Hydrogels based on poly(styrene-*co*-maleic anhydride) for the application of reversible male contraceptive

By

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Lehani Verwey *March 2016*

To my beloved parents, grandparents and sisters

Abstract

The use of a polymeric gel as male contraceptive, injected into the vas deferens of the male, has attracted a considerable amount of interest due to improved criteria over the current available options. The non-invasive character of these gels offer reduced side effects and improved reversibility over a vasectomy, rendering a promising new alternative to satisfy society's ever increasing demand for an ideal contraceptive.

This thesis focuses on the design, synthesis and characterisation of a polymer hydrogel system with improved biocompatibility and reversibility properties for contraception purposes in the male. Poly(styrene-*co*-maleic anhydride) (SMA), a biocompatible, commercially available copolymer that easily undergoes chemical modification through its highly reactive maleic anhydride residues is a candidate system for use in these hydrogel matrices. RAFT (reversible addition fragmentation chain transfer) mediated polymerisation was utilised for the synthesis of well-defined SMA, followed by two post-polymerisation modification approaches. The first, prepared through modification of the anhydride residues in the SMA backbone and the alternative, through chain end modification of the thiocarbonyl thio group of the RAFT-made SMA. Preference was given to the first approach during the present study, as side-reactions turned out to hamper the disulphide formation of thiol terminal-SMA.

Water-soluble boronic acid functionalised polymers (SMI-BA) were prepared by partial modification of the reactive maleic anhydride residues in SMA with 3-aminophenylboronic acid (BA). SMI-BA was combined with poly(vinyl alcohol) (PVA) to prepare the required hydrogels. Gelation is achieved within minutes after mixing of aqueous solutions of SMI-BA and PVA, based on reversible ester crosslink formation between the boronic acid groups in SMI-BA and the 1,3 diols in PVA. The structural properties of the gels were characterized by oscillatory rheometry as a function of composition and degree of modification (SMI-BA). Adequate gelation times of approximately 1-5 minutes (37 °C, PBS, pH = 7.4), with storage moduli (G') of 12 kPa – 50 kPa were obtained. Rapid reversibility of the gels is achieved within a few minutes by the addition of glucose in combination with sodium bicarbonate ($NaHCO_3$). The results indicate that the polymeric hydrogel is a promising candidate for further investigation as an injectable gel for male contraception purposes.

Opsomming

Die gebruik van 'n polimeriese jel as manlike voorbehoedmiddel deur inspuiting in die vas deferens buis van die man het aansienlike belangstelling gelok as gevolg van verbeterde kriteria oor huidige beskikbare opsies. Die nie-indringende karakter van hierdie jels bied verminderde newe-effekte en verbeterde omkeerbaarheid oor 'n vasektomie, wat as belowende nuwe alternatief kan dien vir die samelewing se toenemende vraag na 'n ideale voorbehoedmiddel.

Hierdie tesis fokus op die ontwerp, sintese en karakterisering van 'n polimeer jel sisteem met verbeterde biologiese geskiktheid en omkeerbaarheid vir voorbehoeding doeleindes in die manlike geslag. Poli(stireen-ko-maleïne anhidried) (SMA), 'n kommersieel beskikbare kopolimeer wat maklik chemiese modifikasie ondergaan deur middel van sy hoogs reaktiewe maleïne anhidried groepe dien as goeie kandidaat vir hierdie jel matriks doeleindes. RAFT bemiddelde polimerisasie is aangewend vir die sintese van goed gedefinieerde SMA, gevolg deur twee sekondere polimerisasie benaderings. Die eerste, voorberei deur modifikasie van die anhidried groepe in die SMA ruggraat en die alternatief deur modifikasie van die tiokarboniel tio groep van die SMA polimeer. Voorkeur is gegee aan die eerste benadering tydens die huidige studie, as gevolg van newe-reaksies wat die disulfied formasie belemmer na die sintese van tiol terminale SMA.

Water-oplosbare boroniese suur getransformeerde polimere (SMI-BA) is voorberei deur die gedeeltelike modifikasie van die reaktiewe maleïne anhidried reste in SMA met 3-amienfenielboroniese suur (BA). SMI-BA is gekombineer met poli(vinielalkohol) (PVA) om die vereiste jels te vorm. Jel formasie is binne minute na die toevoeging van water oplosbare SMI-BA en PVA bereik. Netwerk formasie is gebaseer op omkeerbare ester binding formasie tussen die suur groepe in SMI-BA en die 1,3 diol in PVA. Die strukturele eienskappe van die jels is gekenmerk deur ossillasie reologie as 'n funksie van samestelling en graad van verandering (SMI-BA). Voldoende jel formasie van ongeveer 1-5 minute (37 °C, PBS, pH = 7.4), met die stoor modulis (G') van 12 kPa - 50 kPa is verkry. Vinnige, voldoende omkeerbaarheid van die jels is binne 'n paar minute bereik deur die byvoeging van glukose in kombinasie met natriumbikarbonaat (NaHCO3). Die resultate dui daarop dat die polimeriese jel sisteem dien as belowende kandidaat vir verdere ondersoek as 'n inspuitbare jel vir manlike voorbehoeding doeleindes.

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List of Abbreviations

ACHN	1,1′-azobis(cyanocyclohexane)
AIBN	2,2'-Azobis(isobutyronitrile)
AIDS	acquired immune deficiency syndrome
ARS	alizarin red s
ATR-FTIR	attenuated total reflection fourier transform infrared
ATRP	atom transfer radical polymerization
BA	3-aminophenylboronic acid
BHT	2,6-Di-*tert*-butyl-4-methylphenol
CTA	chain transfer agent
DCM	dichloromethane
DMAc	*N*,*N*-dimethylacetamide
DMF	dimethylformamide
DMPA	medroxyprogesterone acetate
DMSO	dimethyl sulfoxide
DP	degree of polymerisation
EPHP	N-ethylpiperidine hypophosphite
EWC	equilibrium water content
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
HCl	hydrogen chloride
HDL-C	high-density lipoprotein cholesterol
IVD	intra vas device
LH	luteinizing hormone
LNG	levonorgestrel
LVR	linear viscoelastic region
MA	maleic anhydride
MHC	male hormonal contraceptive

MMD	molar mass distribution
MPU	medical grade polyurethane
MS	mass spectroscopy
MSR	medical grade silicone rubber
NCS	neocarzinostatin
NMP	nitroxide mediated polymerisation
NMR	nuclear magnetic resonance
NSV	no-scalpel vasectomy
NVP	*N*-Vinylpyrrolidone
PAA	poly(acrylic acid)
PBS	phosphate buffered saline
PMA	poly(methyl acrylate)
PMMA	poly(methyl methacrylate)
PS	polystyrene
PVA	poly(vinyl alcohol)
PVP	poly(*N*-vinylpyrrolidone)
PVPS	post-vasectomy pain syndrome
RAFT	reversible-addition fragmentation chain-transfer
RDRP	reversible-deactivation radical polymerisation
RISUG	reversible inhibition of sperm under guidance
SEC	size exclusion chromatography
SMA	poly(styrene-*co*-maleic anhydride)
SMA-BA	boronic acid functionalised SMA polymers
TE	testosterone enanthate
TLC	thin layer chromatography
TU	testosterone undecanoate
UV	ultraviolet

Chapter 1: Prologue

1.1 Introduction

Contraception is critical to health, wellbeing, development, and quality of life. Over the past 50 years, birth control for women has been refined to the point that there are now dozens of alternatives, but the lack in progress in male options has raised many concerns. The current state of contraceptive options is far from ideal, offering only two options *i.e.* condoms and a vasectomy. The acceptance of both these methods have been proven challenging due to high failure rate of condoms and the requirement of surgery, negative side effects and reversal issues regarded with a vasectomy.[1] Fortunately, several other male contraceptive alternatives by means of hormonal, non-hormonal systemic and vas-based methods have been under development, yet only a few are close to commercialisation. As such, the design of a safe, effective, reliable, reversible, non-hormonal method for men would meet a critical need in the market.

One promising approach, that has obtained substantial interest over the last couple of years, is the use of an injectable high molar mass poly(styrene-*co*-maleic anhydride) (SMA) gel combined with dimethyl sulfoxide (DMSO) in the vas deferens of the male.[2] The injected gel partially blocks the flow of sperm by anchoring itself to the folds of the inner lining of the vas deferens and has shown to reduce side effects experienced by vasectomised men.[3] Reversibility of the gel by the injection of DMSO solvent, to dissolve the polymer to allow evacuation via the ejaculatory duct, has been successful in animals.[4] However, this reversal approach has required non-favourable squeezing and manipulation of the vas deferens. Shortcomings of the current gel include the undesired use of DMSO solvent during administration and insufficient reversibility, which leaves scope to improve the current hydrogel system.

The design of an enhanced biocompatible hydrogel system to minimise reversal issues can be achieved by injection of two polymeric aqueous solutions to gel *in situ* in the male vas deferens. Chemical crosslinking of low molar mass SMA segments under physiological conditions, readily reversed with exposure to non-harmful additives, will provide an improved alternative of the above system.

Chemical crosslinking is a highly resourceful method for the formation of hydrogels that allow easy control over structural parameters to optimise synthesised gels with desired physical and mechanical properties.[5] These injectable systems will have several advantages over the current system by means of an optimised elastic system with improved biocompatibility and easy reversal.

1.2 The aim and objectives of this work

The aim of this study was to synthesise an injectable biocompatible hydrogel for the use as reversible male contraceptive. This injectable gel should consist of polymers that will gel at physiological conditions in the male vas deferens.

The main objectives of the study can be summarised as follows:

1. To synthesise the SMA copolymer by a reversible-deactivation radical polymerisation (RDRP) technique *i.e.* RAFT polymerisation.
2. To modify SMA via two post-polymerisation techniques by (1) introduction of a boronic acid moiety via the maleic anhydride residues in the SMA backbone or (2) through chain end modification of the thiocarbonyl thio group of the RAFT-made SMA to dimerise low molar mass SMA chains into linear high molar mass polymer chains suitable for hydrogel formation.
3. To form a suitable biocompatible gel with the following requirements: (1) quick, effective gelation under physiological conditions (37 °C, pH = 7.4) (2) easy, quick reversibility with the addition of non-harmful additives.
4. To investigate the structural properties of the gels by means of swelling studies and oscillatory rheology.

1.3 Thesis layout

Chapter 1- Prologue

Chapter 1 gives a brief insight into the male contraceptive market with the introduction of potential injectable polymers as reversible male contraceptive. In addition, the aim and objectives of the study are presented.

Chapter 2 – Historical Background

A comprehensive literature review of the current and future developments in the male contraception market is presented. In addition, the disadvantages and benefits of current contraception methods are compared. Furthermore, the proposed alternative of polymeric gels as male contraceptive is presented.

Chapter 3 - Synthesis and Characterisation of SMA

Chapter 3 introduces RAFT mediated polymerisation as an effective technique to synthesise and modify polymers for biological applications. In addition, the polymer of choice and the methods employed to synthesise, modify and characterise all relevant polymers are discussed.

Chapter 4 – Dimerisation Model Study of SMA

Chapter 4 represents a model study into the ineffective disulphide formation of SMA polymers synthesised in Chapter 3. An alternative method is developed to achieve disulphide formation of SMA. In addition, characterisation of the polymers is discussed.

Chapter 5 – Hydrogel Synthesis and Characterisation

Chapter 5 is dedicated to the formation and characterisation of gels of the polymers synthesised in Chapter 3. Gel formation, swelling studies, rheology and reversibility of the gels are described.

Chapter 6 - Epilogue

Chapter 6 describes conclusions and recommendations for future work.

1.4 References

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Chapter 2: Historical Background

2.1 Introduction

Family planning is essential to the reproductive health and quality of life for families and communities. It is achieved through controlled use of contraceptives, allowing couples to manage their fertility, improving health and securing economic wellbeing. Furthermore, family planning by means of contraceptives prevents unintended pregnancies and ultimately results in the reduction of infant mortality and unsafe abortion, precluding many tragic deaths annually.

Over the last few decades, significant scientific development in contraception methods for women has led to a substantial expansion of commercially available contraception products and procedures. This contraceptive revolution has allowed couples to better plan their families, reducing unintended and unwanted pregnancies. To date, contraception variety for women has been refined to the point that there are dozens of effective options available. The most popular choice among these options are barrier methods, reversible contraceptives and hormonal methods.[1]

In contrast, modern contraceptive methods for men remain limited. Currently, two main options are available to men, *i.e.* condoms and vasectomy, neither being reversible or hormonal methods. In addition, the disadvantages of these two common birth control methods are quite significant. Condoms, which are not covered by medical insurance, have a high failure rate and vasectomies are not readily reversible. This lack of contraception options for men has raised many concerns and has led to numerous research attempts. A number of promising male contraceptive methods are currently in research stages, due to the ever increasing demand and need for male birth control options, despite the challenges associated with their development caused by economic strains and socio-political factors.[2]

Importantly, for a contraceptive to be accepted by society it must be long term, reliable, safe, reversible and have minimal side effects on the body. A non-surgical, non-occlusive, non-hormonal method with easy reversibility and minimal side effects is therefore desired. Both current methods and the majority of other researched methods for male contraception fall short of these criteria and for decades now no new method has reached the market.

However, in the male, the vas deferens has been shown to be a suitable site for contraception intervention, controlling unintentional pregnancy.[3] Therefore, numerous attempts to create an ideal vas-based male contraceptive have been investigated over the last few years, showing improved results over systemic researched methods. In order to design an ideal vas based contraceptive, careful design needs to be implemented to ensure a reversible method suitable for long-term contraception.

2.2 Male contraception methods currently available

The existing male contraception situation represents a massive global concern. Over the past decade, pharmaceutical industries have abandoned financial interest in the field due to fear of disrupting the lucrative oral contraceptive market. These obstacles have led to financial shortcomings and long developing periods of new potential methods. Despite economic and political obstacles hindering development in the field, the primary problem remains the current state of male contraceptive technologies. Methods on the market are far from ideal due to inherent shortcomings relative to society's needs. The following section will introduce the two current male contraceptives commercially available and present the benefits and limitations of both.

2.2.1 Condoms

Condoms, if used consistently and correctly, provide an effective method of contraception. They are low-cost and prevent the spread of sexually transmitted diseases, such as AIDS, during sexual interaction.[4] However, they have a few main drawbacks. Condoms have marginal contraceptive efficacy due to improper use. One significant drawback is their poor long-term compliance, especially in long-term monogamous couples. In addition, men dislike them as they reduce sensation, hampering consistent use. In conventional rather than correct use, the annual pregnancy rate can be up to fifteen percent. Even when used correctly, breakage and slippage occur in 2% - 8% of cases.[5] According to a new study, irregular or no condom use occurred in 33% of 4014 couples in casual relationships and 14% in 2387 serious relationships.[6] Furthermore, allergic reactions experienced by some men and women, make them undesired.[7,8] Condoms play a vital role in disease prevention and the contraceptive market, however, they alone cannot meet everybody's needs.

2.2.2 Vasectomy

Vasectomy is a well-established surgical form of male contraception where the vas deferens is cut, tied and the tubes are sealed to prevent sperm from entering the seminal fluid. In addition, pregnancy cannot occur since the sperm is blocked and absorbed by the body instead of being ejaculated with the seminal fluid. The first case of human vasectomy was reported by Ochner, a United States surgeon, in 1897.[9] Ochner introduced and advocated vasectomy as a method to hinder undesirables such as criminals, perverts and paupers from procreating.[9] One of the earliest practitioners of vasectomy in the 1900s, Harry C. Sharp, reported that he performed the procedure on inmates in an attempt to reduce criminal behaviour and prevent criminal reproducibility.[10]

In recent years, the option of a no-scalpel vasectomy (NSV) technique, that takes up to 15 minutes is now widely available.[11] The procedure is considered simple, safe and convenient, however not immediately effective due to the remainder of sperm present beyond the now-blocked tubes.[12] After surgery, other birth control methods need to be implemented for about 3 months to ensure no more sperm is present in the seminal fluid.

The procedure is considered permanent and beside high costs, success in the reversal of this procedure is minimal. A vasovasostomy, vasectomy reversal, is a delicate microsurgical procedure, and success thereof is greatly dependant on the surgeon's skill.[13] The reversal success is also dependent on the number of years between vasectomy and vasovasostomy.[14] It has been reported that pregnancy success rates are as low as 30%, especially in cases when men have had their vasectomy for many years.[15] Results show that approximately 6% of vasectomised males will eventually seek reversal surgery.[16]

Despite low reversal success being dependant on the surgery itself, the major drawback of the process is the development of antisperm antibodies caused by the continuation of sperm production by the male after a vasectomy. The body responds by absorbing most fluid through the membranes and solid content gets broken down by responding macrophages and reabsorbed through the blood stream. However, sperm maturation takes place in the epididymis over a period of 30 days before leaving the testis and over time the membranes have to increase in size to accommodate the capacity of the fluid. As a result, an increased number of macrophages are recruited to destroy the sperm build-up. The disadvantage of this is that the body will persist to develop these antibodies even after the vas deferens tubes are

joined together by vasovasostomy. Within a year, 60% - 70% of vasectomised men develop antisperm antibodies.[17]

Long term complications of a vasectomy procedure include chronic pain conditions, post-vasectomy pain syndrome (PVPS), which affects 15% -33% of patients either immediately or years after surgery.[18] Postulated etiologies for PVPS include: Epididymal congestion, nerve damage/entrapment, fibrosis from the body's reaction or surgical wounds or sperm granulomas caused by sperm leaking from the severed duct.[19] All of the above complications, a direct result of complete severing of the duct during surgery, can effectively be minimised by the use of a method which intends to keep most of the vas deferens intact either by smaller incision or puncture.

Even with recent improvements of existing methods, the increased demand to develop a new method remains since no current method matches the criteria for an ideal contraceptive. This deficiency of current methods to meet the needs of men leads to disapproval and rejection, resulting in lack of family planning. As pointed out earlier, this can have quite a significant effect of economic wellbeing.

2.3 Need for new male contraceptives

The current male contraceptive situation is far from ideal. To date, an acceptable, reliable, reversible male contraceptive remains elusive. Currently available methods, condoms and vasectomy, don't meet everyone's needs and the continuously increasing abortion rates raises big concerns. In light of this, studies since 1998 show that males seek access to improved contraceptives in order to share the responsibility for contraceptive measures.[20] The studies indicate that over 60% of men in Germany, Brazil, Mexico and Spain are amenable to use a new method of male contraception.[20] In an additional study conducted under British men, 80% picked the idea for a male contraceptive pill as one of their top three choices.[21] Thus, the time is ripe to introduce new methods to the contraceptive market in an attempt to reduce abortion rates and satisfy the contraceptive needs of society.

2.3.1 High abortion rates

In South Africa, over 80,000 registered abortions are performed annually, the majority of which can potentially be avoided by implementing family planning.[22] Furthermore, information on teenage pregnancy indicates that 20% of girls, between the ages of 15 and 19, reported that they had been pregnant.[23,24]

Risks associated with having an unintended pregnancy, whether terminated or not, on the maternal and perinatal health is increased, especially in unsafe termination.[25] According to the WHO, unsafe abortion is one of the leading causes of maternal mortality.[26,27] Unintended pregnancies are associated with poor pregnancy and child health outcomes, especially with closely spaced pregnancies.[28,29,30] Unsafe abortion also results in other consequences, such as stigmatisation, economic costs to families and to health system providers and psychosocial effects on women.

All abortions, both safe and unsafe, are a captivating indicator of unintended pregnancy incidence. The application of particular attention to factors that contribute to unintended pregnancies and unsafe abortion is a crucial step in reducing the international health burden of women. The majority of unintended pregnancies can be potentially avoided with the implementation of family planning by use of contraceptives, which has been shown to decrease abortion rates.

2.3.2 Current contraceptives don't meet everyone's needs

Contraceptives are crucial to the success of family planning and new developments in contraceptive technologies continue to make family planning more accessible to couples looking to space the birth of their children. Current contraceptives for men and women are falling short, and new advancements are required to meet the demands of people in developing countries. Furthermore, men and women have dissimilar reproductive requirements throughout their years, thus it is essential that they have access to a variety of contraceptives to help them plan, space, and restrict pregnancies.

The current state of the contraceptive market leaves two large groups unserved. The first group consists of women who encounter unbearable side effects from usage of hormonal methods, which include breast tenderness, weight gain and diminished libido.[31]

In addition, some modern contraceptives can be difficult to use consistently and correctly, while others result in severe side effects that lead to discontinued use. The second group is composed of young men for whom a vasectomy is not ideal and who want a more reliable method than the use of condoms to control fertility.

Modern contraceptive technologies are needed to overcome these challenges and contribute to an unfulfilled need for family planning. New technologies should be low cost, reversible and widely available. Furthermore, they should have minimal or no side effects on human health. New products or procedures with new improved qualities could help reach a new

market of potential contraceptive consumers and recapture men who have abandoned use after discontent and frustration of current methods. In order to design such a method, careful consideration needs to be implemented to ensure the method will comply with a number of properties desired by society.

2.1.1 The ideal male contraceptive

The acceptability of a male contraceptive is measured by the prevalence and continuation of practice of a specific method. For a contraceptive method to be accepted by society it must satisfy a particular set of properties. Satisfaction of these properties is crucial, and would ultimately lead to an ideal male contraceptive.

The ideal male contraceptive requirements:

- Acceptability by both partners
- Should be safe and preferably long term
- Similarly effective as female methods with rapid onset of contraceptive action
- Applied independent of the sexual act
- No interference with libido or the sexual act
- No unacceptable short and long term side effects on the body
- Fully reversible

Failure of the current contraception techniques is primarily due to the dissatisfaction toward the current techniques. The dissatisfaction of requirements has led society to demand new contraception methods. A number of promising methods are currently in research stages, due to this growing demand and need for male birth control options. In the next section, the male reproductive physiology will be introduced in order to understand the onset of action of hormonal methods, with particular attention to potential hormonal male contraception methods.

2.2 Male contraception approach

In an attempt to introduce new male contraceptives to the market, scientists have focussed primarily on the development of methods that inhibit spermatogenesis through the use of testosterone to induce negative feedback mechanisms.

2.2.1 Male reproductive physiology

The male reproductive system consists of the testes and a series of ducts and glands that produce sperm, fluids, and hormones. The male testes, composed of twin endocrine glands, are the most essential organs of the male reproductive system responsible for sperm and testosterone production. Testosterone, a steroid hormone, is important in the maintenance of muscle, bone mass and libido of the male.[32] Furthermore, testosterone has positive benefits on mood, cognition and general wellbeing.[33]

An intricate feedback loop is responsible for testicular function and the production and regulation of hormones (Figure 2.1). The principle regulator of pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion is gonadotropin-releasing hormone (GnRH), a decapeptide secreted by hypothalamic neurons by part of the brain in a pulsatile fashion.[34] Testosterone is produced by the gonads, Leydig cells within the interstitium of the testes, and its production is stimulated by LH, essential for reproduction.[35] Sperm cells are produced within the seminiferous tubules, where their maturation is supported and nurtured by Sertoli cells under the influence of FSH and high levels of intratesticular testosterone.[36]

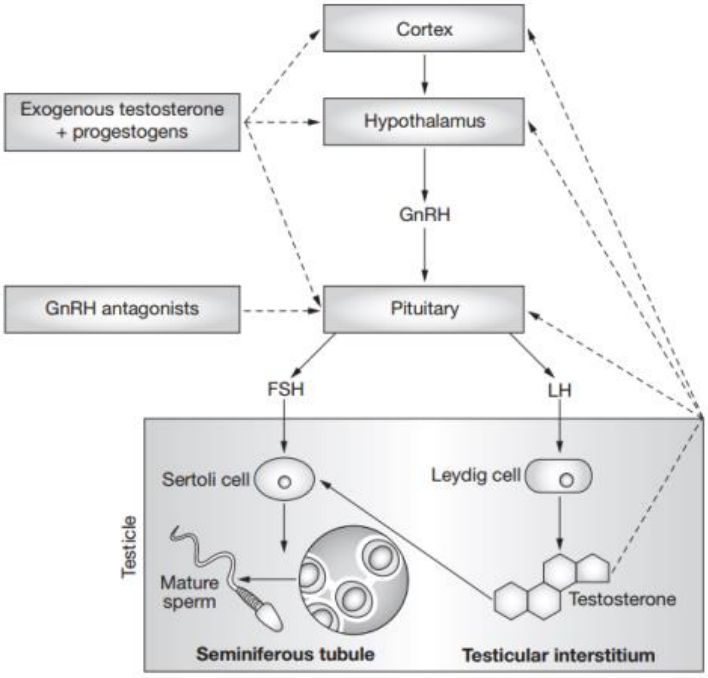

Figure 2.1 The endocrinology of spermatogenesis and male hormonal contraception.[37] Solid arrows promote spermatogenesis; dashed lines indicate inhibition of spermatogenesis.

Based on the male physiology, the use of a male contraceptive can offer success by one of three main methods: (1) the use of physical barriers such as condoms or vasectomy; (2) the prevention of sperm production using hormonal methods; and (3) the inhibition of sperm functions by either sperm vaccines or polymer gel injection. A number of contraceptive techniques that fall into one or more of these categories are currently being developed world wide due to shortcomings of current methods on the market.

They are generally divided into three categories namely: (1) hormonal systemic, (2) non-hormonal systemic and (3) vas-based approaches. Although several of these contraceptives are close to commercial production, none are currently available. The majority is still at the stage of basic scientific research and tested in animals. Only a few systems have entered the process of clinical trials, while others have been abandoned due to negative outcomes. Examples of currently investigated hormonal methods with their benefits and shortcomings will be discussed in the following section.

2.2.2 Hormonal systemic approaches

Over the last four decades, development of hormonal targets for male contraception has been pursued. A male hormonal contraceptive (MHC) would be analogous to female hormonal contraceptive composed of a combination of various hormones that aims to suppress sperm production. Surveys conducted in various countries have shown that a hormonal method, administered by periodic injections or daily pills would be considered by a substantial number of men and women.[38,39]

A hormonal systemic approach can be accomplished by stopping the secretion of male hormones in the brain and testes. Supraphysiological doses of testosterone have the ability to act as a contraceptive by suppression of the pituitary gonadotropins LH and FSH. Low levels of LH and FSH deprive the testis of sperm production, however not in all men. Hormonal contraceptives do not incapacitate existing sperm; they only stop the initiation of sperm. The fastest possible onset of efficacy after taking a MHC would be two to three months since it takes an average of 72 days for the last spermatids produced to travel through and exit the epididymis. Administration of unmodified testosterone (Figure 2A) is impractical because when given orally or by injection it is quickly degraded by the liver.[40] Therefore, most hormonal contraceptive regimens have used longer-acting injectable testosterone esters. There are currently three major classes of MHCs which will be discussed here.

2.2.2.1 **Androgen**

Androgen, a male sex hormone, can be used to block the production of natural testosterone by the addition of a quantity of synthetic testosterone higher than generally found in circulating blood (Figure 2A).[41] Synthetic testosterone in the blood stream prevents the production of GnRH and LH, through the inhibition of crossing over to the testes, keeping testosterone in the testes too low for sperm production. An injectable testosterone ester, such as testosterone enanthate (TE) (Figure 2B) can be given by intramuscular injection on a weekly basis to achieve infertility. On such a regimen, it can be expected that sperm counts will approach zero after 2 to 3 months, with recovery of normal sperm totals around 3 to 4 months after terminated injections.

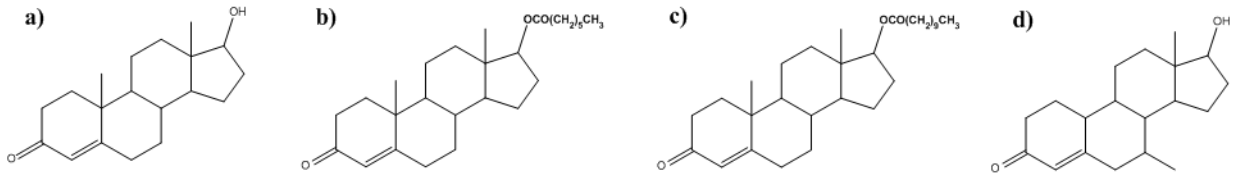

Figure 2.2 Androgens researched as a male contraceptive: (A) testosterone, (B) testosterone enanthate, (C) testosterone undecanoate, (D) 5α-methyl-19-nor-testosterone.

Two multicentre studies of TE, conducted by the WHO, as a male hormonal contraceptive have shown good efficacy above 95%.[42,43] In both trials, no major side effects were reported, and sperm counts returned to normal after discontinued testosterone injections. However, the required weekly injections are a drawback to some patients and resulted in discontinued involvement in the trials. In addition, a delay in full contraceptive action of 3 to 4 months is a deterrent. Despite the drawbacks of the current method, the studies have shown that injected TE is effective, safe, and fully reversible as a contraceptive in the majority of men. A portion of men failed to respond to the hormones and remained fertile. Studies show that an increased amount of TE results in the decrease of high-density lipoprotein cholesterol (HDL-C) serum, which could accelerate atherosclerosis, the narrowing and hardening of arteries.[44,45] Poor acceptability of weekly injections resulted in the development of sustained testosterone delivery methods. In addition, testosterone undecanoate (TU) (Figure 2C), results in normal serum T levels for at least 6 weeks in hypogonadal men.[46,47,48] Despite attempts of two large trials in China, the approach wasn't approved for use in China, and subsequently abandoned.[49]

2.2.2.2 **Progestin with androgen replacement**

A synthetic testosterone progestin combination hormone was shown to be successful in achievement of male contraception. This is attributed to the fact that synthetic progestins (Figure 2.3) inhibit the production of the gonadotropins FSH and LH from the pituitary, which results in the lack of production of spermatids and testosterone in the testes.[50] Synthetic testosterone acts as a replacement to maintain male characteristics. The hormone combination to block sperm production has extensively been tested.[51,52,53] Injections of medroxyprogesterone acetate (DMPA) in combination with testosterone have had good success in Chinese and Australian trials.[54,55] Drawbacks of the progestin, levonorgestrel (LNG), in combination with TE were weight gain and decreases in the HDL-C in comparison to TE alone.

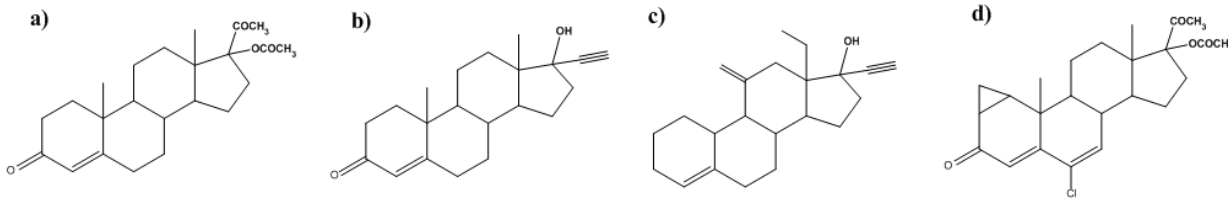

Figure 2.3 Progestins researched as a male contraceptive research: a) medroxyprogesterone acetate (DMPA), b) levonorgestrel, c) desogestrel, d) cyproterone acetate.

2.2.2.3 **GnRH antagonist with androgen replacement**

The addition of GnRH antagonists as adjuvant to androgen therapy has been tested as male contraceptive.[50] The GnRH antagonists bind to the GnRH receptors and block the production of FSH and LH, necessary to produce spermatids and testosterone.[56] However, the need for daily administration renders them impractical for long-term use in males. The GnRH antagonist, cetrorelix in combination with 19-nortestosterone have had promising results, but azoospermia was not maintained after discontinued treatment.[57] Even with promising results, further studies to delineate the effects are crucial before it can be tested in clinical trials. Despite numerous attempts to develop a suitable hormone-based male contraceptive, several side effects and drawbacks are ever present. These drawbacks include increased acne, mood changes and a depressed level of HDL-C in the blood. This potentially means that men with a risk of heart disease should be advised to avoid MHCs.[58]

One major concern researches have to solve, before commercial availability, is the fact that some men don't respond to hormonal treatment at all. This is due to the lack of knowledge regarding the current mechanism, thus they can't predict which men will respond to MHC treatment.[58,59] The biggest disadvantage of the abovementioned approach is the systemic delivery, which results in negative side effects on other parts of the body due to lack of targeted delivery.

2.2.3 Non-hormonal systemic approaches

Non-hormonal approaches of contraception include the use of compounds by targeting either sperm production or sperm mobility to impair or disrupt the production of spermatids. The selectivity, specificity and fewer side effects makes these approaches attractive candidates, as opposed to hormonal methods plagued by side effects. Furthermore, these methods allow more rapid onset of action. Over the last few decades, a variety of new methods have been developed, however progress has been slow, as many of these are still in research stages of development.

Early stages of development include testicular targets such as local application of heat in the scrotal area[60,61]and plant extracts such as gossypol[62,63,64,65] and triptolide.[66,67] Alternatively, the use of compounds such as indenopyridines that target Leydig or Sertoli cells, crucial to sperm production, have also been reported.[68,69] The oral administration of 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide (adjudin) has been shown to disturb the adhesion and interaction of Sertoli cells and germ cells.[70,71,72,73] Epididymal targets prohibit the maturation of spermatozoa in the epididymis.[74,75,76]

There are currently a number of potential non-hormonal male contraceptives with different mechanisms being researched. Although these approaches are still far away from clinical introduction, they offer potential for sperm specific techniques and great promise as reversible, long-term contraceptives with minimal side effects.

2.2.4 Previous Vas-based approaches

The vas deferens, the duct that transports sperm from the testicle to the urethra, has been shown to be a suitable site for male contraception.[3] Vas occlusion methods block the vas deferens with the aim to leave the tube intact, offering several advantages over a vasectomy and systemic methods. By leaving the tube intact, numerous complications experienced by

vasectomy patients are effectively minimized. Easy, successful reversal is achieved by simple removal of the device from the vas.

The vas-based approach offers target site-specific contraceptive control over systemic methods, resulting in limited side effects. In this section, we will introduce various vas-based methods and the benefits they can offer over systemic approaches.

2.3.2.1 Injected plugs

Injected plugs were developed as an attempt to find a potential alternative for vasectomy. The plugs are injected in liquid form, into the vas deferens and harden over time to form a plug to block the flow of sperm. Two types of plugs have been developed: medical grade polyurethane (MPU)[77] and medical grade silicone rubber (MSR).[78] Sponsored research has been ongoing since the 1980s but unfortunately results varied and it was estimated that the device takes 18 to 24 months to work effectively. In addition, the total vas occlusion approach will most likely result in the development of antisperm antibodies, which will lead to irreversibility, even after plug removal. Furthermore, the plug may lead to rupture of the vas.[79] Due to the lack of promising results, the studies have since been abandoned.

2.2.4.1 Intra Vas Device (IVD)

The intra vas device (IVD) is similar in function to a vasectomy, except it leaves the vas deferens intact. The device is comprised of a tiny set of implants that blocks the flow of sperm.[80] The device offers several advantages over injected plugs. Among them the most substantial advantage is the size of the preformed plug in relation to the size of the vas deferens could be controlled. This will relieve stress and avoid the possible rupture of the vas. In addition, the anchoring mechanism can prevent the migration of the plug along the length of the vas. Currently, two types of IVD are employed in clinical trials. The first is soft silicone plugs made by the Shepard Medical Company in the United States. The other consists of a urethane tube lined with a small nylon sieve developed by the Foshan Medical Company in China. The development of both these devices was inspired by the previous work of Zaneveld in 1980, who showed that the use of an IVD is safe and effective for male contraception purposes.[81]

The United States design is sized to the width of the recipient's vas deferens and inserted with a patented insertion tool. Two plugs are inserted in the same vas deferens tube, with a small space in between, to ensure effectiveness. Movement of the plugs is prevented by the insertion of small sutures, anchoring them to the wall.

The Chinese IVD (Figure 2.4) is a urethane tube closed at one end, lined with medical grade nylon mesh, and operates as a filter to capture and trap sperm. One tube is inserted in each vas deferens tube. A small hole at the closed end allows the flow of fluid, however sperm gets trapped. The design aims to prevent the build-up of pressure on the epididymis, as in the case of a vasectomy. Insertion of this device can be performed using a non-surgical procedure in less than 20 minutes. A small opening is made in the wall of the vas to allow insertion of the IVD. The IVD is anchored in place by a tiny suture outside the vas to keep it from dislodging. An additional form of contraception is required for 3 months after IVD insertion, along with follow up visits to the doctor for sperm counts to ensure that the device is working properly.

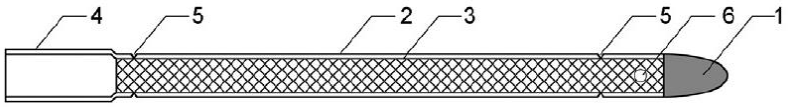

Figure 2.4 Schematic diagram of the Chinese IVD. 1, substantial head; 2, shell; 3, nylon thread (medical grade); 4, dilated tail; 5, sulcus; 6, hole.[82]

Removal of the IVD is accomplished by a small incision over the plugs to obtain access. Reversal, however, will be faster and more cost effective in the case of an IVD compared to vasovasostomy. A study of IVD reversal in primates delivered encouraging results, however reversal has not been tested in humans.[81]

A comparative study was performed on the Chinese IVD to the no-scalpel vasectomy (NSV).[82] Results indicated less side effects of the IVD group than the NSV group with a 50% reduction in tenderness and pain. No issues of spontaneous reversal of the IVD group compared to a few in the NSV group.

2.2.5 Present Vas-based approaches

2.2.5.1 Reversible inhibition for sperm under guidance (RISUG)

Guha *et al*. introduced reversible inhibition of sperm under guidance (RISUG) in 1979, where a solution of poly(styrene-*co*-maleic anhydride) (SMA) in dimethyl sulfoxide (DMSO) solvent is injected into the vas deferens to inhibit the flow of sperm.[83,84] The method has been under research ever since and aims to partake as a promising alternative to a vasectomy, even though the mode of action of the polymeric gel is not fully understood.[85] Guha and co-workers hypothesised that the mode of action might be due to a pH lowering effect caused by hydrolysis of the polymer in the presence of water in spermatic fluid.[86] An additional hypothesis given by Guha is that upon conversion to the hydride, the formed positive charges attract negative charged sperm, causing a charge imbalance, killing the sperm.

This non-surgical procedure is achieved by the injection into the vas deferens through the intact scrotal wall, resulting in minimal recovery time of the patient. Within a few minutes of injection, the polymeric gel anchors itself to the folds of the inner wall of the vas deferens. During the process, the polymer doesn't degrade, as in the case of most contraceptive devices, which makes it suitable for long-term use. The advantages of RISUG over vasectomy are the absence of the formation of antibodies and granulomas. RISUG still allows fluids to pass through, reducing the incidence of back-pressure that causes antibody formation. In addition, the process of injection doesn’t result in sperm leakage into surrounding tissue, the main reason for granuloma formation. Side effects in humans given RISUG included only minor pain and swelling in the testes immediately after treatment, which dissipated with a couple weeks. Granulomas, autoimmune responses, and prostate issues were not observed.

Reversibility is achieved by flushing out the polymer through the injection of a sodium bicarbonate ($NaHCO_3$) or DMSO solution into the vas deferens via the scrotal wall.[87,88,89] Clinical tests were performed in 1979 on male rats, followed by Phase 2 of the clinical trial in 1997 with injection of the polymer into healthy adult male monkeys to establish the degree of fertility control over a period of one year.[90] No pregnancies occurred for the duration of the study. Short term studies on reversal in male rats, by flushing out the polymer with DMSO solution, resulted in 100 % fertility 90 days after reversal.[91] Long-term effects of RISUG and its reversal by DMSO and $NaHCO_3$ have not been thoroughly investigated. Phase 3 clinical trials are currently ongoing in India to gain more insight into long-term effects of this method on humans. Furthermore, clinical trials of RISUG are underway in the United States under the trade name Vasalgel.

2.3 Acceptability of current researched methods

Additional male contraceptive options are required to control population growth and prevent unwanted pregnancy. Existing male methods of contraception are effective but have limitations. Emerging research is opening doors for novel methods regarding male contraception, providing better alternatives other than the existing methods in the near future.[92] However, acceptance of the majority of these methods has been proven challenging due to the side effects or reversal issues. Male hormonal methods as male contraception are effective, reversible, and seem safe. However, for reasons not entirely understood, not all men respond to treatment. This effectively means that men should be screened first in order to determine if they would be eligible for this method. In addition, hormonal methods offer health risks such as loss of androgenicity, affecting the libido and characteristics of the sex organs, resulting in negative psychotropic effects such as irritability, anxiety and depression.[93] Common side effects such as acne and suppression of HDL cholesterol were experienced in clinical trials. Overall, however, the high efficacy of hormonal contraceptives might outweigh the concerns of side effects, which can be suppressed with improved optimised regimens in the near future. Hormonal and non-hormonal systemic methods with the requirement of weekly or monthly injections are the biggest disadvantage to these options, since the mode of delivery is a very important aspect in the acceptability of a contraceptive method.

From the overview of methods in development presented, the most promising new approach appears to be RISUG and Vasalgel, a vasectomy alternative, with the injection of a polymeric gel into the vas deferens. The current results have shown this method to be minimally invasive and with marginal side effects. It satisfies the greater amount of needs required by an ideal contraceptive and has shown superior results in clinical trials. Despite the fact that these gels are still in research stages, they have proven to have a great deal of potential for the near future on contraception in males.

The current global unsatisfied need for more affordable and effective contraceptives indicates the requirement of further technological advancement or innovations to products. Further research in the improvement of potential methods, such as contraceptive hydrogels will be extremely useful due to increased demand that there is yet a male contraceptive to be developed to satisfy all the desired criteria. Further research in this aspect would be extremely valuable to the many couples who do not wish to have children, but do not want to give up their health and future fertility.

2.4 Our Approach

The current chapter is mainly focussed on existing male contraception methods, with the addition of new approaches that have the potential to provide alternative forms of male contraception in the near future. A promising polymeric gel has shown good results in clinical trials, however further improvements of these gels are required due to current inadequacies. Shortcomings of the current gel systems include the undesired use of DMSO solvent during administration and insufficient reversibility. Investigation into alternative gels with improved biocompatible and reversal properties remains elusive and further research is required.

In subsequent chapters, the synthesis of strong, elastic, biocompatible gels with the ability to gel *in situ* at physiologically relevant conditions will be demonstrated. SMA copolymers contain the desired biological properties and chemical modification ability to alter the mechanical properties of the gels in order to obtain a strong, yet elastic gel for the desired application. The crosslinking densities can be controlled by variation of the polymer composition and degree of functionalization of the SMA polymer. It will be shown that reversibility of these gels is easily achieved by the addition of non-harmful additives to readily dissociate the gels.

Ultimately, the synthesis of a suitable polymeric gel with appropriate swelling and viscoelastic properties to satisfy the requirements of an ideal contraceptive would provide an outcome for an improved, reversible vas-based contraception technique for the male.

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Chapter 3: Synthesis Modification and Characterisation of SMA

3.1 Introduction

The discovery of reversible-deactivation radical polymerisation (RDRP) has induced extensive research efforts over the past few decades.[1] RDRP offers control over structural parameters by achieving simultaneous growth of all chains through reduction of irreversible termination reactions. This living behaviour is accomplished by the facile equilibrium of dormant and active propagating radicals and ultimately termination is minimised.[2] As a result, well defined polymers with controlled molecular weight, narrow molecular weight distribution and complex architectures can be synthesised. The most prominent of RDRP techniques used in contemporary polymerisation are reversible addition fragmentation chain-transfer polymerisation (RAFT)[3,4] atom transfer radical polymerisation (ATRP)[5,6] and nitroxide-mediated polymerisation (NMP)[7,8] . RAFT polymerisation is considered the most robust and versatile method of all RDRP techniques. It is compatible with a wide range of monomers, solvents, initiators and has the ability to synthesise well defined complex architectures with high purity.[9] In addition, it is suitable for the synthesis of polymers for biological applications.[10]

The RAFT technique was first reported in 1998 by the Australian group at CSIRO.[3] The technique requires the presence of an organic addition fragmentation chain transfer agent (CTA), also known as a RAFT agent, that contains an active thiocarbonyl thio group.[11,12] The fundamental steps of the mechanism using the RAFT polymerisation method are extensively reported in literature.[13] The success of the RAFT process in terms of controlled molecular weight and dispersity is dependent on the effectiveness of the CTA governed by the nature of the R and Z substituent on the RAFT agent.[14,15] The R substituent must be a good leaving group stabilising the radical in the pre-equilibrium stage, but also efficiently reinitiate the growth of a new chain.[16] An effective Z group stabilises the intermediate radicals to result in reversible fragmentation and activates the C=S bond towards radical addition.[17] Keddie *et al*., provides a guideline for the selection of appropriate R and Z groups for controlling various monomer species.[18]

A key feature of the RAFT process is the ability to retain the thiocarbonyl thio groups, present in the initial RAFT agent, in the polymer products.[19] The presence of these groups results in coloured, odorous products which are disadvantageous to certain applications.[20] A variety of effective methods were developed for the post-treatment of RAFT-made polymers in order to remove or transform the thiocarbonyl thio groups.[21] Removal of RAFT end-groups can be achieved by introduction of various compounds such as a) nucleophiles (e.g. primary amines) and reducing agents (e.g. sodium borohydride) to yield thiols[22] b) thermolysis by exposure to heat to leave an unsaturated chain end[21] c) radical induced removal of the thiocarbonyl thio end with hypophosphate salts[23] and tributylstannane[24] to yield a saturated chain end. For further details on various chain end modification methods, the reader is referred to a paper by Moad *et al.*[25]

Poly(styrene-*co*-maleic anhydride) (SMA) copolymers are highly interesting functional polymers with eminent chemical and biological features. Chemical modification of the polymer can easily be accomplished due to the accessible and reactive maleic anhydride residues. Maleic anhydride residues can readily undergo reactions with various compounds such as water, alcohols and (primary or secondary) amines. A variety of biological applications reported for this synthetic biocompatible polymer include a polymer–protein conjugate of SMA with a potent antitumor polypeptide neocarzinostatin (NCS) as drug carrier for targeted treatment of lung and liver cancer.[26,27,28] Also, SMA and its derivatives have been reported in the effective inhibition of HIV-1 infections and as a micellar carrier for ovarian cancer drugs.[29,30,31]As such, it is considered an attractive polymer choice in the design of a polymeric gel to be injected into the vas deferens as male contraceptive.

RAFT polymerisation is one of the few RDRP techniques able to successfully control the polymerisation of SMA. In addition, it offers the option of further modification of the maleic anhydride or chain end functional groups to obtain the desired functionality for hydrogel formation. Numerous successful attempts to synthesise well defined SMA polymers via RAFT polymerisation with pre-determined molar mass and low dispersity have been reported in literature.[32,33,34]

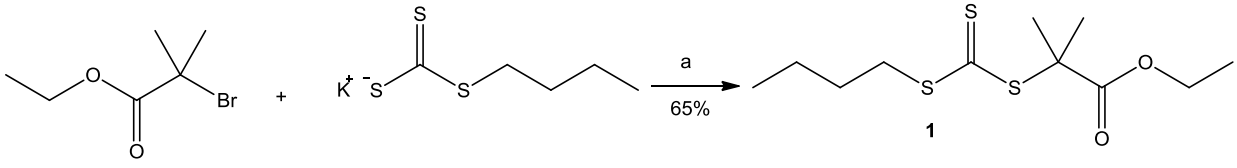
3.2 Results and discussion

In this chapter, a synthetic route towards the controlled polymerisation of SMA is described. In addition, two approaches involving post-polymerisation modification reactions of the SMA were investigated, in an attempt to form two different polymeric gels. The rationale behind demonstrating these two different approaches is to show that SMA can be chemically modified at different sites to introduce new functional groups to the polymer that can lead to hydrogel formation as will be discussed in Chapter 5. For the SMA polymer, this can be accomplished either through modification of the anhydride residues in the SMA backbone or through the chain end modification of the available thiocarbonyl thio groups of the RAFT-made SMA. The former crosslinks by the addition of a multifunctional polymer while the latter will result in chain coupling to form high molar mass chains composed of low molar mass SMA segments, ultimately leading to physical hydrogel formation.

3.2.1 Synthesis and Characterisation of RAFT system

Ethyl 2-(((butylthio)carbonothioyl)thio)-2-methylpropanoate (**1**) was selected as the CTA of choice for synthesis of SMA since the R and Z groups are suitable for effectively controlling styrene and maleic anhydride monomer species.[18]

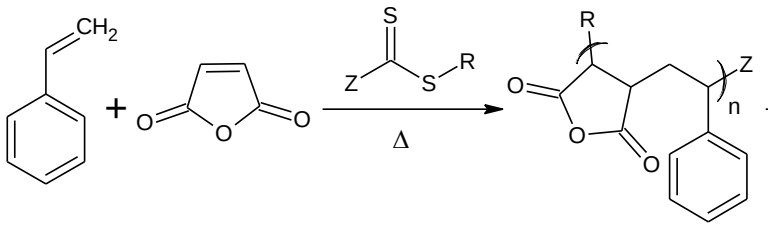
Synthesis of RAFT agent **1** is shown in Scheme 3.1. Ethyl-2-bromo-2-methylpropionate was reacted with potassium butyl trithiocarbonate, through nucleophilic substitution, to attain the desired RAFT agent **1**. The product was purified by column chromatography and ^{1}H NMR spectroscopy confirmed the successful synthesis of RAFT agent **1**.



Scheme 3.1 Synthesis of RAFT agent via nucleophilic substitution reaction: a) acetone, r.t.

3.2.2 Polymerisation of SMA

RAFT agent **1** was employed to control the polymerisation of SMA (Scheme 3.2) at 70 °C, using AIBN as thermal initiator. The RAFT agent/initiator (AIBN) ratio was kept constant at 5:1 to ensure only a negligible amount of chains is initiator-based. Results of various targeted degrees of polymerisation (DP) are summarised in Table 3.1.



Scheme 3.2 RAFT polymerisation of SMA

Table 3.1 Polymerisation conditions and results for SMA synthesised with RAFT agent 1

#	DP	α[a] (%)	Solvent	Reaction time (hr)	Reaction Temp (°C)	$M_{n,\ theo}$[b] (g/mol)	$M_{n,\ SEC}$[c] (g/mol)	$M_{n,\ NMR}$[d] (g/mol)	$Đ$[e]
1a	50	80	Dioxane	4	70	4324	4614	4447	**1.18**
1b	100	75	Dioxane	4	70	7863	9141	7987	**1.11**
1c	200	68	Dioxane	6	70	14030	15767	14443	**1.15**

[a] Conversion.

[b] Theoretical molar mass was calculated as follows : $M_{n,\ theo} = \frac{M_{initial\ monomers}}{M_{initial\ RAFT}} \times \alpha \times M_{r_{(SMA)}} + M_{r_{(RAFT)}}$ …. Equation 1

[c] $M_{n,,\ SEC}$ in *DMAc* based on PMMA standards

[d] $M_{n,\ NMR}$ determined as follows : $M_{n,\ NMR} = \frac{\int Polymeric\ protons\ of\ styrene/5}{\int chain\ end\ protons/2} \times M_{r_{SMA}} + M_{r_{(RAFT)}}$ …… Equation 2

[e] $Đ$ is the molar mass dispersity.

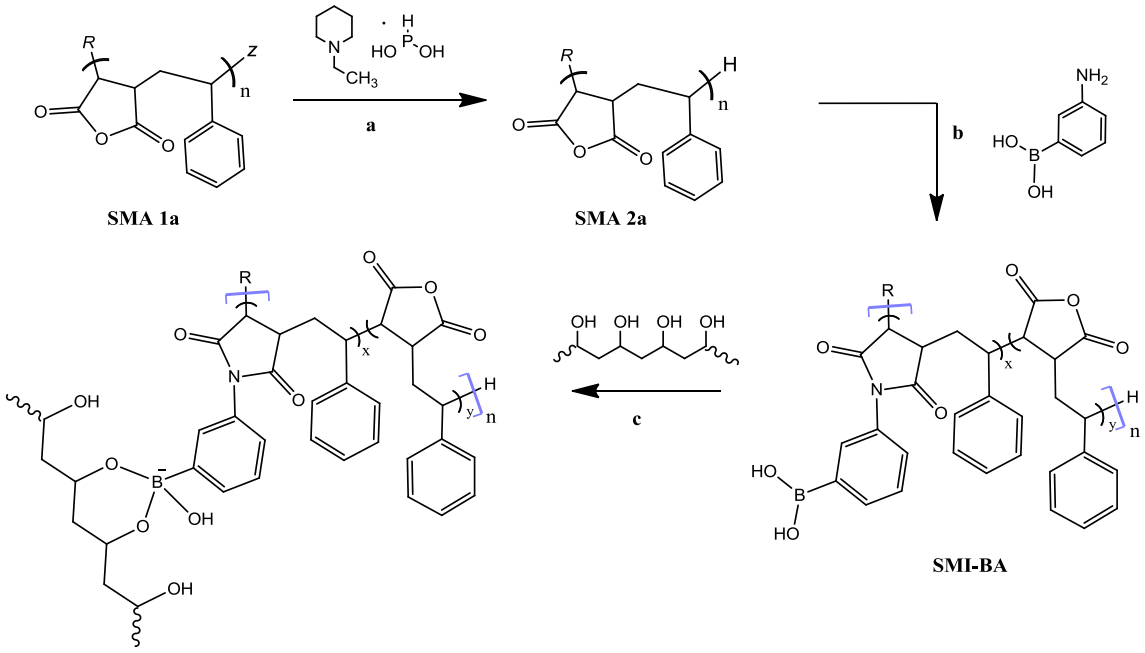
Molar mass (M_n) and dispersity ($Đ$) of the synthesised polymers were determined by size exclusion chromatography (SEC) in *N*,*N*-dimethylacetamide (DMAc), calibrated with PMMA standards. For an RDRP, $Đ$ values below 1.2 from SEC are indicative of good control. Narrow molecular weight distributions and $Đ$ values for all polymerisations indicate that all reactions were effectively controlled. ^{1}H NMR spectroscopy showed that the majority of RAFT end-groups were retained. The minor discrepancy between $M_{n,theo}$ and $M_{n,NMR}$ is due to the latter being based on the assumption that each chain is end-capped with a thiocarbonyl thio moiety. Nonetheless, all M_n data sets were in excellent agreement with each other.

Polymer sample **1a** was chosen as model system for all modification reactions in Approach 1 since quantification through various analytical techniques is more facile with lower molar mass polymers.

3.2.3 Post-polymerisation modification of SMA

3.2.3.1 Approach 1: Modification with 3-aminophenyl boronic acid

This first approach involved the a) radical induced removal of the thiocarbonyl thio end-group on **SMA 1a**, followed by b) chemical modification of the anhydride units with 3-aminophenyl boronic acid (BA) (Scheme 3.3). The reaction between the anhydride units and a primary amine is known to be very rapid and straight forward.[35] Hydrogel formation, **c)** will be studied in Chapter 5 where gelation is achieved by mixing aqueous solutions of the boronic acid modified SMA (SMI-BA) with poly(vinyl alcohol) (PVA). The crosslinking reaction, resulting in gel formation, is based on reversible ester formation between the boronic acid groups, in SMI-BA, and the 1,3 diols in PVA.[36] The formation, swelling behaviour, viscoelastic properties and reversibility of the gels are to be discussed further in Chapter 5.



Scheme 3.3 Approach 1 - Modification of SMA: (a) EPHP, 110 °C ; (b) 3-aminophenyl boronic acid, DMF, 170 °C; (c) PVA, H_2O, r.t.

SMA 1a was treated with N-ethylpiperidine hypophosphite (EPHP) to yield odourless, colourless **SMA 2a** saturated chain ends. The exceptional feature about hypophosphite salts is that it, and all by-products formed in the reaction, are water soluble.[37] This allows for easy removal and purification of the polymer by a simple water wash.

Purified **SMA 2a** was modified with BA, to various degrees, via imidisation of the anhydride groups to yield three different functionalised SMI-BA polymers. Alteration of the percentage of functionalization will allow the synthesis of gels with various cross-linking densities by introducing a controlled amount of functional groups available to crosslink. Change in crosslink density of the gel, upon mixture with PVA, results in gels with various viscoelastic properties and gelation times. This allows us to ultimately narrow down a suitable gel composition with the optimum physical and mechanical properties for contraceptive application. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was used as a technique to characterise and monitor the extent of imidisation of the anhydride peaks of three modified SMI-BA (25%, 50% and 100%) polymers (Figure 3.1).

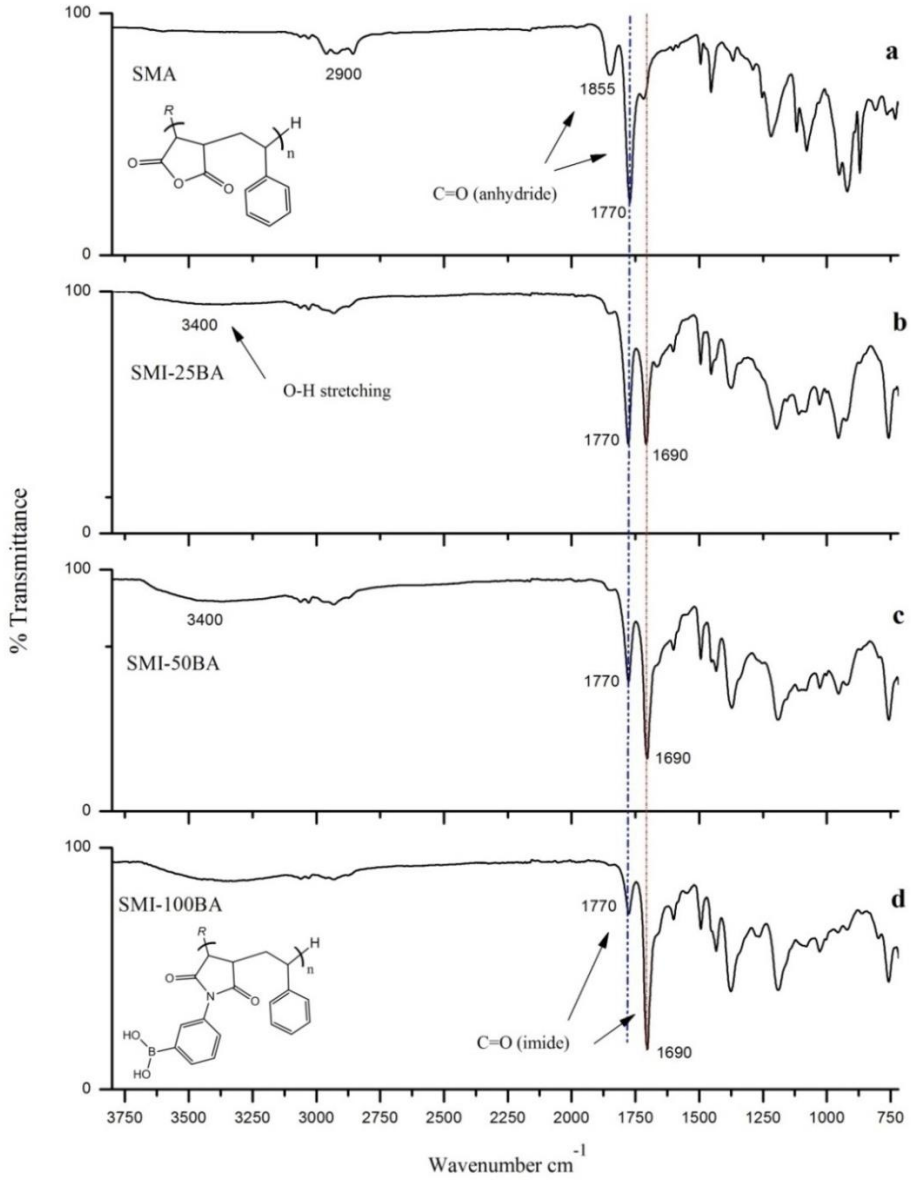


Figure 3.1 Extent of imidisation monitored via ATR-FTIR: (a) SMA (b) SMI-25BA (c) SMI-50BA (d) SMI-100BA

SMA 2a is characterised by a pair of carbonyl absorbing bands at 1770 $cm^{-1}$and 1855 cm^{-1} due to symmetrical and asymmetrical stretching of the anhydride moiety. The small peak at 3044 cm^{-1} is due to the sp^2 aromatic C-H bend vibrations of the styrene unit and the bands at 2900 cm^{-1} is due to the sp^3 aliphatic C-H bending vibrations of the polymer chain.[38,39]

After imidisation of **SMA 2a** with BA, followed by heat-induced ring closure, a new strong band at 1690 cm^{-1} is observed due to successful formation of the imide groups in all three SMA-BA polymers (b-d).[40] Conformation of ring closure is established by the absence of absorption band at 1550 cm^{-1}, indicative of N-H bending.[41] The gradual decrease of the carbonyl anhydride peaks (1770 cm^{-1} and 1885 cm^{-1}) followed by the gradual increase of the absorption band of the imide at 1690 cm^{-1}, after modification with increased amounts of BA (a to d) represent the increased incorporation of BA in the **SMA 2a** polymers. Complete imidisation of SMI-100BA (d) is attributed by the absence of anhydride peaks. The broad band at 3400 cm^{-1} is assigned to the stretching of the hydroxyl groups, present after incorporation of BA, due to diol functionality of BA. The intensities of peaks in ATR-FTIR can be used as a technique to give a qualitative measurement and an estimated quantitative value of modification. Since peak overlapping was evident in the spectra, an alternative method for quantitative chemical analysis can be achieved through analysis of absorbance with UV-vis spectroscopy with the employment of a dye reacting with the functional diol groups on BA.

Boronic acids are well known to form crosslinks with compounds containing diols through reversible ester formation.[42] Alizarin Red S (ARS) is an anthraquinone dye that contains 1,2-cis diols that have the potential to interact covalently with the BA diols.[43] The concentration of BA present in the modified SMI-BA polymers was determined using ARS, by a change in absorbance as a response to the quantity of boronic acid-diol crosslink formation, via UV-vis spectroscopy (Figure 3.2).

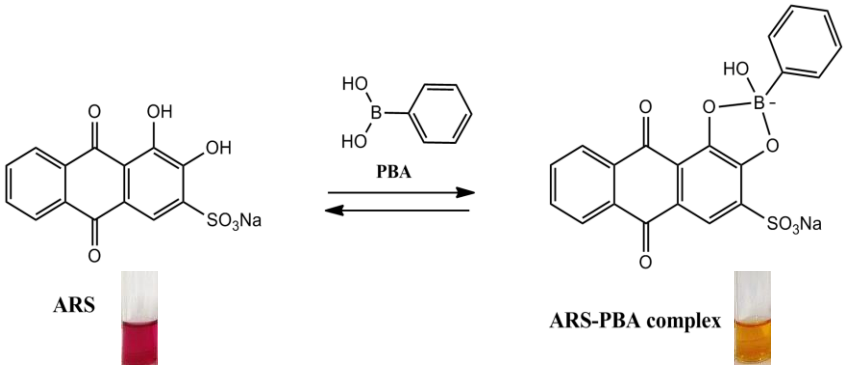


Figure 3.2 Alizarin Red S (ARS) interacts covalently with phenylboronic acid (BA) to form the complex ARS-PBA

The extent of high-affinity complex formation between the diol functionality present in the dye and boronic acid is visually observed by a colour change from deep red to yellow with increase in boronic acid-diol complex formation.[44] As a result, the UV-Vis absorbance spectrum undergoes a hypsochromic shift from λ_{max} = 515 nm to λ_{max} = 468 nm. A solution of ARS was prepared in phosphate buffered saline (PBS) of pH 7.4 and treated with various dilutions of BA (0 μM – 250 μM). The mixtures were equilibrated for 30 minutes before the measurement of every spectrum. The UV-Vis of the solutions are presented in Figure 3.3

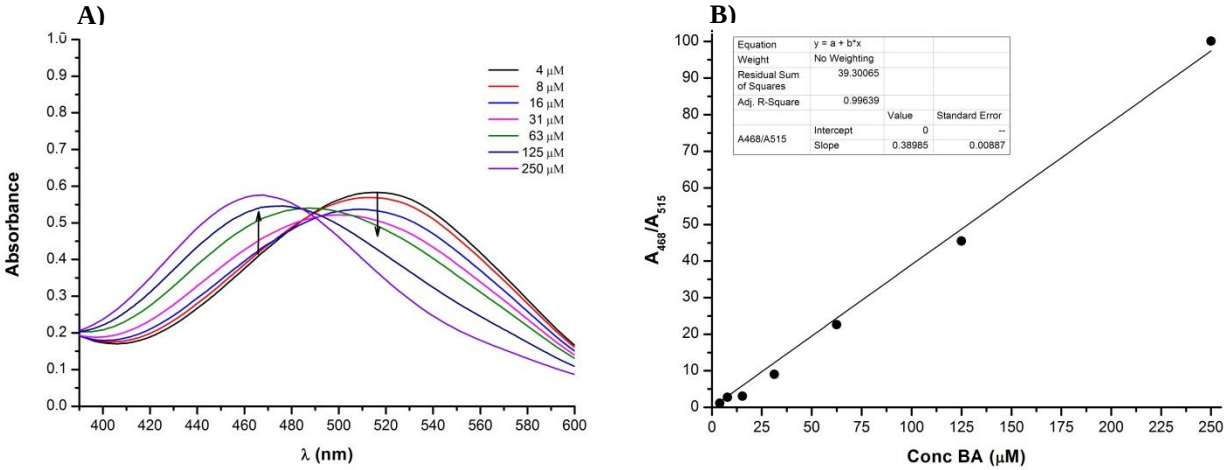


Figure 3.3 (A) UV-Vis absorption spectra changes of ARS (250 μM) with increased concentration of BA (0 μM – 250 μM) in phosphate buffered saline (0.008 M) (B) Calibration curve of the peak area ratio A468/A515 as a function of BA concentration

It is seen from the above spectra that the increased concentration BA up to 31 μM resulted in a decrease in peak intensity followed by a slight shift to shorter wavelength. Further increase in the BA concentration from 31 μM resulted in increased absorbance with a larger shift to lower wavelength. This typical behaviour observed in the UV absorbance has extensively been reported in literature.[45,46] Moreover, the deconvoluted peak area ratio of 468 nm and 515 nm with various BA concentrations is useful for determining the unknown concentration of BA in a sample. Figure 3.3B shows a linear relation of the deconvoluted peak area ratio as a function of BA concentration.

Subsequently, the UV-vis absorption spectra were recorded for SMI-25BA, SMI-50BA and SMI-100BA in Figure 3.4. All SMI-BA solutions in PBS were prepared 24 hours before measurements to obtain clear solutions, due to the requirement of prolonged stirring, solubility dependant on partial hydrolysis of the anhydride groups. The mixtures were equilibrated for 30 minutes before the measurement of every spectrum.

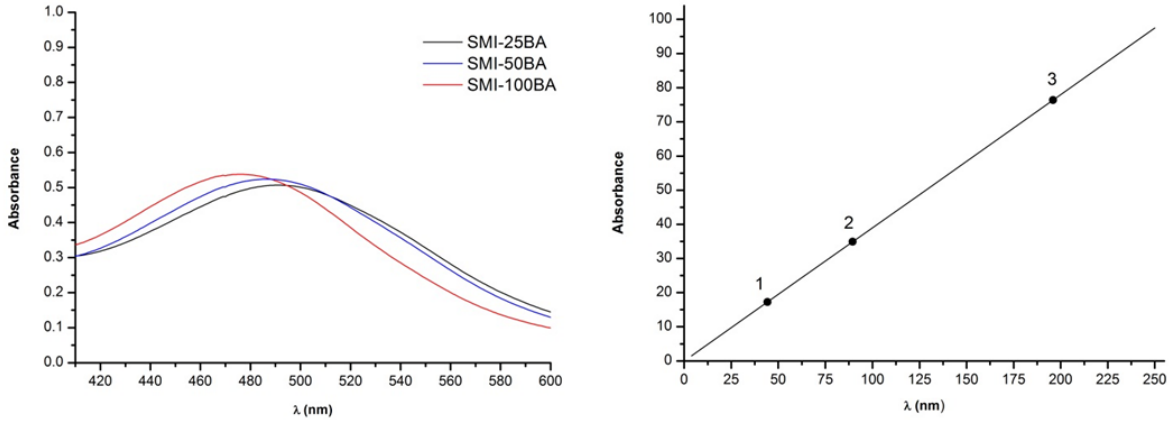


Figure 3.4 Absorption spectra of ARS (250 μM) with SMI-25BA, SMI-50BA and SMI-100BA in PBS to determine the mole fraction of BA in the functionalised polymers

The hypsochromic shift for the modified polymers from λ_{max} = 490 nm to λ_{max} = 475 is typical of increased number of boronic acid-diol crosslink formation with increased BA groups available on the modified polymers. The deconvoluted peak area ratio for the three samples were determined and plotted on the calibration curve to determine a quantitative concentration of BA present in the SMI-BA polymers synthesised. The data obtained from the plots are summarised in Table 3.2.

Table 3.2 UV-vis data obtained from the SMI-BA polymers

#	Sample size (mg)	$MA_{fraction}$ [a] (mol)	$MI_{fraction}$ [b] (mol)	λ_{max}	BA_{PLOT} [b] (μM)	$BA_{fraction}$ [c] (mol)
SMI-25BA	1	4.94×10^{-6}	1.23×10^{-6}	490	44.29	1.10×10^{-6}
SMI-50BA	1	4.94×10^{-6}	2.47×10^{-6}	485	89.49	2.21×10^{-6}
SMI-100BA	1	4.94×10^{-6}	4.94×10^{-6}	475	195.94	4.86×10^{-6}

[a] mole fraction of maleic anhydride (MA) residues in 1 mg SMA polymer before modification

[b] mole fraction of maleimide (MI) residues in SMI-BA polymers at 100% conversion

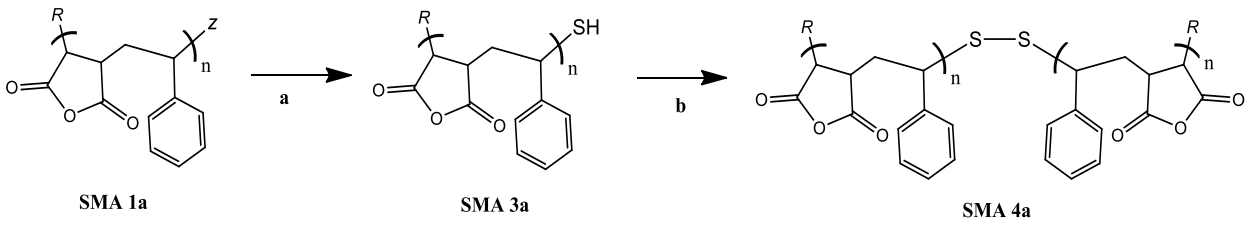
[c] mole fraction of BA in SMI-BA polymers determined from the calibration curve

UV-vis confirmed that SMI-25BA portray the lowest BA concentration, followed by SMI-50BA and ultimately SMI-100BA. The mole fraction of BA in the polymer samples was determined from the calibration plot and compared to the mole fraction of modified maleic anhydride residues in the polymers after modification. The results are in excellent agreement with what would be expected for the modified polymers. From the data it is clear that SMI-100BA is in very close proximity to the expected value for 100% conversion. This is due to the addition of BA in excess to ensure all BA reacted with the available reactive maleic anhydride residues.

These results confirmed the successful incorporation of BA functional groups via the maleic anhydride groups as an effective method for preparation of functionalised SMA polymers with good conversions. UV-vis spectroscopy can effectively be used as a tool for the quantitative determination of BA functional groups along low molar mass polymers.

3.2.3.2 Approach 2: Modification of RAFT end-groups to form thiols

The second approach involves the a) reduction of RAFT-made SMA with sodium borohydride ($NaBH_4$), to yield thiol end-groups. The resulting polymer was b) exposed to air in an attempt to link the thiols into disulphides under oxidising conditions (Scheme 3.4)



SMA 1a SMA 3a SMA 4a

Scheme 3.4 Reduction of RAFT-made SMA to yield thiol end-groups in an attempt to form disulphides under oxidising conditions a) NaBH4, H_20, 24h, r.t. b) O_2, 24h, r.t.

It is readily known in literature that under oxidising conditions the thiol end group termini of most polymers readily oxidise to form dimers containing disulphide bridge-linkages between two polymer segments.[47] The reduction of thiocarbonyl thio end-groups of **SMA 1a** in the presence of $NaBH_4$ was performed in water to yield **SMA 3a**. Successful cleavage of the thiocarbonyl thio groups is visually noted by a solution colour change, from yellow to white. It was hypothesised that the **SMA 3a** polymer with thiol functionality will dimerise into disulphide bridge-linkages, coupling two SMA chains with same approximate molar mass. This will result in polymer **SMA 4a**, double the molar mass of **SMA 3a**.

The use of difunctional RAFT agent such as 1,4-bis(2-(thiobenzoylthio)prop-2-yl) can be employed to prepare α,ω-bis-(dithioester)-functionalised polystyrene, which can be further converted into terminal thiol groups to couple and form linear polymers when subjected to oxygen.[48] This concept can be applied to our polymer system to introduce thiols on both sides of the polymeric chain to form consecutive disulphide bridges, resulting in high molar mass chains, made up of small SMA segments. This will result in a linear increase in molar mass of SMA chains necessary for hydrogel formation. The formation of disulphide linkages can be observed form SEC, by a doubling in the molar mass distribution (MMD) due to a disulfide bridge linking two chains together.[49] The SEC chromatograms obtained for **SMA 1b** before and after reduction with $NaBH_4$ are shown in Figure 3.5

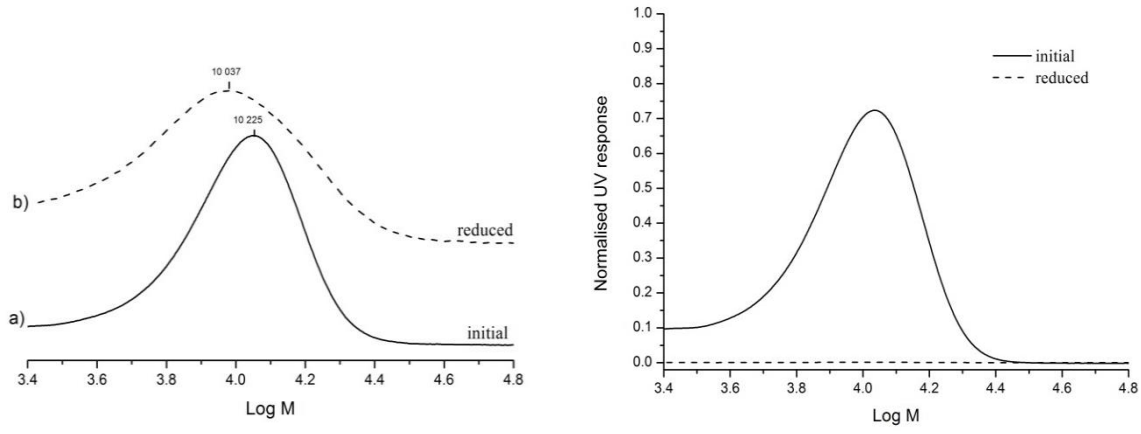


Figure 3.5 SEC chromatogram of SMA 1b Left: a) initial and b) after reduction showing absence of disulphide formation. Right: UV_{320} indicating successful cleavage of the chromophore

The molar mass of the polymer after reduction with $NaBH_4$ and exposure to air have approximately the same molar mass as the polymer prior to reduction. The lack of doubling in molar mass suggests that no disulphide formation took place between polymer chains. This can either be due to unsuccessful reduction of the polymer **SMA 1a** to convert to thiol-terminal **SMA 3a** polymer or a side reaction is hampering the disulphide coupling form **SMA 3a** to **SMA 4a**. The UV signal at 320 nm, attributed to the presence of the thiocarbonyl thio moiety in **SMA 1a** show intense absorbance after the polymerisation, an indication that the polymer chains do contain the RAFT-moiety as an end group.[50] After treatment with $NaBH_4$, the UV signal at 320 nm disappeared, indicative of successful cleavage of the thiocarbonyl thio moiety and the formation of **SMA 3a** polymer. After exposure to air for 24 hours, to the oxidation of the thiol-terminal **SMA 3a** attempt the formation of polymer **SMA 4a** by**,** a lack in the doubling of the molar mass was observed. The result indicates that the thiocarbonyl thio moiety in **SMA 1a** was successfully cleaved to yield the thiol-terminal **SMA 3a** polymer, however after exposure to air for 24 hours to dimerise the polymer, **SMA 4a** was never obtained.

Xu *et al.* reported aminolysis of poly(methyl methacrylate) (PMMA) resulting in formation of a thiolactone terminus, hindering disulphide formation.[49] The thiol end-group generated during aminolysis with hexylamine cyclizes through a backbiting reaction, resulting in the thiolactone structure. The same reaction was observed in poly(*N*,*N*-dimethylaminoethyl methacrylate) and poly(lauryl methacrylate). All of the above polymers have similar structure as ring opened SMA, as such it was speculated that this side reaction might possibly happen during reduction of the SMA polymer. A model study was subsequently carried to test this hypothesis. The model study will be described in Chapter 4.

3.3 Conclusion

It was demonstrated that RAFT agent 1 is sufficient to stabilize the controlled radical polymerisation of SMA. Two synthetic approaches, with very different chemistry routes, were carried out in an attempt to form two different modified SMA samples suitable for hydrogel formation.

In approach one, the successful synthesis of SMI-BA polymers, by introduction of 3-amino phenyl boronic acid (BA), functional polymers were synthesised via the reactive maleic anhydride residues. The polymers were successfully characterised via ATR-FTIR spectroscopy and the degree of modification was quantified using UV-vis spectroscopy.

It was found that the reduction of SMA in the second approach resulted in the successful cleavage of the thiocarbonyl thio groups, however disulphide formation was never observed. It was speculated that this was a result of thiolactone ring formation via the backbiting of the thiol group toward the penultimate unit, eliminating the possibility of disulphide formation. However this requires further investigation.

3.4 Experimental

3.4.1 General experimental details

All chemicals and solvents were purchased from commercial sources and used without further purification, unless stated otherwise. Styrene (Plascon Research Centre, University of Stellenbosch, estimated purity ~99% by ^{1}H-NMR) was washed three times with an aqueous 0.3 M KOH solution to remove the inhibitor and subsequently distilled under reduced pressure. 2,2'-Azobis(isobutyronitrile) (AIBN) (Riedel de Haën) was recrystallized from methanol and dried under vacuum at ambient temperature. Distilled deionised water was obtained from a Millipore Milli-Q purification system and used in all experiments. Reactions were monitored using thin layer chromatography (TLC), utilising Machery-Nagel Silica gel 60 plates (particle size 0.063-0.2mm/ 70-230 mesh) with a UV 254 fluorescent indicator. Moisture and oxygen sensitive reactions were carried out in an inert argon atmosphere.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian VXR-Unity (300 MHz or 400 MHz) spectrometer at room temperature. Samples were prepared in deuterated solvents obtained from Cambridge Isotope Labs. Integration of spectra was carried out using Mestranova 9.0. Chemical shifts were reported in parts-per-million (ppm), referenced to the residual solvent protons. Abbreviations for NMR splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet), whereas coupling constants (J) are reported in hertz (Hz).

Size exclusion chromatography (SEC) was performed on a system consisting of a Shimadzu LC-10AT isocratic pump, a Waters 717+ autosampler, a column system fitted with a PSS guard column (50×8 mm) in series with three PSS GRAM columns (300 × 8 mm, 10 µm, 2 × 3000 Å and 1 × 100 Å) kept at 40 °C, a Waters 2487 dual wavelength UV detector and a Waters 2414 differential refractive index (DRI) detector. *N*,*N*-Dimethylacetamide (DMAc) was used as the eluent, stabilized with 0.05 % 2,6-di-tert-butyl-4-methylphenol (BHT) (w/v) and 0.03 % LiCl (w/v), at a flow rate of 1.0 mL.min^{-1}. All polymer samples were filtered through 0.45 µm GHP filters, to remove impurities, preceding analysis. Molar mass of

polymers were determined by calibration using poly(methyl methacrylate) (PMMA) standard sets (Polymer Laboratories) ranging from 690 to 1.2 × 10^6 g.mol^{-1}. Data acquisition was performed using Millennium software, version 4.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was performed on a Nexus infrared spectrometer equipped with a Smart Golden Gate attenuated total reflectance diamond from Thermo Nicolet with ZnSe lenses. Each spectrum was scanned 32 times with 4.0 cm^{-1} resolution. The data analysis was performed with Omnic Software version 7.2.

UV-Vis spectra were measured using a Perkin Elmer photodiode array spectrophotometer. The model of the spectrophotometer was a Lambda 20 which comprised a holographic monochromator, pre-aligned deuterium and halogen lamps and a photodiode array detector. Samples were measured in a 2 mm × 2 mm cuvette at 25 °C. UV Winlab (version 2.0) software was used for data acquisition and processing.

3.4.2 Synthetic procedures

Synthesis of ethyl 2-(((butylthio)carbonothioyl)thio)-2-methylpropanoate (1):

Potassium phosphate tribasic (16.6 g, 78.2 mmol) was dissolved in acetone in a 500 mL round bottom flask and stirred for 5 hours to form a yellow suspension. 1-butanethiol (8.0 mL, 74.2 mmol) was added and the mixture was stirred for an additional 1 hour. Carbon disulphide (9.1 mL, 15.1 mmol) was slowly added drop-wise and the solution was allowed to stir for 2 hours at 0 °C. ethyl 2 bromo-2-methylpropionate (13.7 g, 70.0 mmol) was added and the solution was allowed to stir for 24 hours at room temperature. The reaction mixture was filtered and concentrated. The residue was diluted with 10% HCl solution and stirred overnight at room temperature. The organic layer was extracted with hexane and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the residual crude product was purified by column chromatography, with petroleum ether/ethyl acetate (9/1) as eluent, to yield a yellow solid (12.78 g, 65% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 4.15 (t, J=7.1 Hz, 2H), 3.27 (t, J=7.4 Hz, 2H), 1.68 (s, 6H), 1.66-1.60 (m, 2H), 1.45-1.34 (m, 2H) 1.23 (t, J=7.1 Hz, 3H), 0.91 (t, J=7.4 Hz, 3H) ^{13}C NMR (151 MHz,$CDCl_3$) δ (ppm) 221.44, 172.98, 62.04, 56.04, 36.67, 30.05, 25.47, 22.17, 14.12, 13.70. MS (ESI): m/z = 281.07 m/z (Calculated 281.07 m/z for [M+H$^+$])

Polymerisation of Styrene-co-maleic anhydride (SMA):

Styrene (843 mg, 8.1 mmol), maleic anhydride (834 mg, 8.5 mmol), AIBN (40.53 mg, 0.247 mmol), RAFT agent 1 (346.18 mg, 1.23 mmol) and dioxane (2.5 mL) were added to a 20 mL pear shape flask. The reaction mixture was degassed with argon for 45 minutes and placed in an oil bath at 70 °C for 4 hours. The solution was precipitated from isopropanol twice and the polymer was dried under reduced pressure overnight at room temperature to yield a the polymer product (1025 mg, 61% yield, M_n = 3717 g/mol, $Đ$ = 1.23)

Synthesis of Styrene-aminophenylbronic acid maleimide copolymer (SMI-100BA):

SMA (0.200 g, 1.00 mmol MA residues) was dissolved in 3 mL DMF in a 50 mL round bottom flask. 3-aminophenylboronic acid (BA) (0.140 g, 1.05 mmol) was placed in a dropping funnel with 5 mL DMF and added drop-wise to the SMA solution, at room temperature, over a period of 30 minutes. The solution was placed in a 155 °C oil bath for 7 hours for complete imidisation of maleic anhydride groups. The solution was cooled and precipitated in isopropanol, re-dissolved in dioxane and precipitated again in isopropanol. The light brown polymer was dried in vacuum at 40 °C for 24 hours to yield SMA-100BA (0.302 g, 95% yield).

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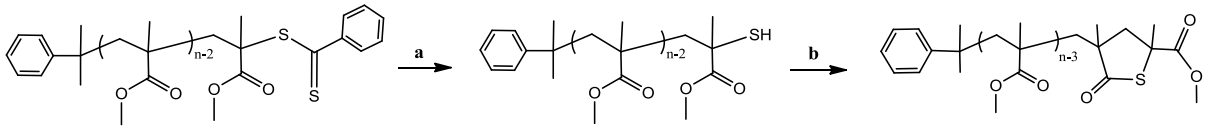
Chapter 4: Dimerisation Model Study of SMA

4.1 Introduction

The reaction of thiocarbonyl thio groups with excess amine (aminolysis) is one of the most versatile and widely used methods of RAFT end group conversion, resulting in the formation of thiol-terminal chain end polymers, capable of undergoing multiple reactions with various functional groups.[1,2] It is well known that thiol moieties readily form disulphides through mild oxidation, followed by easy reversal by the addition of a reducing agent. Whittaker *et al.* reported the ease of disulphide formation of thiol-terminal polystyrene synthesised with a difunctional RAFT agent, followed by aminolysis and oxidation to result in linear, high molecular weight multiblock polymers.[3] This attractive chain end manipulation strategy allows for a variety of polymers to be reversibly crosslinked under mild conditions, with disulphide bridge formation by exposure to air, easily cleaved again under reducing conditions.

It has been shown that the thiols formed during the aminolysis of various methacrylates undergo a side reaction to hinder any possibility of disulphide formation. Thiol-terminal poly(methyl methacrylate) (PMMA) (Scheme 4.1) is found to attach to the penultimate unit in the chain by backbiting, resulting in the formation of a thiolactone ring.[4] In addition, the side reaction is promoted by the stability of the five-membered ring.[5] Furthermore, thiolactone ring formation was also observed by other researchers for poly(acrylic acid) (PAA)[6] polymer.



Scheme 4.1 Thiolactone ring formation via aminolysis of PMMA: a) hexylamine, 20 eq, THF, r.t. b) O_2, 24h, r.t.

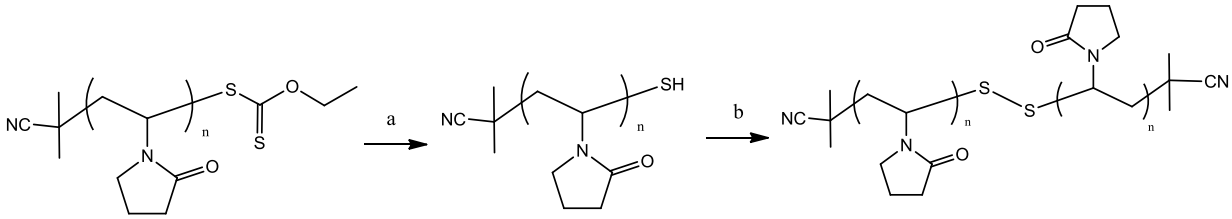
While such cyclisation reactions are undesirable if the aim is to obtain free polymeric thiols, inhibition of thiolactone ring formation in PMMA can be accomplished by the introduction of a short block of polystyrene via chain extension of the PMMA macro-RAFT agent with styrene monomer.[4]

The present chapter investigates the possibility of thiolactone ring formation of thiol-terminal SMA described in Chapter 3. In this model study, the controlled synthesis of RAFT-made polymers, structurally similar to SMA, is described. The polymers chosen for this study represent both possibilities of either a maleic anhydride or styrene monomer next to the thiocarbonyl thio RAFT end group. In addition, reduction of the polymers was carried out to ensure thiol-terminal chain end polymers capable of disulphide formation. The extent of disulphide formation in the polymers is studied in order to determine the structural conditions that subsequently hinder disulphide formation in SMA. Furthermore, an attempt to introduce a short polystyrene block to the SMA macro-RAFT agent to promote disulphide formation is discussed.

4.2 Results and discussion

4.2.1 Model study

It is well known that thiol-terminated poly(*N*-vinylpyrrolidone) (PVP) polymer chains readily form disulphides under oxidising conditions.[7] Xanthate end functional PVP was prepared via (*S*)-(2-cyano-2-propyl)-*O*-ethyl xanthate-mediated polymerisation.[8] A common aminolysis procedure was applied to the synthesised polymer as shown in Scheme 4.2.



1c **2c**

Scheme 4.2 Reaction scheme of intermolecular disulphide starting from functionalised PVP. The applied reaction conditions include a) hexylamine, 20 eq., DCM, r.t b) O_2, 24 h, r.t.

Disulphide formation is observed as a doubling of molar mass which can be seen as a shift in the molar mass distribution (MMD) (Figure 4.1) In addition, the absence of the UV (280 nm) signal attributed to absorbance by the xanthate functionality, is a clear indication of the effective cleavage of the C=S chromophore.[9]

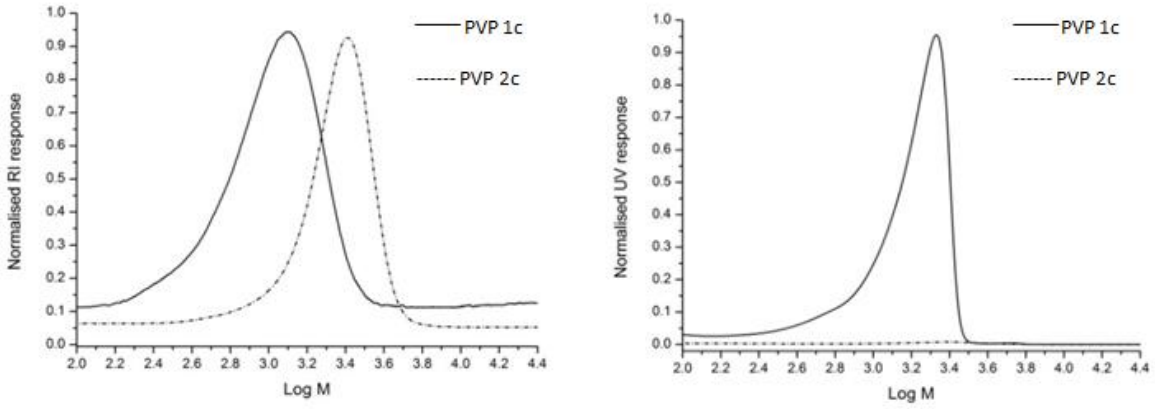


Figure 4.1 DRI and UV SEC molar mass distribution from PVP 1c to PVP 2c confirming successful disulphide formation

Since the SMA copolymer is considered strictly alternating in structure, the monomer unit next to the thiocarbonyl thio group can either be a styrene or a maleic anhydride. As such, the polymers chosen to participate in the model study, structurally similar to SMA (Figure 4.2) are the following: polystyrene (PS) represents the former case where a styrene group is adjacent to the RAFT end group and PMMA and poly(methyl acrylate) (PMA) systems the latter of having a maleic anhydride adjacent to the thiol after reduction. The extent of disulphide formation was investigated with SEC.

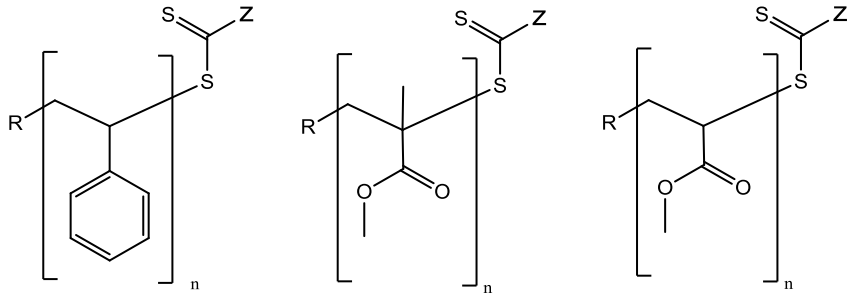


Figure 4.2 Polymers structurally similar to SMA

The above mentioned polymers were successfully synthesised using ethyl 2-(((butylthio)carbonothioyl)thio)-2-methylpropanoate (RAFT agent 1) synthesised in Chapter 3. AIBN and 1,1′-azobis(cyanocyclohexane) (ACHN) were used as thermal initiators. All polymerisations were carried out under an inert atmosphere. Reductions of the polymers were carried out using excess $NaBH_4$ (20 equivalents). Polymerisation conditions and SEC results are summarised in Table 4.1.

Table 4.1 Synthesis and characterisation of model study polymers

#	Polymer	$M_{n,SEC}$[a], (g/mol)	$Đ$[a]	Reducing agent	Time (h)	UV_{320}	Reduced polymer	$M_{n,SEC}$[b], (g/mol)	$Đ$[b]	UV_{320}
1a	SMA	4614	1.18	$NaBH_4$	24	Yes	**2a**	4717	1.23	No
1b	SMA	9141	1.11	$NaBH_4$	24	Yes	**2b**	8617	1.20	No
1c	PVP	1239	1.13	$NaBH_4$	24	No[c]	**2c**	2219	1.14	No[c]
							2d	2191	1.14	No[c]
1d	PS	2488	1.14	$NaBH_4$	24	Yes	**2e**	5518	1.24	No
							2f	5509	1.22	No
1e	PMA	3416	1.16	$NaBH_4$	24	Yes	**2g**	3643	1.20	No
							2h	3476	1.23	No
1f	PMMA	3976	1.18	$NaBH_4$	24	Yes	**2i**	3890	1.31	No
							2j	3743	1.31	No
1g	PMMA	10939	1.26	$NaBH_4$	24	Yes	**2k**	12770	1.25	No

[a] $M_{n,SEC}$ obtained from SEC in DMAc based on PMMA standards before reduction [b] $M_{n,SEC}$ after reduction [c] UV at 280 nm

Low dispersities, $Đ \leq 1.26$, of synthesised polymers **1a-1g** are indicative of good polymerisation control. The UV signals (320 nm), attributed to the thiocarbonyl thio moiety of the synthesised SMA, PS, PMA, and PMMA polymers show intense absorption.[10] It is therefore concluded that all chains do contain the RAFT-moiety as an end group. Reduction of polymers **1a-1g** results in the disappearance of UV absorbance at the characteristic wavelength, indicative of the successful cleavage of the thiocarbonyl thio moiety. Disulphide formation of PVP and PS was confirmed by a doubling in molar mass after exposure to air for 24 hours. PMMA, PMA and SMA exhibit a molar mass approximately the same as the starting material, which suggests the lack of disulphide formation.

^{13}C NMR was used as an additional technique to determine if these low molar mass polymers *i.e.* PMMA, PMA and SMA formed thiolactone ring end species. Thiolactone ring formation of PMMA in Figure 4.3 is confirmed by the disappearance of the carbonyl carbon (–C=S)S- at 221 ppm and the appearance of a new signal at 210 ppm, assigned to the carbonyl carbon of the thiolactone ring. This value of chemical shift is in close proximity to the reported values of Engel *et al.*[11] A similar shift of the carbonyl occurred for the PMA polymer.

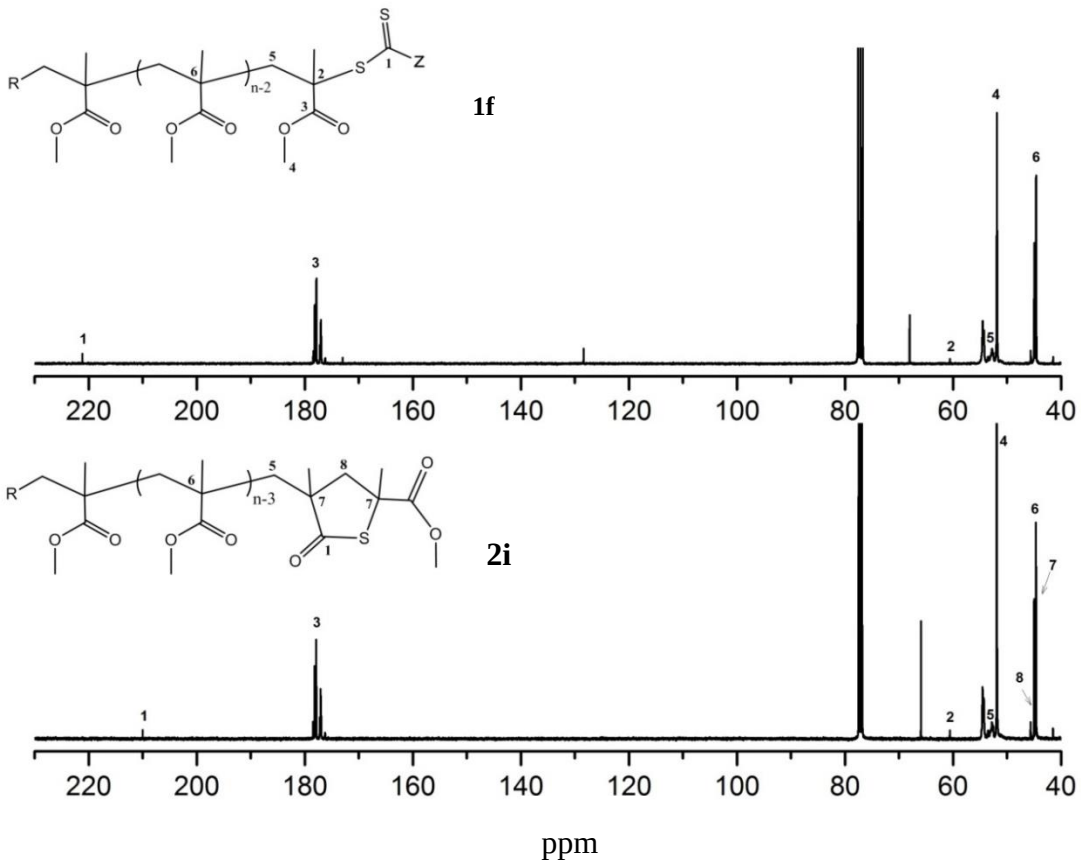


Figure 4.3 ^{13}C NMR spectra confirming thiolactone ring formation after treating PMMA 1f with a reducing agent to result in PMMA 2i

Since ring-opened SMA is structurally similar to acrylates, it was hypothesised that the polymer might also undergo thiolactone ring formation. It was speculated that this side reaction is the main interference in disulphide formation of our polymer after numerous attempts resulted in no doubling in molar mass after confirmed disappearance of the thiocarbonyl thio group from SEC in Table 4.1. If thiolactone ring formation was indeed the cause of this, an expected up field shift of the carbonyl carbon at 222 ppm in close proximity to 210 ppm can be confirmed by ^{13}C NMR spectroscopy.

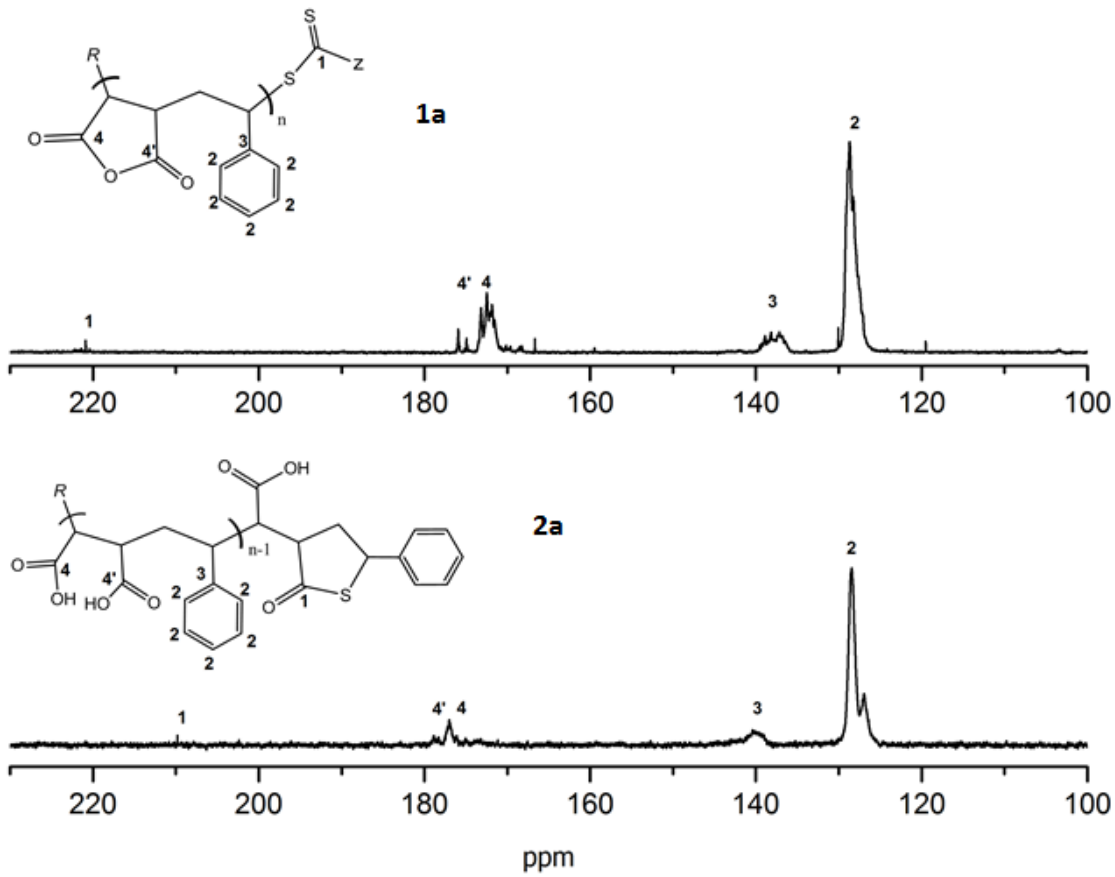


Figure 4.4 ^{13}C NMR spectra confirming thiolactone ring formation after treating SMA 1a with a reducing agent to result in SMA 2a

^{13}C NMR spectroscopy confirmed the disappearance of the carbonyl carbon at 222 ppm and the appearance of a new signal at 209 ppm, assigned to carbonyl carbon of the proposed 5-membered thiolactone ring formation in **SMA 1a** polymer with styrene as the terminal monomer and maleic anhydride as the penultimate unit. The same result was however not observed in synthesis of SMA with maleic anhydride as terminal unit, even though lack of disulphide formation was also observed in this case.

4.2.2 SMA-*b*-PS diblock synthesis and characterisation

Introduction of a small block of styrene monomers after the RAFT polymerisation of SMA was carried out in an attempt for the polymer to undergo reduction according to the mechanism of polystyrene, to result in disulphide coupling under oxidising conditions. Two SMA-*b*-PS diblock polymers were synthesised by chain extending SMA macro-RAFT agent **1a** with approximately five styrene monomer units. Table 4.2 provides a summary of the SEC results of the synthesised block copolymers before and after reduction with $NaBH_4$ and exposure to air for 24 hours.

Table 4.2 SEC results of synthesised SMA-b-PS block copolymers

Macro RAFT agent	*Polymer*	*Target Mn*	*$M_{n,SEC}$[a], (g/mol)*	*Đ[a]*	*Composition*	*Time (h)*	*Reduced polymer*	*$M_{n,SEC}$[b], (g/mol)*	*Đ[b]*
1a	3a	5654	5001	1.22	SMA_{22}-*b*-PS_4	22	4a	10374	1.28
	3b	5654	5147	1.20	SMA_{22}-*b*-PS_5	22	4b	10367	1.28

[a] *$M_{n,SEC}$ obtained from SEC in DMAc based on PMMA standards before reduction* [b] *$M_{n,SEC}$ after reduction*

A maximum of four PS units at the end of polymer chain **3a** is sufficient to prevent cyclisation of the penultimate unit. From the SEC chromatogram in Figure 4.5 the doubling of the molar mass confirms disulphide formation of the block copolymer **3a** to **4a**. This is followed by the successful cleavage of the thiocarbonyl thio unit by the disappearance of the UV signal at 320 nm after treatment with $NaBH_4$.

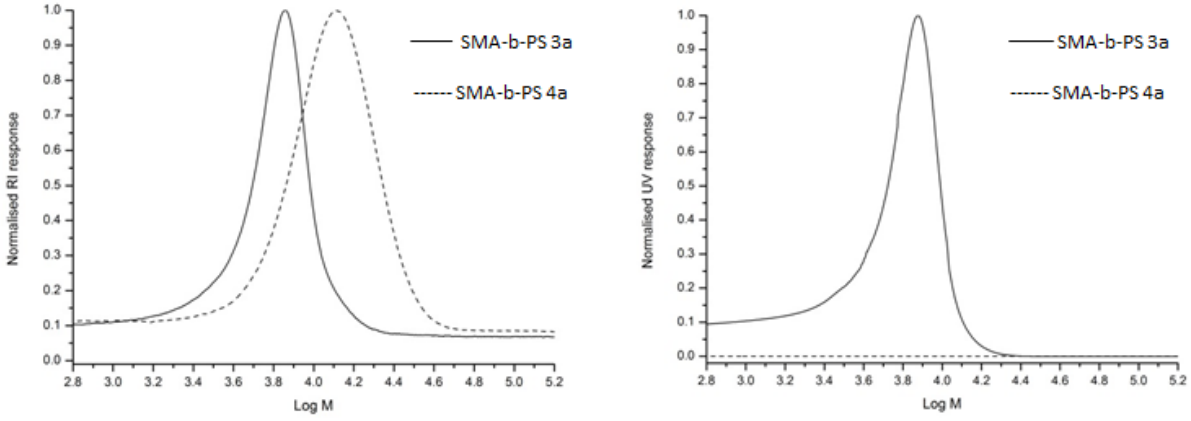


Figure 4.5 DRI and UV SEC molar mass distribution from SMA-b-PS 3a to SMA-b-PS 4a confirming disulphide formation of the block polymer

4.3 Conclusion

It was demonstrated that disulphide formation between two polymeric chains is predominantly dependant on the structural format of the penultimate and last monomeric units next to thiocarbonyl thio group of RAFT-made polymers. The reduction of PVP, PS, PMMA, PMA, and SMA polymers prepared by RAFT polymerisation results in polymers with thiol termini confirmed by the disappearance of the UV signal of the specified chromophore in SEC measurements.

It was shown, through SEC analysis, that PVP and PS couple through the formation of disulphide bonds, however PMMA, PMA and SMA polymers cyclise to a thiolactone end

group formed via the backbiting reaction promoted by the stability of the 5-membered ring. The thiolactone ring formation in these polymers was confirmed by ^{13}C NMR spectroscopy.

It was established by SEC analysis that extension of the SMA macro-RAFT agent with four styrene units was enough to prevent cyclisation and promote disulphide formation of the polymer.

4.4 Experimental

4.4.1 General experimental details

All chemicals and solvents were purchased from commercial sources and used without further purification, unless stated otherwise. *N*-vinylpyrrolidone (Aldrich, 99%) was dried over molecular sieves and purified by distillation under reduced pressure. Styrene (Plascon Research Centre, University of Stellenbosch, estimated purity ~99 % by ^{1}H-NMR), methyl methacrylate (Aldrich, 99%), methyl acrylate (Aldrich, 99%) were passed through an Al_2O_3 column and subsequently distilled under reduced pressure. (2,2'-Azobis(isobutyronitrile) (AIBN) (Riedel de Haën) and 1,1′-azobis(cyanocyclohexane) (ACHN) (Aldrich, 98%) was recrystallized from methanol and dried under vacuum at ambient temperature. Moisture and oxygen sensitive reactions were carried out in an inert argon atmosphere.

^{13}C-NMR spectra were recorded on a Varian VXR-Unity (400 MHz) spectrometer at room temperature. Samples were prepared in deuterated solvents obtained from Cambridge Isotope Labs. Chemical shifts were reported in parts-per-million (ppm), referenced to the residual solvent peaks.

Size exclusion chromatography (SEC) was performed on a system consisting of a Shimadzu LC-10AT isocratic pump, a Waters 717+ autosampler, a column system fitted with a PSS guard column (50×8 mm) in series with three PSS GRAM columns (300 × 8 mm, 10 μm, 2 × 3000 Å and 1 × 100 Å) kept at 40 °C, a Waters 2487 dual wavelength UV detector and a Waters 2414 differential refractive index (DRI) detector. *N*,*N*-Dimethylacetamide (DMAc) was used as the eluent, stabilized with 0.05 % 2,6-di-tert-butyl-4-methylphenol (BHT) (w/v) and 0.03 % LiCl (w/v), at a flow rate of 1.0 mL.min^{-1}. All polymer samples were filtered through 0.45 μm GHP filters, to remove impurities, preceding analysis. Molar mass of polymers were determined by calibration using poly(methyl methacrylate) (PMMA) standard sets (Polymer Laboratories) ranging from 690 to 1.2 × 10^{6} g.mol^{-1}. Data acquisition was performed using Millennium software, version 4.

4.4.2 Synthetic procedures

Synthesis of ethyl 2-(((butylthio)carbonothioyl)thio)-2-methylpropanoate (1):

Potassium phosphate tribasic (16.6 g, 78.2 mmol) was dissolved in acetone in a 500 mL round bottom flask and stirred for 5 hours to form a yellow suspension. 1-butanethiol (8.0 mL, 74.2 mmol) was added and the mixture was stirred for an additional 1 hour. Carbon disulphide (9.1 mL, 15.1 mmol) was slowly added drop-wise and the solution was allowed to stir for 2 hours at 0 °C. ethyl 2 bromo-2-methylpropionate (13.7 g, 70 mmol) was added and the solution was allowed to stir for 24 hours at room temperature. The reaction mixture was filtered and concentrated. The residue was diluted with 10% HCl solution and stirred overnight at room temperature. The organic layer was extracted with hexane and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the residual crude product was purified by column chromatography, with petroleum ether/ethyl acetate (9/1) as eluent, to yield a yellow solid (12.78 g, 65% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 4.15 (t, J=7.1 Hz, 2H), 3.27 (t, J=7.4 Hz, 2H), 1.68 (s, 6H), 1.66-1.60 (m, 2H), 1.45-1.34 (m, 2H) 1.23 (t, J=7.1 Hz, 3H), 0.91 (t, J=7.4 Hz, 3H) ^{13}C NMR (151 MHz,$CDCl_3$) δ (ppm) 221.44, 172.98, 62.04, 56.04, 36.67, 30.05, 25.47, 22.17, 14.12, 13.70. MS (ESI): m/z = 281.07 m/z (Calculated 281.07 m/z for [M+H$^+$])

Synthesis of *S*-(2-cyano-2-propyl)-*O*-ethyl xanthate (2)

This xanthate transfer agent was prepared exactly as described in literature.[12] ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 4.74 (q, J=7.2 Hz, 2H), 1.75 (s, 6H), 1.52 (t, 7.2 Hz, 3H).

Poly(*N*-vinylpyrrolidone) (PVP) synthesis:

NVP (1667 mg, 15 mmol), AIBN (10 mg, 0.06 mmol), RAFT agent 2 (118 mg, 0.60 mmol) were added to a 50 mL pear-shape flask. The reaction flask was degassed with argon for 1 hour and immersed in a 60 °C oil bath for 5 hours. After 5 hours, 1 mL of DCM was added and left to stir for 30 minutes. The solution was precipitated into diethyl ether and centrifuged. The precipitate was re-dissolved in DCM, precipitated in diethyl ether and centrifuged again. The polymer product was dried under reduced pressure overnight at room temperature to yield the desired polymer (756 mg, 45% yield, M_n = 1239 g/mol, $Đ$ = 1.13).

Polystyrene (PS) synthesis:

Styrene (1562 mg, 15 mmol), ACHN (20 mg, 0.08 mmol), RAFT agent 1 (162 mg, 0.57 mmol), and toluene (2.0 mL) were added to a 50 mL pear-shape flask. The reaction flask was degassed with argon for 1 hour and immersed in a 100 °C oil bath for 24 hours. After 24 hours the solution was precipitated into methanol and centrifuged. The precipitate was re-dissolved in THF, precipitated in methanol and centrifuged again. The polymer product was dried under reduced pressure overnight at room temperature to yield the desired polymer product (922 mg, 59% yield, M_n = 2488, $Đ$ = 1.14)

Poly(methyl methacrylate) (PMMA) synthesis:

Methyl methacrylate (700 mg, 7.0 mmol), AIBN (8.50 mg, 0.05 mmol), RAFT agent 1 (70 mg, 0.26 mmol), and dioxane (1.5 mL) were added to a 20 mL pear shape flask. The reaction flask was degassed with argon for 1 hour and immersed in a 70 °C oil bath for 10 hours. After 10 hours the solution was precipitated into diethyl ether and centrifuged. The precipitate was re-dissolved in DCM, precipitated in diethyl ether and centrifuged again. Finally the polymer was dried under reduced pressure overnight at room temperature to yield the desired polymer (416 mg, 59% yield, M_n = 3976, $Đ$ = 1.18).

Poly(methyl acrylate) (PMA) synthesis:

Methyl acrylate (775 mg, 9.0 mmol,), AIBN (9.3mg, 0.06 mmol), RAFT agent 1 (77 mg, 0.28 mmol), and dioxane (1.5 mL) were added to a 20 mL pear shape flask. The reaction flask was degassed with argon for 1 hour and immersed in a 70 °C oil bath for 10 hours. After 10 hours the solution was precipitated into isopropanol and centrifuged. The precipitate was re-dissolved in dioxane, precipitated in isopropanol and centrifuged again. The polymer was dried under reduced pressure overnight at room temperature to yield the desired polymer product (205 mg, 27% yield, M_n = 3416, $Đ$ = 1.16)

Poly(styrene-*co*-maleic anhydride-*block*-styrene) (SMA-*b*-PS) synthesis:

Macro-RAFT **1a** (200 mg, 0.04 mmol), styrene (57 mg, 0.55 mmol) and AIBN (1.7 mg, 0.01 mmol) were added to a 20 mL pear-shape flask. The reaction flask was degassed with argon for 1 hour and immersed in a 70 °C oil bath for 24 hours. The polymer was isolated by precipitation form isopropanol twice and dried under reduced pressure overnight at room temperature to yield the desired polymer product **3a** (42 mg, 21% yield, M_n = 5001, $Đ$ = 1.22)

4.5 References

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Chapter 5: Hydrogel Synthesis and Characterisation

5.1 Introduction

Hydrogels are three-dimensional networks formed by hydrophilic homopolymer or copolymer chains, crosslinked to form water-insoluble matrices.[1,2,3] These polymers have a high affinity for water, but the physical and/or chemical crosslinks prevent the networks from dissolving in aqueous media. Water penetrates the interconnected polymer chain domains and causes swelling of the polymer networks, which results in the formation of hydrogels. Hydrogels have the ability to retain their shape in solution despite the fact that they imbibe excessive amounts of water, in some cases up to thousand times the dry weight of the polymeric matrix.[4,5] Their thermodynamic compatibility with water results in soft, hydrated materials with a degree of elasticity very similar to natural tissue, which makes them appealing candidates for mimicking natural biological tissue.[6,7,8] Since mechanical, solute permeability, elasticity, swelling, and hydrophilic/hydrophobic properties of a hydrogel can be tailored by choice of polymers and synthesis method, it is possible to create gels that fulfil the demands of biomaterials with physical integrity and network structure suitable to be used as wound covers, implant coatings or materials that mimic *in vivo* functionality.[9,10,11] In the past few decades, numerous polymeric hydrogels have been developed for use in tissue engineering,[12,13,14] drug delivery,[15,16,17] wound dressings[16,18] and various other biological systems.[19]

The crosslinks between the polymeric chains are classified as either physical or chemical. Physically crosslinked hydrogels occur due to entanglements between dynamic macromolecules or by linkages held together by non-covalent interactions, *e.g.* hydrogen bonding, ionic and hydrophobic interaction between polymer chains.[20] Chemical gels arise through the formation of covalent bonds between functionalized polymers.[21] These gels are generally easier to modify and have greater mechanical properties compared to physical gels.

The main drawback of the Vasalgel/RISUG hydrogel system as a potential male contraceptive is its reversibility. Procedural difficulties in the reversibility of these gels by flushing with DMSO or $NaHCO_3$ solutions have been encountered in rats and monkeys.[22,23] Additional non-favourable squeezing and manipulation of the vas has been enforced on subjects to dislodge the gel from the vas to the ejaculatory duct, resulting in additional

swelling and bruising. Reversal has been proven difficult by injection of the solvents as a result of viscous solutions in the duct due to use of high molar mass polymers. To date, sterilisation reversal in a human subject has not been reported. As such, this shortcoming in reversibility leaves scope for improvement. Reversibility can effectively be improved by the formation of a hydrogel composed of linkages that can readily breakdown into soluble low molecular weight fragments by the addition of non-harmful additives. This will solve the reversibility problem hampering the current system.

Formation of a hydrogel by combining aqueous solutions of sodium borate (borax) and poly(vinyl alcohol) (PVA), a polyol polymer, have been reported by Duel *et al.* more than half a century ago.[24] Hydrogel crosslinking is achieved by the esterification between the borate ion in borax and the diol groups in PVA.[25] Gelation of other hydroxyl polymers with borax includes poly(glyceryl methacrylate)[26] and a few polysaccharides.[27,28,29] These latter gels are not suitable for biomedical applications since gelation is only achieved at a pH above the pK_a of the boric acid (pK_a = 9), outside the range of physiological pH. However, it has been shown that the crosslinking efficiency can be improved by the use of polymers containing functional groups containing phenyl boronic acid (BA), as the binding of BA to 1,3 diols in PVA is entropically favoured over borax crosslinking.[30,31] The phenyl substituent results in an enhancement of electron deficiency of the boron centre and reduces the pK_a of the boronic acid. Thus, binding of BA to 1,3 diols in PVA via boronic ester formation can happen at relevant physiological pH. A single chain of boronic acid containing polymer can interact with multiple chains of PVA to effectively crosslink through reversible ester formation in aqueous medium.

A novel glucose-responsive drug delivery system based on a polymer gel formed by crosslinking PBA-functionalized poly(N-vinylpyrolidone), p(NVP-co-PBA), and PVA was reported by Kitano *et al.*[32] The hydrogel was developed as a glucose sensitive sensor. It was found that reversible exchange of boronate-diol crosslinks occurs in the presence of glucose, which results in a weakening of the gel and dissociation at high glucose concentrations. This approach provides a potential route for effective gel disruption under near-physiological conditions. It was also found that increasing the PVA molecular weight as well as increasing the polymer concentrations increased the viscosity of the system, and these parameters could be easily adjusted to control the rheological properties of the hydrogel system.

5.2 Gel formation of SMI-BA and PVA

SMI-25BA (32 mg, 3.95 x 10^{-5} mol MI, M_n ~ 15 000) was dissolved in 500 µL phosphate buffered saline (PBS) and PVA (7.6 mg, 17.3 x 10^{-5} mol OH, M_n ~ 130 000,~ 99% hydrolysed) was dissolved in 500 µL PBS, respectively. The SMI-BA solution was added to the PVA solution. A single chain of boronic acid containing polymer can interact with multiple chains of PVA to effectively crosslink. After mixing the viscosity gradually increased until gel formation occurred a few minutes later. Preliminary gels with various composition ranges of the SMI-BA and PVA for making the hydrogel was investigated in order to determine if gelation takes place for a wide variety of concentrations. The results are summarised in Table 5.1

Table 5.1 Preliminary hydrogel formulations

PVA (wt%)	SMI-15BA (wt%)			SMI-25BA (wt%)		
	6.0	**2.5**	**1.5**	**6.0**	**2.5**	**1.5**
2.5	√	√	√	√	√	√
2.0	√	√	√	√	√	√
1.5	√	√	**O**	√	√	**O**
0.6	√	**O**	×	√	**O**	**O**
0.3	**O**	×	×	**O**	**O**	×

√ Firm gel formation **O** Loose gel formation × No gel formation

A variety of firm, loose and no gels were obtained from the above formulations. Gelation occurred for the majority of the formulations. However, SMI-BA gels experienced ineffective gelation with 0.3 wt% and 0.6 wt% PVA formulations. This is primarily due to the reduced molar ratios of PVA to the SMI-BA gels compared to the other formulations with increased wt% PVA. In some instances, the addition of an increased molar ratio of SMI-BA (6 wt%) to increase the crosslinks between the polymers was just enough to notice a slight increase in viscosity, and obtain a loose gel.

The 6 wt% SMA and PVA (1.5 wt% - 2.5 wt%) composition ranges were chosen for further analysis since they consisted of highest concentrations functionalised polymer and managed to form firm gels with relatively quick gelation times (Table 5.1). Gelation times were determined with the tilt test, by mixing the two aqueous polymer solutions in a glass vial and continuously tilting the vial until the gel appears to have formed Figure 5.1 shows the formation of the gel.

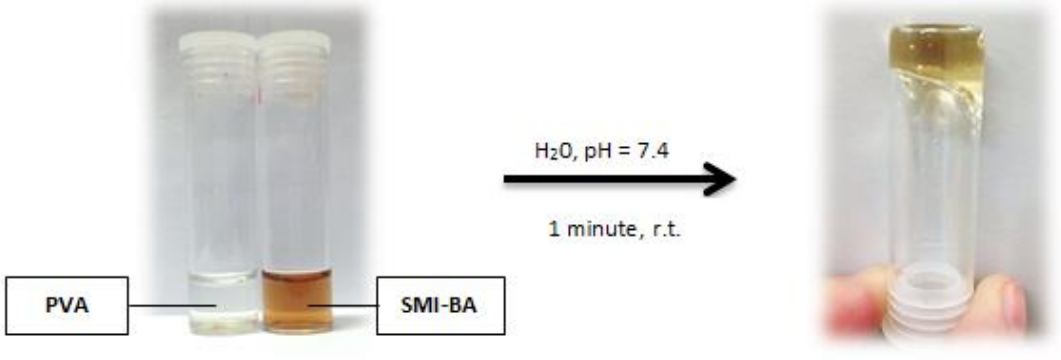

Figure 5.1 Formation of SMI-BA-PVA gel by mixing solution PVA with SMI-BA at physiological conditions

Table 5.2 Gelation times of the various formulations

PVA (wt %)	SMI-BA (6 wt %)	
	SMI-15BA	SMI-25BA
2.5	101 sec	38 sec
2.0	152 sec	60 sec
1.5	270 sec	90 sec

The gelation times of SMI-15BA gels were more than 2 times longer than SMI-25BA gels with the same PVA content. The decrease in gelling times of SMI-25BA are as a result of increased crosslink density caused by the additional reactive BA groups along the polymer chain, leading to tighter network formation in a reduced amount of time. A general trend observed for both functionalised polymers was a decrease in gelling time with increased PVA composition (Figure 5.2). This is primarily due to the introduction of more available diol functional groups in the mixture to crosslink, network formation occurs more readily and viscosity increases more rapidly.

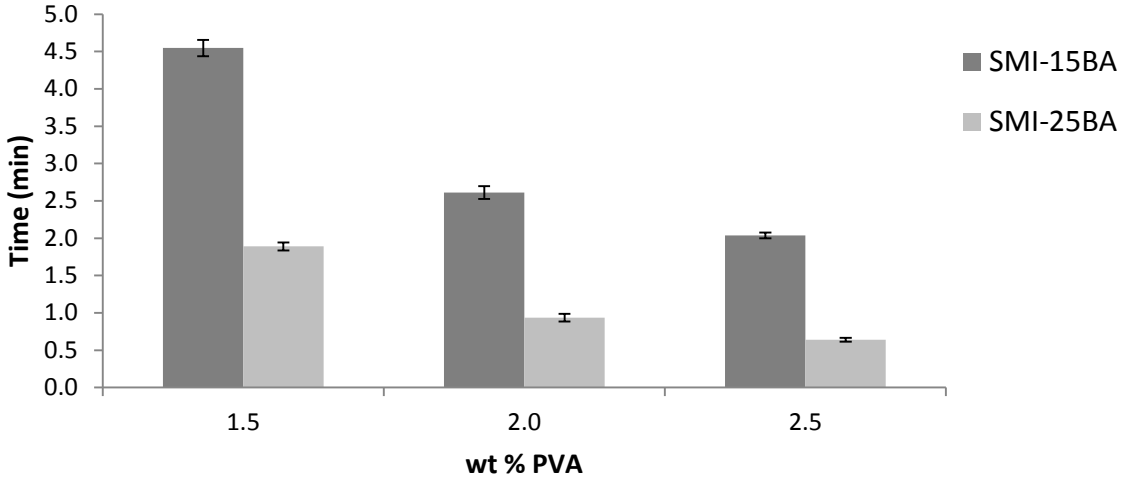

Figure 5.2 Gelation times obtained through tilt test (n = 3, 37 °C, *pH = 7.4)*

It has been shown in literature that an increase the PVA molecular weight and/or polymer concentration results in a viscosity increase of the solution, affecting both gelation times and rheological properties of the gels.[32] Furthermore, the influence of pH on the gelation and mechanical properties of the boronate ester crosslinked gels was investigated by Chantasirichot *et al*.[33] It was found that in an alkaline environment, the phenyl boronic acid groups can readily switch from an uncharged state to an ionic charged state, leading to increased activation towards the hydroxyl groups. The effect of this is faster onset of gelling, higher crosslink densities and harder gels. It was shown that the gel time can significantly be reduced from 35 minutes to 15 minutes by an increase in pH = 7.4 to pH = 9.4. Gelation varied from a couple of seconds to minutes. However, for the purpose of our gels it was only relevant to investigate the behaviour of the gels under physiological conditions.

5.3 Swelling studies

Swelling studies involve the detection of mass increase of a gel as a function of time caused by liquid absorption by the polymeric network submerged in a particular fluid. The physical and mechanical properties of gels are also dependant on the percentage fluid absorbed by the gel. As such, knowledge about the swelling properties of gels is important to determine the potential application of the gel. The degree of swelling depends on many factors such as the nature of the polymer and solvent, polymer-solvent compatibility and degree of crosslinking. A gel will rapidly absorb liquid and continue to absorb until equilibrium is reached.[34] An exponential trend is expected, which will level off once equilibrium has been achieved. This constant swelling % at equilibrium is called the equilibrium water content (EWC %).

The percentage swelling was calculated as follow:

$$\% Sw = \frac{M_t - M_0}{M_0} \times 100$$

M_t – Mass of the swollen gel at time t

M_o – Mass of the gel at time 0

The water uptake of initially dry hydrogels in phosphate buffered saline (PBS, pH = 7.4) was followed gravimetrically by removal of the gel from liquid, dabbing it dry on filter paper and subsequently weighing it at certain time intervals. A graph of % swelling with time was constructed by taking the average measurements of 3 samples.

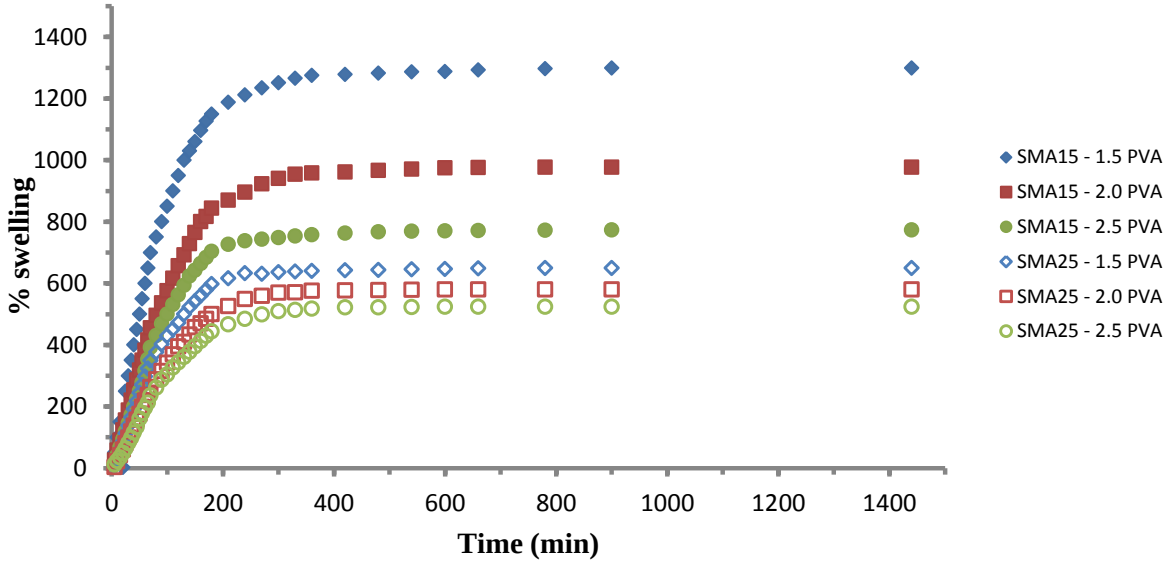

Figure 5.3 Swelling studies of various SMI-BA and PVA compositions

From the graph in Figure 5.3 it is evident that all the gels absorb water rapidly during an initial period of 200 minutes, and reach equilibrium within 300 - 400 minutes of immersion. The water absorbed by the hydrated network is quantitatively represented by the equilibrium water content (EWC), the ratio of weight of water in the hydrogel to the weight of the hydrogel at equilibrium hydration, expressed as a percentage. See Table 5.3 for swelling conditions.

The EWC was calculated as follow:

$$\% EWC = \frac{M_s - M_0}{M_s} \times 100$$

M_s – Mass of the swollen gel at equilibrium

M_o – Mass of the gel at time 0

Table 5.3 Summary of selected hydrogel swelling conditions

Gel	mol BA	mol OH	Solid in solvent (wt %)	Volume (µL)	Dry weight (g)	Equilibrium weight (g)	% EWC
SMI-15BA-1.5 PVA	2.38 x 10^{-5}	17.3 x 10^{-5}	3.75	1000	0.0501	0.7011	93
SMI-15BA-2.0 PVA	2.38 x 10^{-5}	23.2 x 10^{-5}	4.00	1000	0.0567	0.6105	91
SMI-15BA-2.5 PVA	2.38 x 10^{-5}	29.0 x 10^{-5}	4.25	1000	0.0575	0.5020	89
SMI-25BA-1.5 PVA	3.95 x 10^{-5}	17.3 x 10^{-5}	3.75	1000	0.0561	0.4201	87
SMI-25BA-2.0 PVA	3.95 x 10^{-5}	23.2 x 10^{-5}	4.00	1000	0.0590	0.4015	85
SMI-25BA-2.5 PVA	3.95 x 10^{-5}	29.0 x 10^{-5}	4.25	1000	0.0610	0.3805	84

The absorption of water was found to depend on the degree of crosslinking of the gels. The swelling ratios of the gels decrease with increasing boronic acid modification along the SMA polymer backbone, as much tighter networks are able to form for SMI-25BA polymers. In addition the unreacted ring-opened maleic anhydride groups on the polymer chain increase the hydrophilic nature of the gel, resulting in increased water uptake. As the concentration of PVA increased, there is a decrease in the swelling ratio. This is due to the higher viscosity during gelation, leading to smaller pores. Furthermore, chain flexibility decreases, resulting in reduced swelling capacity of the polymer.[35] The values of % EWC of obtained hydrogels (84% - 93%) are greater than the percentage water content of living tissues in the human body (ca 60%).[36,37] As such, SMA-BA hydrogels, crosslinked with PVA are sufficient for biological use. Once the injected gels have swollen they will conform to the shape of the vas deferens, anchoring itself to the longitudinal folds of the of the inner vas deferens wall.

5.4 Rheological characterisation

5.4.1 Gelation time

The storage (G') and loss moduli (G") of SMI-15BA-1.5 PVA (as example) measured during an oscillatory time sweep test are shown in Figure 5.4 over time (min). The incidence of a gel point in rheological curves was defined according to the Chambon and Winter criterion by the crossover of the storage modulus (G') and the loss modulus (G'') congruent over a range of frequencies to a constant phase angle of 45°.[38,39,40] The gel point, occurred at 4.33 ± 0.11 min. The gel point of SMI-15BA-2.0 PVA was determined as 2.36 ± 0.07 min. *In situ* gelation could not be performed on the other gel formulations due to the rapid gelling time.

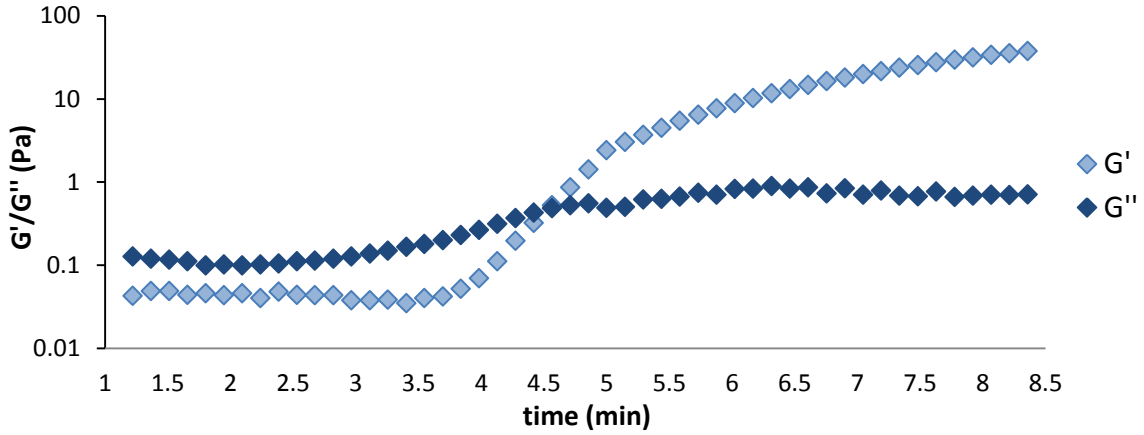

Figure 5.4 Plot of in situ gelation of SMI-15BA–1.5 PVA during oscillatory time sweep (1 Hz, 2 % strain, 37 °C)

5.4.2 Viscoelasticity

When subject to oscillatory stress sweep (Figure 5.5), the storage moduli (G') were constant until the ultimate stress level was reached as evidenced by a sharp decrease in G'. This was done to determine the ranges in which G' of swollen gels are constant when subjected to stress, which are regarded as the linear viscoelastic region (LVR) of the gels. While sample structure is maintained, G' is constant; when the stress is too high, breakdown occurs and G' decrease.[41,42] Once the LVR is determined, a frequency sweep can be used to determine the nature of the material.

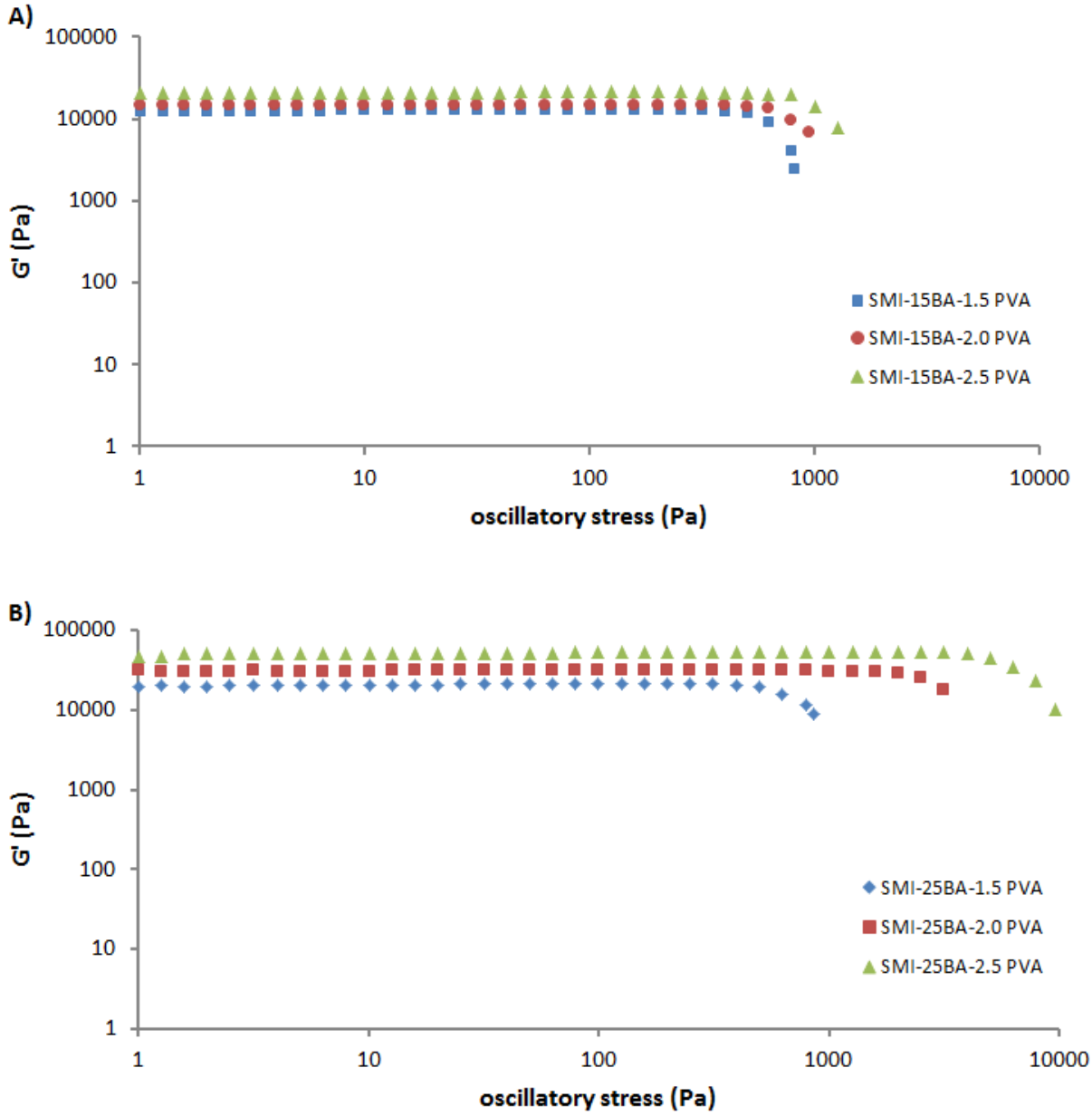

Figure 5.5 Linear viscoelastic region determination of A) SMI-15BA and B) SMI-25BA with different wt % PVA, obtained through oscillatory stress sweep (1 Hz, 37 °C)

Constant G' values ranged for various gel formulations. SMI-15BA gels ranged from (12.5 ± 0.5) x 10^3 to (20.5 ± 0.7) x 10^3 Pa with ultimate stress levels from (0.5 ± 0.04) x 10^3 Pa to (0.8 ± 0.08) x 10^3 Pa. SMI-25BA gels ranged from (20.5 ± 0.8) x 10^3 Pa to (50.5 ± 1.0) x 10^3 Pa with ultimate stress levels from (0.5 ± 0.04) x 10^3 Pa to (4.0 ± 0.1) x 10^3 Pa.

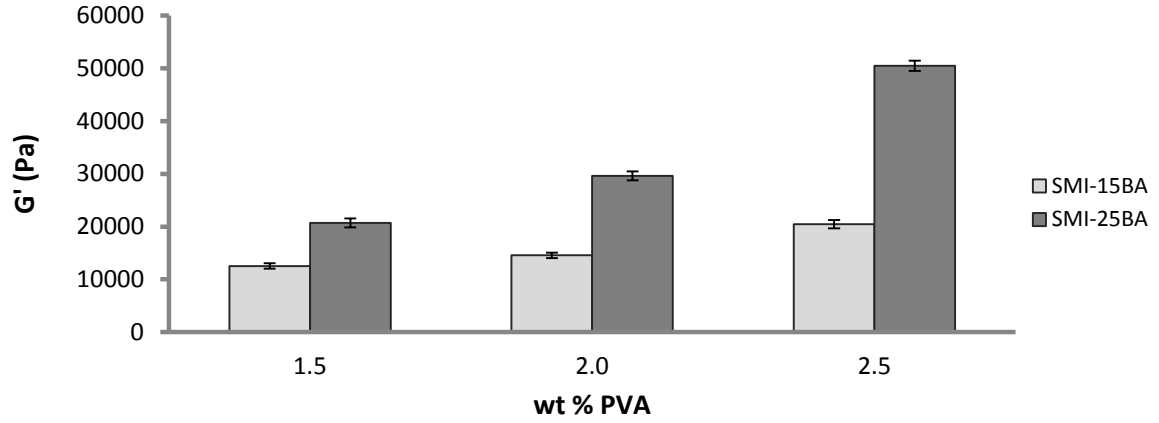

Figure 5.6 Storage moduli of gels obtained through oscillatory stress sweep (n = 4)

Increased storage moduli of type SMI-25BA gels can be ascribed to the increased crosslink density and polymer chain entanglement, thereby enhancing the gel stiffness. Priest *et al.* reported rheology measurements on PVA and phenyl boronic terminated PPO-PEO-PPO spacers, and showed that the introduction of more crosslinking sites can rapidly increase G' at constant pH.[43]

Frequency sweep was performed on gels with constant shear stress to determine the mechanical stiffness of the gels. The G' of SMI-15BA (as example), subjected to frequency sweep at a constant shear stress (250 Pa), exhibits a plateau in the range of 1 – 80 Hz (Figure 5.7). This is followed by a slight increase at higher frequencies (80 - 100 Hz). The loss modulus (G'') is one or two magnitudes lower than G' over all measured frequencies and characteristic for all equilibrium swollen gels of different gel formulations.

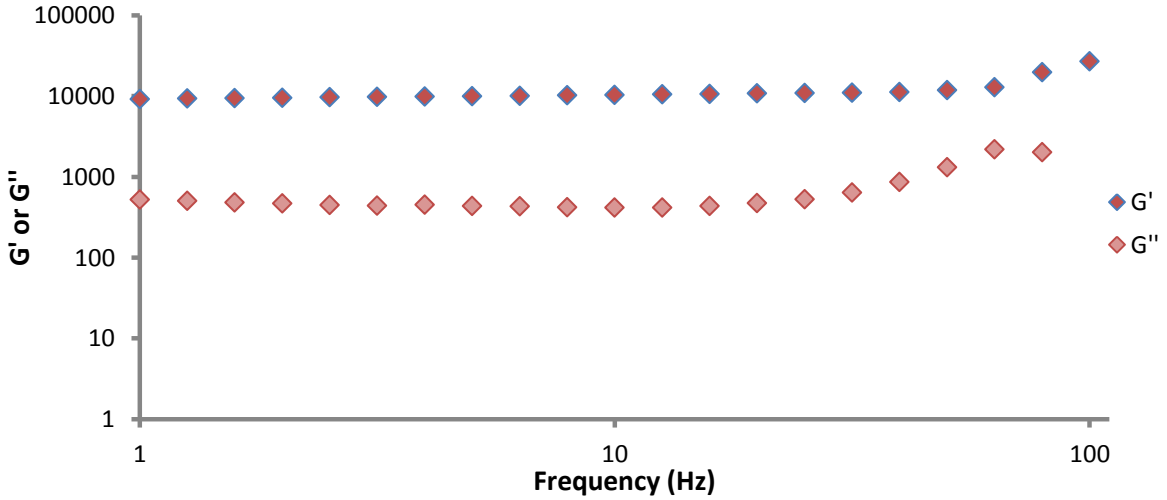

Figure 5.7 Characteristic plot of all equilibrium swollen gels during oscillatory frequency sweep (37 °C)

G′ (the elastic component) and G″ (the viscous component) are independent of frequency in the low frequency range (1 – 80 Hz) studied. This finding confirms that the hydrogels are fully crosslinked and swollen at the time of the measurements.[44] Furthermore, the value of G′ is one to two orders of magnitude larger than the value of G″ indicating that the hydrogels remains intact and elastic during the entire experiment.[45] The slight increase in G' at higher frequency (above 80 Hz) can be ascribed to gel stiffening. [41,44]

5.5 Reversibility

It is also readily known in literature that glucose is able to diffuse through the polymer gel and exchange with the boronate-diol crosslink, resulting in dissociation of the gel.[32] This might hamper the use of the hydrogel as a potential male contraceptive by diabetic men as they may reach higher glucose concentrations in their seminal fluid. It was however noted that glucose sensitivity towards phenyl boronic acid was only observed at high pH values.[46,47] To investigate this further, immersion of the hydrogels with lowest crosslinking densities, SMI-15BA-1.5PVA and SMI-15BA-2.0PVA in glucose mixtures of pH = 7.4 and pH = 9 was carried out. These gels were specifically chosen for the study since they are more likely to dissociate than higher crosslinked gels.

Normal seminal glucose levels in healthy men is estimated to be between 1.02 mmol/L – 5.70 mmol/L *i.e.* 18 mg/dL – 102 mg/dL.[48] The gels were immersed in concentrations within and outside the normal range glucose range at two different pH levels and data is summarised in Table 5.4.

Table 5.4 Reversibility studies of gels in glucose

Glucose level	Glucose concentrations	SMI15BA-15PVA		SMI15BA-20PVA	
		pH = 7.4	pH = 9.0	pH = 7.4	pH = 9.0
Normal	50 mg/dL	Ok	Ok	Ok	Ok
High	100 mg/dL	Ok	Dissociate	Ok	Dissociate
Abnormally high	1000 mg/dL	Swell	Dissociate	Swell	Dissociate

The immersion of gels in normal and high glucose concentration solutions at both pH values resulted in no dissociation. It was however observed that swelling of the gels occurred at glucose concentrations of 1000 mg/dL at pH = 7.4. This swelling behaviour as a result of glucose addition to boronate-diol crosslinks have been extensively reported in literature.[49,50] Higher glucose sensitivity at pH = 9 is observed by dissociation of the gels in a matter of

minutes at the concentrations of 100 mg/dL or higher. An increase in glucose concentration to 1000 mg/dL resulted in faster dissociation of the gels.

At physiological pH, glucose fails to respond to the boronate ester crosslinked gels. This potentially means that diabetic men could potentially use the hydrogel for contraception purposes since no dissociation of the gel will occur under physiological pH conditions. As such, reversibility of the gel will have to rely on injection of a sugar solution at pH = 9 or higher. Injection of a $NaHCO_3$ solution can also be incorporated to achieve reversibility by dissolution of the polymer.

5.6 Conclusion

In this study, a range of SMI-BA polymers were effectively crosslinked with poly(vinyl alcohol) (PVA) in phosphate buffered saline, resulting in reversible gels. Gels formed spontaneously at physiological conditions (37 ºC, pH = 7.4) by the boronate ester crosslinks, without the need for additional chemical reagents that might leave undesirable byproducts.

The gels were immersed in buffered aqueous solution to investigate the ability of the gels to absorb fluid. The swelling results delivered promising results for biological application. The biocompatibility was further enhanced by the ability of the polymeric network to swell in water without the need of harmful solvents.

Gel properties (gelation times and elastic moduli) were readily influenced by the crosslinking densities. For the SMI-BA-PVA hydrogels, the cross-link density predominantly depends on two factors: (1) the fraction of BA groups in the gel precursor *i.e.* extent of modification of SMI-BA and (2) the concentration of PVA added to the solution. Alteration of physical and mechanical properties can easily be accomplished by the variation of these two parameters, in an attempt to obtain the desired properties. Reversibility of the gels could not be achieved by the addition of high concentrations of glucose at pH = 7.4. However, easy and fast reversibility is achieved by addition of glucose in the presence of $NaHCO_3$ solution.

5.7 Experimental

5.7.1 General experimental details

An AR-1500 Rheometer (TA Instruments Inc.) with 20 mm diameter standard steel parallel-plate geometry was used for the characterization of all samples. Oscillatory time, stress and frequency sweep tests were employed.

Time sweep monitored the *in situ* formation of gels (100 µL, n = 4) over 10 min (50 µm gap thickness) at constant strain (2%), temperature (37 °C) and frequency (1 Hz). Storage (G′) and loss moduli (G″) were recorded over time.

Gels (1000 µL, n = 4, 22 mm diameter, 2.0 mm thick) used in stress and frequency sweep tests were formed for 1 hour at 37 °C. The formed gels were individually immersed in PBS to swell to equilibrium (37 °C, overnight). To prevent slippage, the plate height was adjusted, starting from the original sample height and compressing the sample to reach a normal force of 30% compression.

Stress sweep (G' as a function of oscillatory stress) was performed by shearing the gels (1-10,000 Pa) at constant frequency (1 Hz) and temperature (37 °C) until structure breakdown.

Frequency sweep (G' and G'' as a function of oscillatory frequency) was performed at a constant shear stress (50% of the LVR) by varying frequency from 1 to 100 Hz, to determine the mechanical stiffness of the gels.

5.8 References

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Chapter 6: Epilogue

6.1 General conclusions

This thesis focussed on the design of an improved polymeric hydrogel system for the potential use as a reversible male contraceptive in the vas deferens of the male. SMA has previously shown to be a potential polymer for this application, due to its attractive chemical and biological properties, easy availability and low cost. The proposed outcome was to synthesise an improved biocompatible hydrogel system with enhanced reversibility and optimised mechanical properties in an attempt to overcome the shortcomings of the current injectable SMA hydrogel systems. The main findings of this study are summarised in the current chapter and recommendations for future work are discussed.

In Chapter 3, RAFT mediated polymerization was utilized for its ability to synthesize SMA copolymers of well-defined molar mass and low dispersity. In addition, two different post-polymerisation modification approaches of SMA were investigated to achieve hydrogel formation. The first approach involved the successful chemical modification of the anhydride residues of the polymer using 3-aminophenylboronic acid (BA). All boronic acid functionalised polymers (SMI-BA) were successfully characterised by ATR-FTIR spectroscopy and the degree of modification was quantified using UV-vis spectroscopy. In the second approach it was found that the reduction of the thiocarbonyl thio end-groups of SMA resulted in side-reactions that prevented the intended disulphide formation.

Chapter 4 presented a model study into the dimerisation of polymers structurally similar to SMA to gain insight into the unsuccessful disulphide formation of SMA in Chapter 3. It was shown that PVP and PS readily couple to form disulphides, while the PMA, PMMA and SMA polymer cyclise to a thiolactone end group formed via the backbiting reaction promoted by the stability of the 5-membered ring. The thiolactone ring formation in these polymers was confirmed by ^{13}C NMR spectroscopy. It was demonstrated that disulphide formation between two polymeric chains is predominantly dependant on the nature of the penultimate and terminal monomer units next to thiocarbonyl thio group of various RAFT-made polymers. As such, this side reaction was overcome by the chain extension of the SMA macro-RAFT agent with four styrene units to prevent cyclisation and promote disulphide formation of the

polymer. The potential use of these systems as hydrogels was not investigated further due to time constraints.

Chapter 5 describes the formation and characterisation of SMI-BA-PVA hydrogels. A range of SMI-BA polymers were effectively crosslinked with poly(vinyl alcohol) (PVA) in phosphate buffered saline, resulting in reversible gels. Gels formed spontaneously at physiological conditions (37 °C, pH = 7.4) by the boronate ester crosslink formation. The swelling behaviour of the gels, immersed in buffered aqueous solution delivered promising results for biological application. Enhanced biocompatibility of the gels is achieved by the use of aqueous solutions as opposed to DMSO as a solvent in Vasalgel. The structural properties of the gels were characterized as a function of composition and degree of modification (SMI-BA) by oscillatory rheometry. For the SMI-BA-PVA hydrogels, the crosslink density predominantly depends on two factors: (1) the fraction of BA groups in the gel precursor *i.e.* extent of modification of SMI-BA and (2) the concentration of PVA added to the solution. It was found that the mechanical properties of the gels can easily be tuned by the variation of these two parameters, in an attempt to obtain gels with desired gelation times and elasticity. Adequate gelation times of approx. 1-5 minutes (37 °C, PBS, pH = 7.4), with storage moduli (G') 12 kPa – 50 kPa, were obtained. Rapid reversibility of the gels is achieved by the addition of glucose in combination with sodium carbonate ($NaHCO_3$), to overcome the reversibility issues Vasalgel currently faces.

6.2 Future recommendations

These encouraging results suggest that the injectable hydrogels developed in this project have considerable potential as male contraceptive. However, further development of the system needs to be considered.

Further research in terms of *in vitro* studies is required for the SMI-BA-PVA hydrogels developed so far in this study. This should be done in order to evaluate the efficacy of the hydrogel system as a contraceptive in the presence of spermatozoa. In addition, the motility and vitality of the sperm needs to be assessed. Furthermore, the stability of the hydrogel needs to be evaluated in order to establish if such a system will be able to achieve contraception over long periods of time (10 years) before any advancement towards *in vivo* studies can be carried out.

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