of a bulky amine accelerator, which acts as a good leaving group, by activating the acid chloride as seen in Figure 3.5. The pendant tertiary amino group on **3** or **4** may also act as an accelerator, thus becoming positively charged (by-products **1** and **2**) and further decreasing its solubility in the organic media (Figure

3.6). In the present case, TEA (pK_a = 11.01) or pyridine (pK_a =5.21) were added to the reaction mixture to promote acylation, but no difference was observed.

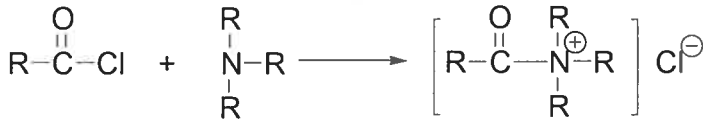


Figure 3.5 Activation of an acid chloride by a tertiary amine.

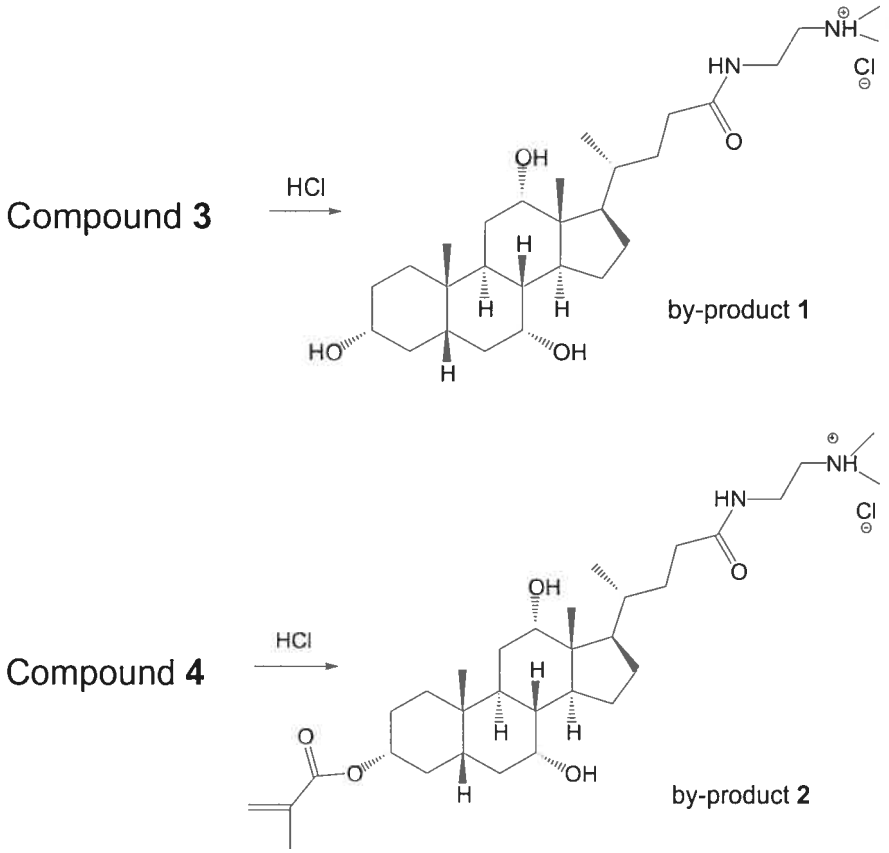


Figure 3.6 Representation of the ionization of the tertiary amino group on **3** and **4** during the methacrylation. **3** and **4** are poorly soluble in DMF, by-products **1** and **2** are insoluble in DMF.

(3) Purification

Finally, because of the polarity of compounds **3** and **4**, isolation by column chromatography using silica gel was difficult. Generally, at the end of the methacrylation reaction, as many as 7 spots were seen on the TLC plate. The purification by silica gel column chromatography was carried out 3 times. Impurities with large differences in polarity can be first removed by a preliminary flash column. Then the pure compound was collected after the second and third columns. However, those products with similar polarities often had long trails as shown on the TLC, and consequently the isolation of individual compounds was difficult.

The overall yield for the synthesis of monomer **4** from cholic acid **1** was low (< 6%). Insufficient quantities of monomer **4** were obtained for in depth characterization of its properties (aside from ^{1}H NMR spectroscopy and mass spectrometry) or polymerization. Consequently, cationic polymers containing cholic acid derivatives were not prepared.

3.2 Synthesis of a monomer bearing a primary amino group

3.2.1 Synthetic procedures

Polymers with pendant amino groups are expected to be pH sensitive. The strategy used to prepare such polymers involved the synthesis of a monomer possessing amino groups protected with *tert*-butyloxycarbonyl (Boc) groups. The presence of a Boc-ylated amine on the monomer rather than a free tertiary amine should greatly simplify synthesis, purification, and polymerization of the resulting monomers.

The general scheme used to synthesize monomer **7** in four steps from cholic acid **1** is shown in Figure 3.7. As for the previous section, cholic acid **1** was easily esterified to a methyl ester **2** with a 91% yield. Transesterification of compound **2**

with EDA (as reactant and solvent) obtained **5** with an 85 % yield. Compound **5** was easily recovered in high purity by filtration. The pendant primary amino group generated on this molecule was protected using Boc_2O to give **6** in 5 h (yield 90%). The isolation and purification of this compound were straightforward by column chromatography.

Acylation of compound **6** to give monomer **7** was done with methacryloyl chloride and TEA in THF with a yield of 57%, substantially better than the yield for monomer **4** (9%). But, this moderate yield for monomer **7** was not as high as what was observed for similar reactions previously in our group (the yield of similar reactions was around 70%)[3] due to difficulties in purification caused by the similar polarities of compounds **6** and **7**. This will be further discussed (see 3.2.3 aspects of the synthesis).

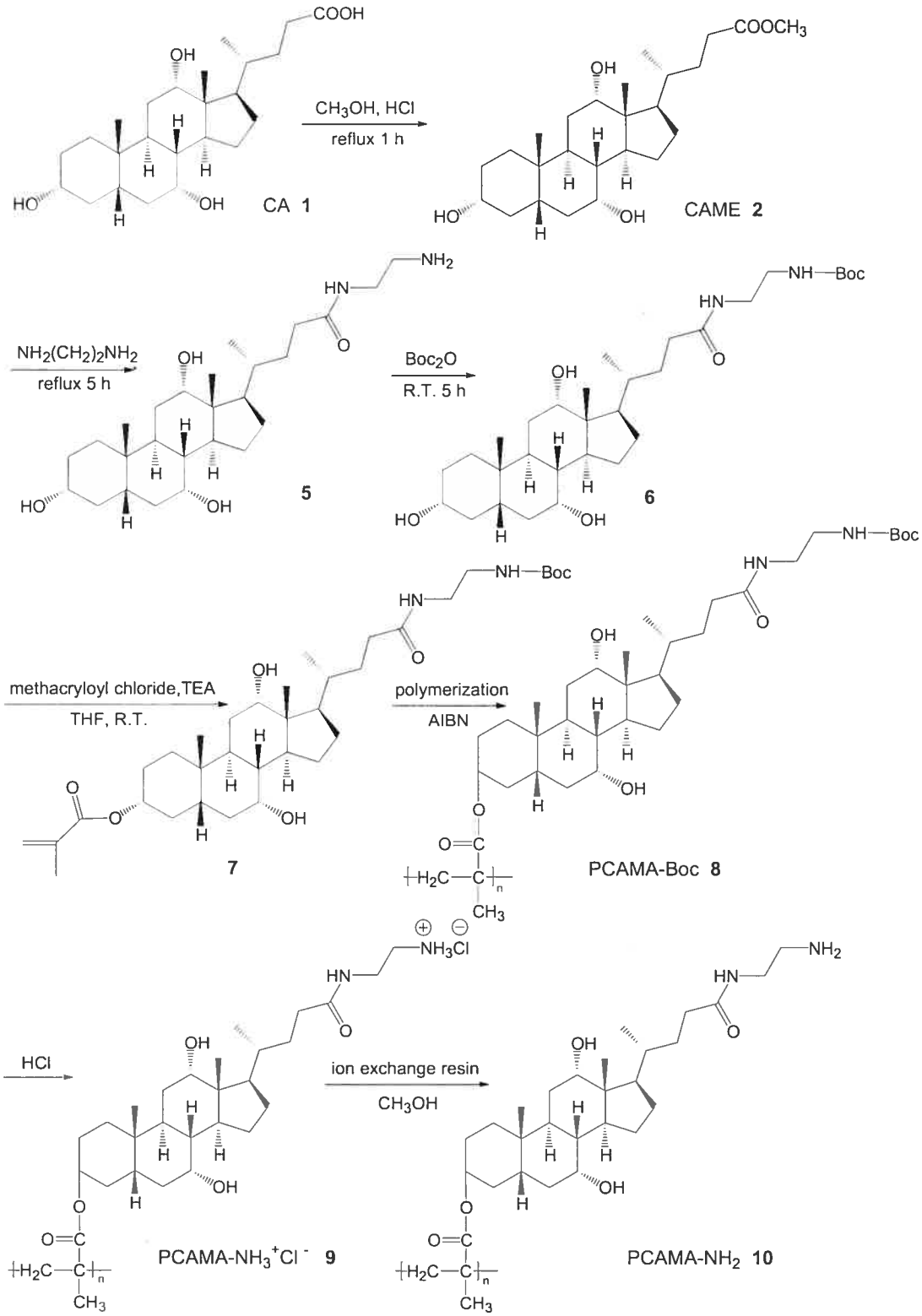


Figure 3.7 Synthesis of primary amine polymer.

3.2.2 NMR spectroscopy and mass spectrum

The ^{1}H NMR spectra of monomer **7** and the intermediate compounds **5** and **6** synthesized from CAME are observed in Figure 3.8 respectively. Also, the ^{13}C NMR spectrum of monomer **7** is shown in Figure 3.9.

The trans-esterification on compound **5** can be easily observed by ^{1}H NMR spectroscopy (Figure 3.8) by the appearance of two triplets at 2.75 and 3.26 ppm that correspond to the ethylene group from EDA. Concurrently, the singlet of the methyl ester at 3.76 ppm disappeared due to the replacement by an amide group.

After adding the Boc group to protect the terminal amino group, the ^{1}H NMR spectrum of compound **6** on Figure 3.8 shows a sharp singlet at 1.46 ppm which integrates for the protons of the *tert*-butyl group. Also peak b was shifted down field (from 2.75 to 3.37 ppm) because of the influence of the carbonate group attached the amine group.

In the ^{1}H NMR spectrum, the presence of singlets at 5.53 (vinyl group), 6.09 (vinyl group), and 1.94 ppm (methyl group) confirms the presence of a methacrylate group on position 3 of monomer **7**. The location of the methacrylate group at monomer **7** is confirmed by the shift of the proton on the position from 3.48 to 4.65 ppm caused by the esterification of the alcohol group. The ^{13}C NMR spectrum of monomer **7** (Figure 3.9) presents two peaks at 125 and 138 ppm identifiable as vinyl groups (C31 and C32). Three carbonyl groups (C25, C24 and C30) are observed at and 157, 168 and176 ppm, respectively. A remarkable δ shift of the tertiary carbon from the Boc group is shown at 69 ppm. The structure of monomer **7** is confirmed by both ^{1}H and ^{13}C NMR spectrum.

The detailed values of the ^{1}H NMR chemical shifts of the final monomer **4** and **7** and their intermediates (compound **3, 5** and **6**) are listed on Table 3.1.

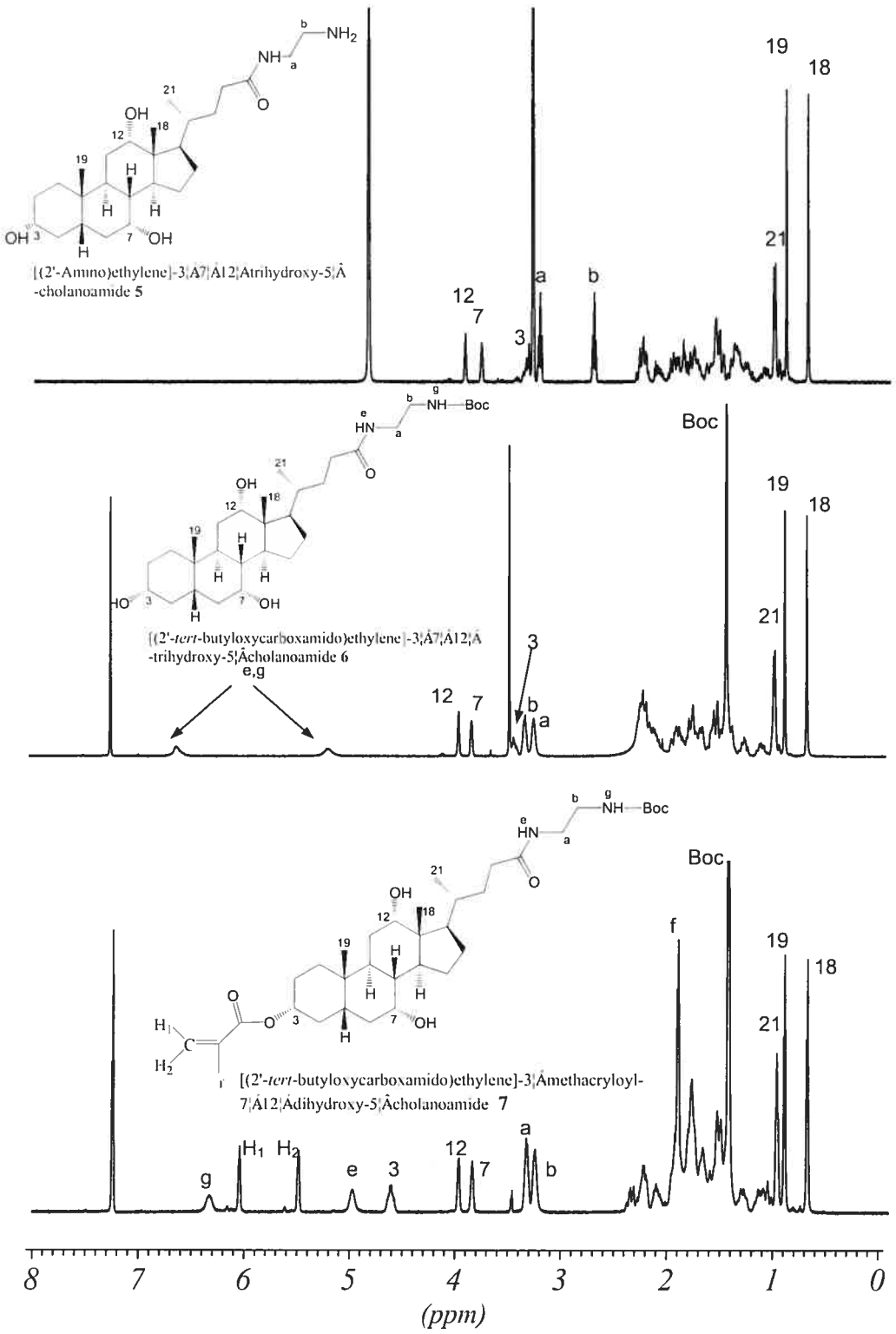


Figure 3.8 ^{1}H NMR spectra of compound **5** in CD_3OD, compound **6** in $CDCl_3$ and monomer **7** in $CDCl_3$.

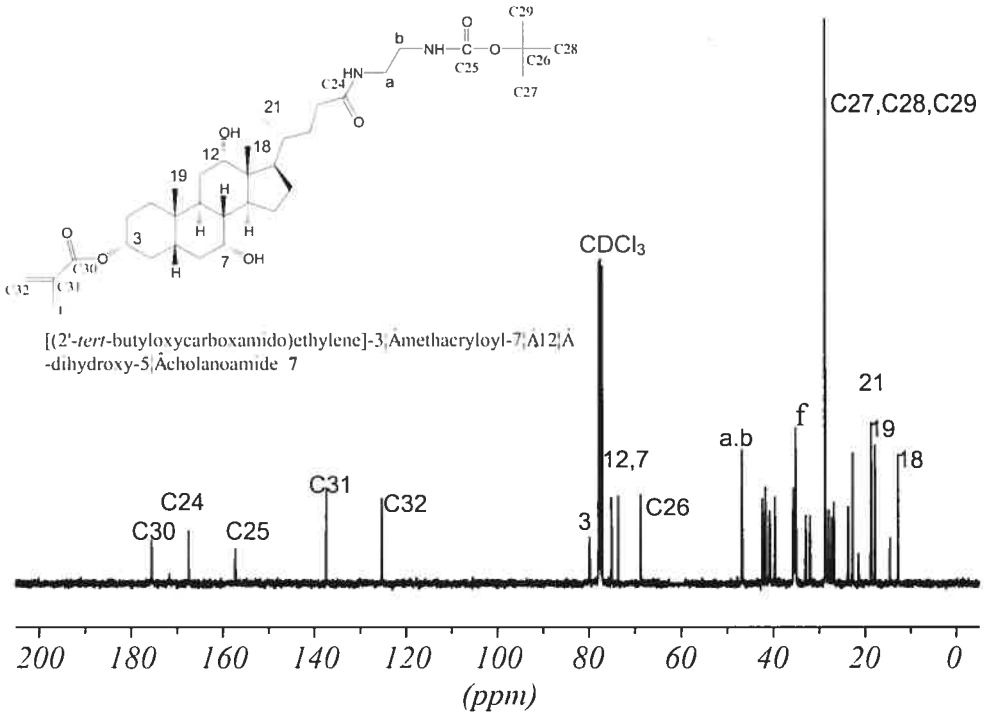


Figure 3.9 ^{13}C NMR spectrum of monomer **7** in $CDCl_3$.

Table 3.1 ^{1}H NMR chemical shifts of final monomers and intermediates.

Proton	^{1}H NMR chemical shifts (ppm)				
	Intermediates			Final monomers	
	3	**5**	**6**	**4**	**7**
3-CH	3.52	3.47	3.48	4.62	4.65
7-CH	3.84	3.81	3.81	3.87	3.88
12-CH	4.01	3.97	3.97	4.01	4.01
18-CH_3	0.73	0.73	0.73	0.73	0.73
19-CH_3	0.94	0.94	0.94	0.94	0.94
21-CH_3	1.05	1.06	1.05	1.05	1.05
H_2C=C-C$\mathbf{H_3}$		N/A		1.94	1.94
C=CH_2		N/A		5.52 & 6.08	5.53 & 6.09

Note; **3**, **4**, **6** in $CDCl_3$ and **5**, **7** in CD_3OD

The structure of monomer **7** was confirmed by elemental analysis and mass spectrometry (Figure 3.10). The mass spectrum of monomer **7** (Figure 3.10) shows a peak at 619.4 amu corresponding to **7** with the molecular ion +H [618.4 + 1 = 619.4]. The principal peak at 641.4 amu, corresponding to **7** with a sodium ion [618.4 + 23 = 641.4] is also observed. When the unstable molecular ion decomposes before it arrives at the ionization chamber, a second peak appears at 563.4 amu [619.4 – 57 + 1 = 563.4] (minus a *tert*-butyl group). In a similar way, when one Boc group is lost, a third peak appears at 519.4 amu [619 – 101 + 1 = 519.4].

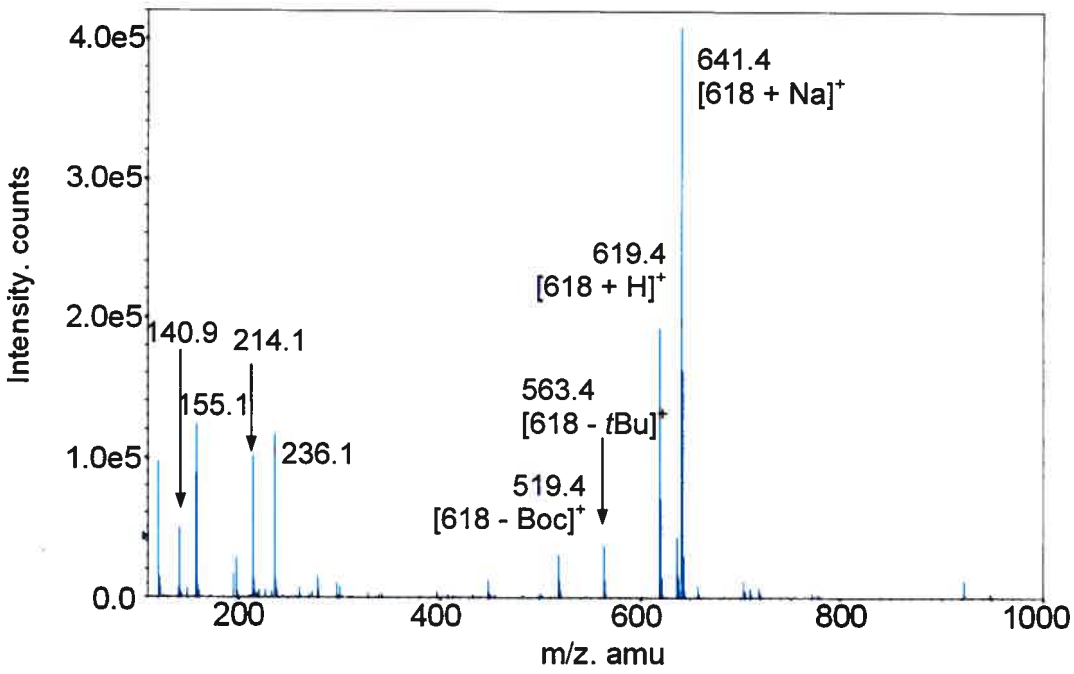


Figure 3.10 Mass spectrum of monomer **7**.

3.2.3 Aspects of the synthesis

During the synthesis of compound **5**, EDA reacts with CAME at position 24, producing amide **5** with a yield of 85%.[2] The liquid EDA acts as both reactant and solvent since CAME is easily solubilized at a temperature close to 60°C. To

maximizing the yield of **5**, allowing time for precipitation was very important after the reaction.

In order to protect the primary amine group, Boc group was selected. Methanol diluted reagent Boc_2O was slowly added dropwise into the system and CO_2 was produced simultaneously. By releasing the pressure of CO_2 in the system, the speed of the reaction was controlled carefully. 2-Methylpropan-2-ol was also produced which could be removed by evaporation. Following up the reaction by TLC after 2 h, we obtained compound **6** with reasonable purity . Small amounts of by-products could be removed by column chromatography on silica gel. All the steps before arriving to the final monomer **7** were successful with high yields.

Although many monomers with structures similar to monomer **7** have been synthesized by this method with yields around 85%,[2,4] in this reaction the final monomer **7** had a lower yield (57%). All the reported monomers were not as polar as monomer **7**. Because of the polarity of monomer **7**, it is difficult to purify by column chromatography silica gel. There are two aspects to be focused: reaction control and purification.

(1) Reaction control

Compound **6** can be completely dissolved in THF upon heating. Cooling the solution in an ice bath preserved the internal temperature of the reaction flask around 0 °C while methacryloyl chloride was slowly introduced to the system. This step can prevent the reaction of hydroxyl groups on positions 7 and 12, thereby reducing the by-products. It is well known that the hydroxyl group on position 3 is more reactive than the other hydroxyl groups. At a lower temperature, this hydroxyl group is more reactive towards nucleophilic substitutions.

The use of fresh methacryloyl chloride was also very important for the syntheses of the final monomers. Methacryloyl chloride was purchased from Aldrich. It must be distilled under reduced pressure before use, kept in a refrigerator

and used within two weeks. Otherwise, the colorless liquid gradually becomes yellowish and slowly polymerizes.

(2) Purification:

The hydroxyl group on compound **6** was converted to the methacryloyl group, which should be more soluble with a lower polarity than monomer **7**. On the TLC plate, monomer **7** has $R_f = 0.3$ with a mixture of methanol and ethyl acetate (v/v = 1:10) as the eluent. However, compound **6** and monomer **7** had very similar polarities. They are very closer on the TLC plate which caused problems for their separation. Column chromatography on silica gel must be carried out more than twice to obtain pure monomer **7**. The first column will remove most of the by-products, the rest will separate compound **6** from monomer **7**. Moreover, it should be noted that lowering the eluent polarity as much as possible will help in the separation.

Because of the similar polarities between compound **6** and monomer **7**, there was an unexpected separation difficulty in obtaining monomer **7**. Enough monomer **7** was nevertheless synthesized to carry on with the polymerization.

Due to the relatively higher chemical reactivity of the OH group at position 3 of CA and the excess amount of methacryloyl chloride, it is very possible to obtain a product with two methacrylate groups as a by-product of the reaction.[1] Several spots with slightly higher R_f than compound **7** (observed by TLC after the reaction) indicated that the other alcohol groups on **6** may have participated in the methacrylation reaction. This is a further explanation for the lower than expected yield for monomer **7**.[1] The yield depended on the ratio of methacryloyl chloride and CA. The NMR integration of monomer **7** indicated a ratio of cholate to methacrylate of 1:1. Thus, it is established that only one methacrylate double bond was introduced into the molecular structure of the compound. Monomer **7** was pure enough to carry out the polymerization reaction.

3.3 Preparation of the polymers

3.3.1 pH-sensitive polymer

The synthetic route for PCAMA-$NH_3^+Cl^-$ **9** is illustrated in Figure 3.7. Free radical polymerization of monomer **7** to PCAMA-Boc **8** was achieved with AIBN (3 mol%) as the initiator. In order to obtain high molecular weight for the polymer, the temperature of the reaction was raised gradually from room temperature to 70 °C and maintained at this temperature for 48 h. The reaction was considered complete when ^{1}H NMR spectra taken of aliquots of the reaction mixture no longer displayed protons characteristic of the unreacted double bond on the methacrylate. Polymer PCAMA-Boc **8** was then precipitated from THF by the addition of petroleum ether followed by filtration (yield 87%).

The ability to carry out a thermodynamically feasible polymerization depends on whether the process proceeds at a reasonable rate under a proposed set of reaction conditions. In this polymerization, a slow heating rate (room temperature to 70 °C takes place during 2 h) and efficient temperature in long reaction time (70 °C for 48h) were required.

To determine the effect of temperature on the molecular weight of the polymer produced in a free radical polymerization, two factors have to be considered: ν, the kinetic chain length and $\overline{X}_n$, the number-average degree of polymerization.[5] For polymerization initiated by the thermal homolysis of an initiator, ν can be expressed by

$$\nu = \frac{k_p[M]}{2(fk_dk_t[I])^{1/2}} \qquad (1)$$

where $[M]$ is the concentration of the monomer, $[I]$ is the concentration of the initiator, f is the initiator efficiency, k_d, k_p, and k_t are the rate constants for initiation, propagation and termination, respectively. If the propagation radicals are terminated by coupling of two chains, the number-average degree of polymerization $\overline{X}_n$ can be expressed by

$$\overline{X}_n = 2v \tag{2}$$

and if the chains are terminated by disproportionation, $\overline{X}_n$ can be expressed by

$$\overline{X}_n = v \tag{3}$$

In both cases

$$\overline{X_n} \sim \frac{k_p[M]}{(fk_d k_t[I])^{1/2}} \tag{4}$$

This equation describes the number-average degree of polymerization $\overline{X}_n$ to be inversely dependent on the square root of the initiator concentration. Increasing initiator concentration leads to short polymer chains and lower degree of polymerization. Moreover, initiator concentration at the beginning increases when the temperature is high. Therefore, gradually increasing the temperature lowers the initiator concentration which decreases radical formation and consequently may yield polymers of higher molecular weight.

The quantitative effect of temperature is complex since the rate and degree of polymerization depend on a combination of the three rate constants (k_d, k_p and k_t). Each of these constants can be expressed by Arrhenius-type relationship[5]

$$k = Ae^{-E/RT} \text{ or } \ln k = \ln A - \frac{E}{RT} \tag{5}$$

k is the rate constant, A is the collision frequency factor, E is the Arrhenius activation energy, R is the rate of polymerization, and T is the temperature. Some studies have evaluated the values of E_p (activation energy for propagation) and A_p (frequency factor for propagation).[5] There are remarkable differences between the values A_p of more or less steric hindered monomers. The more hindered monomers (e.g., methyl methacrylate) have much lower A_p values than the less hindered ones. Long reaction times may be needed for the polymerization with slow kinetics of the propagation step due to the steric effects of the methacrylate derivatives.[3]

After polymerization, the Boc group was removed by reacting with hydrochloric acid to yield PCAMA-$NH_3^+Cl^-$ **9** as an ammonium salt. The reaction

was done at room temperature overnight with a yield of 93%. ^{1}H NMR spectra of aliquots of the reaction mixture were used to follow this reaction and to confirm the absence of residual Boc groups.

PCAMA-$NH_3^+Cl^-$ **9** was passed through an ionic exchange resin to give the polyamine, PCAMA-NH_2. The resin was pretreated with sodium hydroxide aqueous solution to contain exchangeable OH^- anions. While the polymers passed through the resin, the ions OH^- were exchanged with Cl^- and neutral polymer PCAMA-NH_2 **10** could then be collected. Because PCAMA-$NH_3^+Cl^-$ **9** was not soluble in water, methanol was used as the eluent.

Given that these polymers have potential applications as environmentally sensitive drug vectors, molecular weight and polydispersity are important variables because they affect the range and abruptness of transitions. Because of the insolubility of polymer PCAMA-$NH_3^+Cl^-$ **9** in water or THF (except when heated), (they was not suitable for SEC measurements on our system). The molecular weight and polydispersity was estimated from the values obtained for the Boc-ylated polymer PCAMA-Boc **8** (no breakdown of the polymer chains was assumed). PCAMA-Boc **8** had a M_w of 53,700 (equivalent to about 87 monomer units) and a polydispersity index of 1.42 (a value which is typical for free radical polymerizations). In principle, the molecular weight of this polymer could be increased by reducing the amount of AIBN used to initiate the polymerization, though it is known that the polymerization of bulky monomers is difficult. While PCAMA-$NH_3^+Cl^-$ **9** is soluble in methanol, DMF and THF (heated), PCAMA-NH_2 **10** is insoluble in these solvents. After conversions of the ammonium salt group on PCAMA-$NH_3^+Cl^-$ **9** to an amine group, the resulting polymer PCAMA-NH_2 **10** was insoluble in all solvent tested. All the physical properties were studied in methanol.

3.3.2 Thermo and pH-sensitive copolymers

The copolymerization of monomer **7** with NIPAM followed the same procedure as the previously described pH-sensitive polymers. The synthetic route for poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** is illustrated in Figure 3.11.

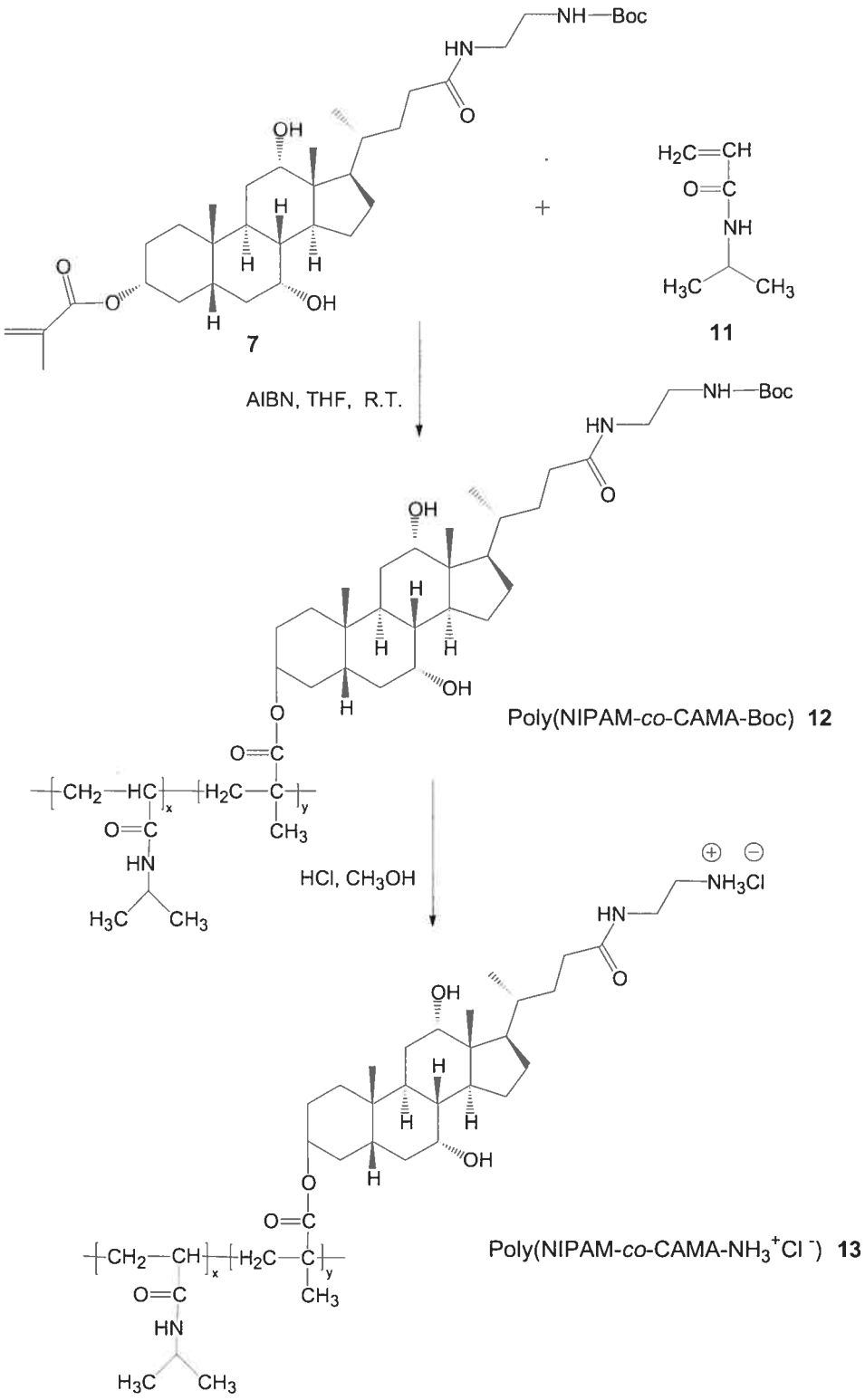


Figure 3.11 Scheme of synthesis for poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$).

The copolymers had different solubilities. The NIPAM polymer has a random coil conformation leading to enhanced dissolution in water. All the Boc-ylated and hydrolyzed copolymers were soluble in methanol, but became difficult to solubilize or insoluble in THF. Poly(NIPAM-*co*-CAMA-Boc) **12** could only be dissolved in THF under heating and poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** copolymers were insoluble in THF. When the content of monomer **7** increased, the solubility of poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** in H_2O decreased. These copolymers were soluble in aqueous media up to 10 mol% monomer **7** in poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13**, after which the copolymer (poly(NIPAM-*co*-20%CAMA-$NH_3^+Cl^-$)) became insoluble. Because of the aforementioned problem of solubility only poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$)s **13** containing 3, 6 and 10 mol% of monomer **7** were analyzed in water for their physical properties.

The molecular weights of the copolymers were measured by SEC in THF and are reported in Table 3.2. Like the homopolymer, only poly(NIPAM-*co*-CAMA-Boc) **12** was soluble in the eluent THF. The molecular weight was measured for the poly(NIPAM-*co*-CAMA-Boc) copolymers.

Table 3.2 The molecular weight of poly(NIPAM-*co*-CAMA-Boc)s.

Ratio of monomer **7** in copolymer	M_w	M_n	M_w/M_n	Yield %
poly(NIPAM-*co*-3%CAMA-Boc)	40,300	27,300	1.48	92
poly(NIPAM-*co*-6%CAMA-Boc)	37,200	23,700	1.57	88
poly(NIPAM-*co*-10%CAMA-Boc)	35,100	21,200	1.65	88
poly(NIPAM-*co*-20%CAMA-Boc)	38,800	22,800	1.70	86

3.4 Characterization of pH-sensitive polymers

3.4.1 NMR spectroscopy

NMR has been used for checking reaction completion and for confirming the structures of the homopolymers. As shown in Figure 3.12, the NMR spectrum of PCAMA-Boc **8** shows the reduction of double bonds on the monomer **7** at 5.53 and 6.09 ppm to undetectable levels indicating that the polymerization was complete. The remaining chemical shifts remained the same except for the broadened peaks.

The NMR spectrum of PCAMA-$NH_3^+Cl^-$ **9** is shown in Figure 3.12. After deprotection, the peak of *tert*-butyl group at 1.46 ppm disappeared. The peak b (CH_2-NH-Boc) shifted from 3.36 to 3.52 ppm because the Boc group was replaced by ammonium chloride group.

The NMR spectra of the homopolymers confirmed that reactions of polymerization and deprotection were successfully completed.

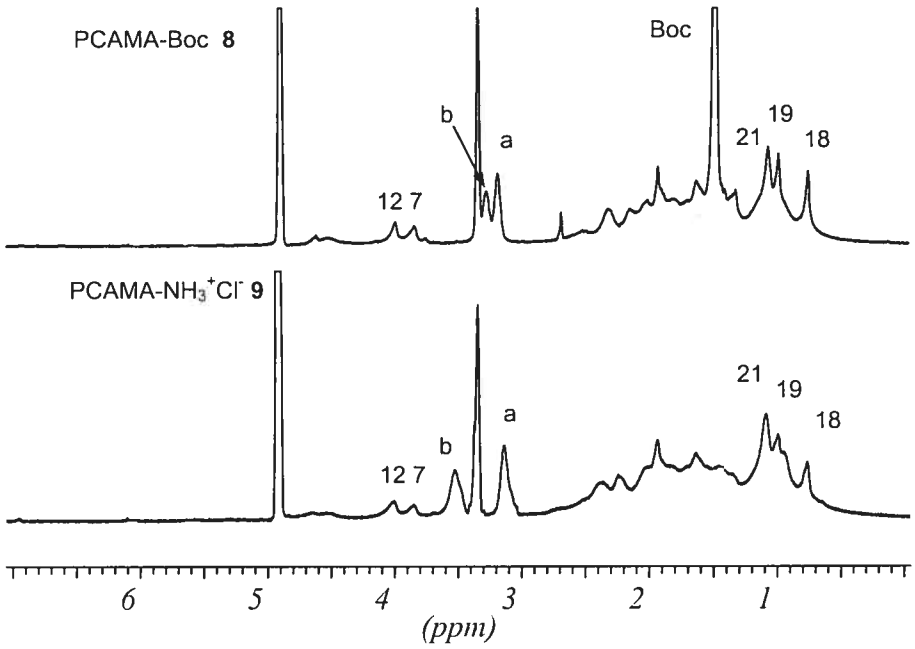


Figure 3.12 ^{1}H NMR spectra of PCAMA-Boc **8** and PCAMA-$NH_3^+Cl^-$ **9** in CD_3OD.

was done at room temperature overnight with a yield of 93%. ^{1}H NMR spectra of aliquots of the reaction mixture were used to follow this reaction and to confirm the absence of residual Boc groups.

PCAMA-$NH_3^+Cl^-$ **9** was passed through an ionic exchange resin to give the polyamine, PCAMA-NH_2. The resin was pretreated with sodium hydroxide aqueous solution to contain exchangeable OH^- anions. While the polymers passed through the resin, the ions OH^- were exchanged with Cl^- and neutral polymer PCAMA-NH_2 **10** could then be collected. Because PCAMA-$NH_3^+Cl^-$ **9** was not soluble in water, methanol was used as the eluent.

Given that these polymers have potential applications as environmentally sensitive drug vectors, molecular weight and polydispersity are important variables because they affect the range and abruptness of transitions. Because of the insolubility of polymer PCAMA-$NH_3^+Cl^-$ **9** in water or THF (except when heated), (they was not suitable for SEC measurements on our system). The molecular weight and polydispersity was estimated from the values obtained for the Boc-ylated polymer PCAMA-Boc **8** (no breakdown of the polymer chains was assumed). PCAMA-Boc **8** had a M_w of 53,700 (equivalent to about 87 monomer units) and a polydispersity index of 1.42 (a value which is typical for free radical polymerizations). In principle, the molecular weight of this polymer could be increased by reducing the amount of AIBN used to initiate the polymerization, though it is known that the polymerization of bulky monomers is difficult. While PCAMA-$NH_3^+Cl^-$ **9** is soluble in methanol, DMF and THF (heated), PCAMA-NH_2 **10** is insoluble in these solvents. After conversions of the ammonium salt group on PCAMA-$NH_3^+Cl^-$ **9** to an amine group, the resulting polymer PCAMA-NH_2 **10** was insoluble in all solvent tested. All the physical properties were studied in methanol.

3.3.2 Thermo and pH-sensitive copolymers

The copolymerization of monomer **7** with NIPAM followed the same procedure as the previously described pH-sensitive polymers. The synthetic route for poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** is illustrated in Figure 3.11.

3.4.2 Fourier transform infrared spectroscopy

The FTIR spectra for homopolymers are shown in Figure 3.13. No peak was observed at 1633 cm^{-1} (stretching of alkene double bonds) confirming the absence of double bonds in the polymers. The broad bands, ranging from 3600 to 3000 cm^{-1}, are due to the characteristic OH bond and hydrogen bond.[6] The two peaks at 2930 and 2865 cm^{-1} were attributed to the C-H, asymmetric and symmetric stretching vibrations. The stretching of the C=O group is visible around 1712 cm^{-1}, and the vibration of the *tert*-butyl group is at 1375 cm^{-1}. The peaks from 500 to 1500 cm^{-1} region are quite similar for PCAMA-$NH_3^+Cl^-$ **9** and PCAMA-NH_2 **10**. The FTIR absorption peaks are listed in Table 3.3.[7]

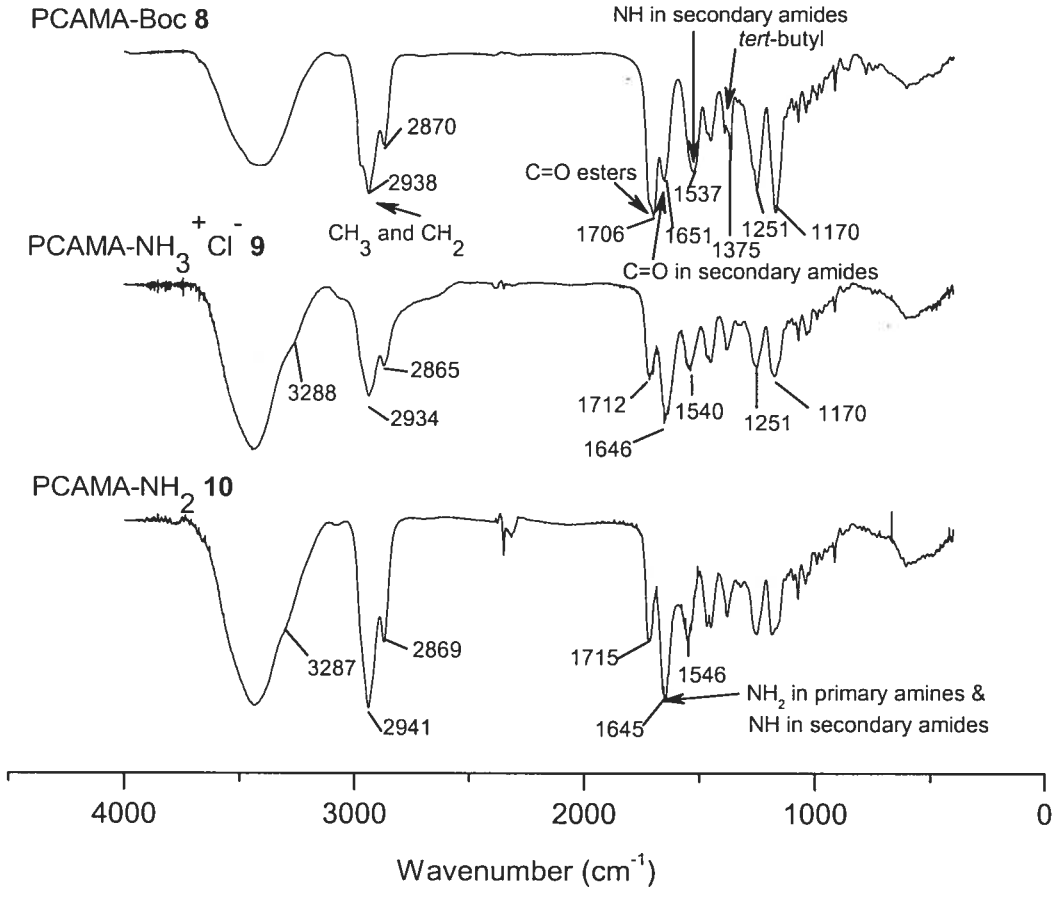


Figure 3.13 FTIR spectra of PCAMA-Boc **8**, PCAMA-$NH_3^+Cl^-$ **9** and PCAMA-NH_2 **10**

Table 3.3 FTIR adsorption of selected functional groups and classes of compounds.[5]

Wavenumber (cm^{-1})	Group and class
3700-3200 (s)	-OH in alcohols
3300-3280 (s)	-NH in secondary amides
2990-2850 (m-s)	$-CH_3$, $-CH_2-$, -CH-
1720-1700 (vs)	C=O esters
1680-1630 (vs)	C=O in secondary amides
1650-1580 (m-s)	NH_2 in primary amines
1565-1475 (vs)	NH in secondary amides
1400-1370 (m)	*tert*-butyl group

Note: s = strong, vs = very strong, m = medium

Comparing PCAMA-Boc **8** and PCAMA-$NH_3^+Cl^-$ **9**, there are significant signals differences. In Figure 3.13, the peak at 1375 cm^{-1} (*tert*-butyl group) disappeared after the hydrolysis, and the intensities of peaks at 1706 (C=O esters) and 1537 cm^{-1} (NH in secondary amides) reduced, indicating that the Boc group has been converted into ammonium chloride.

The resulting charged polymer PCAMA-$NH_3^+Cl^-$ **9** was passed through an ion exchange resin column (pre-treated with sodium hydroxide) to deprotonate the pendant amino groups. This was done in methanol since the polymers PCAMA-$NH_3^+Cl^-$ **9** and PCAMA-NH_2 **10** (charged and uncharged) were insoluble in water. FTIR spectra were taken before (compound **9**) and after (compound **10**) the ion exchange column. Due to spectral overlap in the 1500-1800 cm^{-1} region (the NH_2 in primary amine and C=O in secondary amides), identification of the peaks was difficult. No characteristic peaks of the symmetric or asymmetric stretching of compound **10** were observed.

3.4.3 Thermogravimetric analysis

The thermal stabilities of homopolymers were established by TGA measurement. The TGA decomposition curves are shown in Figure 3.14.

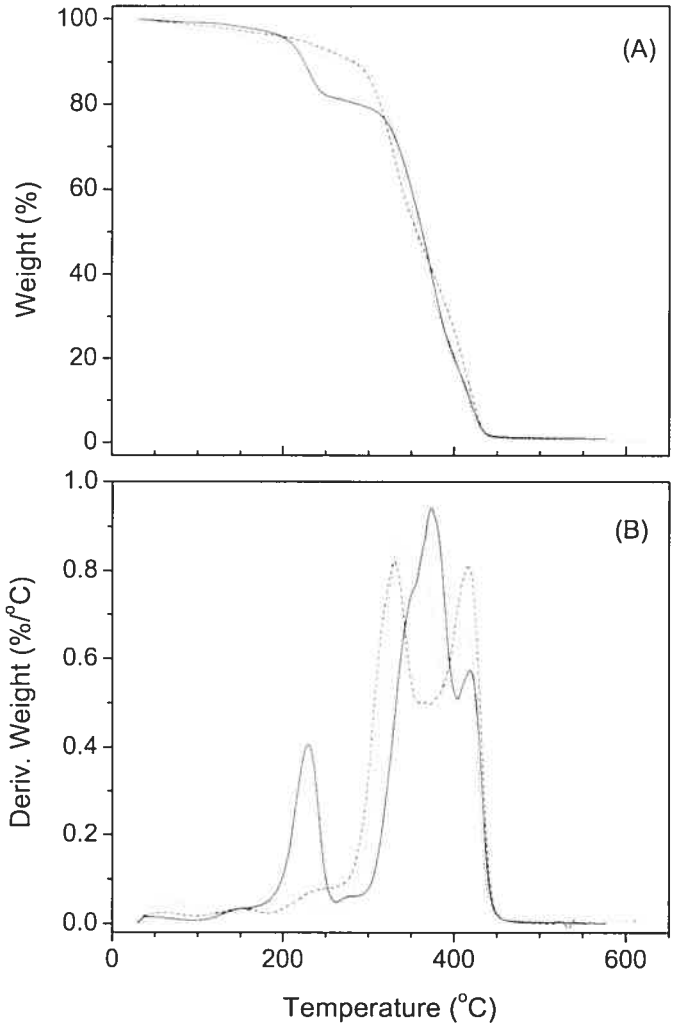


Figure 3.14 Thermogravimetric analysis of homopolymers: PCAMA-Boc **8** (—— solid line), PCAMA-$NH_3^+Cl^-$ **9** (--- dashes) and PCAMA-NH_2 **10** (··· dots). Temperature dependence of (A) weight loss percentage and (B) derivative of the weight loss percentage. The decomposition of all the homopolymers was complicated. PCAMA-Boc **8** loses 15.7 wt% around 200°C because of the loss of the Boc group.

Thermogravimetric analysis of polymers PCAMA-Boc **8**, PCAMA-$NH_3^+Cl^-$ **9** and PCAMA-NH_2 **10** revealed that decomposition occured between 300 and 400 °C, except for PCAMA-Boc **8**, which lost its Boc group at 200 °C (Figure 3.15). This

was confirmed by the similarity between the expected (16.3 wt%) and observed (15.7 wt%) weight loss (the molecular weight of the monomer **7** is 618 g/mol while the molecular weight of the Boc group is 101 g/mol). Thus, the percentage of the Boc group on monomer **7** by weight was 16.4 wt% [101/618=16.4]) when one calculates the relative mass of the monomer **7** and Boc group. The decomposition was a complicated process as shown in Figure 3.14. TGA experiment indicated that all of the homopolymers were not very thermally stable.

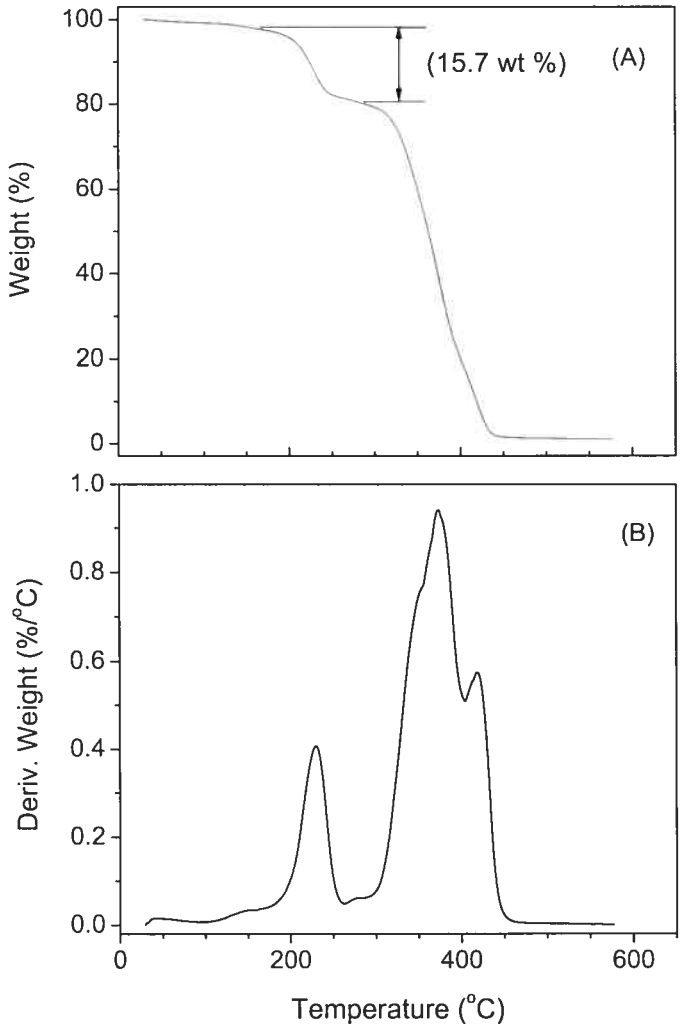


Figure 3.15 Thermogravimetric analysis of PCAMA-Boc **8**. Temperature dependence of (A) derivative of the weight loss percentage and (B) derived weight loss percentage.

3.4.4 pH-sensitivity

The pH-sensitivity of polymer PCAMA-$NH_3^+Cl^-$ **9** was evaluated by measuring the absorbance of a polymer solution (1.5 wt/v% in methanol) at a wavelength of 500 nm. In a general procedure, the pH and the absorbance of the solution containing the polymer was measured, then the pH was readjusted using NaOH. This was carried out incrementally until the polymer precipitated (pH = 7.35). The pH sensitivity of polymer PCAMA-$NH_3^+Cl^-$ **9** is shown in Figure 3.16.

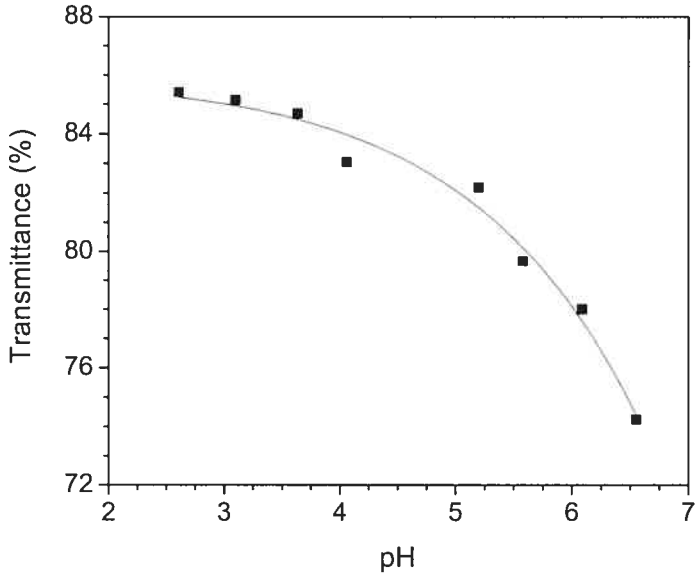


Figure 3.16 PCAMA-$NH_3^+Cl^-$ **9** transmittance changes at 500 nm as a function of pH at 25 °C in methanol.

However, these results must be interpreted in light of the fact that pH values of non-aqueous solutions cannot be accurately compared to those of an aqueous buffer. The activity of the hydrogen ion varies, depending on the background medium because of differences in dielectric constants, solvent acidities, and ion mobilities.[8] The pH values obtained can only be used as relative measurements. In other words, the measurement can be used to compare acid-base qualities of similar solvents, and to indicate when adjustments in pH are needed. It is essential that solvent backgrounds of samples and buffers be as similar as possible. The development of pH scales for non-aqueous solutions is a formidable task. Some work has been done

on methanol and ethanol mixtures.[9] When the pH value increased from 2.61 to 6.55, the percent transmittance (%T) decreased from 85.4 to 74.3%. It gave a rough idea that the polymer **9** had pH sensitivity in the organic solvent.

3.5 Characterization of copolymers

3.5.1 NMR spectroscopy

The relative amounts of NIPAM and monomer **7** in poly(NIPAM-*co*-CAMA-Boc) **12** were measured by ^{1}H NMR spectroscopy (Figure 3.17). As shown in Figure 3.17, poly(NIPAM-*co*-CAMA-Boc) copolymers did not show the presence of the double bonds (monomer **7** at 5.53 and 6.09 ppm, or NIPAM at 5.64 and 6.28 ppm). This indicates that the copolymerization was complete.

Figure 3.18 shows the ^{1}H NMR spectra of all poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) copolymers. After deprotection, the peak of Boc at 1.46 ppm disappeared. Peak b (C**H**$_2$-NH-Boc) shifted from 3.36 to 3.52 ppm because the Boc group was replaced by ammonium chloride group. The rest of the spectra remained the same for poly(NIPAM-*co*-CAMA-Boc) **12**.

One of the important facts that can be extracted from NMR data is the experimental ratio of the monomer **7** to NIPAM within the copolymer. Four copolymers were made containing 3, 6, 10 and 20 mol% of monomer **7**. Considering the integrations of monomer **7** and NIPAM, the ratio of these monomers within the copolymers could be obtained. Two signals which were easily defined and which did not overlap with other peaks were taken into account to obtain the ratio of the monomers. These peaks were the methyl peak at position 18 of CA and the peak from the proton on the tertiary carbon [NH-C**H**-$(CH_3)_2$] on NIPAM. The results are shown in Table 3.4. It seems that the experimental molar ratios of the monomers are slightly higher than the values in the feed. The NMR

spectra of the homopolymers confirm that the polymerization and deprotection were successfully completed.

Table 3.4 The molar percent of monomer **7** in the copolymers.

Monomer **7** in the feed (mol%)	3	6	10	20
Monomer **7** in poly(NIPAM-*co*-CAMA-Boc) measured by NMR experiment (mol%)	3.5	7.5	15.4	22.2
Monomer **7** in poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) measured by NMR experiment (mol%)	2.8	6.1	13.3	18.3

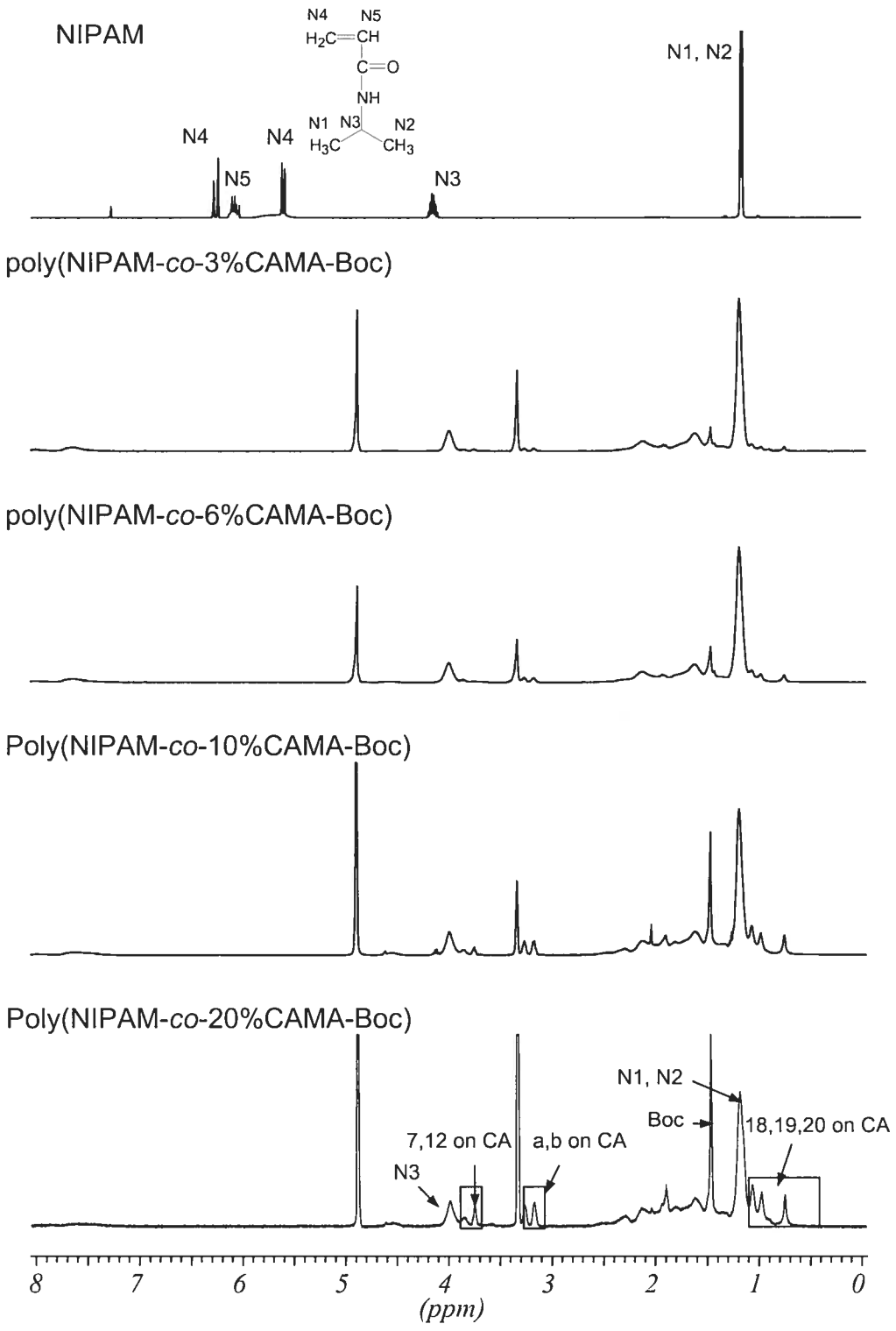


Figure 3.17 ^{1}H NMR spectra of NIPAM in $CDCl_3$ and 3, 6, 10 and 20 mol% of monomer **7** in poly(NIPAM-*co*-CAMA-Boc) **12** in CD_3OD.

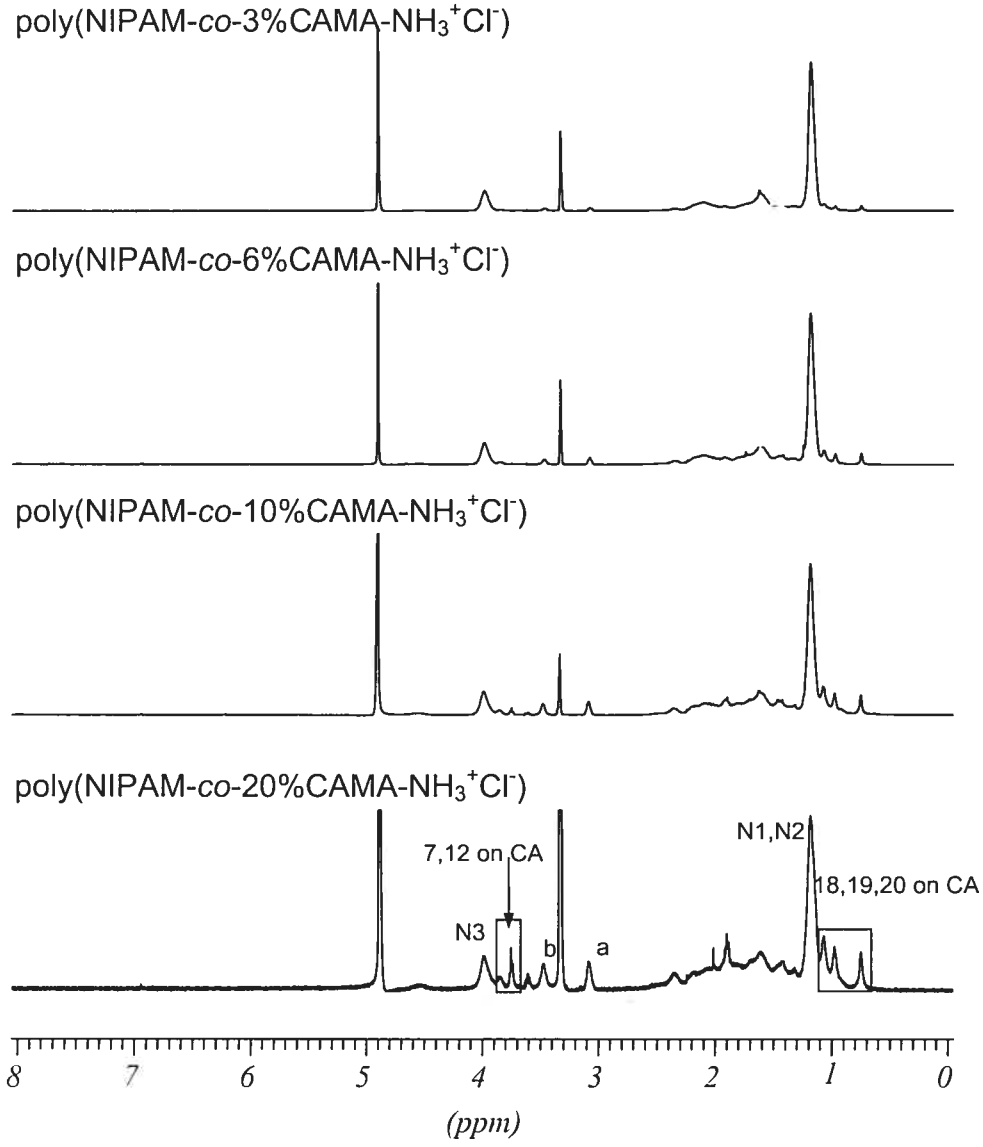


Figure 3.18 ^{1}H NMR spectra of 3, 6, 10 and 20 mol% of monomer **7** in poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** in CD_3OD.

3.5.2 Thermogravimetry

The thermal stabilities of all the copolymers were established by TGA measurements. The decomposition of poly(NIPAM-*co*-CAMA-Boc) is shown in Figure 3.19 while that of poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) is shown in Figure 3.20.

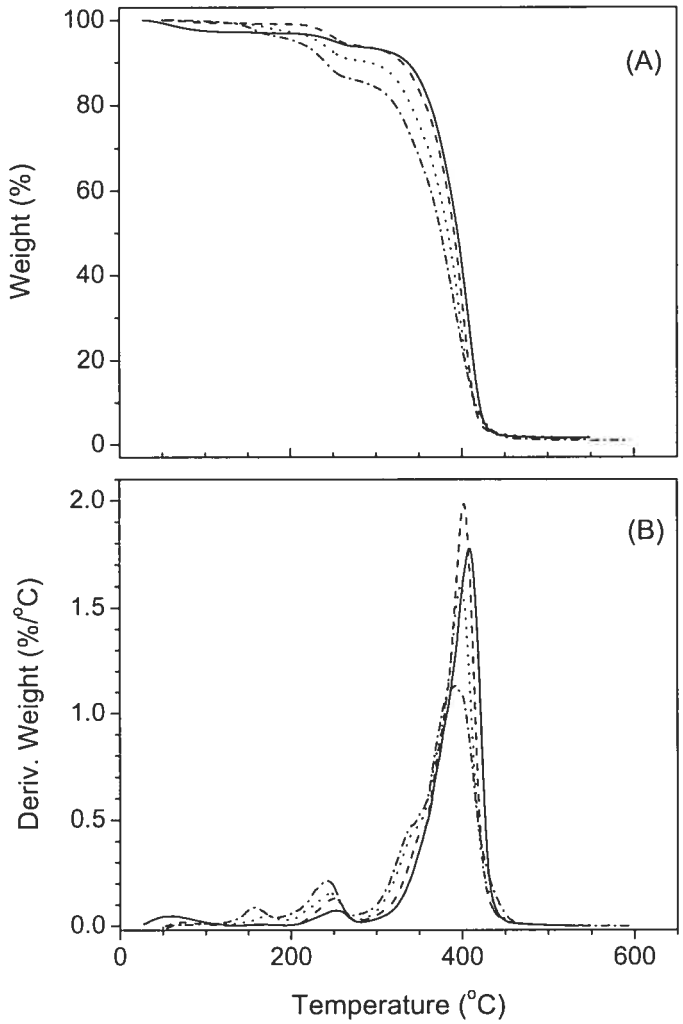


Figure 3.19 Thermogravimetric analysis of poly(NIPAM-*co*-CAMA-Boc)s. poly(NIPAM-*co*-3%CAMA-Boc) (— solid line), poly(NIPAM-*co*-6%CAMA-Boc) (--- dashes), poly(NIPAM-*co*-10%CAMA-Boc (··· dots) and poly(NIPAM-*co*-20%CAMA-Boc) (-- dash dot). Temperature dependence of (A) weight loss percentage and (B) derivative of the weight loss percentage.

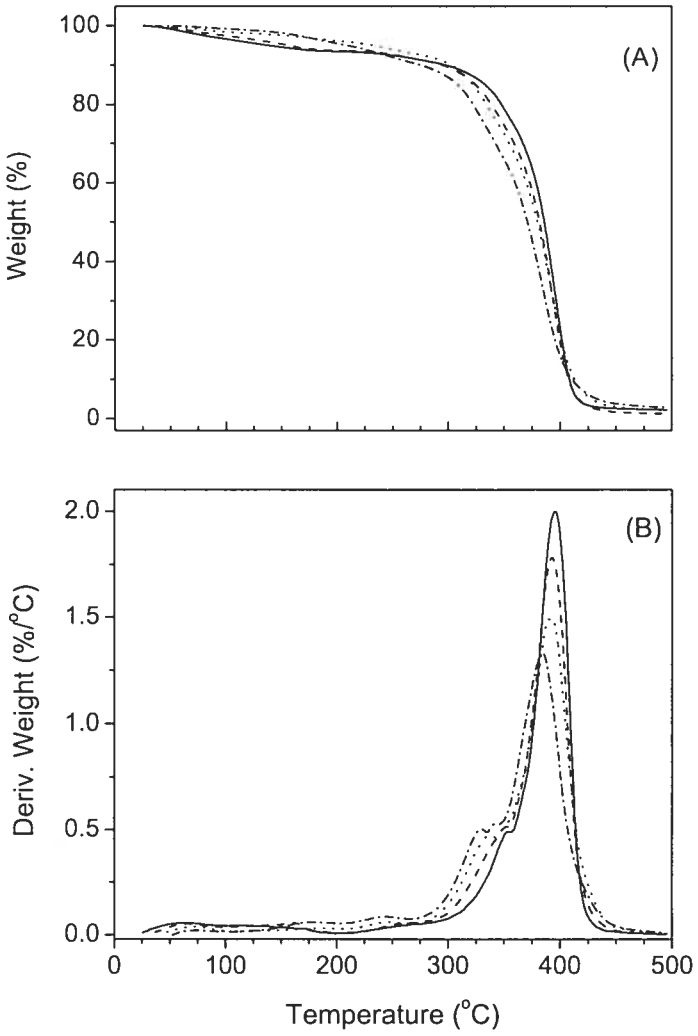


Figure 3.20 Thermogravimetric analysis of poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$)s. poly(NIPAM-*co*-3%CAMA-$NH_3^+Cl^-$) (—— solid line), poly(NIPAM-*co*-6%CAMA-$NH_3^+Cl^-$) (--- dashes), poly(NIPAM-*co*-10%CAMA-$NH_3^+Cl^-$) (··· dots) and poly(NIPAM-*co*-20%CAMA-$NH_3^+Cl^-$) (-- dash dot). Temperature dependence of (A) derivative of the weight loss percentage and (B) derived weight loss percentage.

The comopolymers have large range of decomposition temperatures. Almost all the weight changes took place between 300 and 450 °C. The temperature at the highest derivative weight loss was at 400 °C. The TGA experiments indicate that poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$)s had good thermal stability (Figure. 3.20). It can be seen from Figures 3.19 and 3.20 that between 300 to 360 °C the amount of decomposition increased with the ratio of monomer **7** in the copolymers

(poly(NIPAM-*co*-CAMA-Boc) and poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$)). This indicated that decomposition began on the steroid structure.

Integration of the weight loss (Figure 3.19) showed the departure of the Boc-group at 200 °C was selected as representative of the quantity of monomer **7** in polymer **12**. This approach was deemed justified given the good accord between the calculated and measured weight loss (of the Boc group) for the homopolymer **8** (Figure 3.15), indicative that there is no loss of the Boc group during polymerization. Hydrolysis of the Boc group in acidic conditions was followed by ^{1}H NMR spectroscopy (Figure 3.18) and verified by thermogravimetric analysis (no weight loss associated with the departure of the Boc group; Figure 3.20).

The ratio of monomer **7** in the Boc-ylated copolymer can be calculated by percentage of the weight loss of Boc groups (W_{Boc}%). We suppose that, in 1 g of the sample, there are x_{NIPAM} mol of NIPAM and $x_{monomer\,7}$ mol of monomer **7** ($x_{monomer\,7} = x_{Boc} = x_{CAMA}$; CAMA is defined as monomer **7** without Boc group).

$$x_{NIPAM} = \frac{1 - W_{Boc}\% - \dfrac{W_{Boc}\%}{M_{w(Boc)} / M_{w(monomer\,7)}}}{M_{w(NIPAM)}} = \frac{1 - W_{Boc}\% - \dfrac{W_{Boc}\%}{101/618}}{113} \tag{6}$$

$$x_{CAMA} = \frac{1 - W_{Boc}\% - M_{w(NIPAM)} \times x_{NIPAM}}{M_{w(CAMA)}} = \frac{1 - W_{Boc}\% - 113x_{NIPAM}}{517} \tag{7}$$

Where are the molecular weights of NIPAM ($M_{w(NIPAM)} = 113$), monomer **7** without Boc ($M_{w(CAMA)} = 517$), Boc ($M_{W(Boc)} = 101$) and monomer **7** ($M_{w(monomer\,7)} = 618$). Because $x_{monomer\,7} = x_{Boc} = x_{CAMA}$

$$x_{monomer\,7}\%(mol\%) = \frac{x_{monomer\,7}}{x_{NIPAM} + x_{monomer\,7}} \times 100\% \tag{8}$$

The calculated results are shown in Table 3.5. The ratios of monomer **7** in the copolymers obtained by TGA were higher than the values in the feed, but in agreement with the NMR results (Table 3.4).

Table 3.5 Theoretical and experimental ratios of the monomer **7**, and weight loss of Boc groups (W_{Boc}%) measured by TGA.

Monomer **7** in the feed (mol%)	3	6	10	20
Percentage of weight loss of Boc groups (W_{Boc}%) measured by TGA (wt%)	3.07	4.79	6.58	9.38
Monomer **7** in poly(NIPAM-*co*-CAMA-Boc measured by TGA (mol%)	4.99	8.91	14.23	27.41

3.5.3 pH- and thermo sensitivity

Polymers made with NIPAM are known to be thermo sensitive. Inclusion of NIPAM as comonomer for compound **12** and **13** was done to increase the hydrophilicity of the polymers (and thus its solubility in aqueous media), but may also make the latter thermo sensitive. This section describes the solution behaviour of poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** copolymers containing 3, 6 and 10 mol% of monomer **7**.

(1) Salt and concentration effects

In order to accurately interpret the potential pH and thermo sensitivity of the copolymers, the effects of polymer concentration and salt concentration (factors which are known to influence pH and thermo sensitivity) were first investigated.

The effect of concentration on the thermo sensitivity of polymer **13** was evaluated by measuring the cloud points of various aqueous solutions with concentrations ranging from 0.25 to 2.0 wt/v%. Assumption is made that the effect of concentration was the same for all copolymers. Results are plotted in Figure 3.21 and show that above 1.2 wt/v%, the thermo sensitivity became independent of polymer concentration (over the range examined). All subsequent measurements were made at a polymer concentration of 1.5 wt/v%.

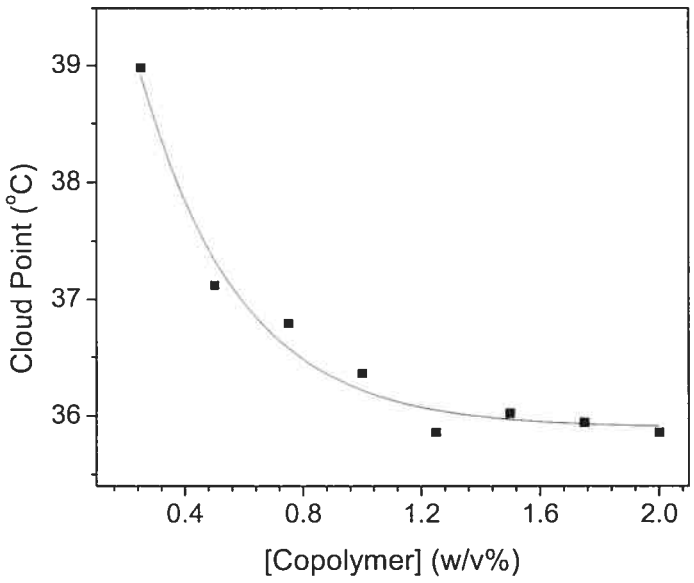


Figure 3.21 The correlation of poly(NIPAM-*co*-10%CAMA-$NH_3^+Cl^-$) concentration and cloud point in aqueous solution.

The effect of salt concentration was examined for poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** containing 3, 6 and 10 mol% of monomer **7** because, in subsequent sections, the pH was adjusted with NaOH, which can increase the concentration of salts (Figure 3.22).

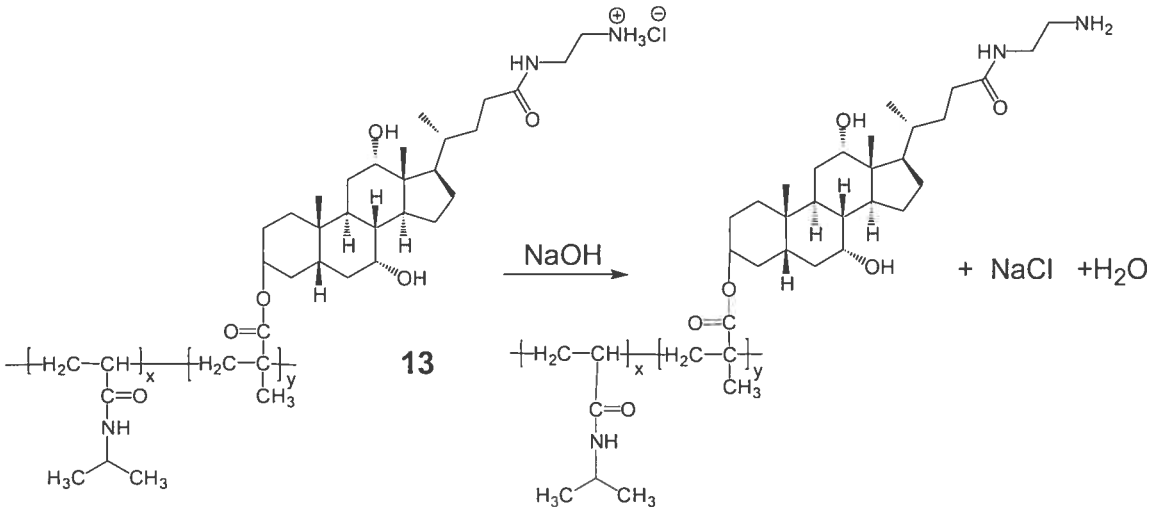


Figure 3.22 Salt is generated with the addition of NaOH to solutions containing poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$).

The variation of cloud point with salt concentration was linear as can be seen in Figure 3.23. Linear regression of this data provided a means for correcting the cloud point measured at discrete salt concentrations.

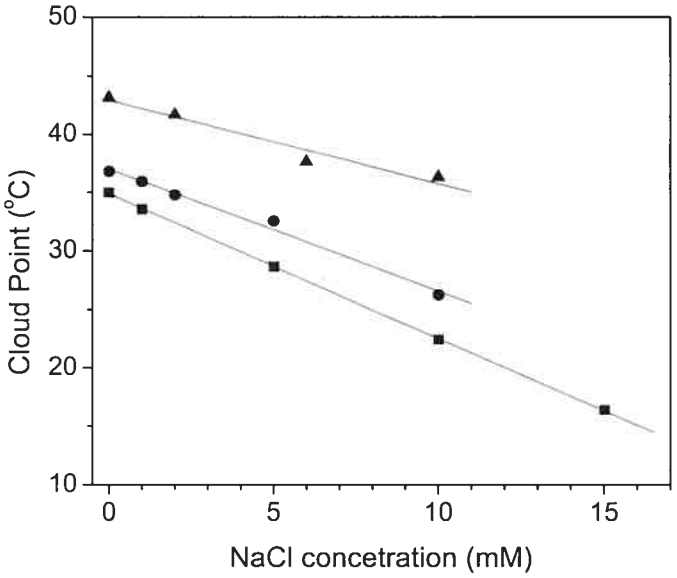


Figure 3.23 Correlation of NaCl concentration with cloud point of poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) (1.5 wt/v% in water). (▪) poly(NIPAM-*co*-3%CAMA-$NH_3^+Cl^-$), (•) poly(NIPAM-*co*-6%CAMA-$NH_3^+Cl^-$), (▲) poly(NIPAM-*co*-10%CAMA-$NH_3^+Cl^-$). The linear correlation functions of the copolymers obtained as CP = 42.95-0.72[NaCl], CP = 37-1.04 [NaCl] and CP = 34.86-1.24 [NaCl] for poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) containing 3, 6 and 10 mol% of monomer **7,** respectively.

(2) Thermo and pH-sensitivity

The thermo sensitivity of the copolymers poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$) **13** containing 3, 6, and 10 mol% monomer **7** was evaluated at different pHs up until the pH at which precipitation occurred (the deprotonated form of the copolymer is less soluble than the charged form). The pHs at which precipitation occurred were 8.2, 8.32, and 8.06 for the copolymers containing 3, 6 and 10 mol% of monomer **7**, respectively. The copolymer containing 20 mol% of monomer **7** was insoluble at all pHs in water and was therefore omitted from these experiments. The pHs of the solutions being initially acidic, discrete amounts of NaOH were added to increase

the pH (which was measured using a pH meter). The concentration of salt at each different pH was calculated and compiled in Table 3.6.

Table 3.6 The concentration of NaCl under different pH for all the poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$).

poly(NIPAM-*co*-3%CAMA-$NH_3^+Cl^-$)		poly(NIPAM-*co*-6%CAMA-$NH_3^+Cl^-$)		poly(NIPAM-*co*-10%CAMA-$NH_3^+Cl^-$)	
pH	[NaCl] (10^{-3} mM)	pH	[NaCl] (10^{-3} mM)	pH	[NaCl] (10^{-3} mM)
1.87	0	1.93	0	2.14	0
2.94	3.3	3.18	4.1	3.10	2.5
3.92	5.5	4.22	6.1	4.51	3.5
4.81	6.4	5.44	6.5	5.33	3.8
5.84	6.5	6.64	6.6	6.49	4.0
7.04	6.9	7.74	7.8	7.41	4.6
7.83	7.2	-	-	-	-

From this table, it can be seen that even at high pHs, the concentration of salt was insufficient to affect the cloud point of the solutions. This parameter can therefore be neglected.

Figure 3.24 shows the pH dependence of the cloud point of all three copolymers. Generally, the cloud point decreased as the pH increased. This can be attributed to the lower solubility of the polymers with increasing pH (less protonated amine groups). However, over the entire pH range examined, the change was relatively small: about 2.5, 1, and 6 °C for the poly(NIPAM-*co*-CAMA-$NH_3^+Cl^-$)s **13** containing 3, 6, and 10 mol% of monomer **7**, respectively. The lower values observed with increasing monomer **7** content can potentially be attributed to a lower pK_a of monomer **7**. This would be consistent with the lower solubility of the copolymer at lower pHs.

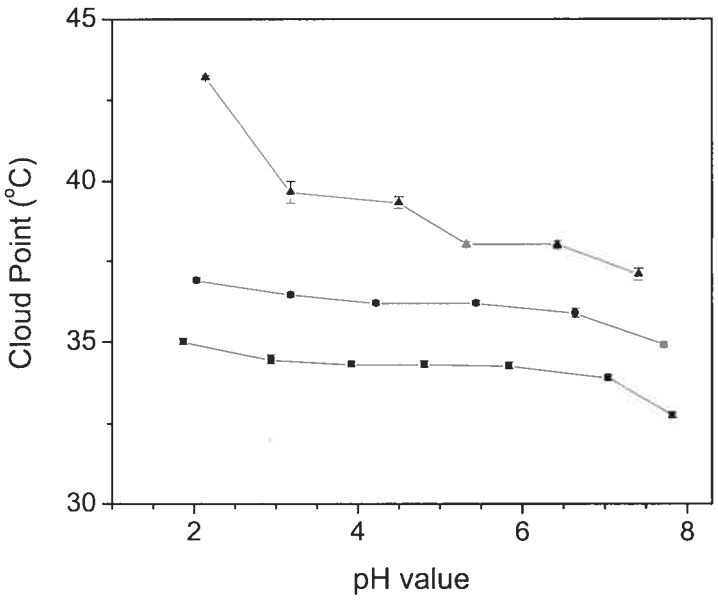


Figure 3.24 Correlation of pH value and cloud point of poly(NIPAM-*co*-CAMA- $NH_3^+Cl^-$) (1.5 wt/v% in water). (■) poly(NIPAM-*co*-3%CAMA-$NH_3^+Cl^-$), (•) poly(NIPAM-*co*-6%CAMA-$NH_3^+Cl^-$), (▲) poly(NIPAM-*co*-10%CAMA-$NH_3^+Cl^-$).

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CHAPTER 4
CONCLUSIONS

4.1 Synthesis

The syntheses of the monomers were not trivial because of the charges desired on these compounds. The monomers were only soluble in polar solvents which made the synthetic approaches and separations very difficult.

Although a small amount of the monomer **4** was made and its structure confirmed, the synthesis of a polymer with permanent positive charges met serious challenges. The structure of monomer **4** may have to be reconsidered in order to facilitate the purification of the products. The monomer could be designed to have a less polar structure (such as adding alkyl spacer on position 24). An alternative synthetic strategy is shown in Figure 4.1. In this case, a bromo alcohol on position 24 would reduce the polarity of the monomer relative to the monomers containing an amine group. This bromo alcohol can be transformed into a quaternized amine after polymerization. This synthetic strategy may help solve the central problems surrounding solubility, ionization and purification.

We have overcome the synthetic difficulties of monomer **7**. In addition, the cationic polymer and copolymers have been successfully prepared. Poor solubility of monomer **7** led to the use of many polar solvents since the choice of the right solvent was very important for these experiments. The solubility of such polymers in their present form may also limit their uses.

The challenges of this project involved a good understanding of the solubility, ionization and purification of the compounds. It is imperative that these challenges be taken care of before initiating further experiments.

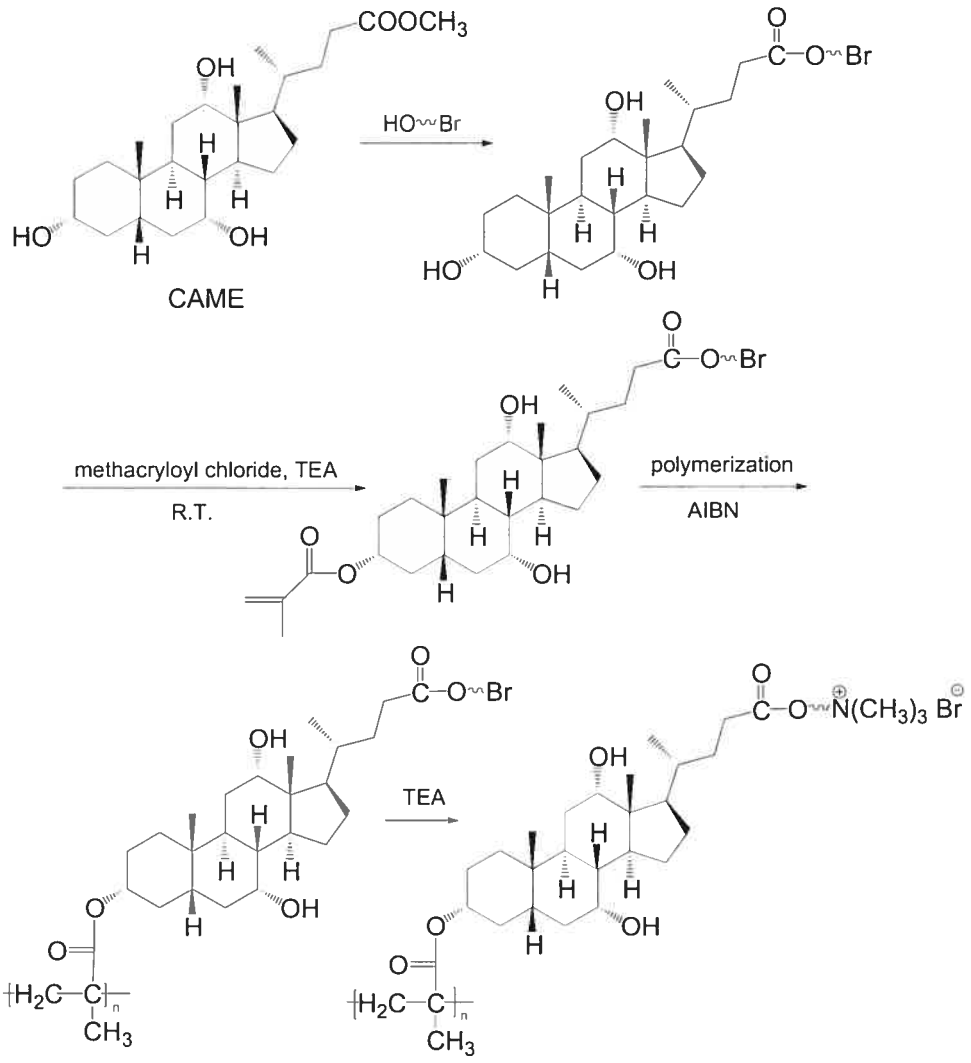

Figure 4.1 Alternative synthetic strategy of a cationic polymer containing cholic acid.

4.2 Physical properties of the polymers

Two classes of polymers were synthesized: a primary amine cationic homopolymer and thermo sensitive cationic copolymers.

The cationic homopolymer **9** bearing primary amines was not soluble in water but was soluble in organic solvents such as methanol. The pH-sensitivity of

this polymer was clearly observed by cloud point experiments. The polymer, although expected to be a hydrogel, did not show swelling characteristics in water. A more hydrophilic polymer could be made by copolymerization with a hydrophilic comonomer to obtain interesting materials for bio-related applications.

In order to improve the solubility of polymers containing monomer **7** and to also confer thermo sensitivity to these polymers, NIPAM was used as comonomer. These copolymers were found to be pH- as well as thermo sensitive and were soluble up to 10 mol% of monomer **7**. Copolymer concentration and salt concentration were also shown to affect the transition temperatures of a selected copolymer. The cloud point was found to decrease with increasing pH of the cationic copolymers. The ratio of monomer **7** in the copolymer increased the cloud point as well. Over the entire pH range examined, the change in cloud point was relatively small (about 2.5 °C, 1 °C and 6 °C for polymers containing 3, 6, and 10 mol% of monomer **7**, respectively). The copolymers exhibited the general properties expected, which can be further developed with structure modification, cross-linking, etc.

Although the solubility and the narrow range of the thermo- and pH-sensitivities of polymers may limit their applications, future work may help to overcome some of the problems encountered.