SYNTHESIS, CHARACTERIZATION, MICELLAR AND DRUG RELEASE PROPERTIES OF DIHYDROPHILIC/AMPHIPHILIC POLY(N-ISOPROPYLACRYLAMIDE)-B-POLY(VINYL ACETATE)/POLY(VINYL ALCOHOL) DIBLOCK COPOLYMERS

by

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To all whom I hold dear …

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ABSTRACT

SYNTHESIS, CHARACTERIZATION, MICELLAR AND DRUG RELEASE PROPERTIES OF DIHYDROPHILIC/AMPHIPHILIC POLY(N-ISOPROPYLACRYLAMIDE)-B-POLY(VINYL ACETATE)/POLY(VINYL ALCOHOL) DIBLOCK COPOLYMERS

In this study, poly(vinyl acetate)-b-poly(N-isopropylacrylamide) block copolymers, which were previously synthesized by macromolecular design via interchange of xanthates (MADIX) process, are partially or fully hydrolyzed to yield poly(vinyl acetate)/poly(vinyl alcohol)-b-poly(N-isopropylacrylamide) thermoresponsive amphiphilic/dihydrophilic block copolymers for the first time. In the next stage, the aqueous micellar solution and drug release properties of the polymeric products are studied.

The products are characterized by ^{1}H NMR and Fourier Transform Infrared Spectroscopy (FTIR) for proving and calculating per cent hydrolysis values for partial and full hydrolysis. About 70% partial hydrolysis for obtaining dihydrophilic thermoresponsive block copolymers and full hydrolysis for obtaining amphiphilic thermoresponsive block copolymers are successfully perfromed. The aqueous micellar solutions are analyzed by Dynamic Light Scattering Particle Size Analyzer (Nano-PSA) and Environmental Scanning Electron Microscope (ESEM) for checking stability of micelles and determining micelle sizes and temperature effect on micelles There is a good agreement between results derived from both analyses. Differential Scanning Calorimetry (DSC) analyses are performed to find T_g of block copolymers, and lower critical solution temperature (LCST) values, which are not necessarily influenced by hydrolysis. A very low CMC value, which is typically exhibited by block copolymers, is found by Fluorescence Spectroscopy. Drug loading capability and *in vitro* release properties of micelles are analyzed via UV-Visible Spectroscopy with the usage of a hydrophobic drug, Ezetimibe. The experiment exhibits an expected high loading and slow release type behavior of the block copolymer micelles.

ÖZET

DİHİDROFİLİK/AMFİFİLİK POLİ(N-İZOPROPİLAKRİLAMİD)-B-POLİ(VİNİLASETAT)/POLİ(VİNİLALKOL) DİBLOK KOPOLİMERLERİNİN SENTEZİ, KARAKTERİZASYONU, MİSEL VE İLAÇ SALINIM ÖZELLİKLERİ

Bu çalışmada, daha önce ksantat değişimi yoluyla makromoleküler tasarım (MADIX) yöntemi ile sentezlenmiş olan poli(vinilasetat)-b-poli(N-izopropilakrilamid) diblok kopolimerleri, kısmen veya tamamen hidrolizlenerek bir dizi yeni ısıya duyarlı poli(vinilasetat)/poli(vinilalkol)-b-poli(N-izopropilakrilamid) amfifilik/dihidrofilik blok kopolimerleri ilk defa elde edilmiştir. Bir sonraki adımda, ürünlerin su içinde misel solüsyon ve ilaç salınım özellikleri incelenmiştir.

Ürünler, kısmi ve tam hidrolizi kanıtlamak ve hesaplamak amacıyla ^{1}H NMR ve Fourier Transform Infrared Spektroskopisi (FTIR) ile karakterize edilmiştir. Dihidrofilik ısıya duyarlı blok kopolimer eldesi için yaklaşık %70'lik hidroliz ve amfifilik ısıya duyarlı blok kopolimer eldesi içi tam hidroliz başarıyla gerçekleştirilmiştir. Sulu misel solusyonlar, misellerin boyutsal ölçümleri, kararlılık özellikleri ve ısıya duyarlılıkları açısından Dinamik Işık Saçımı Partikül Boyut Analizörü (Nano-PSA) ve Çevresel Taramalı Elektron Mikroskopu (ESEM) analizleriyle her iki yöntemden alınan parallel sonuçlar ışığında incelenmiştir. Blok kopolimerlerin T_g ve düşük kritik solüsyon sıcaklık değerleri (LCST) Diferansiyel Taramalı Kalorimetre (DSC) analizleri ile incelenmiş ve hidrolizden pek de etkilenmedikleri tespit edilmilştir. Kritik misel konsantrasyonunun (CMC) tayini için Florasans Spektroskopi tekniği kullanılmış ve blok kopolimerlerde sıkça görülen düşük bir değer bulunmuştur. Misellerin ilaç yükleme ve *in vitro* ilaç salınım özellikleri, Ezetimibe adında bir hidrofob ilaç kullanılarak UV-Görünür Spektroskopi yöntemiyle analiz edilmiş ve blok kopolimer miselleri beklenen yönde yüksek yükleme ve yavaş salınım özelliği göstermişlerdir.

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LIST OF SYMBOLS/ABBREVIATIONS

% (w/v)	Mass-volume Percentage
Bp	Boiling Point
C	Concentration
g	Gram
h	Hour
I.I.	Integrated Intensity of NMR Peak
min	Minute
mg	Miligram
ml	Millilitre
μm	Micrometer
mmHg	Millimeter of Mercury
M_n	Number Average Molecular Weight
Mp	Melting Point
Mt / Mi	Final to Initial Weight Fraction
M_w	Weight Average Molecular Weight
M_w/M_n	Polydispersity Index
MW	Molecular Weight
RT	Room Temperature
T_g	Glass Transition Temperature
V	Volume

AFM	Atomic Force Microscopy
ATRP	Atom Transfer Radical Polymerization
-b-	Block copolymer
$CDCl_3$	Deuterated chloroform
CLRP	Controlled/Living Radical Polymerization
CMC	Critical Micelle Concentration
CTA	Chain Transfer Agent

Cu	Copper
D_2O	Deuterated water
d_6-Acetone	Deuterated acetone
d_6-DMSO	Deuterated dimethylsulfoxide
DLS	Dynamic Light Scattering
DSC	Differential Scanning Calorimetry
ESEM	Environmental Scanning Electron Microscope
FTIR	Fourier Transform Infrared Spectroscopy
LCST	Lower Critical Solution Temperature
MADIX	Macromolecular Design via Interchange of Xanthates
Nano-PSA	Nano Particle Size Analyzer (Dynamic Light Scattering)
NIPAM	N-isopropylacrylamide
NMP	Nitroxide Mediated Polymerization
NMR	Nuclear Magnetic Resonance Spectroscopy
P2VP	Poly(2-vinylpyridine)
PBS	Phosphate Buffered Saline
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PLGA	Poly(L-glutamic acid)
PNIPAM	Poly(N-isopropylacrylamide)
PVAc	Poly(vinyl acetate)
PVA	Poly(vinyl alcohol)
RAFT	Reversible Addition-Fragmentation Chain Transfer
SEC	Size Exclusion Chromatography
UV-Vis	Ultraviolet-Visible
VAc	Vinyl acetate

1. INTRODUCTION

1.1. Amphiphilic Block Copolymers

The advances in polymer chemistry in recent years show that interest in amphiphilic copolymers with blocks exhibiting different physical and chemical properties is increasing. Block copolymers, consisting of homopolymer sequences such as AB in diblock copolymers or ABA/ABC in triblock copolymers, having special properties such as amphiphilicity enables them to undergo conformation and phase transitions, and consequently hydrophilic/hydrophobic properties of the polymer changes. Amphiphilic block copolymers exhibit these different behaviors in solvents with different polarities, and by adjustments done on pH and temperature, depending on molecular interactions.

Amphiphilic block copolymers having hydrophilic and hydrophobic segments in their structures are used in many applications such as surface modification, stabilization of emulsions and polymer blends and phase transfer catalysis [1].

1.1.1. Micellar Block Copolymer Structures

Amphiphilic block copolymers can form micelles in selective solvents thermodynamically favorable for the shell and unfavorable for the core of the micellar structure. This type of systems has been used in controlled drug release studies for more than two decades [2]. The interactions between blocks, structure and solvent effects which play an important role in the formation of micelles are in constant investigation [3]. Depending on the shape and size of the particles, structural characterization of block copolymer micelles has been extended to a vast variety of morphologies and it is possible to name a few such as spheres, star, crew-cut, rods, cylinders, worm-like, hollow circles, vesicles and lamellae type micelles [4]. Crew-cut aggregates are micellar structures that have larger cores than their shells and are not water soluble [5-7].

The threshold for individual polymeric chains to come together and just start forming micelles, called critical micelle concentration (CMC), is very important in the aspect of drug loading and release capabilities of these structures. As the concentration of block copolymer increases in aqueous solution and comes closer to CMC of the specific block copolymer, associations start. When the concentration is above CMC, stable micellar structures form [8]. Block copolymer micelles prepared by experimental methods, such as dialysis against water, have very low CMC's and easily associate/dissociate to bond to hydrophobic drugs into the core of the micelles through non-covalent hydrophobic interactions at very low concentrations [9,10]. Thus, hydrophobic drugs are physically soluble in water with the help of polymeric micelles and they pose an advantage over surfactants with their high thermodynamic stabilities. Dialysis technique is conducted generally for obtaining block copolymers micelles, incorporating the usage of organic solvents that are water miscible [5].

For determining the size of the micelles in aqueous solution, dynamic light scattering (DLS) instruments are generally used. There have been many reported cases in which it can be concluded that an increase in the molecular weight and hydrophobic components of diblock copolymers produce larger micelles [11].

The dissociative properties of polymeric micelles (Figure 1.1) also enable them to be excreted or broken down from the body as they reform single polymer chains after the completion of release of the drug [12]. As a real life usage, polymeric micelles that are loaded with a hydrophobic drug remain stable and do not dissociate under rapid high dilution after injection due to their low CMC's [13].

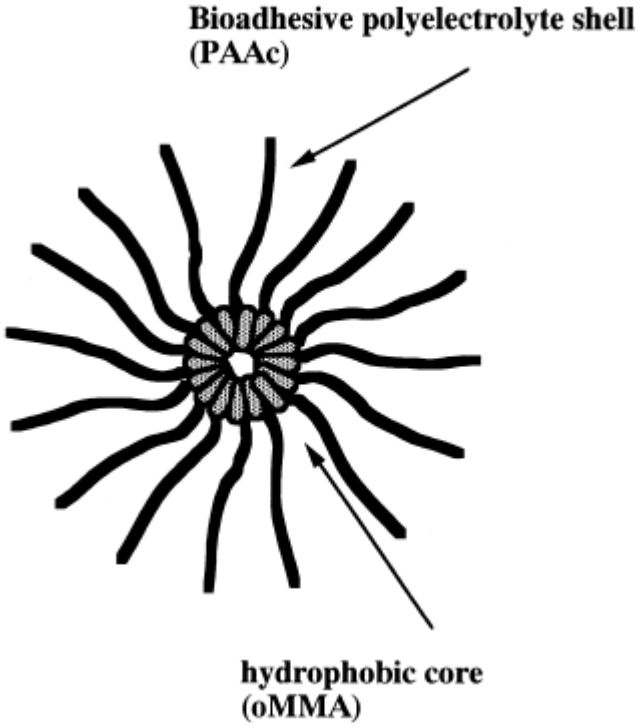

Figure 1.1. An example of the structure of an amphiphilic block copolymer micelle in aqueous media (oligo(methyl methacrylate) and poly(acrylic acid)) [12]

In a study by Shin et al., methoxy poly(ethylene glycol)/e-caprolactone amphiphilic block copolymer micelle system is successfully used for the delivery of a hydrophobic drug, indomethacin, that was loaded to the hydrophobic core of the micellar structure. *In vitro* drug release studies are done using UV-Visible spectroscopy in phosphate buffered saline (PBS) medium and CMC is determined by using fluorescence spectroscopy with pyrene as a fluorescence probe. This study reports that nature and the length of the hydrophobic block determines the onset of micellization and due to good structural stability of amphiphilic micelles, drug delivery is feasible and release of the drug is efficient [11].

1.2. Dihydrophilic Block Copolymers

Block copolymers with two hydrophilic blocks, also denoted as double hydrophilic block copolymers, can exhibit special characteristics when one or both of the blocks have amphiphilic properties. The uses of these block copolymers vary greatly in biological and medicinal science [14].

The so-called stimuli responsive polymers, such as poly(N-isopropylacrylamide), poly(L-glutamic acid), poly(2-vinylpyridine), poly(ethylene oxide), under certain temperature, pH or ionic strength adjustments in the aqueous media, can switch between hydrophilic and hydrophobic (insoluble) states [15, 16]. The blocks can be ionic or non-ionic type and due to the given external stimuli, electrostatic or hydrophobic interactions cause the formation of micellar structures at certain settings [4].

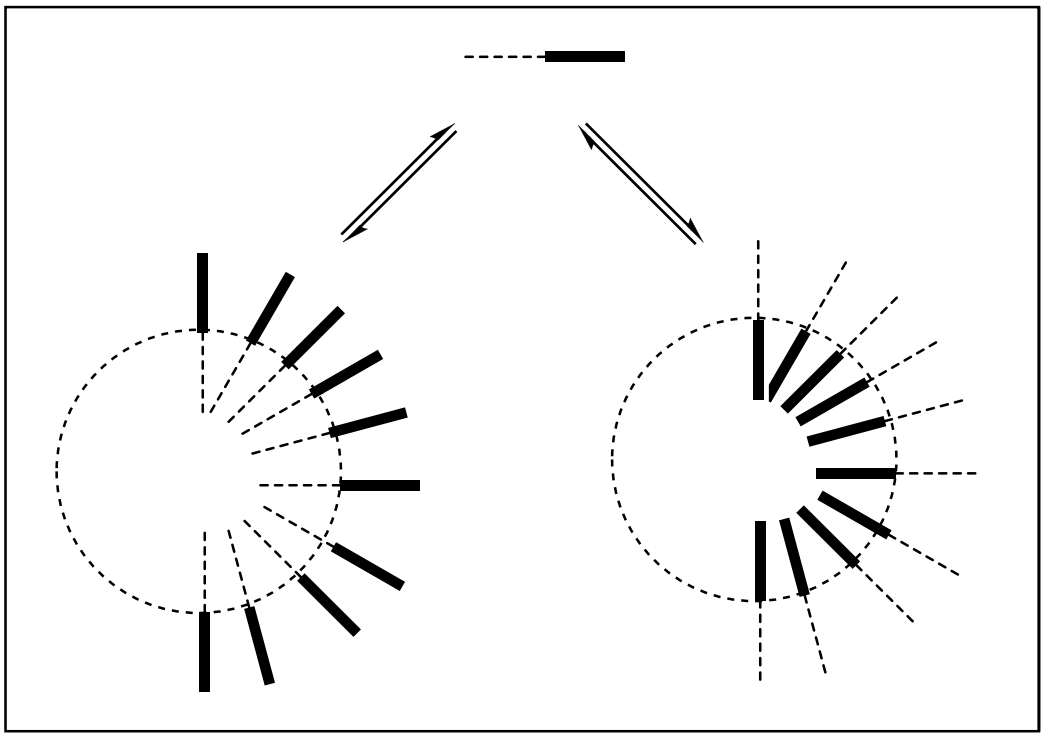

Figure 1.2. Inversion of micellar structures in amphiphilic dihydrophilic block copolymers upon external stimuli

When both of the blocks are amphiphilic, with certain stimuli adjustments, the block copolymers can form micelles, deform back to dihydrophilic state and then inversion of the micelle by the route of inversion of the core and shell of the micelle can be achieved (Figure 1.2). In the study by Zeng et al., it is reported that poly(N-isopropylacrylamide)-b-poly(2-vinylpyridine) dihydrophilic stimuli-responsive block copolymers are synthesized and investigated for their micellar properties in aqueous medium. Poly(2-vinylpyridine) is water-soluble below pH 5 and poly(N-isopropylacrylamide) is a thermoresponsive polymer with a lower critical solution temperature of 32 °C. With adjustment of pH and temperature, the dihydrophilic/amphiphilic block copolymer exhibits 'schizophrenic' behavior by the inversion of the micellar structure (Figure 1.3) [15].

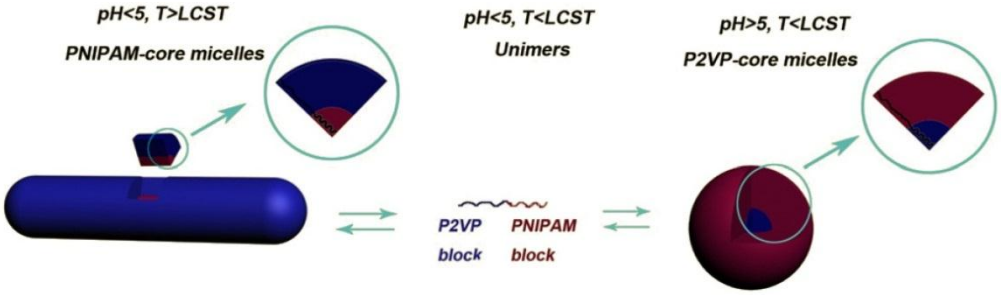

Figure 1.3. Representation of the 'schizophrenic' micellar behavior of the PNIPAM-b-P2VP block copolymer in aqueous medium [15]

1.3. Controlled/Living Radical Polymerization (CLRP)

Controlled/Living Radical Polymerization (CLRP) is a technique that brings many advantages over traditional free radical polymerization leading to its increase in popularity in the recent years. With conventional free radical polymerization, preparing high molecular weight commercial polymers and copolymers, with the usage of many vinyl monomers via mild reaction conditions and in a wide temperature range was made possible. Possible draw-backs can be listed as poor control on end-group functionalities, chain architectures, polydispersities and molecular weight. As an alternative, ionic living

polymerizations can be used to obtained well-defined polymers. On the other hand, suitable monomers are limited and reaction conditions are extensively strict.

As a solution to all these problems, CLRP offer polymerization techniques resulting in well-defined polymer architectures under mild reaction conditions for synthesizing homo and copolymers. As an advantage over free radical and ionic living polymerizations, linear propagation of number average molecular weight with extent of conversion, narrow polydispersities and ability of 'living' chain growth after consumption of initial monomer feed can be exemplified [17, 18].

CLRP enables synthesis of copolymers by sequential addition of comonomers due to 'living' character of the polymers. This property opens possibilities for various structural designs of polymers, broadly shown in Figure 1.4 [18].

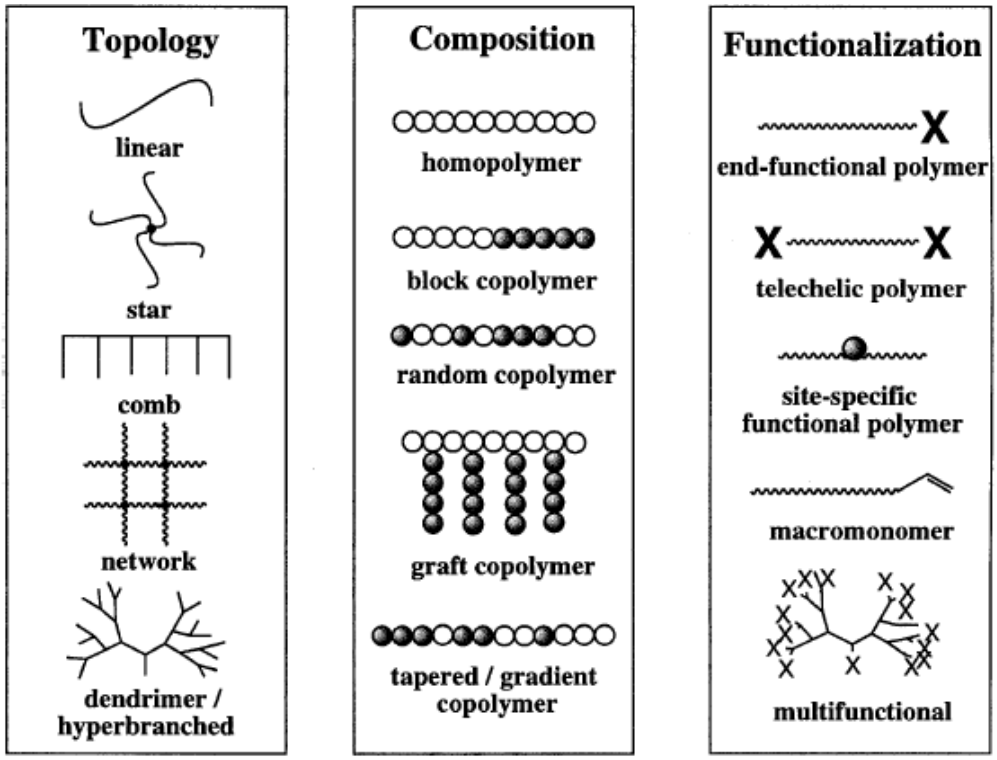

Figure 1.4. Architecture and functionality in polymers generated by CLRP [19]

Controlled/Living Radical Polymerizations techniques can be classified as follows:

- Nitroxide mediated polymerization
- Atom transfer radical polymerization (ATRP)
- Degenerative transfer
  - (i) Reversible addition-fragmentation chain transfer (RAFT) process
  - (ii) Macromolecular design via interchange of xanthates (MADIX)
  - (iii) Initiator-transfer agent-terminator (Iniferter) process
  - (iv) (Reversible) Iodine transfer polymerization [(R)ITP]

The most common technique of CLRPs is the reversible addition-fragmentation chain transfer (RAFT) polymerization which is used for synthesizing living polymers via reversible transfer [20].

1.3.1. RAFT Polymerization

In the present years, synthesis of polymers via reversible addition-fragmentation chain transfer (RAFT) polymerization has become an important tool because of its wide range of usability with many monomers, good control on polydispersities and molecular weights on polymers synthesized [21]. Apart from its advantages, in RAFT polymerization, same polymerization temperatures, initiators, monomers and solvents can be used as in traditional free radical polymerization [22, 23].

The RAFT polymerization is carried out by the usage of thiocarbonylthio compounds with a general structure Z–C(═S)–S–R that act as reversible addition-fragmentation chain transfer agents in a conventional monomer and initiator containing free radical polymerization system. The polymer products obtained are in the form of end-functionalized living polymers with predetermined molecular weights and low polydispersities, mostly in the range of $M_w/M_n < 1.2$ [21, 24].

The key point in the mechanism is the transfer of CTA between growing radical chains, which are at very low concentrations, and dormant polymeric chains, which are present at higher concentrations, controls the growth overall polymer chains and thus

molecular weight can be predetermined with the help of termination reactions kept to a minimum. This mechanism is in many ways different than that of atom transfer radical polymerization (ATRP) or nitroxide mediated polymerization (NMP), which are basically monomolecular reversible reactions of radical capping. In the RAFT polymerization, chain growth depends on the cooperative bimolecular reactions of chain transfer between polymeric chains.

The proposed overall mechanism of RAFT polymerization is given in Figure 1.5 [20].

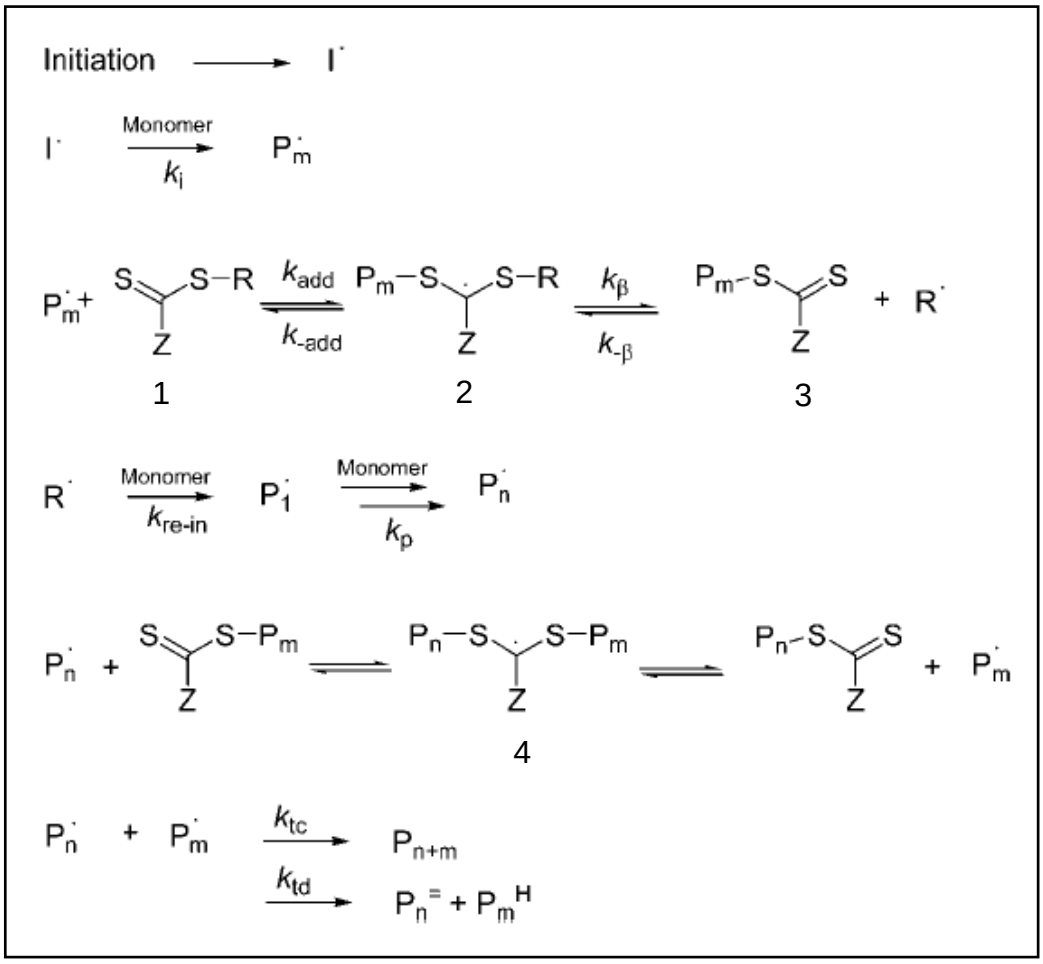

Figure 1.5. Proposed general mechanism of RAFT polymerization

The xanthate mediated reversible addition fragmentation polymerization is named as MADIX (macromolecular design via interchange of xanthates) [25- 28]. In comparison of

the reaction mechanisms, the RAFT and MADIX processes are identical with the only difference being xanthates used in MADIX and thiocarbonylthio compounds used in RAFT technique as CTA's (Figure 1.6).

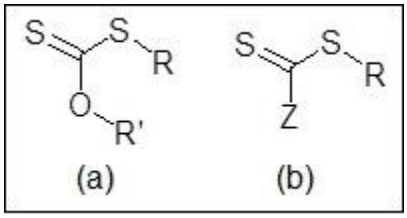

Figure 1.6. (a) Xanthate-based and (b) general thiocarbonylthio-based CTA's

1.4. N-Isopropylacrylamide Polymers

N-Isopropylacrylamide (NIPAM) is an important water soluble nonionic acrylamido monomer (Figure 1.7). Because of the special properties of its polymer, extensive research over the years have been spent on NIPAM [29-32].

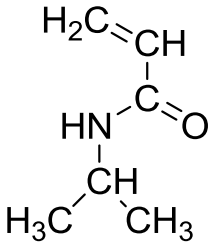

Figure 1.7. N-Isopropylacrylamide (NIPAM)

Poly(N-isopropylacrylamide) (PNIPAM) (Figure 1.8) is a thermoresponsive amphiphilic polymer that undergoes coil-to-globule transition in aqueous solution at about 32 °C, defined as the lower critical solution temperature (LCST) [33]. The reason for this behavior is attributed to a balance of hydrogen bonding and hydrophobic interactions in between polymer-polymer (intramolecular) and solvent-polymer molecules [34].

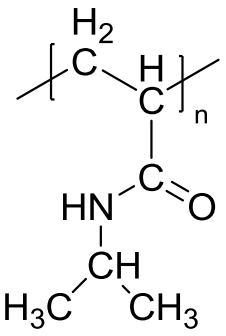

Figure 1.8. Poly(N-Isopropylacrylamide) (PNIPAM)

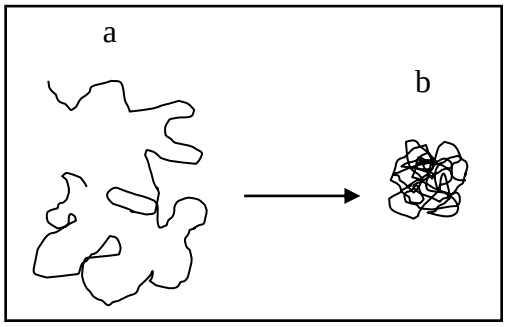

Figure 1.9. Conformation transition of PNIPAM from (a) a polymer coil to (b) a polymer globule

Absorption of water molecules via hydrogen bonding to the amide functional group below LCST gives PNIPAM its water solubility. On the other hand, as temperature is increased above LCST, hydrogen bonds are broken and intramolecular interactions dominate causing hydrophobic collapse of NIPAM units through coil to globule transition (Figure 1. 9) [35].

The thermoresponsive amphiphilic behavior of PNIPAM is used in areas such as drug delivery, surface modification, reaction catalysis, biomedical applications, etc. [29-32, 34].

In a 2007 study by Rao et al., thermo- and pH-responsive micellization behavior of PNIPAM-b-PLGA block copolymers are experimented and inversion of micellar structures can be successfully detected (Figure 1.10) [36].

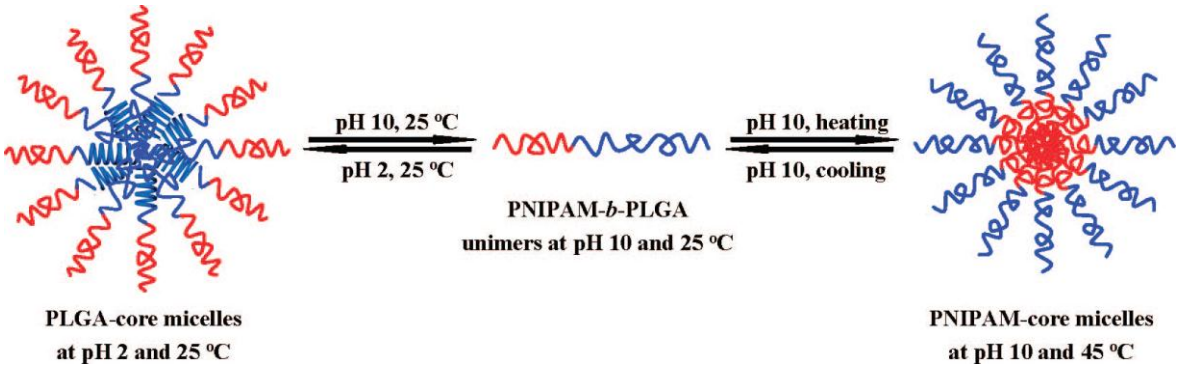

Figure 1.10. Schematic illustration of the thermo- and pH-responsive micellization of PNIPAM-b-PLGA [36]

For the preparation of NIPAM copolymers by conventional free radical polymerization leads to polymers with undefined end-group chemistries and high polydispersities that may cause instabilities in the LCST of PNIPAM due to effect of chain length [32]. To be able to synthesize well-defined and end-functionalized PNIPAM block copolymers, an important technique called the reversible addition-fragmentation chain transfer (RAFT) polymerization (1.3, 1.3.1) process can be used. RAFT is a powerful tool for synthesizing polymers with narrow molecular weight distributions and various NIPAM copolymers have been synthesized successfully with this technique. Due to the fact that LCST of PNIPAM is close to body temperature (37 °C), varying the comonomer type and content, LCST can be tuned to be used more practically in biomedical applications such as drug delivery [32, 37, 38].

As an example for the LCST tuning of PNIPAM, a previous study by Yan et al., investigated how LCST of PNIPAM within a PEO-b-PNIPAM block copolymer micelle varies greatly to a range of 28-42 °C, when compared to the LCST of 32 °C with a PNIPAM homopolymer (Figure 1.11) [39].

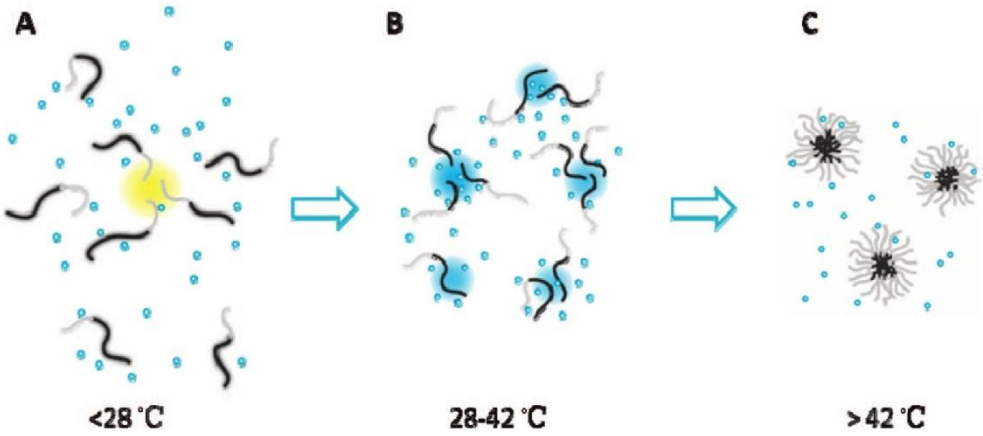

Figure 1.11. Dihydrophilic block copolymers forming micelles as temperature increases: (A) associates at temperatures below 28 °C, (B) loose aggregates formed between 28 °C and 42 °C, and (C) micelles at above 42 °C [39]

1.5. Vinyl Acetate Polymers

Interest in hydrophobic polymers such as poly(vinyl acetate) (Figure 1.12) has gained these materials many application areas. Poly(vinyl acetate) is used in the manufacture of paints, adhesives, pharmaceuticals and packaging [40, 41].

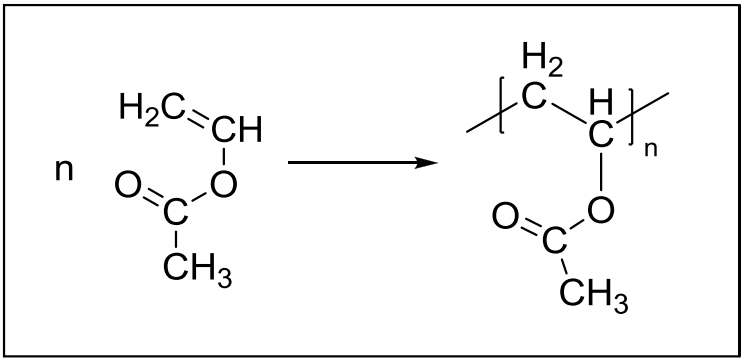

Figure 1.12. Vinyl acetate (VA) polymerization to form poly(vinyl acetate) (PVAc)

Vinyl acetate is one of the most typical and important monomers that is polymerized only via a radical mechanism. The propagating radical of VAc is highly reactive and

unstable due to the absence of a conjugating substituent. Therefore, the VAc radical especially tends to undergo chain transfer and termination reactions resulting in polymers with high polydispersities and broad molecular weight distributions. Moreover, poor control on end-functionalities prevents the synthesis of PVAc block copolymers [42]. To overcome these undesired problems and to be able to synthesize well-defined homopolymers and furthermore, block copolymers of PVAc, CLRP techniques were experimented over the years. It has been proved that using NMP, ATRP or RAFT using dithioester RAFT CTA's, control over the polymerization is difficult [43]. In fact, dithioesters CTA's inhibit the polymerization due to the poor homolytic leaving group property of the VAc propagating chain during the fragmentation of the RAFT-adduct radical chain (3) shown in Figure 1.5 [43]. Fragmentation is very slow in this case and feasibility of the polymerization reaction is low. On the other hand, using dithiocarbamates or xanthates as CTA's, polymerization is successful due to the increase in electron density on the macro RAFT radical (shown as (3) in Figure (1.5)) causing destabilization and eventually increasing fragmentation rates [40, 43, 44]. As a result, well-defined PVAc homopolymers and block copolymers can be successfully synthesized.

In the recent years, Charmot et al. performed the controlled living polymerization of poly(vinyl acetate) using the MADIX technique, this being the first example of CLRP process of vinyl acetate reported [45].

1.6. Preparation of Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) is a hydrophilic polymer that is used in many important areas such as in the controlled delivery of drugs and hydrogels [46, 47]. With conventional polymerization techniques, synthesizing poly(vinyl alcohol) by usage of vinyl alcohol as the monomer is rendered impossible because of the keto-favored form of the keto-enol tautomerism of vinyl alcohol and acetaldehyde.

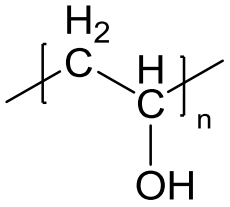

Figure 1.13. Poly(vinyl alcohol)

In literature, it is given that for preparing PVA (Figure 1.13), hydrolysis reactions are used on its precursor, PVAc, previously polymerized [46, 48-51]. One of the oldest hydrolysis techniques is the usage of transesterification by base catalysis in which acetate pendant groups of PVAc are replaced basically with hydroxyl groups.

By keeping the PVAc polymer under hydrolysis reaction for excess times such as 24 hours, full hydrolysis can be obtained at room temperature with potassium hydroxide in methanol solutions [52].

In addition to the full hydrolysis reaction, partial hydrolysis for obtaining PVAc/PVA random copolymers can be achieved. Adjustment of the reaction times is one of the easiest and straight-forward ways of accomplishing partial hydrolysis [53]. If base concentration or the polydispersity of the polymer is increased, hydrolysis rate can be increased [54]. On the other hand, for the sake of usefulness of the technique, adjusting just the reaction times is much simpler and generally kept as the only variable in partial hydrolysis reactions.

In an early study by Ahmed et al., partial and full hydrolysis of PVAc homopolymers were done by the methoxide-catatlyzed transesterification reaction with the use of sodium methoxide as base in methanol solution. Partial hydrolysis is achieved by stopping the reaction at earlier stages by neutralizing the medium with acetic acid before the completion of full hydrolysis (Table 1.1) [49].

Table 1.1. Time elapsed for partial and full hydrolysis of PVAc [49]

Reaction time (minutes)	Per cent mole conversion of PVAc to PVA
90	34.9
120	51.8
165	62.7
180	69.7
> 240	99.6

The mechanism of the hydrolysis reaction follows a simple transesterification mechanism by the replacement of an acetate group with the base catalyst and a final hydrogenation from the solvent molecule (Figure 1.14). The overall reaction is autocatalytic and unzipping effect due to hydroxyl groups formed influencing adjacent acetate groups into hydrolysis. In other words, the reaction rate constant increases as reaction proceeds and the reactivity of acetate groups adjacent to hydroxyl groups are enhanced towards hydrolysis (alcoholysis). Therefore, per cent hydrolysis is exponentially increasing as reaction time increases [48, 49, 55].

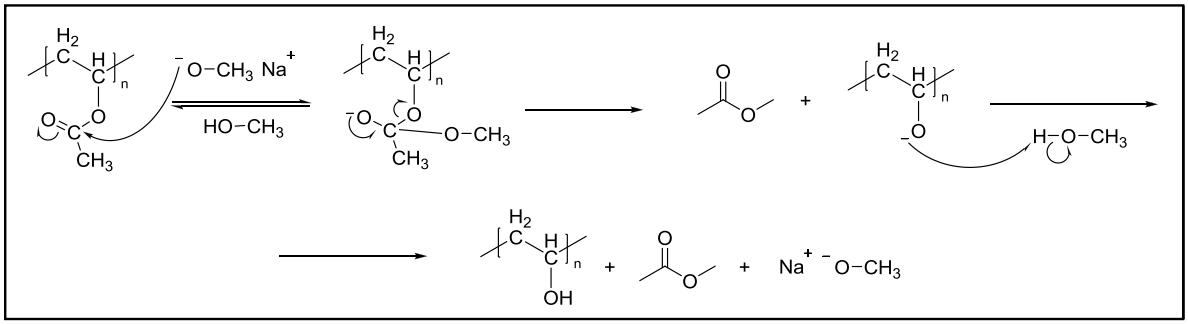

Figure 1.14. Schematic representation of the mechanism of methoxide-catalyzed transesterification reaction

1.7. Poly(N-Isopropylacrylamide)-b-Poly(Vinyl acetate) Block Copolymers

It is known that diblock copolymers consisting of a PNIPAM block and a hydrophobic block can form core–shell micellar structures below the LCST. This polymeric micellar structure forms a hydrophilic outer shell of hydrated PNIPAM blocks and a hydrophobic inner core. Moreover, the structure and abovementioned micellar structure dependent properties can be tuned by changing the temperature and the composition of the block copolymers [56].

Attaching a hydrophilic component to PNIPAM also serves to modulate the LCST of the polymer. Accordingly, approach on raising the LCST of PNIPAM above 32 °C as a block copolymer is under experimentation in many studies. For example, attaching PEG with 24 repeating units increases the LCST of the copolymer by 26 °C [57]. More hydroxyl and polar groups causes LCST of PNIPAM to increase and hydrophilicity increases (due to stronger hydrogen bonding with water) and phase transition slows down. This can benefit the LCST being closer to body temperature and the inversing of micelles causes the loaded drug to be released in a slower controlled release manner. In addition to hydrophobic drug loading, there is a possibility of loading a hydrophilic drug as well with PNIPAM block copolymers [58].

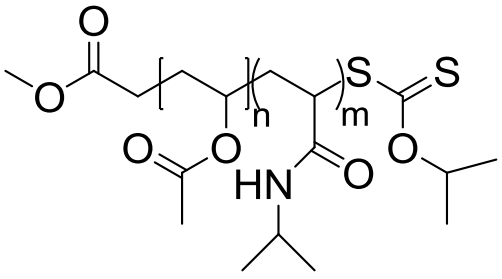

Figure 1.15. Methyl(isopropoxycarbonothioyl)sulfanyl acetate end-functionalized poly(N-isopropylacrylamide)-b-poly(vinyl acetate) (PNIPAM-b-PVAc)

In a study reported by Ozguc et al., well-defined poly(N-isopropylacrylamide)-b-poly(vinyl acetate) diblock copolymers (Figure 1.15) are successfully synthesized via the macromolecular design via interchange of xanthates (MADIX) process [59]. In the first

step, methyl (isopropoxycarbonothioyl)sulfanyl acetate (MIPCTSA) was used as chain transfer agent to synthesize the PVAc macro-chain transfer agent. Next, the second monomer, NIPAM was introduced and block copolymers with different PNIPAM block lengths were synthesized based on the PVAc macro-chain transfer agent. Characterizations of the well-defined block copolymers were done using ^{1}H NMR spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Size Exclusion Chromatography (SEC), Scanning Electron Microscopy (ESEM) and Atomic Force Microscopy (AFM).

The products can be used in the thermoresponsive aqueous micelle studies owing to the dehydration and hydrophobic collapse of the PNIPAM chain to globule form at temperatures above its LCST.

For preparing poly(N-Isopropylacrylamide)-b-poly(vinyl acetate/vinyl alcohol) random block copolymers, aforementioned hydrolysis procedure can be followed. It is shown in literature that either methoxide-catalyzed, base-catalyzed or even acidic hydrolysis does not affect PNIPAM and only poly(vinyl acetate) block can be hydrolyzed safely [60, 61].

The solution properties of poly(N-Isopropylacrylamide)-b-poly(vinyl alcohol/vinyl acetate) random block copolymers differ greatly. Poly(vinyl alcohol) is soluble in aqueous medium above 70-75 °C and 70-90% partially hydrolyzed PVAc is water soluble at any temperature [46, 48, 49, 62, 63]. Bearing in mind the LCST of PNIPAM and the solubility variation of the partial or full hydrolyzed PVAc/PVA block, with the poly(N-Isopropylacrylamide)-b-poly(vinyl alcohol/vinyl acetate) random block copolymers, amphiphilic, dihydrophilic and dihydrophobic behaviors with the addition of possible inversion of micelles in aqueous medium can be all achieved.

2. OBJECTIVES

In this study, the aim is to synthesize thermoresponsive amphiphilic/dihydrophilic poly(N-isopropylacrylamide)-b-poly(vinyl acetate)/poly(vinyl alcohol) diblock copolymers by partial or full hydrolysis from pre-synthesized and characterized well-defined diblock copolymers of poly(N-isopropylacrylamide) and poly(vinyl acetate) via MADIX technique. The next step is to prepare micellar solutions of the samples and performing spectroscopic characterizations, studying solution properties and finally conducting drug delivery experiments on these solutions.

3. EXPERIMENTAL

3.1. The Reagents, Solvents and Samples

The reagents and solvents used in the experiments are listed in Table 3.1.

Glacial acetic acid, methanol, acetone, 1,4-dioxane, pyrene and sodium metal are used as received without purification.

Table 3.1. Properties of reagents and solvents used in all experiments

Name	Formula	MW (g/mol)	Supplier	Bp (°C)	Density (g/ml)
Glacial Acetic Acid	$C_2H_4O_2$	60.05	Merck	118	1.049
Methanol	CH_4O	32.04	Merck	65	0.791
Acetone	C_3H_6O	58.08	Merck	56	0.792
1,4-Dioxane	$C_4H_8O_2$	88.11	Merck	101	1.034
n-butylamine	$C_4H_{11}N$	73.14	Merck	77	0.737
PVAc	$(C_4H_6O_2)_n$	100,000	Aldrich	–	1.191
PVA (>98%)	$(C_2H_4O)_n$	72,000	Merck	230 (Mp)	0.4-0.6
Pyrene	$C_{16}H_{10}$	202.25	Merck	145-148 (Mp)	1.271

The block copolymer samples used in the experiments are listed in Table 3.2.

Poly(N-isopropylacrylamide)-b-poly(vinyl acetate) diblock copolymers and poly(N-isopropylacrylamide) homopolymer (PNIPAM-E1) are previously synthesized and purified. Poly(vinyl acetate) and poly(vinyl alcohol) homopolymers are used as received from the supplier.

Table 3.2. Compositions, M_n and M_w/M_n (determined by SEC) of block copolymer samples used

Sample Name	Block length with respect to PVAc:PNIPAM	M_n	M_w/M_n
C-AN1	1:1	19,253	1.77
C-AN4	1:1	19,531	1.88
C-AN2	1:0.5	28,109	1.82
C-AN8	1:0.5	27,023	1.84
C-AN3	1:2	21,382	1.88
C-AN9	1:2	21,595	1.81

3.2. Methoxide-Catalyzed Transesterification (Hydrolysis) of PVAc

About 1.2000g PVAc beads (MW:100,000) is dissolved in 25 ml dry methanol at 65 °C with mixing for 1 hour in a round bottom flask under a Teflon-tape-sealed reflux condenser with a $CaCl_2$ (anhydrous) trap attached onto its open end to avoid moisture getting into the reaction mixture. Solution is taken, cooled down to 22 °C while stirring. Separately, Na metal in dry methanol (5% (w/v); 2.174M) solution is prepared freshly (1.2614g Na / 25 ml methanol) in a graduated balloon flask by adding methanol drop wise onto thin-cut strips of Na metal and 0.5 ml of this prepared solution is added into the dissolved PVAc solution drop wise afterwards. At desired times of reaction for partial hydrolysis of PVAc, 2 ml of samples are taken by Pasteur pipettes and transferred into vial bottles. Each reaction sample is quenched by 1-2 drop(s) of glacial acetic acid and pH is checked for neutrality. Temperatures at which each sampling is done are additionally measured, but the temperature effect on rate of hydrolysis is not considered and experimented. Due to the higher ambient temperatures, as time elapsed increases, solution temperature eventually increases.

To be able to monitor early stages of hydrolysis, lower reaction times are followed as well. Therefore, hydrolysis reaction times that are experimented are 10, 20, 30, 60, 90, 120, 165, 180, 360 minutes and lastly for assuring full hydrolysis: 1320 minutes.

Procedure is followed to remove unreacted methoxide and present NaOH so that hydrogen bonding of PVA is not influenced and it can remain soluble in water as solvent. Sample solutions collected at specific times of the hydrolysis reaction are poured into test tubes and depending on their degree of hydrolysis, which influences the water or methanol-insolubility of the products, the solutions are added drop by drop with vigorous mixing onto appropriate non-solvent achieve precipitation of the solid products. Precipitates are washed with non-solvent twice, decanted, dissolved in the according solvent and followed by reprecipitation by non-solvent and aforementioned washing and decantation steps. Non-solvent volume is about 20ml, approximately 10 times the amount of solvent in each part of the purification. Finally, solid precipitates are dried over phosphorous pentoxide inside a desiccator at 22 °C under vacuum conditions.

Water-insoluble products, i.e. HD1-10, HD1-20, HD1-30 and HD1-60, which are up to 70% hydrolyzed, are precipitated by adding onto distilled water, washed twice, decanted, reprecipitated from methanol, and rewashed lastly with distilled water. Methanol-insoluble products, i.e. HD1-90, HD1-120, HD1-165, HD1-180, HD1-360 and HD1-1320, which are more than 70% hydrolyzed, are precipitated from aqueous solution by adding onto acetone as the non-solvent, washed with acetone twice, decanted, reprecipitated from distilled water and rewashed lastly with acetone.

After taking the precipitates into vial bottles, freeze-drying method is applied to dry solid products completely. The drying process under vacuum is conducted for 24 hours. For the next step, solid precipitates are taken into a polycarbonate desiccator and stored over phosphorous pentoxide used as the desiccant, for 2 days at room temperature under a mild vacuum of 15mmHg owing to a water-tromp vacuum system. The microstructures of the polymers are determined by ^{1}H NMR spectroscopy.

^{1}H NMR (d_6-DMSO): δ= 1.2-1.5 (m, 2H, C*H*$_2$CHOH), 1.6-1.8 (d, 2H, C*H*$_2$CHO), 1.8-2.0 (s, 3H, CO_2C*H*$_3$), 3.7-4.7 (m, 1H, CH_2C*H*OH), 4.7-5.0 (s, 1H, CH_2C*H*O).

3.3. Methoxide-Catalyzed Hydrolysis Trial of PNIPAM

0.24g PNIPAM (PNIPAM-E1) (previously synthesized via MADIX, purified and dried) is weighed and dissolved in 5ml dry methanol at 65 °C with mixing for 1 hour in a 25 ml-round bottom flask under a Teflon-tape-sealed reflux condenser with a $CaCl_2$ (anhydrous) trap attached onto its open end to avoid moisture getting into the reaction mixture. As the white solid is dissolved into almost colorless solution, the round bottom flask is taken, cooled down to 25 °C (RT) while stirring. Separately, 0.5ml of previously prepared sodium methoxide solution (5%(w/v); 2.174M) is diluted with the addition of 2ml of methanol and 0.5ml of this diluted solution is added into the dissolved PNIPAM solution drop wise. The suspected reaction is quenched after 22 hours, by adding 2 drops of glacial acetic acid and pH is checked for neutrality. Quenched reaction mixture is taken into a glass vial bottle and methanol is let to evaporate at RT at open atmosphere overnight. For precipitating out the polymeric sample, hot (45 °C - 50 °C) distilled water that is above the LCST of PNIPAM is used as the nonsolvent that is at least 10 times the volume of the reaction mixture. After cloudy-white precipitates of PNIPAM are formed, hot filtration over a sintered glass with fine pore size is done.

Lastly, the precipitate is washed with hot distilled water. Solid precipitate of the suspected hydrolysis reaction of PNIPAM on the sintered glass is dried overnight in a desiccator over phosphorous pentoxide under a mild vacuum of 15mmHg owing to a water-tromp vacuum system. Final product after drying in the desiccator appears as a white and fine powder. The microstructure of the polymer is determined by ^{1}H NMR spectroscopy.

^{1}H NMR ($CDCl_3$): δ= 1.1 (s, 6H, $CH(CH_3)_2$), 1.5-1.9 (d, 2H, CH_2CH), 2.0-2.4 (s, H, CH_2CH), 4.0 ppm (s, 1H, $NHCH(CH_3)_2$).

3.4. Methoxide-Catalyzed Partial and Full Hydrolysis of Poly(N-Isopropylacrylamide)-b-Poly(Vinyl Acetate) with the Addition of Amine (HD-NH2-C-AN4-30 & HFULL-NH2-C-AN4)

0.4981g of C-AN4 sample is weighed into a 25 ml-round bottom flask and 10 ml methanol is added. Complete dissolving of the sample is observed within a couple of minutes by mixing the solution at RT. 1.48 ml n-butylamine is added drop wise into the solution with a syringe and all contents are let to mix for 0.5h. 2.5 ml of the prepared sodium methoxide solution (5%(w/v); 2.174M, in methanol) is added drop wise into the reaction mixture over 15 minutes. Reaction is conducted for 30 minutes for partial (HD-NH2-C-AN4-30) and 24 hours for full hydrolysis (HFULL-NH2-C-AN4) followed by glacial acetic acid quenching. The reaction mixture is added onto excess hot distilled water (150 ml) and hot filtration over a sintered glass with fine pore size is done, but due to the inefficiency and slowness of the technique, freeze-drying (at a vacuum of 10^{-3}mmHg) is done without filtration or washing the product. The solution is first dialyzed against distilled water (1500 ml) for purification of the product by removing methanol, methoxide, acetate anions and other salts from the reaction media. Then, the dialyzed solution is freeze-dried overnight and product in the form of white solid flakes is obtained. During the dialysis, salt precipitates on the outer surface of dialysis tubings are observed, indicating successful purification.

The pH of the product is checked by dissolving the white solid product in a small amount of distilled water.

^{1}H NMR (D_2O): δ= 1.0 (s, 6H, $CH(CH_3)_2$), 1.3-1.7 (d, 2H, CH_2CH; d, 2H, CH_2CHOH), 1.8-2.1 (s, H, CH_2CH), 3.8 ppm (s, 1H, $NHCH(CH_3)_2$), 3.7-4.0 (m, 1H, CH_2CHOH), 4.5-4.8 ppm (s, 1H, CH_2CHOH; s, D_2O).

^{1}H NMR (d_6-DMSO): δ= 1.0 (s, 6H, $CH(CH_3)_2$), 1.3-1.7 (d, 2H, CH_2CH; d, 2H, CH_2CHOH), 1.8-2.1 (s, H, CH_2CH), 3.8 ppm (s, 1H, $NHCH(CH_3)_2$), 3.7-4.0 (m, 1H, CH_2CHOH), 4.2-4.8 ppm (m, 1H, CH_2CHOH).

FTIR: 3294.61 (N–H, O–H (PVA)), 2970.46 (−CH_3, PNIPAM), 1640.64 (C=O, PNIPAM), 1543.01 (N–H), 1039.58 cm^{-1} (C−O).

3.5. Preparation of Micelle Solutions of Poly(N-Isopropylacrylamide)-b-Poly(Vinyl Acetate) Block Copolymers with or without the Dialysis Procedure

Without the dialysis technique, appropriate amount of solid PNIPAM-b-PVAc block copolymer samples are taken and directly dissolved in distilled water according to the concentration wanted.

For preparing micelle solutions with the dialysis technique, samples are first separately dissolved in 1,4-dioxane with vortex mixing in a concentration of 0.2% (w/v) and put into dialysis tubings after filtration through Millipore filters with 0.45μm pore size (ProFill HPLC Syringe Filter, hydrophilic, Regenerated Cellulose). The type of dialysis tubing is Fisherbrand regenerated cellulose nominal MW 3,500Da (pore size) with flat width of 19mm, volume/cm ratio of 1.15 ml, wall thickness of 25μm and dry circumference of 12.1mm. Before transferring the polymer solutions in 1,4-dioxane into the tubings, the tubes are swollen in distilled water for 20 minutes to be able to transfer the contents into them.

Dialysis is conducted in an excess amount distilled water bath, about 1200 ml - 2000 ml, and stirred mildly. Time elapsed for dialyzing is 24h to 72h and dialysis time is not tested as a variable. After this procedure, the completed dialysis solutions are cloudy and are transferred into glass vial bottles, sealed and stored at RT for analysis.

Block copolymers used are C-AN1 or C-AN4 (1:1 composition), C-AN3 (PVAc:PNIPAM composition of 1:2 respectively) and C-AN2 (PVAc:PNIPAM composition of 1:0.5 respectively).

3.6. Characterization of Samples

The microstructures of the products are analyzed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy using a Varian Gemini 400 MHz spectrometer.

Fourier Transform Infrared (FTIR) analysis is performed by Thermo Nicolet, FTIR 380 Spectrometer, using diamond ATR accessory.

Thermal behavior and lower critical solution temperatures (LCST) of the samples are investigated by Differential Scanning Calorimetry (DSC) using TA Instruments Q200, under nitrogen atmosphere with usage of a liquid nitrogen cooling system. For determining glass transition temperatures (T_g) of polymer samples, about 4-5mg in aluminium pans are heated from 10 °C to 180 ^{o}C at a rate of 3 °C/min. The Tg are determined from the first heating scan. For the LCST measurement procedure, about 4mg of dry sample with 1 drop (50μL) of distilled water is added into an aluminum pan and aluminium lid is not pinched. 5 °C/min temperature increment is followed and only one run is done.

The morphology of the micellar structures are examined by Scanning Electron Microscopy (ESEM), by ESEM-FEG/EDAX Philips XL-30 instrument. High magnification photographs of the prepared micelle solutions are taken and micellar structures are detected and observed. Micelle solutions prepared are dropped onto Cu grids and dried overnight in open atmosphere before analysis. Solutions are filtered through Millipore filters with 0.45μm pore size (ProFill HPLC Syringe Filter, hydrophilic, RC) prior to dropping on to grids.

Particle size analysis is done for determining micelle sizes in water with the Brookhaven Instruments Corporation 90Plus Dynamic Light Scattering Particle Size Analyzer (Nano-PSA). PMMA cuvettes are used throughout the analyses. During analysis, consequent dilutions are done using the initial micelle solutions. In dilutions, deionized water that is filtered through Millipore filters of 0.1μm pore size is used.

Fluorescence spectroscopy measurements for determining critical micelle concentration (CMC) are conducted using Varian-Cary Eclipse Fluorescence

Spectrophotometer. Pyrene as the fluorescence probe and slit width of 2.5nm settings are used in order to calculate CMC from data of excitation peaks at 339nm and 334nm with respect to emission wavelength of pyrene at 390nm. Pyrene solution is prepared by dissolving pyrene in acetone and adding deionized water with adjusting concentration as $6x10^{-7}$M. Block copolymer solution is prepared by dissolving the sample in acetone and adequate amounts are added onto pyrene stock solution, following with evaporation of acetone at open atmosphere. Consequent dilutions are done to obtain very dilute solutions.

In vitro drug release tests are monitored by TU-1880 UV-Visible Spectrophotometer using quartz cuvettes. 50 mg block copolymer and 50mg of drug is dissolved in 10ml acetone and dialyzed against 2000 ml of deionized water for 24 hours for the loading of the drug. Release media used is 100 ml of phosphate buffered saline (PBS) adjusted to pH 7.4 which resembles the environment of blood plasma. Aliquots of 3ml are taken from the release media at specific time intervals, such as 1-6h, 24h and 48h, for UV-Visible absorption analysis and transferred back once the analysis was done. A control test with free drug release, in which only drug dissolved in acetone, is subjected to *in vitro* release. Drug loading efficiency is calculated by the absorption values from UV-Visible measurements of drug loaded block copolymer micelle solution versus same amount of pure drug dissolved in acetone.

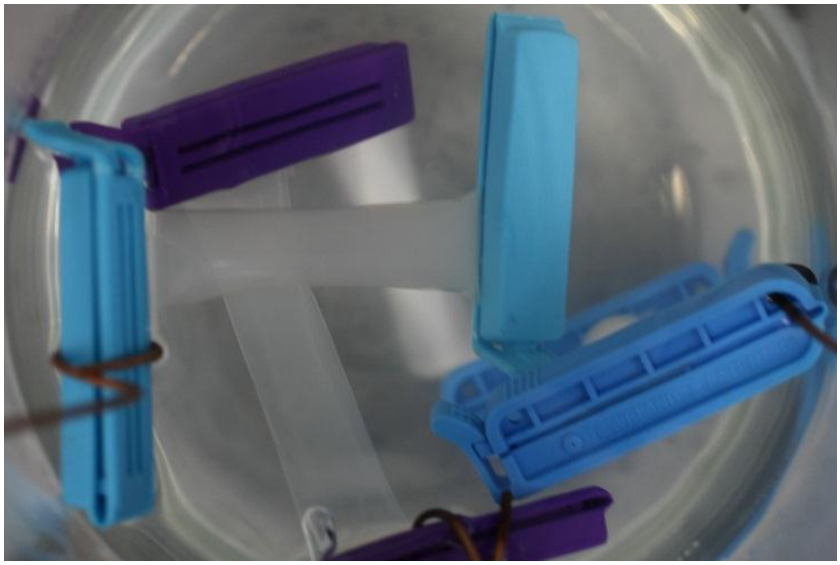

Figure 3.1. Dialysis using dialysis tubings – 1

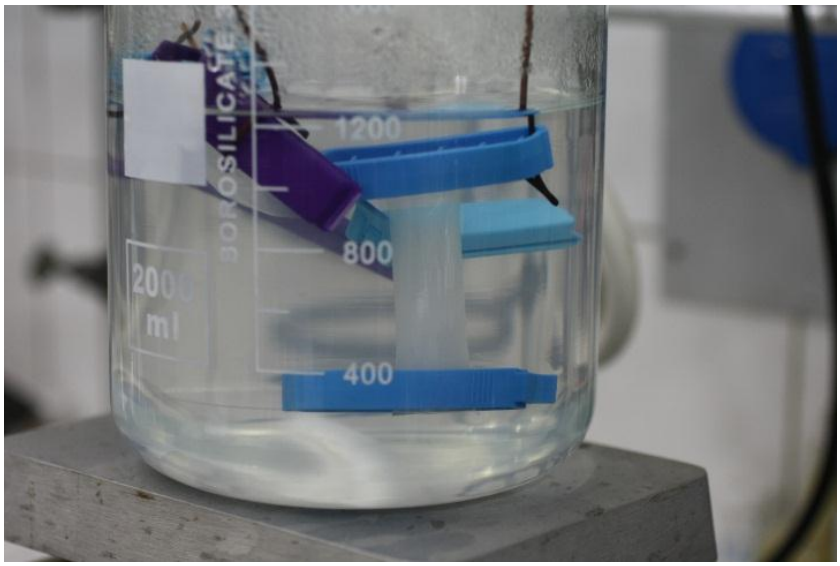

Figure 3.2. Dialysis using dialysis tubings – 2

Figure 3.3. Reflux setup used in PVAc hydrolysis

Figure 3.4. Reactor setup for hydrolysis with amine usage

4. RESULTS AND DISCUSSION

4.1. Methoxide-Catalyzed Transesterification (Hydrolysis) of PVAc

The success of hydrolysis procedure (3.2) which is given in literature [49] is first examined by using commercial PVAc homopolymer. The results of the sampling times and per cent hydrolysis calculated by ^{1}H NMR are given in Table 4.1.

Table 4.1. Sample names, sampling times and per cent hydrolysis values of PVAc

Sample Name	HD1-10	HD1-20	HD1-30	HD1-60	HD1-90	HD1-120	HD1-165	HD1-180	HD1-360	HD1-1320
Sampling time (minute)	10	20	30	60	90	120	165	180	360	1320
Per cent hydrolysis (%) (High Field/Low Field)	3.3/ 2.6	5.5/ 3.9	11.9/ 10.5	42.6/ 38.5	81.6/ 78.9	91.0/ 92.1	95.8/ 95.2	96.5/ 96.1	99.1/ 99.4	99.6/ 99.2

Deuterated-chloroform ($CDCl_3$) and deuterated-dimethyl sulfoxide (d_6-DMSO) are good solvents for PVAc and PVA respectively for ^{1}H NMR analyses as stated by Bernard et al. [64]. Similarly, Debuigne et al. provides the information that $CDCl_3$ is a solvent for PVAc and D_2O/H_2O/2-propanol (1:3:traces) mixture can be used as a solvent for PVA [52]. According to these information, before using d_6-DMSO as NMR solvent for calculating per cent hydrolysis of the samples, solubility of pure PVAc and PVA in DMSO is checked.

PVAc and PVA exhibits complete dissolution in DMSO after 3-4 hours. The solutions become highly viscous. In spite of this difficulty, to prevent different shifting of the peaks and to avoid overlapping of sample peaks with a solvent peak, the same solvent, DMSO, is used in all NMR analysis of the hydrolysis products.

Correct correlation of the NMR peaks could be done when all the ^{1}H NMR spectra are overlaid on each other (Figure 4.1). By this method, increasing and decreasing peaks can be clearly identified and calculations are done based on these findings.

Conversion (hydrolysis amount) percentages are calculated from high and low fields of the NMR spectra based on the calculations of integrated intensity of NMR peaks (I.I.) in the ranges provided such as:

$$\text{Low Field Calculation} = \frac{I.I.(5.2ppm-4.7ppm)}{x + I.I.(5.2ppm-4.7ppm)} \quad (4.1)$$

(Where x stands for I.I.(4.7ppm-4.2ppm) or I.I.(4.2-3.7ppm))

$$\text{High Field Calculation} = \frac{I.I.(1.6ppm-1.2ppm)}{2} / \text{x} \quad (4.2)$$

$$\text{(Where x stands for } \left[\frac{I.I(2.0ppm-1.6ppm)}{5} + \frac{I.I.(1.6ppm-1.2ppm)}{2}\right] \text{)} \quad (4.3)$$

Ahmed et al. states that as hydrolysis proceeds, C*H*-OH peaks in ^{1}H NMR are triple singlets due to hydrolyzed segments with different proximities to unhydrolyzed acetate groups [49]. The mechanism is autocatalytic due to hydroxyl groups formed influencing adjacent acetate groups into hydrolysis (alcoholysis) and enhancing their reactivity [48, 49, 55]. Therefore, per cent hydrolysis is exponentially increasing as reaction time increases [49].

As a result, from the calculated hydrolysis percentages, it is found that degree of hydrolysis increases exponentially in agreement with literature results [49] as the reaction proceeds (Figure 4.2). The ^{1}H NMR results indicate that 360 minutes is adequate for almost full hydrolysis of the acetate groups of PVAc. For 70% hydrolysis 75-90 minutes and for low hydrolysis percentage of 25% of PVAc 40-50 minutes are suitable.

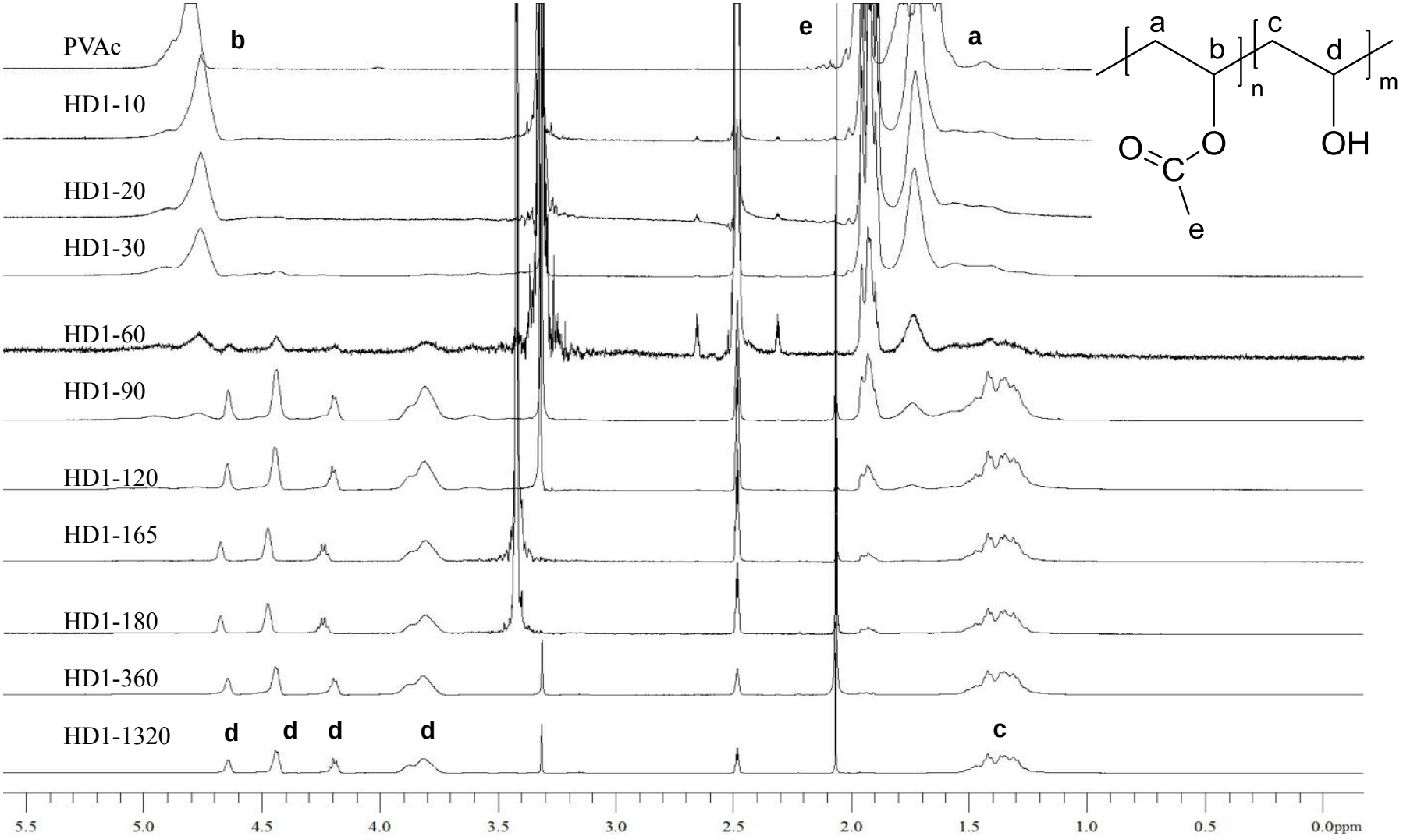

Figure 4.1. ^{1}H NMR (d_6-DMSO) results as overlays of PVAc homopolymer and hydrolyzed samples

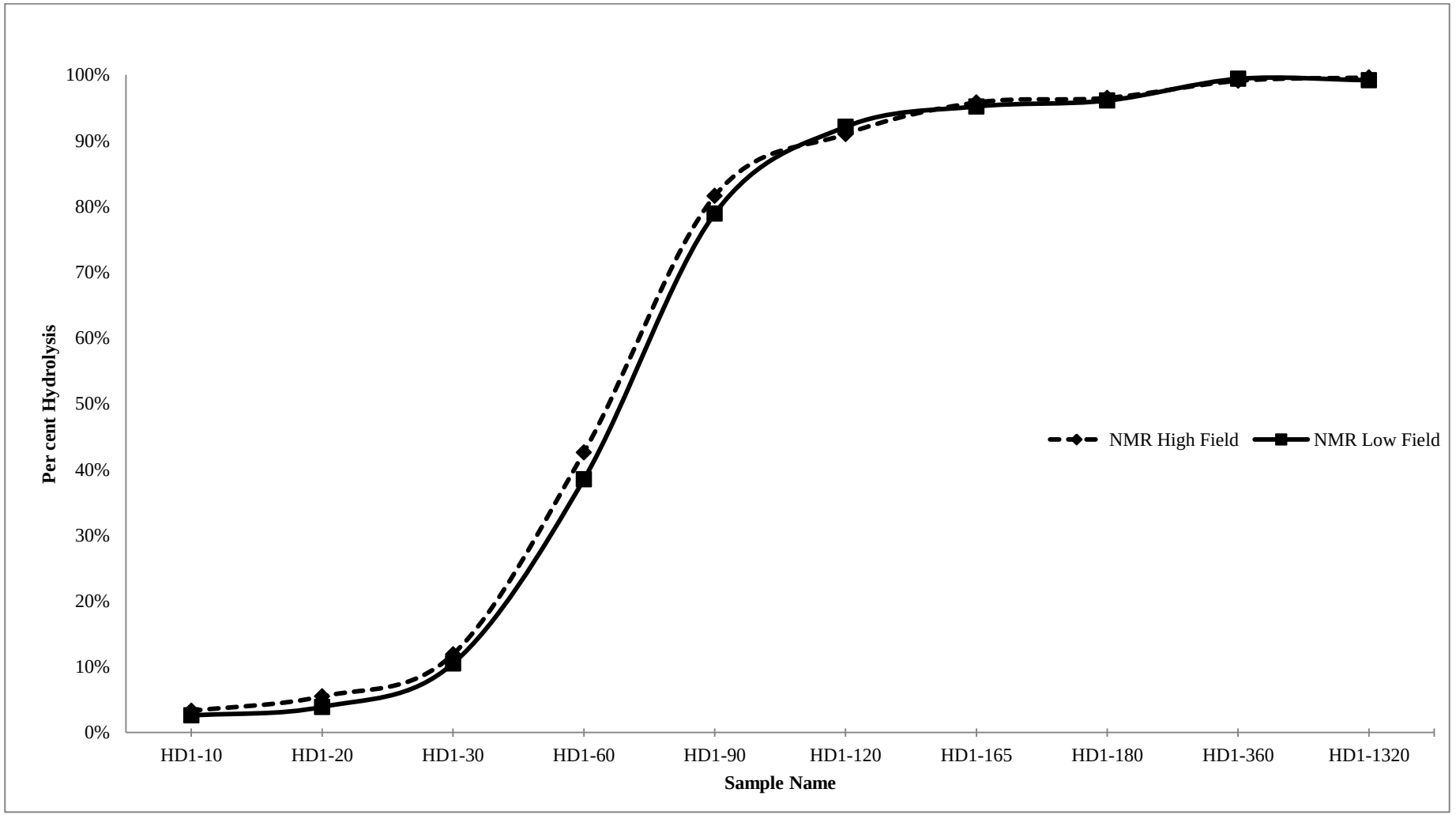

Figure 4.2. Conversion percentages of PVAc homopolymer hydrolysis calculated from ^{1}H NMR analysis

4.2. Methoxide-Catalyzed Hydrolysis Trial of PNIPAM

In this study, PNIPAM block in PVAc-b-PNIPAM is wanted to be kept unhydrolyzed throughout the methoxide-catalyzed transesterification of PVAc block. To achieve this task successfully, it must be proven that NIPAM pendant groups do not react with sodium methoxide in methanol. It was reported by Nuopponen [60] that the basic hydrolysis of PNIPAM is not feasible in ethoxide solution. Moreover, no hydrolysis in acidic medium is also observed [61]. This was attributed to the stabilization effect of the isopropyl group on the amide carbon, avoiding the attack of the basic or acidic catalyst onto this site [65]. In order to confirm these results, PNIPAM hydrolysis trial is conducted under the same reaction conditions used in the methoxide-catalyzed transesterification of PVAc homopolymers.

After this treatment, ^{1}H NMR results of PNIPAM-E1 and HD-PNIPAM-E1 are exactly the same (Figure 4.3). This confirms that, there is no hydrolysis of PNIPAM taking place as in abovementioned conditions. The peak at 3.0 ppm indicates the moisture and methanol content in the sample due to poor drying.

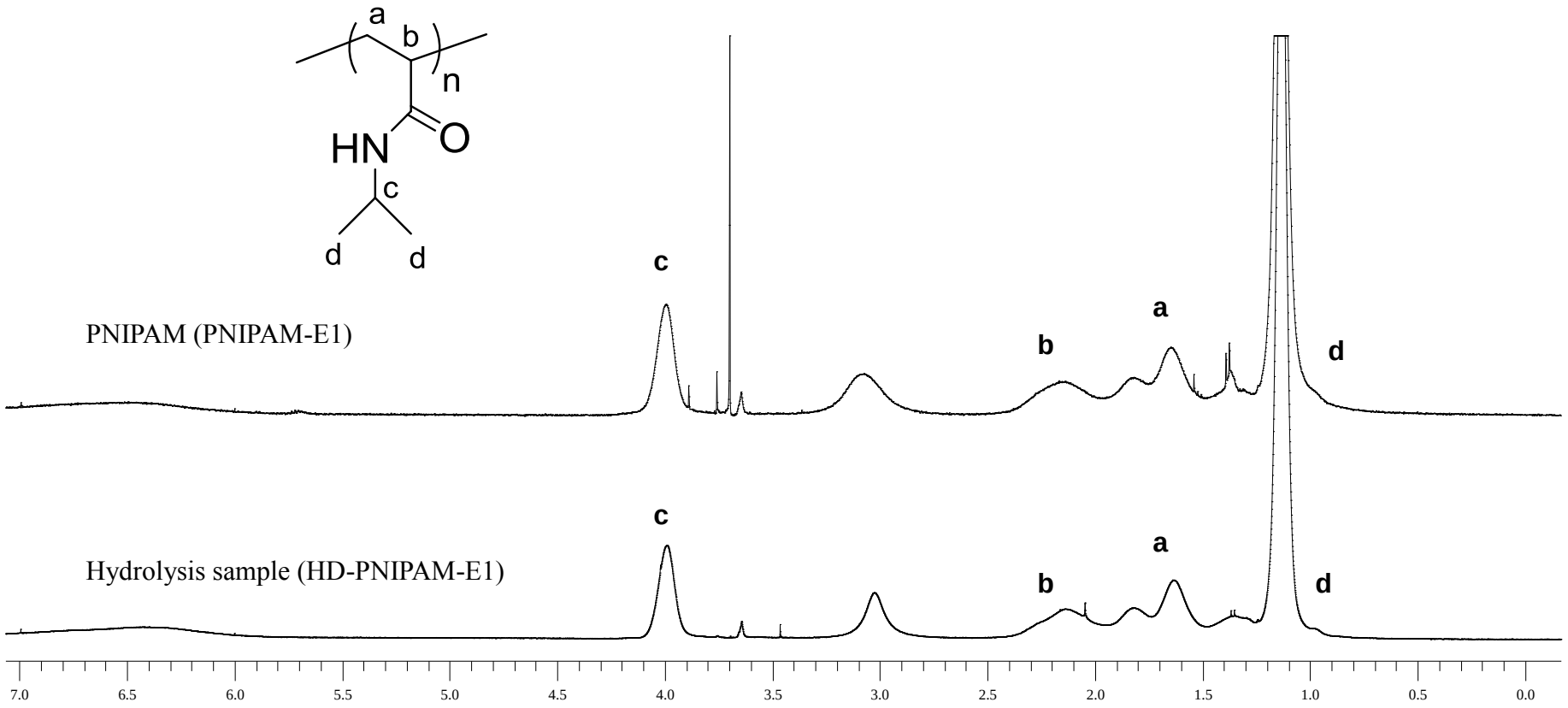

Figure 4.3. ^{1}H NMR ($CDCl_3$) results as overlays of PNIPAM homopolymer and hydrolysis sample

4.3. Methoxide-Catalyzed Partial and Full Hydrolysis of Poly(N-Isopropylacrylamide)-b-Poly(Vinyl Acetate) with the Addition of Amine (HD-NH2-C-AN4-30 & HFULL-NH2-C-AN4)

As stated by Tong et al. [46], the xanthate group at the end of the PVAc macro chain transfer agent is removed by the methoxide (NaOH) hydrolysis and conjugated double bonds on the main polymer chain and aldehyde group at the chain end form (Figure 4.4 (a)). The polymer chains collapse onto themselves and in ^{1}H NMR analysis, C*H*-OH or C*H*-acetate group specific peaks cannot be detected in either of the DMSO, acetone or the D_2O solvents, thus leading to inability to calculate per cent hydrolysis values of the hydrolyzed samples. The reason for a broad band of conjugated double bonds observed at the 6-7 ppm region in ^{1}H NMR is this phenomenon. The products appear yellow. On the other hand, if n-propylamine is added into the reaction mixture during the hydrolysis reaction, xanthate groups are broken to form Schiff base structure at the chain end and chain integrity is preserved without alternating conjugated double bond formations randomly (Figure 4.4 (b)). This leads to good NMR peak resolutions in comparison to hydrolyzed products via no amine usage, which may be an indication for the presence of the xanthate- terminated PVAc homopolymer. Obtaining the white powder product instead of a yellow or brown one can be accepted as an indication of successful approach.

Additionally, pH of the solid product is determined as 7.5. This result shows that quenching followed by dialysis removed all the residual methoxide out of the reaction mixture effectively.

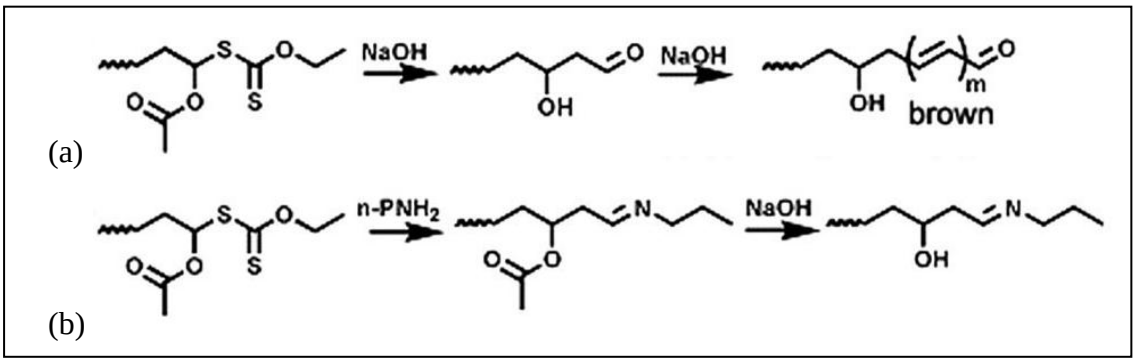

Figure 4.4. Formation of hydrolyzed products with and without amine usage [46]

^{1}H NMR analysis (D_2O) of the product after dialyzing and freeze-drying the reaction mixture suggest that hydrolysis is successful due to only two C*H* specific peaks present at about 4.0 ppm and 3.5 ppm of the PVA and PNIPAM groups (Figure 4.5 (a)). Peak integration values used for calculations in combination of high and low field ^{1}H NMR results are proving full hydrolysis. Additionally ^{1}H NMR analysis in d_6-DMSO is done due to the overlapping of D_2O peak with polymer peaks at about 5.0 ppm. These results also show the success of full hydrolysis of the PVAc block (Figure 4.5 (b)).

For the calculation of per cent hydrolysis from both D_2O and d_6-DMSO ^{1}H NMR of partial hydrolysis (HD-NH2-C-AN4-30), combination of high and low field peaks are used (Figure 4.6). These calculations are based on the integrated intensity of NMR peaks (I.I.) in the ranges provided such as:

High and Low Field Combined Calculation:

$$\frac{\left[I.I.(4.2-3.6\text{ppm}) - \left[\frac{I.I.(1.1-0.8\text{ppm})}{6}\right]\right]}{I.I.(4.2-3.6\text{ppm})} \text{ x } 100 \qquad (4.4)$$

The calculations indicate that the per cent hydrolysis of HD-NH2-C-AN4-30 is 67.7%. 70% hydrolyzed PVAc/PVA is water-soluble at room temperature and 67.7% hydrolysis percentage is an acceptable hydrolysis degree for obtaining block copolymers with dihydrophilic properties.

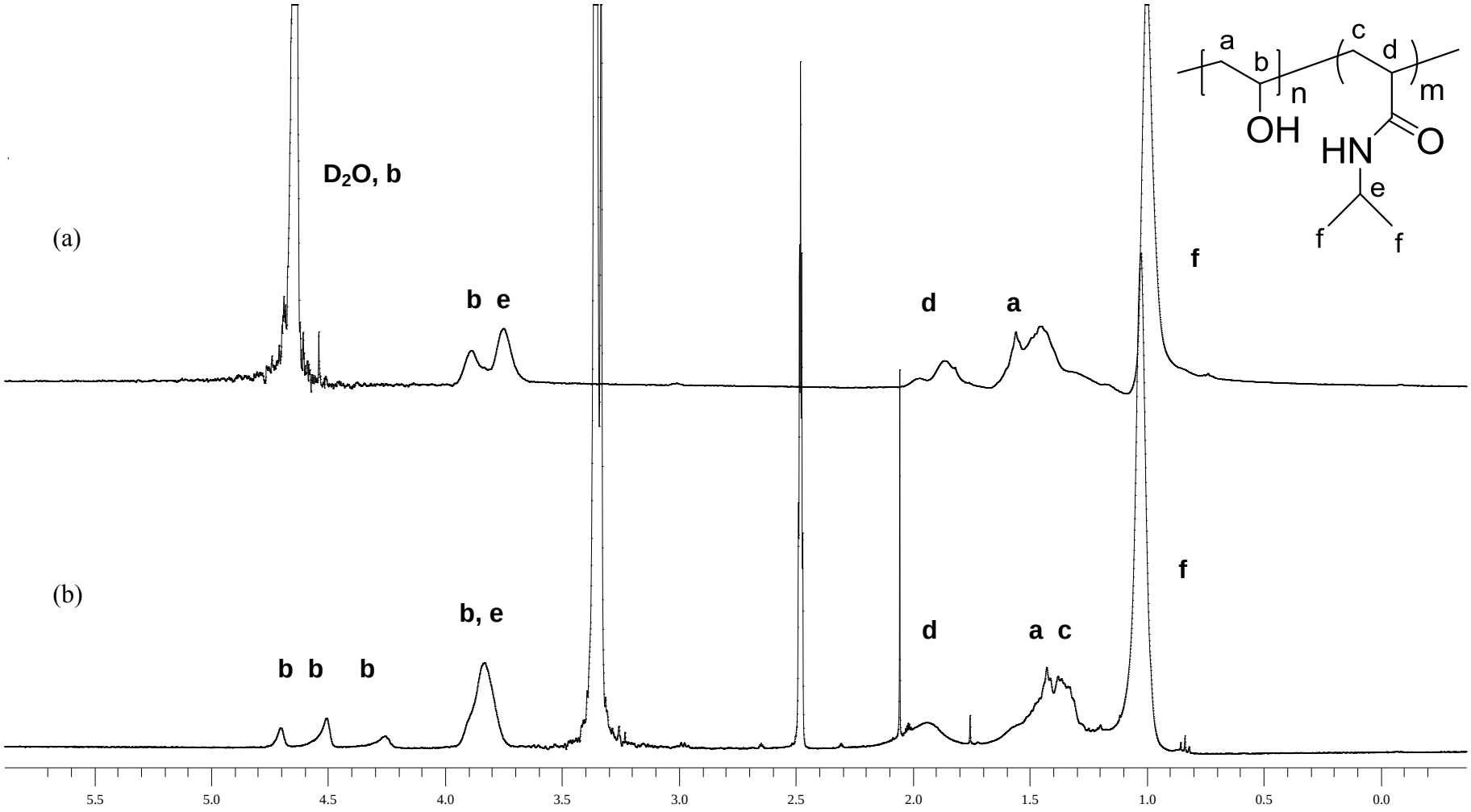

Figure 4.5. D_2O and d_6-DMSO ^1H NMR results as overlays of HFULL-NH2-C-AN4

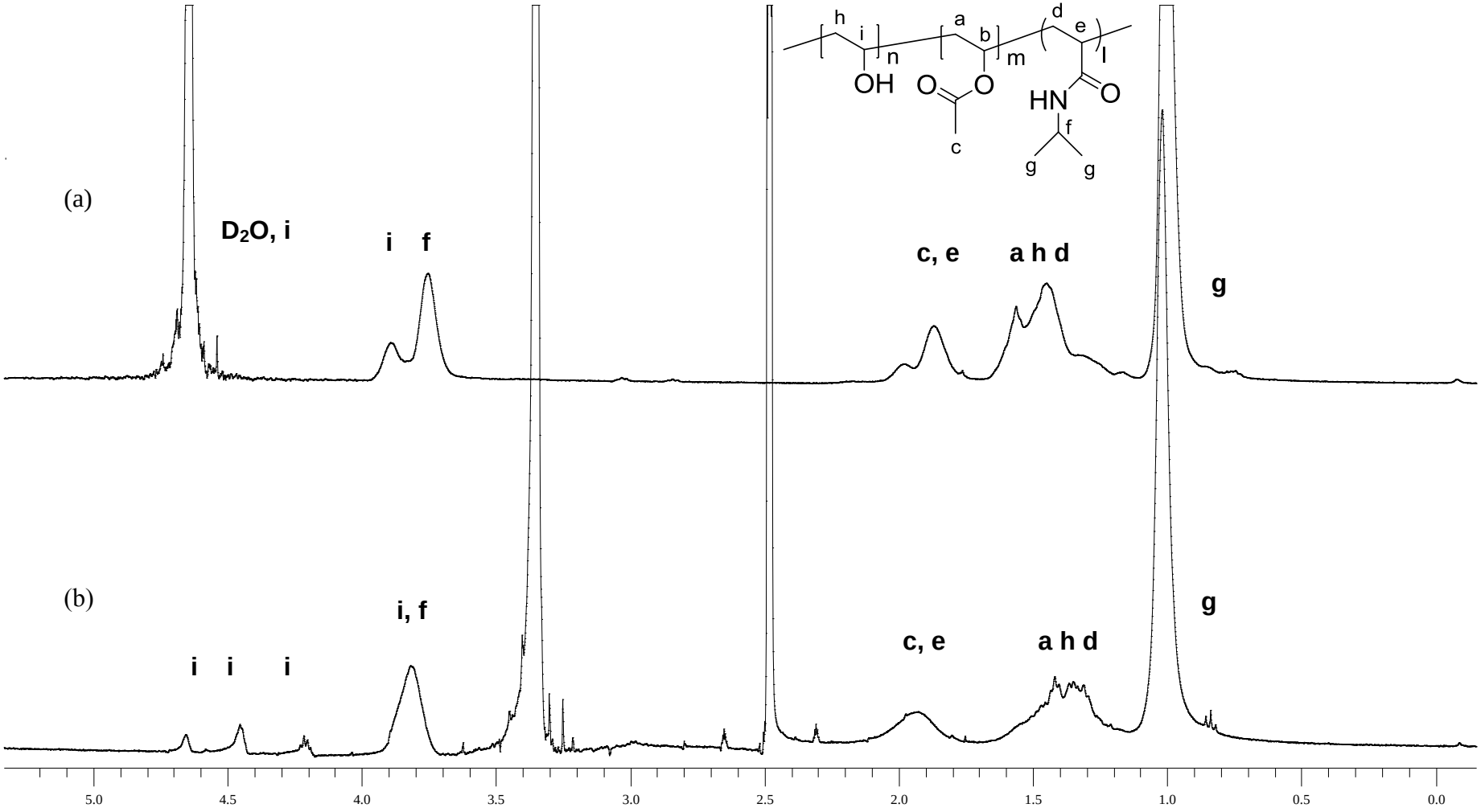

Figure 4.6. D_2O and d_6-DMSO ^1H NMR results as overlays of HD-NH2-C-AN4-30

4.4. Preparation of Micelle Solutions of Poly(N-Isopropylacrylamide)-b-Poly(Vinyl Acetate) Block Copolymers with or without the Dialysis Procedure

With the incorporation of dialysis technique, micelle solutions are expected to be homogeneous without any secondary aggregate formations [66]. Therefore, dialyzed solutions are well-defined compared to solutions prepared without dialysis, as proven by ESEM and Nano-PSA analyses (4.5.1 and 4.7.1). The solution concentration is also important because it is recorded in literature that micelle formation of PNIPAM block copolymers is most efficient in the range of 0.2-2.0% (w/v) concentration and after 6% (w/v) concentration, gel formation occurs in the micelle solution [60]. The mechanism of dialysis follows as 1,4-dioxane goes out of the dialysis tubings and water enter in. At RT, PVAc blocks are insoluble in water and they form the core and the soluble PNIPAM blocks form the shell of the micelles.

The obtained solutions are characterized by ESEM and Nano-PSA analyses.

4.5. Morphological Characterizations

4.5.1. Environmental Scanning Electron Microscopy (ESEM) Analysis of Micelles Prepared with or without the Dialysis Procedure (C-AN2, C-AN3, C-AN4)

ESEM analyses are done with solutions prepared with or without dialysis technique and are filtered through 0.45μm Millipore filters to remove any large impurities. Additionally, evaporation effect on micelle structures during sample preparation (40 °C) is examined on the C-AN2 and C-AN4 samples prepared with dialysis.

All ESEM results for the micelles prepared without dialysis show that large agglomerate formations are observed due to poor solubility of samples in water. Besides, the size distributions in all results are unfavorably broad, meaning both large and small micelles are present in the solutions (Figure 4.7). To back up this argument, the study by

Debuigne et al. states that self-assembly of block copolymers into aggregates due to hydrogen bonding is possible [63]. Average sizes of micelles of the samples increase as shell block length increases (Table 4.2). On the other hand, C-AN2 sample has a larger average micelle size compared to that of C-AN4. Since, water is dried off from the micelle solution on Cu grid, micelles of C-AN2 sample adjoin and form tubular structures due to thin shell stabilization effect of shorter PNIPAM block in the shell of the micelles. In this sample, association and aggregation of micelles due to less steric repulsion of the shell is also observed [67].

Table 4.2. ESEM analysis results for average micelle sizes for solutions prepared without dialysis

Sample Name	C-AN4 (1:1)	C-AN3 (1:2)	C-AN2 (1:0.5)
Average Micelle Sizes	100nm	200nm	175nm

Low magnification ESEM images for all block copolymers prepared by dialysis (Figure 4.8) well exhibit the quite broad particle size distributions for short and long PNIPAM segments (2:1 and 1:2 compositions) and very narrow distribution for the 1:1 composition. These results were also confirmed by Nano-PSA data given in Table 4.8. As it is clearly seen from the higher magnification images of C-AN4 (1:1 composition) also exhibits stable and perfect spherical micelle structures with *ca.* 600 nm effective diameter. C-AN2 sample, on the other hand, forms mainly vesicles and rod-like aggregates with *ca.* 1500 nm diameter, most probably resulting from the relatively shorter block lengths of PNIPAM in the block copolymer, similar to results of C-AN2 sample prepared without dialysis. It is well known that if the shell forming block is shorter than the core forming block, the crew-cut aggregates such as vesicles, rods, spheres, are expected to be formed [67]. Compared with results of samples prepared without dialysis, dialyzed samples present fewer agglomerations, larger sizes and more homogeneous micellar structures (Table 4.3).

Table 4.3. ESEM analysis results for average micelle sizes for solutions prepared via dialysis

Sample Name	C-AN4 (1:1)	C-AN3 (1:2)	C-AN2 (1:0.5)
Average Micelle Sizes	500-600nm	1000-1200nm	*ca*. 1500nm

With the dialyzed samples, to see the evaporation effect on micelle structures during sample preparation, the dialyzed micelle solutions of C-AN4 and C-AN2 (0.2% (w/v) and unfiltered) are analyzed after drying on Cu grids at 40 °C. The C-AN4 micrographs showed that many well-dispersed micelle structures with 400-500nm average sizes are present. In the C-AN2 micrographs, deformed vesicles and smaller micelles with 400nm average sizes, compared to results taken at RT previously, due to high temperature drying are seen (Figure 4.9). Large circles seen in micrographs are due to water evaporation during drying.

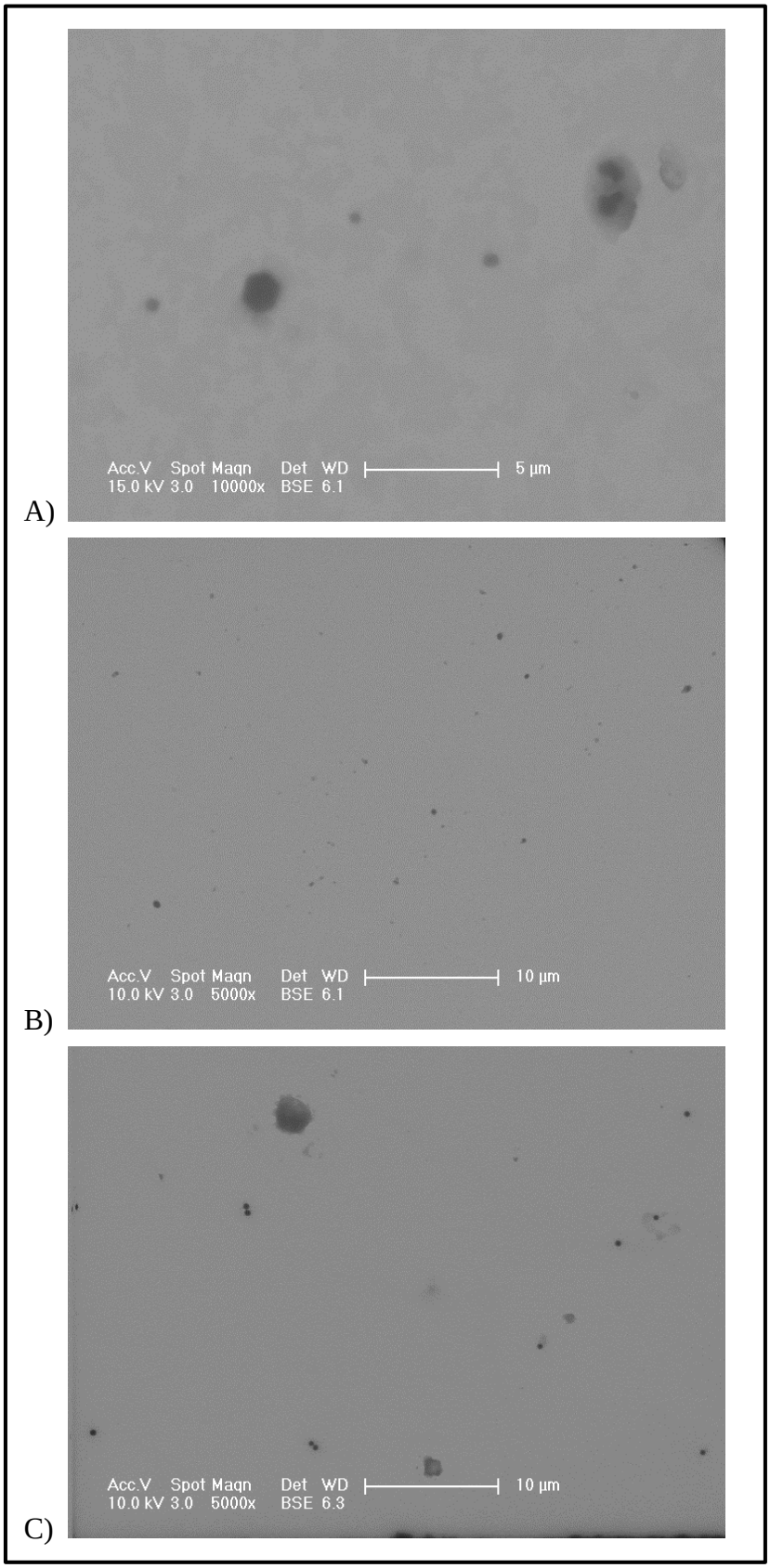

Figure 4.7. Low magnification ESEM micrographs of filtered samples prepared without dialysis: A) C-AN2 (1:0.5), B) C-AN4 (1:1), C) C-AN3 (1:2)

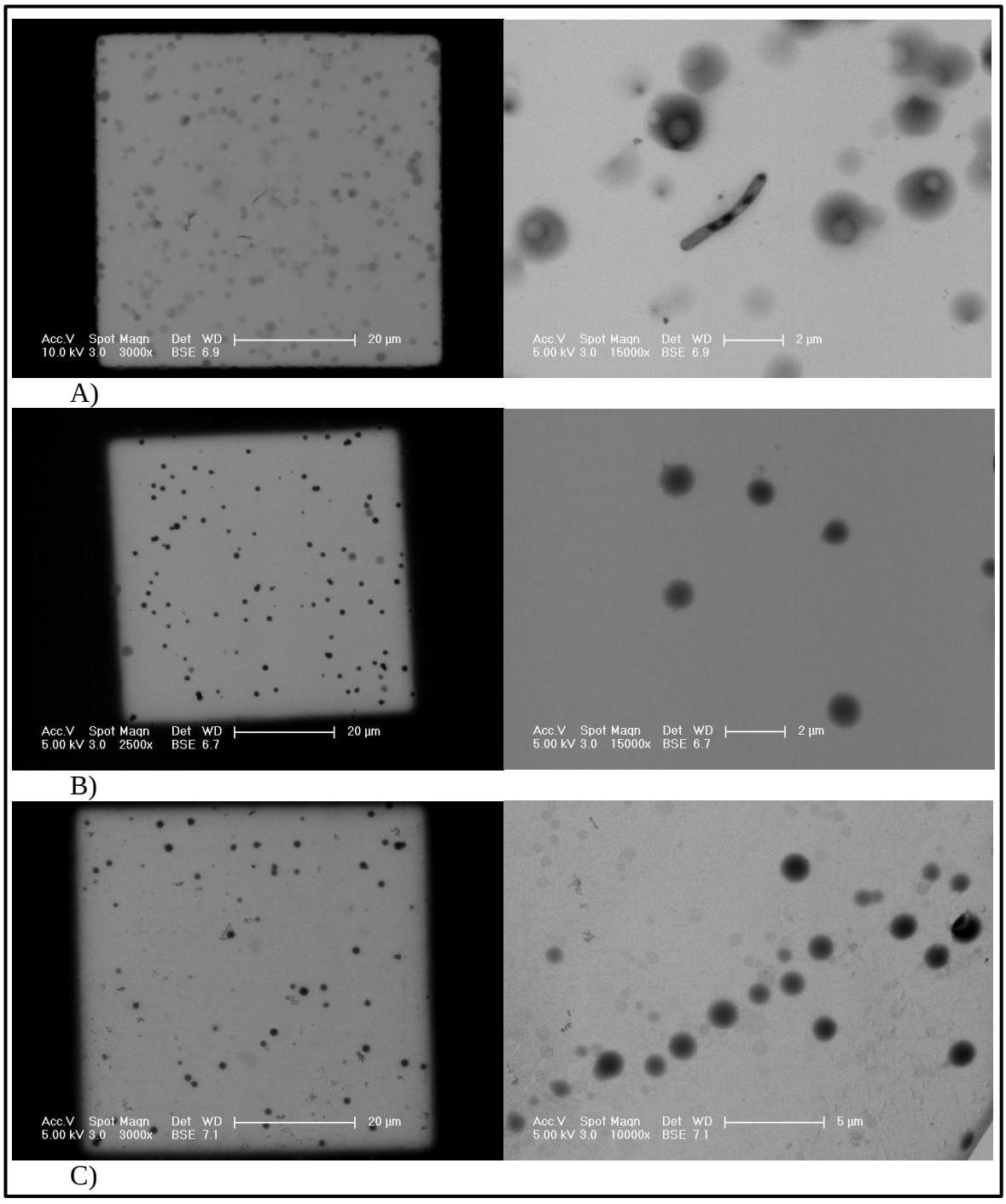

Figure 4.8. Low and high magnification ESEM micrographs of dialyzed and filtered samples: A) C-AN2 (1:0.5), B) C-AN4 (1:1), C) C-AN3 (1:2)

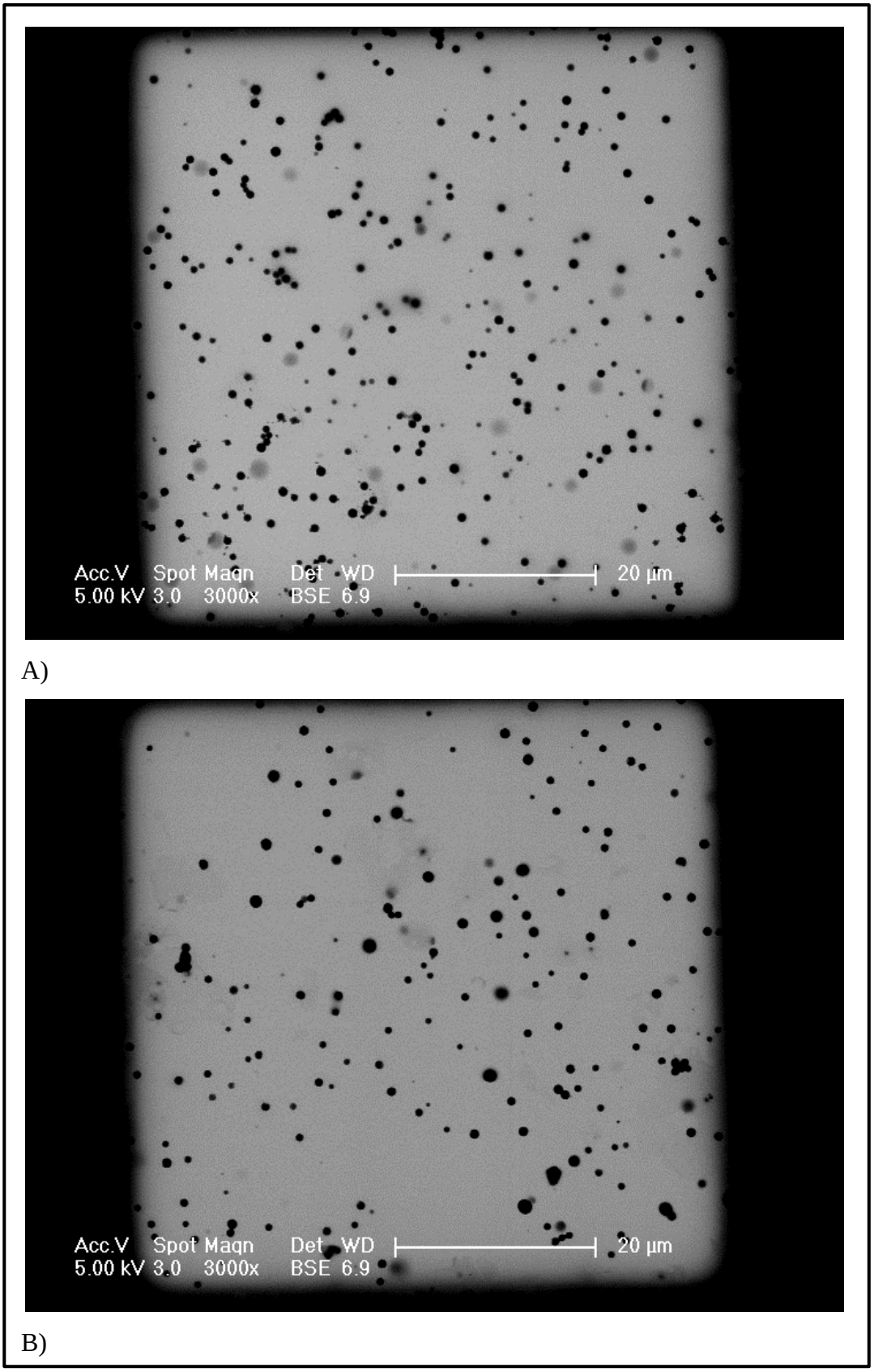

Figure 4.9. Low magnification ESEM micrographs of samples dried on Cu grids at 40 °C: A) C-AN4 (1:1), B) C-AN2 (1:0.5)

4.6. Thermal Characterizations

4.6.1. Differential Scanning Calorimetry (DSC) Analysis for Glass Transition Temperature (T_g) Determination

The average glass transition temperatures of the synthesized diblock copolymers of PVAc-b-PNIPAM are collected in Table 4.4, as well as the T_g of PVA homopolymer [59]. When thermal characterization is performed for hydrolyzed block copolymers, the T_g values for PVA and PVAc segments of hydrolyzed samples cannot be seen due to unsuccessful drying (Table 4.5). The large endothermic peak in this range causes the T_g values to be undetectable. Fortunately, T_g of PNIPAM block can be clearly detected in all samples (Figure 4.10). Obviously after hydrolysis, the strength of inter/intramolecular interactions in terms of hydrogen bonding increases, consequently, the T_g of PNIPAM is a little higher in hydrolyzed samples and also the peak becomes broader in appearance [68].

Table 4.4. Glass Transition Temperature (T_g) values of PVAc, PVA and PNIPAM

Sample Name	T_g Value
PVAc	36.46 °C
PVA	85 °C (Theoretical)
PNIPAM	136.05 °C

Table 4.5. Glass Transition Temperature (T_g) results for hydrolyzed samples

Sample Name	T_g Value for PVAc/PVA block	T_g Value for PNIPAM block
HD-NH2-C-AN4-30	–	141.15 °C
HFULL-NH2-C-AN4	–	140.72 °C

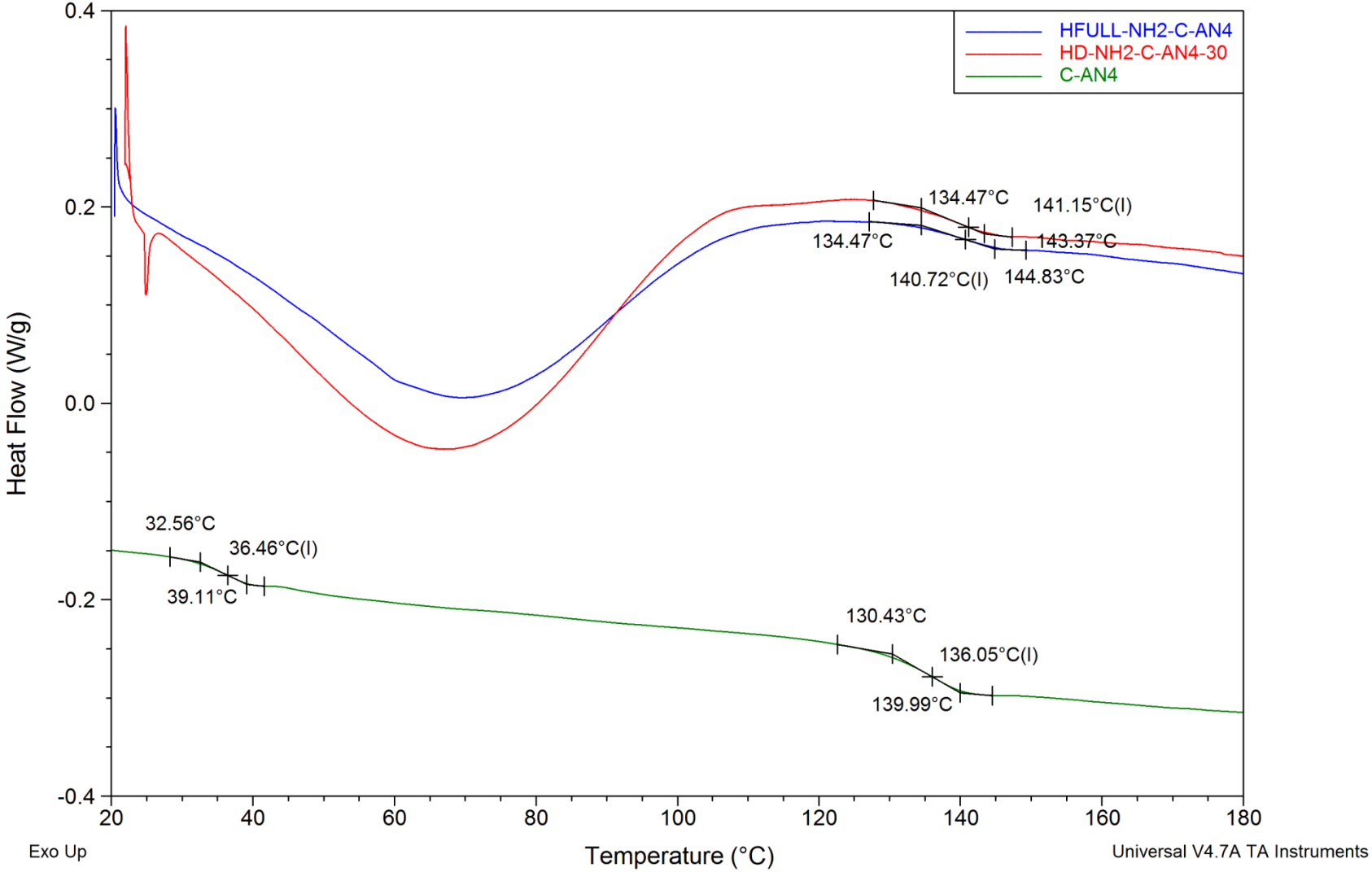

Figure 4.10. DSC curves of samples

4.6.2. Differential Scanning Calorimetry (DSC) Analysis for Lower Critical Solution Temperature (LCST) Determination of the PNIPAM Block

DSC technique is frequently performed to define lower critical solution temperature (LCST) of PNIPAM in literature [69]. It is seen that when water is released with increase in temperature over LCST, a sharp endotherm is detected. The endotherm is explained by the release of water molecules from the hydrophobic N-isopropyl groups of PNIPAM via hydrophobic interactions of these groups. The minimum of the endothermic peak is read as the LCST of the whole block copolymer. Solutions in the pan are very concentrated (~25%) to be able to get good signals.

LCST values are recorded from the graphs as (Figure 4.11 and Figure 4.12); 30.66 °C for C-AN8, 31.96 °C for C-AN9, 31.74 °C for C-AN4, 31.04 °C for PNIPAM-E1, 31.97 °C for HD-NH2-C-AN4-30 and 30.80 °C for HFULL-NH2-C-AN4 samples.

The results of unhydrolyzed samples, C-AN4, C-AN8 and C-AN9, show that there is no change in LCST of PNIPAM block as a function of PNIPAM content (Figure 4.12). The most probable reason for that is the relatively broad molecular weight distribution of block copolymers.

Partially hydrolyzed sample has the highest LCST due to water solubility and hydrogen bonding. An increase in the overall hydrophilicity of the block copolymer increases the LCST [70, 71]. This rule is not preserved when partial and full hydrolysis results are compared. This result may be attributed to strong hydrogen bonding between polymer chains.

As a conclusion, hydrolysis does not affect LCST of block copolymers, but merely changes the dissolving properties of the partially or fully hydrolyzed block copolymer samples in water.

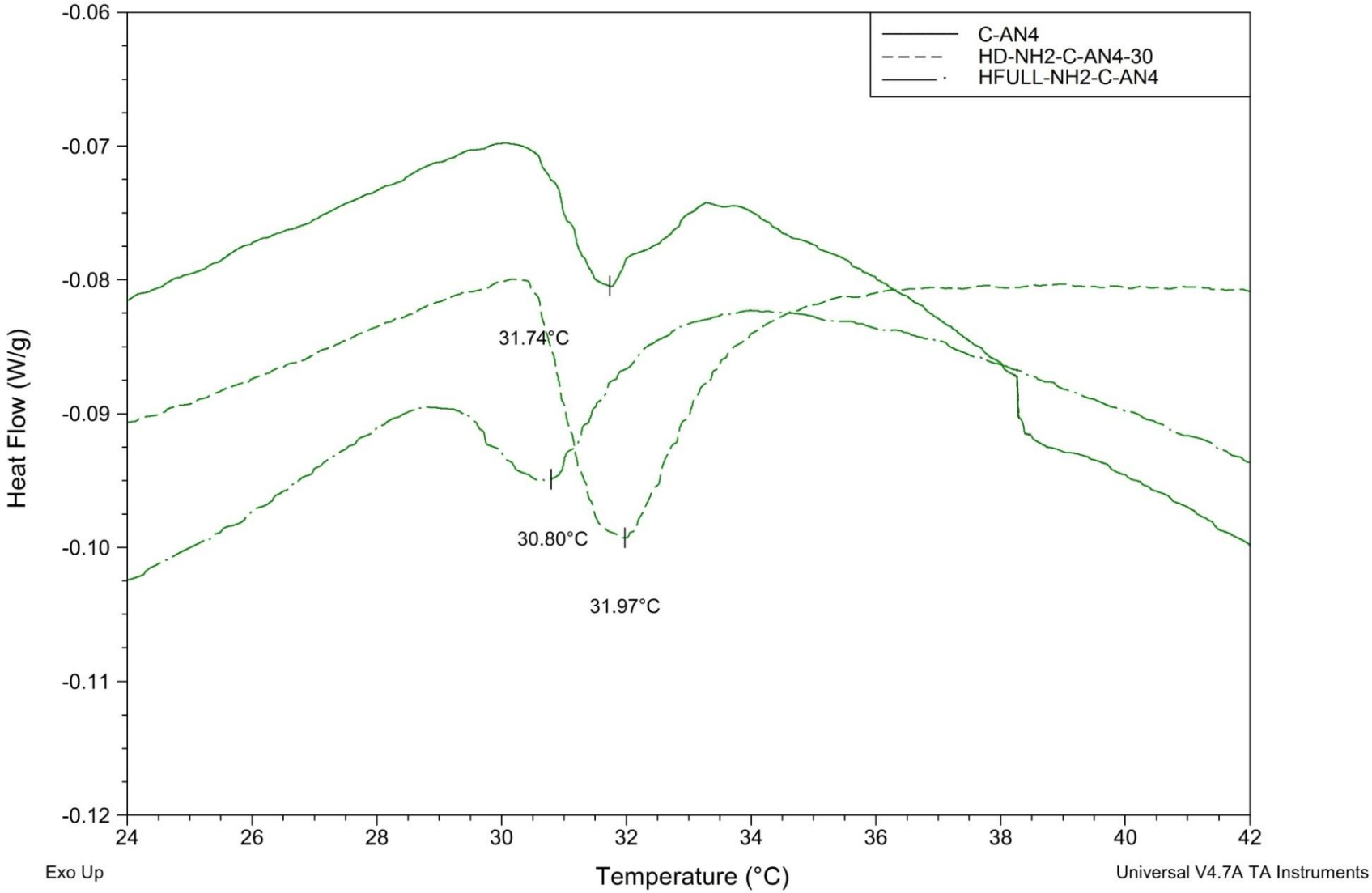

Figure 4.11. DSC endotherms indicating LCST of partial and full hydrolyzed samples

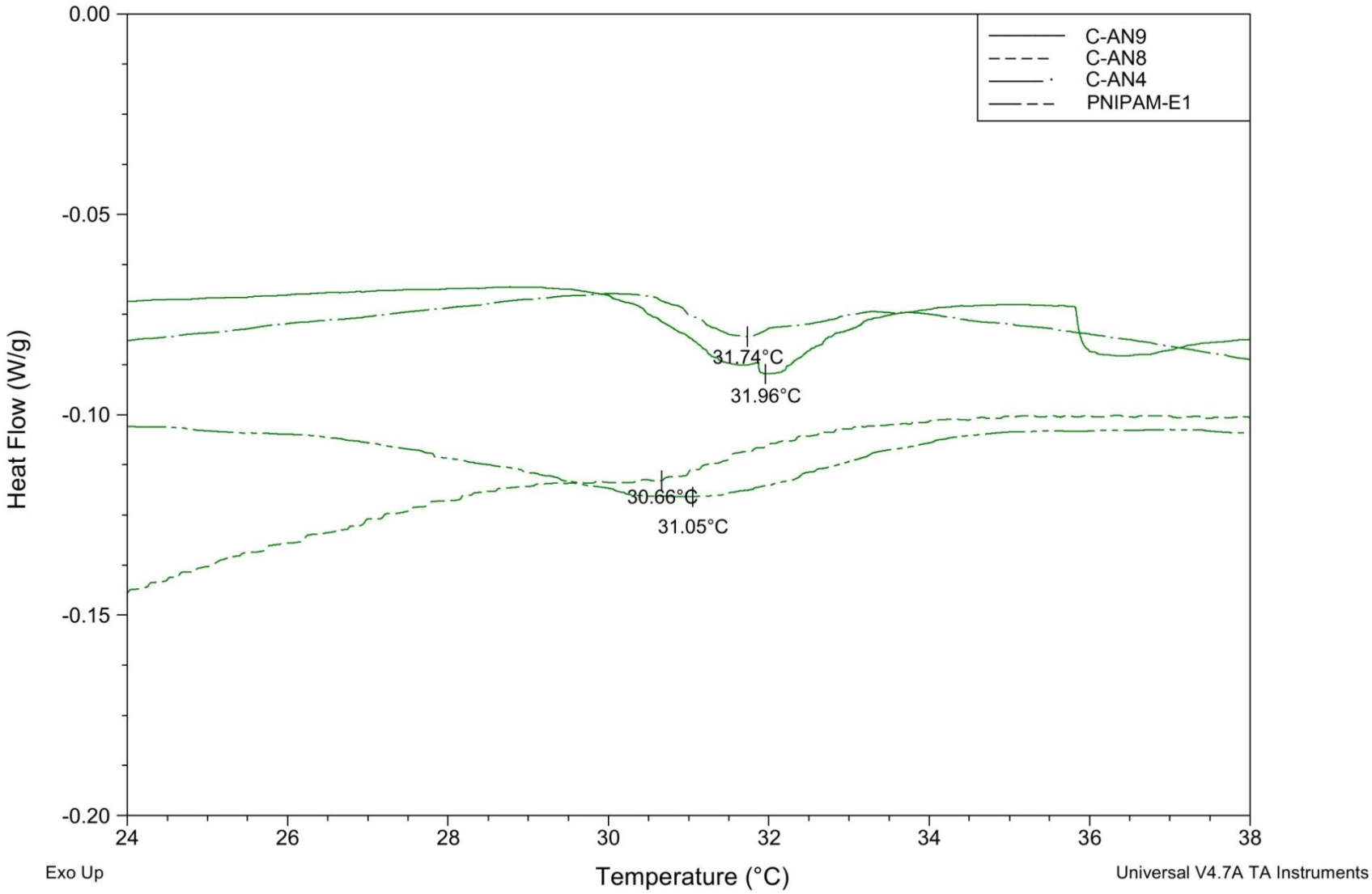

Figure 4.12. DSC endotherms indicating LCST of unhydrolyzed samples

4.7. Spectroscopic Characterizations

4.7.1. Nano Particle Size Analysis of Micelles Prepared with or without the Dialysis Procedure (C-AN2, C-AN3, C-AN4)

The Nano-PSA results (Table 4.8) indicate that micelles obtained via the dialysis method have bigger sizes and are more stable than micelles prepared by direct dissolution. Stability of the dialyzed samples is proved by the fact that when samples prepared without dialysis are diluted, no measurement of micellar size could be obtained by the Nano-PSA (Table 4.6). When dialyzed samples are diluted up to a concentration of 0.025 % (w/v), micelle sizes are almost the same with the initial results obtained from the 0.2 % (w/v) solutions (Table 4.7). On the other hand, when further dilution is done, micelle sizes decrease. This is shown in the case when C-AN4 (dialyzed and filtered) is further diluted to 0.005 % (w/v) (190nm average particle size), 0.0005 % (w/v) (170nm average particle size), $5x10^{-6}$ % (w/v) (180nm average particle size), $2.5x10^{-6}$ % (w/v) (184nm average particle size) and lastly to $2.5x10^{-7}$ % (w/v) (184nm average particle size). These results can be explained by the stability of micelles even in very dilute concentrations. The same stability property is observed for C-AN2 and C-AN3 samples as well.

Another test for stability of micelles is done by ultrasonic bath treatment of micellar solutions. The dialyzed and filtered C-AN4 (0.2 % (w/v)) sample is put into ultrasonic bath for a couple of minutes and this resulted in the breakage of micellar aggregate structures to form smaller micelles. Unexpectedly, measured size after ultrasonic bath is 200nm and within a few minutes, this result changed from 200nm to 300nm and as more time passed, the resulting average micelle size was 500nm. This proves that small micelles are associating by time to form bigger and more stable micelle structures.

The filtration through filters also has an effect on micelle sizes. Without filtration through 0.45μm Millipore filters, the results have high per cent deviations and micelle sizes exhibit broad size distributions. After filtration, all micelle sizes decrease (Table 4.8).

Table 4.6. Average particle size results from Nano-PSA for micelle solutions prepared without dialysis

Sample Name	C-AN4	C-AN3	C-AN2
Sample concentration	0.2 % (w/v); filtered	0.2 % (w/v); filtered	0.2 % (w/v); filtered
Results for average size distributions	113nm	200nm	160nm
Results for average size distributions after consequent dilutions	No Results	No Results	No Results

Table 4.7. Average particle size results from Nano-PSA for micelle solutions prepared with dialysis

Sample Name	C-AN4	C-AN3	C-AN2
Sample concentration	0.2 % (w/v)	0.2 % (w/v)	0.2 % (w/v)
Results for average size distributions	730nm	612nm	1100nm
Results for average size distributions after consequent dilutions	0.1% (w/v) = 777nm 0.05% (w/v) = 770nm 0.025% (w/v) = 770nm	0.1% (w/v) = 593nm 0.05% (w/v) = 597nm 0.025% (w/v) = 586nm	0.1% (w/v) = 920nm 0.05% (w/v) = 860nm 0.025% (w/v) = 870nm

Table 4.8. General results from Nano-PSA at RT for samples that are in the concentration of 0.2 % (w/v), unless otherwise indicated

Sample Name	Min Size (nm)	Max Size (nm)	Average Size (nm)	Effective Size (nm)
C-AN4 not dialyzed, filtered	16	170	127	113
C-AN3 not dialyzed, filtered	198	204	200	195
C-AN2 not dialyzed, filtered	30	289	225	189
C-AN4 dialyzed, not filtered	743	754	750	780
C-AN3 dialyzed, not filtered	496	1606	705	617
C-AN2 dialyzed, not filtered	739	3756	1506	1073
C-AN4 dialyzed, filtered	88	295	236	209
C-AN3 dialyzed, filtered	42	293	231	213
C-AN2 dialyzed, filtered	49	241	186	168
C-AN4 dialyzed, not filtered (40 °C)	153	565	442	387

The temperature effect on self-assemblies is investigated on 0.2 % (w/v) dialyzed and filtered C-AN4 sample. The results indicate that the effective diameter of micelles change from *ca.* 780 nm at 25 ºC to *ca.* 380 nm at 40 ºC which is thought to be well above LCST of PNIPAM (Table 4.8, Figure 4.13). This behavior of the micellar solution is due to the collapse of PNIPAM block and micelle size shrinkage occurring as temperature is increased.

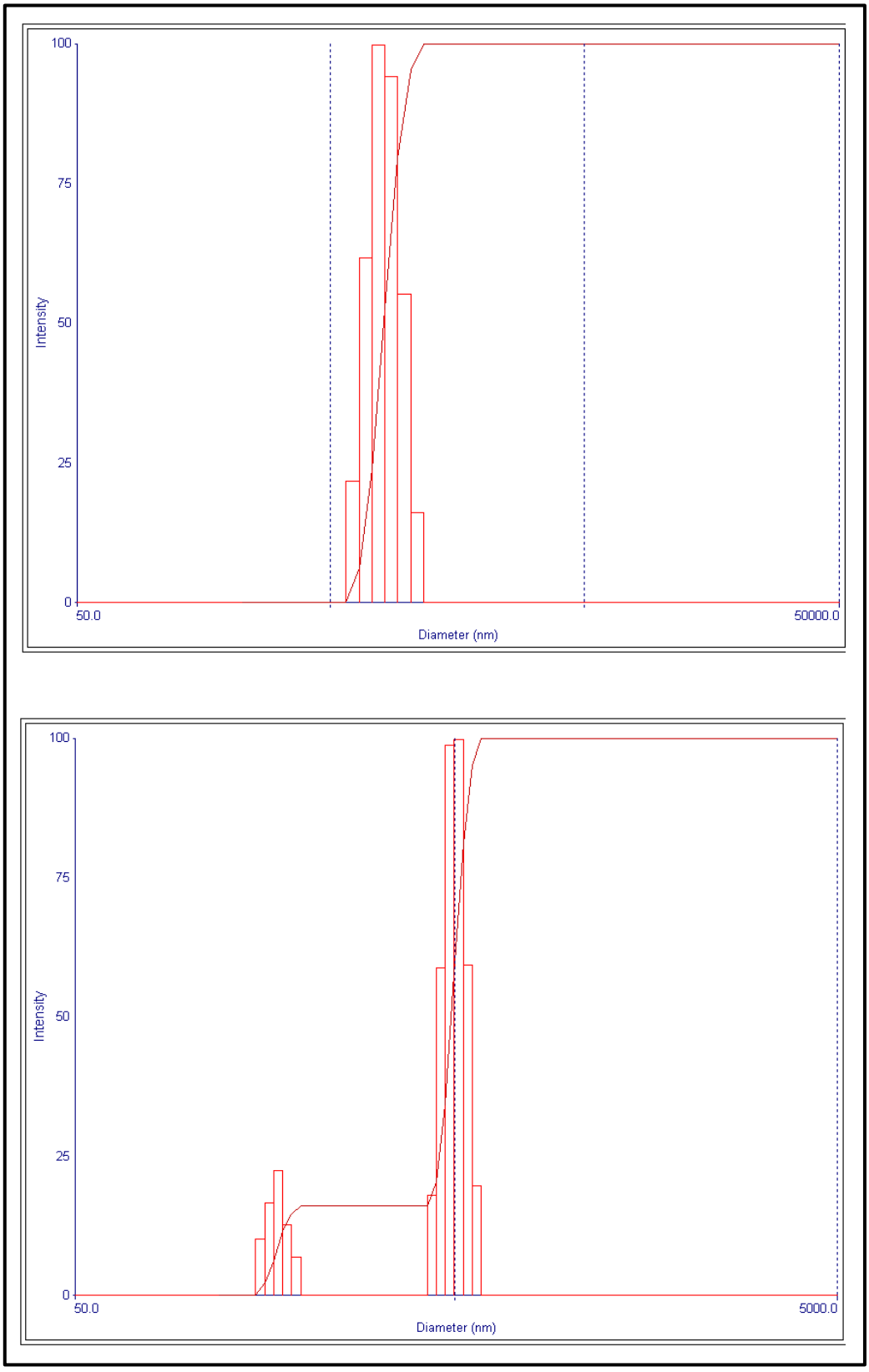

Figure 4.13. Nano-PSA results for C-AN4 at 25 ºC and 40 ºC, respectively

4.7.2. FTIR Analysis

C-AN4 and HFULL-NH-C-AN4 samples are analyzed by FTIR. Full hydrolysis sample exhibits an overwhelming broad band of water, indicating moisture due to their hygroscopic nature of hydroxyl groups of PVA. There are no carbonyl stretches (1737.67cm^{-1}) and CH_3 peaks (1367.77cm^{-1}) of the acetate groups in HFULL-NH2-C-AN4 compared to C-AN4 (Figure 4.14, Figure 4.15). Only the hydroxyl broad band (*ca.* 3300 cm^{-1}) from the PVA segments and the carbonyl stretch of the PNIPAM block can be characterized. These results show that hydrolysis is successfully conducted.

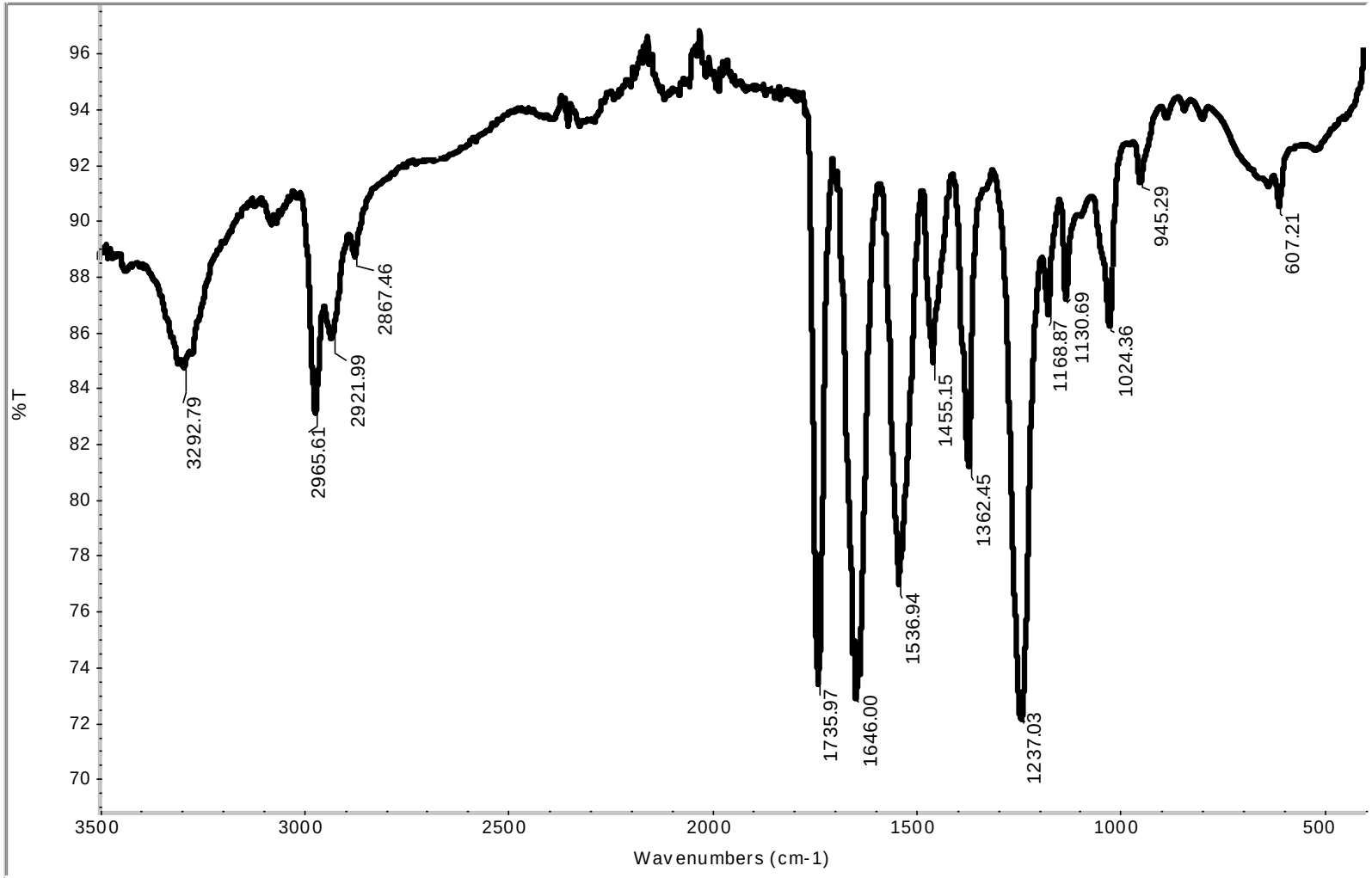

Figure 4.14. FTIR spectrum of C-AN4

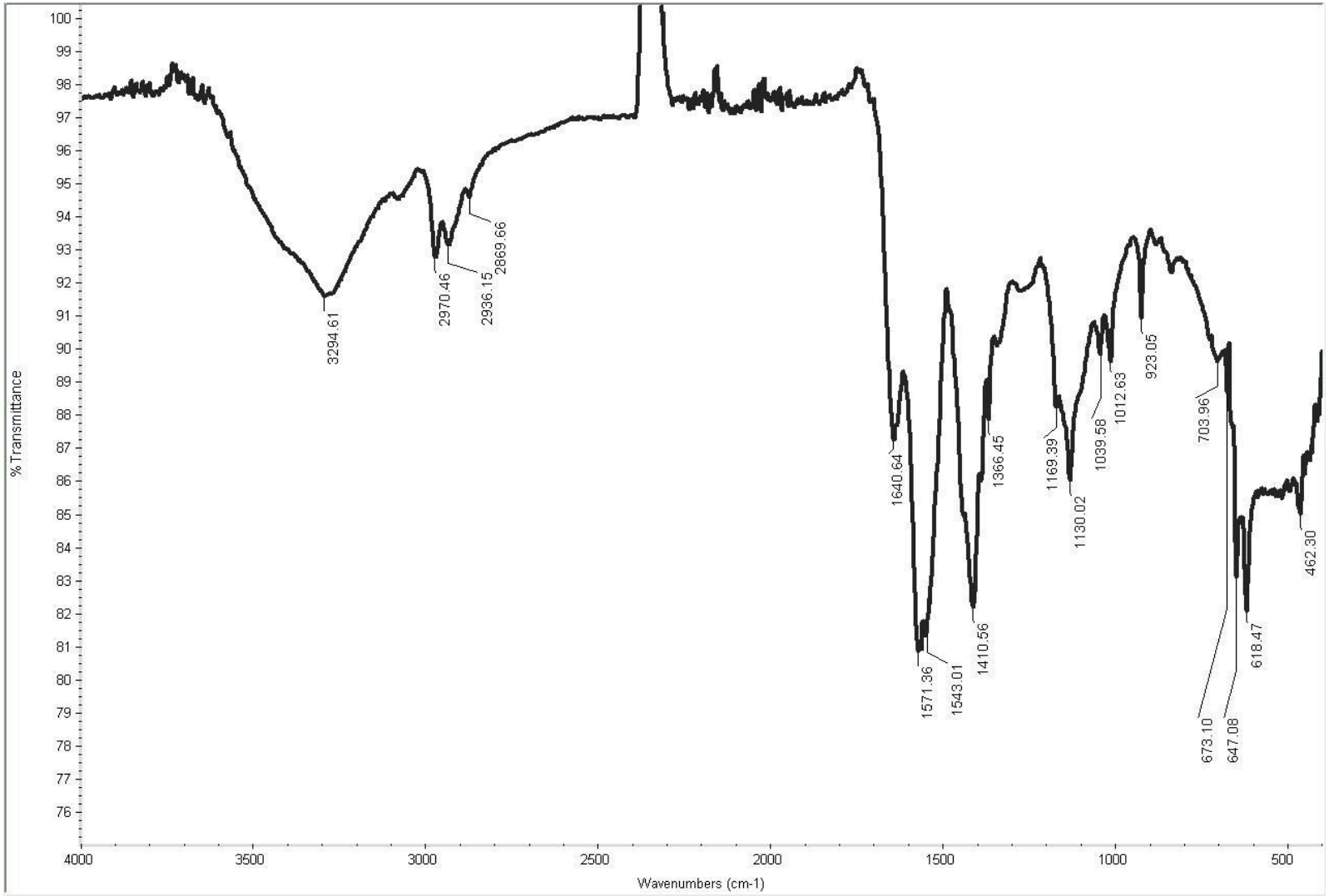

Figure 4.15. FTIR spectrum of HFULL-NH2-C-AN4

4.7.3. Fluorescence Spectroscopy Analysis Using Pyrene as a Fluorescence Probe for Determining Critical Micelle Concentration (CMC) of Micelles

Pyrene, which has a very low solubility in water ($6x10^{-7}$M), is hydrophobically attracted and attached to the micellar cores with stability and thus solubilized in water [72, 73]. As a result, pyrene can be detected even at very low concentrations via micelles in water by the fluorescence spectroscopy technique and with changing micelle concentrations, fluorescence intensity changes proportionally [11].

The data acquired with respect to emission wavelength at 390nm, are overlaid as shown in Figure 4.16. CMC of PVAc-b-PNIPAM block copolymer (1:1 with respect to PVAc and PNIPAM block lengths) is found from the curve of ratios of excitation intensities at 337nm and 334nm versus minus logarithm of concentrations (Figure 4.17). The convergence point of two tangent lines drawn on the curve indicated a –log C value which equals to 2.39. Further calculations using this value gave a concentration of $4.07x10^{-3}$ g/L which is the CMC of the block copolymer.

The expected low CMC of PVAc-b-PNIPAM block copolymer is in well agreement with the fact that high molecular weight block copolymers exhibit low CMC's due to early start of significant micellar associations. Thus, formation of micellar structures occurs even in very dilute solutions [10].

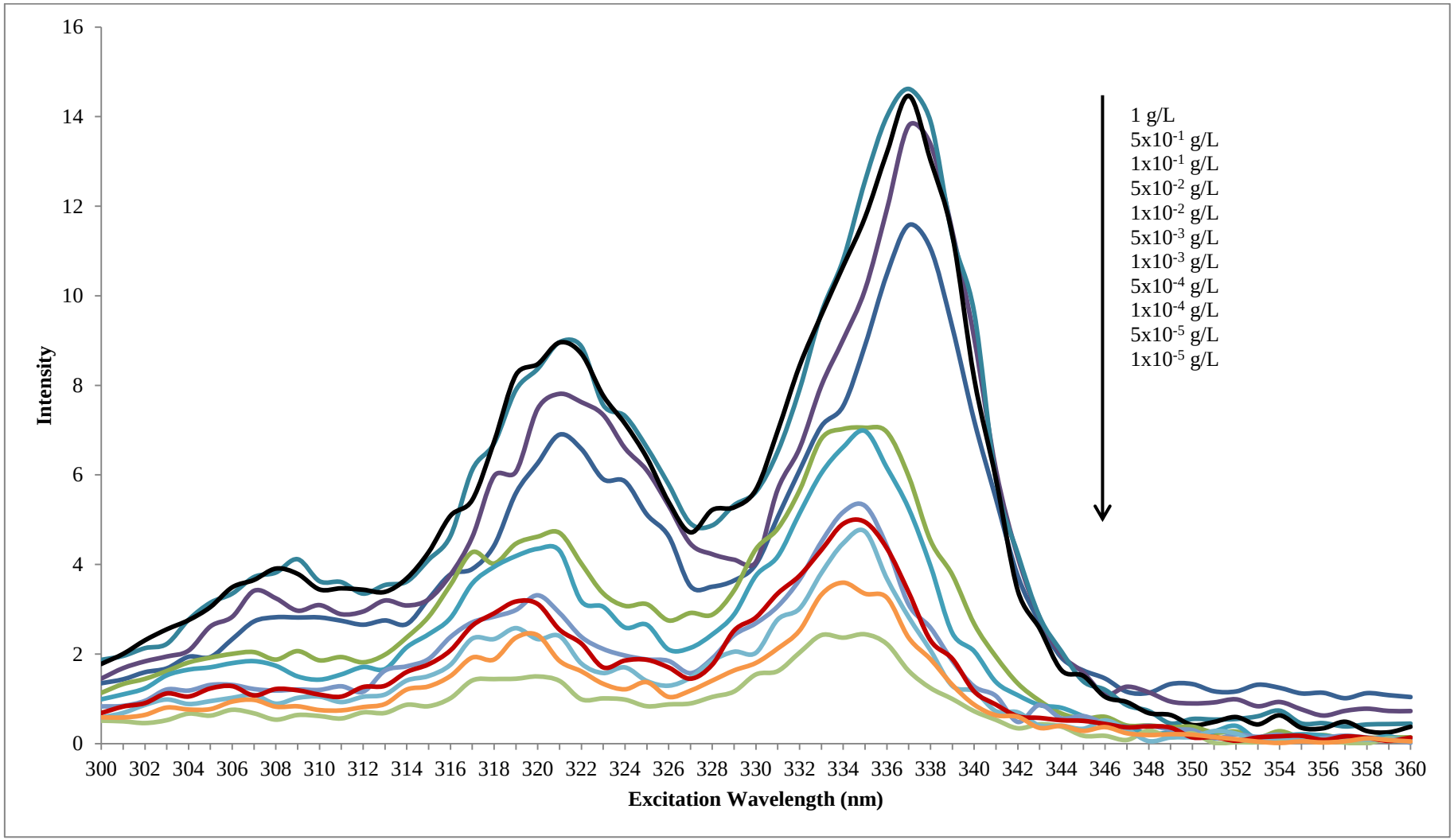

Figure 4.16. Fluorescence pyrene excitation curves, with respect to decreasing concentrations

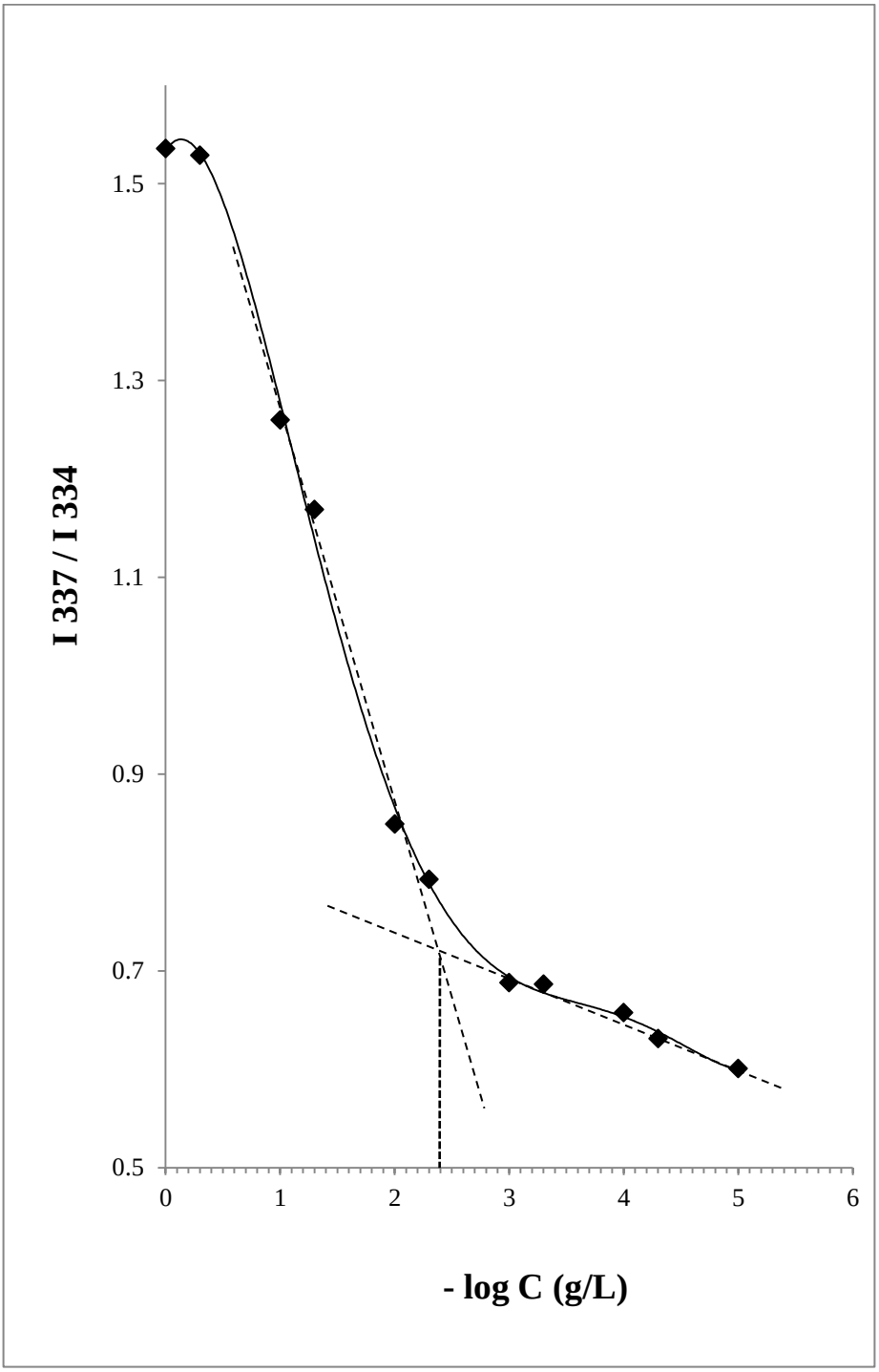

Figure 4.17. Fluorescence excitation spectrum intensity ratios of I_{337}/I_{334} (with pyrene as fluorescence probe) as a function of minus log of polymer concentration (- log C)

4.7.4. Drug Loading and *In Vitro* Release Analyses of Micelles Loaded with a Hydrophobic Drug

The hydrophobic drug used throughout the analyses is a water insoluble anti-cholesterol drug called Ezetimibe (Figure 4.18). The maximum absorbance of the drug is determined at a wavelength of 296nm by the UV-Visible spectrophotometer (Figure 4.19).

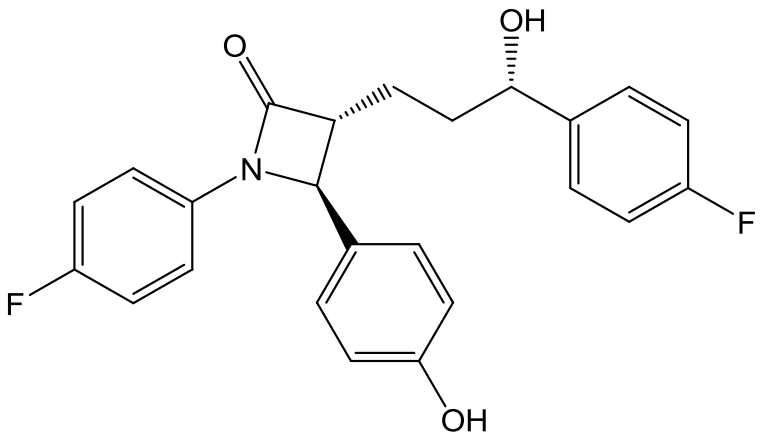

Figure 4.18. Structure of Ezetimibe ((3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one)

Drug loading efficiency is expressed as the amount of hydrophobic drug that could be hydrophobically entrapped inside the core of block copolymer micelles during dialysis. This efficiency is calculated from the absorbance ratios of drug loaded micelles and solution of free drug dissolved in acetone at 296nm. The result indicates a 49% drug loading efficiency (Figure 4.19).

Drug release results show that the absorbance values from the release media for 1-6 hours are almost the same and absorbance ratios indicate about 31% by weight of Ezetimibe is released from polymeric micelles. In the control experiment, free drug releases about 86% by weight of the drug from the dialysis tubing quickly within 2 hours (Figure 4.20). It is observed that drug is slowly released from block copolymer micelles in these experiments [12]. On the other hand, in 48 hours, amount of Ezetimibe decreases to 20% by weight in the release media for the drug loaded micelle experiment. The same trend of decreasing values is also observed in free drug release experiment. These

unexpected results can be attributed to slow decomposition of Ezetimibe at neutral or basic media, such as the phosphate buffer release solution in this case [74].

Contrary to the fact of decomposition of Ezetimibe occurring in the release media, *in vitro* drug release studies are successfully conducted with high drug loading efficiency and slow release behavior owing to the block copolymeric micelle system.

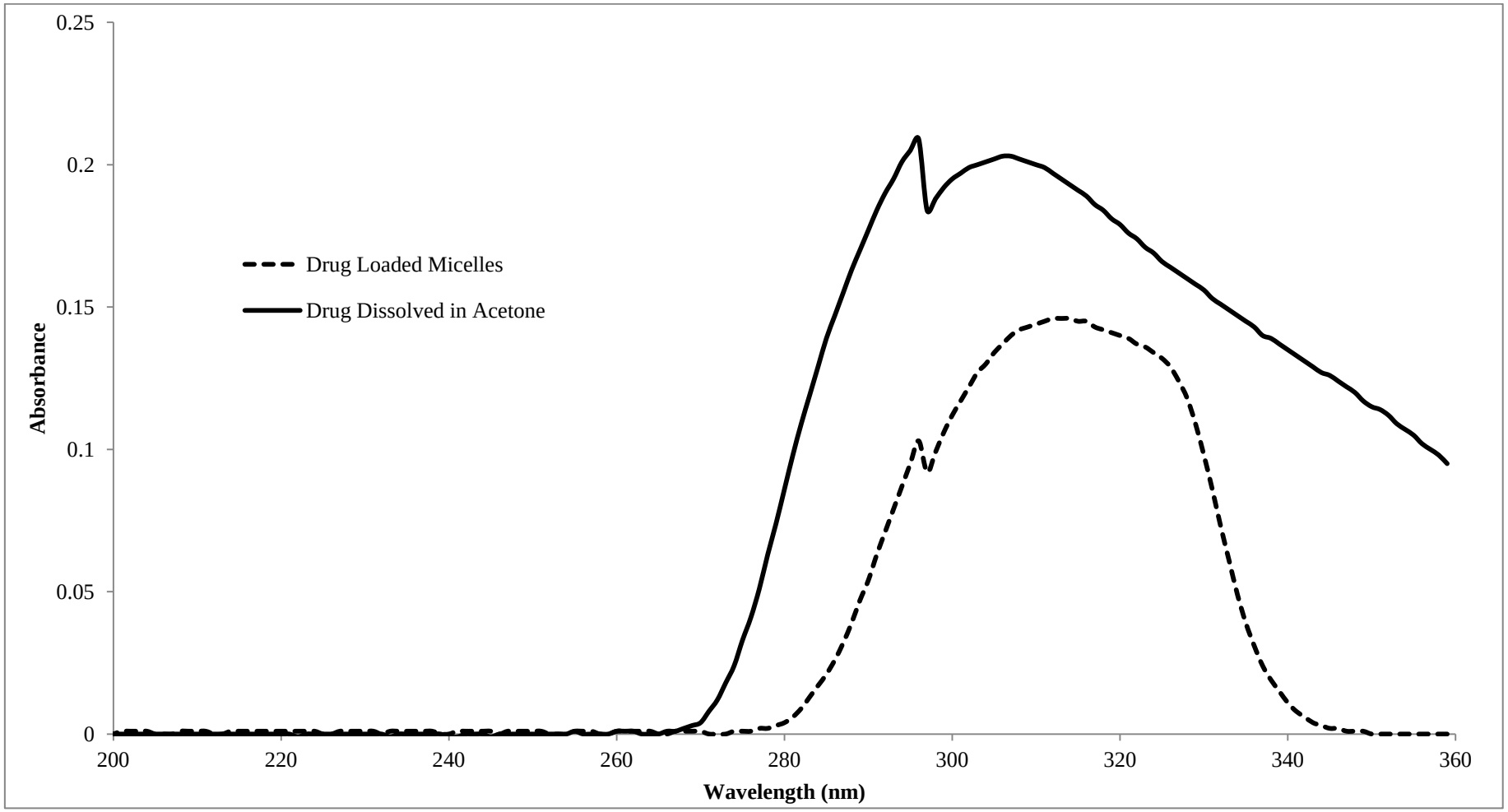

Figure 4.19. UV-Visible curves for drug loaded micelles and free drug (drug dissolved in acetone) as absorbance versus wavelength (nm)

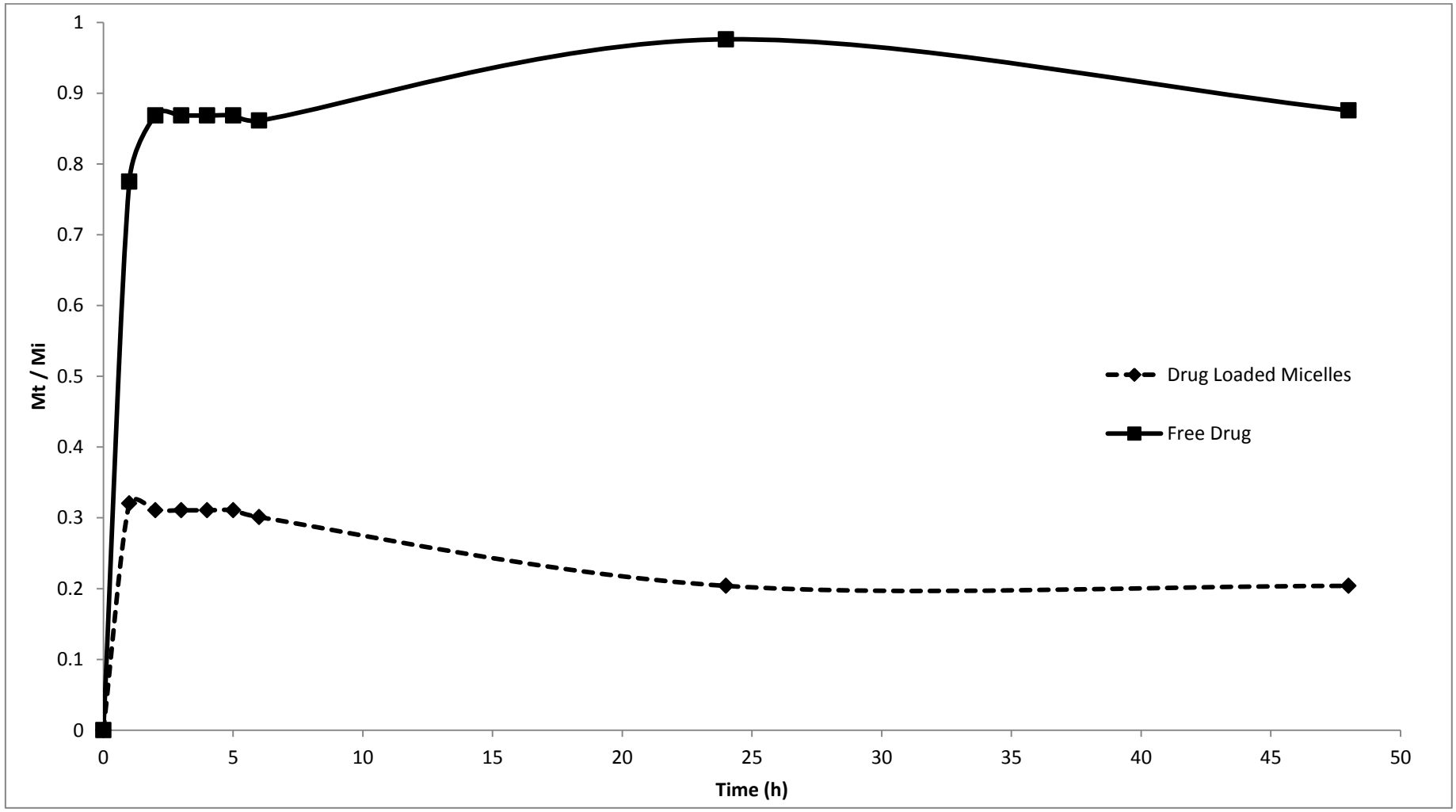

Figure 4.20. *In vitro* drug release amounts in PBS for drug loaded micelles and free drug with respect to weight fraction versus time

5. CONCLUSIONS

In this study, PVAc-b-PNIPAM block copolymers, which are previously synthesized by MADIX, are successfully hydrolyzed by methoxide-catalysis for the first time, to obtain PVAc/PVA-b-PNIPAM thermoresponsive amphiphilic and dihydrophilic block copolymers, while preserving back-bone structures owing to n-butylamine usage.

Success of hydrolysis is confirmed and solution and drug release properties of the products are investigated by using ^{1}H NMR, FTIR, ESEM, DSC, Nano-PSA, Fluorescence and UV-Visible Spectroscopy techniques.

^{1}H NMR analysis results showed the autocatalytic trend of PVAc homopolymer hydrolysis. Using the same technique, per cent hydrolysis values for block copolymer hydrolysis is confirmed. Partial hydrolysis of 67.7% within 30 minutes and full hydrolysis within 360 minutes of reaction time was achieved. PNIPAM block was not affected by hydrolysis.

The occurrence of hydrolysis is proved by the FTIR spectra with respect to characteristic peaks.

ESEM and Nano-PSA analyses are done using micellar solutions prepared with or without dialysis technique. ESEM results indicated good particle size distribution for block copolymer solution with 1:1 block lengths prepared with dialysis. Without dialysis, agglomerate formations are observed and smaller micelle sizes are measured. It is also found that with increasing PNIPAM shell lengths, micelle sizes increase. On the other hand, due to thin-shell stabilization effect, block copolymer with shorter PNIPAM block exhibited tubular micelles and larger micelles due to associations. Nano-PSA results also showed that larger micelle sizes and better size distributions are observed for solutions prepared with dialysis. The micelles are more stable when dialyzed solutions are diluted. Temperature has an effect of reducing micelle sizes due to collapsing of PNIPAM shell above its LCST.

With the DSC technique, glass transition temperatures (T_g) and LCST of the block copolymers are measured. The T_g of the PVAc/PVA block in the hydrolyzed block copolymer could not be measured due to incomplete drying. Fortunately, T_g of PNIPAM block is measured and an increment by a few degrees Celcius after hydrolysis is found. LCST of block copolymers with different compositions and the hydrolyzed samples do not deviate much compared to the LCST of the PNIPAM homopolymer.

Fluorescence spectroscopy is used to find the critical micelle concentration (CMC) of the block copolymer with 1:1 block length composition. The result, which is 4.07×10^{-3} g/L, is in well agreement with the fact that block copolymers have very low CMC's.

In-vitro drug release studies of the drug-loaded micelles are done successfully by monitoring the release media via UV-Visible spectroscopy. Even though the hydrophobic drug, Ezetimibe, decomposes over time, high drug loading capability and slow-release profile of the block copolymer micelle solution is proven.

6. FUTURE WORKS

As future works, investigation of solution properties of hydrolyzed block copolymers and synthesizing hydrogels from these samples are planned. Block copolymers with 1:2 and 1:0.5 compositions with respect to block lengths will be partially and fully hydrolyzed with the usage of amine for preserving polymer back-bone. Other hydrolysis methods such as NaOH-catalyzed transesterification with amine usage are under ongoing investigation.

It is anticipated that depending on the degree of hydrolysis and ambient temperatures, micellar and thermoresponsive dihydrophilic, dihydrophobic, amphiphilic and inverse-micellar structures will be achieved using hydrolyzed block copolymers in aqueous media.

A new RAFT procedure will be followed in order to decrease the formation of PVAc homopolymer during the block copolymer synthesis.

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