REACTIVE POLYMERIC PLATFORMS

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To My Family

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ABSTRACT

REACTIVE POLYMERIC PLATFORM

Synthesis of polymers bearing reactive functional groups is an active area of research due to their potential applications in interdisciplinary areas such as biomaterials, material science and drug delivery. Most of the methods for accessing polymers containing reactive maleimide groups as side chains have relied on a single or multistep transformations of the functional groups in the parent polymer obtained after the polymerization reaction. Non-quantitative nature of such transformations and possible side reactions, coupled with the difficulty in reliable quantification makes it highly desirable to develop alternative methods.

The methodology developed provides easy access to polymers decorated with maleimides as 'reactive' functional groups. The design involves synthesis of a conceptually novel acrylate based 'latent – reactive' monomer where the maleimide double bond is protected using a Diels-Alder reaction. After the polymerization reaction, thermally induced retro Diels-Alder cycloreversion reaction quantitatively converts the protected maleimide units to their reaction form. Thus obtained reactive polymer was conjugated with octanethiol to demonstrate efficient reactivity of the maleimide side chains of the polymer.

As an extension of this work, polymers containing two orthogonal functional groups as side chains, namely thiol reactive maleimide and amine reactive succinimide units were synthesized. Selective functionalization of the maleimide side chains in presence of succinimide groups by addition of octanethiol was achieved.

In the second part of the study, the reactive polymers were used to fabricate reactive micro arrays via a low-cost and efficient method, namely, micro contact printing. These micro arrays were labeled with a thiol containing fluorescent-dye to show that these surfaces can be used for immobilization purposes.

ÖZET

REAKTİF POLİMERİK PLATFORMLAR

Reaktif guruplara sahip polimerlerin sentezi biyokonjugasyon alanındaki uygulamalar nedeniyle çok aktif bir araştırma konusudur. Polimer zinciri üzerindeki reaksiyonları yapabilmek için en az iki veya üç adım gerektiren dönüşümlerin yapılması gereklidir. Polimer zincirleri üzerinde tepkime yapmak ise veriminin ölçülememesi, istenmeyen yan tepkimeler oluşması gibi zorluklar doğurur. Bu zorluklara çözüm bulmak adına, bu araştırmada gerek biyolojik alanda gerekse malzeme biliminde uygulama alanı bulabilecek polimerler sentezlenmiştir.

Bu araştırmada kullanılan yöntem ile reaktif grup olarak maleimid fonksiyonel grubunu barındıran ve biokonjugasyon alanında kullanılabilecek yeni monoblok kopolimerler sentezlenmiştir. Bu yönteme ek olarak, iki farklı reaktif grubu bünyesinde barındıran polimerlerin sentezi üzerinde de çalışılmıştır. Bu sentez ile, reaktif grup olarak maleimid ve succinimid birimlerini kullanarak farklı iki molekülle konjugasyon olanağı sağlayan polimerler elde edilmiştir.

Bu çalışmanın bir uzantısı olarak bünyesinde iki farklı reaktif fonksiyonel grup barındıran polimerler sentezlenmiştir. Bu fonksiyonel gruplar, tiol reaktif meleimide ve amin reaktif succinimid grupları olarak seçilmiştir. Bu polimerin oktan tiol ile reaksiyonu sonucunda tiol gruplarının maleimid grupları ile seçici olarak konjugasyon yaptığı gözlemlenmiştir.

İkinci bölümde, sentezlenen polimerler biyomoleküllerin bağlanabilmesi için farklı yüzeyler üzerinde ince filmler olarak hazırlanmıştır. Bu ince filmlerle konjugasyon yapılabileceğini göstermek amacıyla, yüzeyler ilk olarak floresan özelliğe sahip boyalar ile konjuge edilmiş ve başarılı sonuçlar gözlemlenmiştir.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv
ABSTRACT v
ÖZET vi
LIST OF FIGURES ix
LIST OF TABLE xiv
LIST OF PICTURES xv
LIST OF SYMBOLS/ABBREVIATIONS xvi
1. INTRODUCTION. 1
2. DIELS-ALDER REACTION IN POLYMER CHEMISTRY 4
2.1. Polymerization via Diels-Alder Reaction. 5
2.2. Cross Linking via Diels-Alder Reaction 8
3. MALEIMIDES IN BIOMOLECULAR IMMOBILIZATION 15
3.1. Maleimide Terminated Surfaces 15
3.2. Functionalization of Maleimide on the Polymers 19
4. AIM OF THE STUDY 21
5. RESULTS AND DISCUSSION. 23
5.1. Latent-Reactive Maleimide based Monomer: Synthesis and Characterization . 23
5.2. General Methods and Materials. 24
5.2.1. Synthesis of the Furan-Maleic Anhyride Adduct 24
5.2.2. Synthesis of the Maleimide Adduct 25
5.2.3. Synthesis of Furan-Maleimide Monomer 25
5.3. Maleimide Based Copolymers: Synthesis, Characterization and Functionalization 27
5.3.1. General Polymerization Procedure 28
5.3.2. General Procedure for retro Diels-Alder Reaction. 32
5.3.3 Addition of Thiol to Reactive Polymer. 36
5.4. Orthogonally Functionalizable Polymers. 38
5.5. Copolymers Containing Maleimide and Succinimide Side Chains: Synthesis, Characterization and Functionalization. 39
5.5.1 Synthesis of the Succimide Monomer. 40

5.5.2 Polymerization of Succinimide Monomer. 41
5.5.3 Polymerization of Succinimide and Maleimide Monomer. 42
5.5.4 Retro Diels-Alder Reaction of Succinimide-Maleimide Copolymer. . . . 43
6. MICRO CONTACT PRINTING 47
6.1. General Methods for μCP 48
6.1.1. Preparation of Mold for the Preparation of PDMS Stamp 48
6.1.2. Preparation of the PDMS Stamp and μCP Process. 49
6.3.1. Coupling of the Fluorescent Dye to the Patterned Surfaces. 52
7. CONCLUSIONS 54
APPENDIX A: SPECTROSCOPY DATA 55
REFERENCES. 76

LIST OF FIGURES

Figure 1.1. Polymeric microarrays with immobilized DNA strands on them 3

Figure 1.2. A graphic overview of a biochip 3

Figure 2.1. Representation of the DA and rDA reactions 4

Figure 2.2. Diels-Alder polymerization of AA and BB type of monomers 6

Figure 2.3. Diels-Alder polymerization of AB type of monomer 6

Figure 2.4. Diels-Alder polymerization of 2-furfurylmaleimide 7

Figure 2.5. Diels-Alder polycondensation of difunctional monomers 7

Figure 2.6. Post modification strategy. 9

Figure 2.7. DA strategy in hydrogel formation 10

Figure 2.8. Modification of sytrene polymers for crosslinkling 11

Figure 2.9. Use of bismaleimides as crosslinkers 13

Figure 2.10. Croslinking of anthracene polymers. 14

Figure 3.1. Self-assembled monolayer used to immobilize thiol-terminated ligands. . 16

Figure 3.2. SAMs bearing succimidyl ester and maleimide groups and a bridge peptide structure on the gold surface. 17

Figure 3.3. Schematic representation of the procedure for preparing PEG-grafted, maleimide terminated surfaces for the immobilization of thiolated DNA oligonucleotides 18

Figure 3.4. Synthesis of biomolecule conjugated initiator 19

Figure 3.5. Post functionalizaiton of 1° amine terminated polymer 20

Figure 3.6. Synthesis of maleimide terminated polymer 20

Figure 4.1. Illustration of polymer synthesis via DA / rDA sequence. 21

Figure 5.1. Latent –reactive monomer synthesis 23

Figure 5.2. Synthesis of furan-maleic anhyride adduct. 24

Figure 5.3. Synthesis of the maleimide adduct 25

Figure 5.4. Synthesis of latent-maleimide containing monomer. 26

Figure 5.5. HPLC of the latent-maleimide containing monomer. 26

Figure 5.6. ^{1}H NMR spectrum of latent-maleimide containing monomer 27

Figure 5.7. ^{13}C NMR spectrum of latent-maleimide containing monomer. 27

Figure 5.8. Synthesis of reactive copolymer 28

Figure 5.9. Synthesis of latent-maleimide containing polymer 29

Figure 5.10. ^{1}H NMR spectrum of latent-maleimide polymer 29

Figure 5.11. ^{13}C NMR spectrum of latent-maleimide polymer. 30

Figure 5.12. Comparison of ^{1}H NMR spectrum of monomer and polymer 31

Figure 5.13. Synthesis of the reactive polymer. 32

Figure 5.14. ^{1}H NMR spectra of polymer before and after rDA. 34

Figure 5.15. ^{13}C NMR spectrum of reactive polymer 34

Figure 5.16. Thermogravimetric analyses of polymers 35

Figure 5.17. DSC thermogram of P2 36

Figure 5.18. Addition of thiol to maleimide polymer 37

Figure 5.19. ^{1}H NMR spectra of polymer before and after thiol addition 38

Figure 5.20. Orthogonal functionalization 39

Figure 5.21. Synthesis of polymers bearing two reactive moeities. 40

Figure 5.22. Synthesis of succinimide containing monomer 41

Figure 5.23. Synthesis of succinimide containing polymer 41

Figure 5.24. ^{1}H NMR spectrum of succinimide containing copolymer. 42

Figure 5.25. Synthesis of succinimide-maleimide containing polymer 43

Figure 5.26. ^{1}H NMR spectrum of succinimide-maleimide copolymer 43

Figure 5.27. Retro DA reaction of succinimide-maleimide containing copolymer . . 44

Figure 5.28. ^{1}H NMR spectrum of succinimide-maleimide copolymer after rDA. . . 44

Figure 5.29. Thiol addition to the succinimide-maleimide containing copolymer. . 45

Figure 5.30. ^{1}H NMR spectrum of thiol conjugated succinimide-maleimide copolymer. 46

Figure 6.1. Preparation of mold for PDMS stamp 48

Figure 6.2. Preparation of PDMS stamp 49

Figure 6.3. μCP process 51

Figure A.1. ^{1}H NMR spectrum of diels-alder adduct. 56

Figure A.2. ^{1}H NMR spectrum of of maleimide adduct 57

Figure A.3. ^{1}H NMR spectrum of maleimide monomer 58

Figure A.4. ^{13}C NMR spectrum of maleimide monomer. 59

Figure A.5. ^{1}H NMR spectrum of maleimide copolymer **P1** 60

Figure A.6. ^{1}H NMR spectrum of maleimide copolymer **P2** 61

Figure A.7. ^{13}C NMR spectrum of maleimide copolymer **P2** 62

Figure A.8. ^{1}H NMR spectrum of maleimide copolymer **P3** 63

Figure A.9. ^{1}H NMR spectrum of maleimide copolymer **P4**. 64

Figure A.10. ^{1}H NMR spectrum of maleimide copolymer **P5** 65

Figure A.11. ^{13}C NMR spectrum of maleimide copolymer 66

Figure A.12. ^{1}H NMR spectrum of octane thiol conjugated maleimide copolymer. . 67

Figure A.13. ^{1}H NMR spectrum of succinimide monomer 68

Figure A.14. ^{1}H NMR spectrum of succinimide copolymer 69

Figure A.15. ^{1}H NMR spectrum of succinimide-maleimide copolymer 70

Figure A.16. ^{1}H NMR spectrum of succinimide-maleimide copolymer after rDA . . 71

Figure A.17. ^{1}H NMR spectrum of thiol conjugated succinimide-maleimide polymer. 72

Figure A.18 IR spectrum of maleimide monomer 73

Figure A.19. IR spectrum of maleimide polymer 74

Figure A.20. IR spectrum of maleimide polymer after rDA reaction. 75

LIST OF TABLES

Table 5.1. Synthesis of latent-reactive monomer containing random copolymers . 32

LIST OF PICTURES

Picture 6.1. Microscopic view of the mold used for the preaparetion of PDMS stamp. 50

Picture 6.2. Microscopic view of the PDMS stamp. 50

Picture 6.3. Polymer islands on the surface. 52

Picture 6.4. Dye labelled maleimide based polymeric arrays on the surface. 53

LIST OF SYMBOLS / ABBREVIATIONS

J	Coupling constant
υ	Frequency
M_n	Number average molecular weight
AIBN	2,2'-azobisisobutyronitrile
BMI	Bismaleimide
BSA	Bovine Serum Albumin
DA	Diels Alder
DCC	N,N'-Dicyclohexylcarbodiimide
DIPEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
FTIR	Fourier transform infrared
GPC	Gel Permeation Chromatography
GPS	3-glycidoxypropyl trimethoxysilane
MHz	Mega hertz
MMA	Methyl methacrylate
NMR	Nuclear Magnetic Resonance
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PET	Polyethyleneterephthalate
rDA	retro Diels Alder
RNA	Ribonucleic acid
SAM	Self Assembled Monolayer
SEC	Size-Exclusion Chromatography
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
UV	Ultraviolet
μCP	Micro Contact Printing

1. INTRODUCTION

Micro arrays containing groups of biomolecules immobilized to a surface via bioconjugation between the substrates and biomolecules has given rise to the field of biochips. The development in biochip fabrication has dramatically increased the rate and scope of discoveries in basic and applied science. Examples include the development of DNA chips for genome analysis [1, 2], the preparation of protein chips for evaluation of protein-substrate interactions [3], and the construction of peptide [4], and carbohydrate chips [5] for the evaluation of ligand-receptor interactions and enzymatic activities. All these developments in biochip technology need reliable and reproducible chemistries for the immobilization of ligands to a single substrate.

The methods used for immobilization of biomolecules to surfaces or other molecules usually involve addition and substitution reactions with amine groups of lysine or the thiol of cysteine. The most widely used functional group for such attachments is the N-hydroxysuccinimide activated ester that reacts with amine containing molecules through amide bond formation [6]. In some cases, the maleimide group is chosen in biomolecular immobilization as an alternative due to the facile conjugate addition of thiols across the electron-deficient double bond without the need for any additional reagents [7]. Functionalization of biomolecules via thiols is often a much sought after approach in biomolecular immobilization since cystein residues can be engineered at specific sites of bimolecules.

Interfaces such as self assembled monolayers (SAMs) on metal surfaces are promising candidates for the fabrication of biochips surfaces. Metal surfaces like gold have been coated with self assembled monolayers (SAMs) containing maleimide by deposition of maleimide containing disulfide [7-9]. The maleimide containing SAMs were shown to undergo efficient functionalization with polypeptides and carbohydrate molecules. Silicon surfaces have also been recently modified with terminal maleimide containing ligands. These were shown to undergo efficient Diels-Alder reaction for immobilization and retro Diels-Alder reaction for analysis using mass spectroscopy [10].

While maleimide group have been extensively exploited in biomolecular immobilization on to gold surfaces, reports of polymeric surfaces modified by maleimides are rare. Recent work by Howorka and coworkers highlight the potential such platforms hold towards DNA oligonucleotide micro arrays [11]. Micro arrays refer to a two dimensional arrays of small reaction cells (each on the order of 100 X 100 μm or less) fabricated on a solid substrate. The solid substrate can be a silicon wafer, a thin sheet of glass, plastic or a nylon membrane. Biochips are similar to semiconductors, except that instead of having electronic circuits, they have biological material, DNA or RNA or protein, immobilized on the substrate via chemical bonds.

A DNA biochip can be explained in the following way, molecules from a specific sequence of single strand DNA fragment are anchored to the surface of the biochip in the multiple wells or on the hills that form the array [Figure 1.1]. The DNA fragments can either be short, about 20 to 25 sequences or longer strands of complementary DNA. The specific sequence of bases in each cell is preselected or designed based on the intended applications. The known sequences of single strand DNA fragments immobilized on the substrates are often called probes [Figure 1.2]. When unknown fragments of single strand DNA samples, called the target, react or hybridize with the probes on the chip, double strands DNA fragments are formed where the target and the probe are complementary according to the base-pairing rule. To facilitate the diagnosis or analysis of the hybridized chip, the target samples are often labeled with tags, such as fluorescent dyes, or radioisotope molecules. When the targets contain more than one type of sample, each is labeled with its own distinguishable tag. Depending on the size of the array, this kind of DNA micro array chip provides a platform where the unknown target or targets can potentially be identified with very high speed and high throughput by matching with tens of thousands of different types of probes via hybridization in parallel.

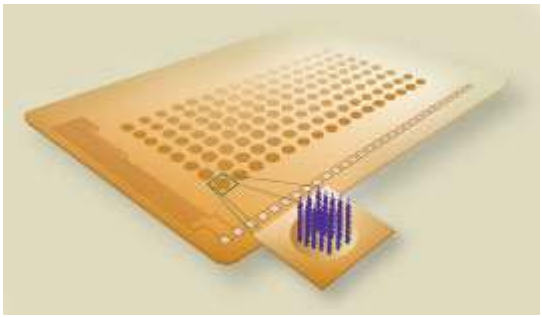

Figure 1.1. Polymeric micro arrays with immobilized DNA strands on them

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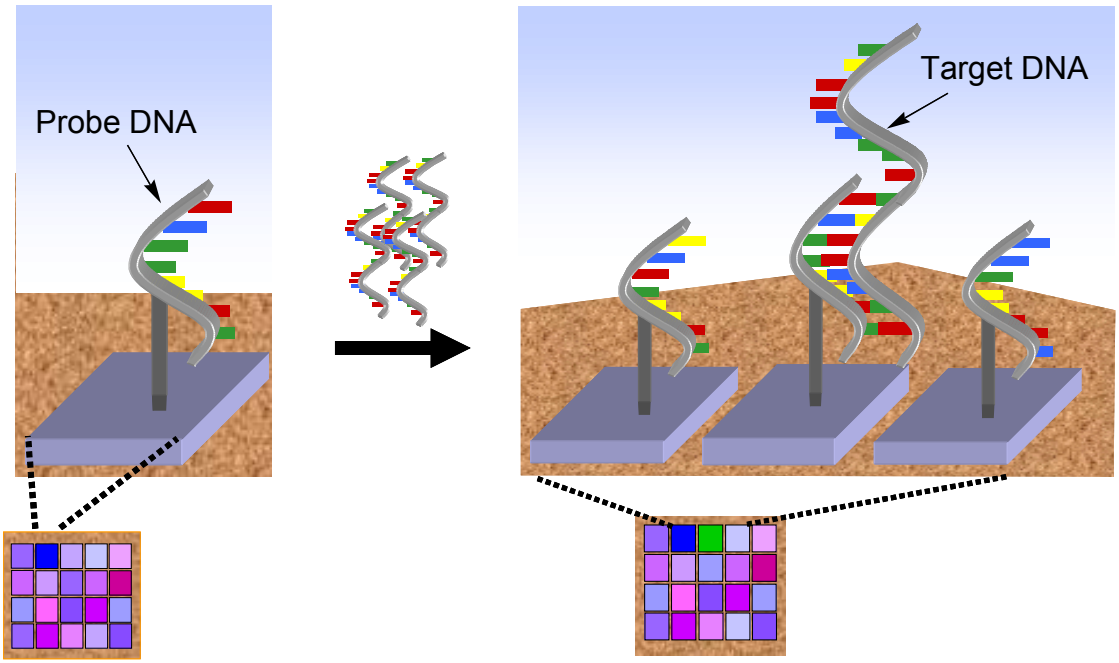


Figure 1.2. A graphic overview of a biochip

A variety of specific techniques are present that allows access to surfaces with polymeric micro arrays. The technique we are interested in our project is Micro-Contact Printing (μCP). Micro-Contact Printing (μCP) is a high-resolution printing technique in which a pattern is transferred from the channels of an elastomeric stamp to a solid substrate by conformal contact. This is one of several "Soft Lithography" technologies that are under development for high resolution patterning of a variety of materials for advanced applications. The related methodology and examples are disscussed in Chapter 6.

2. DIELS-ALDER REACTION IN POLYMER CHEMISTRY

The Diels-Alder (DA) reaction is one of the most widely used synthetic methods in organic synthesis [12, 13]. In this reaction a "diene" functional group reacts with a "dienophile" unit and generates new bonds by inter- or intramolecular cycloaddition reactions. In this cycloaddition reaction, addition of a dienophile to a conjugated diene results in a cyclic product referred as an adduct (Figure 2.1). Furan and anthracene derivatives have been widely explored as diene components in macromolecular construction whereas maleimide due to their high reactivity and wide structural variability through the nature of the nitrogen substituents, are preferred as the choice of dienophile (Figure 2.1). One of the most relevant and attractive aspects of the DA reaction is the thermoreversibility, which implies that the adducts can be readily converted to the starting material via retro-Diels-Alder (rDA) reaction [14].

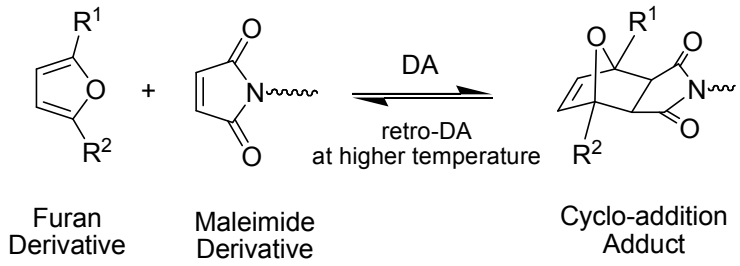


Figure 2.1. Representation of the DA and rDA reactions

In macromolecular chemistry, the DA reaction has been utilized to synthesize linear polymers as well as crosslink structures. Linear polymers have been synthesized through successive DA cycloadditions involving multifunctional complementary monomer, e.g. a di-furan derivative and a bismaleimide. For the crosslinked macromolecules, DA reaction have been utilized to induce the cross-linking to the polymer structure by taking the advantage of the inter-macro-molecular couplings with a difunctional complementary reagent such as furan copolymer plus bismaleimides or polymer bearing maleimide moieties plus difurans. The thermal sensitivity of the DA reaction makes it an attractive

candidate for thermally reversible cross-linking. These polymers can revert to their precursors through the rDA reaction and this feature can be exploited in many applications, like the possibility of recycling or “mending” network-based materials.

2.1. Polymerization via Diels-Alder Reaction

Utilization of the DA reaction in polymerization dates back to an early report about formation of oligomers of cyclopentadiene by Alder and coworkers in 1932 . In 1961, Stille and co-workers successfully accomplished the utilization of DA reactions in polymerizations to produce high molecular weight polymers [15]. In this study, they utilized the DA reaction between cyclopentadiene based dimers and bismaleimides. Although a seminal contribution, the study did not promise further developments due to the utilization of biscyclopentadienyl compounds that are highly prone to homopolymerize.

Quarter of a century later a report by Sastri and coworkers disclosed the synthesis of linear polymers from A-A and B-B type of monomers such as difurans and bismaleimides, respectively [16]. Sastri’s group used different difuran compounds and bismaleimides bearing aliphatic, aromatic, dimethylsiloxane spacers to synthesize heat resistant polymers. Since their aim was to obtain heat resistant polymeric structures, instead of retro Diels-Alder reaction, these polymers were converted to more stable structures via aromatisation. Since then several other groups have explored the utilization of furan and maleimide based building blocks for synthesis of novel polymeric materials.

In 1990s, DA polymerization has given rise to the synthesis of variety of polymers such as polyimides, polyurethanes and acrylic copolymers [17-18]. A series of papers which show the DA polymerization between difuran derivative and bismaleimide have been published in the early nineties. One of the studies involving the DA polymerization between a difuran and a bismaleimide has been done by Diakoumakos and Mikroyannidis in 1992 [19]. This work shows the application of the DA reaction for the preparation of polyimides. Certain AB or AA and BB type of monomers bearing maleimide and furan segments were used to obtain polyimides via DA reaction. The AA and BB type monomers, namely bismaleimide and bisfurfurylpyromellitimide, were polymerized via DA reaction to afford a polyimide which was then dehydrated to an aromatic structure

upon heating relatively higher temperatures (Figure 2.2). The second curing at a higher temperature has been achieved to improve thermal stability of the polymer.

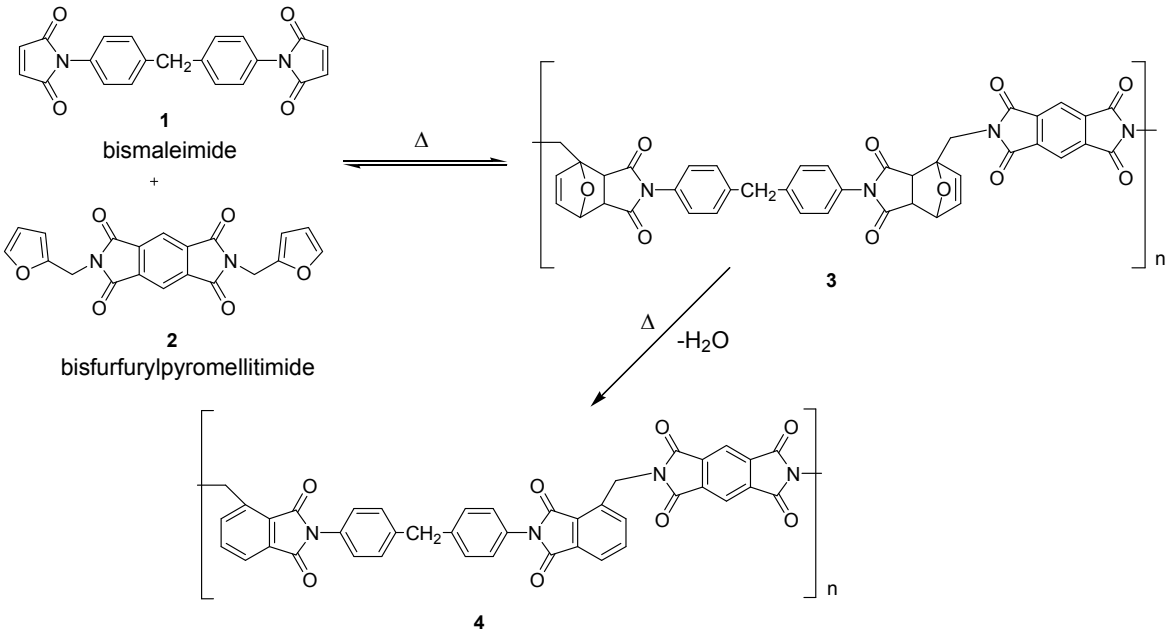


Figure 2.2. Diels-Alder polymerization of AA and BB type of monomers

AB type monomer which contained stoichiometric amount of diene and dienophile on the same molecule, namely furan and maleimide, was capable of intermolecular, thermally induced DA polymerization which resulted in a linear polyimide. Then, this polyimide was dehyrated at higher temperatures (Figure 2.3). This study was one of the first examples to prepare monomer bearing both a furan and a maleimide moeity.

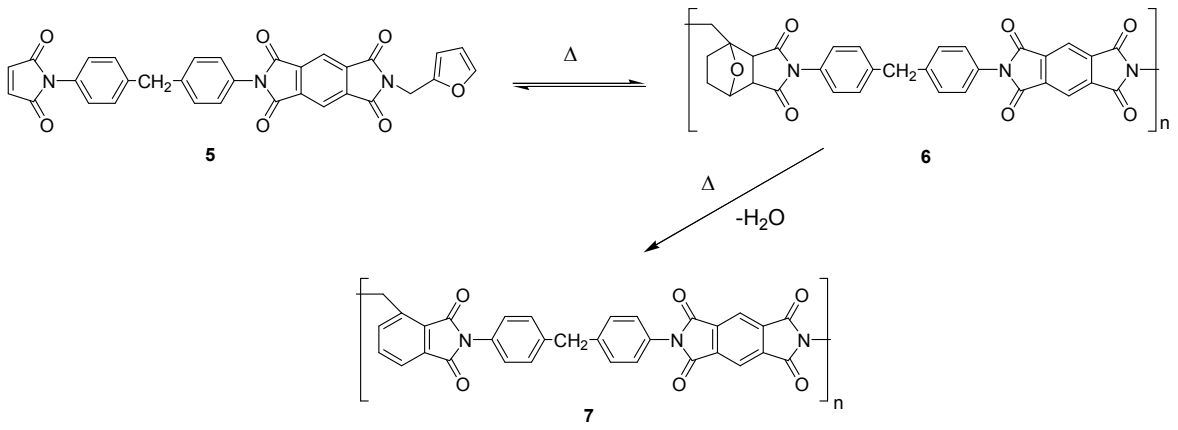


Figure 2.3. Diels-Alder polymerization of AB type of monomer

A new AB type monomer, 2-furfurylmaleimide **(8)**, was polymerized to afford partially soluble products, suggesting that the high temperatures necessary to induce self-condensation through the DA reaction were also responsible for some aromatization of the adducts [14] (Figure 2.4).

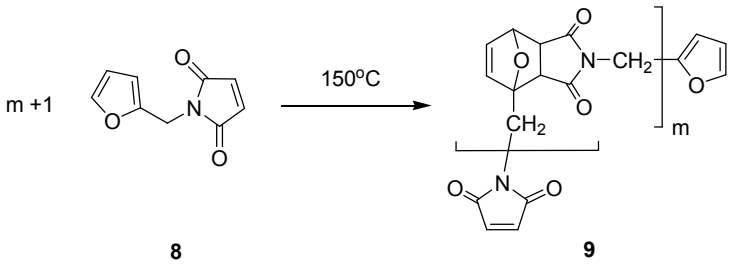


Figure 2.4. Diels-Alder polymerization of 2-furfurylmaleimide

Gandini's group with their broader and long-standing interest in furan polymers has been engaged in different polymer applications of the Diels-Alder reaction involving furanic structures. In their more recent studies, they investigated the DA polymerization of a difuran diacetal **11** with either aliphatic or aromatic bismaleimides **10** [20] (Figure 2.5). This AA & BB type monomers were polymerized via Diels-Alder type polycondensation in THF yielding the polymer **12**.

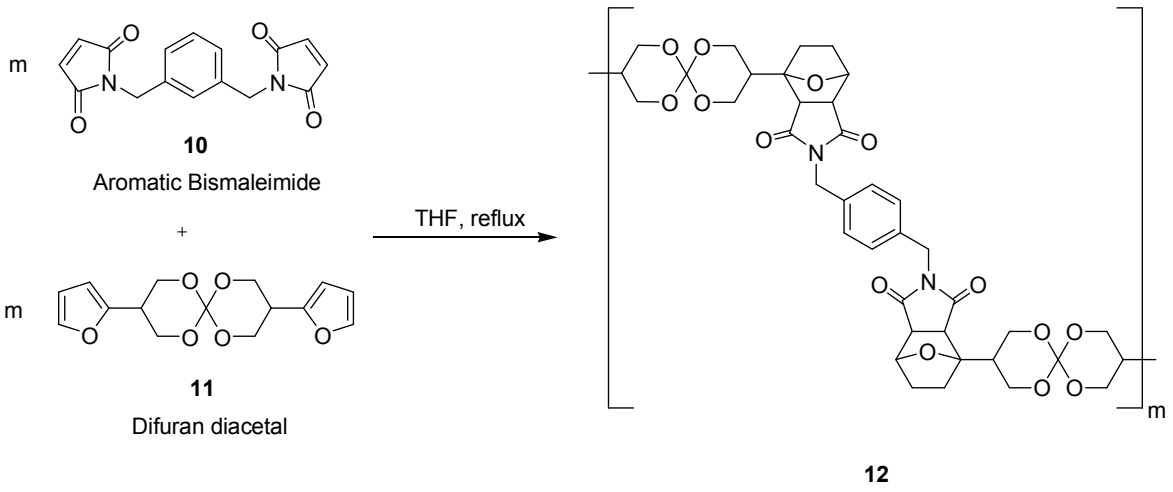


Figure 2.5. Diels-Alder polycondensation of difunctional monomers

Bismaleimides (BMIs) have been widely used as high performance composite matrices [21]. The Diels-Alder polymerization of bismaleimides is important in the field of thermally stable polymers. However, the poor solubility of BMIs in low boiling point solvents and the brittlenes of their cured networks are drawbacks for applications in many fields, especially as high thermal-oxidative stable adhesives. To overcome these problems BMIs containing silicon linkages were investigated by many research groups [22].

2.2. Cross Linking via Diels-Alder Reaction

Diels-Alder chemistry has also been explored to synthesize various crosslinked materials. The first study which explained the formation of thermally reversible networks from linear polymers bearing reactive dienes & dienophiles, namely furan and maleimide functionalities, has been done by Saegusa and Chujo in 1990 [23]. It was also the first example of a thermally reversible chemical hydrogel. The same study has been extended to the formation of the organic-inorganic polymer hybrids by Chujo in 2000. Chujo and co-workers introduced the maleimide and furan functionalities to the side chain of polymer **14** [24]. To achieve this they first hydrolyzed 10% of the amide groups of polymer **14** to the amine moiety (Figure 2.6). Then, this polymer was modified via DCC-DMAP coupling by maleimide and furan bearing groups.

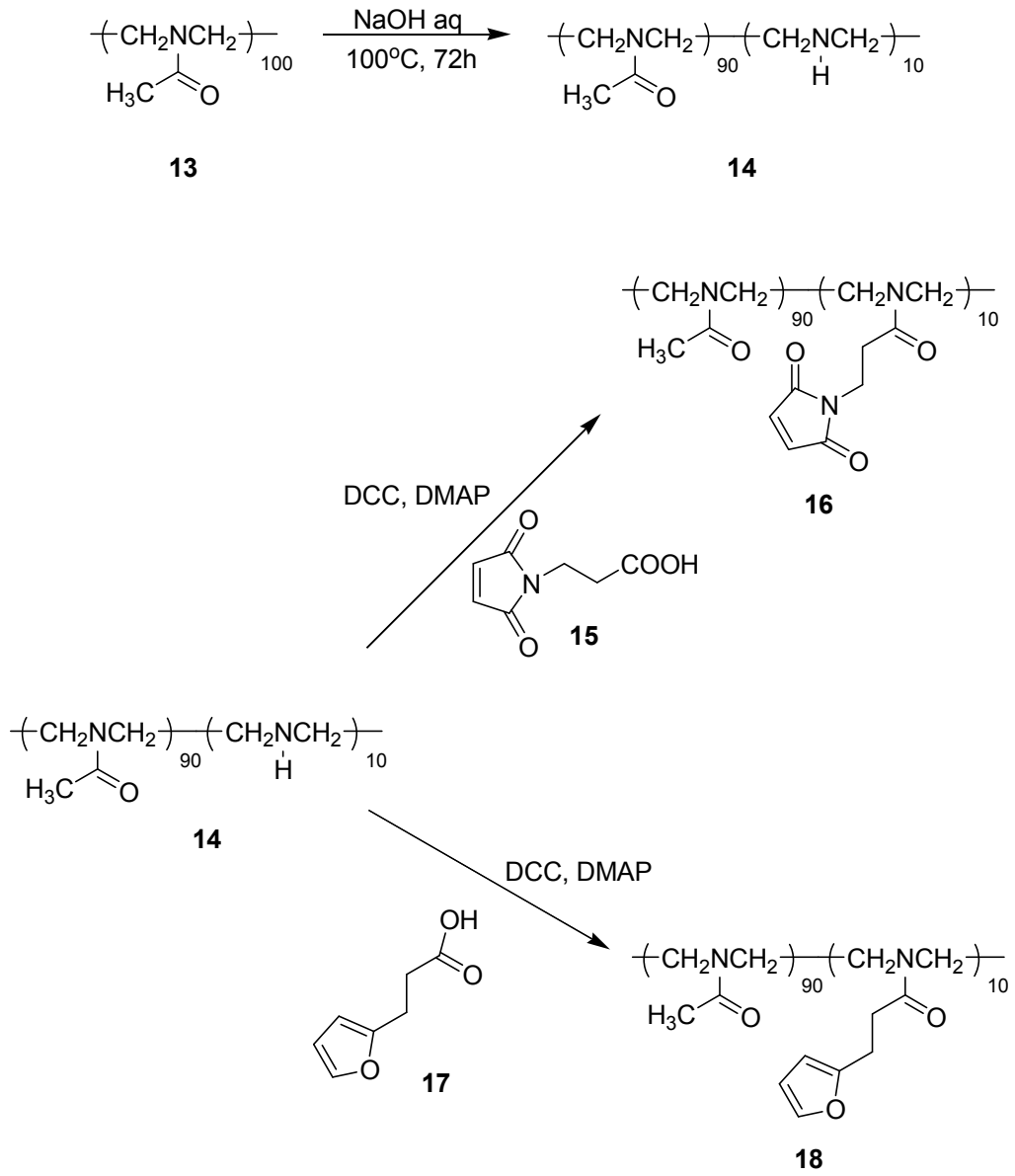


Figure 2.6. Post modification strategy

Polymers **16** & **18** were cross-linked with each other via DA reaction between maleimide and furan moieties of the linear polymers as mentioned in the first study (Figure 2.7) [23]. The resulting hydrogel did not dissolve in water but rather swelled in water as expected from a hydrogel. The rDA reaction took place at 80°C in a polar solvent and the degraded hydrogels become soluble.

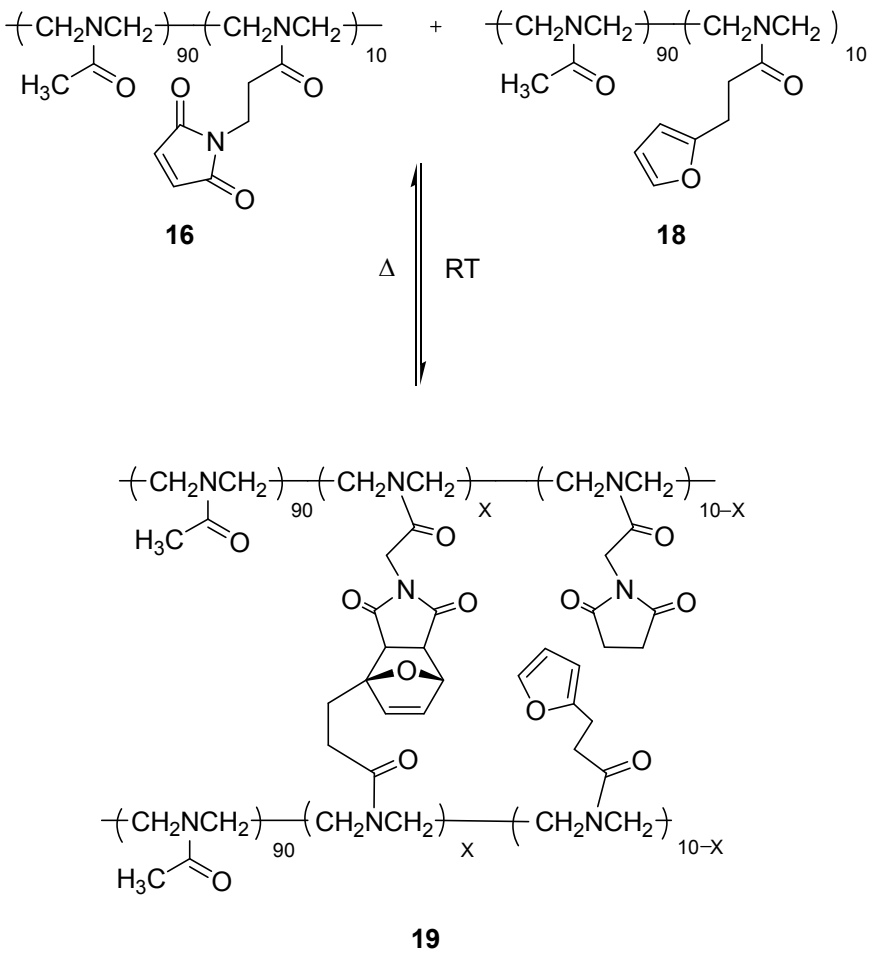


Figure 2.7. DA strategy in hydrogel formation

In 1992, Stevens used tin (IV) chloride-catalyzed Friedel-Crafts reaction to substitute the polystyrene polymer with N-chloromethylmaleimide [25]. To obtain the furan containing polymer, poly [styrene-co-(maleic anhydride)] was modified with furfuryl alcohol. Maleimide and furan modified styrene copolymers **26** & **27** were then used to synthesize the crosslinked polymer which was thermally reversible (Figure 2.8).

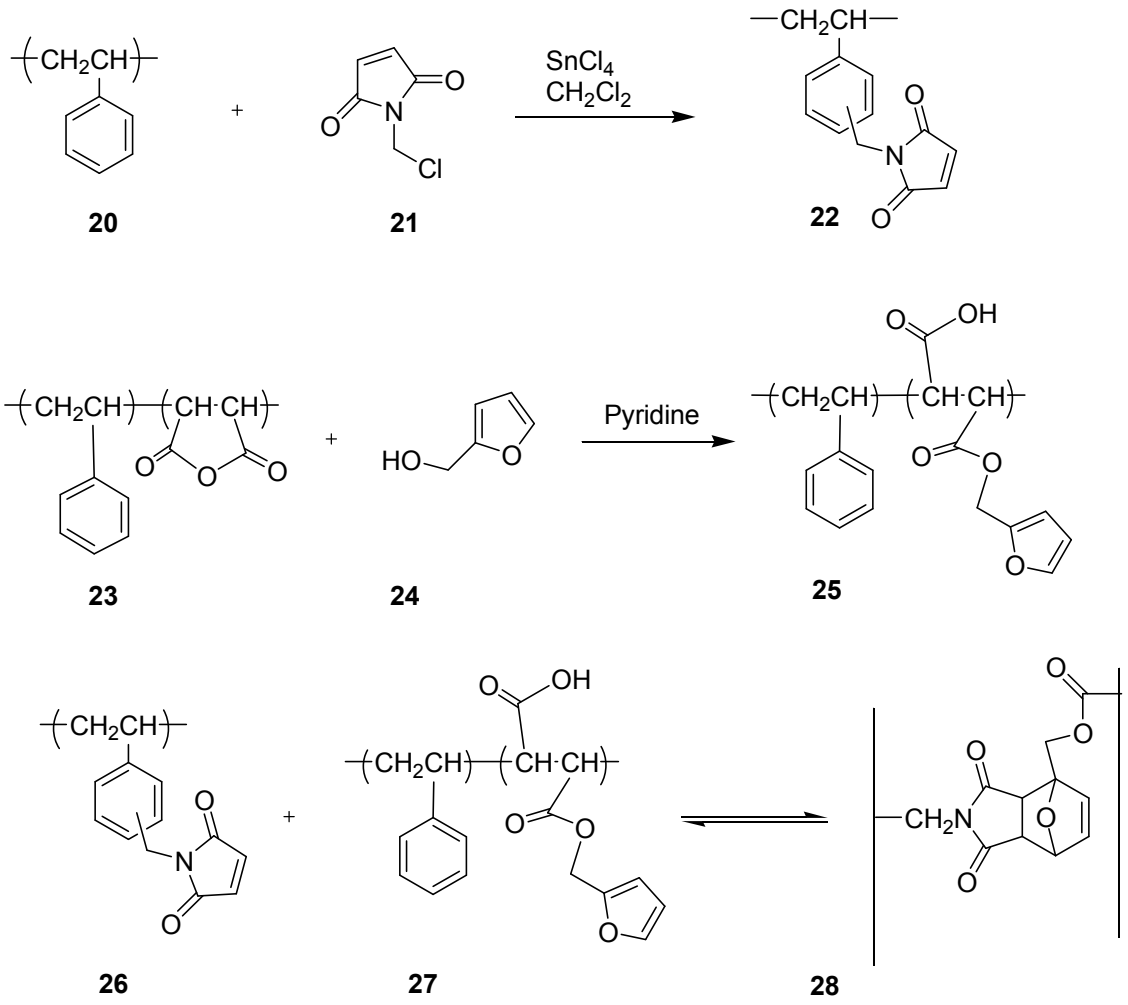


Figure 2.8. Modification of sytrene polymers for crosslinkling

Even when the thermally reversible crosslinking was achieved, substituted polymers posed stability problems because of the light sensitivity of the furfuryl groups. Moreover, insolubility of the maleimidomethylated polystyrene was observed upon standing for extended periods which might be due to free radical or photocycloaddition crosslinking. Furthermore, methods based on the strategy of post modification of the parent polymers to obtain maleimide contained polymer also limits the variability of polymer backbone since the addition of groups on the polymer backbone is not straightforward.

The thermoreversible nature of the Diels-Alder reaction makes it an attractive candidate for self healing polymeric materials. A crosslinked polymeric material was shown to possess such remendable property by Wudl *et. al.* [26, 27]. The authors demonstrated that cracks induced in the crosslinked material result in the fragmentation of

the furan-maleimide adduct. Upon heating the cracks in the damaged material were sealed and the material regained its mechanical properties to a large extent. Various examples of thermoreversible crosslinked polymeric materials have been reported by Gandini and Chujo [28, 29]. Since the reactive double bond of maleimide does not allow the use of a monomer containing this functional group, Gandini used a furan containing styrene copolymer and a bismaleimide crosslinking agent to synthesize thermally reversible crosslinked materials [30]. The retro-DA reaction of the polyadducts, by the use of excess furan trap, has been successfully carried out to obtain the original polymer. (Figure 2.9) This study may be considered as the first systematic approach to a reversible DA - rDA cross-linking system showing the validity of the DA – rDA strategy in the synthesis of elastomeric networks which have recycling properties by their convertion to thermoplastic properties through a rDA reaction.

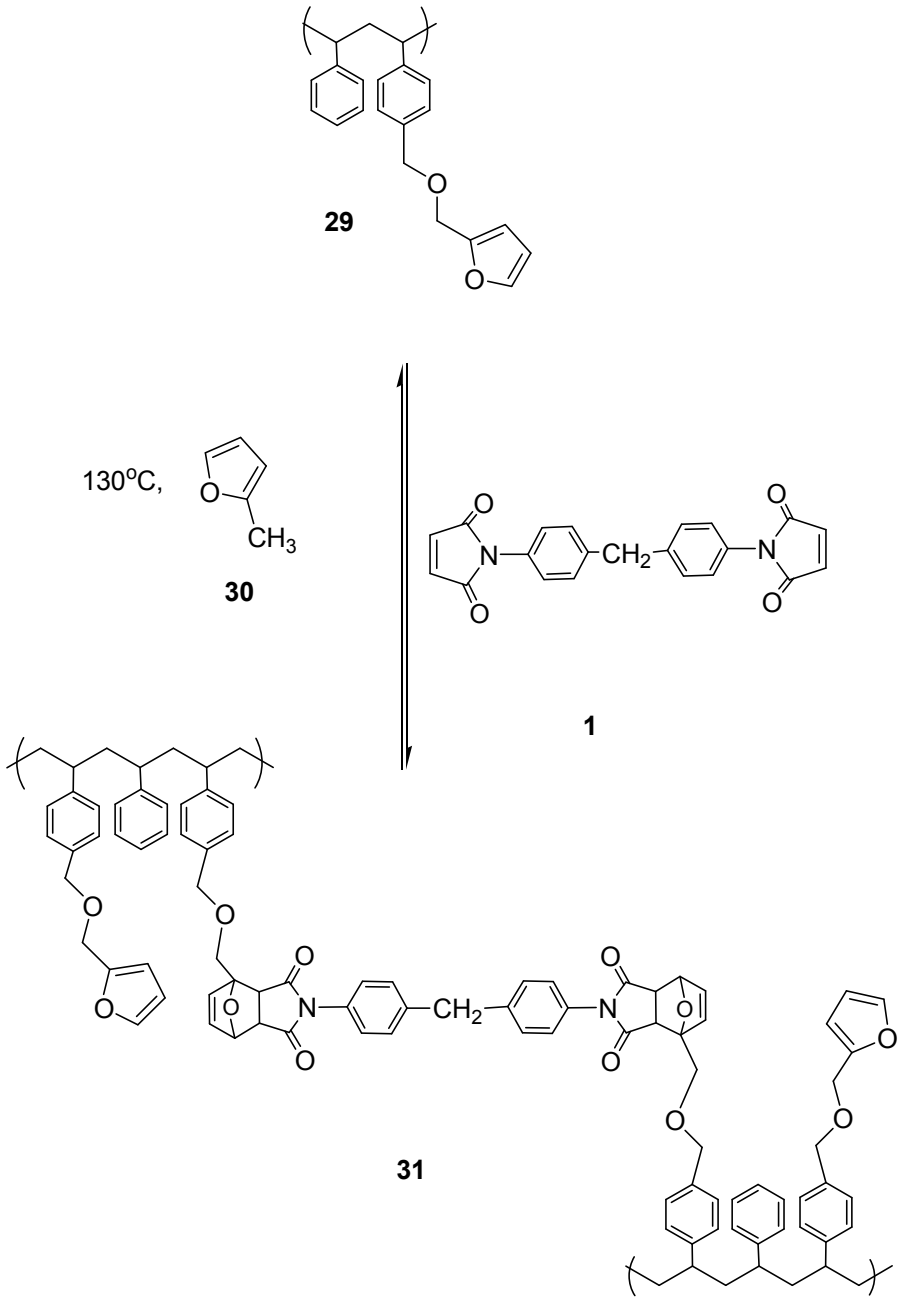


Figure 2.9. Use of bismaleimides as crosslinkers

In an other study, Schiraldi preferred to use aromatic and thermally stable anthracene unit which also acts as a diene in DA reactions [31]. Poly(ethylene terephthalate) PET copolymers containing 2,6-anthracenedicarboxylate were modified by DA reaction with bismaleimides to obtain crosslinked PET. Modification of the PET is necessary just to improve the properties such as hydrophilicity, soil resistance, and dyeability. On the other hand, necessary comonomer which contains the modification unit

should be thermally stable since high temperatures are required for the thermal transesterification polymerization of dimethyl terephthalate. The choice of anthracene moeity in this study reflects on the thermal stability.

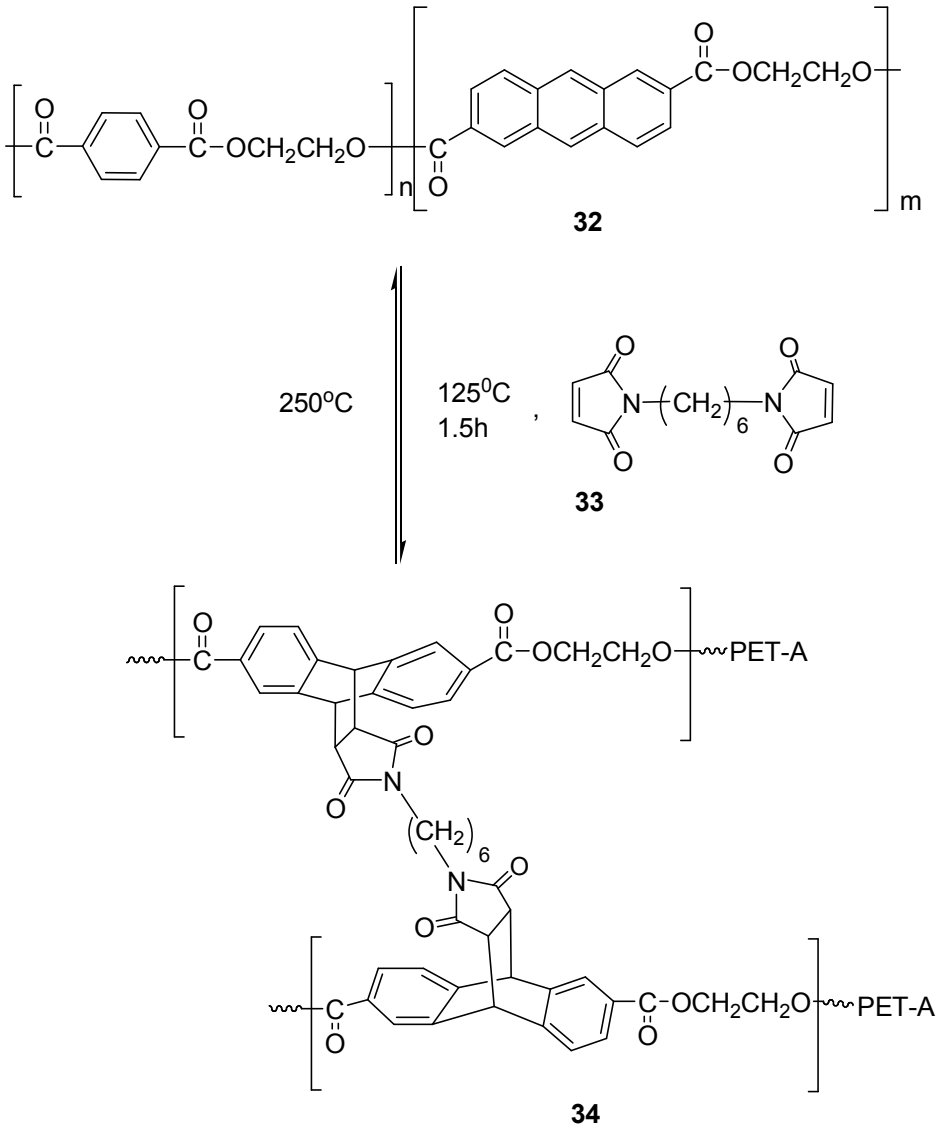


Figure 2.10. Crosslinking of anthracene polymers

Polymer **32** was heated to 125°C to afford the corresponding Diels-Alder adduct **34** (Figure 2.10). The studies on model polymers showed that the reverse reaction occurred only slowly at 250°C, where the PET-anthracene copolymers were prone to thermal decomposition.

3. MALEIMIDES IN BIOMOLECULAR IMMOBILIZATION

An immobilization reaction should have several characteristics for broad utilization in preparation of a wide variety of biochips. First, the reaction should occur rapidly and therefore allow the use of low concentrations of reagents for immobilization. Second, the chemistry should require little, if any, post-synthetic modifications before immobilization to maximize the number of compounds that can be generated by solution or solid-phase synthesis and minimize the cost of reagents. Third, the immobilization process should occur selectively in the presence of common functional groups, including amines, thiols, carboxylic acids, and alcohols, to ensure homogenous ligand immobilization. In this context, maleimides are important class of substrates as reactive surfaces for biochips. Because of the electron-deficient double bond, they are able to make facile conjugations with thiols which are either prevalent or can be engineered in biomolecules. Because of their selective reactivity towards thiols in the presence of other functional groups, maleimide terminated surfaces are attractive platforms.

3.1. Maleimide Terminated Surfaces

Recent work by Mrksich and coworkers demonstrated that the maleimide modified self-assembled monolayer (SAM) can act as a biochip platform for immobilization of proteins and carbohydrates [7]. In 2003, they reported a convenient method for immobilizing biologically active ligands to self-assembled monolayers of alkanethiolates on gold. In this study, gold surfaces were modified with maleimide terminated penta(ethylene glycol) groups and free penta(ethylene glycol) groups by deposition of disulfides (Figure 3.1). Penta(ethylene glycol) groups were used to prevent the nonspecific adsorption of biologically active ligand, while maleimide groups react selectively and efficiently with thiol-containing molecule. The characterization of the surfaces after conjugation showed that the reaction between soluble thiol and surface-bound maleimide groups occured efficiently.

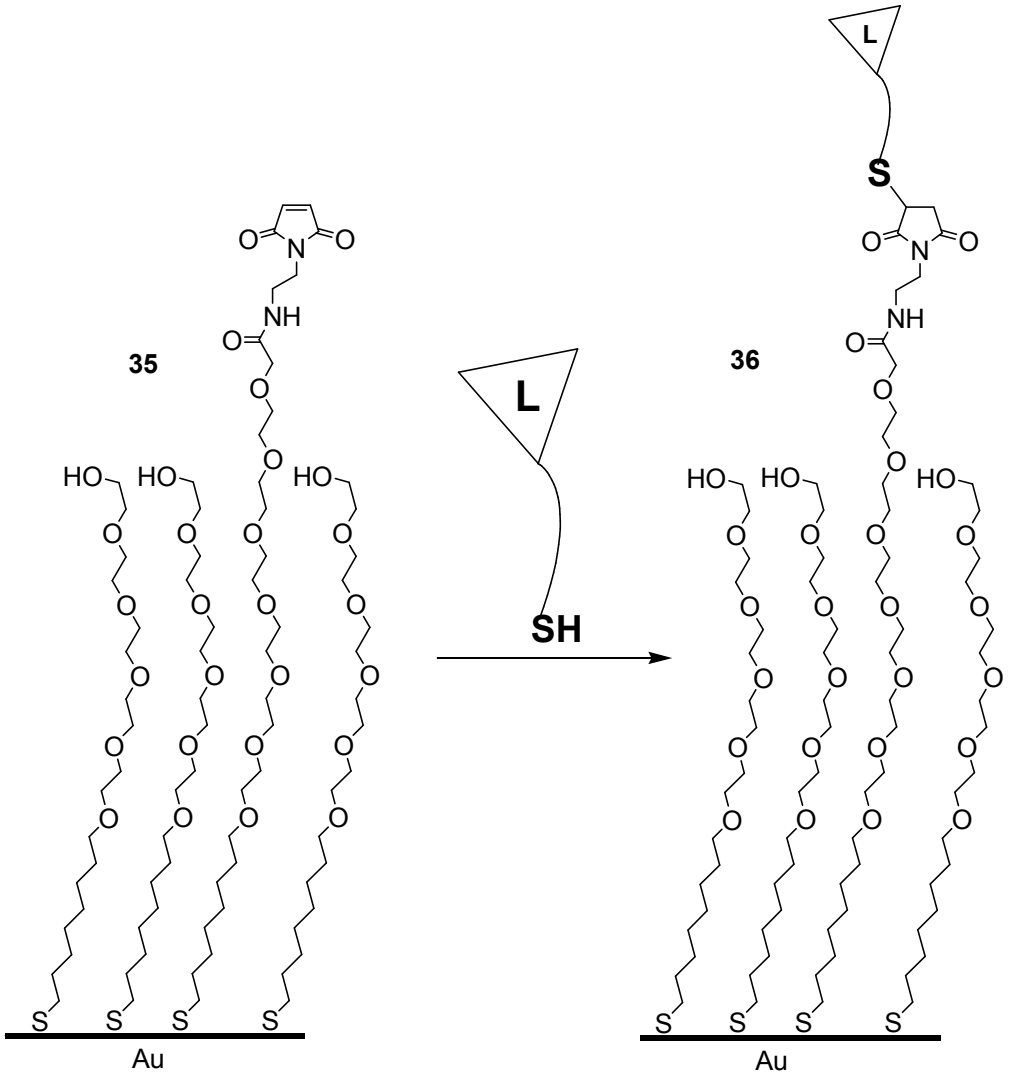


Figure 3.1. Self-assembled monolayer used to immobilize thiol-terminated ligands.

In 2005, Xiao *et. al*. also showed efficient functionalization of maleimide and succinimide containing SAMs with polypeptide molecules [6]. The succinimide and maleimide groups were attached on a gold surface via reaction of 4-aminothiophenol with heterobifunctional crosslinker bearing succinimidyl ester and maleimide groups (Figure 3.2). Surface reactions of amines with crosslinker (N-succimidyl-6-malemidyl hexonate) **38,** genereated heterogenous surfaces with both maleimidyl and succimidyl ester groups. A simple peptide, GRGDSPC, with both free amino and thiol groups was attached to maleimide and succinimide moieties, respectively, resulting in a bridged peptide structure.

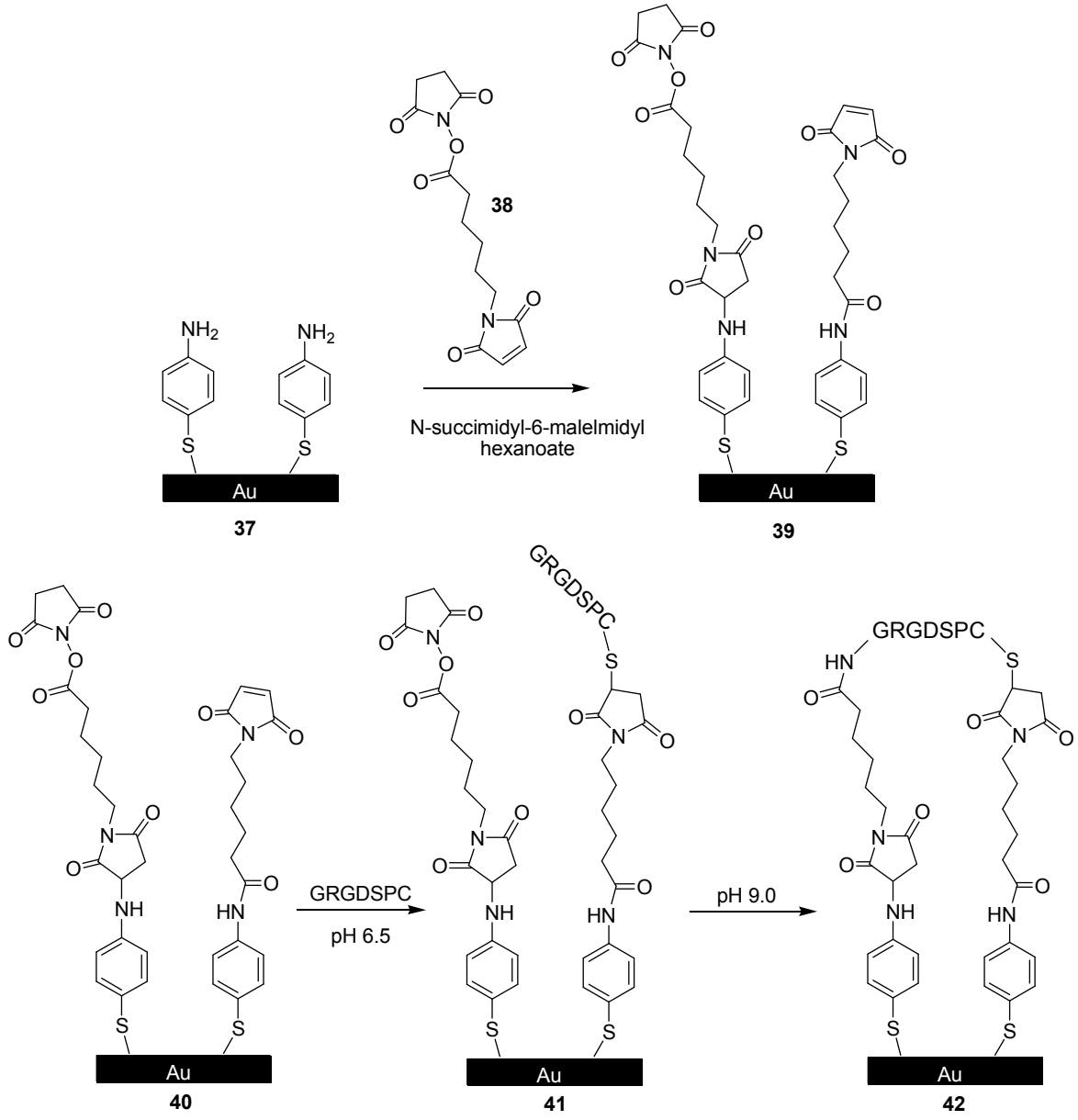


Figure 3.2. SAMs bearing succimidyl ester and maleimide groups and a bridged peptide structure on the gold surface

As mentioned before, DNA micro arrays are important biomedical research tools with the ability of capturing target DNA specifically and simultaneously onto thousands of probe spots. The substrate most widely used for the fabrication of micro arrays is silica or glass. In 2006, Howorka and co-workers prepared glass surfaces carrying a dense layer of poly(ethylene glycol) (PEG) which are potential platforms for DNA oligonucleotide micro arrays [11]. The glass slides were first silanized by using 3-glycidoxypropyl trimethoxysilane (GPS). The epoxide groups of the GPS-silanized surface were hydrolyzed

to diols which were subjected to oxidative cleavage resulted in the formation of aldehyde slides (Figure 3.3). To obtain a homogeneous thin layer of PEG, a solution of PEG-diamine was applied onto the slides. PEG-grafted slides displaying terminal amino groups were incubated with succinimidyl 4-[p-maleimidophenyl] butyrate resulted in maleimide terminated surfaces which were then used to immobilize DNA oligonucleotides.

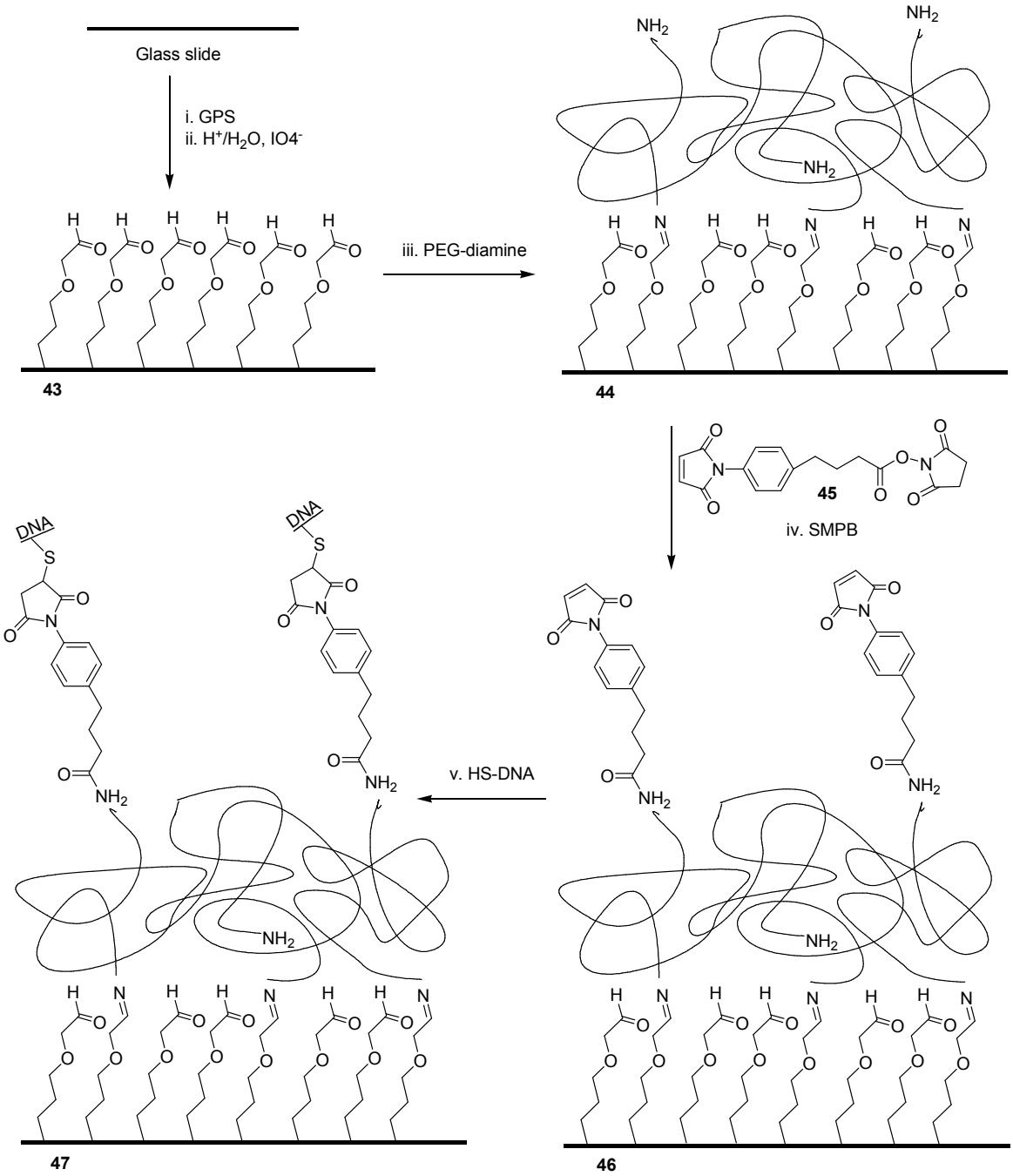


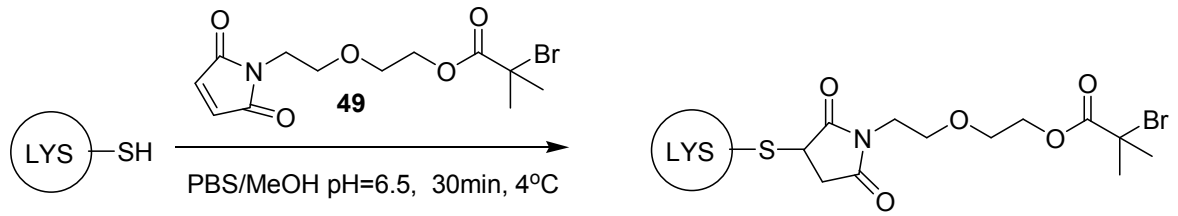
Figure 3.3. Schematic representation of the procedure for preparing PEG-grafted, maleimide terminated surfaces for the immobilization of thiolated DNA oligonucleotides

While the utility of maleimide based conjugation of biomolecules to surfaces have been extensively demonstrated, SAM based coatings are thermally labile. Such monolayers are also unstable over long periods of time due to slow chemical oxidation of the thiol functional groups. This pushes the need to search for more robust alternatives.

3.2. Functionalization of Maleimide on the Polymers

Apart from the potential applications in material science where cycloaddition chemistry has been widely explored to synthesize various crosslinked materials, maleimide containing polymers have attracted considerable interest in biotechnology. Derivatization of biomolecules with soluble polymers is widely used strategy for increasing the circulation time of therapeutic agents. Unfortunately, in most cases the study is limited to maleimide end functionalized polyethylene glycol polymers since the synthesis of polymers containing maleimide functionalities is not straightforward. The complication arises due to presence of the free radical reactive double bond of the maleimide which does not allow use of monomers containing this functional group. Very recently a few strategies have applied to overcome this problem.

In 2005, Maynard and co-workers demonstrated that bioactive “smart” polymer conjugates could be synthesized by polymerizing from defined initiation sites on proteins, thus preparing the polymer conjugates in situ [32]. They first synthesized a maleimide containing initiator and conjugated a biomolecule prior to polymerization (Figure 3.4).



Lysozyme

48

Lysozyme-M-I

50

Figure 3.4. Synthesis of biomolecule conjugated initiator

During our own investigations in this area, Haddleton and co-workers disclosed two retrosynthetic approaches to obtain maleimide end functionalized macromolecules [33]. They introduced the maleimide moiety into the polymers by following two independent approaches: (a) a post functionalization of a preformed primary amine-terminated polymer (Figure 3.5), and (b) the use of a "protected" maleimide initiator for the polymerization step followed by deprotection to give the expected maleimide terminated polymers (Figure 3.6). These α-functional methacrylate polymers have been succesfully employed in coupling reactions with a tripeptide and a model protein Bovine Serum Albumin (BSA).

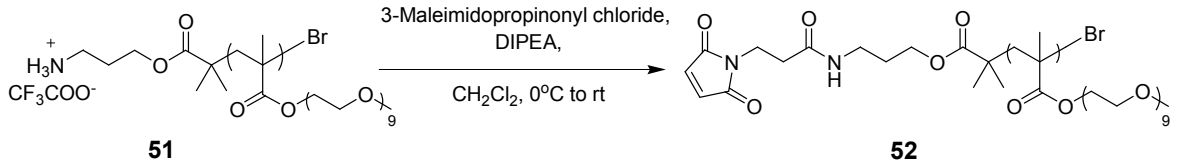


Figure 3.5. Post functionalizaiton of 1° amine terminated polymer

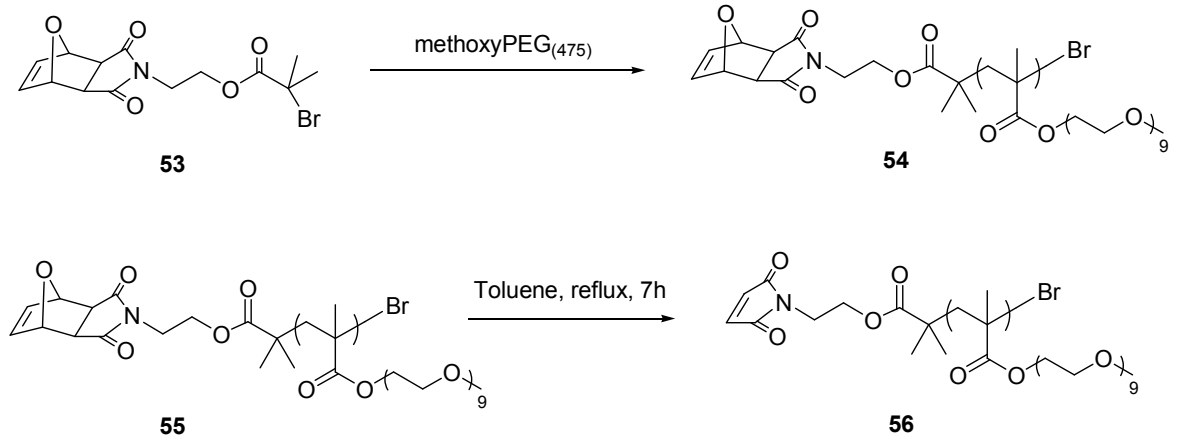


Figure 3.6. Synthesis of maleimide terminated polymer

Recent examples from literature establish that the maleimide moeity has an important role in the conjugation of biomolecules both to surfaces and directly to the polymer backbone. These studies while still in there infancy, highlights the promise held by maleimide based materials as bridging element between biology and material science.

4. AIM OF THE STUDY

The aim of this study is to synthesize well defined maleimide containing reactive polymeric entities that should find applications both in the area of biology and material science. The proposed methodology will provide direct access to polymers that are decorated with maleimide as “reactive” functional groups. To date no attempts have been reported to directly obtain polymers that contain maleimide groups on their side-chain since the maleimide group has a reactive double bond that participates in polymerization reactions. In order to retain the reactive maleimide double bond, a protection-deprotection strategy is proposed in this work. This novel approach will allow the synthesis of polymers bearing a reactive maleimide group along the backbone of a monoblock copolymer (Figure 4.1). In this research, polymers containing thiol-reactive maleimide groups in their side chains will be synthesized by utilization of a novel acrylate monomer containing a masked maleimide. Diels-Alder reaction between furan and maleimide was adapted for the protection of the reactive maleimide double bond prior to polymerization. AIBN catalyzed free radical polymerization reaction would provide copolymers containing masked maleimide groups. The maleimide groups in the side chain of the polymers can be unmasked into their reactive form by utilization of the retro Diels-Alder reaction.

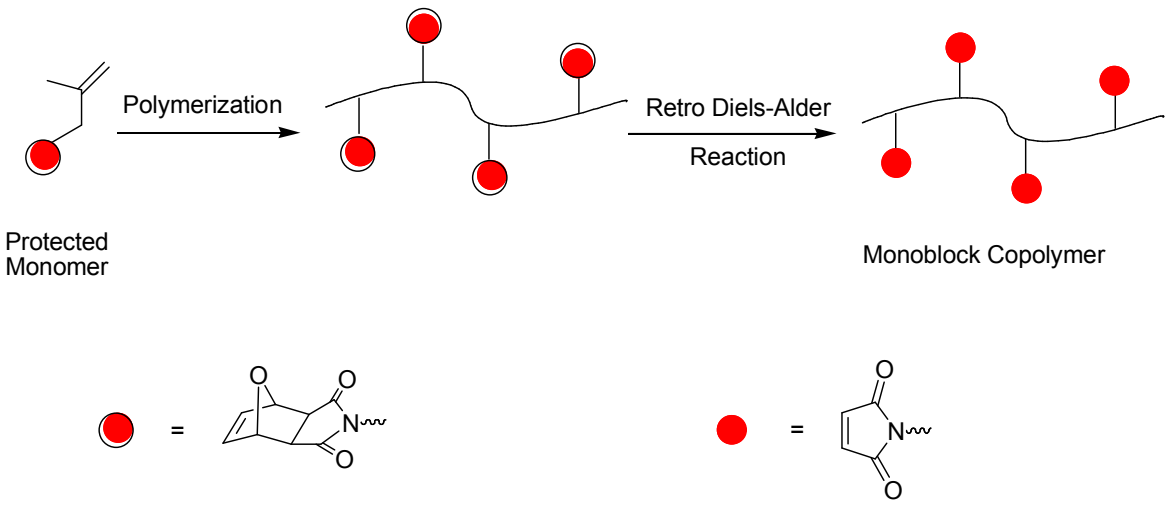


Figure 4.1. Illustration of polymer synthesis via DA / rDA sequence

As an extension of this research, synthesis of copolymers containing two orthogonal reactive groups that would allow multifunctionalization in a controlled fashion is undertaken. In other words, these polymers will enable attachment of two different molecules to the same polymeric backbone with control over their relative amounts. Copolymers containing the novel latent-reactive maleimide monomer and previously known succinimide monomer will be synthesized. Thiol containing nucleophiles will selectively add to the maleimide units on the polymer while leaving the succinimide moieties intact. Subsequent exposure to amine group containing molecules would allow further functionalization via amidation reaction with the succinimide activated esters.

Finally, fabrication of micron size arrays of maleimide and succinimide containing polymers on a glass surface by using micro contact printing technique will be addressed. Exposure of these arrays to thiol group containing fluorescent dye molecules will allow further characterization of physical and chemical properties of these constructs.

5. RESULTS AND DISCUSSION

5.1. Latent-Reactive Maleimide based Monomer: Synthesis and Characterization

The synthesis of the maleimide based latent-reactive monomer starts with a DA reaction of furan (**57**) and maleic anhydride (**58**) which results in the protection of the double bond of the maleic anhydride (Figure 5.1). As a next step, furan-maleic anhydride cyclo- adduct **59** is reacted with 3-amino-1-propanol (**60**) according to previously reported literature [34] to obtain the maleimide adduct **61**. The desired monomer **63**, containing the protected maleimide unit and a polymerizable double bond, is synthesized by the reaction of the alcohol **61** with methacryloyl chloride (**62**) in the presence of triethylamine at 0°C. The acrylate **63** is obtained as white waxy solid after passing through a short silica gel column. Satisfactory NMR (^{1}H and ^{13}C) and FTIR data are used to confirm the monomer structures and purity.

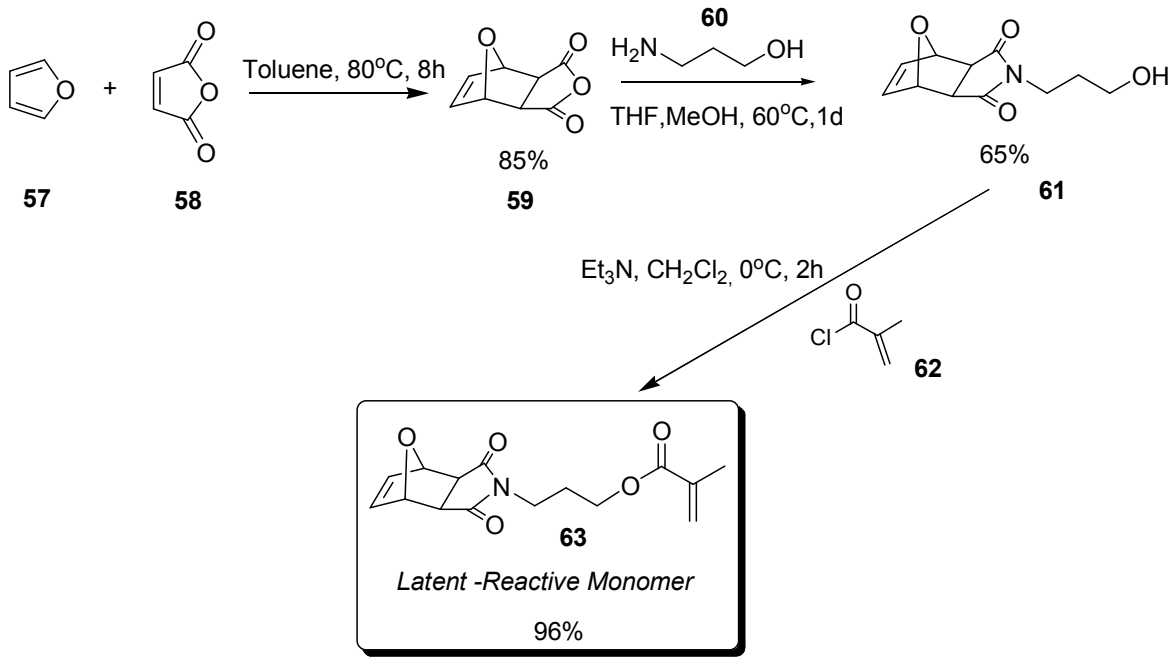


Figure 5.1. Latent –reactive monomer synthesis

5.2. General Methods and Materials

All reagents were obtained from commercial sources (Merck, Aldrich, Lancaster, Alfa Aesar, Avocado, Riedel de Haen) and were used as received unless otherwise stated. Methyl methacrylate was obtained from commercial resources and distilled prior to use. Solvents used for purification, hexane, ethyl acetate, methanol and dichloromethane were distilled prior to use (Akkimya). The monomer and polymer characterizations involved ^{1}H and ^{13}C solution NMR spectroscopy (Varian 400 MHz), Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer 1600 Series). Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were done on Rheometric Scientific (Software V5.42) and TA instrument respectively. The molecular weights were estimated by gel permeation chromatography (GPC) with polystyrene as a standard and with refractive index detector, and the sample was eluted with dry THF.

5.2.1. Synthesis of the Furan-Maleic Anhyride Adduct (59)

Maleic anhydride (10.00 g, 99.92 mmol) was suspended in toluene (50 mL) and the mixture was warmed to 80°C. Furan (11.00 mL, 150.00 mmol) was added via syringe and the turbid solution was stirred overnight. Then, the mixture was cooled to room temperature. After 1 h, the resulting white crystals were collected by filtration and washed with hexane (2 x 30 mL). The white solid (14.09 g, 84.56 mmol) was obtained with 85% yield. ^{1}H NMR ($CDCl_3$, ppm) 6.56 (s, 2H, CH=CH), 5.44 (s, 2H, CH bridgehead protons), 3.17 (s, 2H, CH-CH, bridge protons) corresponding to the literature values [33].

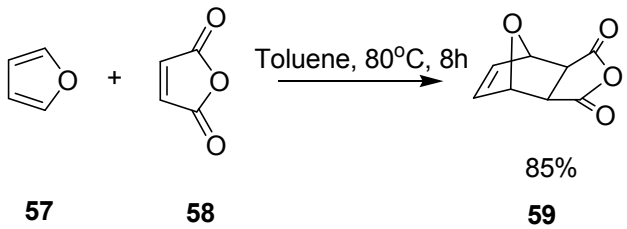


Figure 5.2. Synthesis of furan-maleic anhyride adduct

5.2.2. Synthesis of the Maleimide Adduct (61)

The anhydride **59** (5.00g, 30.00 mmol) was suspended in THF:MeOH mixture (150 mL, 1:1). 3-Amino-1-propanol (**60**) (3.43 mL, 45.00 mmol) was added dropwise to the solution. The solution was stirred at 60°C for 1 d, and then concentrated to yellow oil. This oil was purified by dissolving it in CH_2Cl_2 (100 mL), which was washed with H_2O (2 x 30 mL). The organic layer was dried over Na_2SO_4, and concentrated to give 3.50 g (15.50 mmol, 65% yield) of **61** as a white solid. ^{1}H NMR ($CDCl_3$, ppm) 6.50 (s, 2H, CH=CH), 5.25 (s, 2H, CH bridgehead protons), 3.64 (t, 2H, J=6.2 Hz, OCH_2), 3.50 (t, 2H, NCH_2), 2.85 (s, 2H, CH-CH, bridge protons), 2.41 (s, 1H, OH), 1.75 (m, 2H, $CH_2CH_2CH_2$) corresponding to the literature values [34].

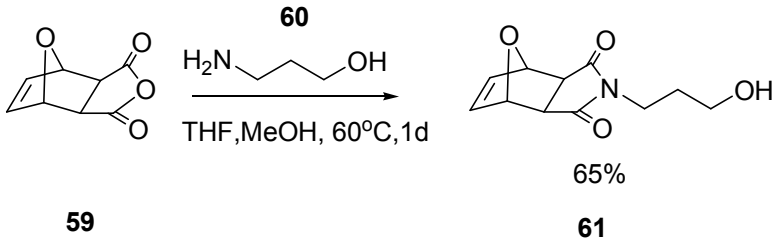


Figure 5.3. Synthesis of the maleimide adduct

5.2.3. Synthesis of Furan-Maleimide Monomer (63)

To a solution of the alcohol **61** (2.00 g, 8.86 mmol) and the triethylamine (1.05 mL, 10.63 mmol) in dichloromethane (120 mL) at 0 °C, was added methacryloyl chloride (**62**) (0.91 mL, 9.39 mmol) in 0.1 mL portions over 30 min. The clear solution was stirred for 3 h at 0°C. The reaction mixture was washed with saturated $NaHCO_3$ (2 x 40 mL) and H_2O (2 x 40 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to give a yellow residue that was purified by flash chromatography on SiO_2 (EtOAc: CH_2Cl_2 1:1) affording 2.50 g (96 % yield) of **63** as a white waxy solid. Purity has been determined by HPLC (98.4%). ^{1}H NMR ($CDCl_3$, ppm) 6.49 (s, 2H, CH=CH), 6.11 (s, 1H, CH_2=C), 5.55 (m, 1H, CH_2=C), 5.24 (s, 2H, CH bridgehead protons), 4.09 (t, 2H, J=6.2 Hz, OCH_2), 3.59 (t, 2H, J=7.0 Hz, NCH_2), 2.82 (s, 2H, CH-CH, bridge protons), 1.98

– 1.91 (m, 2H, $CH_2CH_2CH_2$), 1.93 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) 176.0, 167.1, 136.4, 136.14, 125.41, 80.8 , 61.4, 47.3, 35.67, 26.6, 18.2; IR (KBr): v = 1705.8 cm^{-1} .

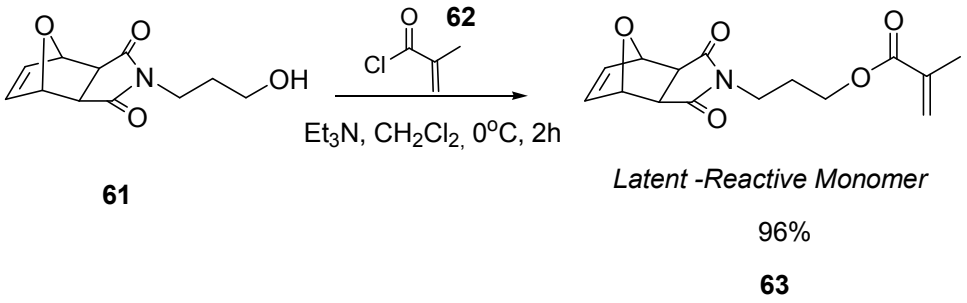


Figure 5.4. Synthesis of latent-maleimide containing monomer

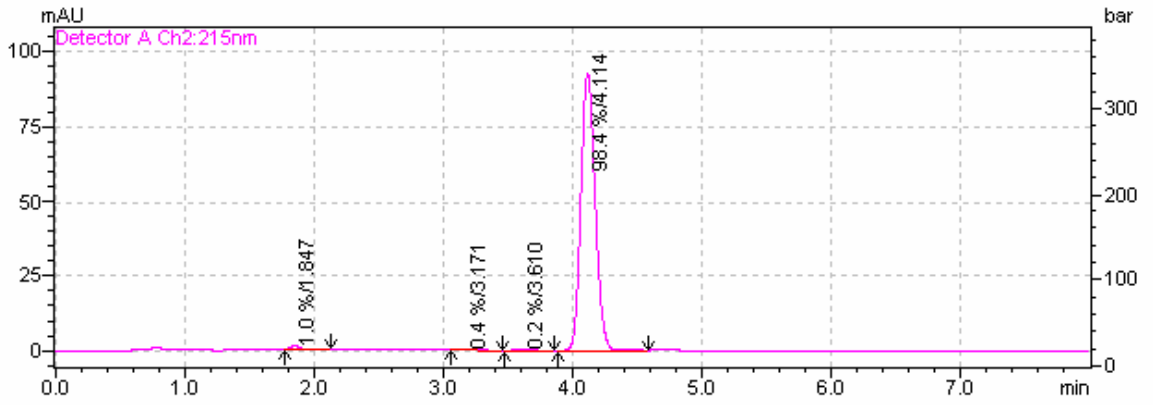


Figure 5.5. HPLC of latent-maleimide containing monomer **63**

In the ^1H NMR spectrum of the monomer (Figure 5.6), the two types of vinylic protons are readily distinguishable. The resonances of the vinylic protons on the acrylic part appear at 5.55 and 6.11 ppm (protons **i** & **h**) whereas the vinylic protons on the bicylic oxanorbornene moeity appear at 6.49 ppm (protons **a**). Additionally, the resonances of the carbon atoms of the acrylate bond appear at 125.4 and 136.1 ppm (**k** & **i**) whereas the carbon atoms belonging to the oxanorbornene part appear at 136.4 ppm (**a**, Figure 5.7).

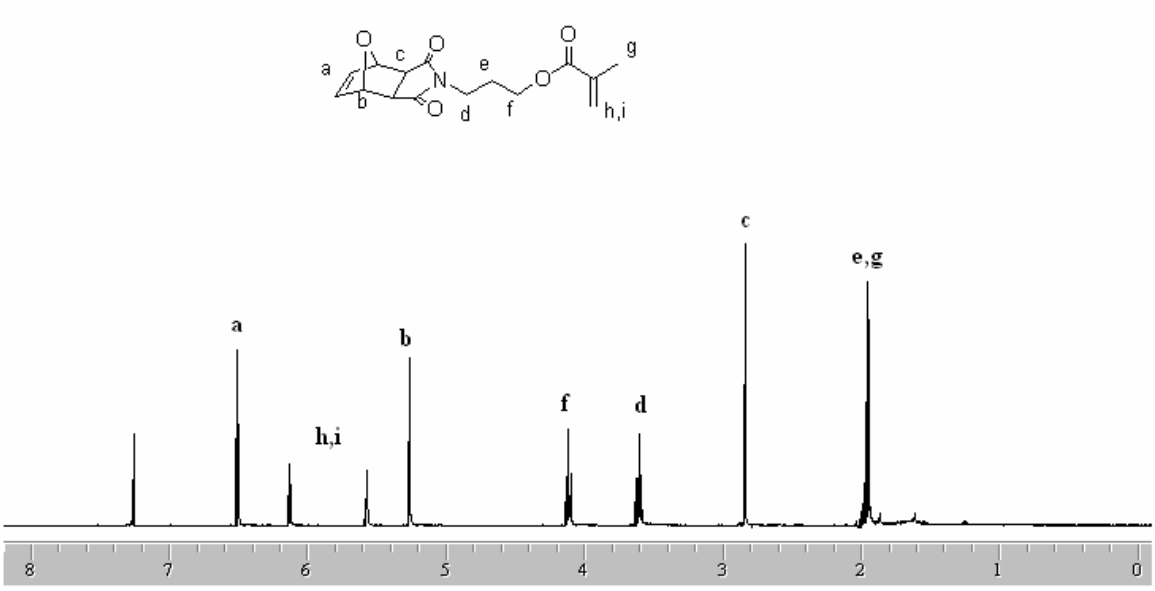


Figure 5.6 ^{1}H NMR spectrum of latent-maleimide containing monomer

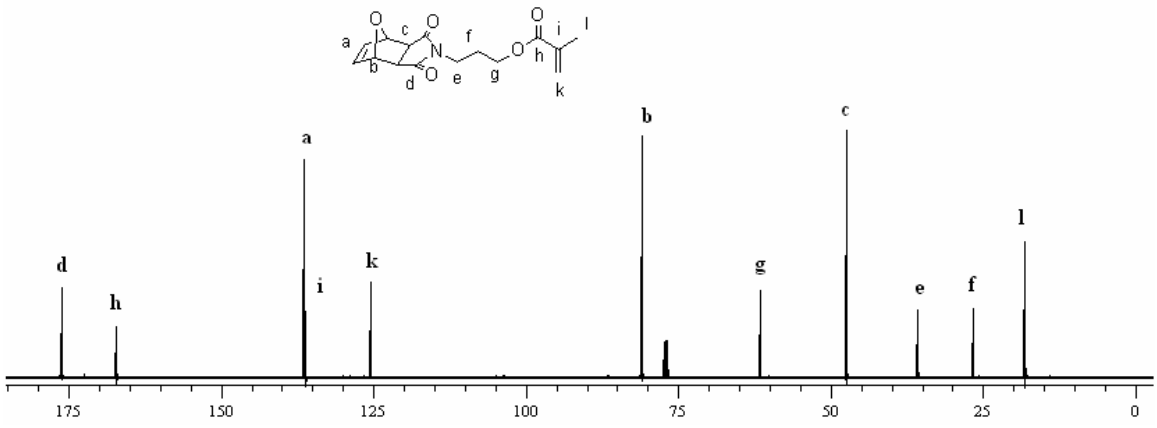


Figure 5.7. ^{13}C NMR spectrum of latent-maleimide containing monomer

5.3. Maleimide Based Copolymers: Synthesis, Characterization and Functionalization

The novel acrylate monomer containing masked maleimide **63** is copolymerized with methyl methacrylate (**64**) via AIBN catalyzed free radical polymerization. The masked maleimide moeity is converted to the reactive maleimide functionality via rDA reaction. In order to prove the efficiency of maleimide groups in thiol addition, octane thiol is reacted with reactive copolymer **66** (Figure 5.8).

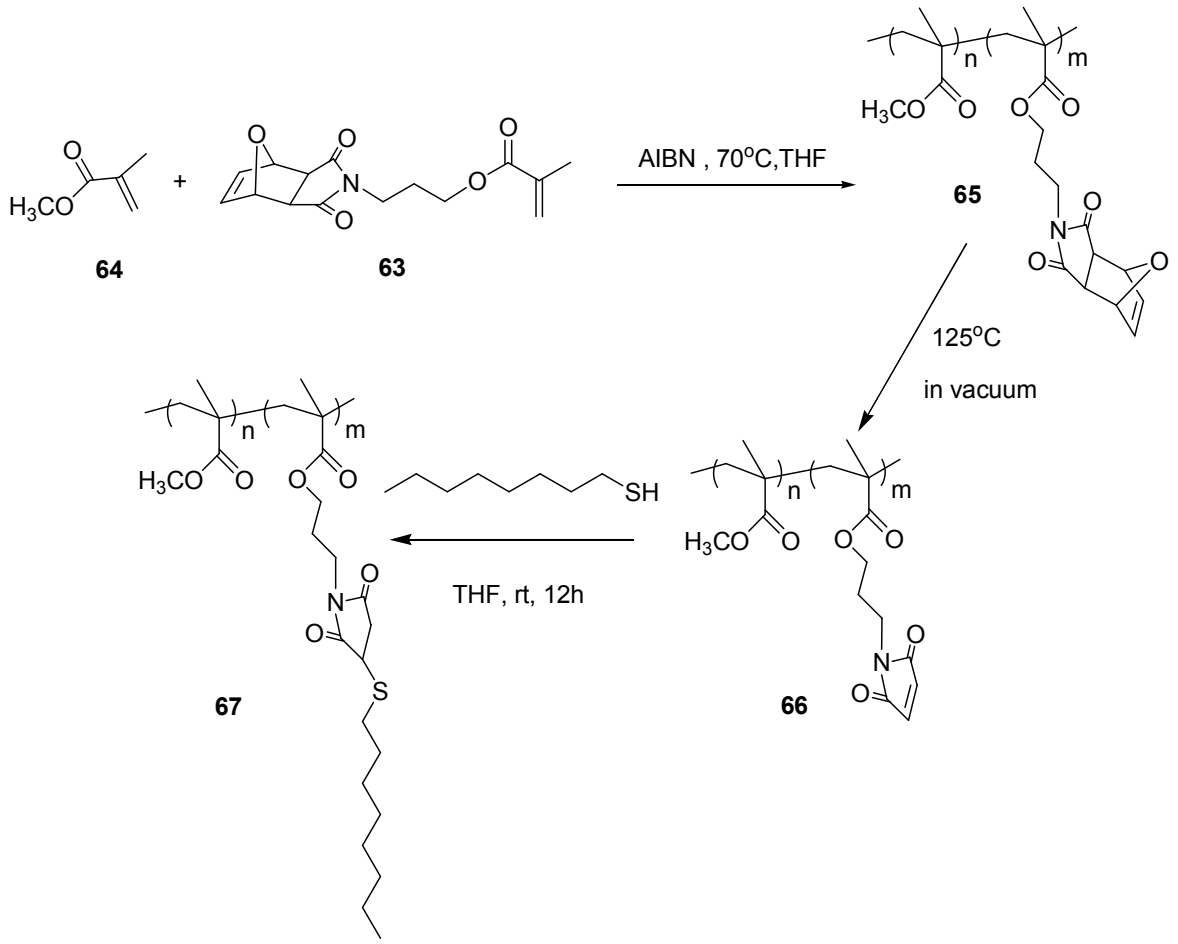


Figure 5.8. Synthesis of reactive copolymer

5.3.1 General Polymerization Procedure

In a typical experiment, to a solution of methyl methacrylate (1.24 mL, 12,3 mmol) and compound **63** (1.80 g, 6,12 mmol) in dry THF (35 mL), was added 2,2'-azobisisobutyronitrile (AIBN, 0,11 g, 0,62mmol). The mixture was degassed and then heated to reflux. At the end of the reaction, THF was evaporated under *vacuo*. The polymer was dissolved in minimum amount of dichloromethane and added to cold methanol to precipitate the polymer as a solid (50% yield). NMR for polymer **P2**: ^{1}H NMR ($CDCl_3$, ppm) 6.51 (s, 2H, CH=CH), 5.25 (s, 2H, CH bridgehead protons), 3.93 (br s, 2H, OCH_2), 3.57 (br s, 5H, OCH3 and NCH_2), 2.85 (s, 2H, CH-CH, bridge protons), 1.89–0.82 (m, 7H, $NCH_2CH_2CH_2O$, CH_2 and CH_3 along polymer backbone); ^{13}C NMR ($CDCl_3$) 178.1, 177.1, 176.0, 136.6, 81.0 , 62.1, 54.4, 51.8, 47.4, 44.9, 44.6, 35.9, 26.5, 18.8, 16.6; IR (KBr): v = 1728.8, 1703.9 cm^{-1} .

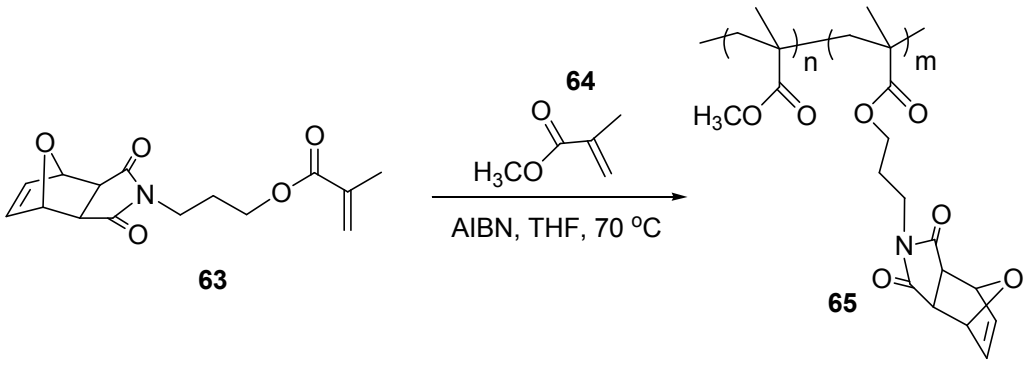


Figure 5.9. Synthesis of latent-maleimide polymer

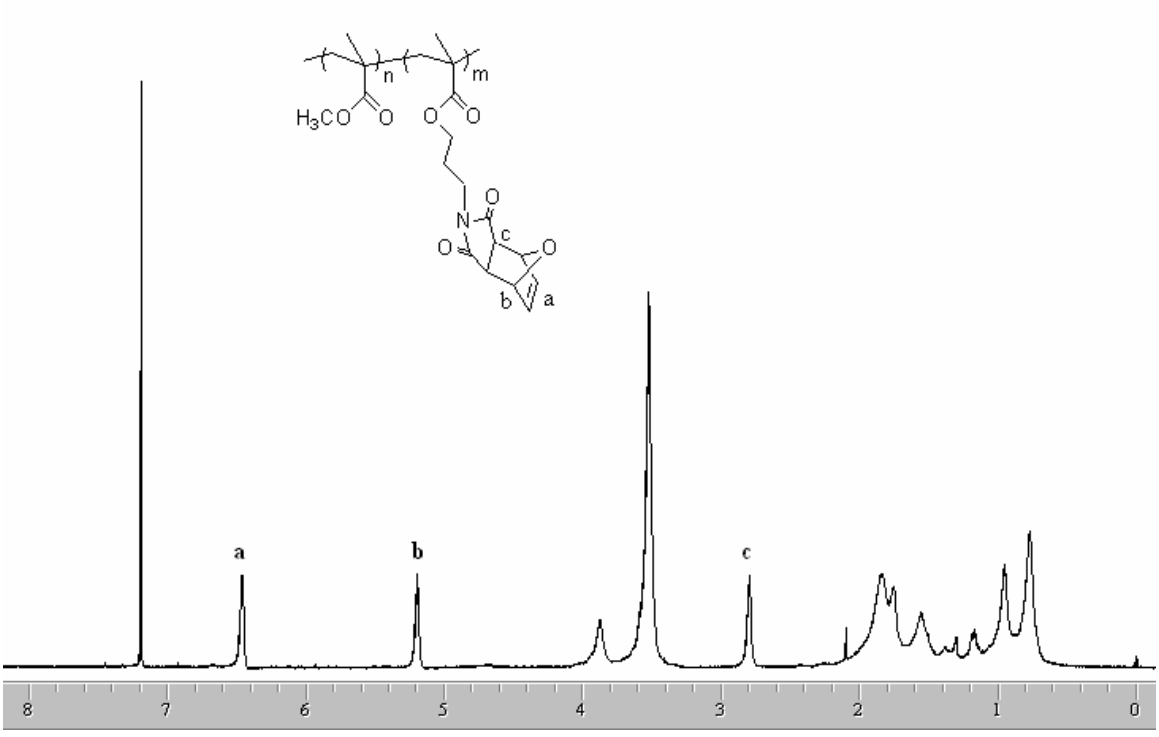


Figure 5.10. ^{1}H NMR spectrum of latent-maleimide polymer

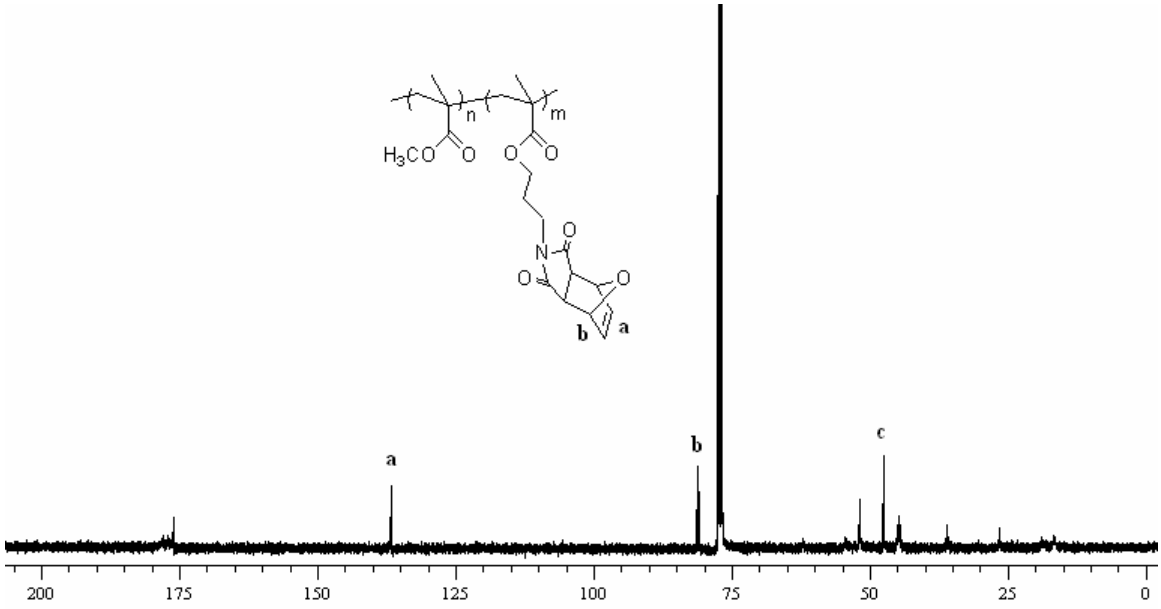


Figure 5.11. ^{13}C NMR spectrum of latent-maleimide polymer

Polymerization of the monomer in the desired manner should result in disappearance of the proton atom resonances (**h** & **i**) from the acrylate unit while leaving the signals due to the bicylic oxanorbornene (**a,b,c**) unperturbed (Figure 5.10). Several conditions for the copolymerization reactions were investigated using AIBN as the initiator. Polymerizations with MMA were examined under both bulk and dilute conditions. Among the different solvents surveyed for polymerizations, lower polydispersities were obtained upon using THF as solvent. Table 5.1 shows a summary of different polymerization conditions that were investigated and the results obtained. The composition of the copolymers could be easily determined from the integration of the ^{1}H NMR spectra. The ratio of area under the peak 6.51 ppm corresponding to the alkene protons of the monomer **63** to the area under the methyl ester group of MMA at 3.57 ppm was used in the determinations of copolymer compositions.

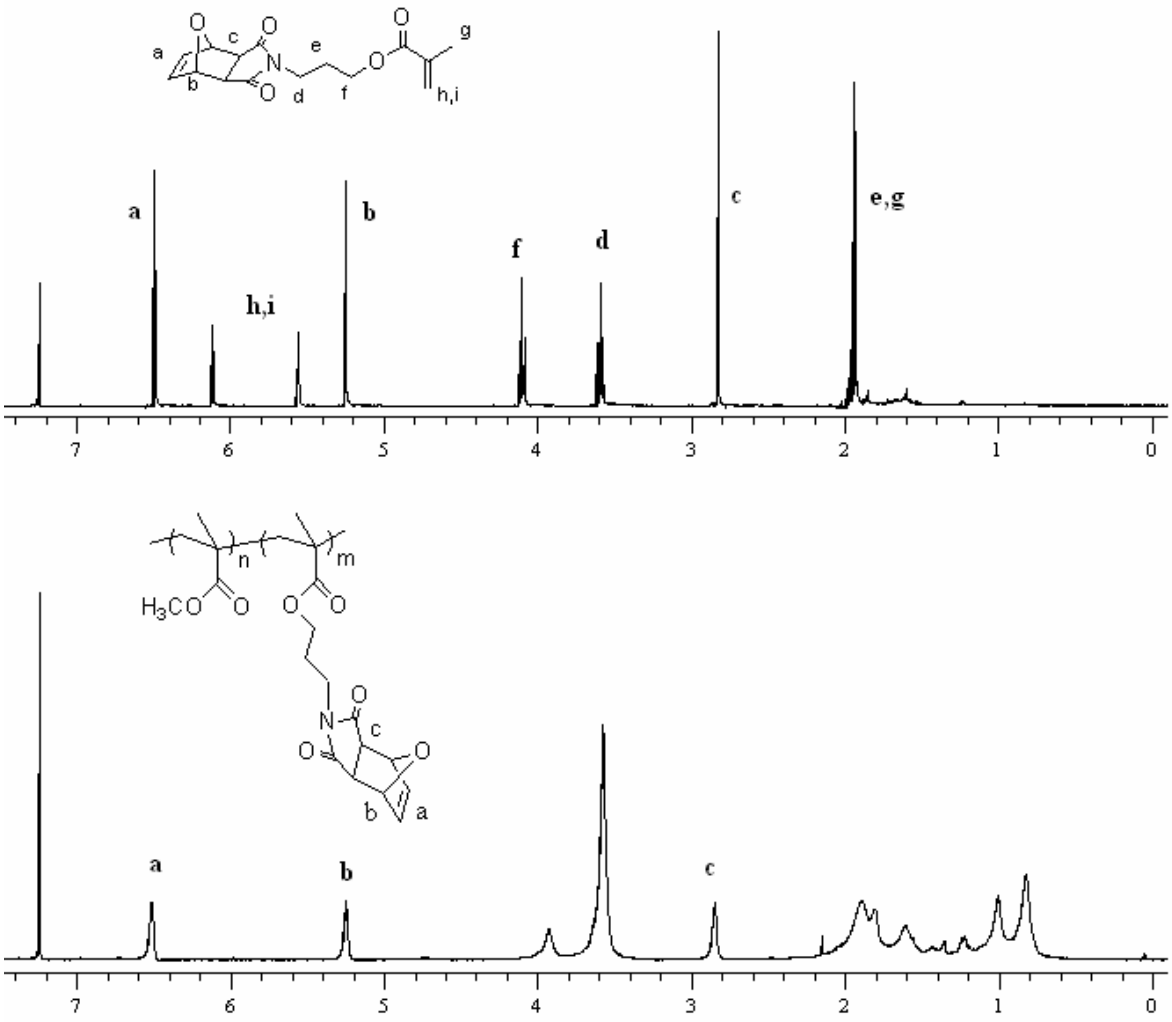


Figure 5.12. Comparison of ^{1}H NMR spectrum of monomer and polymer

It was crucial to conduct the polymerization under dilute conditions. It was observed that extensive crosslinking occurs in bulk and concentrated solutions of THF. In much more dilute solutions (< 0.14g/mL), no crosslinking was observed even after 24 hours. Polymerization in other solvent systems such as anisole and toluene resulted in higher polydispersities. Higher M_n such as 11K of the polymer has been obtained in THF with an increase in polydispersity to 2.4 . Under the same conditions other solvent systems also resulted in higher M_n like around 11K but with higher polydispersity indexes such as 2.5 and 3.8 for anisole and toluene, respectively.

	$F_{theoretical}$ [b]	time	% yield	$F_{observed}$ [c]	M_n [d]	M_w/M_n [d]
P1	50.0%	7.5h	36	31.0%	2334	1.35
P2	33.3%	7.5h	50	20.53%	3395	1.38
P3	25.0%	7.5h	59	12.3%	3265	1.79
P4	12.5%	7.5h	61	6.8%	4220	1.72

Table 5.1. Synthesis of latent-reactive monomer containing random copolymers[a]

[a] Initiator, AIBN; solvent, THF; temperature, 70 ^oC; [b] feed ratio M:MMA; [c] Ratio of monomers in the copolymer; [d] Estimated by SEC eluted with THF, using polystyrene calibrations

5.3.2 General Procedure for retro Diels-Alder Reaction:

Polymer **65** (20.0 mg) was heated under vacuum at 125°C for 3 hours. NMR analysis showed that quantitative conversion of the oxabicyclic moiety to the maleimide functional group. NMR for polymer **66** : ^{1}H NMR ($CDCl_3$, ppm) 6.71 (s, 2H, CH=CH), 3.96 (s, 2H, OCH_2), 3.57 (br s, 5H, OCH3 and NCH_2), 1.93–0.82 (m, 7H, $NCH_2CH_2CH_2O$, CH_2 and CH_3 along polymer backbone); ^{13}C NMR ($CDCl_3$) 177.9, 176.8, 176.0, 136.6, 81.0 , 62.1, 54.4, 51.8, 47.4, 44.9, 44.6, 35.9, 26.5, 18.8, 16.6; IR (KBr): v = 1728.8, 1703.9 cm^{-1} .

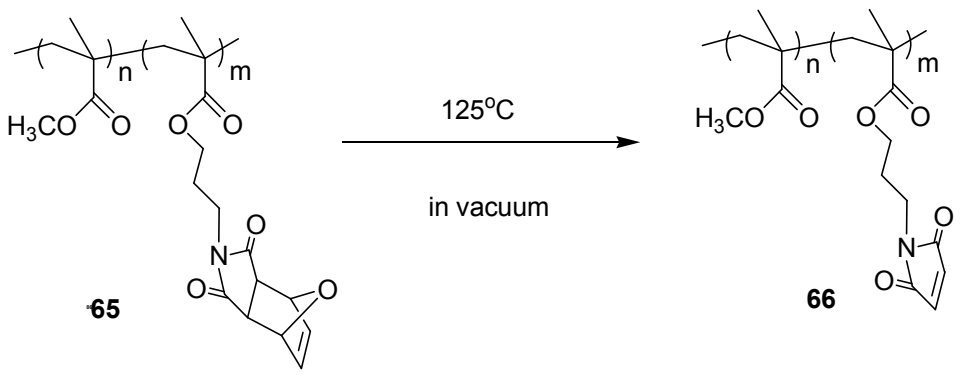


Figure 5.13. Synthesis of the reactive polymer

The polymers at hand were converted to their reactive form upon subjecting them to the retro Diels Alder reaction. The polymers were heated under vacuum at 125 ^{o}C for 3 hours. This resulted in complete cycloreversion of the furan-maleimide adducts to afford a maleimide side chain containing polymers. HNMR analysis confirmed that the cycloreversion was almost quantitative (Figure 5.14). Appearance of a new peak at 6.71 ppm, accompanied by disappearance of peaks at 5.25 and 6.51 ppm corresponding to the oxabicyclic moiety revealed to the successful cycloreversion. Additionally, the resonances of the carbon atoms belonging to the oxanorbornene part appear at 81.0 and 136.6 ppm disappeared and a new peak corresponding to the maleimide double bond appeared at 134.2 ppm (Figure 5.15). GPC analysis proved that there was no detrimental effect on the polymer as confirmed by presence of a monomodal peak with low polydispersity, e.g. when polymer **P2** was subjected to the cycloreversion for 3 h at 125 ^{o}C, maleimide functionalized polymer **P5** was obtained. As expected, presence of a monomodal peak (M_n = 3464 , M_w/M_n = 1.452) in SEC analysis revealed that no degradation takes place during the cycloreversion.

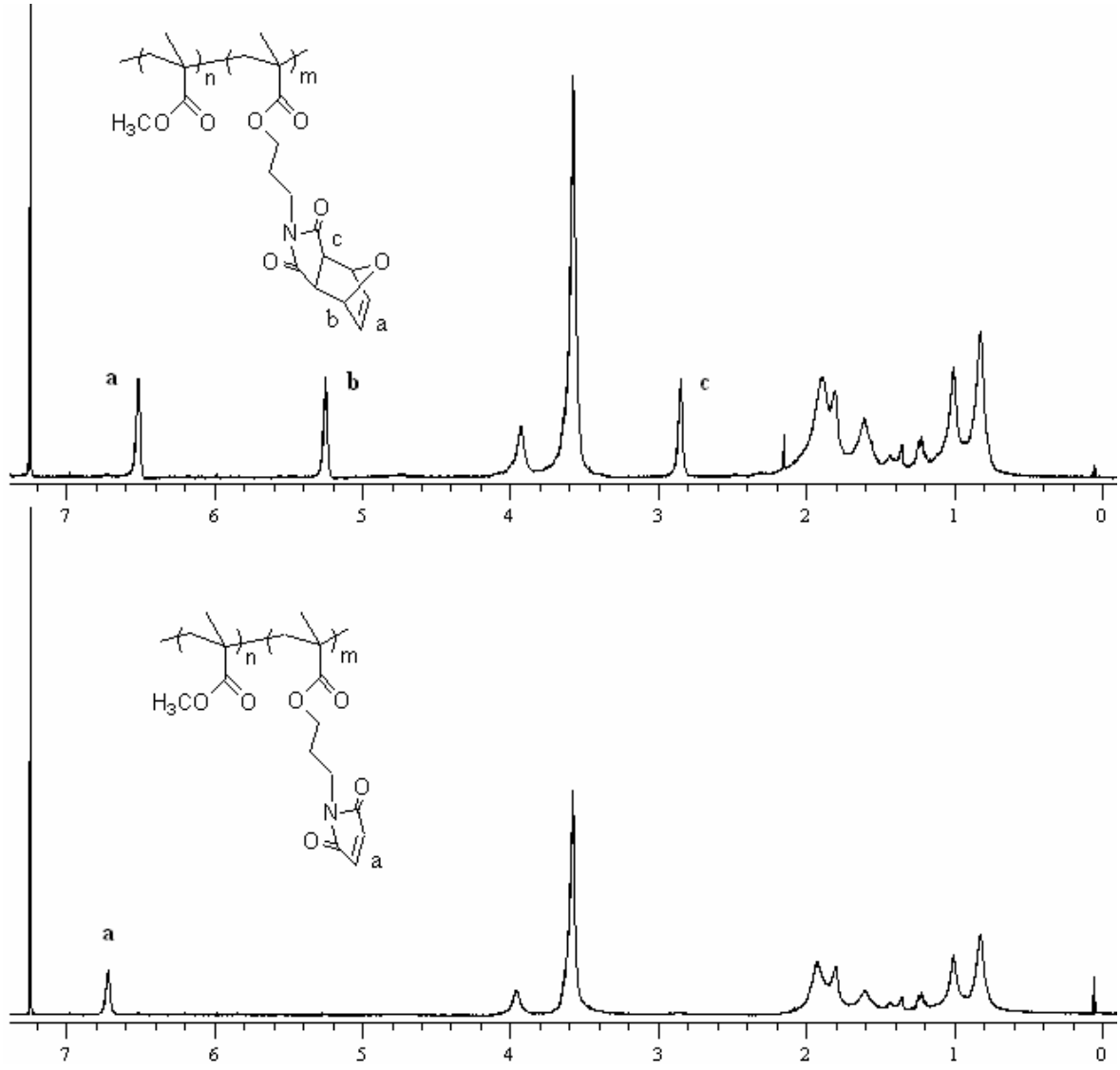


Figure 5.14 ^{1}H NMR spectra of polymer before and after rDA

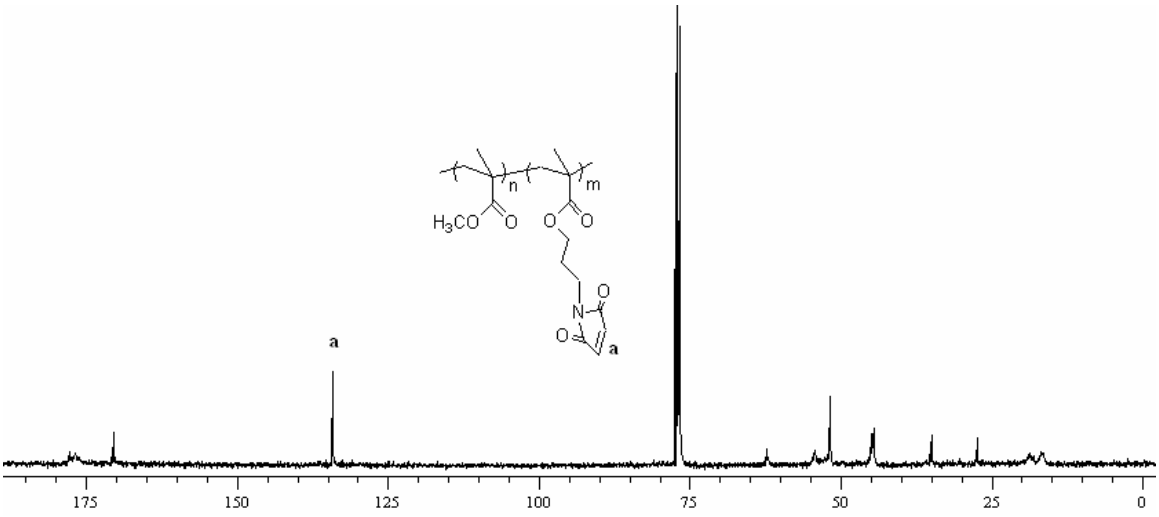


Figure 5.15. ^{13}C NMR spectrum of reactive polymer

Thermogravimetric analysis (TGA) was used to determine the thermal stability of the copolymer and monitor the activation of the maleimide groups via loss of furan during rDA reaction. TGA of the polymers **P(1 – 4)** showed a weight loss starting at 120 ^{o}C (Figure 5.16). A consistent increase in weight loss of the polymers was observed upon increasing the amount of furan based monomer. According to the TGA analysis the observed weight losses were 11.5%, 9.5%, 7.5% and 3.5% for polymers **P1**, **P2**, **P3** and **P4** respectively. These were lower than the expected weight losses (17.4%, 13.8%, 9.8% and 6.2%). Similar observations have been reported before and the discrepancies have been attributed to the broad range of local environment around the cycloadducts [35]. Quantitative cycloreversion and elimination of furan was additionally confirmed by the NMR analysis of the polymers subjected to retro Diels-Alder reaction. As expected, no weight loss in the region of 120 ^{o}C was observed upon subjecting the polymer **P5** obtained after the cycloreversion step, due to lack of any furan adducts in the side chains.

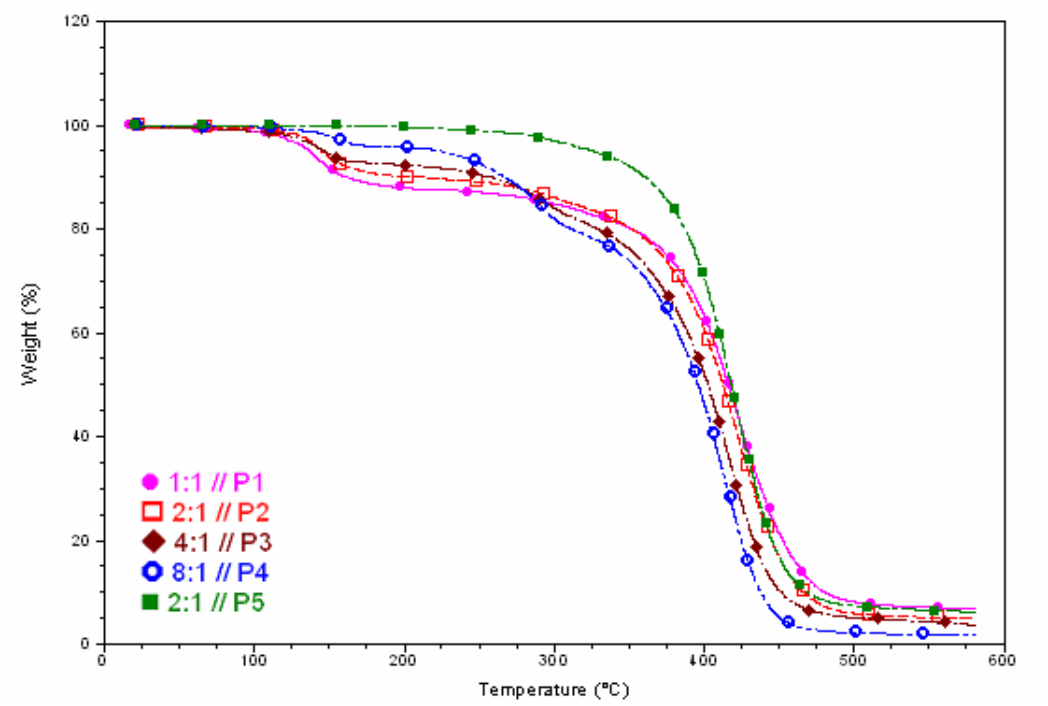


Figure 5.16. Thermogravimetric analyses of polymers

Thermal cycloreversion was further probed using DSC (Figure 5.17). The sample was first heated upto 200 ^{o}C (1st heating), followed by cooling to room temperature and then reheated to 200 ^{o}C (2nd heating). During the first heating a broad endotherm was

observed between 115 – 165 ^{o}C, which is ascribed to the cycloreversion reaction. During the cooling step and 2nd heating step, no peaks were observed. As expected, similar lack of endotherm was observed in DSC thermogram of a maleimide polymer obtained after cycloreversion.

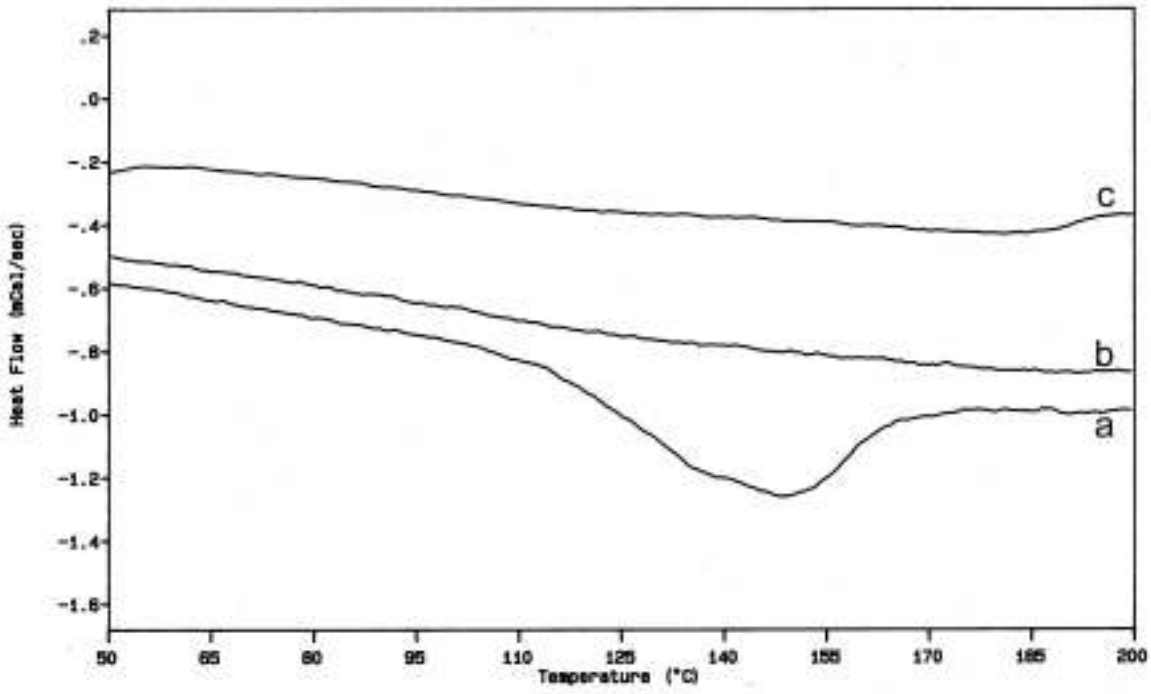


Figure 5.17. DSC thermogram of P2 (a: 1st heating, b: 2nd heating) and P5 (c)

5.3.3 Addition of Thiol to Reactive Polymer

Polymer **66** (**P5**) (10.0 mg, 0.003 mmol) has been dissolved in 10 mL of THF and octane thiol (**67**) (30µl, 1.73*10^{-4} mmol) has been added to the solution. The mixture was stirred overnight at room temperature. After evaporation of THF, the solid residue was dissolved in minimum amount of polymer and precipitated in methanol affording to yellowish polymer (**68**). ^{1}H NMR ($CDCl_3$, ppm) 3.94 (s, 2H, OCH_2), 3.72 (s, 1H, SCH), 3.57 (br s, 5H, OCH3 and NCH_2), 3.11 (s, 1H, CH of succinimide), 2.85, 2.72 (s, 2H, SCH_2), 2.52 (d, 1H, CH of succinimide), 1.90–0.81 (m, 22H, $NCH_2CH_2CH_2O$, CH_2-CH_3 along polymer backbone, and CH_2-CH_3 of octane thiol).

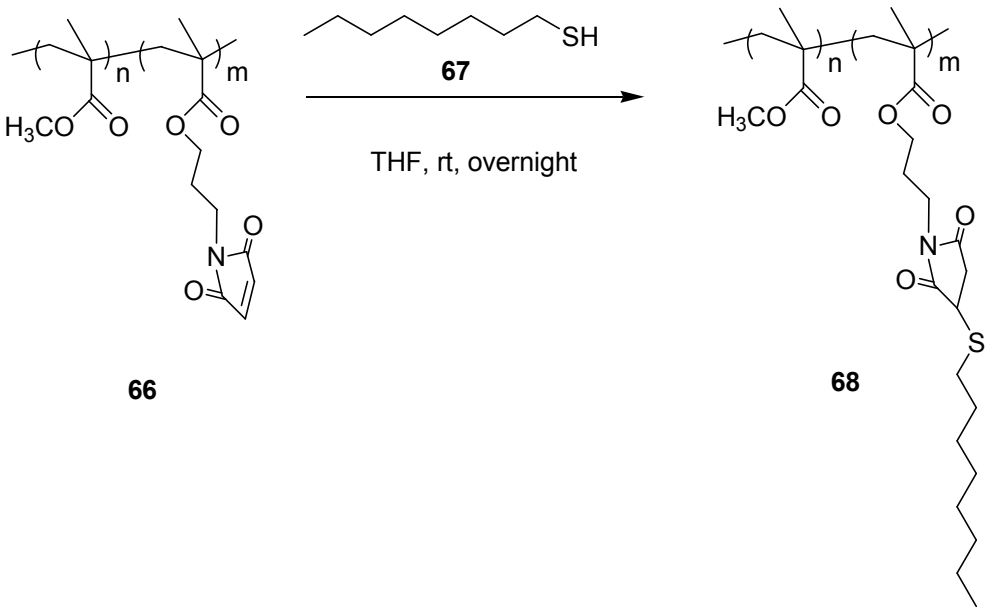


Figure 5.18. Addition of thiol to maleimide polymer

Octanethiol has been added to the reactive polymer **66**, to show that the conjugation with thiol functionality works properly. The disapperance of the peak at 6.71 corresponding to the double bond of the maleimide functionality along with appearance of expected peaks implied the succesful addition of octanethiol (Figure 5.19).

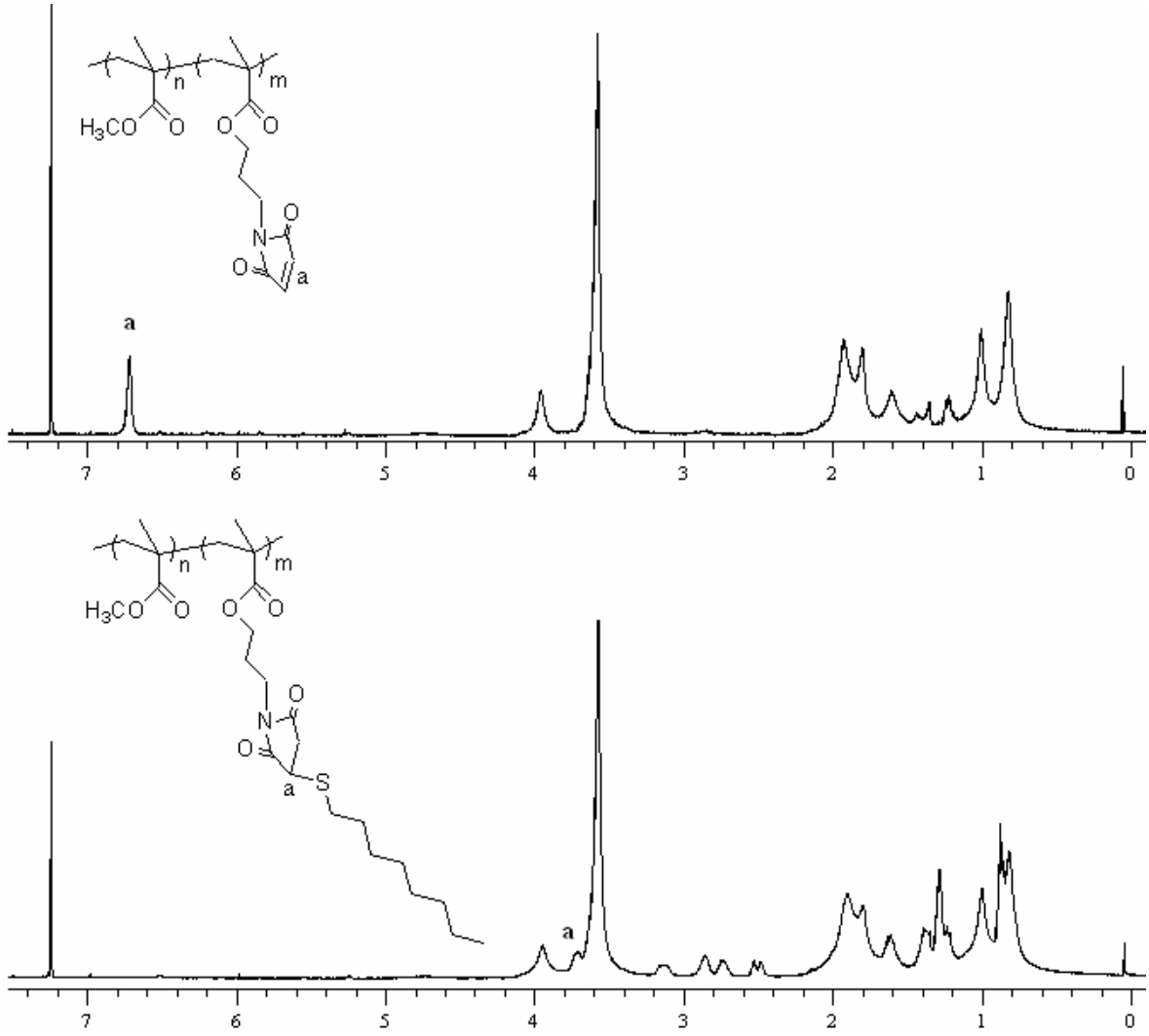


Figure 5.19. ^{1}H NMR spectra of polymer before and after thiol addition

In summary, a conceptually novel maleimide based monomer was synthesized. Successful polymerization and subsequent functionalization with thiol based nucleophile under mild conditions, without any need for addditional reagents was demonstrated.

5.4. Orthogonally Functionalizable Polymers

In recent years synthesis of polymers that would allow orthogonal functionalizations of side chains has attracted attention since it allows access to multifunctional platforms for the delivery of therapeutics [36], smart materials [37], and nano-sized devices [38]. Orthogonal functionalization refers to the ability to attach two different molecules to the same polymer in a single step or stepwise manner (Figure 5.20).

The relative ratio of the different functional groups is controlled by the ratio of orthogonal reactive groups in the parent polymer.

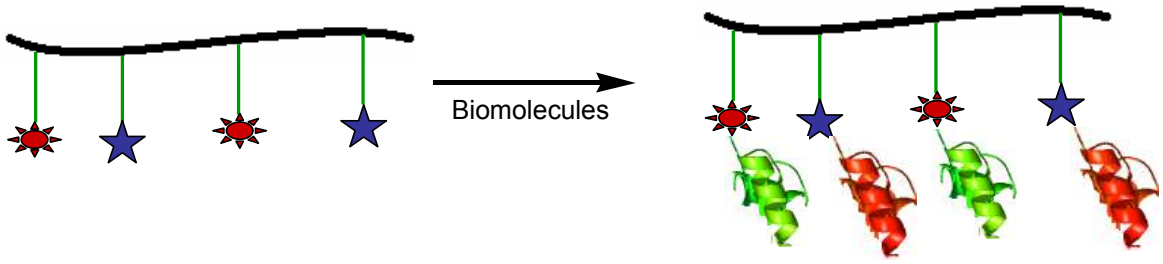


Figure 5.20. Orthogonal functionalization

5.5. Copolymers Containing Maleimide and Succinimide Side Chains: Synthesis, Characterization and Functionalization

As an extension of the research, succinimide based monomer **70** has been copolymerized with the maleimide containing monomer **63** resulting in the polymer **72**. Upon subjecting copolymer **72** to the rDA reaction conditions polymer **73** which accommodates the amine reactive succinimide group and thiol reactive maleimide moeity along the same polymer backbone is obtained (Figure 5.21). Copolymer **71** was synthesized to serve as a reference polymer for elucidation of NMR spectra and optimization of reaction conditions for further derivatization via amidation.

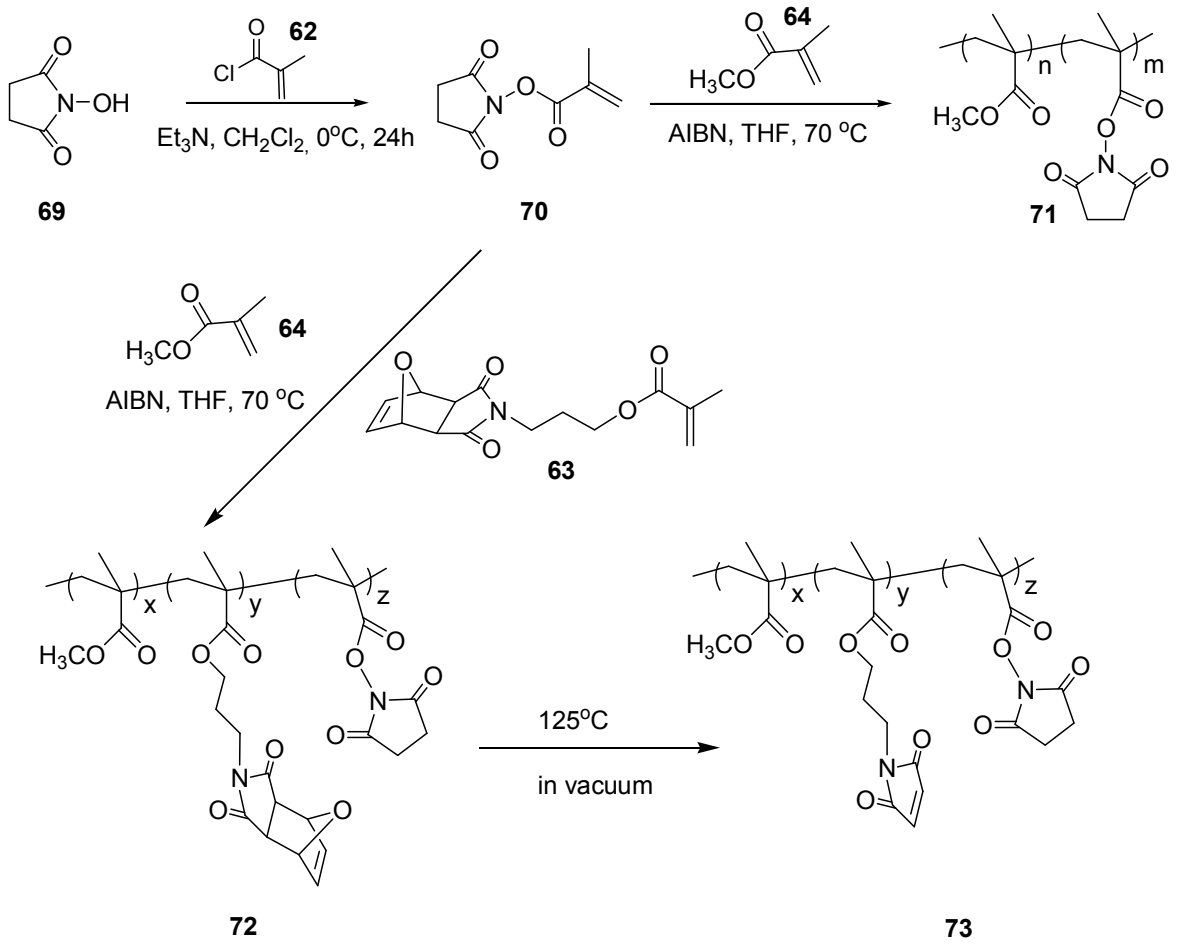


Figure 5.21. Synthesis of polymers bearing two reactive moeities

5.5.1 Synthesis of the Succimide Monomer (70)

The specific amount of N-hydroxysuccimide (**69**) (1.00 g, 8.70 mmol) and triethylamine (1.00 mL, 10.12 mmol) in dichloromethane (125 mL) was cooled to 0°C, and methacryloyl chloride (**62**) (1.00 mL, 10.44 mmol) was added to the flask over 30min. The reaction was stirred under N_2 for 24 h. The reaction mixture was washed with saturated $NaHCO_3$ (2 x 40 mL) and H_2O (2 x 40 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to give a white solid which was recrystallizated with hexane: ethyl acecate (70:30) mixture leading to pure monomer with 50% yield. ^{1}H NMR ($CDCl_3$, ppm): 6.4 (s, H, CH_2=C), 5.9 (s, H, CH_2=C), 2.83 (s, 4H, CH_2-CH_2), 2.04 (s, 3H, CH_3) corresponding to the literature values [39] .

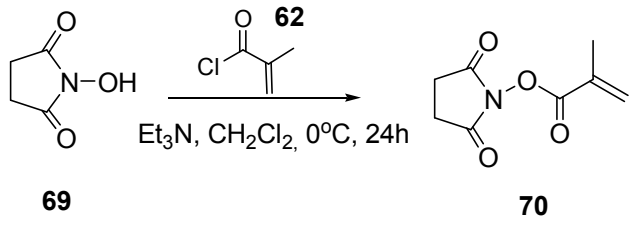


Figure 5.22. Synthesis of succinimide containing monomer

5.5.2 Polymerization of Succinimide Monomer

To a solution of methyl methacrylate (**64**) (1.00 mL, 10.00 mmol) and compound **70** (0.29 g, 2.50 mmol) in dry THF (20 mL), was added 2,2'-azobisisobutyronitrile (AIBN, 20.5 mg, 0,125 mmol). The mixture was degassed and then heated to reflux for 18h. At the end of the reaction, THF was evaporated under *vacuo*. The polymer was dissolved in minimum amount of dichloromethane and added to cold methanol to precipitate the polymer as a solid (43% yield). Mn: 4909, Mw/Mn: 1.5, incorporation: 22%.^{1}H NMR ($CDCl_3$): 3.60 (s, 3H, OCH_3), 2.80 (s, 4H, CH_2-CH_2), 0.83-1.80 (CH_2 and CH_3 along the polymer backbone).

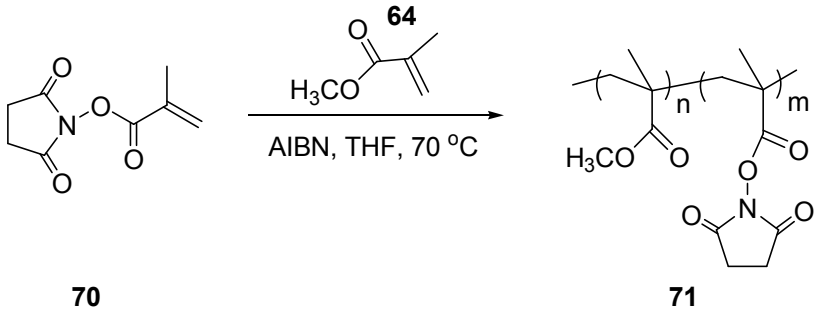


Figure 5.23. Synthesis of succinimide containing polymer

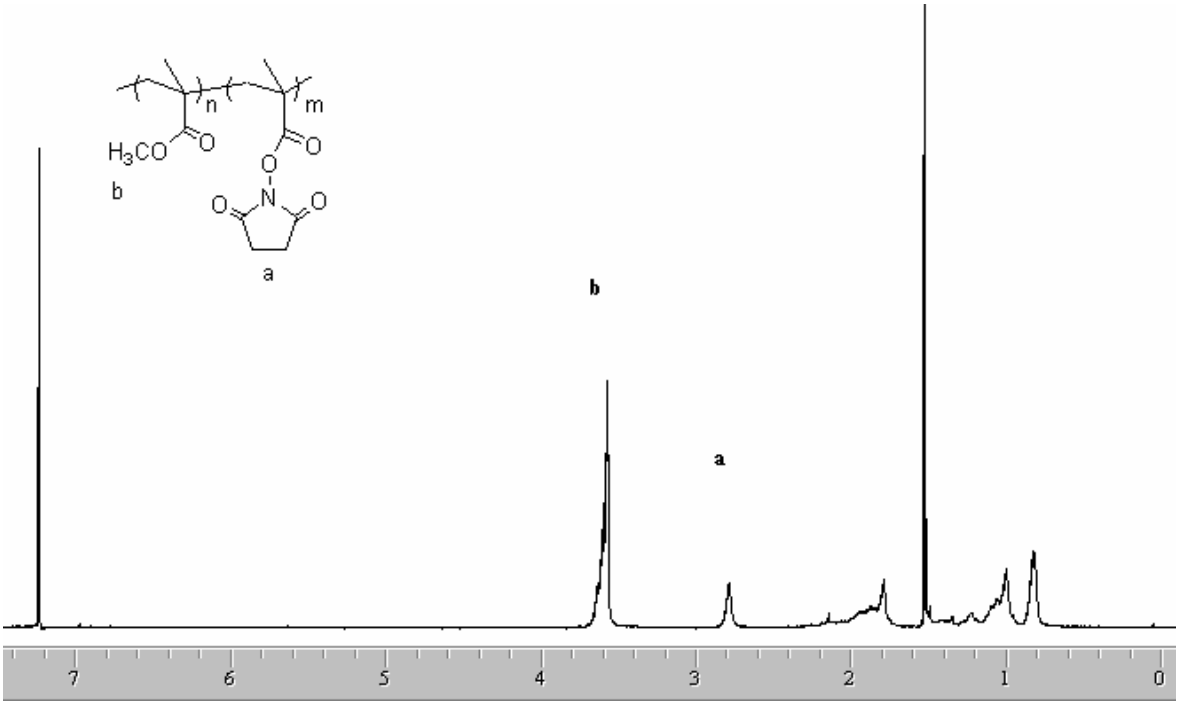


Figure 5.24. ^{1}H NMR spectrum of succinimide containing copolymer **71**

5.5.3 Polymerization of Succinimide and Maleimide Monomer

To a solution of methyl methacrylate (**64**) (0.77 mL, 7.72 mmol), compound **70** (0.22 g, 1.93 mmol) and compound **63** (1.13 g, 3.86 mmol) in dry THF (50 mL), was added 2,2'-azobisisobutyronitrile (AIBN, 18.5 mg, 0.11 mmol). The mixture was degassed and then heated to reflux. At the end of the reaction, THF was evaporated under *vacuo*. The polymer was dissolved in minimum amount of dichloromethane and added to cold methanol to precipitate the polymer as a solid (25% yield). Mn: 3358, Mw/Mn: 1.398, incorporation: 14% maleimide, 20% succinimide. ^{1}H NMR ($CDCl_3$, ppm) 6.51 (s, 2H, CH=CH of maleimide), 5.24 (s, 2H, CH bridgehead protons of maleimide), 3.94 (br s, 2H, OCH_2 of maleimide), 3.57 (br s, 5H, OCH_3 and NCH_2), 2.85 (s, 2H, CH-CH, bridge protons of maleimide), 2.78 (s, 4H, CH_2-CH_2 of succinimide), 1.90–0.82 (m, 7H, $NCH_2CH_2CH_2O$, CH_2 and CH_3 along polymer backbone).

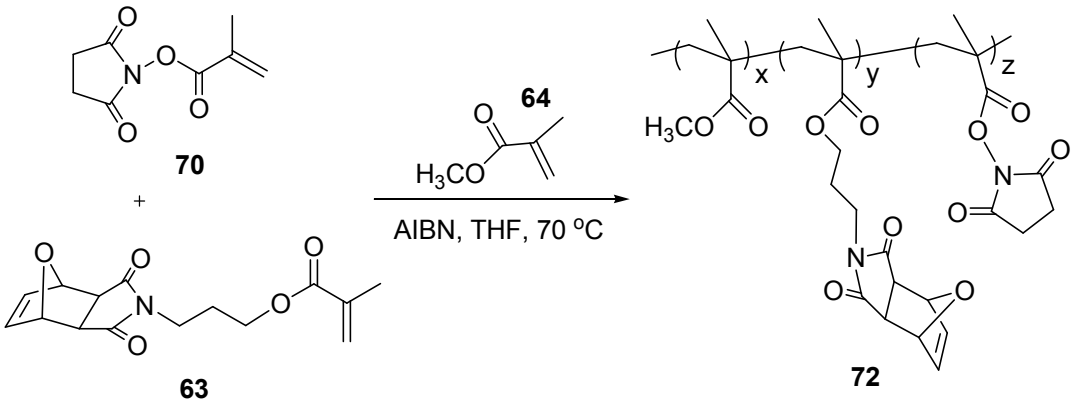


Figure 5.25. Synthesis of succinimide-maleimide containing polymer

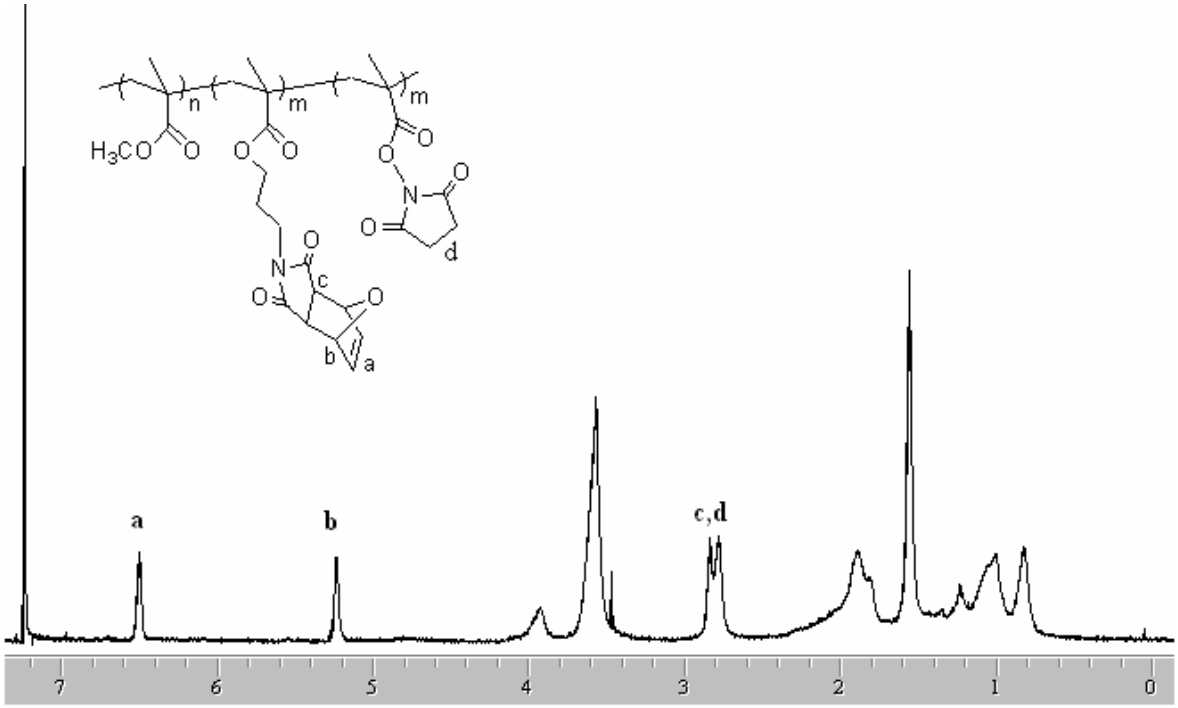


Figure 5.26. ^{1}H NMR spectrum of succinimide-maleimide copolymer

5.5.4 Retro Diels-Alder Reaction of Succinimide-Maleimide Copolymer 72

Polymer **72** (20.0 mg) was heated neat under vacuum at 125°C for 3 hours. ^{1}H NMR ($CDCl_3$, ppm) 6.71 (s, 2H, CH=CH of maleimide), 3.97 (s, 2H, OCH_2), 3.57 (br s, 5H, OCH_3 and NCH_2), 2.79 (s, 4H, CH_2-CH_2 of succinimide), 1.93–0.84 (m, 7H, $NCH_2CH_2CH_2O$, CH_2 and CH_3 along polymer backbone).

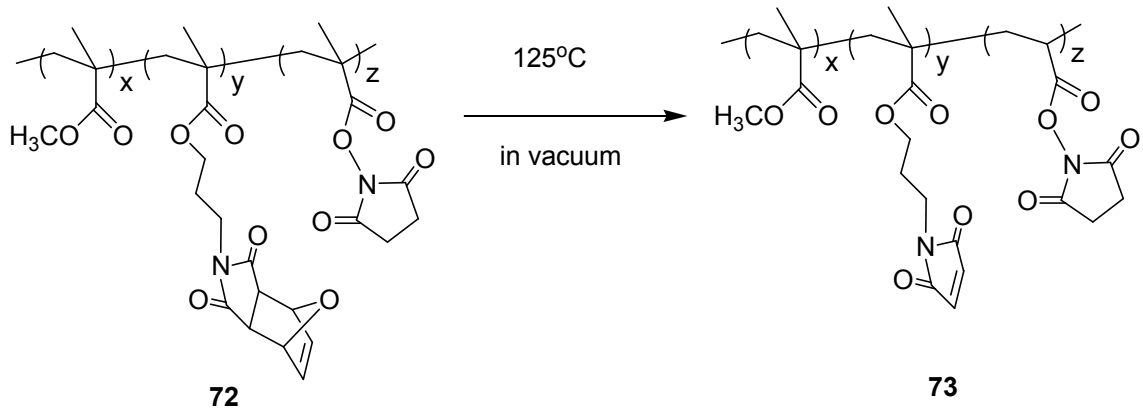


Figure 5.27 Retro DA reaction of succinimide-maleimide containing copolymer

Effective conversion of the oxabicyclic moiety to the reactive maleimide functional group while keeping the succinimide moeity intact during the cycloreversion step was clear from the NMR spectrum of the copolymer **73** (Figure 5.28). Relative stoichiometry of both the reactive groups in the same polymer can be accertained by integration of the proton resonances of the maleimide double bond (6.71 ppm) and succinimide unit (2.79 ppm).

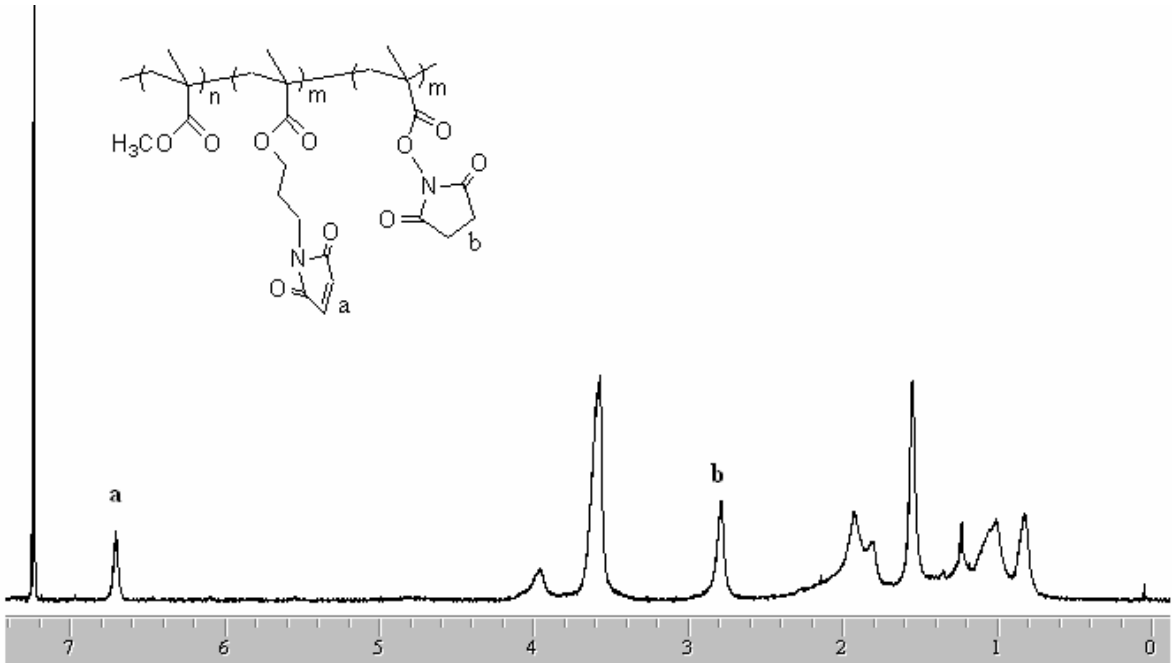
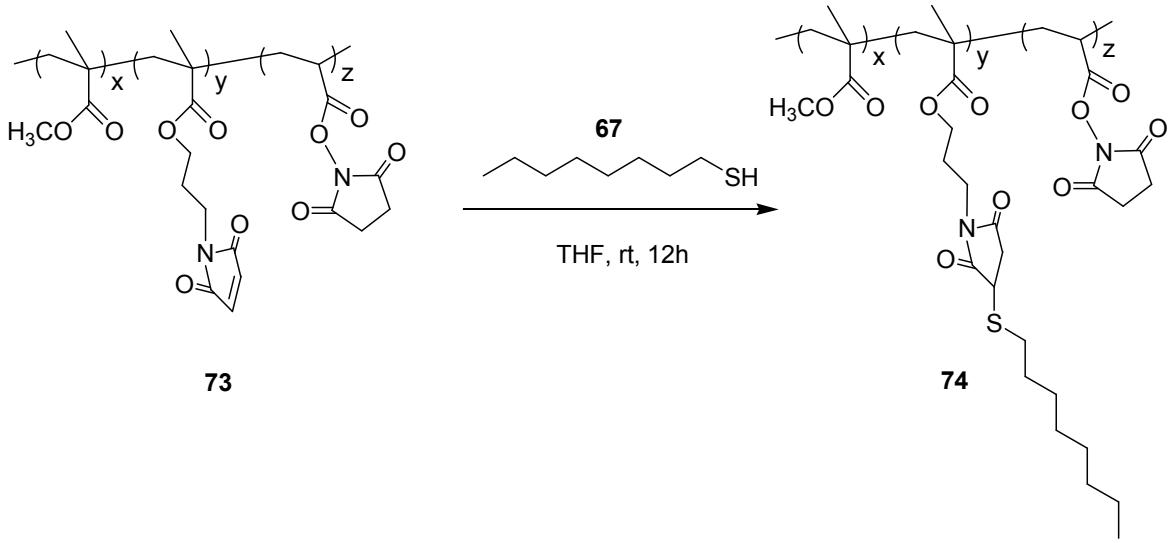


Figure 5.28. ^{1}H NMR spectrum of succinimide-maleimide copolymer after rDA

Selective stepwise functionalization of the maleimide-succinimide containing copolymer was probed by reacting it with octanethiol (Figure 5.29). Disappearance of the peak due to maleimide double bond in the ^{1}H NMR spectra and presence of expected new peaks due to attachment of the octanethiol moiety implied successful Michael addition (Figure 5.30). Furthermore, no effect of the thiol addition step on the succinimide unit was clear from no change in its signature proton resonance at 2.79 ppm. Further reaction of this polymer with amine containing nucleophiles can be expected to smoothly provide the second functionalization.



73 **74**

Figure 5.29. Thiol addition to the succinimide-maleimide containing copolymer

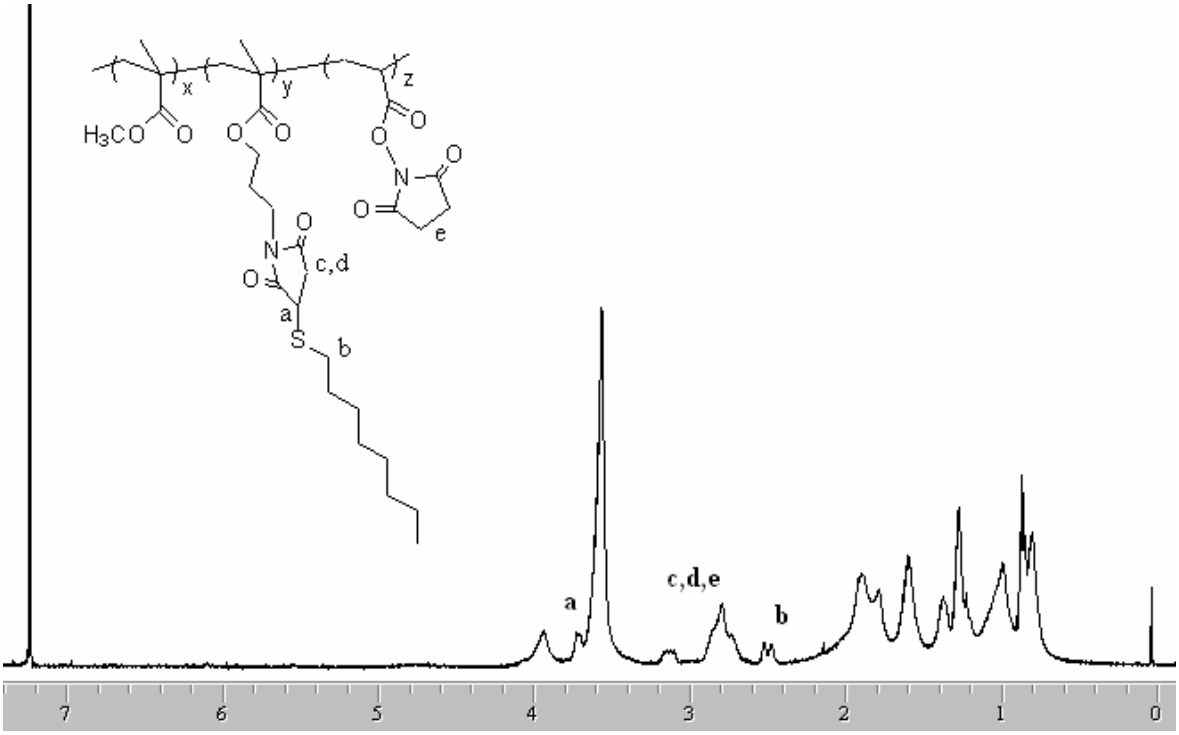


Figure 5.30. ^{1}H NMR spectrum of thiol conjugated succinimide-maleimide copolymer

In summary, a polymer capable of orthogonal functionalization by a thiol based nucleophile and an amine based nucleophile was sucessfuly synthesized. Selective functionalization was demonstrated by selective addition of thiol nucleophile to the maleimide group while leaving the succinimide group intact for further reaction with amine containing molecules.

6. MICRO CONTACT PRINTING (μCP)

Micro arrays provide a powerful analytical tool for the simultaneous analysis in a single experiment, by making use of the improved sensitivity that results from miniaturized binding. Micro arrays were first demonstrated for immunological ligand-binding assays and they were then quickly embraced and developed by genomics community [40]. Challenges in creation of microfabricated arrays involve addressing controlled and selective immobilization of molecules in defined positions on a surface. In the development of rapid, robust screening micro arrays, several strategies have been succesfully applied such as robot-based high-precision contact printing [41], multistep photolithography [42], selective molecular assembly patterning [43], and soft lithographic approaches, such as the micro contact printing (μCP) method. The μCP technigue which was developed by Kumar and Whitesides [44], is widely used for the fabrication of monolayer-based micrometer- and sub-micrometer patterns due to the simplicity of the method. Low cost, flexibility, and the possibility to pattern curved substrates are among other advantages that make μCP very attractive for a variety of applications such as the development of certain biosensors [45].

Micro contact printing is a high-resolution printing technique in which a pattern is transferred from surface of an elastomeric stamp to a solid substrate by conformal contact. This is one of several "Soft Lithography" technologies that are under development for high resolution patterning of a variety of materials for advanced applications. In μCP, an elastomeric stamp, usually a PDMS (polydimethylsiloxane) stamp, with patterned structures on its surface is used to generate patterns and structures on the surface of a substrate. Micro contact printing gives the advantage of transfering a variety of molecules with submicrometer resolution to substrates without the need for any harsh chemical treaments.

6.1. General Methods for μCP

In our study, we investigated the fabrication of bioreactive micropatterns of the maleimide containing polymer **P5** and succinimide containing polymer **71** via micro contact printing.

6.1.1 Preparation of Mold for the Preparation of PDMS Stamp

PDMS (polydimethylsiloxane) stamp, with patterned structures on its surface were obtained by using molds which are prepared by UV photolithograpy. To prepare the mold for PDMS stamp, a photoresist which is a UV sensitive organic polymer is spin-coated to the surface of a silicon wafer. The photoresist thickness can be adjusted by controlling the speed and duration of the spin coating process. Then this silicon surface is gently heated to remove the solvent. After this soft baking step, the photoresist is exposed to UV light through a photomask. In certain areas, this mask allows the light to pass through, and in other areas does not let the light penetrate to the resist surface. After treatment, photoresist is washed vith commercially available developer which dissolves the uncrosslinked parts of the photoresist, leaving a topographic template for molding stamp (Figure 6.1).

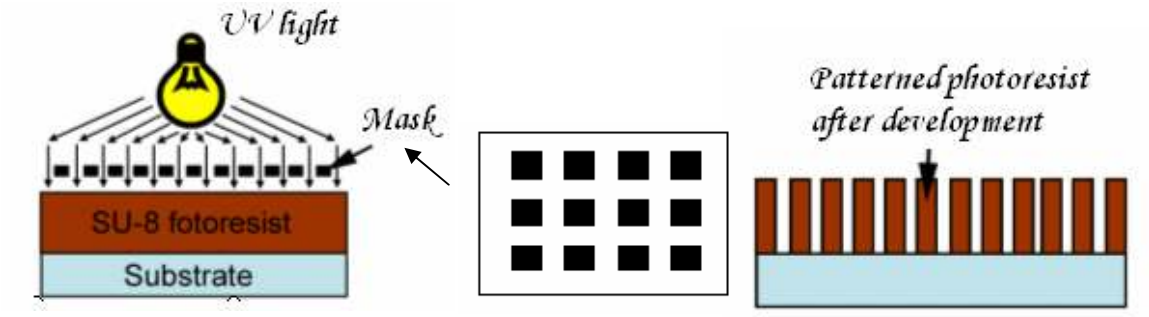


Figure 6.1. Preparation of mold for PDMS stamp

In this study, SU-8 2100 was used as a photoresist. First the SU-8 photoresist was poured over the silicon wafer. After that it was spread at 500 rpm for 10 seconds and spinned at 3000 rpm for 30 seconds. The film thickness was expected as nearly 100μm according to the processing guideline of SU-8 photoresist. After spinning process, the

photoresist spin-coated substrate was dried via a soft baking step. In this process, the substrate was kept on a hot plate at 95°C for 25min. After 25min, substrate was exposed to UV light. We used a UV lamp with a power of around 1 mW/cm^2. We exposed it for 5 minutes. After exposure we kept the substrate on a hotplate at 95°C for 12 minutes and the image of the mask became visible. The next step was the development step, Mrdev 600 SU-8 developer containing 2-methoxy-1-methylethyl acetate is used to develop the photoresist. This process dissolves the uncrosslinked parts of the photoresist. SU-8 is a negative photoresist. It means the polymer stays where it sees UV light. After development, we rinsed the substrate with isopropanol, dried it and obtained the mold to prepare PDMS stamp.

6.1.2 Preparation of the PDMS Stamp and μCP Process

The template prepared by photolithography was used as a mold for the preparation of the PDMS stamp. In this step, PDMS was poured into the photoresist mold and left to cure. After the curing step, it was peeled off from the surface easily and was ready for the patterning process (Figure 6.2).

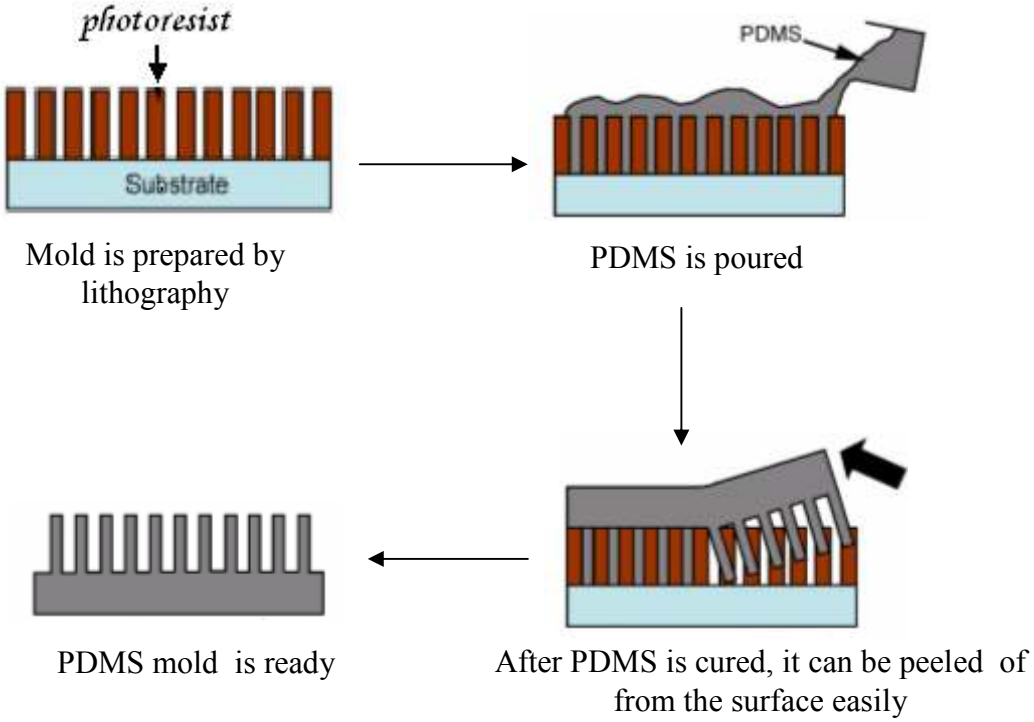
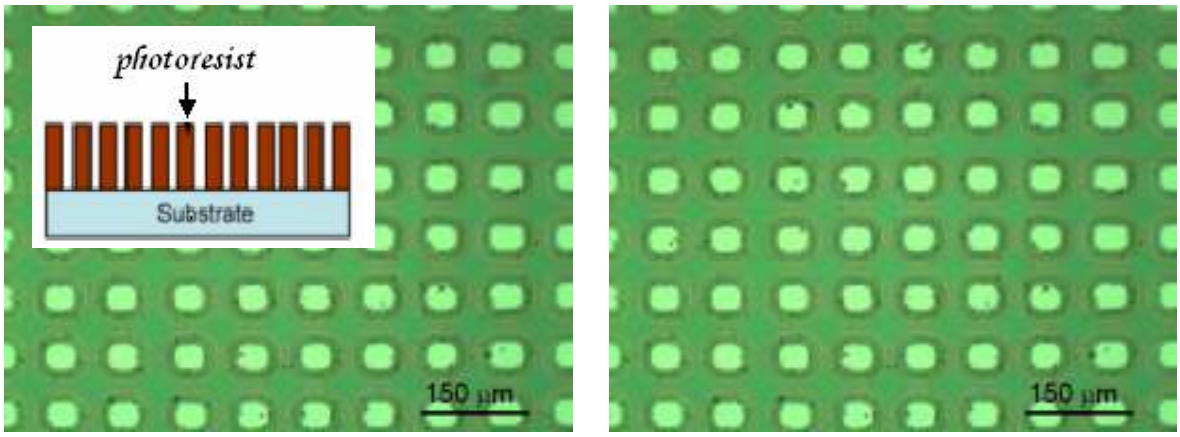
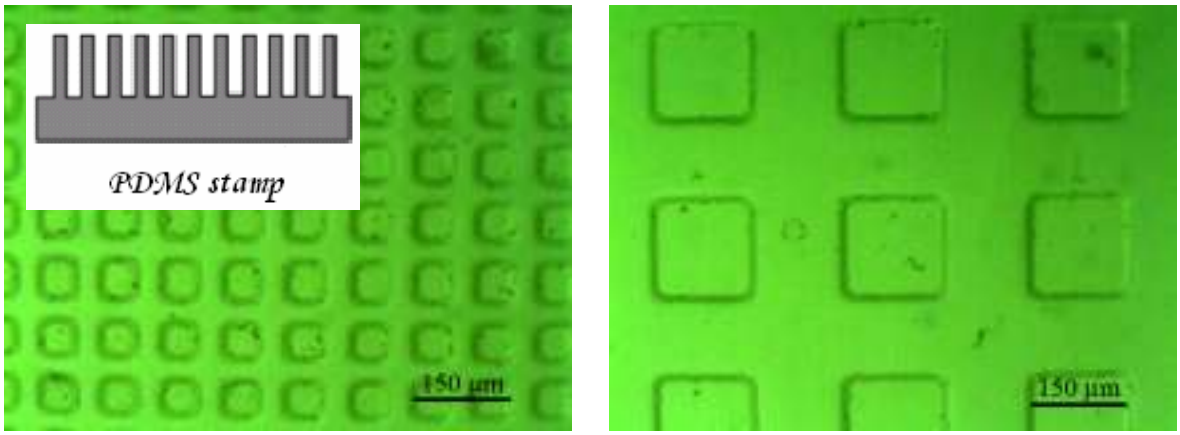


Figure 6.2. Preparation of PDMS stamp

The pictures 6.1 and 6.2 shows how the mold and the PDMS stamp are seen under the optical microscope. The square holes on the photoresist mold forms the hills on the PDMS surface. The photocuring methodology allows to obtain reliable patterns of the photoresist component in a way that corresponds to predefined topography of the PDMS stamp namely the square hills on the stamp.



Picture 6.1. Microscopic view of the mold used for the preaparetion of PDMS stamp



Picture 6.2. Microscopic view of the PDMS stamp

During the µCP process, a solution of our reactive polymer was applied to the surface of the PDMS stamp and after evaporation of most of the solvent, the stamp was placed in contact with a clean substrate. Peeling the stamp off the substrate revealed the printed pattern (Figure 6.3). Several solvents were screened for the printing process. In our

case, best patterns were obtained when THF was used as a solvent to prepare the polymer solutions. In essence, a series of solutions with different concentrations were screened, and it was observed that as the solution concentration increased the patterns on the surface became clear and darker. Moreover, we should wait at least 1min after we washed the stamp with polymer solution just to get rid of excees solvent. This time interval was optimized for each polymer solution in every case. Also the duration of stamping was also important, it was observed that after 15 or 20 seconds of stamping, the reverse images on the surface were also observed. Picture 6.3 and 6.4 shows the microscopic views of the surfaces after patterning of the polymers.

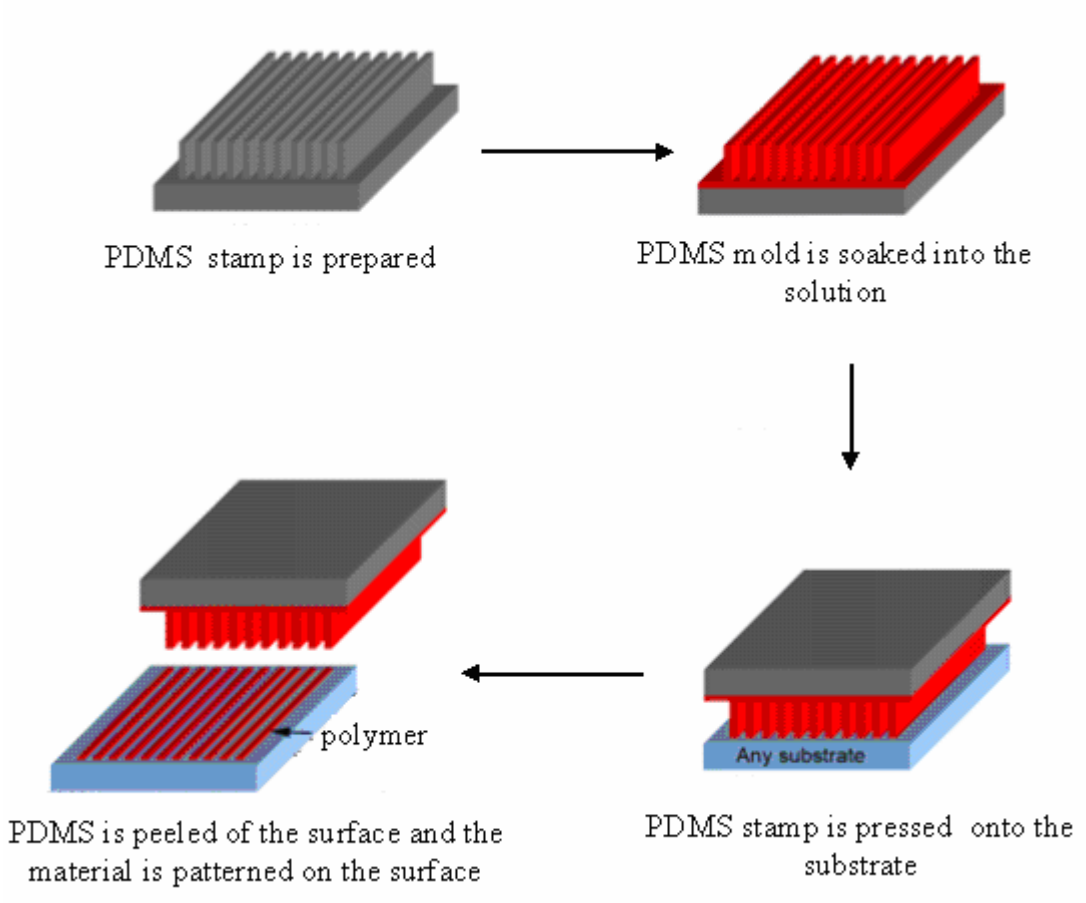
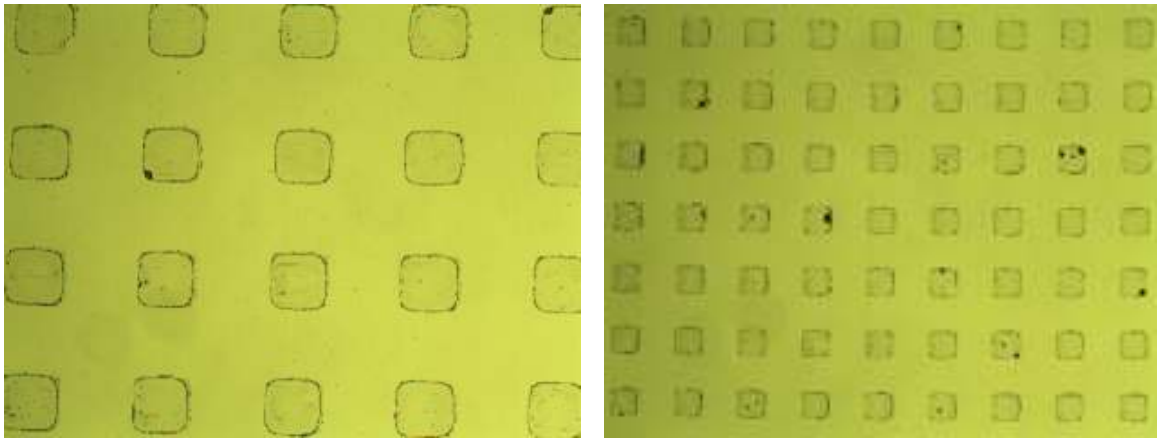


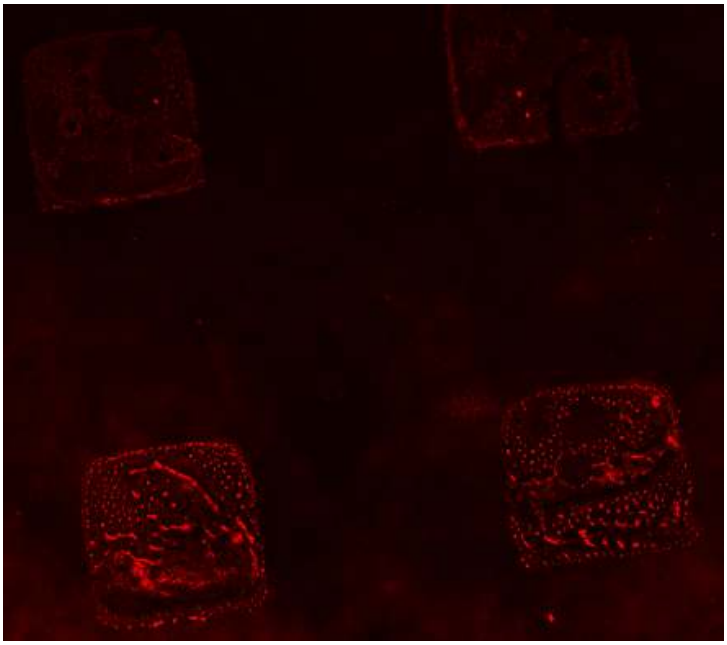
Figure 6.3. μCP process



Picture 6.3. Polymer islands on the surface

6.1.3 Coupling of the Fluorescent Dye to the Patterned Surfaces

The next step after the fabrication of patterned surfaces was to show that conjugation of molecules can be acheived. Immobilization of fluorescent dyes was chosen as model studies to observe the successful covalent immobilization. The surfaces prepared using maleimide based homopolymer immersed in a solution of thiol derivatised BODIPY dye (PBS:pH=7.2) for 4h. Fluoresceinamine was also used to label surfaces patterened using the succinimide based polymers (200μM , PBS: pH=7.2). After incubating in the dye solution, the surfaces were washed with copious amounts of water and dried under vacuum. The fluorescence microscopy image allowed detection of successful attachment of the thiol containing BODIPY dye to the maleimide surface (Picture 6.4). Although it has been realized that immobilization strategy works for a dye molecule, the conditions would require further optimization before addition of a biomolecule.



Picture 6.4. Dye labelled maleimide based polymeric arrays on the surface

7. CONCLUSIONS

A new strategy towards direct synthesis of polymers incorporating a maleimide group in the side chain of a polymer was designed. New acrylate monomer containing a protected maleimide unit was synthesized using furan-maleimide based cycloadducts. This new monomer was copolymerized with MMA using free radical polymerization. The maleimide groups were obtained quantitatively in their native form by heating the polymer. To show that this novel polymer could be functionalized with desired functional groups using a thiol based nucleophile, a thiol containing compound was reacted with malemide polymer. The maleimide groups in the polymers were derivatized with thiol moeities efficiently at room temperature without need of any extra reagents. As an extension, polymers bearing both malemide and succinimide units were successfuly synthesized to enable orthogonal functionalization. In the second part, maleimide containing polymers were patterned on the surface via μCP. Thiol containing BODIPY dye was immobilized on these maleimide based patterned surfaces. Although successful immobilization was apparent from the images obtained using confocal microscopy, it was realized that improvements in the quality of the printed surfaces are desirable. Futher optimization in the printed process followed by enzyme immobilizations will be future goals.

APPENDIX A: SPECTROSCOPY DATA

^{1}H, ^{13}C NMR and IR spectroscopy data for the synthesized compounds are given. Required regions of NMR data are expanded.

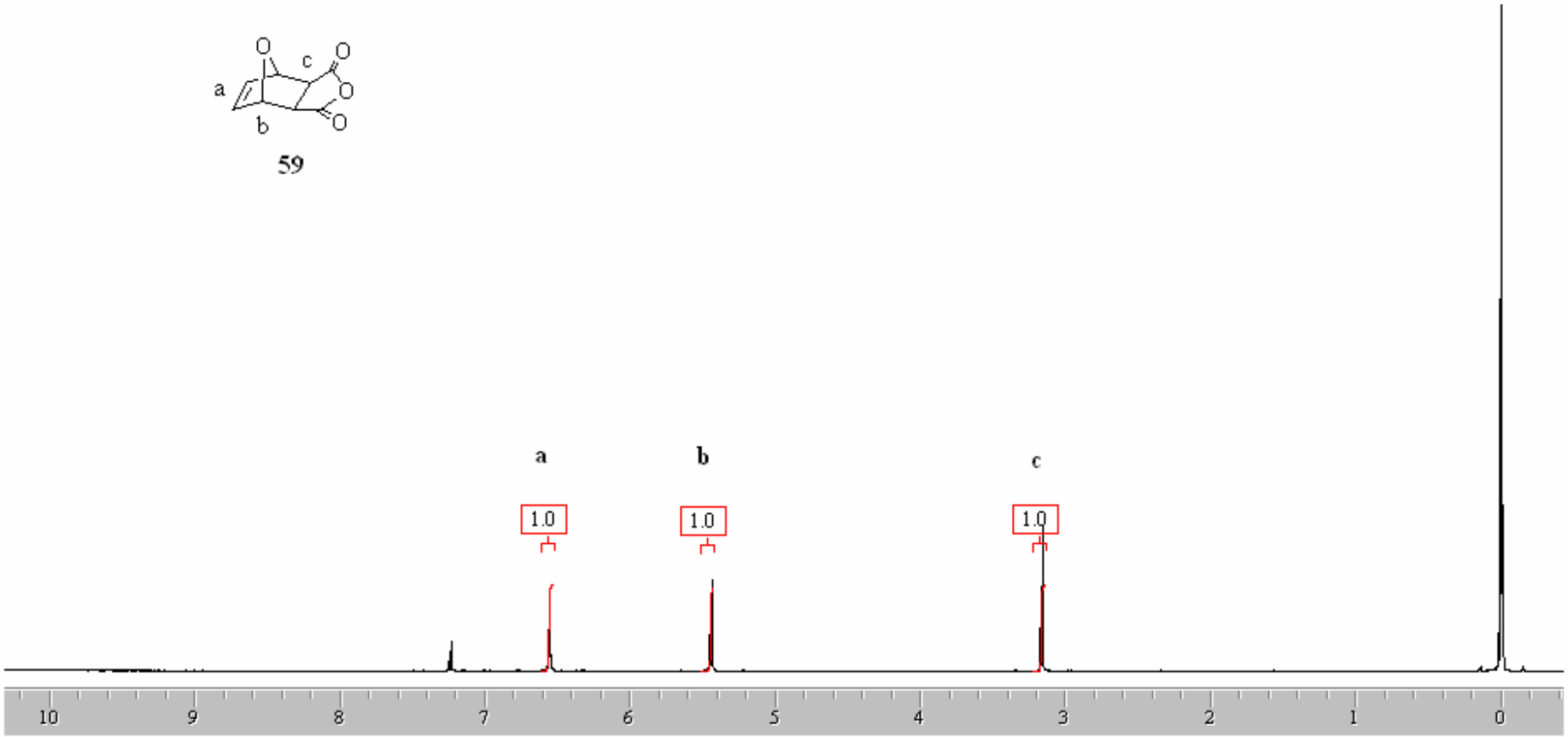


Figure A.1. ^{1}H NMR spectrum of diels-alder adduct **59**

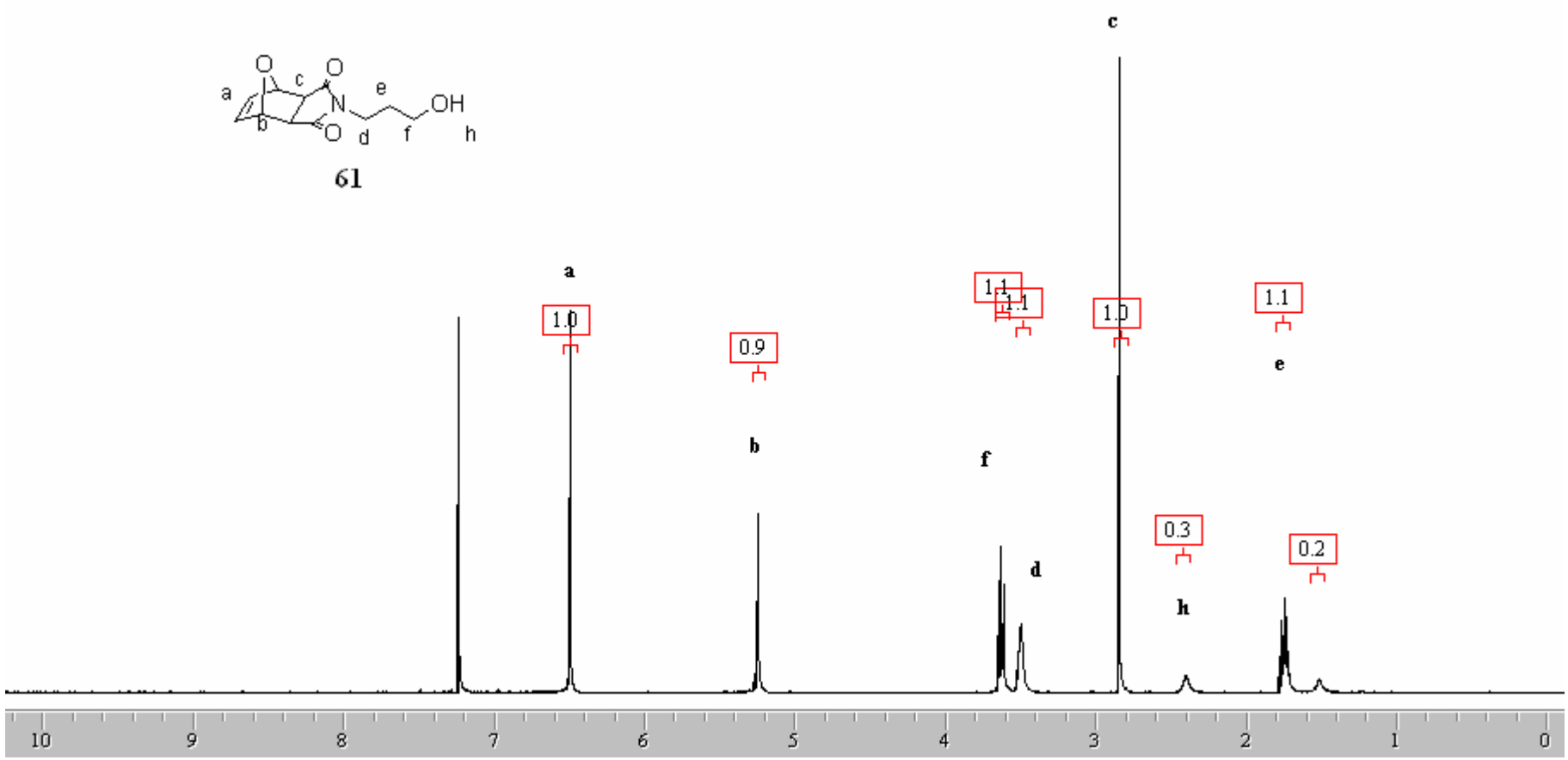


Figure A.2. ^{1}H NMR spectrum of maleimide adduct **61**

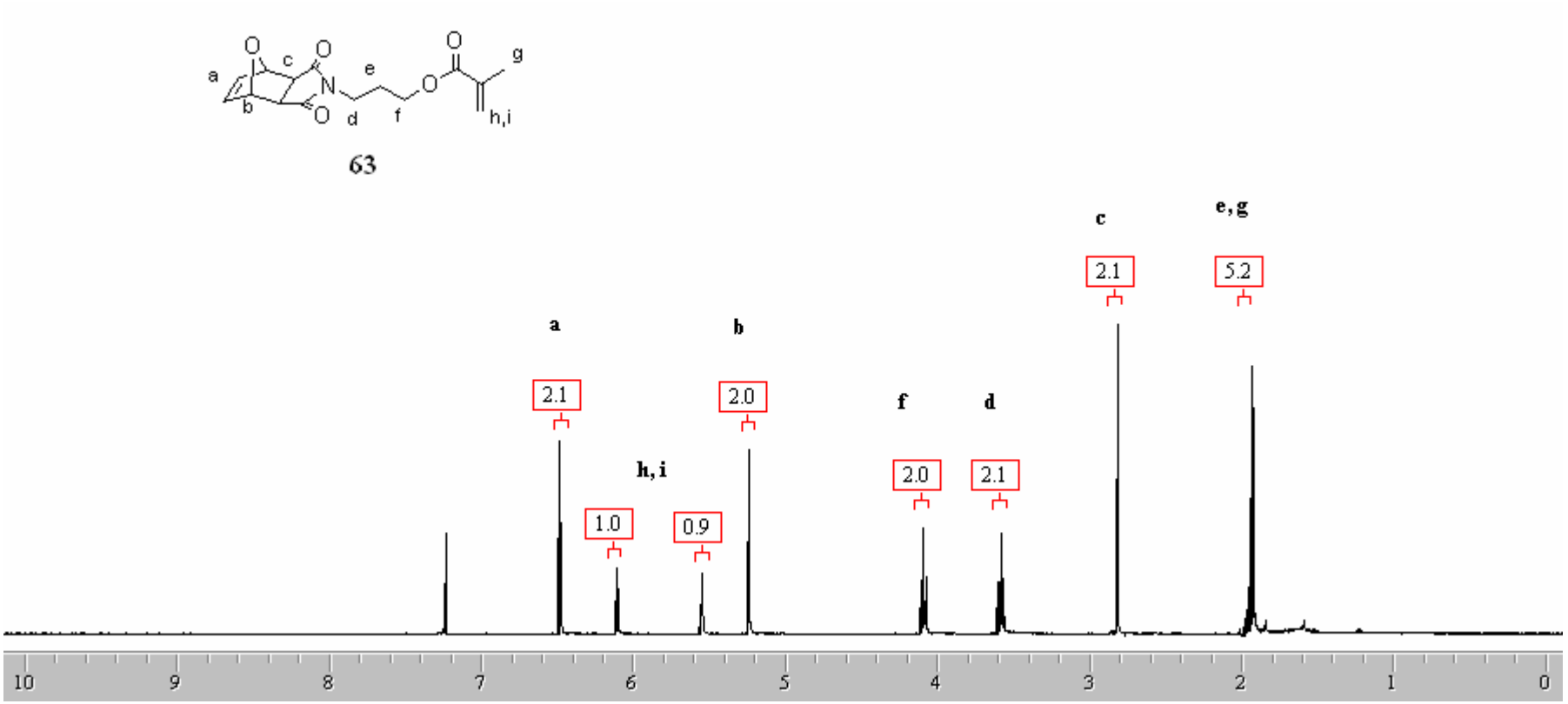


Figure A.3. ^{1}H NMR spectrum of maleimide monomer **63**

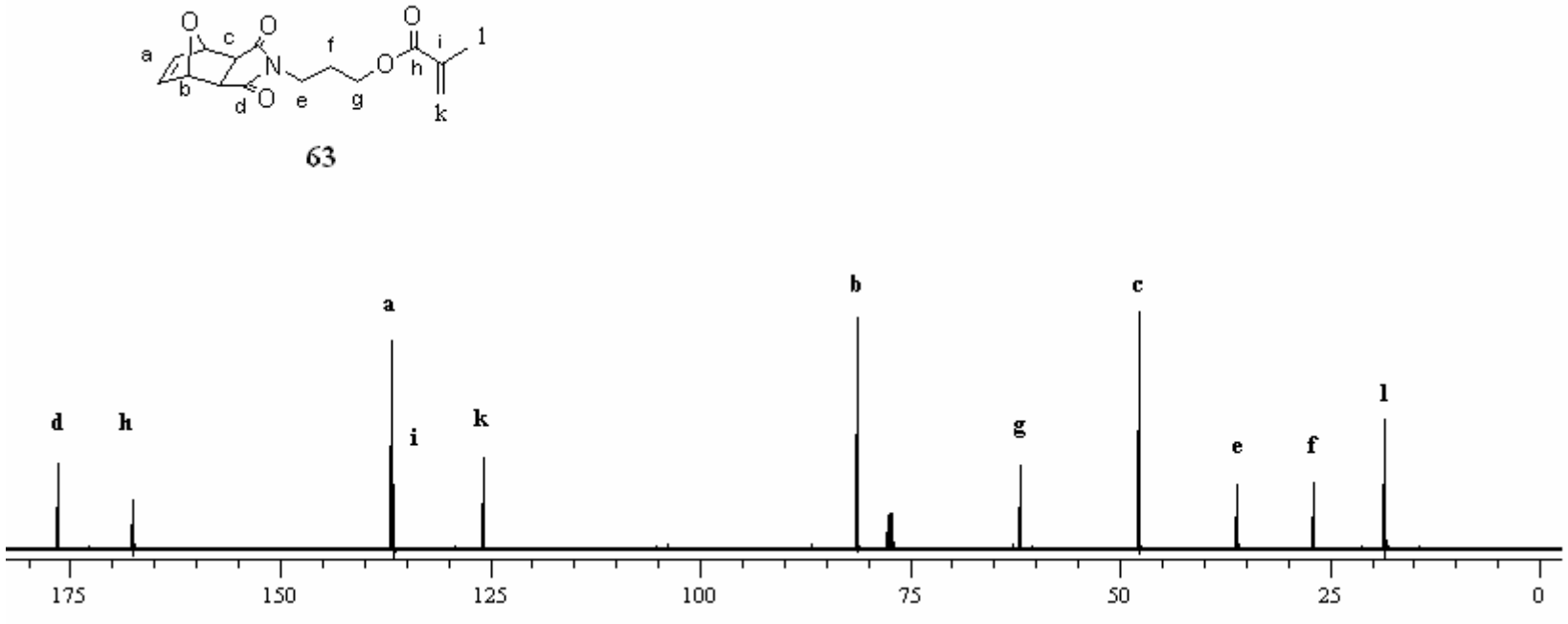


Figure A.4. ^{13}C NMR spectrum of maleimide monomer **63**

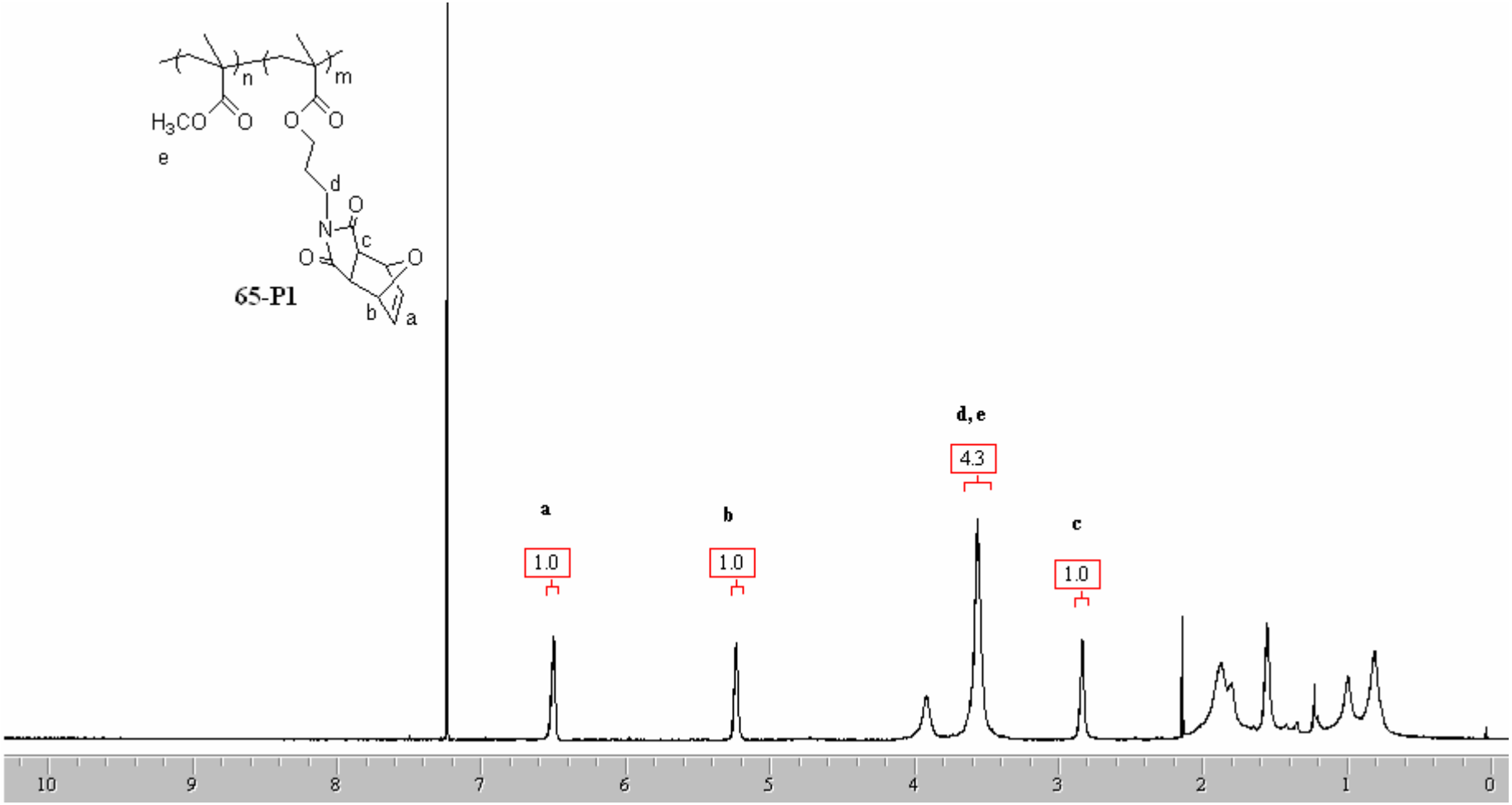


Figure A.5. ^{1}H NMR spectrum of maleimide copolymer **P1**

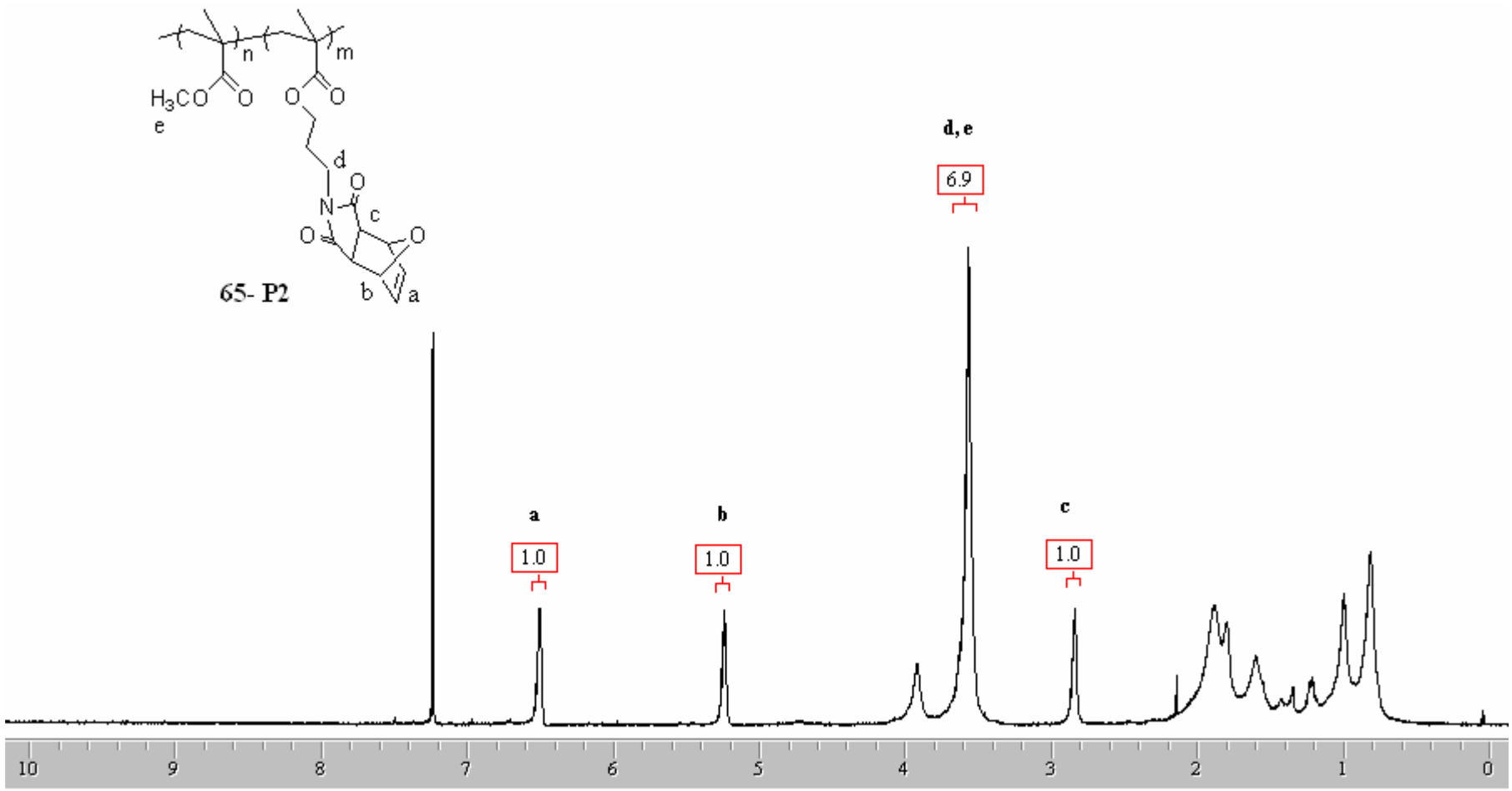


Figure A.6. ^{1}H NMR spectrum of maleimide copolymer **P2**

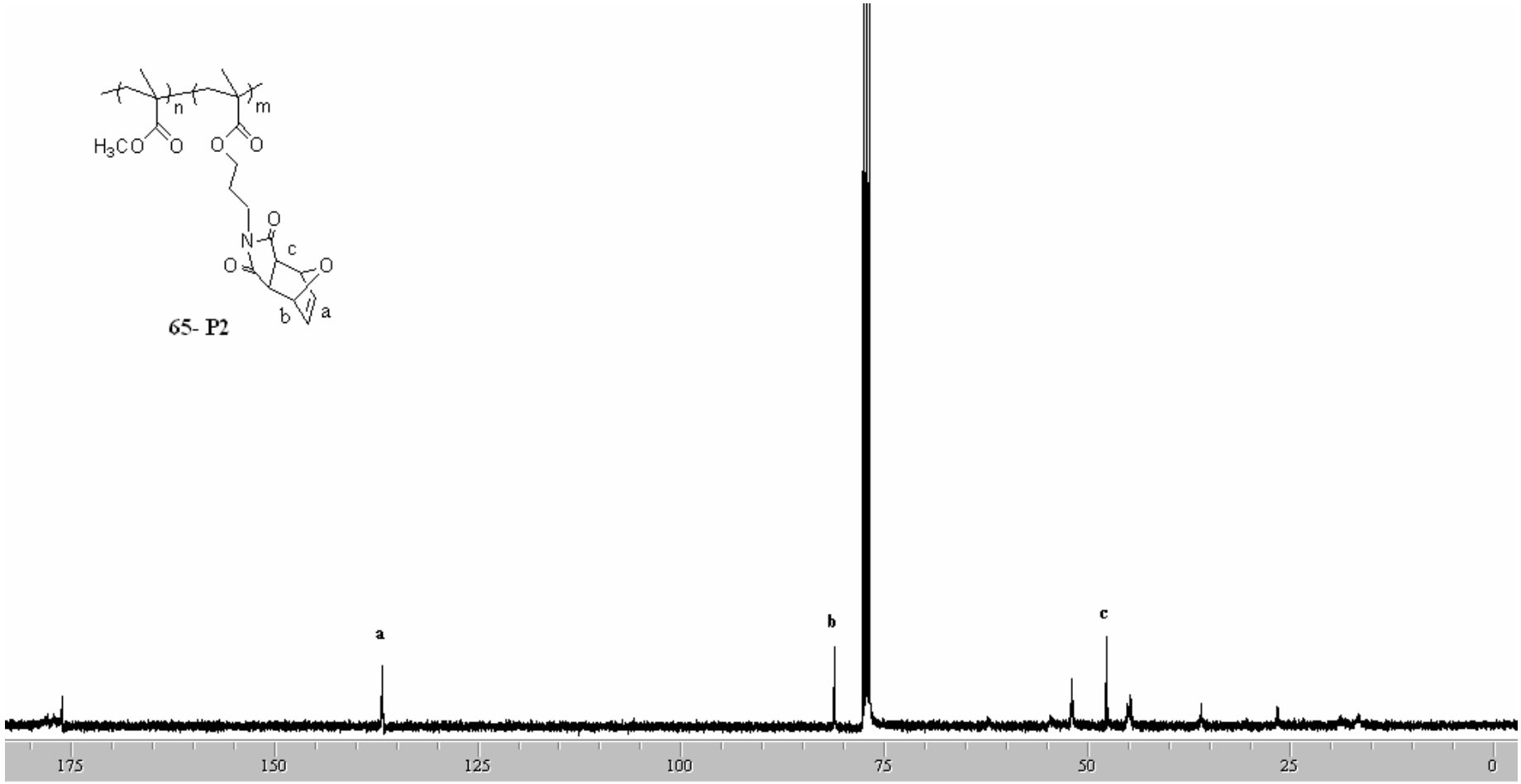


Figure A.7. ^{13}C NMR spectrum of maleimide copolymer **P2**

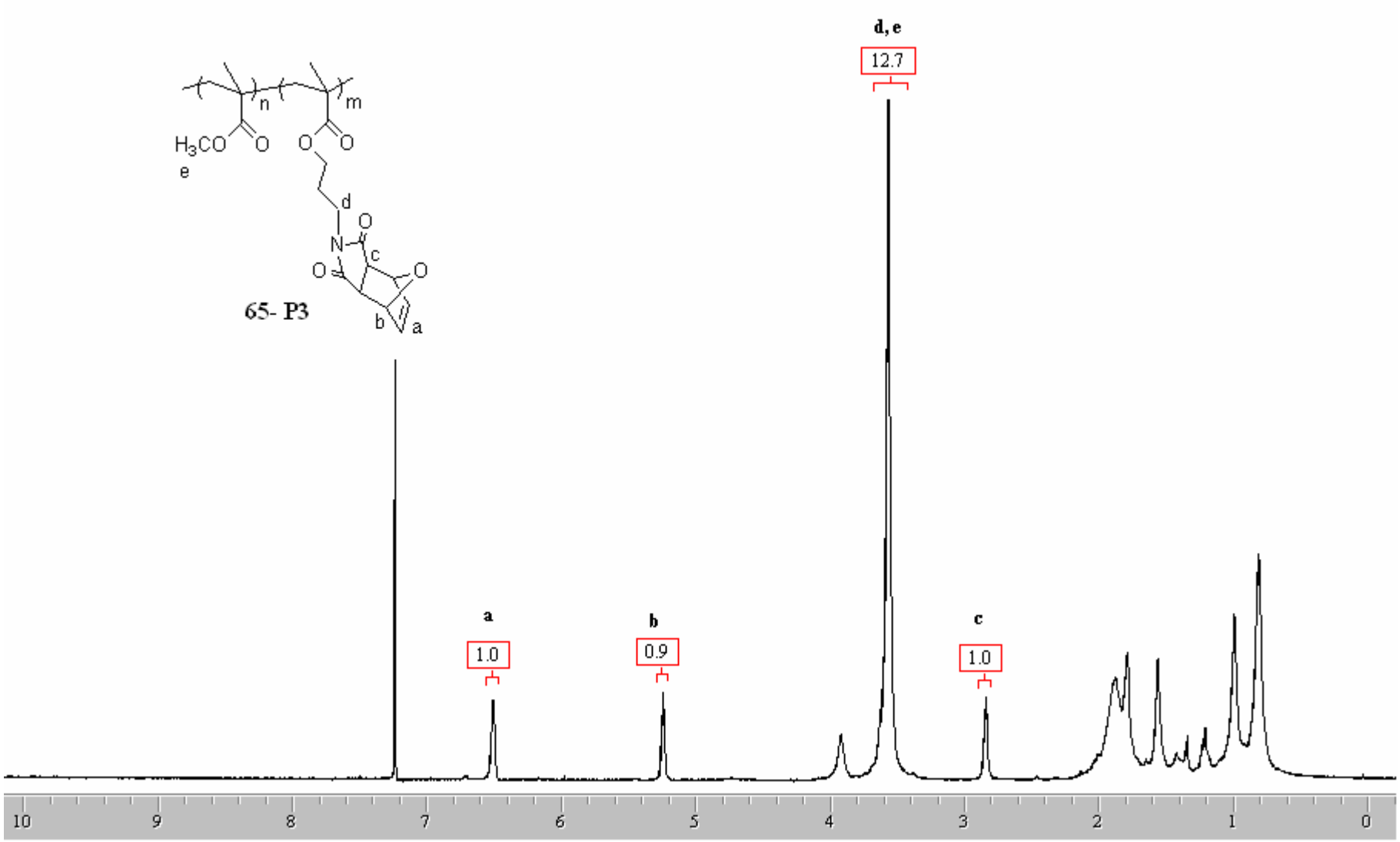


Figure A.8. ^{1}H NMR spectrum of maleimide copolymer **P3**

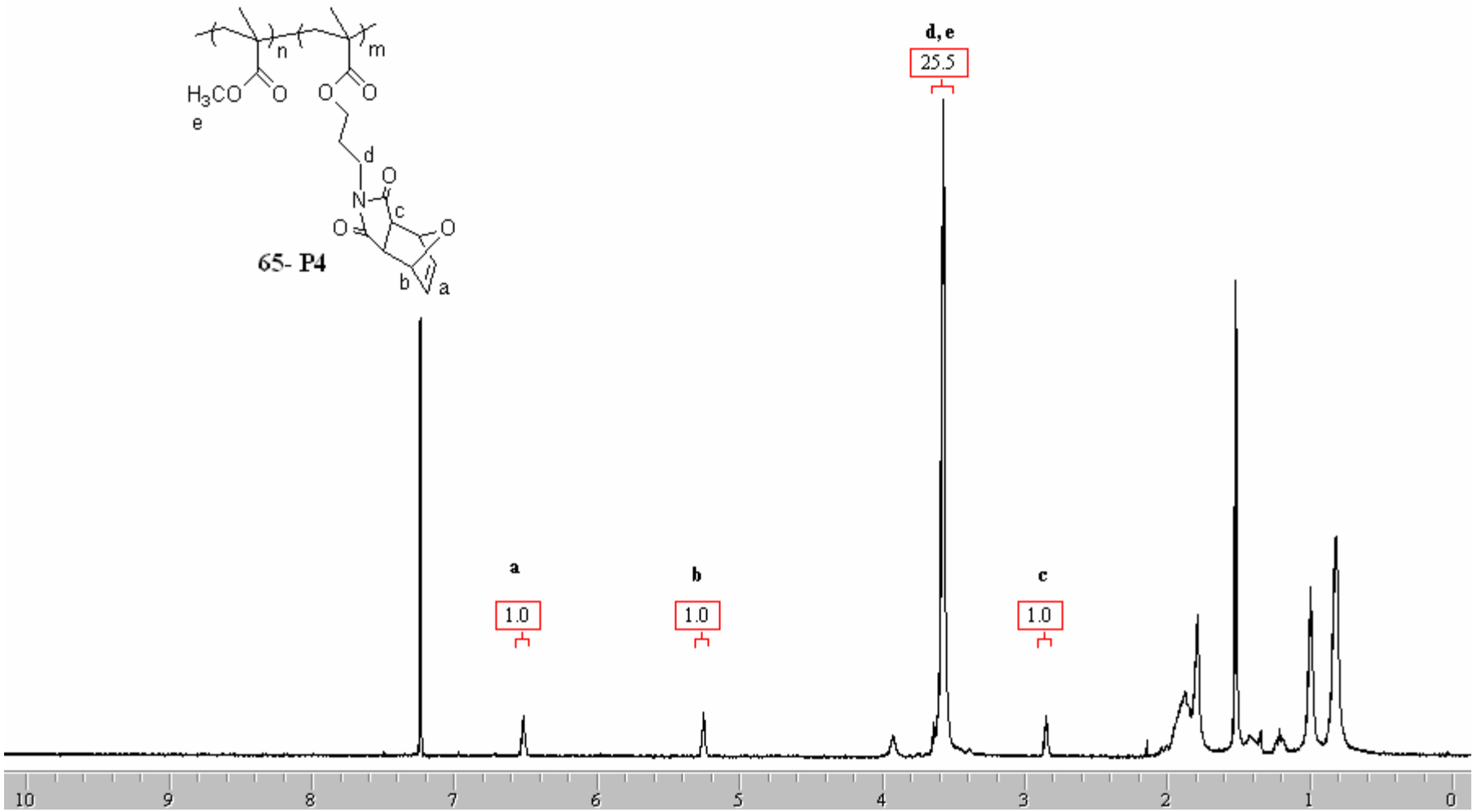


Figure A.9. ^{1}H NMR spectrum of maleimide copolymer **P4**

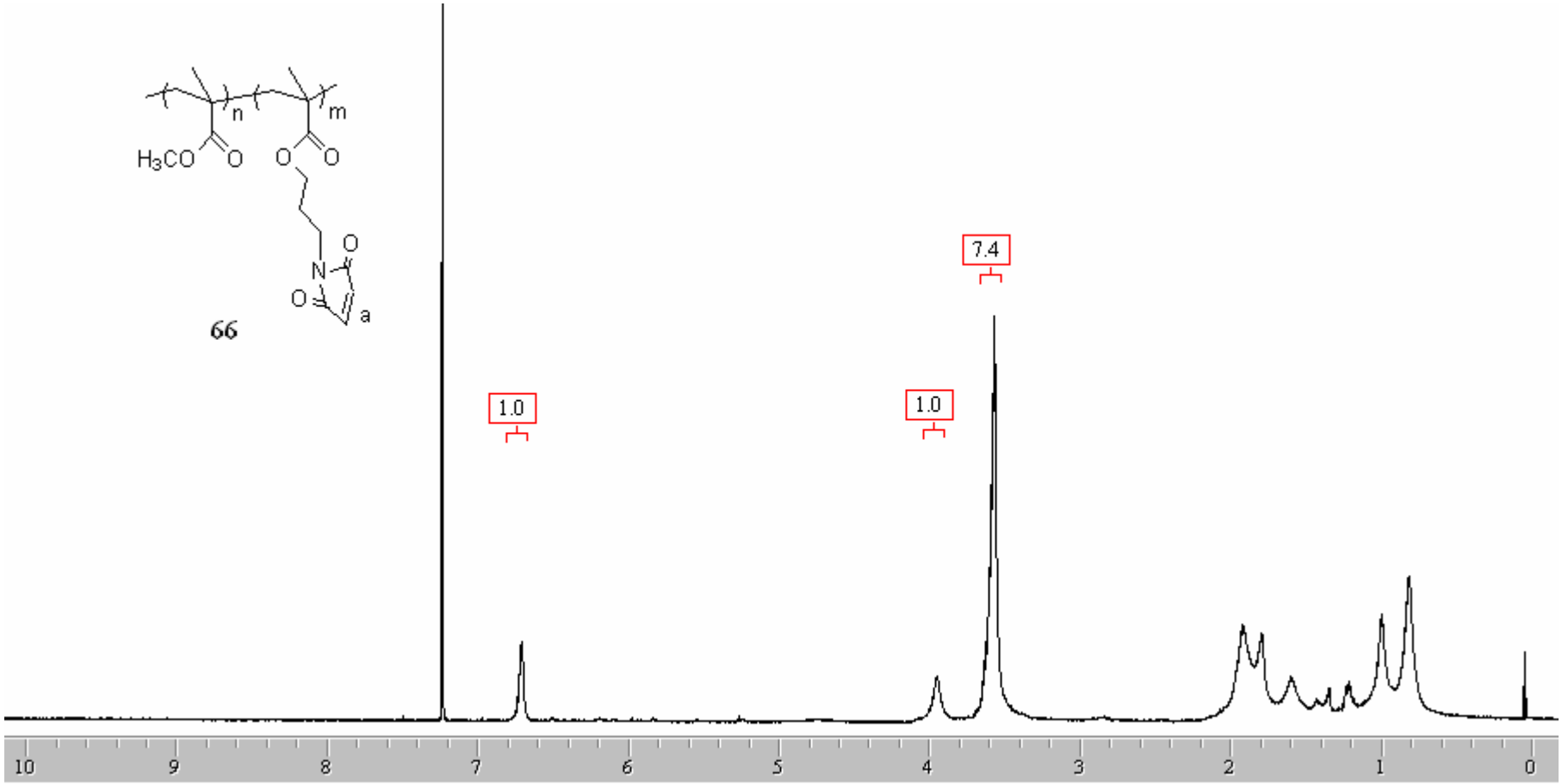


Figure A.10. ^{1}H NMR spectrum of maleimide copolymer **P2** after retro Diels-Alder reaction, (**P5**)

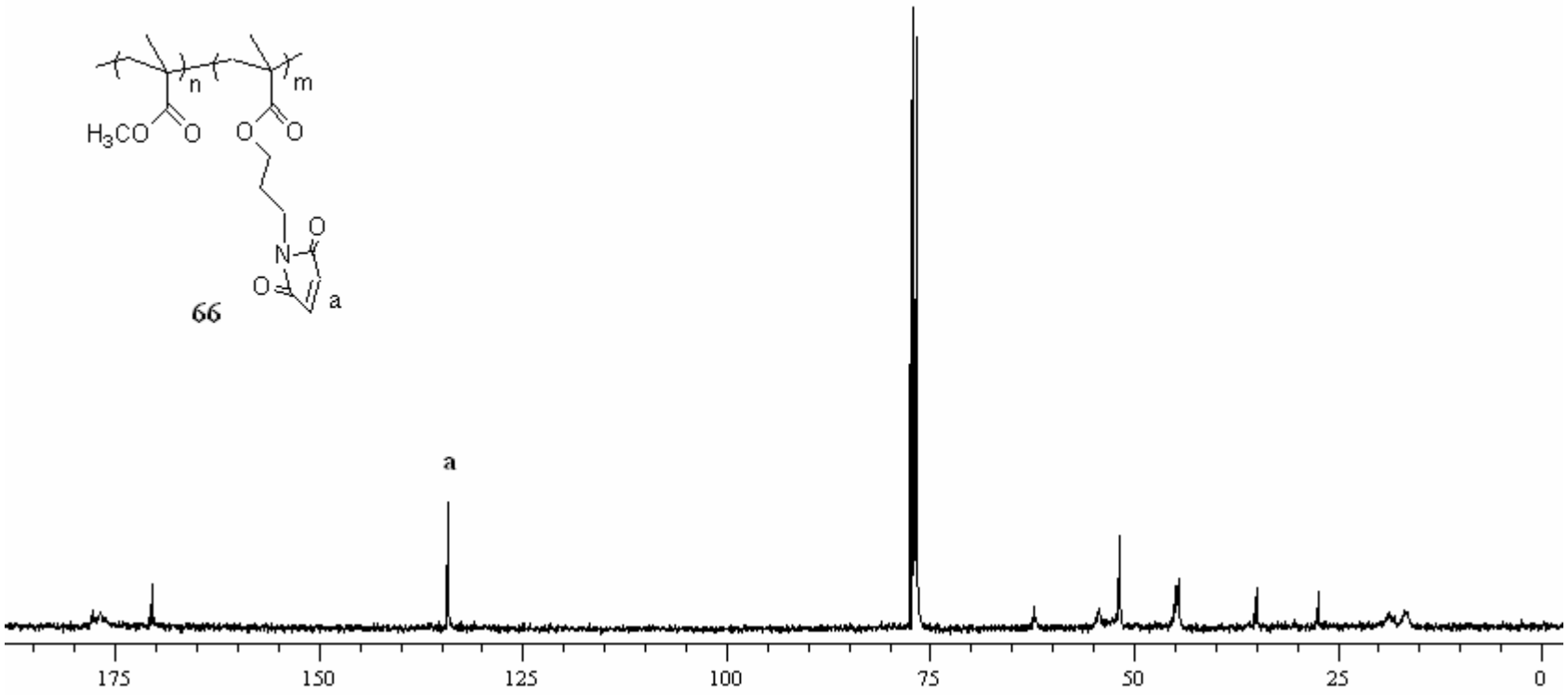


Figure A.11. ^{13}C NMR spectrum of maleimide copolymer **P2** after retro Diels-Alder reaction **(P5)**

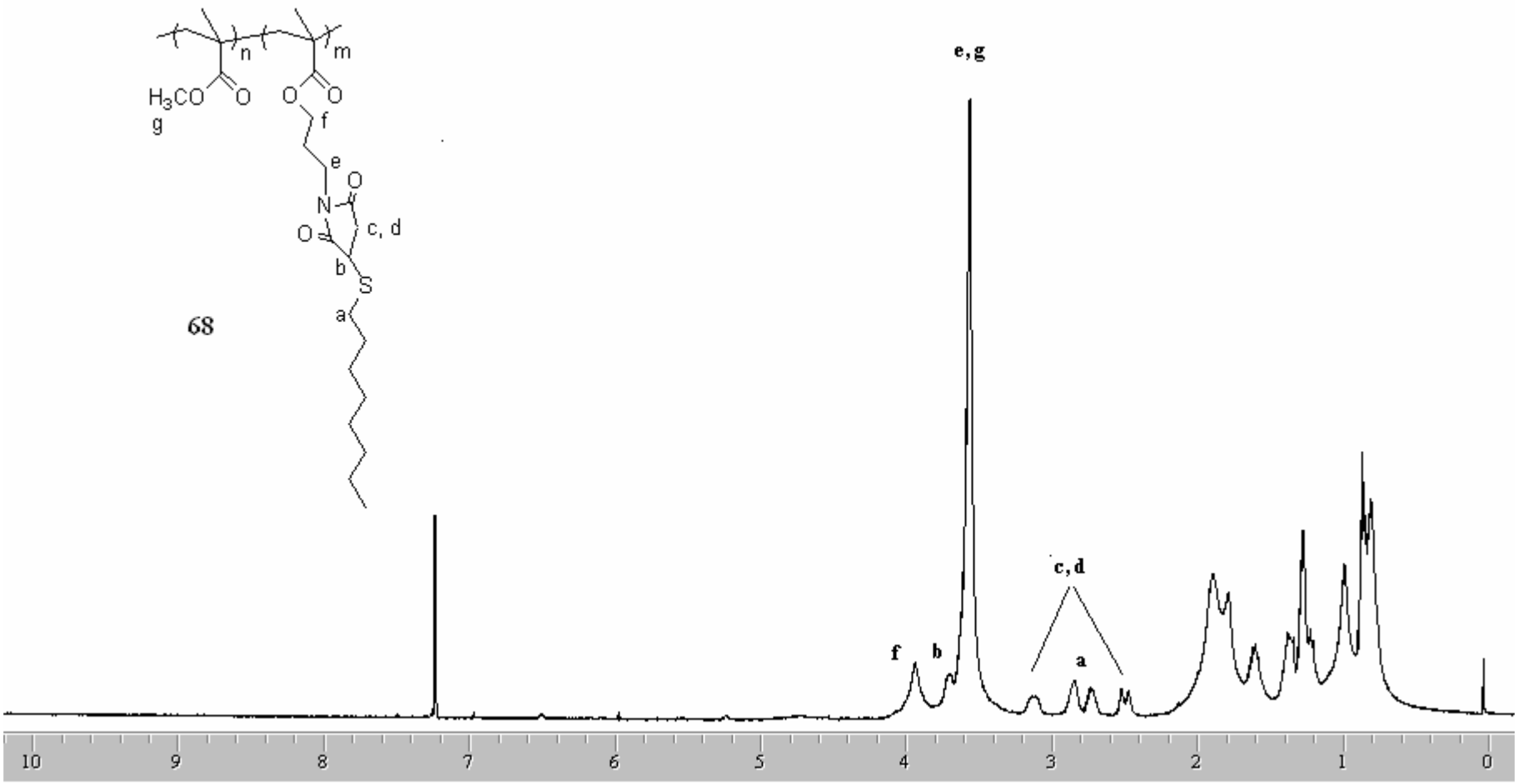


Figure A.12. ^{1}H NMR spectrum of octane thiol conjugated maleimide copolymer **(68)**

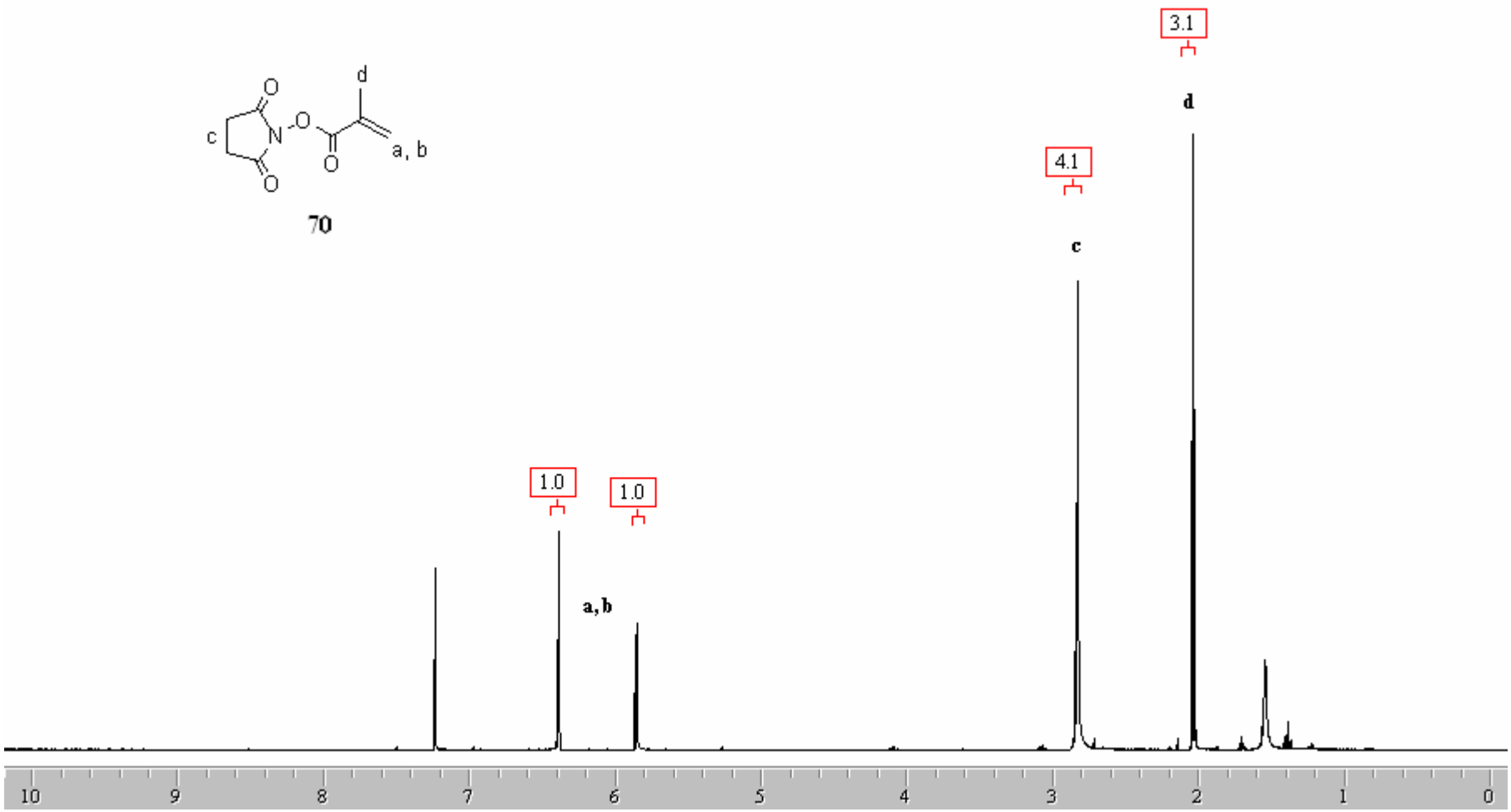


Figure A.13. ^{1}H NMR spectrum of succinimide monomer **70**

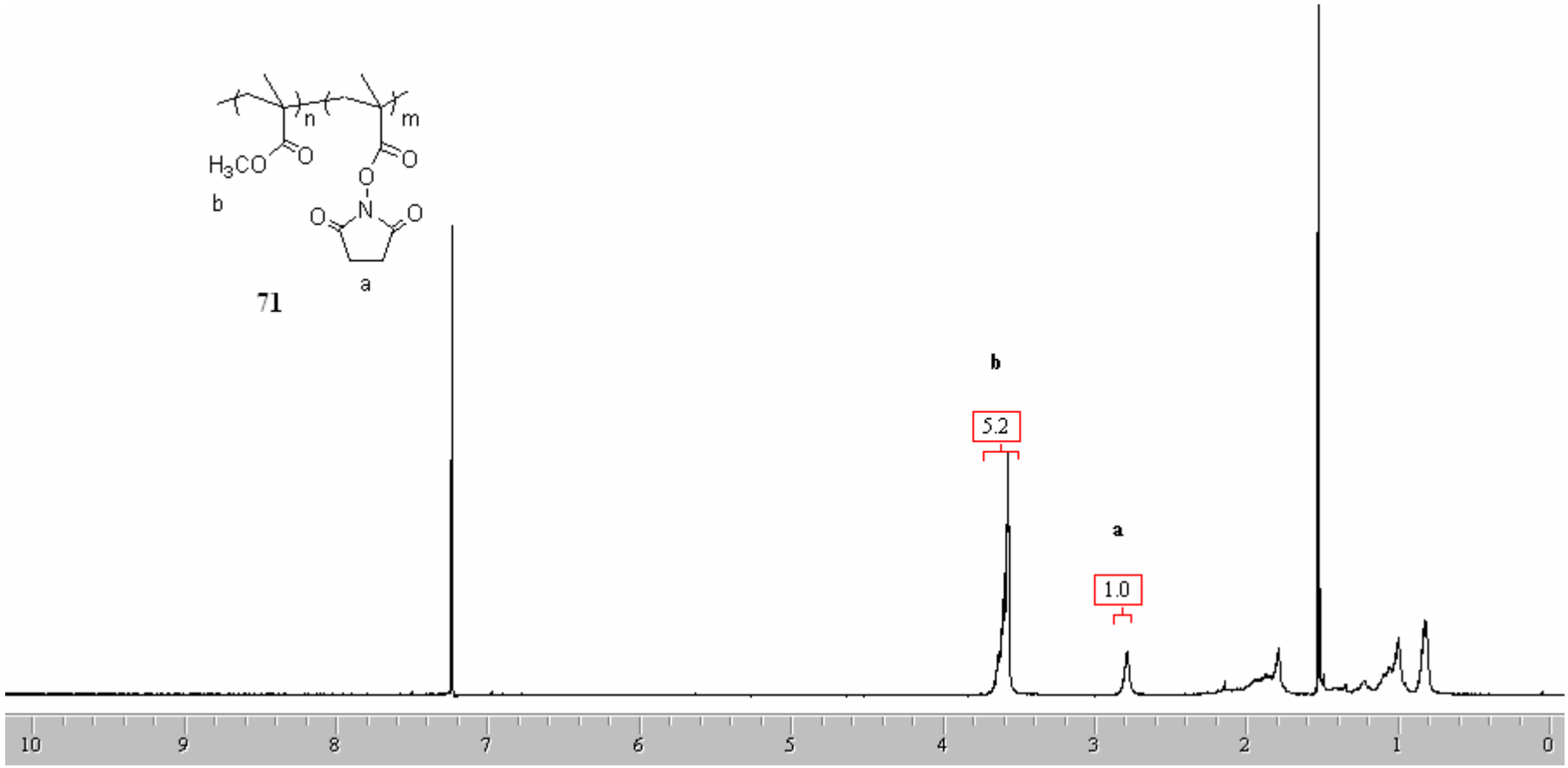


Figure A.14. ^{1}H NMR spectrum of succinimide copolymer **71**

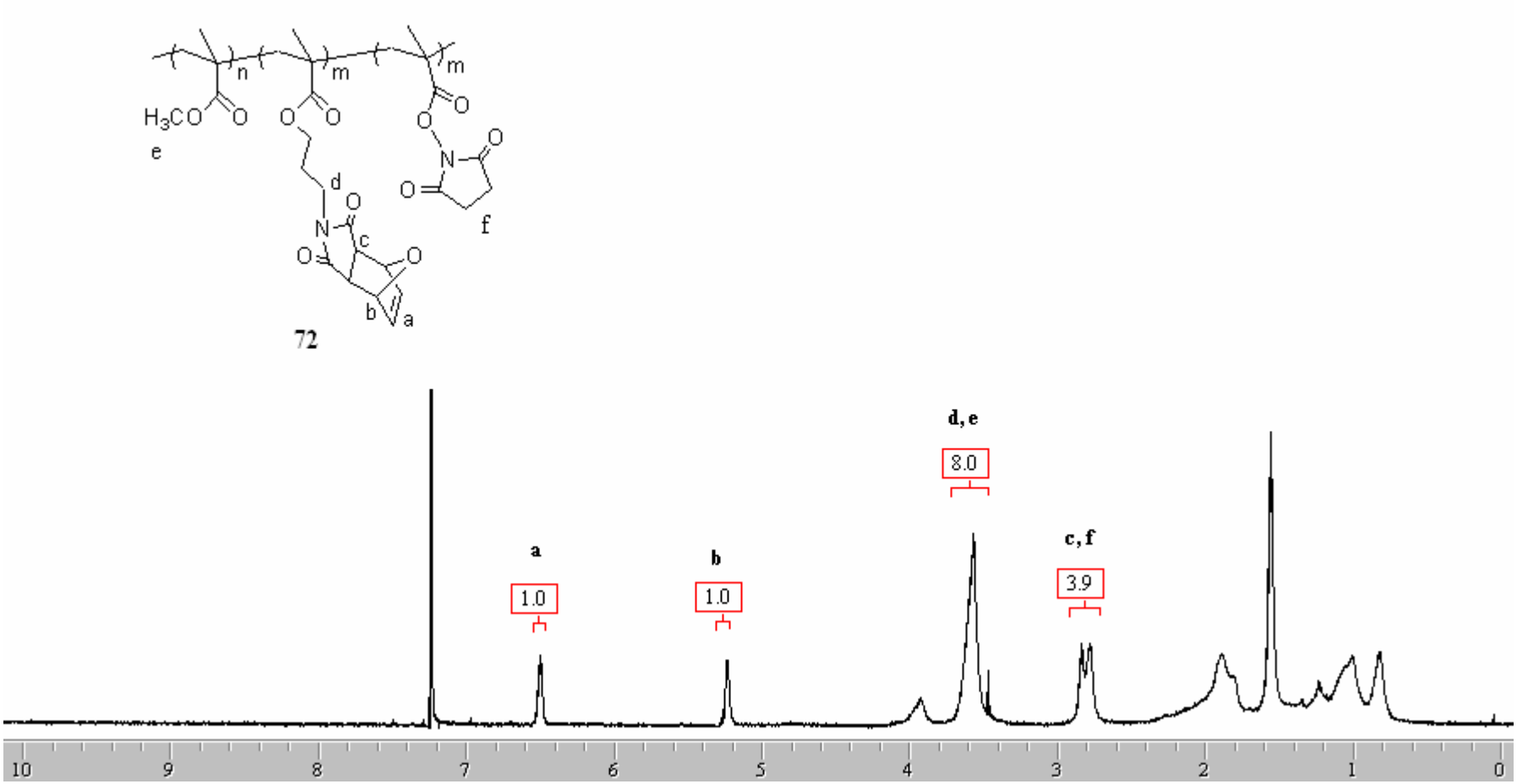


Figure A.15. ^{1}H NMR spectrum of succinimide-maleimide copolymer **72**

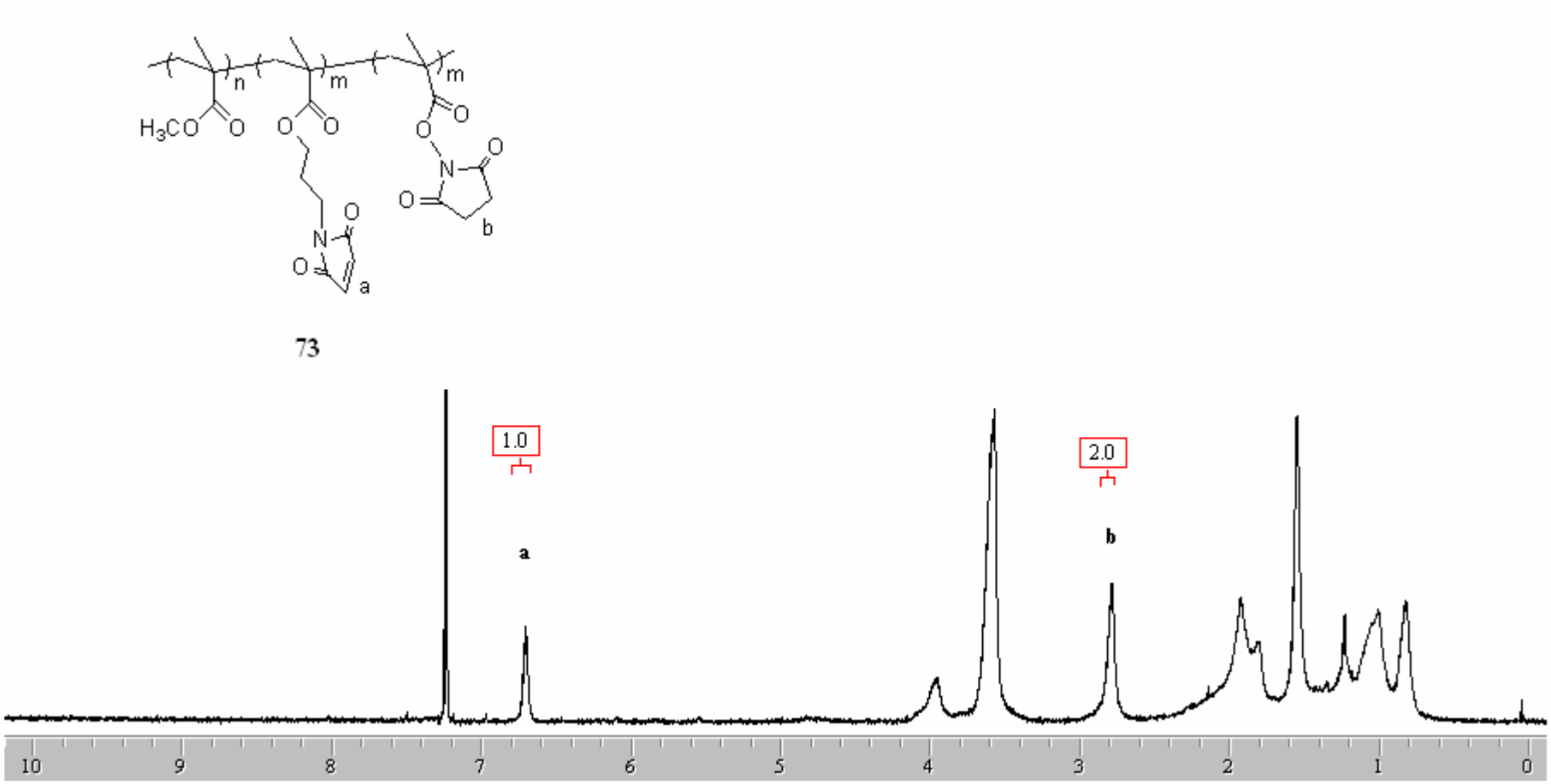


Figure A.16. ^{1}H NMR spectrum of succinimide-maleimide copolymer (**72**) after retro-Diels-Alder **73**

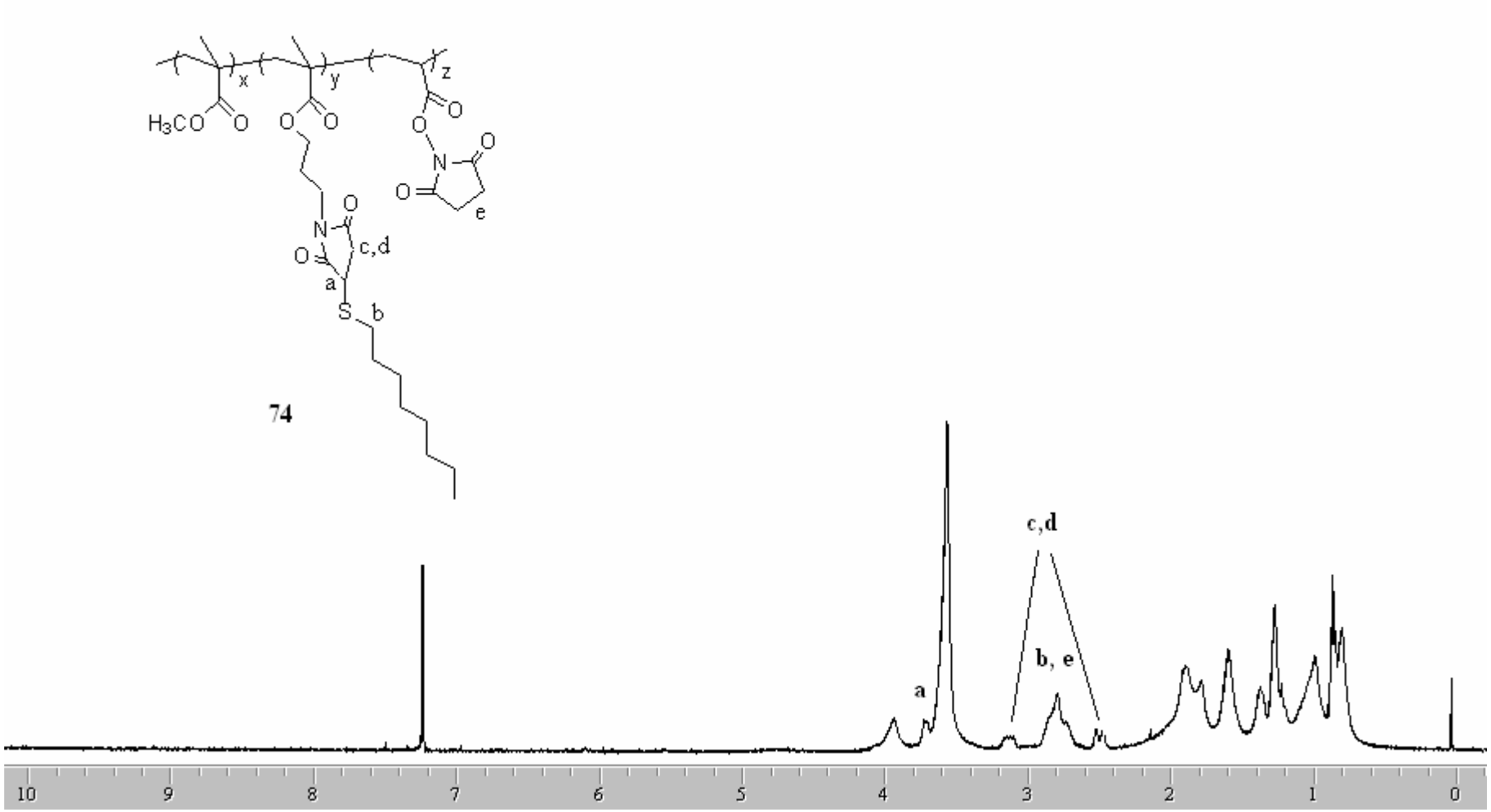


Figure A.17. ^{1}H NMR spectrum of thiol conjugated succinimide-maleimide copolymer **74**

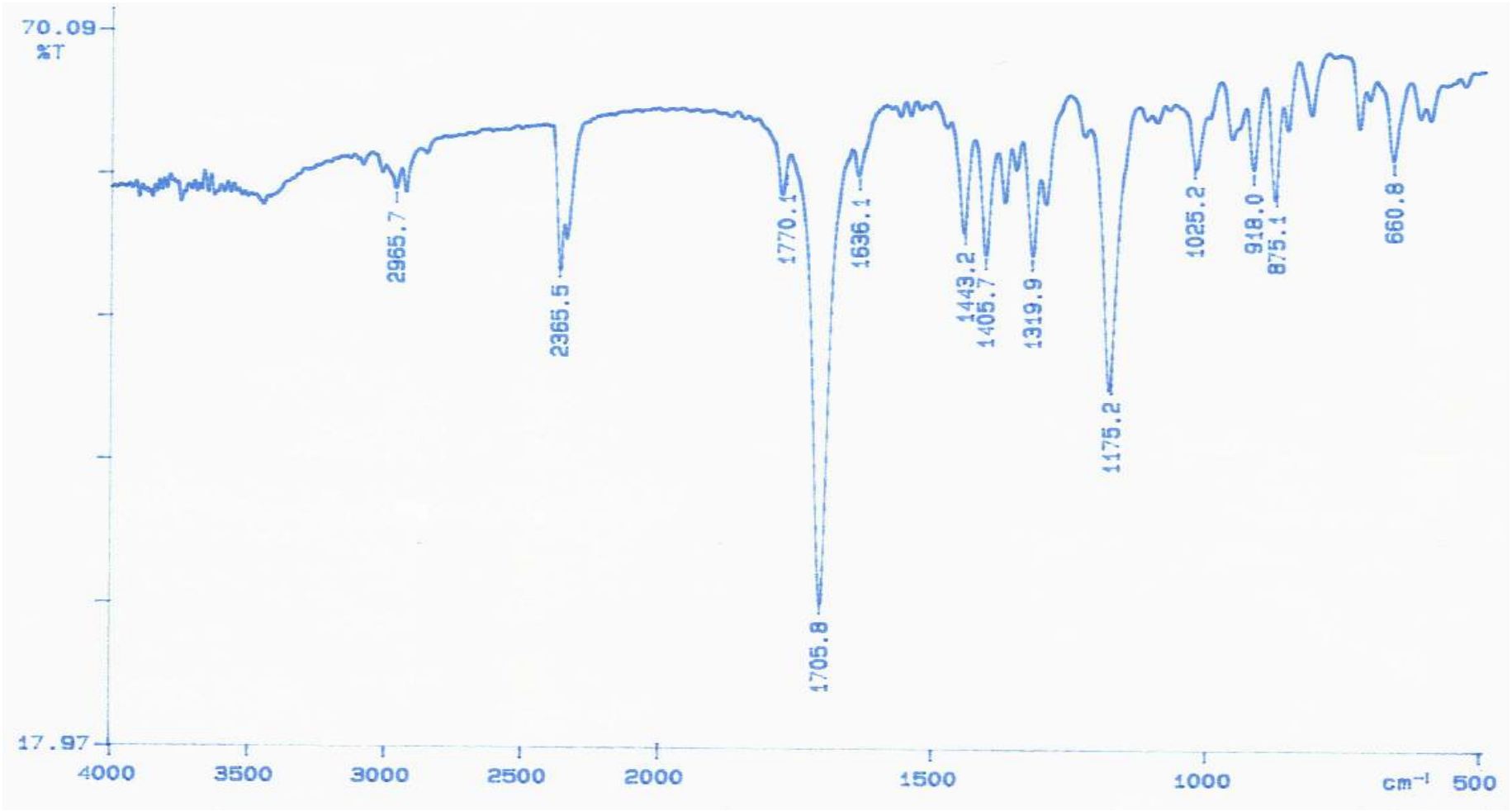


Figure A.18. IR spectrum of maleimide monomer **63**

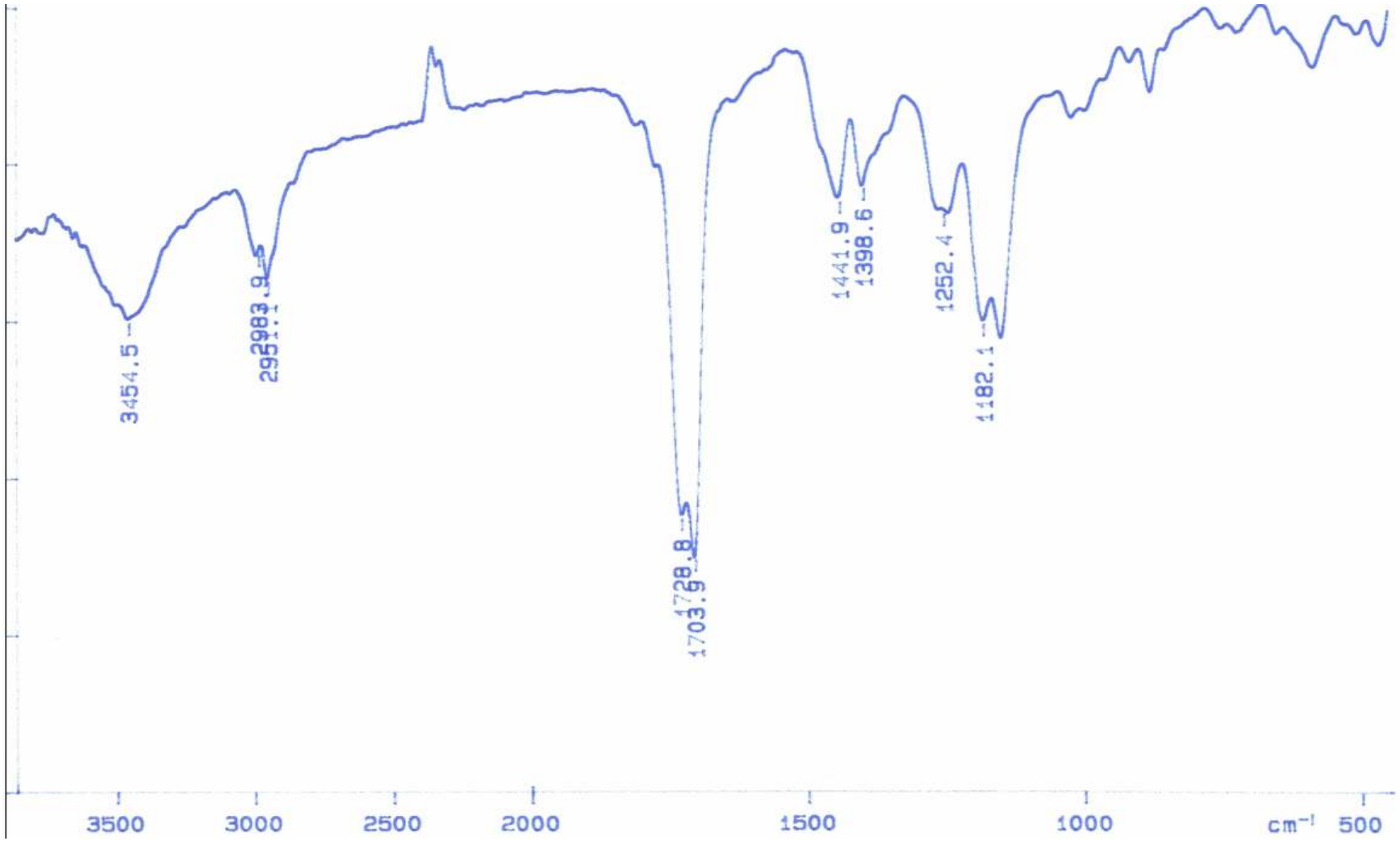


Figure A.19. IR spectrum of maleimide polymer **65**

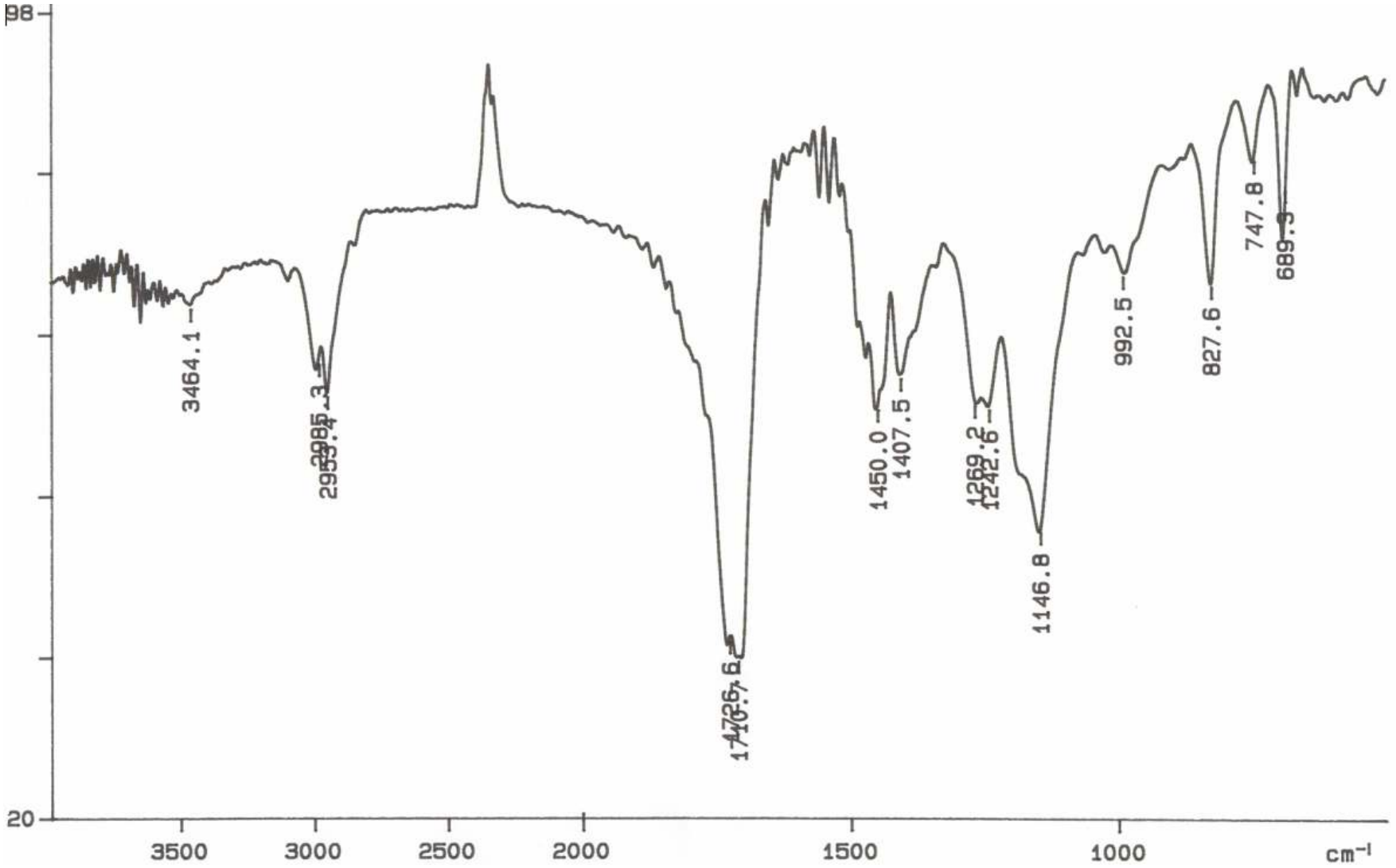


Figure A.20. IR spectrum of maleimide polymer after rDA reaction **66**

REFERENCES

1. Blohm, D. H., A. Guiseppi-Elie, Curr. Opin. Biotechnol., **2001**, 12, 41-47.

2. Eisen, M. B., P. O. Brown, Methods Enzymol., **1999**, 303, 179-205.

3. MacBeath, G., S.L. Schreiber, Science, **2001**, 289, 1760-1763.

4. Houseman, B.T., J. H. Huh, S. J. Kron, M. Mrksich, Nature Biotechnology, **2002**, 20, 270-274.

5. Wang, D., S. Liu, B. J. Trummer, C. Deng, A. Wang, Nature Biotechnology, **2002**, 20, 275-281.

6. Xiao, S-J., M. Wieland, S. Samuel Brunner, Journal of Colloid and Interface Science, **2005**, 290, 172-183.

7. Houseman, B.T., E. S. Gawalt, M. Mrksich, Langmuir, **2003**, 19, 1522-1531.

8. Yonzon, C. R., E. Jeoung, S. Zou, G. C. Schatz, M. Mrksich, R. P. Van Duyne, J. Am. Chem. Soc., **2004**, 126, 12669-12676.

9. Xiao, S-J., S. Samuel Brunner, M. Wieland, J. Phys. Chem. B., **2004**, 108, 16508-16517.

10. Meng, J-C., C. Averbuj, W. G. Lewis, G. Siuzdak, M. G. Finn, Angew. Chem. Int. Ed. Engl., **2004**, 43, 1255-1260.

11. Schlapak, R., P. Pammer, D. Armitage, R. Zhu, P. Hinterdorfer, M. Vaupel, T. Fruhwirth, S. Howorka, Langmuir, **2006**, 22, 277.

12. Fringuelli, F., A. Taticchi, Dienes in the Diels-Alder Reaction, John Wiley and Sons: New York, **1990**.

13. Carruthers, W., Cycloaddition Reactions in Organic Synthesis, Pergamon Press: Oxford, **1990**.

14. Gandini, A., Polimeros: Ciéncia e Tecnologia, **2005**, 15, 95-101.

15. Stille, J. K., L. Plummer, J. Org. Chem., **1961**, 26, 4026-4029.

16. Tesoro, G. C., V. R. Sastri, Ind. Eng. Chem. Prod. Res. Dev., **1986**, 25, 444-448.

17. Diakoumakos, C. D., J. A. Microyannidis, J. Polym. Sci. Polym. Chem., **1992**, 30, 2559-2567.

18. Ahlakimi, G., E. Klemm, H. Görls, J. Polym. Sci. Polym. Chem., **1995**, 33, 1133-1142.

19. Laita, H., S. Boufl, A. Gandini, Eur. Polym. J., **1997**, 33, 1203-1211.

20. Goussé, C., A. Gandini, Polym. Intern., **1999**, 48, 723-731.

21. Gu, A., Polym. Adv. Technol., **2005**, 16, 563-566.

22. Hao, J., W. Wang, B. Jiang, X. Cai, L. Jiang, Polym. Int., **1999**, 48, 235-243.

23. Chujo, Y., K. Sada, T. Saegusa, Macromolecules, **1990**, 23, 2636-2641.

24. Imai, Y., H. Itoh, K. Naka, Y. Chujo, Macromolecules, **2000**, 33, 4343-4346.

25. Canary, S. A., M. P. Stevens, J. Polym. Sci. Polym. Chem., **1992**, 30, 1755-1760.

26. Chen, X., M. A. Dam, K. Ono, A. K. Mal, H. B. Shen, S. R. Nutt, K. Sheran, F. Wudl, Science, **2002**, 295, 1698-1702.

27. Chen, X., F. Wudl, A. K. Mal, H. B. Shen, S. R. Nutt, Macromolecules, **2003**, 36, 1802-1807.

28. Gheneim, R., C. Perez-Berumen, A. Gandini, Macromolecules, **2002**, 35, 7246-7253.

29. Adachi, K., A. K. Achimuthu, Y. Chujo, Macromolecules, **2004**, 37, 9793-9797.

30. Goussé, C., A. Gandini, P. Hodge, Macromolecules, **1998**, 31, 314-321.

31. Jones, J. R., C. L. Liotta, D. M. Collard, D. A. Schiraldi, Macromolecules, **1999**, 32, 5786-5792.

32. Heredia, K. L., D. Bontempo, T. Ly, J. T. Byers, S. Halstenberg, H. D. Maynard, JACS, **2005**, 127, 16955-16960.

33. Mantovani, G., F. Lecolley, L. Tao, D. M. Haddleton, J. Clerx, J. J. Cornelissen, K. Velonia, J. Am. Chem. Soc. **2005**, 127, 2966.

34. Neubert, B. J., B. B. Snider, Org Lett, **2003**, 5, 765 - 768.

35. Bailey, G. C., T. M. Swager, Macromolecules, **2006**, 39, 2815 - 2818.

36. Sahoo, S. K., W. Ma, V. Labhasetwar, Int. J. Cancer **2004**, 112, 335-340.

37. Dujardin, E., S. Mann, Adv. Mater., **2002**, 14, 775-788.

38. Hammond, P. T., Adv. Mater., **2004**, 16, 1271-1293.

39. Shunmugam, R., G. N. Tew, J. Poly. Sci., Part A: Polym. Chem. **2005**, 43, 5831-5843.

40. Singhvi, R., A. Kumar, G. P. Lopez, G. N. Stephanopoulos, D. I. Wang, G. M. Whitesides, Science **1994**, 264, 696-698.

41. MacBeath, G., L. Schreiber, Science, **2000**, 289, 1760-1764.

42. Brack, H. P., C. Padeste, M. Slaski, S. Alkan, H.H. Solak, J. Am. Chem. Soc., **2004**, 126, 1004-1005.

43. Lussi, J. W., R. Michel, I. Reviakine, D. Falconnet, A. Goessl, et, al., Prog. Surf. Sci., **2004**, 76, 55-69.

44. Kumar, A., G. M. Whitesides, Appl. Phys. Lett., **1993**, 63, 2002-2004.

45. Crooks, R. M., A. J. Ricco, Acc. Chem. Res. **1998**, 31, 219-227.