

Development and Investigation of Polymer Brush-Based Prodrugs and Micropatterned Lipid Bilayer Membranes

DISSERTATION

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M.Sc. Joshua Schumacher

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Betreuer und erster Gutachter

Prof. Dr. Holger Schönherr

Universität Siegen

Zweite Gutachterin

Prof.in Dr. Manuela S. Killian

Universität Siegen

Tag der mündlichen Prüfung

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***„Ob mir durch Geistes Kraft und Mund,
Nicht manch Geheimnis würde kund;
Daß ich nicht mehr mit saurem Schweiß
Zu sagen brauche, was ich nicht weiß;
Daß ich erkenne, was die Welt
Im Innersten zusammenhält“***

Faust – Faust. Der Tragödie erster Teil

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List of Abbreviations and Acronyms

A	absorbance
a	monomer size according to the mesh model suggested by de Gennes
ACVA	4,4'-azobis(4-cyanovaleric acid)
AFM	atomic force microscopy
APTES	(3-aminopropyl)triethoxysilane
ATR	attenuated total reflection
ATRP	atom transfer radical polymerization
BHT	2,6-di- <i>tert</i> -butyl-4-methylphenol
c	concentration
CAP	chloramphenicol
CFU	colony-forming unit
CHNS	combustion analysis
CPS	counts per second
CTA	chain-transfer agent
D	distance between grafted polymer chains
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	dichloromethane
DEGMA	di(ethylene glycol) methyl ether methacrylate
DMAP	4-dimethylaminopyridine
DMSO	dimethyl sulfoxide
DS	diclofenac sodium
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ESI-MS	electrospray ionization mass spectrometry
eV	electron volt
FAO/WHO	Food and Agriculture Organization of the United Nations/World Health Organization
FDA	U.S. Food and Drug Administration
GC	gas chromatography
GMA	glycidyl methacrylate
GSH	glutathione
H	layer thickness
HMF	hydroxymehtylfurfural
HPLC	high-performance liquid chromatography
JECFA	Joint Expert Committee on Food Additives
I	optical path length
LB/LS	Langmuir-Blodgett/Langmuir-Schaefer
LCST	lower critical solution temperature
LRF	log reduction factor
M	monomer
m%	mass percent
MIC	minimum inhibitory concentration
Mn	number average molar mass
N	number of bond segments

NMR	nuclear magnetic resonance
OEGMA	oligo(ethylene glycol) methyl ether methacrylate
PAAm	polyacrylamide
PBS	phosphate-buffered saline
PDEGMA	polydi(ethylene glycol) methyl ether methacrylate
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
pHBA	<i>p</i> -hydroxybenzaldehyde
PJI	periprosthetic joint infections
Pm	polymer chain
POEGMA	poly(oligo(ethylene glycol) methyl ether methacrylate
PVP	polyvinylpyrrolidone
RAFT	reversible addition-fragmentation chain transfer
RAIRS	reflection-absorption FTIR
R _g	radius of gyration
RGD	arginylglycylaspartic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SI-(ARGET-)ATRP	surface-initiated (activator regenerated by electron transfer) atom transfer radical polymerization
SI-NMP	surface-initiated nitroxide-mediated polymerization
SI-PIMP	surface-initiated photoiniferter-mediated polymerization
SI-RAFT	surface-initiated reversible addition-fragmentation chain transfer
SMX	sulfamethoxazole
SWCA	static water contact angle
TCPS	tissue culture polystyrene
TEA	triethylamine
TMP	trimethoprim
XPS	X-ray photoelectron spectroscopy
γ_l	air-liquid interfacial energy
γ_s	solid-air interfacial energy
γ_{sl}	solid-liquid interfacial energy
ϵ	extinction coefficient
θ	contact angle
ν	Flory exponent
ρ	bulk polymer density
σ	grafting density

Abstract

Advanced implant coatings that can prevent bacterial attachment to avoid subsequent biofilm formation while simultaneously enhancing host cell adhesion to promote successful tissue integration are urgently required to minimize implant-associated infections. Materials designed to modulate this competitive colonization of the implant surface, known as the “race for the surface”, in favor of tissue integration must satisfy stringent biomedical criteria, such as biocompatibility, long-term stability and antibacterial efficacy. This Thesis seeks to advance the understanding of polymer brushes as biomedical coatings to address both pathways of this “race for the surface”. First, the feasibility of creating prodrugs from polymer brushes is explored, giving rise to a novel class of materials referred to as “polymer brush prodrugs”. Two monomeric prodrug systems were developed based on the antibiotics chloramphenicol and sulfanilamide. The first system relied on ester bond hydrolysis for drug release and resulted in minimal chloramphenicol release even at extreme pH. The second system featured an imine linkage and released sulfanilamide under physiological conditions at concentrations sufficient to eradicate three strains of planktonic *Staphylococcus aureus*.

The second part of this Thesis establishes the foundation for novel cell culture platforms based on hybrid systems composed of polymer brush–modified supported lipid membranes. By translating the knowledge of surface-initiated brush synthesis via reversible addition–fragmentation chain transfer (RAFT) polymerization into a novel UV-induced approach, polymer brushes were synthesized onto patterned lipid bilayers. The investigation of cellular responses to these surfaces demonstrated that the antifouling properties of the modified membranes could be tuned by varying the length of the ethylene glycol side chains, thereby enabling or preventing cellular attachment. Furthermore, chain extension of the polymer brushes with a second block of glycidyl methacrylate, which contains a functionalizable epoxide ring, was performed, and could provide the opportunity to introduce specific biochemical motifs for systematic studies of cellular responses. Ultimately, this platform could enable detailed investigations of cell behavior at polymer brush interfaces, improving the understanding of how to encourage host cell proliferation on polymer brush-coated implants.

Zusammenfassung

Verbesserte Implantatbeschichtungen werden dringend benötigt, um die Besiedlung durch pathogene Bakterien zu verhindern und damit die Bildung infektionsverursachender Biofilme zu unterbinden. Gleichzeitig sollte die Adhäsion von Wirtszellen gefördert werden, um eine erfolgreiche Integration des Implantats in das umliegende Gewebe zu ermöglichen. Materialien, die diese konkurrierende Besiedlung der Implantatoberfläche – bekannt als „*race for the surface*“ – zugunsten der Gewebeintegration steuern, müssen dabei hohe biomedizinische Anforderungen erfüllen, darunter Biokompatibilität, Langzeitstabilität und antibakterielle Wirksamkeit.

Ziel dieser Arbeit ist es, das Verständnis von Polymerbürsten als biomedizinische Beschichtungen zu vertiefen, indem beide Aspekte des „*race for the surface*“ systematisch untersucht werden. Im ersten Teil wird die Kombination von Polymerbürsten mit Polymer-Prodrugs betrachtet, wodurch eine neue Materialklasse entsteht, die als „Polymerbürsten-Prodrugs“ bezeichnet wird. Hierfür wurden zwei monomere Prodrugs auf Basis der Antibiotika Chloramphenicol und Sulfanilamid synthetisiert. Diese enthalten entweder eine Ester- oder eine Iminbindung als hydrolysierbare Verknüpfung und wurden anschließend zur Herstellung entsprechender Polymerbürsten verwendet. Die resultierenden Materialien wurden umfassend charakterisiert und hinsichtlich ihrer Fähigkeit untersucht, unter physiologischen Bedingungen durch hydrolytische Spaltung der Bindungen das jeweilige Antibiotikum freizusetzen. Eine verlängerte Wirkstofffreisetzung konnte ausschließlich für das hydrolytisch labile Imin-System beobachtet werden. Dabei wurden Wirkstoffkonzentrationen erreicht, die ausreichten, um drei planktonische Stämme von *Staphylococcus aureus* zu inaktivieren.

Der zweite Teil der Arbeit legt die Grundlage für neuartige Zellkulturplattformen auf Basis hybrider Systeme, die aus mit Polymerbürsten modifizierten Lipidmembranen bestehen. Durch die Übertragung der oberflächeninitiierten Bürstensynthese mittels reversibler Additions-Fragmentierungs-Kettenübertragungspolymerisation (RAFT) auf einen neu entwickelten UV-induzierten Ansatz konnten Polymerbürsten für die Untersuchung der Zelladhäsion auf diesen Oberflächen hergestellt werden. Es zeigte sich, dass sich die Antifouling-Eigenschaften der modifizierten Membranen gezielt über die Länge der Ethylenglykol-Seitenketten einstellen lassen, wodurch Zelladhäsion entweder ermöglicht oder verhindert werden kann. Darüber hinaus eröffnet die

Kettenverlängerung der Polymerbürsten mit einem zweiten Block aus funktionalisierbarem Glycidylmethacrylat die Möglichkeit, spezifische biochemische Motive einzuführen und so zelluläre Reaktionen gezielt zu untersuchen. Insgesamt bietet die entwickelte Plattform neue Möglichkeiten, das Zellverhalten an Polymerbürsten-Grenzflächen detailliert zu analysieren.

1. Introduction and Motivation

Medical implants are routinely used for the surgical replacement of damaged or aged hips (>270,000 hip endoprosthesis replacements, 5th most common surgery in Germany in 2023) and knees (>225,000 knee endoprosthesis, 8th most common surgery in Germany in 2023).¹ Implant materials can be categorized depending on their chemical composition into metallic, ceramic and polymeric devices.² Among these, titanium and its alloys are frequently employed due to their corrosion resistance, mechanical strength and biocompatibility.^{2,3} A major limitation of implant materials, however, is their susceptibility to bacterial colonization. Planktonic bacteria, such as *Staphylococcus aureus* (*S. aureus*), can adhere to implant surfaces and subsequently form biofilms, leading to implant-related infections.⁴ These infections occur in up to 5% of cases, depending strongly on implant location, and often necessitate revision surgeries with associated costs exceeding USD 150,000.⁵

In addition to preventing bacterial adhesion, implant surfaces must also support bone formation around the implant, a process referred to as osseointegration.⁶ For successful integration of the implant into the body, macrophages must cover the implant surface before bacteria can adhere and proliferate.⁷ This competitive situation is termed the “race for the surface”.^{7,8} By encouraging host cell attachment over bacterial colonization, this race can be steered to facilitate successful implant incorporation.

A wide range of strategies have been explored to improve implant performance and reduce bacterial colonization through chemical modifications or structural surface changes.⁹ These include polymeric coatings designed to inhibit initial protein attachment to the titanium surface,^{10,11} release of antibiotics from polymeric coatings^{12,13} and surface-grafted antimicrobial peptides that exhibit anti-adhesive properties and prolonged contact-killing activity.^{14,15} Polymeric coatings have been developed containing enzymes e.g. DNase I which are capable of preventing adhesion of *S. aureus* with reported reductions of 95%,¹⁶ or with inorganic compounds based on transition metals which act as potent antibiotics, with copper and silver being renowned agents.^{17,18} Additionally, the surface morphology and wettability play important roles in battling biofilm formation.^{19,20}

Polymeric coatings are particularly attractive due to their chemical and morphological versatility and their capacity for drug loading.²¹ Hydrogels, in particular, have received significant attention owing to their potential biodegradability, biocompatibility and three-dimensional network structure which allows incorporation of therapeutics.^{22–24} Nevertheless, hydrogels have several disadvantages including difficulties with sample handling and sterilization, burst release of loaded drugs and insufficient mechanical stability at the target site.^{25–27} These limitations motivate the exploration of alternative, more precisely tunable polymeric systems.

An interesting alternative to hydrogels is polymer brushes, which are polymer chains densely end-grafted to a surface. Their conformation is governed by parameters such as grafting density (σ) and the radius of gyration (R_g).^{28,29} Polymer brush-modified surfaces exhibit highly diverse properties which depend on the intrinsic chemical properties of the polymer, film thickness, dispersity, synthesis method and the resulting morphology.³⁰ Consequently, polymer brushes have been investigated in diverse research fields, including lubrication and friction reduction,^{31–33} biomedical applications,^{34–37} sensing,^{38–40} catalysis^{41–43} and the generation of antifouling surfaces.^{44–46}

Antifouling polymer brush coatings are of particular interest for implant applications, as they can prevent bacterial adhesion and therefore inhibit biofilm formation.⁴⁷ Approaches that combine contact-killing motifs with integrin-recognizable peptide sequences to support cell adhesion have been reported.⁴⁷ While these systems show some antibacterial and cell-adhesive properties, they are inherently vulnerable to mechanical damage, as the functionality is lost if the coating gets damaged. In contrast, polymer brushes that act as drug reservoirs enable the continuous release of antibacterial agents, eradicating planktonic bacteria in the vicinity of the implant, especially in the early stage of implantation.¹³ However, existing systems often exhibit rapid drug release and relatively low drug-loading capacities.¹³

An ideal antibacterial coating would therefore combine an initial large release of antibiotic upon implantation, followed by a sustained release, maintaining drug concentrations within the therapeutic window.⁴⁸ Polymer brush prodrugs, in which an antibiotic is chemically linked to the polymer chain via a hydrolytically labile bond, could offer a promising strategy to achieve this goal, but have so far not been reported in the literature. Such a system would enable high drug-loading capacities, an initial burst

release of surface-accessible drug molecules and prolonged release of drug from deeper polymer layers, governed by sterically hindered diffusion through the polymeric film.

In addition to antibiotic properties, the interaction of host cells with the implant is crucial. While cellular responses to polymer brush-modified solid surfaces have been investigated previously, a more advanced model system is required to study the physicochemical reactions of cells to external stimuli at polymeric interfaces under well-defined conditions.^{49–51} Surface-supported lipid bilayers are excellent candidates for biomimetic model membranes to mimic and study cell surface functions. In particular, photopolymerizable diacetylene lipids can be patterned using photolithographic techniques to create membranes with well-defined, micrometer-sized corrals that can incorporate fluid lipid bilayers for the study of membrane-associated processes, such as the behavior of actin nucleation required for cellular migration.^{52–54}

These patterned membranes can be further modified by attaching elastomeric polydimethylsiloxane (PDMS) sheets via an adhesion layer, creating a nanometric volume defined by the corral size and the adhesion layer thickness.⁵⁵ This so-called nanogap-junction enables the spatial confinement and therefore highly sensitive detection of chemical or biological species.⁵⁵ Previous studies have demonstrated that amine-terminated diacetylene lipids can be chemically modified to enable polymer brush synthesis via atom transfer radical polymerization (ATRP).^{56,57} However, this approach relies on cytotoxic reagents and yields polymer brushes with limited thickness and compositional diversity.^{56,57} An ideal brush-membrane hybrid system would immobilize cells on the brush surface, spanning across a corral in the continuous polymeric bilayer and generating a nanometer-sized volume. Within this confined space, cellular responses to environmental changes, in the form of secreted chemical or biological species, could be detected with sensitivities comparable to those reported for nanogap-junction systems.⁵⁵ However, initial attempts by Hebel et al. revealed cells attached preferentially to the underlying substrate rather than the antifouling polymer brush-modified lipid membrane.⁵⁶

In this Thesis, the potential of polymer brushes as prodrug coatings on model implant surfaces was investigated. Two monomeric prodrugs were synthesized and characterized and a synthetic pathway for each monomer was developed for the

generation of antibiotic-loaded surface-grafted polymer brush films. Drug release properties were systematically investigated, demonstrating the feasibility of this previously unexplored concept.

First, a polymerizable chloramphenicol-based prodrug was synthesized and comprehensively characterized. This monomer was then used to synthesize polymer brushes via RAFT polymerization on titanium-coated glass substrates serving as model implant surfaces, yielding brush thicknesses of up to 45 nm. Drug release studies revealed no significant chloramphenicol release under the investigated conditions, attributed to the highly stable ester bond.

To address this limitation, a second monomeric prodrug based on a hydrolytically less stable imine bond was synthesized. Sulfanilamide was conjugated to *p*-hydroxybenzaldehyde to form an imine precursor, followed by esterification with acryloyl chloride to introduce the polymerizable moiety. Successful synthesis of the monomeric prodrug was confirmed. RAFT polymerization on titanium-coated glass substrates yielded polymer brushes with thicknesses up to 80 nm. Drug release profiles were investigated in aqueous solutions of different pH levels, with the release of sulfanilamide confirmed and quantified using chromatographic, spectroscopic and thickness-derived techniques. This first-reported bottom-up polymer brush prodrug system released sulfanilamide at concentrations able to kill planktonic *S. aureus*.

In parallel, polymerized and patterned lipid membranes were prepared by M.Sc. Yu Yoshimura under the supervision of Prof. Dr. Kenichi Morigaki (University of Kobe, Japan). These supported lipid membranes, composed of photopolymerizable diacetylene lipids, served as substrates for the RAFT-based synthesis of polymer brushes, an approach not yet reported in the literature. Phosphatidylethanolamine-containing supported lipid membranes were modified in a carbodiimide-mediated coupling of the chain transfer agent (CTA). Subsequent UV-induced RAFT polymerization enabled the growth of polymer brushes from di(ethylene glycol) methyl ether methacrylate (PDEGMA) or oligo(ethylene glycol) methyl ether methacrylate (POEGMA). Investigation of the adhesion behavior of NIH 3T3 mouse fibroblasts to the brush-modified lipid membranes exhibited a polymer-dependent adhesion behavior. Together, this Thesis demonstrates the potential of RAFT polymerization to yield antimicrobial implant surfaces and platforms for understanding cellular responses at polymer brush interfaces.

2. State of Knowledge

2.1 Biofilm Development on Medical Implants

By 2030, approximately 2 million joint arthroplasties will be performed annually in patients in the US, with the number of implant-related infections expected to increase with the number of performed surgeries.⁵⁸ Knee and hip replacements are among the most common joint arthroplasties with infections occurring in up to 2% of the cases.^{59,60} These periprosthetic joint infections (PJI) are particularly difficult to diagnose and treat and put a significant financial burden on healthcare systems as well as physical and mental burdens on the patient.⁶¹ PJI are difficult to prevent as these post-operative infections can occur in an acute (less than three weeks), early (within less than 4 weeks), or chronic fashion (up to several years).^{62,63} These different temporal stages of PJI are related to the ability of bacteria to form biofilms, in which the bacteria can remain in a dormant state and occasionally release bacteria, which can then cause infections.⁶⁴

Bacterial biofilm formation follows a typical pattern: bacterial adhesion to a surface is followed by accumulation of a microcolony, maturation of the biofilm and detachment of bacteria, which transform back into the planktonic phenotype, as can be seen in Figure 1.⁵ Adhesion of bacteria to a surface is a consequence of Brownian motion, leading the organism towards the implant and a subsequent transition from reversible to irreversible adhesion.^{65,66}

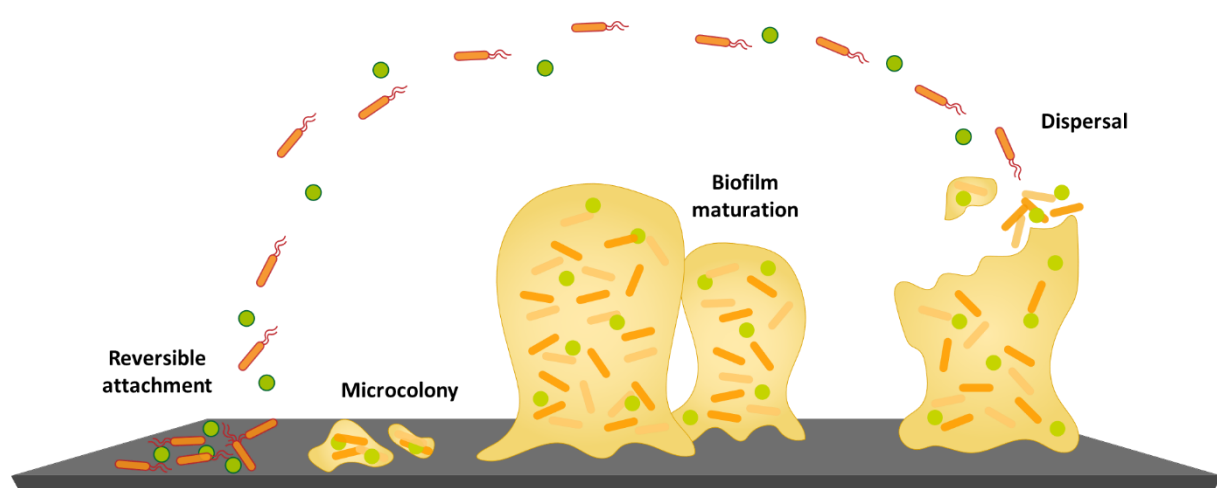


Figure 1 Stages of bacterial biofilm formation on a surface.

Proliferation or attachment of further planktonic bacteria leads to the accumulation of bacteria on the surface of the implant, accompanied by a change in their phenotype to

a sessile phase and production of the so-called extracellular matrix (ECM).^{65,67,68} This matrix, consisting of different organic molecules and biopolymers, including DNA, both protects the bacteria within it from host cell responses, typically requiring 1000-fold increases in antibiotic concentrations, but also allows further adhesion of planktonic bacteria.^{69–71} Channels in the biofilm allow the transportation of necessary nutrients and the removal of wastes.^{65,72}

Antibiotic resistance is a common feature of a mature biofilm.^{73,74} Within the biofilm, key parameters, including pH, oxygen and ion concentration, change, reducing cell metabolism and hence the susceptibility towards antibiotics.^{74,75} The expression of genes that play a role in antimicrobial resistance is also prioritized.^{76,77} A fraction of cells in the biofilm develop into so-called persister cells, which show even higher antibiotic resistance due to uptake of antibiotic-resistance genes, allowing potential repopulation of the biofilm.^{78,79} In the final stage of biofilm growth, the release of toxins decreases the interactions between bacteria in the biofilm, leading to a loss of surface tension and a detachment of bacterial cells, which undergo a transformation back into the planktonic type.^{80,81} These planktonic bacteria can cause further infections, where *Staphylococci* are the predominant implant-associated species alongside *Streptococcus* and *Enterococcus*.^{82–85} The pH values in the periphery of hip and knee implants upon infection were found to decrease from 7.8 in healthy patients down to values of 6.9 in infected patients, even decreasing to 4.4 in patients with septic loosening.^{86,87} This is attributed to the accumulation of metabolic byproducts such as lactic acid, formed from the fermentation of carbohydrates including glucose and sucrose.⁸⁸

A commonly applied treatment of developed biofilms involves exposing the implant to enable mechanical and chemical debridement and irrigation, resulting in the retention of the implant.⁵⁸ Nevertheless, this procedure is only applicable in acute and early-stage infections, for microbes with a low virulence and in suitable surgical candidates.⁸⁹ Failure rates for this treatment are reported in the range of 56 to 72%, highlighting the need for an implant treatment that prevents the initial bacterial attachment to the surface of the implant.⁹⁰

2.2 Implant Modification and Surface Coatings

The term “race for the surface” was coined by Gristina to highlight the competition between host and bacterial cells for colonization of an implant surface.⁸ To prevent biofilm development on medical implants, the suppression of bacteria adsorbing to the surface is critical. A manifold of topographical implant modifications is available, which exert force on the bacterial membrane, leading to cell rupture and death, commonly referred to as the mechano-bactericidal effect.⁹¹ Such nanostructured systems have been reported to show an increase in bone integration when compared to the current gold standard, hydroxyapatite-modified implants.⁹² The dimensions of these structures determine the wettability of the implant; micro- and nanostructures tend to incorporate air bubbles, which significantly increase the contact angle of these surfaces, minimizing bacterial attachment.^{93,94} However, mechano-bactericidal effects cannot completely inhibit bacterial adhesion as each cell type has different interaction processes, and topographical features are incapable of responding to changes in the environment.^{95,96}

A promising alternative for bacteria-repelling surfaces is polymeric coatings.^{97–99} Polymers provide a significant advantage due to their tunability and design opportunities to bind specific biochemical motifs to encourage host cell over bacterial cell attachment.¹⁰⁰ Polyethylene glycol (PEG) is one of the most commonly utilized polymer systems in biomedical applications due to its biocompatibility and antifouling properties.^{101,102} However, PEG-based coatings do not provide intrinsic bactericidal properties and can undergo oxidative degeneration.¹⁰³

The combination of polymeric coatings with antibacterial agents for a temporally controlled release is therefore of particular interest. Polymers with polar amide, ether or alcohol functionalities provide hydrophilic and uncharged surfaces, surrounded by highly hydrated water layers which can act as a physical barrier to bacteria, as described by Faustino et al. for use in catheter coatings.¹⁰⁴ Sophisticated polymer structures can combine antifouling properties to prevent bacterial adhesion with cell-adhesive peptide sequences to enable osteoblast adhesion, which is particularly interesting in load-bearing implants.¹⁰⁵ Three-dimensional, cross-linked networks of water-soluble polymers, so-called hydrogels, offer the advantages of mimicking the ECM and the ability to incorporate drugs.^{106,107} Biodegradable polymers and hydrogels loaded with antibacterial drugs, which are released in a controlled fashion, followed by

the dissolution of the coating, have been proven to reduce the infection rate tenfold.¹⁰⁸ Nevertheless, the low mechanical strength of hydrogels can lead to premature dissolution or dislocation from the target side, as reported by Hoare and Kohane.¹⁰⁹ Furthermore, Burdick et al. have reported on potential issues with the sterilization of such systems.²⁵ In general, drawbacks of polymeric systems include the release of residues from the synthetic procedure remaining in the coating or the release of oxidizing compounds used for the preparation of the surface.¹⁰⁴ Furthermore, prominent polymeric examples such as polyvinylpyrrolidone (PVP) or polyacrylamide (PAAm) are polymers approved by the U.S. Food and Drug Administration (FDA); nevertheless, they are disputed due to the carcinogenicity of their decomposition products.⁹⁷

2.3 Polymeric Prodrugs

The binding of therapeutic agents to polymers is an interesting approach in drug delivery. Utilizing hydrolytically labile chemical linkages enables triggered drug release from different polymeric architectures.¹¹⁰ Typically, therapeutics are either introduced into a pre-synthesized polymer with reactive side or end groups, or the polymeric prodrug is generated from a polymerizable monomeric prodrug.^{111,112} A manifold of drugs for different applications, such as anticancer (doxorubicin, epirubicin, camptothecin, docetaxel, paclitaxel),^{113–117} anti-inflammatory (methyl prednisolone, curcumin, indomethacin, ibuprofen),^{118–121} antiangiogenic and vasodilators (combretastatin A-4, dopamine) have been utilized.^{122,123} As each agent possesses a unique chemical structure, each polymer-drug system must be designed individually. The covalent bond between the drug and the polymer chain (or the chemical bond within the polymeric backbone) is commonly hydrolyzable, enabling release of the antibiotic cargo in the presence of water. Typically, esters,^{124–126} amides^{127,128} and carbamates^{129–131} are used for a stable binding, while imines and hydrazones undergo hydrolysis under milder conditions.^{132,133} Liu et al. utilized cyclic carbonates containing ester-bonded chlorambucil and ester-bonded, disulfide-bridged camptothecin to generate copolymers using ring-opening polymerization, as can be seen in Figure 2.¹³⁴ The release of the drug depended on the concentration of glutathione (GSH) which reduces the disulfide bond, with 15% camptothecin release in the absence and >70% in the presence of GSH.

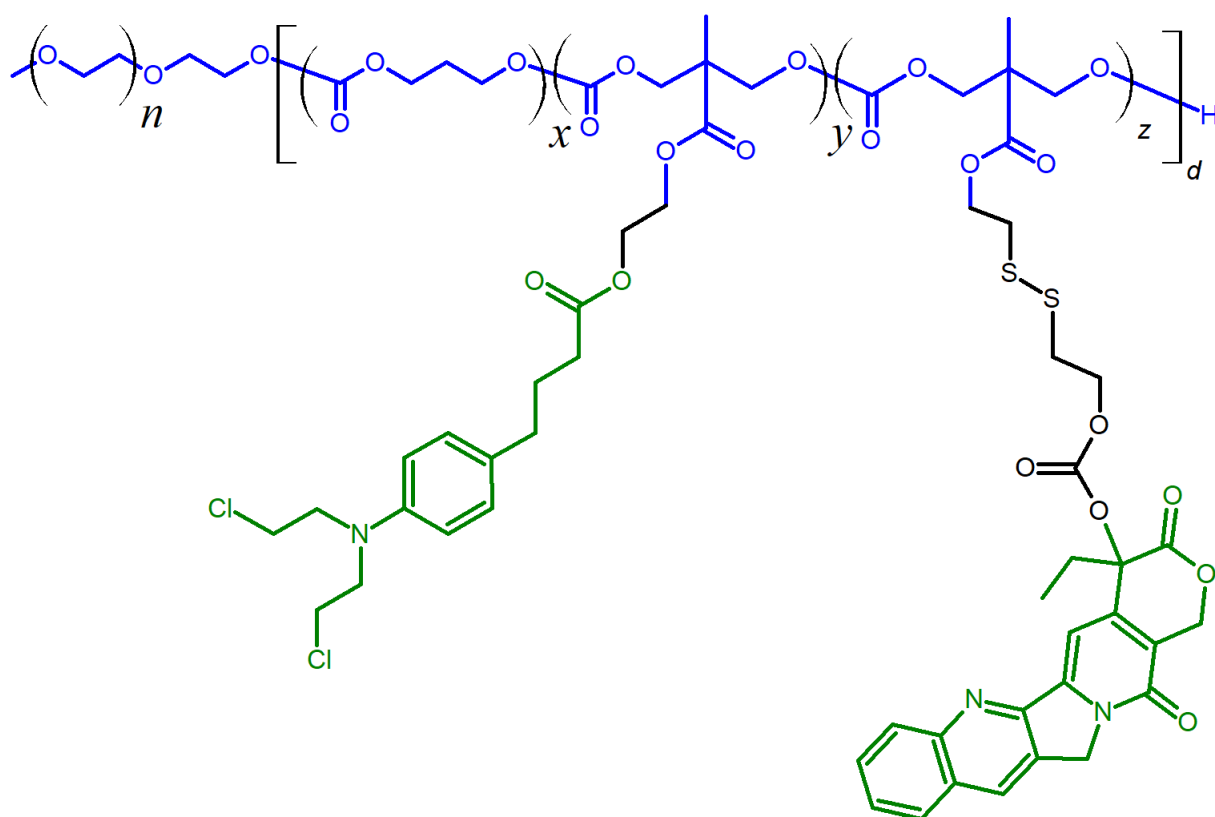


Figure 2 Chemical structure of a polymeric prodrug based on a hydrolyzable ester bond between chlorambucil (green, left) and the polymeric backbone (blue) and a dithio-linked camptothecin (green, right) moiety, sensitive to glutathione.¹³⁴

Under neutral pH conditions, it is estimated that the hydrolysis of purely ester-based prodrugs can require several months for complete release of the cargo.¹²¹ In contrast, imine- or hydrazone-based prodrugs show a faster release, which can be significantly increased in acidic environments, as has been shown by Zhu et al. and Yoo et al., who coupled doxorubicin to PEG, as can be seen in Figure 3, and investigated the release from micelles assembled from those polymers.

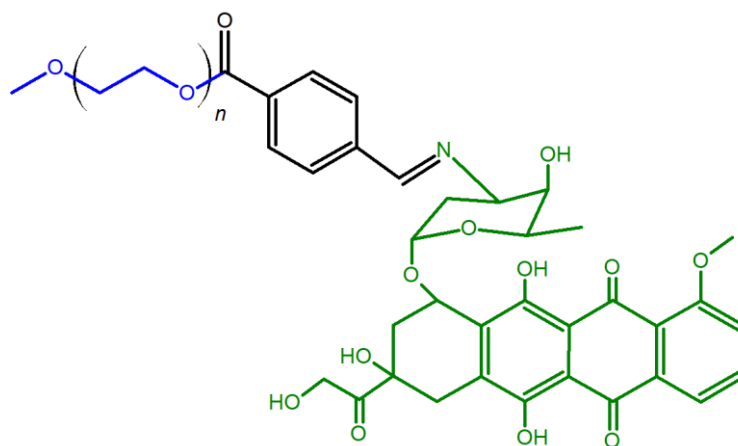


Figure 3 Chemical structure of a polymeric prodrug based on a hydrolyzable imine bond between doxorubicin (green) and the polymeric backbone (blue).¹³⁵

The onset of doxorubicin release was observed at pH 6.5, with the imine hydrolyzed completely only at pH<5, while an alternative hydrazone-based approach yielded twice as much release at pH 3 in comparison to pH 7 after 24 h of release.^{135,136} Kang et al. made similar observations with doxorubicin coupled via a hydrazone bond to a dendritic polyrotaxane, which assembled into micelles and released the drug three times faster at pH 5 in comparison to pH 7.4.¹³⁷ It can therefore be concluded that the stability of these bonds depends on several factors, mainly the pH and the temperature of the environment but also on the types of substituents.

2.4 Surface-Grafted Polymer Brushes

Polymer brushes are polymer chains end-grafted to a surface which, at a sufficiently high grafting density (σ), stretch away from the surface due to steric repulsion.²⁹ Below a threshold value, which was determined to be ~ 0.065 polymer chains/nm² for PAAm, the polymer chains are in a collapsed state, termed the mushroom regime.²⁹ Above this threshold, the brushes start to stretch from the surface.²⁹ However, the conformation of the grafted chains is not only dictated by σ but also R_g which can be estimated through the Flory mean field approach described based on results by de Gennes and Alexander (Eq. 1).^{138,139}

$$R_g \sim N^\nu \quad \text{Equation 1}$$

N represents the number of bond segments and ν the Flory exponent, a measure of the solvent quality (approximately 3/5 for a good solvent, 1/3 for a bad solvent and 1/2 for a theta solvent). The polymer brush layer thickness, H , scales as a function of the cubed root of σ (Eq. 2).¹³⁸

$$H \cong a N \sigma^{1/3} \quad \text{Equation 2}$$

Here, a describes the monomer size according to the mesh model suggested by de Gennes. Calculation of σ (Eq. 3) relies on understanding the distance between grafted polymer chains, D , which depends on H , the bulk polymer density, ρ , Avogadro's number, N_A , and the number average molar mass, M_n , as depicted in Figure 4.

$$\sigma = \frac{1}{D^2} = \frac{H \rho N_A}{M_n} \quad \text{Equation 3}$$

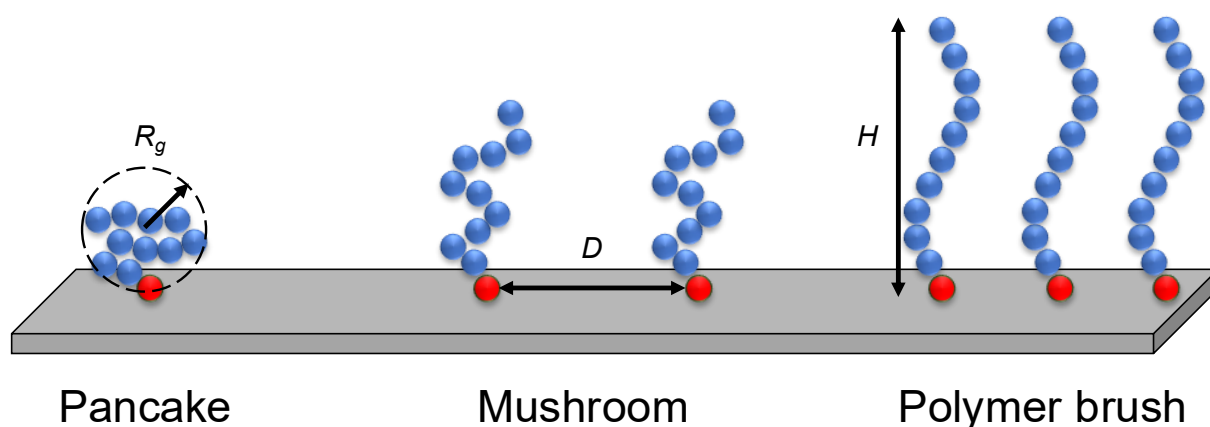


Figure 4 The conformational transition from the pancake to the mushroom to the polymer brush regime, depending on grafting density.

The kinetics of the conformational change can be studied with a quartz crystal microbalance, as studied by Liu et al. using gold-grafted poly(*N*-isopropylacrylamide) brushes.¹⁴⁰

In general, two routes are commonly used to generate polymer brushes, the so-called grafting-to and grafting-from method, as can be seen in Figure 5.³⁰ Both approaches rely on the chemisorption of reactive end-groups of a pre-synthesized polymer (grafting-to) or of a species able to initiate a polymerization reaction (grafting-from) to

the surface to be functionalized. The grafting-from method generates dense polymer brushes more efficiently, and will be the focus of this Thesis.¹⁴¹

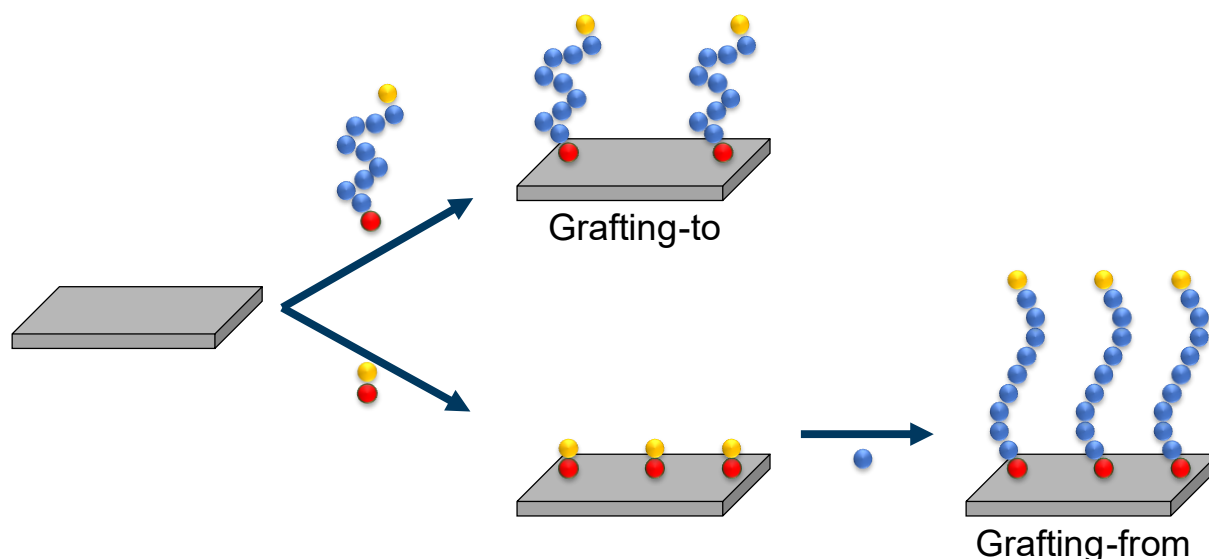


Figure 5 The two main synthetic routes to generate polymer brushes. The grafting-to method involves binding a pre-synthesized polymer to the surface. The grafting-from approach requires the initial attachment of an initiator to the surface, allowing synthesis of a polymer through chain polymerization directly on the surface.

Early approaches to synthesize surface-grafted polymers were achieved by attachment of azo-initiators to oxidic surfaces via silane coupling and thermal decomposition of the grafted azo groups, forming radicals available for the polymerization.¹⁴² As an alternative to such uncontrolled free radical polymerizations, controlled living radical polymerizations can be conducted to form polymer brushes.¹⁴³ These polymerizations have the advantage of producing narrow dispersity polymers with linear polymerization kinetics as well as providing the potential to reinitiate the polymerization, facilitating simple block copolymer synthesis.³⁰ The most commonly employed synthetic approaches are surface-initiated (activator regenerated by electron transfer) ATRP (SI-(ARGET-)ATRP),¹⁴⁴ surface-initiated RAFT (SI-RAFT) polymerization,¹⁴⁵ surface-initiated photoiniferter-mediated polymerization (SI-PIMP)¹⁴⁶ and surface-initiated nitroxide-mediated polymerization (SI-NMP).¹⁴⁷ SI-RAFT polymerization is particularly interesting due to the broad spectrum of polymerizable monomers even under aerobic conditions as well as the broad spectrum of accessible polymeric architectures.^{148,149}

The RAFT polymerization process was first described by Ezio Rizzardo in 1998.¹⁵⁰ Typical radical initiators such as azo compounds and peroxides are commonly employed to initiate the polymerization either via thermal or light-induced decomposition of the initiating species, generating radicals which start growing polymers (Step 1, Figure 6).^{151–154}

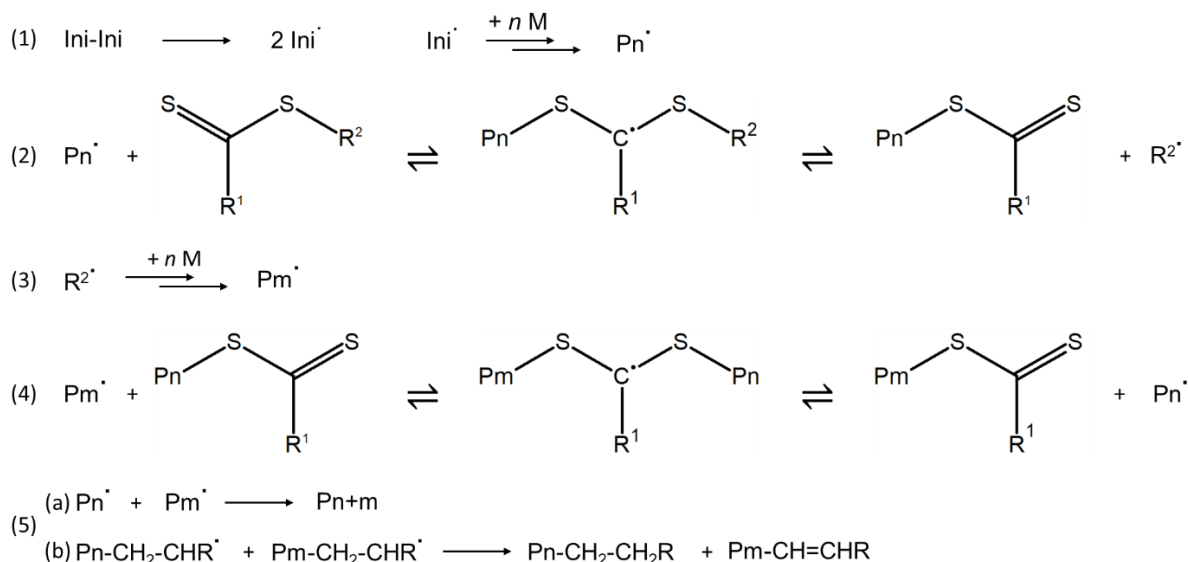


Figure 6 Mechanism of RAFT polymerization. The polymerization is initiated in the first step (1), followed by the transfer of the radical to the attached group R² of the CTA (2), which grows the polymer Pm (3), and is followed by the reverse transfer of the radical to the initial growing polymer, Pn (4). Recombination (5a) and disproportionation (5b) are the typical termination reactions.

Figure 6 Step 2 describes the so-called RAFT pre-equilibrium in which a growing polymer chain radical reacts with a thiocarbonylthio compound. The thiocarbonylthio compound acts as a CTA, where a bond between the thiocarbonyl sulfur atom and the polymeric radical is formed, transferring the radical to the thiocarbonyl carbon. Subsequently, the radical can be either transferred to the original thioester group or back onto the attacking species. The transfer of the radical to another functional group via the CTA happens in an equilibrium reaction, which assures a slow and balanced growth of the polymer. The lifetime of the tertiary carbon radical is determined by the ability of the R¹ group to stabilize the radical and the stability of the bond between the thiocarbonylthio group and the functional group R². Here, the reaction can proceed either by the homolytic bond scission of R¹ or R², and in the case of bond scission of R², a polymeric CTA and a low molecular weight fragment R²[•] are formed.

In Step 3, a re-initiation takes place in which $R^{2\bullet}$ reacts with monomers (M) to form another propagating polymeric chain $Pm^\bullet$. The main RAFT equilibrium is described in Step 4, in which $Pm^\bullet$ reacts with the polymeric CTA, forming the intermediate carbon radical, which might react via homolytic bond cleavage of either attached Pm or Pn . The tendency of bond cleavage is equal, rendering the probability of either reaction equal. If the initiation happens simultaneously within the polymerization solution, the equilibrium will yield polymer chains with a narrow molar mass distribution typical for living radical polymerizations. The chemical structure of the CTA determines the position of the RAFT equilibrium and therefore influences the growth kinetics, dispersity and molar mass of the synthesized polymer. Changing the substituent R^1 will determine the stability of the intermediate radical, while the substituent R^2 must be able to sufficiently stabilize a radical $R^{2\bullet}$ to undergo the homolytic bond cleavage necessary to obtain a balanced RAFT equilibrium. By chemically binding the CTA to a surface, the synthesis of polymer brushes becomes feasible, as the surface-grafted CTA can transfer a radical to the surface-bound functional group, which can then undergo a polymerization reaction.

A variety of polymer brushes has been synthesized based on the SI-RAFT polymerization technique.^{145,149,151} While such polymer brushes were utilized in studies of cell-surface interactions, no attempts have been reported for the synthesis of a surface-grafted polymer brush made from an antibiotic-containing monomer, which would combine polymer brushes and polymeric prodrugs, forging a novel class of so-called polymer brush prodrugs. Using SI-(ARGET)-ATRP for the synthesis of thick polymer brushes, Choi et al. loaded thermoresponsive brushes with levofloxacin, a broad-spectrum antibiotic, and observed the temperature-dependent release of the drug.¹³ It was observed that approximately 50% of the drug was released within the first five minutes, indicating a burst release. *In vivo* experiments showed that the developed coating was able to eradicate pathogenic bacteria, successfully preventing bacterial infections. Nevertheless, the fast release of the relatively low amount of drug (achieved concentrations were estimated to be just above the minimum inhibitory concentration (MIC) of a model strain of *S. aureus*) is unlikely to provide sufficient, long-term protection from bacterial infections.^{10,155} A polymer brush prodrug would combine the advantages of polymeric prodrugs, which are the high drug loading capacity together with the high level of control over the synthesis achieved in SI-

polymerizations, yielding a highly controlled drug-releasing polymer brush film. In this manner, the bacterial side of the “race for the surface” could be approached.

2.5 Supported Lipid Membranes and Cell Culture Systems

Approximately 50% of the mass of animal cell membranes is composed of phospholipid bilayers.¹⁵⁶ These lipids are amphiphilic molecules with polar head groups (e.g., phosphatidylcholine) connected via ester bonding to a glycerol unit bound to apolar hydrocarbon chains of varying length (commonly 14 – 24 carbon atoms), with one chain usually containing at least one or more double bonds in cis-conformation.^{156,157} The general chemical structure of phospholipids can be seen in Figure 7.

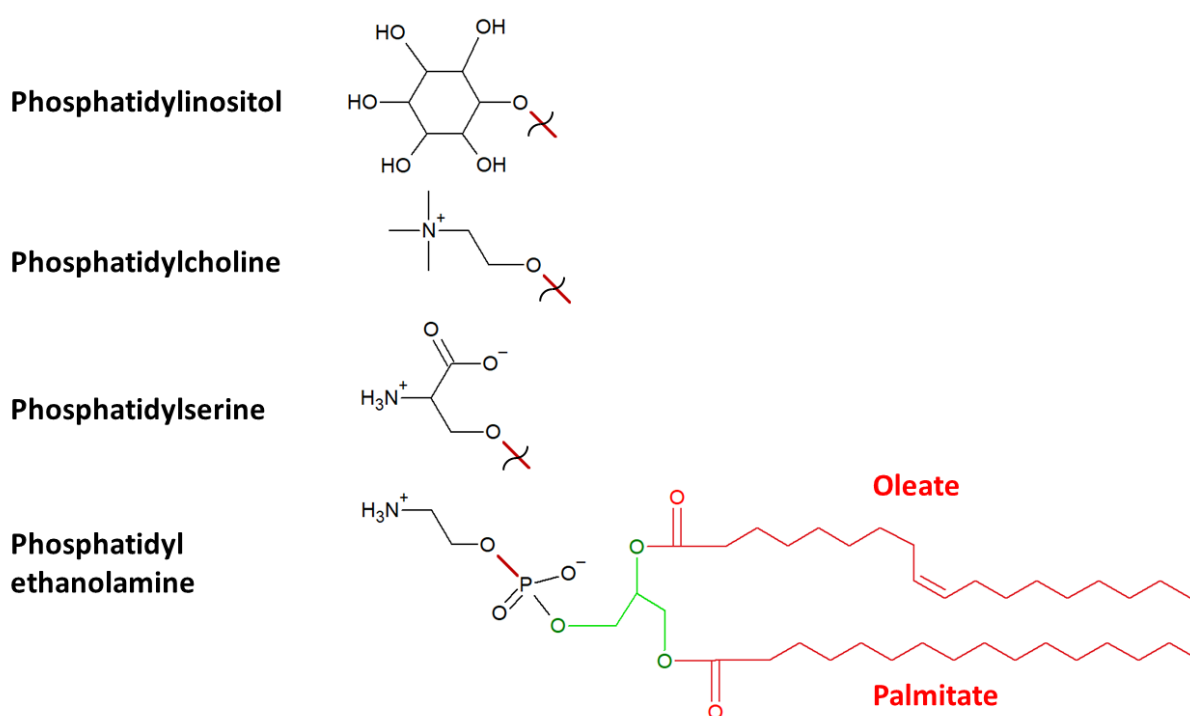


Figure 7 Chemical structures of common phospholipids.

As cell membranes are composed of not only different lipids, proteins and cholesterol-enriched lipid rafts, the study of the function of each component of these complex systems requires a simplified model.^{158–160} Early attempts to generate substrate-supported lipid membranes were based on the generation of lipid monolayers on alkylated glass by von Tscharner and McConnell and were later improved by depositing phospholipid bilayers on silicon wafers through sequential monolayer deposition using a Langmuir trough.^{161,162} While the sequential deposition of lipid monolayers is possible with a Langmuir trough, it is sensitive to contamination due to the reliance on surface

tension measurements. As an alternative, several other techniques are available in the synthesis of supported lipid bilayers.¹⁵⁹ These include: spin-coating, solvent-exchange deposition and vesicle fusion.^{163–165} Vesicle fusion is of particular interest because of its simplicity and versatility, commonly performed on siliceous substrates like glass or mica.¹⁶⁶ The process is described in a simplified fashion by the adsorption of lipid vesicles to a solid substrate, subsequent rupture of the vesicle and fusion of the lipid bilayer with neighboring bilayers, leading to growth of the membrane (Figure 8). Nevertheless, the complexity of naturally occurring membranes composed of several hundred lipid species makes the formation of artificial lipid membranes via vesicle fusion difficult, due to the necessary complexity of the lipid vesicles.¹⁶⁷ A key ingredient in most plasma membranes is cholesterol, which can contribute up to 50% of the lipid composition, but due to its rigidizing effect on lipid vesicles, the fusion of such vesicles on a surface, forming lipid bilayers, is energetically unfavored.^{167,168}

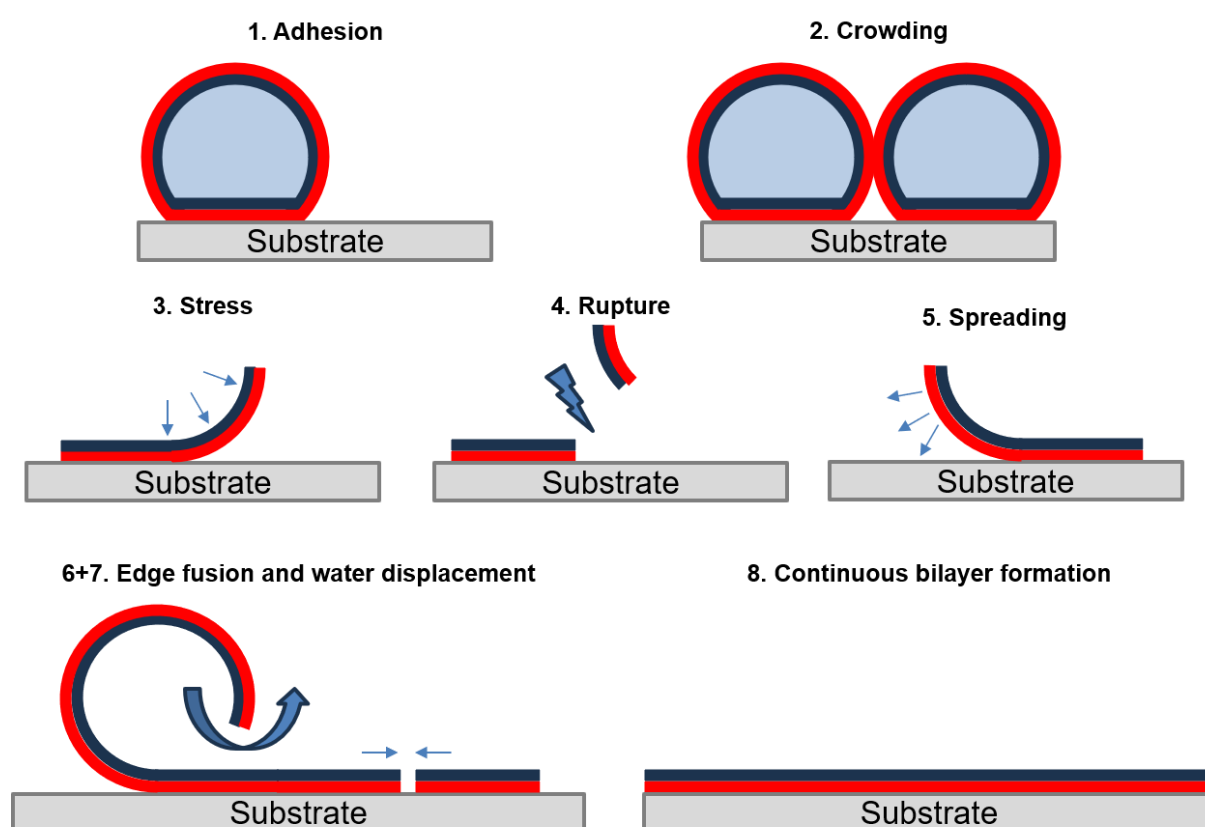


Figure 8 The process of vesicle fusion on a solid support.

The mechanism of vesicle fusion can be deconvoluted into eight stages, starting with the adhesion of lipid vesicles to a surface (1), followed by crowding of adsorbed vesicles with weak adhesion forces (2). At a critical concentration of adhered lipid vesicles, deformation leads to stress on the bilayer (3) and ultimately rupture of the

vesicle wall (4) and the subsequent spreading of the ruptured membrane on the surface (5). The next step involves the growth of the supported lipid bilayers and the fusion between growing membranes at the edges, potentially acting as nucleation sites, lowering the energy necessary for further adsorbed vesicles to rupture (6). The last steps include the displacement of entrapped water (7) and the formation of a continuous lipid bilayer (8). The fusion of the vesicles to form the continuous bilayer is the most critical part and depends on various factors, including the size of the vesicles, temperature, pH as well as the substrate surface chemistry.^{169–171} Nevertheless, the incorporation of essential cell membrane components, including cholesterol and transmembrane proteins, via vesicle fusion is difficult, due to the nanometer-sized water layer between the membrane and the surface.¹⁷² Furthermore, the potential detachment of the membrane from the surface due to competing surface-active agents has led to the development of surface-tethered lipid bilayers.¹⁷³

A major breakthrough in membrane research was achieved through the patterning of lipid bilayers via photopolymerization of diacetylene phospholipids, facilitating the generation of lipid-free corrals in a frame of polymerized bilayer, which enables the incorporation of fluid bilayers into the corral.^{52,53} Powerful tools in the analysis of these polymerized lipid membranes are atomic force microscopy (AFM), which unveils changes in the thickness and lattice formation of the phospholipid head groups, and fluorescence microscopy, which can detect the autofluorescence of the poly(diacetylene) frame or diffusion of incorporated fluorescent species in the fluid lipid bilayer within the corrals.^{174–177} In recent years, the decoration of photolithographically-patterned supported lipid membranes with microfluidic channels in PDMS has been used as a transportation and selection system able to separate different components within the nanometric space between the PDMS sheet and the bilayer generated through extruded lipid vesicle spacers.⁵⁵ This system relied on the use of nanoparticles to control the distance between the membrane and the underlying support, and therefore relies on available nanoparticle dimensions. An alternative approach to control this distance has been suggested as the use of ATRP-synthesized polymer brushes.⁵⁷ By utilizing the Langmuir-Blodgett/Langmuir-Schaefer (LB/LS) deposition technique, the formation of bilayers containing functionalizable lipids facilitates the synthesis of brushes on the membrane.⁵⁷ Although the brush synthesis via ATRP works successfully on both sides of the membrane, drawbacks inherent to the polymerization technique are observed, including a limited brush thickness and adsorption of polymer

formed in solution on the membrane.⁵⁷ Furthermore, the use of high copper concentrations poses a potential threat for further biological applications.⁵⁷

The versatility of polymer brushes for cell culture studies has been previously reported.^{45,178,179} By systematically changing the grafting density of the initiating species on gold, Lilge et al. found a sigmoidal trend of the thickness of the generated PAAm brushes with the fraction of initiator on the surface, corresponding to a mushroom-to-brush transition.⁴⁵ A low fractional content of the initiator in the mixed self-assembled monolayer was sufficient to render the system antifouling. Using soft lithography, patterned polymer brush layers were generated, which forced cells to adapt to the patterned shape, due to the antifouling effect of the polymer brush, while the attachment of cell-adhesive arginylglycylaspartic acid (RGD)-containing peptide sequences to the brushes allowed enhanced cell adhesion.¹⁷⁸ The dependence of antifouling properties on the conformation of the surface-grafted polymer was studied by Großhans et al. using thickness gradients achieved through controlled immersion of the initiator-modified substrates into the polymerization solution.¹⁷⁹ A sharp transition was found for brushes of PDEGMA, where the onset of antifouling behavior was determined at 7 nm. PDEGMA, which undergoes a temperature-dependent phase-volume transition related to the lower critical solution temperature (LCST), was found to have reversible antifouling properties, as the conformation of the polymer can be switched via a temperature shift.¹⁸⁰ This behavior of the antifouling properties was explained with the adsorption of fibronectin to the polymer surface, which then renders the surface adhesive for cells, if the brush was in a collapsed state above 32 °C. As observed, by decreasing the temperature below the threshold value, cells which were adhering to the brush-modified surface could be gently detached and reseeded on tissue culture polystyrene (TCPS).¹⁸⁰ Jiang et al. utilized this effect to culture cell monolayers and cell spheroids and by a simple cooling step, detached the cells from the substrate.¹⁸¹ Based on this knowledge, pancreatic tumor cells in a co-culture with fibroblasts could be selectively separated based on the different binding affinity to PDEGMA brushes below the LCST.¹⁸² Not only monolayers and spheroids can be detached, but also cell sheets were successfully detached on demand, as reported by Schulte et al.¹⁸³ This approach was used to separate spontaneously differentiated stem cells from undifferentiated stem cell sheets after culturing for 5 days.¹⁸⁴ Hence, the combination of lipid membranes and polymer brushes promises a dynamic and versatile platform in cell culture studies.

Hebel et al. reported on the use of supported lipid membranes as potential substrates for cell culture studies.⁵⁶ It was hypothesized that supported lipid membranes could act as ideal candidates to mimic basic cell surface functions and, in combination with polymer brushes, be used in advanced systems to study cell responses to external stimuli. As expected, the functionalization with PDEGMA and POEGMA brushes rendered the membranes antifouling and led to the cells attaching to the polymer-free corrals in the membrane. However, to generate an advanced cell culturing platform based on a lipid membrane, polymer brushes should be synthesized to facilitate immobilization of cells to study cellular responses upon stimulation. No such system has been reported yet, and while ATRP was proven to be capable of generating such systems on solid substrates, RAFT polymerization offers an interesting alternative in the synthesis of functionalizable polymer brushes on lipid membranes due to the use of less toxic chemicals and a broad range of polymerizable monomers. The potential of RAFT polymerization for the synthesis of polymer brushes on lipid membranes will therefore be investigated and discussed. In this manner, the cellular side of the “race for the surface” will be approached, which will, in the future, help to investigate cellular behavior at the interface of polymer-coated implant surfaces.

3. Aims of this Thesis

This Thesis approaches the “race for the surface” from two complementary perspectives. Firstly, the feasibility of generating a drug-releasing polymer brush film in which a therapeutic agent is chemically linked to the polymer chain via a hydrolyzable linker is investigated. This concept, termed a polymer brush prodrug, is designed to eradicate harmful planktonic bacteria, thereby preventing bacterial adhesion and inhibiting subsequent biofilm formation. The development of a polymer brush prodrug will be approached as follows:

1. Reproducible synthesis of a pristine monomeric prodrug
2. SI-polymerization of thick polymer brush prodrugs
3. Time-, temperature- and pH-dependent drug release studies with spectroscopic and chromatographic detection
4. Microscopic and spectroscopic observation of changes in the remaining polymer brush film
5. Testing of bacterial susceptibility to the antibiotic released from the brushes

This previously unreported system is hypothesized to yield a higher drug loading capacity than existing polymer brush-based drug reservoirs and yield a prolonged drug release. In this way, the bacterial dimension of the “race for the surface” is specifically targeted.

Secondly, to generate a novel cell culture platform that can immobilize cells on a polymer brush functionalized surface, a hybrid system of a supported lipid membrane with grafted polymer brushes will be investigated. This hybrid system will be evaluated based on the following criteria:

1. Establishing a RAFT polymerization compatible with lipid membranes
2. Investigation and comparison of cell-brush interactions for well-known polymer brush compositions
3. Extension of homopolymer brushes to facilitate functionalizable block copolymers for future cell immobilization

Such advanced cell culture platforms would lay the foundation for future cell biosensing studies, which would enable the investigation of responses of immobilized cells at the brush interface to chemical and physical triggers. This groundwork serves as an approach to the cellular side of the “race for the surface”.

4. Development of an Ester-Based Polymer Brush Prodrug

4.1 Introduction and Objectives

Polymer brushes on solid substrates have been studied in different subdisciplines of surface science, but are primarily investigated for biological applications.^{185,186} Antifouling brushes have been developed to prevent cellular, bacterial and protein adhesion to the underlying substrate, which can be utilized to prevent biofilm formation.^{151,187,188} By coupling antimicrobial peptides to the end-group of polymer brushes, contact-killing surfaces have been developed.¹⁰⁰ Stimuli-responsive functionalities have also been incorporated to afford polymer brushes that undergo conformational changes upon changes in environmental conditions.¹⁸⁹ Thermoresponsive polymer brushes take advantage of temperature-dependent phase-volume transitions, a behavior that is related to the LCST behavior of the bulk polymer.¹⁸⁰ Antibiotic-loaded thermoresponsive polymer brushes on model titanium implant surfaces were developed by Choi et al., in which the drug was loaded by post-polymerization adsorption to the polymer film.¹³ The drug release kinetics were both time- and temperature-dependent, while the released antibiotic achieved concentrations capable of preventing the formation of bacterial biofilms.¹³ However, such approaches, in which the drug is only loosely adsorbed within the polymer brush film, exhibit burst drug release and have low drug loading capacities, likely due to the need for the drug to diffuse into the dense polymer brush film, which will be sterically hindered.^{13,190}

An alternative approach to achieve high drug-loading capacities is the usage of polymeric prodrugs.¹¹⁰ These prodrugs can be formed either through the formation of a polymerizable monomeric prodrug which contains the active agent coupled to a polymerizable unit, or through the bond formation between a pre-synthesized polymer with reactive side- or end-groups.^{110,112} The bond connecting the drug with the polymer typically undergoes hydrolysis to liberate the drug. Commonly applied bond types include amides, esters and imine bonded systems, where the stability of the linker determines the drug release kinetics.¹¹⁰ While such systems are frequently studied in drug delivery applications based on polymeric nanoparticles or polymersomes, no surface-grafted polymeric prodrug made from a monomeric prodrug has been reported in the literature yet. Such a system would open a new branch of polymeric prodrugs,

namely polymer brush prodrugs. Similarly to polymeric prodrugs in solution, there are two ways of achieving polymer brush prodrugs, either through the synthesis and subsequent polymerization of a monomeric prodrug or the post-polymerization modification of a polymer brush (Figure 9).

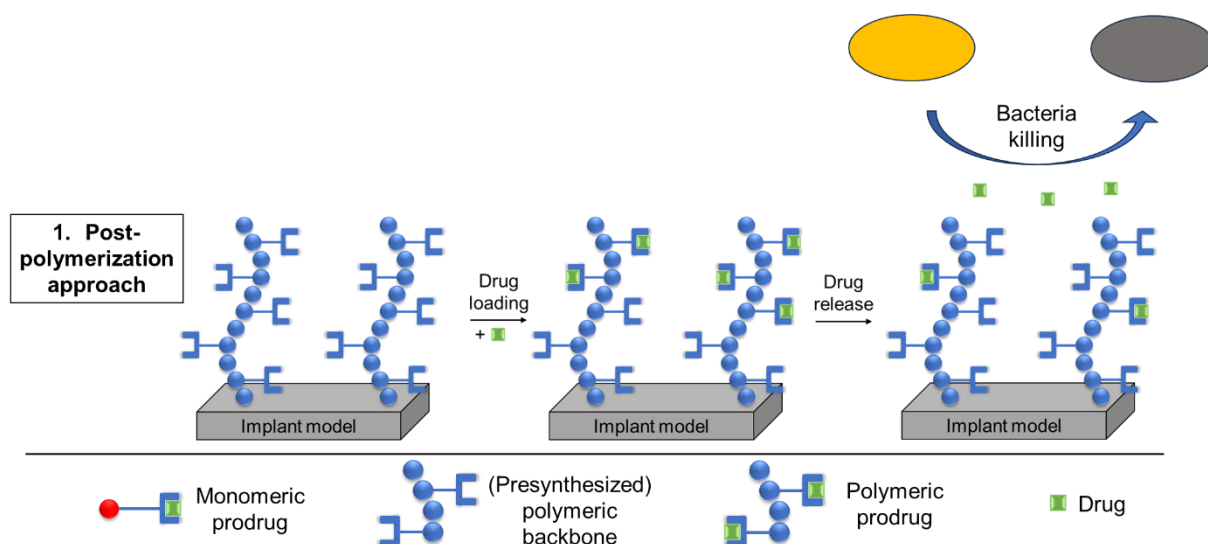


Figure 9 The formation of a polymer brush prodrug via the post-polymerization approach, in which an antibiotic is chemically linked to a pre-synthesized polymer brush.

The post-polymerization approach is hypothesized to suffer from the same disadvantages as described for the absorbed drug-polymer brush systems. The formation of a polymer brush prodrug through the diffusion of a drug into the film and the subsequent bond formation would be sterically disfavored, leading to a low drug loading.¹⁹⁰ In contrast, the bottom-up approach facilitates higher drug loading, as each repeat unit contains a drug molecule (Figure 10). A variety of cleavable bonds, including esters, imines or disulfides, can be used to link the drug to the polymer.¹⁹¹ Esters are particularly interesting as ester hydrolysis is pH-responsive and can be catalyzed by bacterial enzymes.^{192,193}

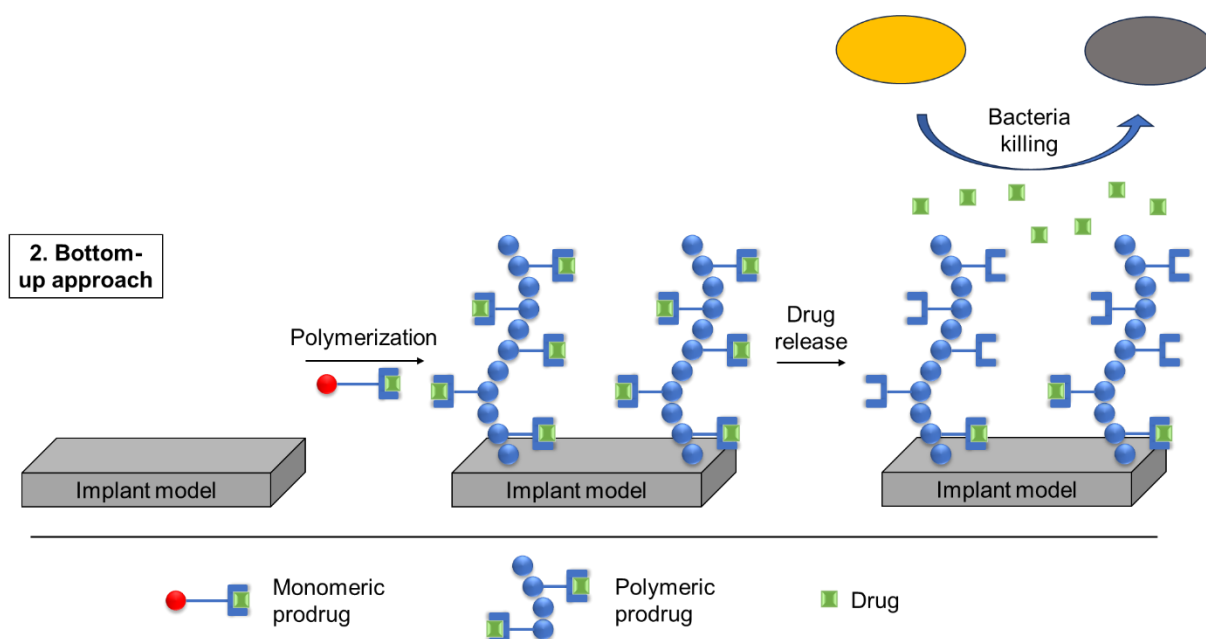


Figure 10 The formation of a polymer brush prodrug via the bottom-up approach, using a polymerizable monomeric prodrug.

In this Chapter, the incorporation of the antibiotic chloramphenicol (CAP) into a polymer brush film is investigated, based on the polymerization of a monomeric prodrug onto titanium substrates to form a polymer brush prodrug. The polymer brushes are then investigated to determine the CAP release behavior. The aims of this Chapter are:

1. Synthesis of a monomeric prodrug from CAP through the introduction of the polymerizable methacrylic unit
2. Formation of a poly(CAP methacrylate) brush prodrug on a model titanium surface
3. Kinetic drug release studies under varying conditions

4.2 Results and Discussion

4.2.1 Monomer Synthesis

CAP was reacted with methacryloyl chloride to form a polymerizable monomeric prodrug, based on work reported by Shiraishi et al. (Figure 11).¹¹¹

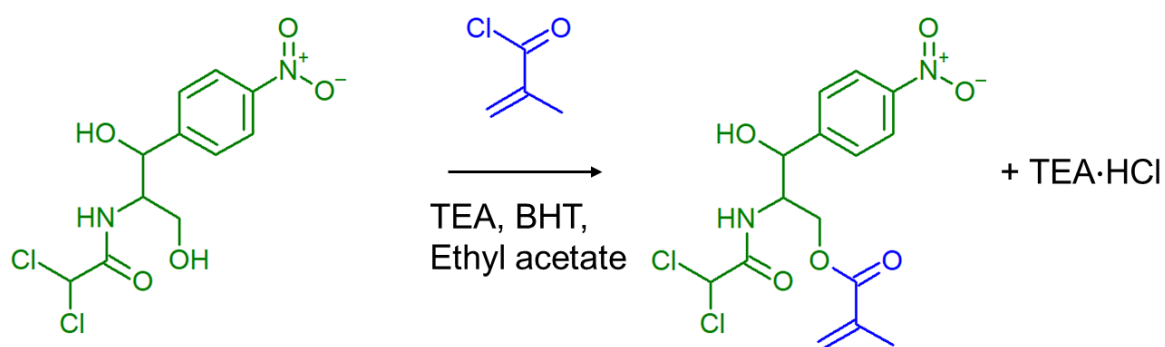


Figure 11 CAP methacrylate synthesis from CAP (green) and methacryloyl chloride (blue) in the presence of triethylamine (TEA) and butylated hydroxytoluene (BHT).

The synthesis of CAP methacrylate afforded a yellow crude product, which was recrystallized from a mixture of water and ethanol to obtain the white, crystalline monomeric prodrug. The monomeric prodrug was then tested by means of $^1\text{H-NMR}$, CHNS analysis and ATR-FTIR. The successful synthesis of the monomeric prodrug was confirmed by $^1\text{H-NMR}$ (Figure 12), confirming the structure proposed in Figure 11.

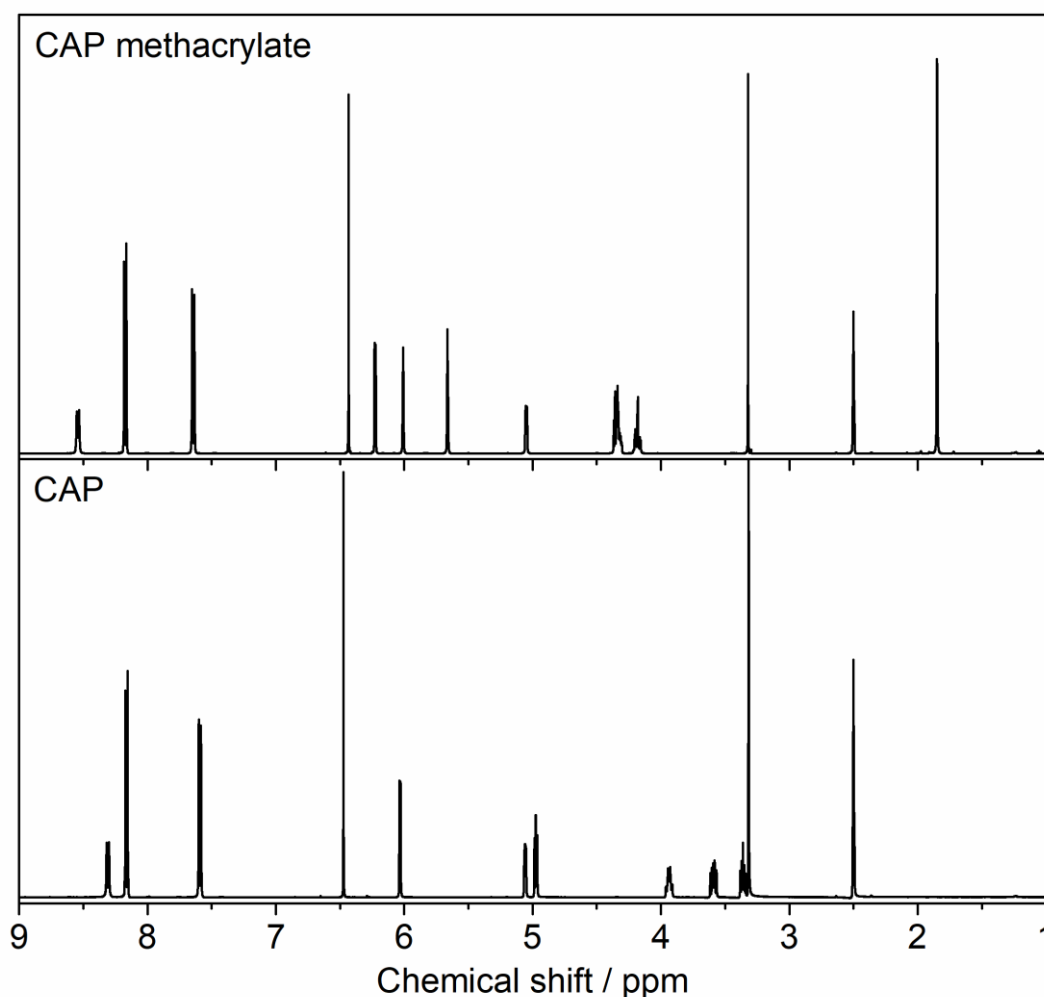


Figure 12 ^1H -NMR spectrum of CAP and CAP methacrylate measured in DMSO- d_6 and referenced to the DMSO- d_5 signal at 2.5 ppm.

The ^1H -NMR analysis of CAP revealed the expected chemical signals for CAP without further impurities. The successful introduction of the polymerizable methacrylic unit is confirmed by the presence of olefinic signals between 5 and 6 ppm and the methyl signal positioned at 1.85 ppm. All signals from the CAP remained present in the CAP methacrylate, except the signal at 4.98 ppm corresponding to the primary alcohol, confirming that the drug remained intact under these reaction conditions. The assigned signals for CAP and CAP methacrylate are listed in Appendix 1 and Appendix 2.

Further analysis of the monomeric prodrug involved the ATR-FTIR investigation of CAP and CAP methacrylate. The resulting spectra can be seen in Figure 13. The introduction of the methacrylic unit introduces additional signals into the IR spectrum compared to neat CAP, including ester signals (1718 cm^{-1} and 1200 cm^{-1}) as well as a signal for the olefinic group (949 cm^{-1}).

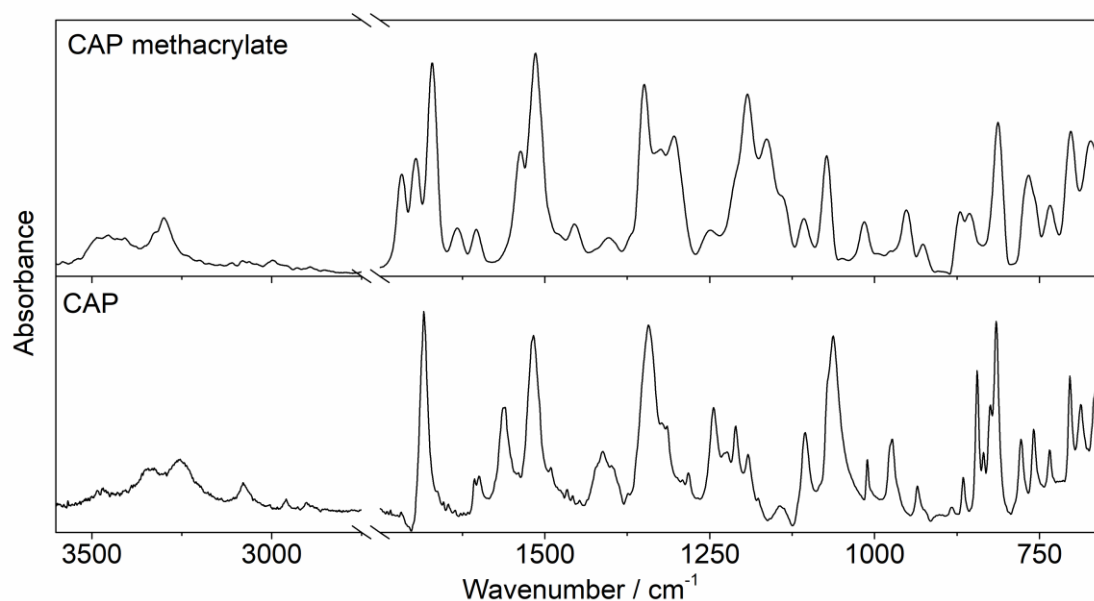


Figure 13 ATR-FTIR spectra of CAP and CAP methacrylate.

Further signals corresponding to CAP or the CAP moiety in CAP methacrylate are listed in Table 1.

Table 1 IR absorption bands of CAP and CAP methacrylate.

Wavenumber / cm ⁻¹	Functional group	Vibration
814	C-Cl	C-Cl stretching
949	C=C-H	C-H out-of-plane bending
1200	RCO-OR'	C-O stretching
1344	NO ₂	N-O stretching
1516	Aromatic C=C	C=C stretching
1683	R ² CONR ³	C=O stretching
1693	RCOOR ¹	C=O stretching
1718	RCOOR ¹	C=O stretching
3256, 3355	R ² CONR ³	N-H stretching

Finally, to further confirm the successful monomer formation and to provide an indication of purity, CHNS analysis of the monomeric prodrug was employed. This provided the chemical composition of the monomer related to the carbon, nitrogen and hydrogen content (Table 2).

Table 2 Theoretical and measured carbon, hydrogen and nitrogen content in CAP methacrylate according to CHNS analysis. The mean value and standard deviation from 4 measurements of one specimen are shown.

Element	Theoretical composition / m%	Experimental composition / m%
C	46.05	46.27 ± 0.34
H	4.12	4.06 ± 0.03
N	7.16	6.92 ± 0.14

As observed in the CHNS analysis, only minor deviations of <0.24% of the measured values relative to the theoretical composition were observed, proving the necessary purity (generally accepted deviation of <0.4%) of the synthesized monomer.

4.2.2 Polymerization of CAP Methacrylate onto Titanium

To mimic an implant surface, titanium-coated glass was used, as titanium is among the most commonly used implant materials. The surface was directly modified with the CTA in a DMAP/DCC-mediated mechanism as reported by Choi et al. and Yildirim et al.^{145,151} The schematic surface modification can be seen in Figure 14.

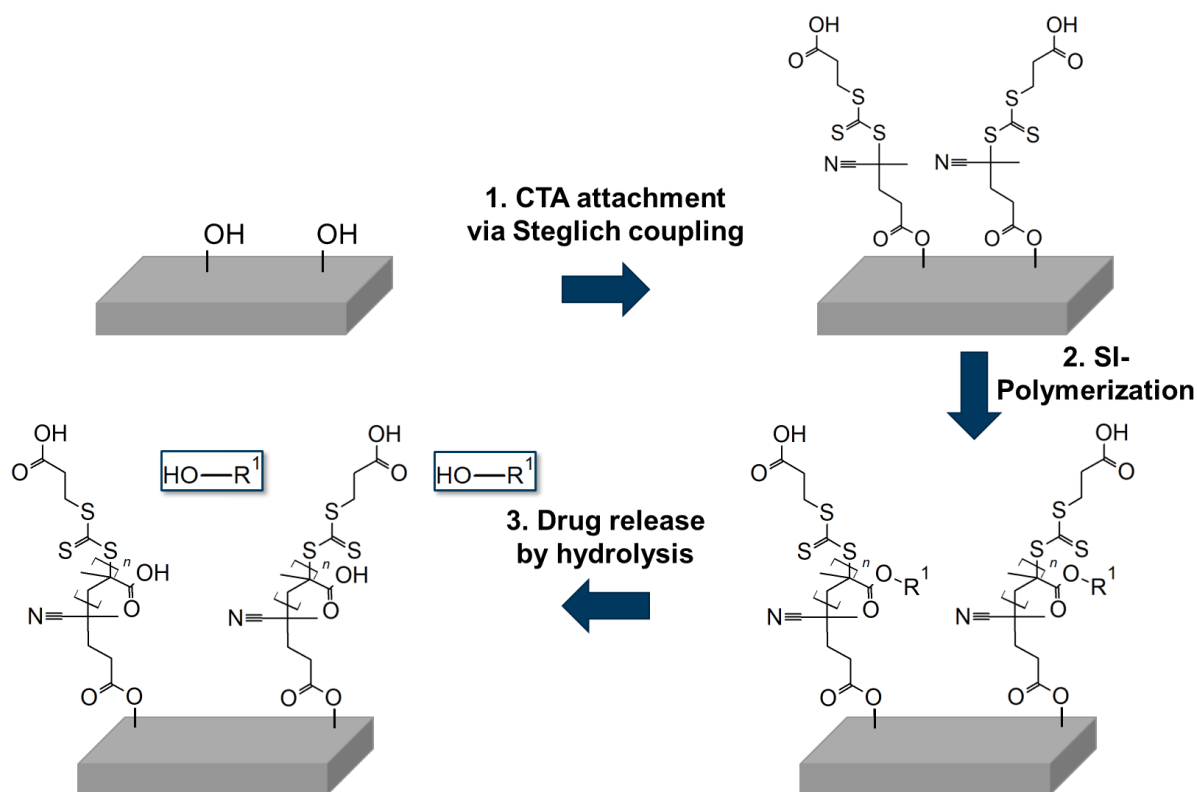


Figure 14 Surface modification with polymer brush prodrugs via SI-RAFT. Step 1, CTA coupling; Step 2, SI-RAFT polymerization; Step 3, drug release based on hydrolysis of the ester bond.

The CTA was directly coupled to the titanium surface, utilizing surface-grafted hydroxy groups.¹⁹⁴ Afterwards, the CTA-modified titanium was immersed in a solution of the monomer and the initiator under argon, then the sealed reactor was immersed in a hot oil bath. This led to the decomposition of the initiator, which started the polymerization in solution. The growing polymeric radicals react with the surface-grafted CTA, which transfers the radical onto the surface-bound group, leading to polymer growth away from the surface. The polymerization was then quenched by the addition of acetone. The resulting polymer brush-modified substrates were investigated by X-ray photoelectron spectroscopy (XPS) and static water contact angle (SWCA) measurements to observe changes in the surface chemistry, while ellipsometry was employed to quantify the growth kinetics of the polymer layer. The measured XPS spectra can be seen in Figure 15.

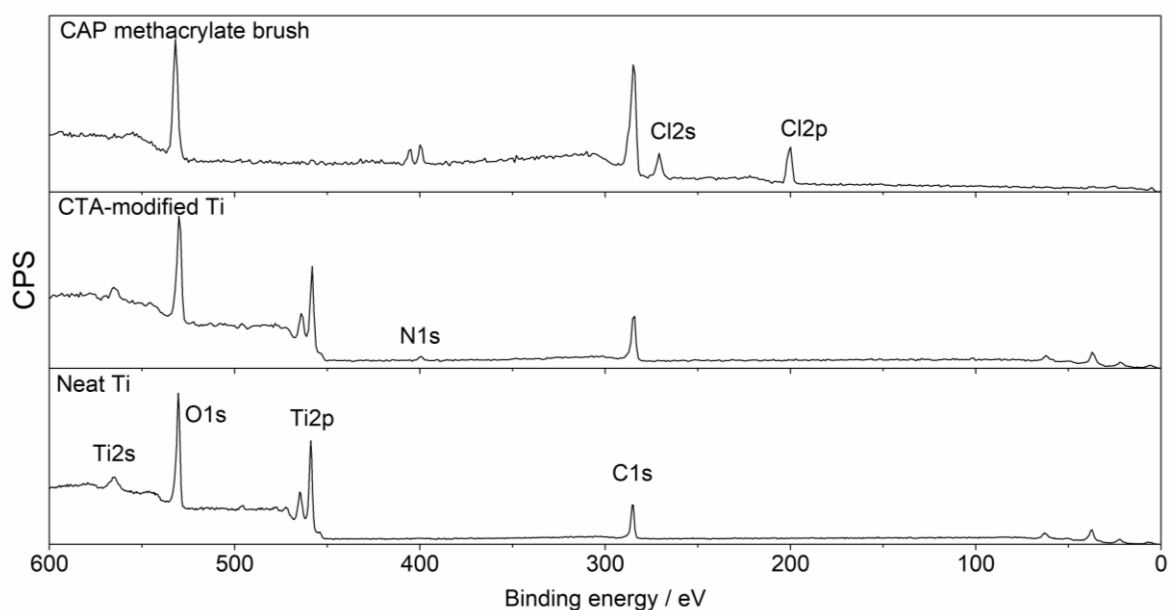


Figure 15 XPS survey spectra of neat titanium, CTA-modified titanium and poly(CAP methacrylate) brushes on titanium, with important signals highlighted. The counts per second (CPS) are plotted against the binding energy.

For the neat titanium substrate, several signals are visible in the survey spectrum, corresponding to titanium and titanium dioxide (454 eV – 459 eV, 2p; 34 eV, 3p; 59 eV, 3s; 564 eV, 2s), adventitious carbon species adsorbed from the atmosphere (285 eV, 1s) and oxygen (529 eV – 533 eV, 1s; 24 eV, 2s) stemming from titanium dioxide and adsorbed species from the atmosphere (Appendix 3). Adventitious carbon species on titanium are well-known and can be employed for charge corrections during referencing.¹⁹⁵ After attachment of the CTA, an additional signal can be seen due to the introduction of nitrogen (400 eV, 1s), while the signal intensity of the substrate-related signals decreases due to the attenuating effect of the CTA layer (Appendix 4). The polymerization afforded thick polymer brush layers, which exceed the escape depth of photoelectrons; hence, the substrate-related signals disappear in the survey spectrum. The high-resolution spectra for the CAP brush-modified surface can be seen in Figure 16.

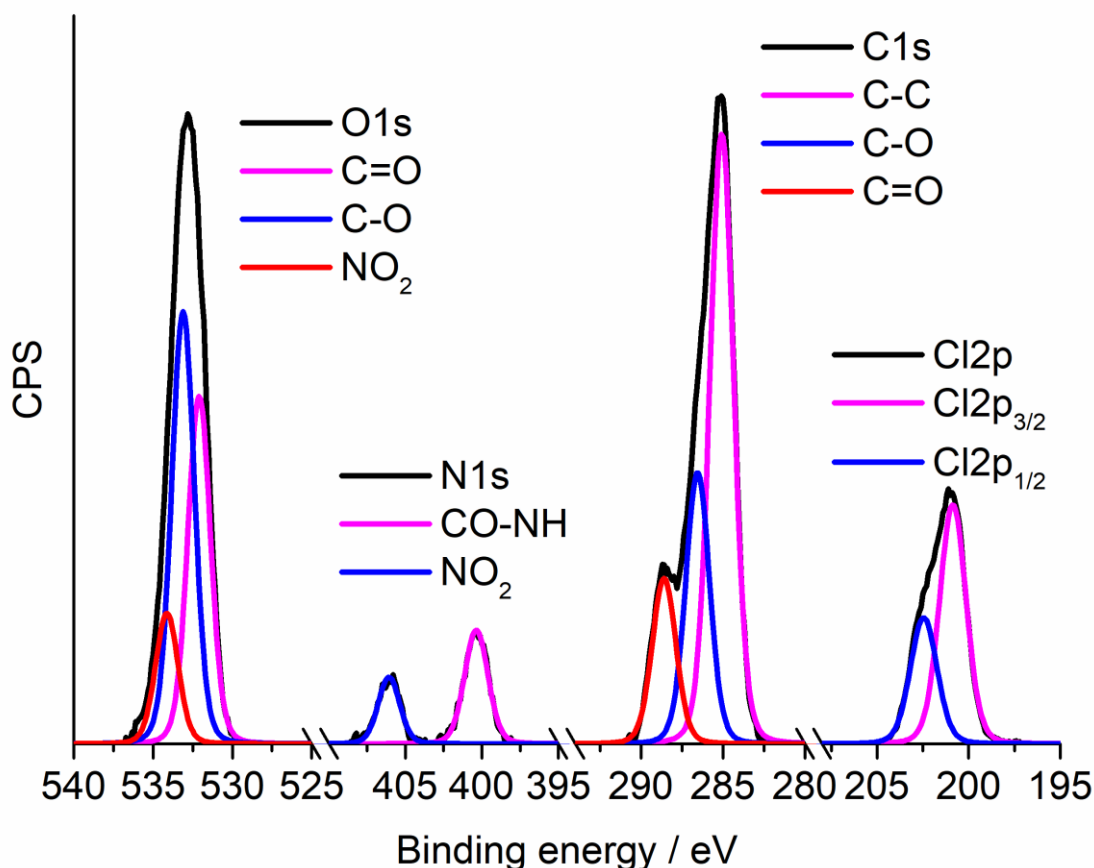


Figure 16 High-resolution XPS spectra of poly(CAP methacrylate) brushes on titanium (dry ellipsometric thickness 45 nm).

The presence of chlorine signals (201 eV, 2p; 271 eV, 2s) confirms the successful incorporation of CAP into the polymer brush film. In the carbon high-resolution spectrum, aliphatic carbon species (C-C), glycolic carbon (C-O) and carbonylic carbon (C=O) signals can be deconvoluted. The nitrogen high-resolution signal indicates the presence of two different nitrogen species, corresponding to the amide (400 eV, 1s) as well as the nitro functionality (406 eV, 1s). The large shift of the nitro functionality is typical for strong positive polarization of the nitrogen atom.^{196,197} In the oxygen spectrum, carbonylic oxygen (C=O), glycolic oxygen (C-O) as well as oxygen species from the nitro group (NO₂) can be deconvoluted. From the high-resolution scans of each step of the surface modification, the chemical composition was determined and can be seen in Table 3.

Table 3 Chemical composition of each reaction step based on the deconvoluted high-resolution XPS spectra.

Element	Titanium		poly(CAP methacrylate) brush	
	Neat / %	CTA-modified / %	Theoretical / %	Experimental / %
Ti	18.0	17.7	0	0
C	34.1	35.4	60	64.0
O	47.9	44.3	24	21.7
N	0	2.1	8	6.5
Cl	0	0	8	7.8
S	0	0.6	0	0

Based on the deconvoluted high-resolution scans of the neat titanium substrate, a titanium: titanium oxide ratio of 0.55 was determined, which is close to the theoretical value of 0.5 for titanium dioxide, indicating an almost complete oxidation of the uppermost titanium layer. After the coupling of the CTA, the substrate-related signals decrease in intensity, while the appearance of nitrogen and sulfur signals confirms the presence of the CTA. The measured signal intensity ratio of nitrogen to sulfur (3.5:1) does not match the theoretical ratio (1:3), likely due to X-ray damage to the CTA layer upon measurement, to which sulfur-containing groups are particularly sensitive.¹⁹⁸

After the polymerization, all substrate-related signals disappear, as the layer thickness exceeds the photoelectron escape depth. All signals, therefore, correspond to the chemical structure of the polymer brush. The values obtained deviate slightly from the expected chemical composition but are in good agreement ($\pm 4\%$). Deconvolution of XPS spectra is typically associated with errors for the determined peak areas up to 4%, even at high signal-to-noise ratios.¹⁹⁹ As can be seen in the nitrogen high-resolution spectrum, the signal of the amide at 400 eV is larger than the signal of the nitro group at 406 eV, indicating X-ray damage to the nitro group. This is supported by the lower oxygen content than expected. Apart from errors occurring from peak deconvolution, the well-known degradation of chlorine-bearing and nitro-group-containing organic materials due to X-ray damaging influences the determined composition of the polymer brush.²⁰⁰

The polymerization kinetics were observed by ellipsometry and showed an initial fast increase in the polymer brush growth, which leveled off after approximately 30 min and yielded a maximum brush thickness of ~45 nm after 120 min (Figure 17).

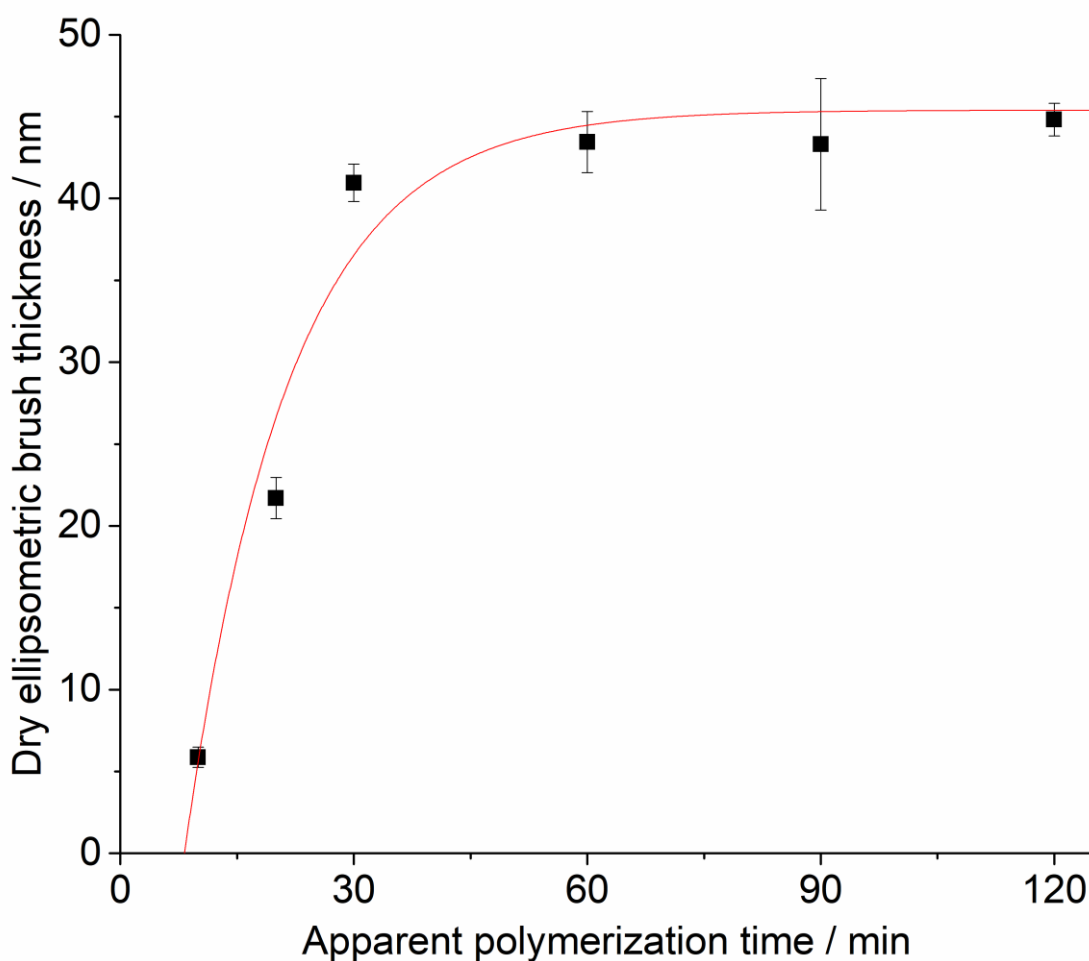


Figure 17 Brush growth kinetics for CAP methacrylate polymerized via SI-RAFT on titanium. The thickness was determined by ellipsometry by measuring three spots per sample over three samples for each polymerization time. The mean value and standard deviations are shown. An asymptotic fit was used to guide the eye (red curve).

The temporal offset of the initiation of the polymerization reaction can be explained by the definition of the start of the polymerization. When the cold reactor is immersed in the hot oil bath, the decomposition of the initiator requires a certain threshold temperature, which is only achieved after approximately 7 min. Termination reactions, including recombination and the loss of CTA, then lead to the death of growing polymer chains, effectively limiting the achievable polymer brush thickness. The growth of the polymer film, which levels off after some time, is a commonly observed feature in SI-RAFT polymerizations.¹⁴⁹

To study the change in the surface chemistry upon modification of the model implants, SWCA measurements were performed (Figure 18). Young's equation defines the contact angle (θ) as can be seen in equation 4.

$$\cos(\theta) = \frac{\gamma_s - \gamma_{sl}}{\gamma_l} \quad \text{Equation 4}$$

The contact angle depends on the interfacial energies of the solid-liquid (γ_{sl}), solid-air (γ_s) and air-liquid (γ_l) interfaces, and is a quantitative measure of the hydrophilicity of a surface.

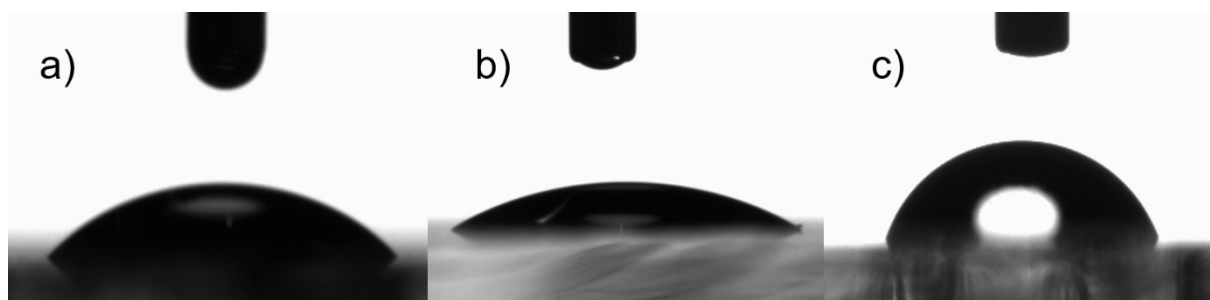


Figure 18 SWCA photographs of a) titanium, b) CTA-coupled titanium and c) poly(CAP methacrylate) brushes on titanium.

As can be seen in the SWCA photographs, the neat titanium surface before UV/ozone cleaning possessed a SWCA of around $47^\circ \pm 2^\circ$. After the attachment of the CTA, the contact angle decreased to around $31^\circ \pm 2^\circ$, and for the final polymer brush prodrug, the SWCA was determined to be around $71^\circ \pm 3^\circ$. These measurements confirm the successful chemical modification of the surface, first with the rather hydrophilic CTA, as described by Choi et al. and Yildirim et al., and secondly of the polymer brush, which is hydrophobic in nature, like the monomer, which is insoluble in water.^{145,151}

In addition to the characterization of the surface-grafted polymer brush, the polymer generated in solution during the polymerization was purified by reprecipitation and analyzed by $^1\text{H-NMR}$ spectroscopy (Figure 19). This allows the direct confirmation of the successful polymerization of the synthesized monomer.²⁰¹

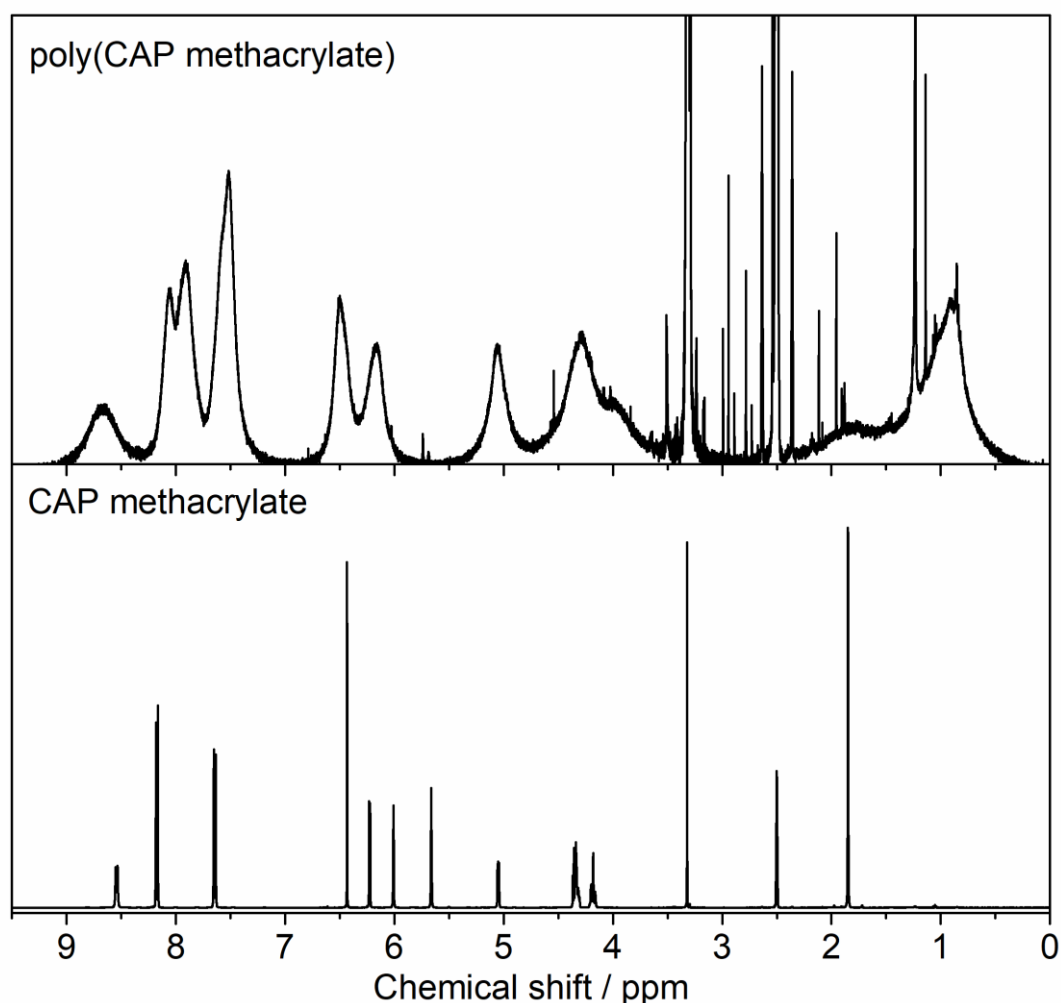


Figure 19 ^1H -NMR spectra (measured in DMSO-d_6) of monomeric CAP methacrylate and the poly(CAP methacrylate) formed in solution, referenced to the DMSO-d_5 peak at 2.5 ppm.

As can be seen in the spectrum for the polymeric prodrug, the peak positions correspond to those of the monomeric prodrug, with the peaks broadened. This broadening itself is an indication of polymer formation, as the reduced molecular mobility increases intramolecular interactions and reduces the relaxation time, resulting in peak broadening.²⁰² The absence of olefinic signals of the methacrylic unit (at 5.66 ppm and 6.01 ppm) and the presence of alkyl signals in the low ppm regime (0.5 - 2.3 ppm) indicate a successful transformation of the monomer into the polymer. Furthermore, integration of the NMR signals shows a total of 16 hydrogen atoms, in agreement with the number expected for the polymer prodrug (Appendix 5).

The reprecipitated polymer was also subjected to CHNS analysis, and the results can be seen in Table 4. The formation of polymer in the polymerization solution allows for a more precise determination of the chemical composition than XPS analysis, as there

are no effects such as X-ray damage. CHNS analysis confirmed the proposed composition of the polymerized CAP methacrylate with deviations below 0.2%, confirming the hypothesized polymerization mechanism.

Table 4 CHNS analysis results for the reprecipitated poly(CAP methacrylate) formed in the polymerization solution. The mean value and standard deviation from two measurements are shown.

Element	Theoretical / m%	Experimental / m%
C	46.05	46.02 ± 0.08
H	4.12	4.06 ± 0.06
N	7.16	7.00 ± 0.01

4.2.3 Investigation of CAP Release from Poly(CAP Methacrylate) Brushes

The pH-dependent drug release behavior of poly(CAP methacrylate) brushes was investigated at a fixed temperature of 37 °C via the immersion of coated substrates into solutions of different pH values. Beforehand, a calibration curve of CAP in phosphate-buffered saline (PBS) was acquired by serially diluting a stock solution and observing the concentration-dependent absorbance (Figure 20). By applying Beer-Lambert law, the absorbance (A) of a solution can be used to calculate the concentration (c) using the extinction coefficient (ϵ) if the optical path length (l) is known (Equation 5).

$$A = \epsilon c l \quad \text{Equation 5}$$

The absorption spectrum of CAP was also measured in HCl at the pH used for the drug release study and showed no significant change compared to the spectrum in PBS, consistent with the absence of acidic functional groups affecting the chromophore. The determined extinction coefficient differed by only 1%, likely due to errors from weighing or during dilution of the stock solution. The spectra, as well as the calibration curve determined for CAP in HCl, can be seen in Appendix 6.

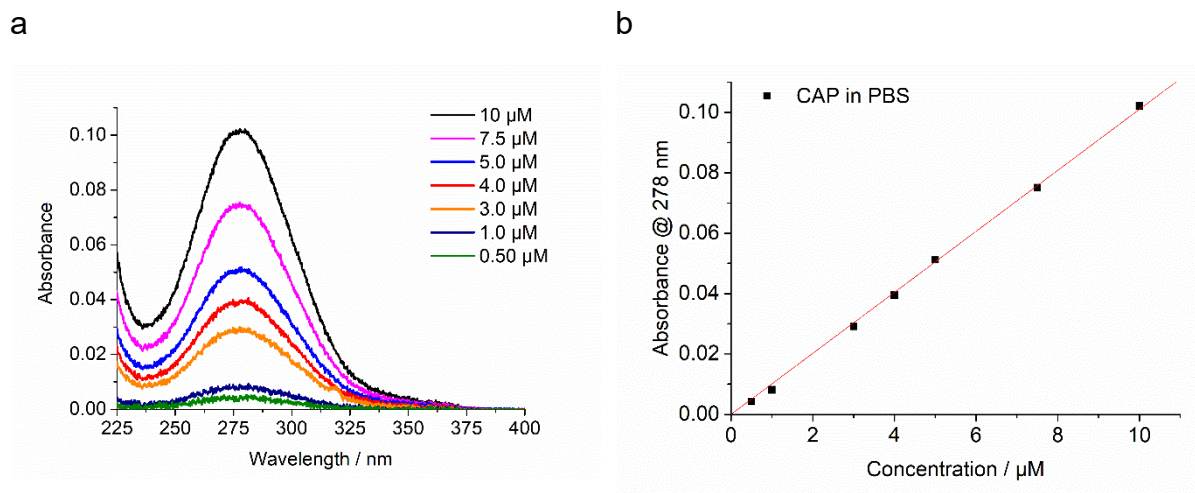


Figure 20 (a) UV-Vis absorption spectra of CAP in PBS and (b) the corresponding calibration curve. The extinction coefficient was determined to be $10.1 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ with a regression coefficient $R^2 = 0.9995$.

The absorbance of CAP was found to scale linearly with the concentration of CAP in PBS at the absorption maximum of 278 nm. The extinction coefficient was found to be $10.1 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, which is in perfect agreement with previously reported values.²⁰³

Release of CAP from poly(CAP methacrylate) brushes was then investigated by measuring UV-Vis spectra of the drug release supernatants in this range. The resulting spectra of the drug release after 72 h at pH 4 and pH 7.4 can be seen in Figure 21.

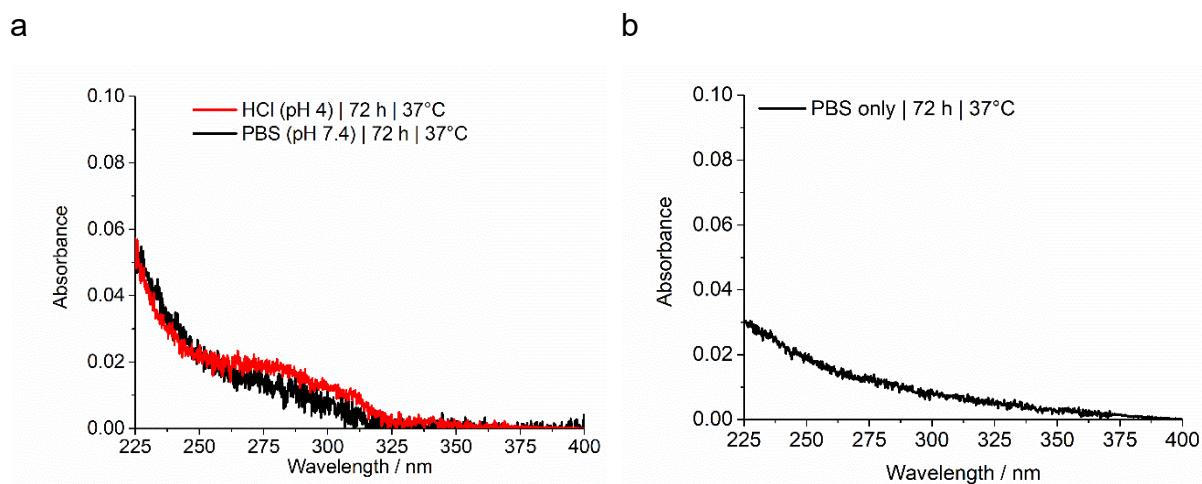


Figure 21 (a) Drug release from poly(CAP methacrylate) brushes polymerized for 120 min after 72 h in PBS (pH 7.4, black) and HCl (pH 4, red) at 37 °C and (b) PBS incubated in a six-well plate for 72 h at 37 °C.

As becomes apparent from the drug release data obtained after three days of incubation, only a minute signal, which can be assigned to CAP, could be detected in the drug release supernatant of the sample incubated in HCl. The release of CAP is expected to be faster in acidic environments than at pH 7.4, as the hydrolysis of esters

is usually catalyzed at low or high pH values.¹¹⁰ Independent of the pH of the drug release solution, the absorbance increases towards shorter wavelengths ($\lambda < 250$ nm). In a control experiment, where PBS alone was incubated in a well plate without a sample, this same absorption behavior was observed (Figure 21b), indicating that there is a minute release of an unidentified species from the well plate into the supernatant. The signal at 278 nm, which can be seen in the drug release supernatant of the sample incubated in HCl, has an absorbance ~ 0.006 higher than the PBS control. This can be calculated with the calibration curve into a relatively small CAP mass concentration of 0.2 $\mu\text{g/mL}$.

To confirm whether CAP was released from the brushes, the chemical composition of the brush surface was measured by XPS after the drug release experiment. The high-resolution spectra of the incubated samples can be seen in Appendix 7. Complete hydrolysis of the ester bond with subsequent release of CAP would yield a strong change in the chemical composition, leaving behind brushes of methacrylic acid, effectively visible as decreasing nitrogen and chlorine signals. As there are no significant changes in the chemical composition visible before and after the incubation, the hydrolysis of the ester seems to be too slow to yield sufficient release of CAP under the tested conditions (Appendix 8).

Considering that most *S. aureus* strains require an MIC of CAP above 8 $\mu\text{g/mL}$, the small amount of CAP released over the course of three days under acidic conditions can be assumed to be insufficient for inhibition of bacterial growth within this timeframe.²⁰⁴ This lack of release indicates the high stability of the ester bond under physiological conditions, rendering the generated polymer brush prodrug unsuitable for drug-releasing applications in the body. Potentially, the drug release could be triggered by applying non-physiological conditions, e.g., under extreme pH or temperature conditions. Furthermore, by applying esterases, the energetic barrier for the ester hydrolysis could be lowered. Such esterases are secreted by bacteria to break down nucleic acids, lipids and esters.²⁰⁵ However, this would necessitate the presence of bacteria in the proximity of the implant, which is against the desired purpose of the implant coating.

4.3 Summary and Conclusions

In this Chapter, a monomeric prodrug based on CAP was synthesized and used for the formation of polymer brushes on a model titanium substrate by SI-RAFT polymerization. The monomer was obtained by esterification of CAP using methacryloyl chloride, introducing a potentially hydrolyzable ester bond and the polymerizable double bond. Recrystallization of the monomer yielded a clean, polymerizable monomer. By binding the CTA directly to the titanium surface, the monomer was used to generate polymer brush-coated titanium surfaces in a thermally-triggered polymerization. These brushes had the expected chemical composition, proving the successful polymer brush prodrug formation.

The release of CAP over the course of three days was observed; however, no significant drug release was observed, neither under neutral nor acidic conditions. This could be related to the inherent stability of aliphatic esters, while esters of substituted phenols are reported to be less stable depending on the substitution pattern of the aromatic system.²⁰⁶ The results of this Chapter indicate that the ester-based polymer brush prodrug is too stable to yield release of CAP under physiological conditions in the magnitude required for inhibition of bacterial growth. The synthesized polymer brushes were therefore not considered applicable for further drug release studies under physiological conditions, and the potential use of CAP methacrylate for the generation of active polymer brush prodrugs could not be achieved.

To generate an efficient polymer brush prodrug from CAP, a different bond between CAP and the polymer backbone that facilitates drug release under physiological conditions would be necessary. A potential CAP-based system could be based on an acetal or ketal, a system where two CAP molecules react with an aldehyde or a ketone in a dehydration reaction. Such systems are known to be much more sensitive towards hydrolysis, potentially enabling the release of CAP under milder conditions.^{207,208}

4.4 Materials and Methods

4.4.1 Materials

Chloramphenicol (CAP) ($\geq 98\%$ (HPLC), Sigma-Aldrich, Germany), methacryloyl chloride ($\geq 97.0\%$ (gas chromatography (GC)), Sigma-Aldrich, Germany) (distilled

before use under ambient atmosphere), triethylamine (TEA) ($\geq 99.5\%$, Sigma-Aldrich, Germany), ethyl acetate ($\geq 99.8\%$ (analytical reagent grade), Fisher Scientific, USA), dichloromethane (DCM) ($\geq 99.8\%$ (analytical reagent grade), Fisher Scientific, USA) (dried over silica gel), 2,6-Di-tert-butyl-4-methylphenol (BHT) (99% (GC), Sigma-Aldrich, Germany), ethanol ($\geq 99.8\%$ (denatured), Carl Roth, Germany), absolute ethanol ($\geq 99.8\%$, VWR, Darmstadt, Germany), acetone (analytical reagent grade, Fisher scientific, USA), 4-((((2-carboxyethyl)-thio)carbonothioyl)thio)-4-cyanopentanoic acid (CTA) (95%, Sigma-Aldrich, Germany), *N,N'*-dicyclohexylcarbodiimide (DCC) ($\geq 99\%$ (for synthesis), Merck, Germany), 4-(dimethylamino)pyridine (DMAP) ($\geq 99\%$ (ReagentPlus®), Sigma-Aldrich, Germany), 4,4'-azobis(4-cyanovaleric acid) (ACVA) ($\geq 98\%$, Sigma-Aldrich, Germany), 1,4-dioxane ($\geq 99.5\%$ (stabilized), Carl Roth, Germany), tetrahydrofuran (THF) ($\geq 99.5\%$ (laboratory reagent grade), Sigma-Aldrich, Germany), HCl ($\sim 37\%$, analytical reagent grade, Fisher Scientific, USA) and DMSO-d₆ (99.8%, Deutero, Germany) were used as received if not mentioned otherwise. Milli-Q water was obtained from a Millipore Direct Q8 device (Millipore, Schwalbach, Germany) equipped with a Millimark Express 40 filter (Merck, Germany) (referred to as water). Phosphate-buffered saline (PBS) solution was prepared by dissolving one tablet of PBS (Sigma-Aldrich, Germany) in 200 mL of water.

4.4.2 Methods

¹H-Nuclear magnetic resonance (¹H-NMR) spectroscopy. ¹H-NMR spectra were recorded for confirmation of the successful monomer and polymer synthesis. ¹H-NMR spectra were measured at room temperature in DMSO-d₆ using a JEOL ECZ 500 MHz device (JEOL Ltd., Tokyo, Japan) and analyzed using MestReNova software (Version 6.0.2-5475). All chemical shifts (δ) are given in ppm.

Combustion analysis (CHNS analysis). CHNS analysis was performed using a HEKAtech EuroEA combustion analyzer (HEKAtech GmbH, Wegberg, Germany). The dried powders of the precursor and the monomeric prodrug were measured, and the relative composition of carbon, hydrogen, nitrogen and sulfur was compared to the theoretical composition.

Static water contact angle (SWCA) measurements. SWCA measurements were conducted using an OCA-15 contact angle microscope (Dataphysics, Filderstadt, Germany). In the sessile drop conformation, water (2 μ L) was dispensed onto the substrate at room temperature and the SWCA was determined based on the profile of the drop. For each surface modification, the SWCA was determined in triplicate over at least 3 samples for the determination of the mean value and the standard deviation.

X-ray photoelectron spectroscopy (XPS). XPS spectra were recorded using an ESCA spectrometer (SSX-100 S-probe, Surface Science Instruments, Mountain View, USA). All spectra were calibrated based on the aliphatic carbon signal, which was shifted to 284.8 eV. The instrument was equipped with a 200 W Al K α X-ray source. Survey spectra were obtained from 0 to 1200 eV with an energy resolution of 1 eV. High-resolution spectra were recorded in the energetic range of the respective components, with a window size of 20 eV and a resolution of 0.1 eV. All spectra were analyzed and quantified using the Casa XPS software (version 1.3.19.1).

UV-Vis spectroscopy. The release of CAP from the poly(CAP methacrylate) brush-modified titanium substrate was quantified via UV-Vis spectroscopy. UV-Vis spectra of the drug release supernatants were recorded at 25 °C in UV-transparent single-use cuvettes (Sarstedt AG & Co. KG, Nümbrecht, Germany) using a Varian Cary 50 Bio UV-Visible Spectrophotometer (Varian, Mulgrave, Victoria, Australia). Measurements were collected from 200 to 400 nm at a scan rate of 200 nm/min with automatic background subtraction of the PBS spectrum measured beforehand. The concentration of CAP was quantified based on an acquired calibration curve (serial dilution of CAP in PBS), correlating the absorption maximum at 278 nm with the concentration of the measured solution.

Ellipsometry. The dry thickness of the polymer brushes was measured using an alpha-SE ellipsometer (J.A. Woollam Co., Inc., Lincoln, USA) at different angles of incidence (65, 70, and 75°), with the wavelength ranging from 380 to 900 nm. The Cauchy model with a refractive index of 1.45 at 632.8 nm was used for the determination of the initiating layer thickness, while the polymer brush thickness was fitted according to the Sellmeier model with a refractive index of 1.52 at 632.8 nm. For the determination of the polymerization kinetics, 9 substrates per polymerization were utilized with 3 separate polymerizations per polymerization time, yielding 27 samples

per polymerization time. The mean values and standard deviations across all samples for a given polymerization time were calculated and used to determine the time-dependent brush growth.

Fourier transform infrared (FTIR) spectroscopy. FTIR spectra were acquired using a Vertex Neo R spectrometer (Bruker Optics GmbH & Co. KG, Ettlingen, Germany). Powdered samples were measured using an ATR-FTIR setup ranging from 600 cm^{-1} to 4000 cm^{-1} with the aperture set to 6 mm and 16 scans.

4.4.3 CAP Methacrylate Synthesis

Synthesis of monomeric prodrug. CAP (10 g) was mixed with TEA (5.1 mL) in ethyl acetate (70 mL) under an argon atmosphere and cooled externally using an ice bath. Freshly distilled methacryloyl chloride in ethyl acetate (7.4% v/v, 50 mL) containing BHT (5 mg) was added dropwise to the reaction mixture via a dropping funnel under argon atmosphere then the reaction mixture was stirred for 2 hours in an ice bath under argon atmosphere. To remove the precipitated TEA hydrochloride, the resulting mixture was filtered through filtering paper (round filter type MN 616, Macherey-Nagel, Carl Roth, Germany) and the filtrate containing the product was dried under a constant air stream. The crude product was recrystallized by heating in water (200 mL) to 90 °C and slowly adding ethanol until complete solvation. Subsequently, the solution was allowed to cool down slowly in the hot oil bath for enhanced crystallization. The recrystallization was repeated, refreshing the solvents until white, needle-like crystals of CAP methacrylate were obtained with a yield of 78%.

4.4.4 SI-RAFT Polymerization of CAP Methacrylate on Titanium

Titanium deposition on glass substrates. Glass microscope slides (1 mm thickness, VWR, Darmstadt, Germany) were cut into 1.5 cm × 2.5 cm pieces with a diamond tip glass cutter. The substrates were sonicated in soap water (detergent, Ecolab, Monheim am Rhein, Germany) for 15 min, followed by sonication in water for 15 min. Afterwards, the substrates were subjected to the steam of boiling isopropanol, then coated with

100 nm titanium through electron beam evaporation under high vacuum (Edwards E306 coating system, Moorfield, UK).

Attachment of the CTA. The titanium-coated substrates were sonicated in acetone, ethanol and water for 5 min each, followed by drying using a nitrogen gun. Subsequently, the cleaned substrates were placed in a BioForce UV/Ozone Pro Cleaner (Bioforce Nanoscience, Ames, USA) for 20 min to activate the surfaces. In the meantime, the reaction mixture for attachment of the CTA was prepared by dissolving the CTA (70 mg) and DMAP (60 mg) in DCM (30 mL) under an argon atmosphere and cooling externally via an ice bath. The UV/Ozone-activated substrates were placed vertically in a Teflon sample holder and immersed in the reaction mixture under constant argon flow. The reaction chamber was closed to maintain the argon atmosphere. After 30 min, DCC (120 mg) dissolved in DCM (8 mL) was added to the solution containing the immersed substrates and the reaction was stirred overnight under an argon atmosphere at room temperature. Then, the substrates were thoroughly washed using ethanol and water, followed by drying using a nitrogen gun.

Polymerization of brushes via SI-RAFT. CAP methacrylate was used to form polymer brushes on the CTA-modified substrates. For this purpose, the substrates were placed vertically in a Teflon sample holder. The monomeric prodrug (2 g) and ACVA (24 mg) were dissolved together in 1,4-dioxane (20 mL) and added to the reaction chamber. Afterwards, the reaction mixture was sparged with argon via a metal needle with a secondary argon flow directly above the reaction mixture. After removal of the metal needle, the reactor was closed, maintaining the argon atmosphere. By immersing the reactor in an 80 °C oil bath, the polymerization reaction was initiated and conducted for specified time intervals. To stop the reaction, the reactor was removed from the oil bath and acetone was added to dissolve the precipitated polymer. The polymer brush-modified substrates were thoroughly washed with acetone, followed by immersion in THF (150 mL) for 45 min. Afterwards, the samples were washed with acetone, ethanol and water and dried using a nitrogen gun.

poly(CAP methacrylate) purification. After the reaction, the quenched reaction solution was precipitated in 1,4-dioxane (10× solvent volume). The supernatant was discarded, and the precipitated and sedimented polymer was dissolved in acetone and filled into a crystallization dish under constant air flow, allowing evaporation of the

solvent. After complete evaporation of acetone, the remaining polymer was dissolved and reprecipitated again. This process was repeated 5× in total to ensure the removal of contaminants and remaining monomer. To remove traces of dioxane, the polymer was dissolved again in acetone and then reprecipitated in chloroform (10× solvent volume), which was allowed to evaporate. Finally, the polymer was dissolved in acetone, reprecipitated in water (10× solvent volume) and freeze-dried until dry.

5. Investigation of an Imine-Based Polymer Brush Prodrug

This Chapter investigates the synthesis of, and drug release from, an imine-based polymer brush prodrug system. The drug released from the system was tested against *S. aureus*. Part of the work presented in this Chapter has been published in Polymer.ⁱ

5.1 Introduction and Objectives

Imine-based polymeric prodrugs rely on an imine bond formed between an antibiotic agent and a polymer chain, allowing release of the antibiotic based on the hydrolysis of the imine, forming an amine and a carbonyl, which is typically either a ketone or an aldehyde.¹¹⁰ Unlike the ester-based approach, imine-based systems are much more sensitive towards hydrolysis, promising a faster release of the antibiotic cargo.¹¹² Therefore, the synthesis of a monomeric prodrug that couples an antibiotic to a polymerizable moiety is hypothesized to enable the synthesis of a polymer brush prodrug, that can release the antibiotic based on the hydrolysis of the imine linker. The stability of the imine linker is expected to be susceptible to changes in the temperature and the pH of the aqueous environment, leading to a pH- and temperature-responsive release profile.¹¹² Sulfanilamide (SA), the first developed antibiotic of the group of sulfonamides, is a broad-spectrum antibiotic that is known to inhibit bacterial growth through the competitive inhibition of *p*-aminobenzoic acid.^{209,210} The main motif of all sulfonamides is the amide of *p*-aminobenzenesulfonic acid, which shows structural similarities to *p*-aminobenzoic acid in terms of the molecular dimensions (Figure 22).²¹¹ The primary aromatic amine offers a potential binding site to form imines, making sulfonamides excellent candidates for the synthesis of prodrugs.

ⁱ **Schumacher, J.**; Cusick, N.; Schütz, D.A.M.; Engelhard, C.; Schönherr, H. A polymer brush prodrug for antibiotic delivery from implant surfaces. *Polymer* **2026**, *349*, 129750.

Author contributions: **JS**: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **NC**: Writing – review & editing, Visualization, Methodology, Investigation, Data curation. **DAMS**: Writing – review & editing, Visualization, Methodology, Investigation. **CE**: Writing – review & editing, Supervision, Funding acquisition. **HS**: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

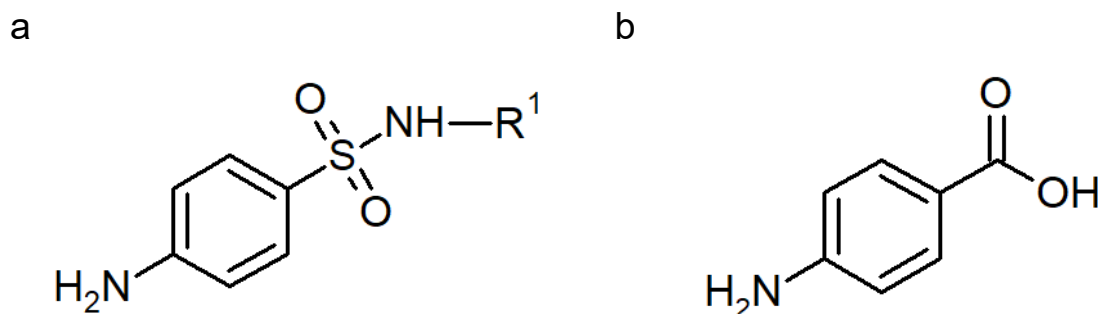


Figure 22 (a) General chemical structure of sulfonamide antibiotics with $R^1 = H$ for sulfanilamide and (b) the structure of *p*-aminobenzoic acid.

Sulfonamides act by inhibiting the formation of dihydrofolate and tetrahydrofolate, ultimately hindering the synthesis of folate, which is critical in the steps necessary for DNA replication.²¹¹ While folate is also essential for human cell growth and development, humans rely on dietary uptake rather than folate biosynthesis. Therefore, sulfonamides have no effect on human cells.²¹¹ However, due to rising antibiotic resistance in bacteria, many sulfonamides have become ineffective in treating bacterial infections.^{211,212} Nowadays, sulfonamides can still be applied for bacterial infections by co-administration with diaminopyrimidines, which act synergistically as dihydrofolate reductase inhibitors. The most commonly applied sulfonamide/diaminopyrimidine combination, co-trimoxazole, contains the sulfonamide derivative sulfamethoxazole (SMX) and the diaminopyrimidine trimethoprim (TMP) in a fixed mass ratio of 5:1 (SMX:TMP, Figure 23).

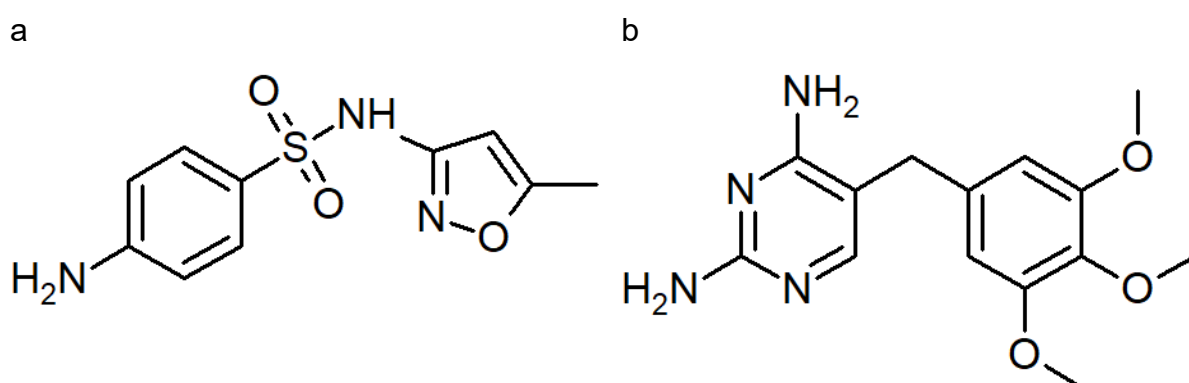


Figure 23 Chemical structures of (a) SMX and (b) TMP.

SA was chosen as a model drug as it is the fundamental specimen of the group of sulfonamides with the simplest chemical structure, a low price, as large quantities of the monomer are required to develop a working polymer brush synthesis, and its ease of detection via the UV-active benzene core in UV-Vis and HPLC. Furthermore, the relatively high solubility in aqueous media renders drug release experiments simpler

due to the ease of fulfilling perfect-sink conditions. To synthesize a polymerizable SA-based monomeric prodrug, an imine bond can be formed via a condensation reaction between the aromatic amine of SA and a polymerizable aldehyde.¹³³ However, polymerizable aldehyde-containing monomers suitable for radical polymerization are not readily available. Aldehyde functionalities are more commonly introduced into polymers by oxidative treatment of hydroxy groups.²¹³ Consequently, a two-step strategy is required, involving the formation of an imine precursor followed by the introduction of a polymerizable acrylic unit.

Alcohol-containing aldehydes (hydroxyaldehydes) offer both an aldehyde for imine formation and a hydroxyl group that can be esterified with acryloyl chloride. Among commercially available hydroxyaldehydes, hydroxymethylfurfural (HMF) and *p*-hydroxybenzaldehyde (pHBA) were considered for the formation of an imine precursor. HMF is a Maillard reaction byproduct present in heated sugar-containing foods, while pHBA is a flavoring agent listed by the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA), with an established safe daily intake threshold of 1800 µg. Both compounds are associated with low biological concern and are structurally suitable. However, practical considerations favor pHBA, as, unlike HMF, it does not require cooled storage and is significantly less expensive. Consequently, pHBA was selected as the aldehyde linker for the synthesis of the imine-based monomeric prodrug. Since SA contains only one reactive amine group (the amide functionality is considered unreactive under mild reaction conditions), direct reaction of the SA moiety in the imine precursor with acryloyl chloride can be excluded.

5.2 Results and Discussion

5.2.1 Synthesis of an Imine-Based Monomeric Prodrug from SA

The overall reaction equation for the formation of the monomeric prodrug is shown in Figure 24. In the first step, pHBA was dissolved together with SA under constant stirring in methanol, forming the imine precursor through the condensation reaction between the aldehyde functionality and the aromatic amine. The precursor precipitated from the reaction solution, enabling convenient filtering and obtaining the pristine precursor by further washing. To form a polymerizable monomeric prodrug, acryloyl chloride was

used to introduce the acrylic unit in an esterification of the phenol. For that, the imine precursor was stirred in acetone with triethylamine (TEA), and under dropwise addition of acryloyl chloride in acetone, the acrylate was obtained. Due to the formation of large amounts of TEA·HCl, strong stirring using a large stirring bar and a powerful stirrer was required. After the reaction, the solid residues were filtered and washed with methanol, leaving behind the pristine monomer.

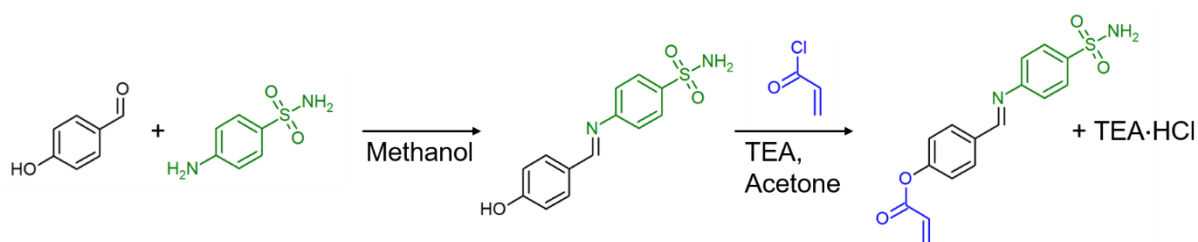


Figure 24 Reaction equation for the synthesis of the imine-based monomeric prodrug from pHBA and SA via the formation of an imine precursor and the subsequent esterification with acryloyl chloride.

The successful synthesis of the monomeric prodrug was confirmed through ^1H -NMR spectroscopy, ATR-FTIR spectroscopy, electrospray ionization mass spectrometry (ESI-MS) and CHNS measurements. The stacked ^1H -NMR spectra of the educts, the precursor and the monomeric prodrug can be seen in Figure 25. The integrals, as well as coupling constants, are given in Appendix 9.

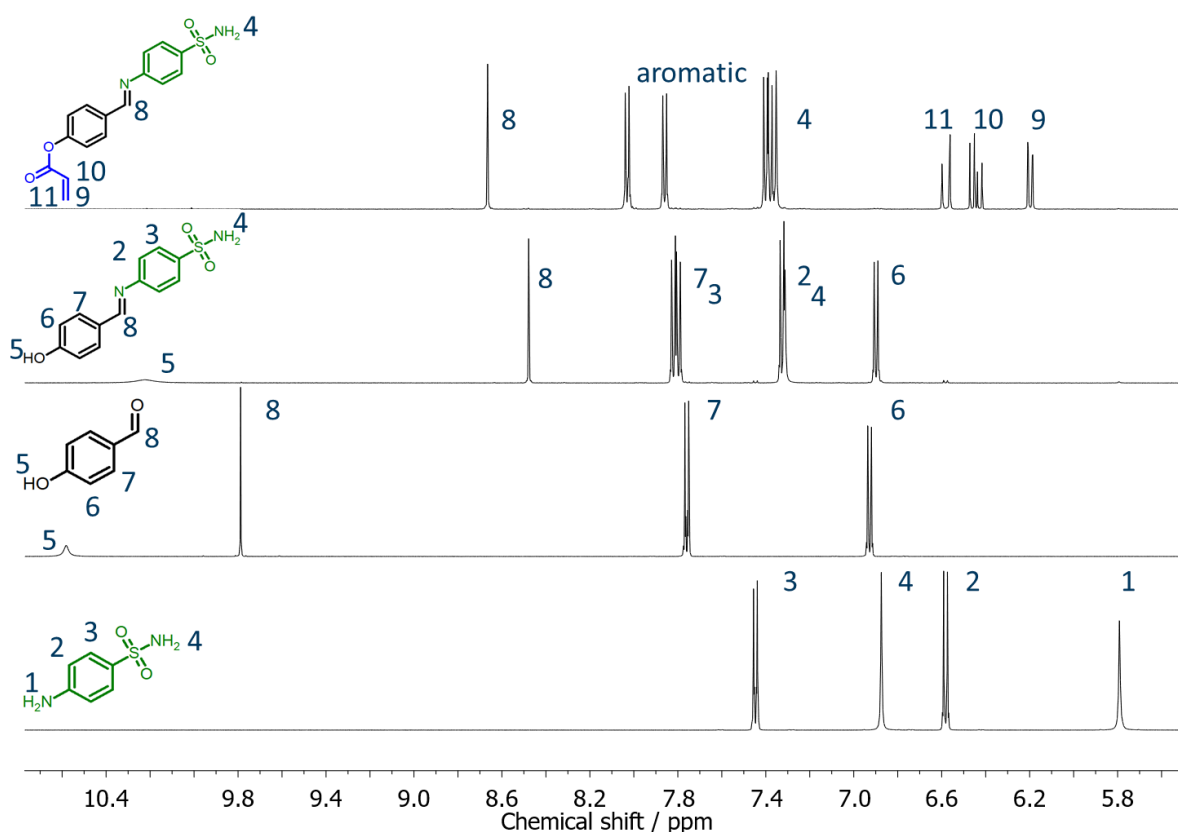


Figure 25 ^1H -NMR spectra of (from bottom to top) SA, pHBA, the imine precursor and the imine monomer, recorded in DMSO- d_6 and referenced to the DMSO- d_5 signal at 2.5 ppm.

In the ^1H -NMR spectrum, SA shows two signals that split into multiplets and show the typical shift of aromatic hydrogen atoms between 6 and 8 ppm, which simplifies the assignment of the signals. Two further signals appear as singlets (5.79 ppm and 6.87 ppm), which can be assigned to the amide and the amine. As expected, all signals possess similar peak areas, corresponding to the number of hydrogen atoms present in SA. As no further signals are present, the purity of SA can be confirmed. pHBA shows two aromatic signals with a similar multiplet splitting as SA, as both contain 1,4-substituted aromatic rings, a sharp signal at 9.79 ppm corresponding to the aldehyde, and a broad signal further downfield coming from the hydroxy group. The aromatic signals can be easily assigned based on increment calculations, while the sharp signal with a strong downfield shift is typical for aromatic aldehyde groups. A broad signal further downfield is common for phenolic hydrogen atoms. The integration of the signals further proves the assignment of the signals, as the aromatic signals yield peak areas twice as large as the assigned aldehyde signal. Only the phenol signal is smaller than the aldehyde signal, which is explained by the exchange of the acidic

hydrogen with deuterium from deuterated water commonly present in deuterated solvents.

After the formation of the imine precursor, the overlap of aromatic signals from the two benzene moieties, together with an overlapping amide signal, makes the assignment of the separate peaks difficult. However, the sharp signal at 8.48 ppm can be easily attributed to the imine hydrogen, which can be used to integrate the signals present. Based on the peak area of the imine signal, the overlapping aromatic and amide signals can be quantified, corresponding to 10 hydrogen atoms. A further broad signal, sitting at 10.23 ppm, can be assigned to the phenolic hydrogen, making the total hydrogen count 12. Based on the integration of signals, the successful synthesis of the imine precursor could be confirmed.

To turn the imine precursor into a polymerizable monomeric prodrug, the phenol group was reacted with acryloyl chloride. This reaction introduces three further olefinic hydrogens, which can also be seen in the ^1H -NMR spectrum of the obtained compound, with the corresponding signals between 6.2 – 6.6 ppm. Further proof of the successful synthesis is the disappearance of the phenolic hydrogen signal, indicating a successful esterification. Due to the strong overlap of the aromatic and the amide signal, a complete peak assignment proves difficult. Nevertheless, the strong singlet signal of the imine at 8.67 ppm can be used to integrate all signals present, which confirms the expected total hydrogen count of 14 hydrogen atoms, confirming the successful monomer synthesis.

Further analysis of the generated monomeric prodrug was performed by measuring IR spectra for each educt, the imine precursor, and the final monomeric prodrug, yielding additional confirmation for the successful synthesis (Figure 26).

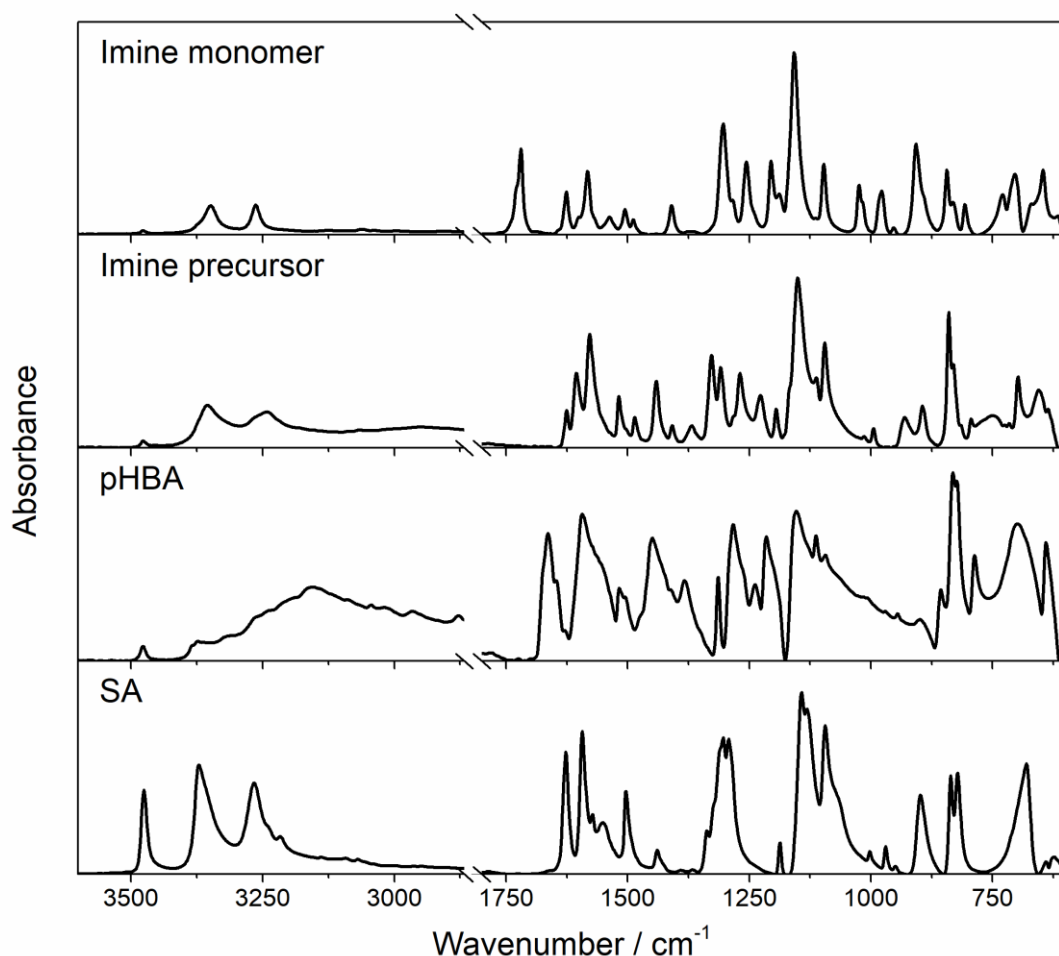


Figure 26 ATR-FTIR spectra of (bottom to top) SA, pHBA, the imine precursor and the imine monomer.

SA shows a typical amine absorption band at 3475 cm^{-1} , and two bands related to the amide functionality, located at 3250 cm^{-1} and 3370 cm^{-1} . Further prominent bands arise from the sulfonamide group, which shows absorption bands around 1300 cm^{-1} and 1140 cm^{-1} . The spectrum of pHBA shows a broad OH stretching band from 3400 cm^{-1} to below 2800 cm^{-1} . Another typical absorption band is located at around 1665 cm^{-1} , which corresponds to the aromatic aldehyde. Like SA, a strong signal related to the aromatic core of pHBA is present around 830 cm^{-1} , indicating *para*-substituted benzene moieties.

The imine precursor possesses two sharp bands above 3200 cm^{-1} corresponding to the sulfonamide functional group. A broad absorption band of the hydroxy group at around 3000 cm^{-1} further indicates the successful coupling of SA with pHBA. Another hint for the successful imine formation is the disappearance of the aldehyde signal and the appearance of the imine signal at 1625 cm^{-1} .

After the reaction of the imine precursor with acryloyl chloride, a strong absorption band at 1720 cm^{-1} proves the formation of an α,β -unsaturated ester. Furthermore, the disappearance of the broad hydroxide signal indicates the successful esterification reaction. As the sulfonamide group is not participating in the reaction, the two signals above 3200 cm^{-1} remain unaffected. The signal related to the imine group remains at 1625 cm^{-1} . The typical absorption band for acrylates is observed at 990 cm^{-1} . An overview of the absorption bands of the educts, the precursor and the monomeric prodrug is listed in Table 5.

Table 5 IR absorption bands of SA, pHBA, the imine precursor and the monomeric prodrug.

Wavenumber / cm^{-1}	Assignment	Vibration
SA		
3475	NH_2	N-H Stretching
3370 and 3250	$\text{SO}_2\text{-NH}_2$	N-H Stretching
1300 and 1140	$\text{SO}_2\text{-NH}_2$	N-H Stretching
837 and 822	Aromatic C=C	C=C Stretching
pHBA		
3200	Aromatic OH	O-H Stretching
1665	Aromatic CHO	C=O Stretching
830	Aromatic C=C	C=C Stretching
Imine precursor		
3354 and 3244	$\text{SO}_2\text{-NH}_2$	S=O Stretching
1625	Ar-C=N	C=N Stretching
1150	$\text{SO}_2\text{-NH}_2$	S=O Stretching
840	Aromatic C=C	C=C Stretching
Monomeric prodrug		
3347 and 3264	$\text{SO}_2\text{-NH}_2$	N-H Stretching
1720	RCOOR'	C=O Stretching
1625	C=N	C=N Stretching
1160	$\text{SO}_2\text{-NH}_2$	S=O Stretching
907	C=C-H	C-H out-of-plane Deformation

Together with the ^1H -NMR spectra, the IR data further substantiate the predicted reaction mechanism. To prove the purity of the generated compounds, CHNS analysis was conducted. The obtained compositions were then compared to the theoretical values and can be seen in Table 6.

Table 6 CHNS analysis of the imine precursor and the monomeric prodrug.

Element	Composition (Precursor) / m%		Composition (Monomer) / m%	
	Theoretical	Experimental	Theoretical	Experimental
C	56.51	56.54	58.17	57.99
H	4.38	4.32	4.27	4.43
N	10.14	9.96	8.48	8.54
S	11.6	11.71	9.71	9.66

The CHNS analysis further confirms the purity of the synthesized compounds ($\pm 0.2\%$), indicating the applicability of the monomer for the subsequent polymerization reaction. Additionally, high-resolution ESI-MS was employed to determine the molecular weight of the generated monomer, which has a theoretical mass of 330.0674 g/mol. Measurements of the analyte in positive detection mode revealed the presence of compounds with a mass-to-charge ratio of 331.0735, 332.0769 and 333.0689, with intensities corresponding to the natural isotopic signatures of $^{12}\text{C}/^{13}\text{C}$ and $^{32}\text{S}/^{34}\text{S}$. The measured mass-to-charge ratios and the distribution of the signal intensities confirm the presence of the protonated monomeric prodrug. Additionally, in the negative detection mode, three signals corresponding to the deprotonated monomeric prodrug were detected with a mass-to-charge ratio of 329.0594, 330.0629 and 331.0552, corresponding to the deprotonated molecule with the expected isotopic signatures, further confirming the successful monomer synthesis.

To estimate the density of the polymer, the density of the monomer was measured by immersing the monomeric powder in liquids of different densities. It was found that the monomer floated in trifluoroethanol ($\rho = 1.38 \text{ g/cm}^3$) while in DCM ($\rho = 1.33 \text{ g/cm}^3$) the monomer sank, indicating a density within this range. It was assumed that the polymer has a similar density to the monomer and for further calculations, a density of 1.35 g/cm^3 was assumed.

5.2.2 Synthesis of Poly(SA-pHBA Acrylate) Brush Prodrugs on Titanium

Before coupling of the CTA, the titanium-coated glass substrates were silanized with APTES to introduce amine functionalities to the surface (Figure 27).

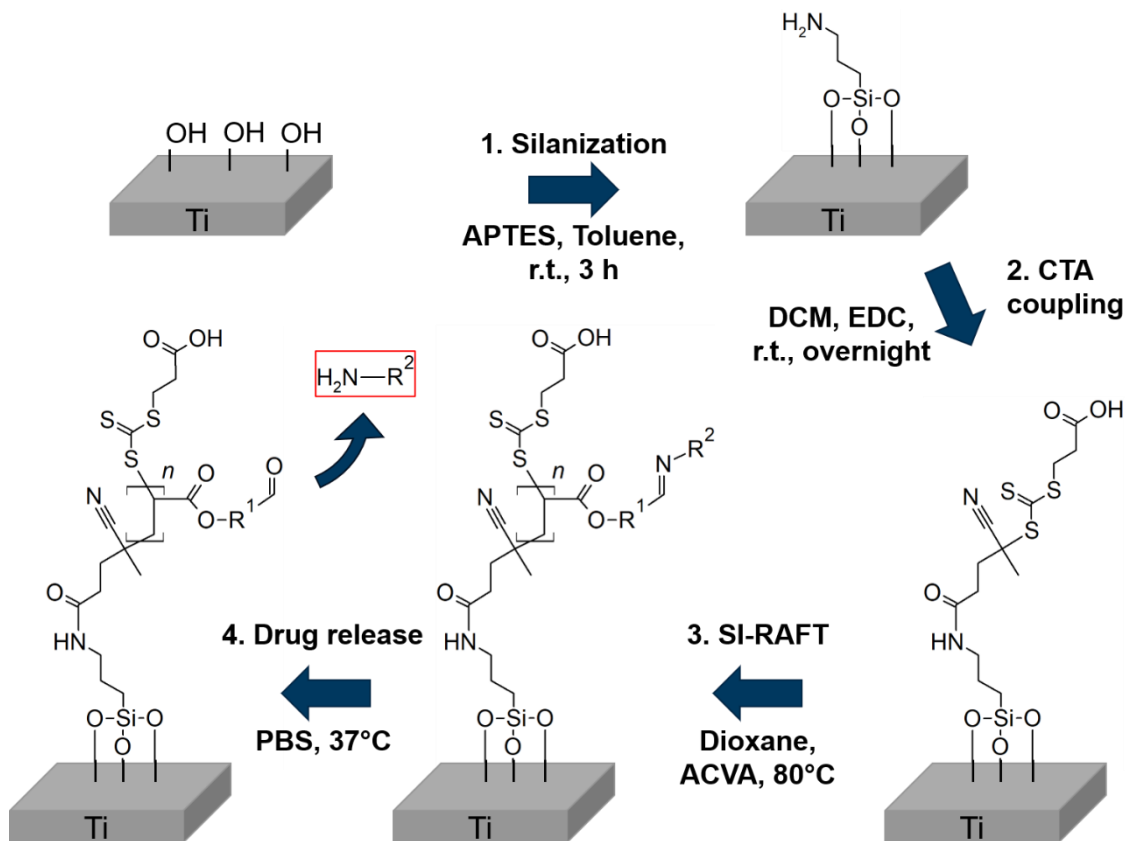


Figure 27 Surface modification with polymer brush prodrugs via SI-RAFT. Step 1, silanization; Step 2, CTA coupling; Step 3, SI-RAFT polymerization; Step 4, drug release based on hydrolysis of the imine bond.

The subsequent coupling of the CTA, which is now based on the formation of an amide, is hypothesized to yield higher stability of the anchoring of the polymer chain to the surface and a higher grafting density of the CTA due to the higher affinity of the EDC-activated carbonyl towards amines than to surface-grafted hydroxy groups. After the CTA-coupling, polymerization of the monomeric prodrug via SI-RAFT yields poly(SA-pHBA acrylate) brushes. The resulting XPS spectra can be seen in Figure 28.

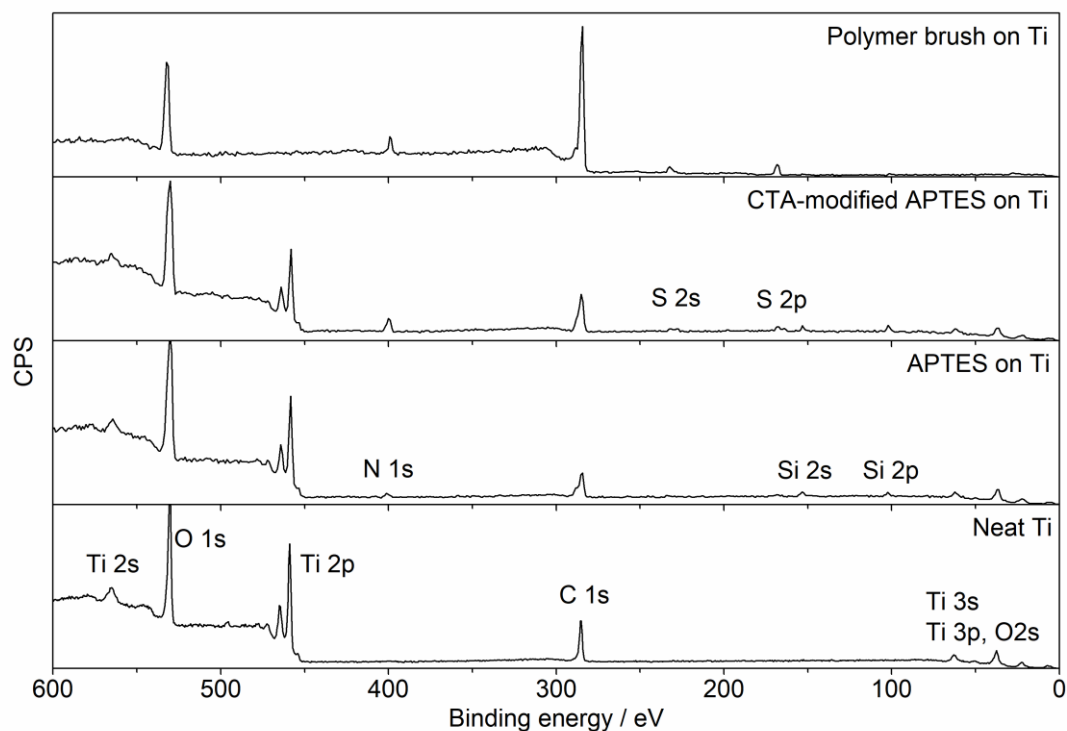


Figure 28 XPS survey spectra of the neat titanium substrate, the silanized titanium, the CTA-coupled substrate and the poly(SA-pHBA acrylate) brush layer on the titanium. All spectra were normalized to the aliphatic carbon contribution at 284.8 eV. Relevant peaks are highlighted.

The XPS spectrum of titanium was discussed in detail on page 29. After silanization, signals related to the silane are observable. Two silicon signals (2s, 2p) are present and together with the nitrogen signal (1s), the formation of an APTES layer was confirmed. Additionally, changes in carbon and oxygen signals indicate a change in surface chemistry. The attenuative effect of the silane layer leads to a decrease in the signal intensity of the underlying titanium substrate. Based on the survey spectrum before the coupling of the CTA, successful silanization can be concluded. The nitrogen-to-silicon ratio (0.7) was found to be lower than the theoretical ratio (1), indicating a potential oxidative loss of amines.²¹⁴ The high-resolution spectra can be seen in Appendix 10.

To generate a polymer brush film on the silanized substrates, the CTA was bound to the free amine of the silane layer, resulting in the formation of an amide bond and therefore the immobilization of the CTA. As the CTA introduces further carbon, nitrogen, oxygen and sulfur species, the change in the surface chemistry is expected to be reflected in the XPS data. After the reaction, further signals appear in the XPS

spectrum, which can be assigned to sulfur (2s, 2p), with further changes visible in the nitrogen and titanium signals. Due to the introduction of a nitrile functional group, the increase in the nitrogen signal can be explained, while the titanium signals simultaneously decrease in intensity. The presence of sulfur signals together with the change of the nitrogen signals and the decrease of the titanium signal confirm the attachment of the CTA. As explained in the previous Chapter, the degradation of sulfur-containing species yields a weaker sulfur signal than theoretically expected. Therefore, the theoretically expected sulfur-to-nitrogen ratio of a completely modified APTES monolayer, which would be 1.5, was instead found to be approximately 0.5. The high-resolution spectra can be seen in Appendix 11.

A detailed analysis of the high-resolution spectra of each reaction step, including deconvolution of each signal into different contributions of each element, allows the precise determination of the chemical composition of the measured surface. The determined chemical compositions for each reaction step are combined in Table 7.

Table 7 Chemical composition of each reaction step measured on the titanium face according to XPS.

Element	Surface modification		
	Ti / %	Silanized Ti / %	CTA on APTES on Ti / %
C	34.1	26.3	34.4
O	47.9	52.8	42.9
N	0	2.6	5.9
S	0	0	2.9
Si	0	3.7	3.5
Ti	18.0	14.6	10.4

After the polymerization, the brush layer exceeds the escape depth of the substrate-related photoelectrons, explaining the disappearance of the titanium signals. The signals detected can therefore be related to the brush layer only, which also matches the signals for a polymer of the expected composition. In the survey spectrum for the polymer brush, signals for sulfur, nitrogen, carbon and oxygen are present, corresponding to the composition of the homopolymer. The high-resolution spectra of the poly(SA-pHBA acrylate) brush prodrug are shown in Figure 29.

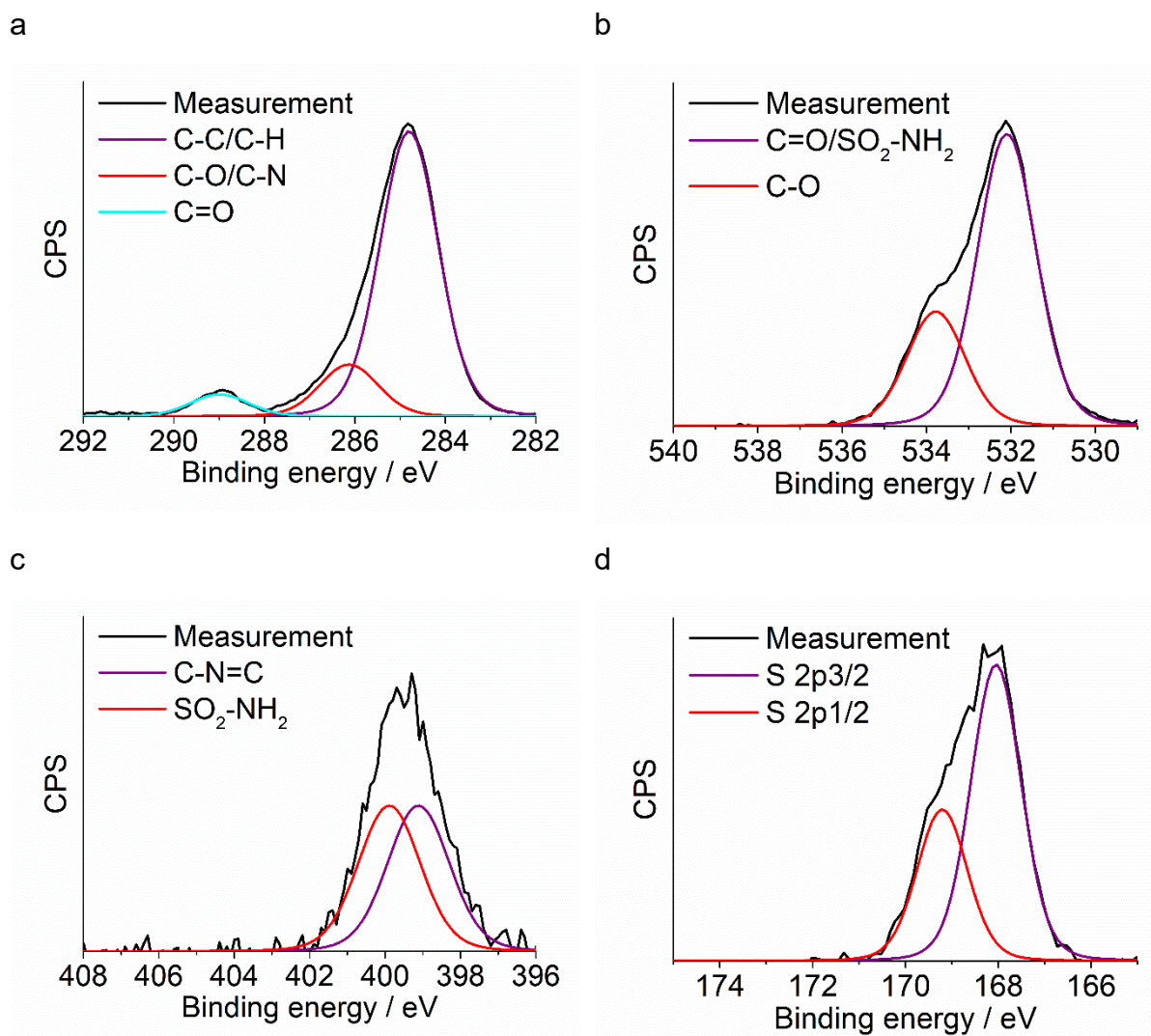


Figure 29 High-resolution XPS spectra of the poly(SA-pHBA acrylate) brush prodrug for (a) carbon, (b) oxygen, (c) nitrogen and (d) sulfur.

In the high-resolution spectrum of carbon of the poly(SA-pHBA acrylate) brush prodrug, three components can be identified, corresponding to the aliphatic polymer backbone and aromatic carbon species in the side chain (284.8 eV), oxygen- or nitrogen-bound carbon species (286 eV) and carbonyl species (289 eV). As the polymer contains mostly aliphatic and aromatic carbon species, the corresponding signal appears strongest in the high-resolution scan. In the oxygen spectrum, two signals can be differentiated, one corresponding to oxygen photoelectrons coming from the carbonyl and the sulfonamide (532 eV), while the other, weaker oxygen signal can be assigned to the phenol-ester species (533.8 eV). The deconvolution of the nitrogen signal allows assignment of two equally strong nitrogen contributions, which can be assigned to the imine (399 eV) and the amide (400 eV). In the high-resolution spectrum of sulfur, the signal can be deconvoluted into two components, corresponding to S 2p_{3/2} and S 2p_{1/2}.

This assignment can be explained by the typical spin-orbit splitting for photoelectrons from orbitals with an angular momentum quantum number >0 . The determined chemical compositions for the poly(SA-pHBA acrylate) brush prodrug and the theoretical chemical composition, as well as that of a hydrolyzed poly(SA-pHBA acrylate) brush, where all SA molecules have been liberated, are combined in Table 8.

Table 8 Theoretical and measured composition of the poly(SA-pHBA acrylate) brush prodrug and theoretical composition of the hydrolyzed brush according to XPS.

Element	Polymer brush prodrug		Hydrolyzed brush
	Theoretical / %	Experimental / %	Theoretical / %
C	69.6	70.0	76.9
O	17.4	21.4	23.1
N	8.7	5.1	0
S	4.3	3.5	0

Deconvolution of the high-resolution signals of the polymer brush yielded values matching the expected composition of the homopolymer of the synthesized prodrug with deviations up to 4%, which can be an artifact of peak-shape analysis as discussed in the previous Chapter. For carbon, a value of 70% is expected, which matches the measured value, while the observed content of oxygen (21.4%) is slightly higher than the expected value of 17.4%. Nitrogen and sulfur are similarly lower than the expected values (5.1% vs. 8.7% and 3.5 vs. 4.3%, respectively), indicating the partial hydrolysis and release of SA from the uppermost layer, potentially from remaining water in the solvent during the polymerization or the absorption of atmospheric water post-polymerization into the polymer film. A polymer brush which is completely hydrolyzed with all SA removed from the film would consist of only carbon, hydrogen and oxygen, with a theoretical chemical composition according to Table 8. As the carbon and oxygen content is much higher in the case of a polymer brush that has released the SA-cargo, a partial hydrolysis of the neat polymer brush prodrug can be assumed based on the observed lowered values for sulfur and nitrogen.

Further spectroscopic characterization of the polymer film was performed by IR spectroscopy to prove the successful incorporation of SA via a hydrolyzable imine bond and the successful polymerization. The resulting IR spectrum can be seen in Figure 30.

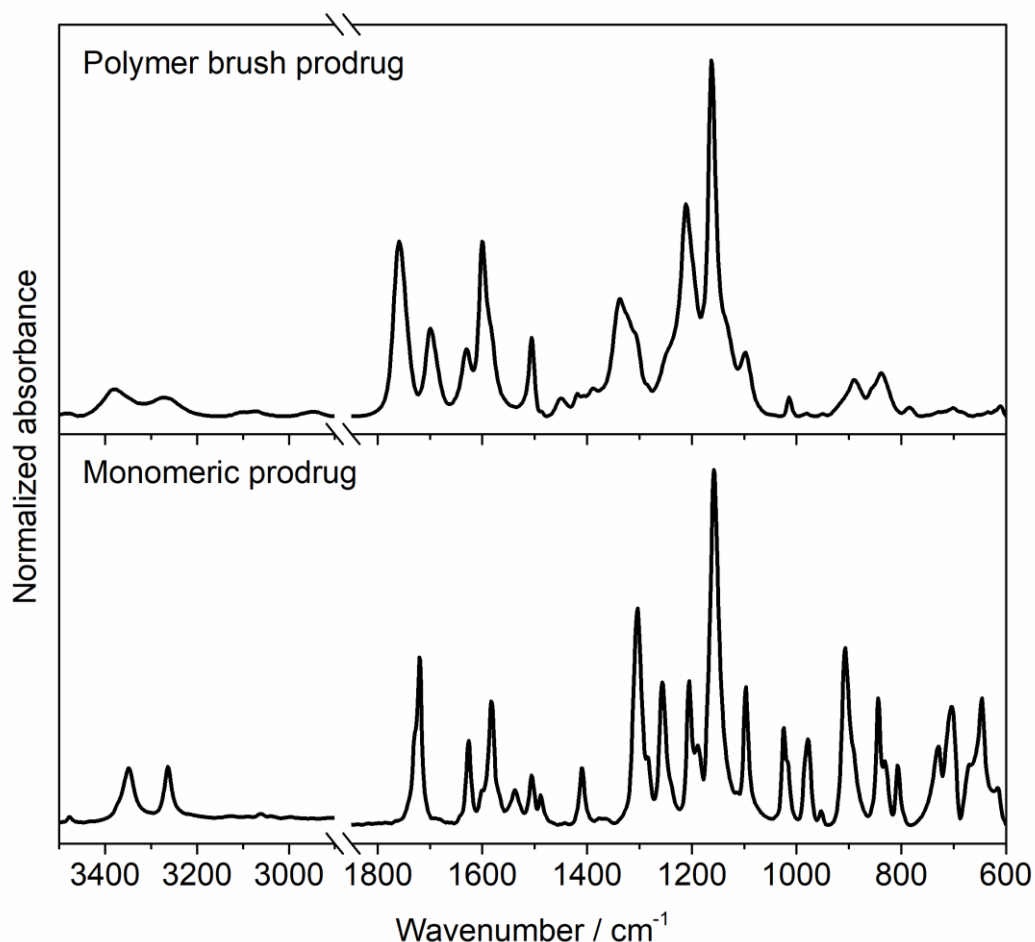


Figure 30 ATR-FTIR data of the monomeric SA acrylate prodrug and the RAIR spectrum of the poly(SA-pHBA acrylate) brush prodrug. Both spectra were normalized to the sharp absorption band of the sulfonamide at 1160 cm^{-1} .

Comparing the IR spectra of the monomeric prodrug with the poly(SA-pHBA acrylate) brush prodrug indicates successful incorporation of the monomer into the polymer brush. The absorption signals of the sulfonamide above 3200 cm^{-1} are present, while the absorption band of the ester shifts from 1720 cm^{-1} to 1760 cm^{-1} , due to the transformation from an α,β -unsaturated ester to an aliphatic ester. A direct proof of the incorporation of SA via an imine bond is the imine signal visible at 1631 cm^{-1} . Nevertheless, the strong signal at 1700 cm^{-1} indicates the presence of an aromatic aldehyde, which can only be explained by the partial hydrolysis of the film, which agrees with the observed XPS data. The absence of an olefinic signal at around 907 cm^{-1} confirms the lack of unreacted monomers adsorbed in the film.

Table 9 IR absorption bands of the monomeric SA acrylate prodrug and the poly(SA-pHBA acrylate) brush prodrug.

Wavenumber / cm⁻¹	Assignment	Vibration
Monomeric SA acrylate prodrug		
3347 and 3264	SO ₂ -NH ₂	N-H Stretching
1720	RCOOR'	C=O Stretching
1625	C=N	C=N Stretching
1160	SO ₂ -NH ₂	S=O Stretching
907	C=C-H	C-H out-of-plane Deformation
Poly(SA-pHBA acrylate) prodrug		
3376 and 3270	SO ₂ -NH ₂	N-H Stretching
1760	RCOOR'	C=O Stretching
1700	Aromatic CHO	C=O Stretching
1631	C=N	C=N Stretching
1338 and 1160	SO ₂ -NH ₂	S=O Stretching

To determine the thickness of the polymer brush film on the titanium-coated glass, ellipsometry was employed and the film thickness was determined for given polymerization times. The result can be seen in Figure 31.

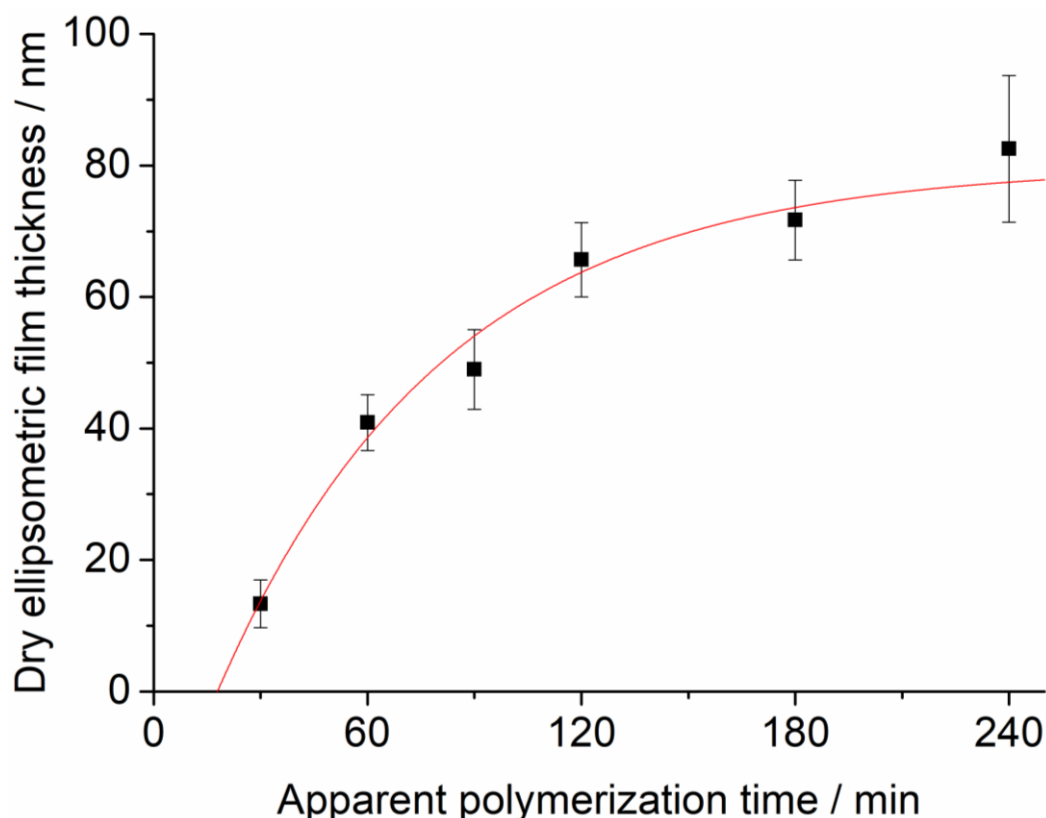


Figure 31 Dry ellipsometric film thickness as determined on the titanium-coated glass substrate for different polymerization times of poly(SA-pHBA acrylate) brushes. An asymptote has been fitted to the data to guide the eye and estimate the maximum film thickness achievable with this approach (red line).

An initial dormant period before the start of the polymerization reaction is observed. This is related to the definition of the start of the observation period, defined as the point where the cold reactor is immersed in the hot oil. The initiation of the polymerization requires the thermal decomposition of the initiator; therefore, brush growth only starts after this dormant period. The asymptote also helps identify the onset of the polymerization, which lies at approximately 17 min. The difference in the onset of the polymerization in comparison to the previous Chapter could stem from a different heat capacity of the polymerization solution. Initially, the brush growth follows a linear trend but levels off and reaches a maximum film thickness according to the fitted asymptote of ~80 nm. This trend indicates the loss of active chain ends, potentially due to loss of the CTA due to oxidation, recombination, or disproportionation reactions of the growing radical chain.

In addition to the growth of poly(SA-pHBA acrylate) brushes on the titanium-coated surface of the glass slide, brush growth was also observed on the glass face of the substrates. Although not considered beforehand, the polymer film on the glass face

can be explained as the coupling chemistry applied to the titanium face also applies to the glass face.²¹⁵ However, as the glass faces of the substrates were not directly exposed to the UV lamp within the UV-ozone chamber, less activation of the glass side is expected, leading to lower grafting densities and subsequently a lower film thickness.²⁹

Determining the film thickness of polymer brushes on glass cannot be achieved using ellipsometry due to the lack of a reflective substrate. Therefore, to determine the film thickness, AFM scratch tests were conducted. The AFM height images of substrates scratched on the glass face can be seen in Figure 32.

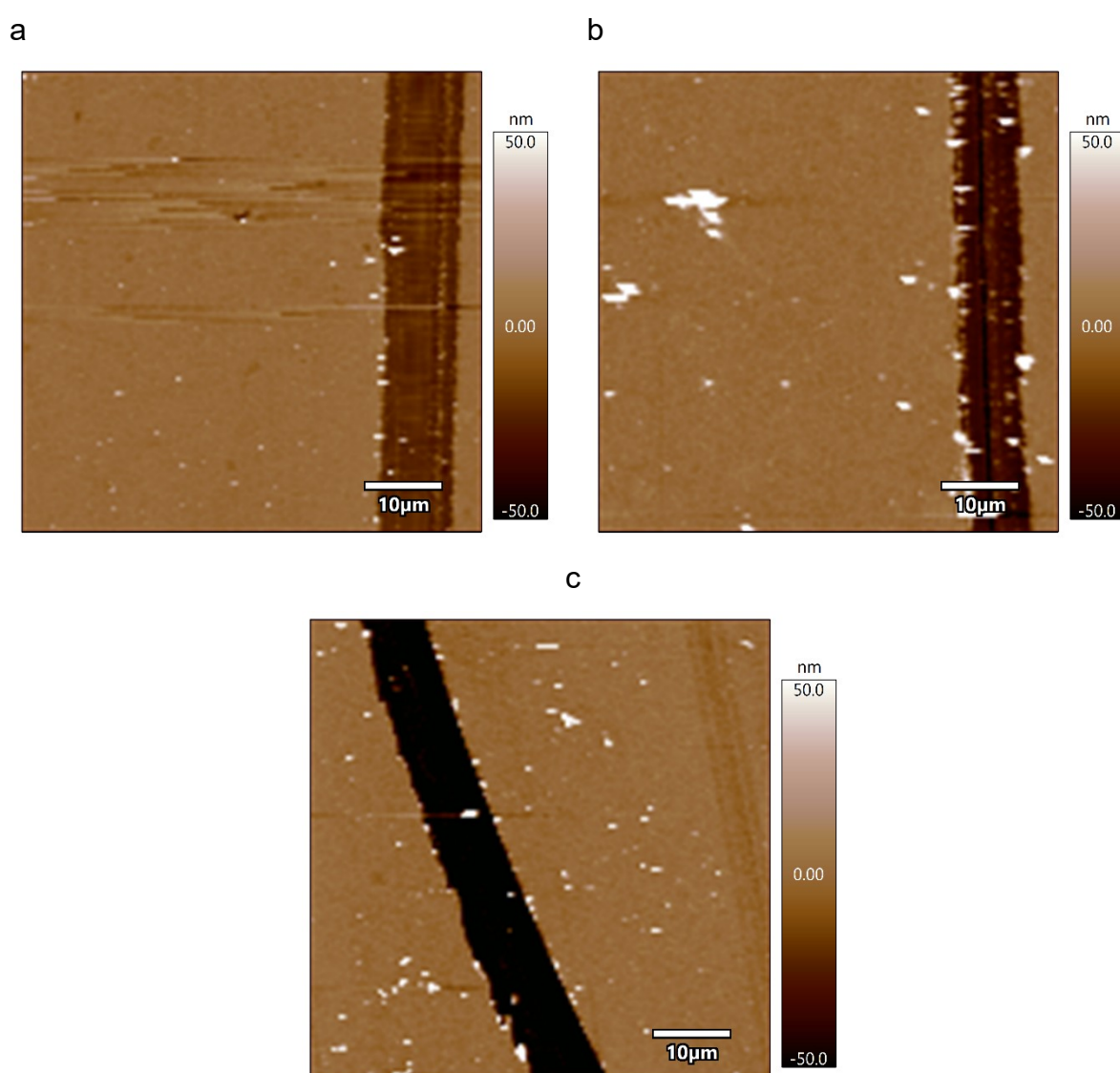


Figure 32 AFM height images of scratched glass face of poly(SA-pHBA acrylate) brush samples polymerized for (a) 60 min, (b) 90 min and (c) 120 min.

In the AFM images, the scratched substrate can be seen with a smooth polymer layer around the scratch. Only a few irregularities can be seen, which very likely stem from

adsorbed polymeric particles left over from the polymerization. However, the roughness of the polymer brush was determined from the AFM height images to be approximately 2 nm, which indicates the overall smoothness and homogeneity of the polymer brushes. Similar values for the roughness of polymer brushes were reported before.¹⁵¹ By plotting the pixel height values in histograms, bimodal distributions were obtained, as can be seen in Figure 33.

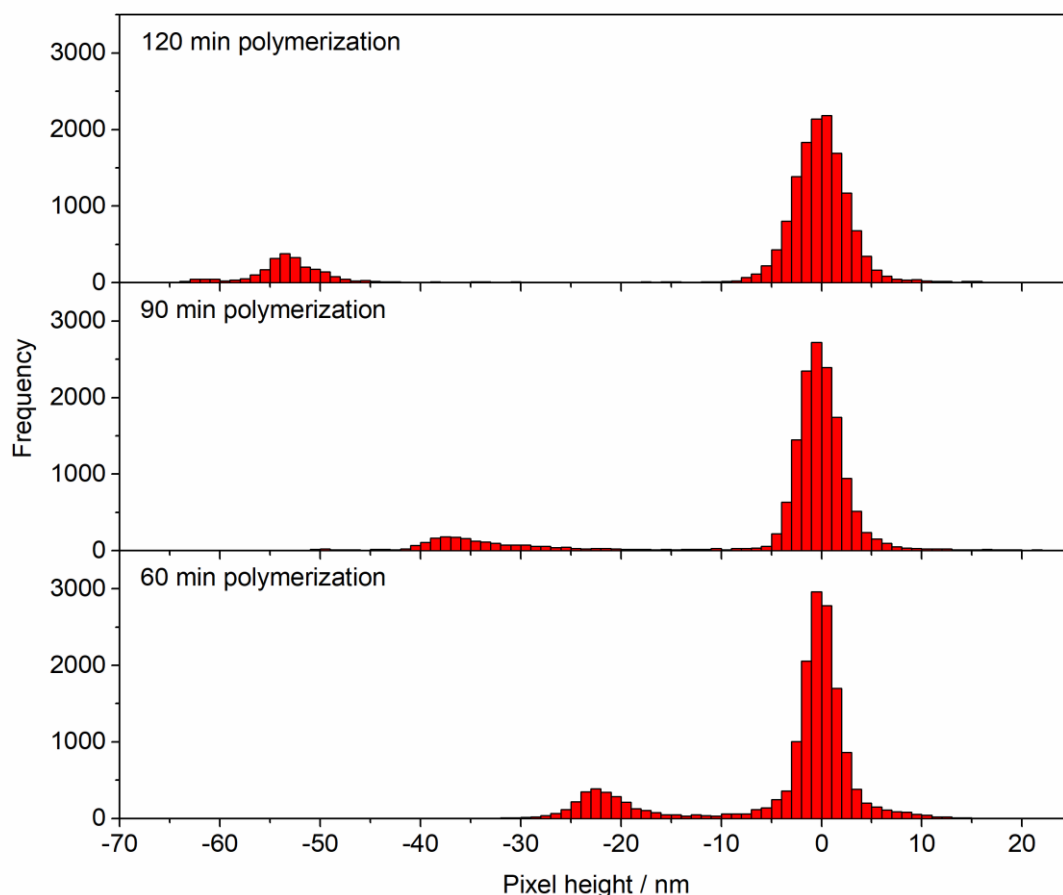


Figure 33 Pixel-height histograms determined from AFM measurements on the glass face of poly(SA-pHBA acrylate) brush samples polymerized for 60 min, 90 min and 120 min.

The step height was determined as the difference between the bimodal pixel height distributions, with one distribution set to 0 nm, corresponding to the polymer layer, and the other distribution corresponding to the scratched area. From the step height measurements, the film thickness was estimated and utilized to calculate the amount of SA in the polymer film on the glass face as well as on the exposed glass edges of the substrates. To confirm that the backside of the substrate is covered in polymer brushes of the same composition, XPS spectra were acquired. Due to the charging

effects of the underlying glass, the spectra are less clear than those measured on the titanium face. The resulting survey spectra can be seen in Figure 34.

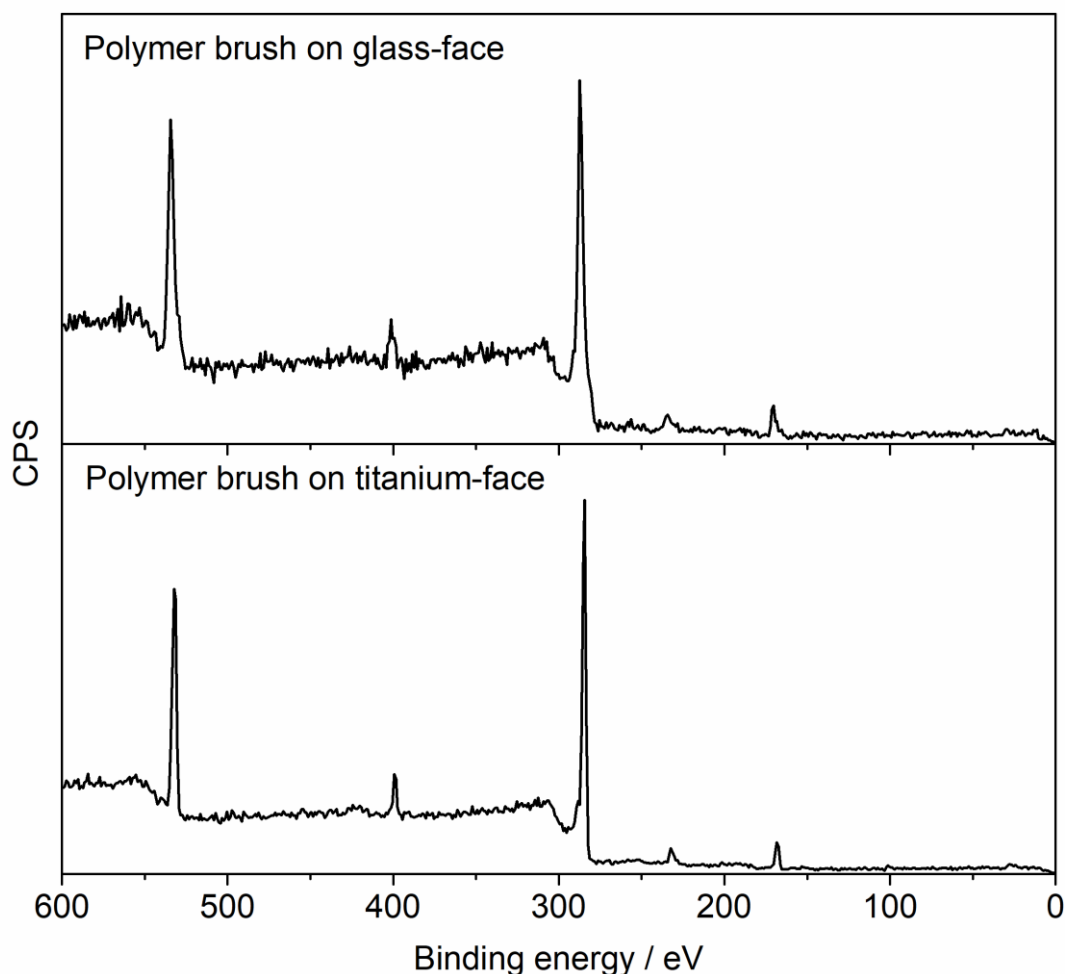


Figure 34 XPS survey spectra of the neat poly(SA-pHBA acrylate) brush prodrug on the titanium face and the glass face of the substrate, polymerized for 120 min.

Based on the high-resolution spectra acquired on glass and titanium, the chemical composition was determined and can be seen in Table 10. As discussed before, deviations observed in XPS can arise from peak shape analysis, partial hydrolysis of the prodrug, and in the XPS analysis of spectra acquired on glass, charging effects will interfere with the precise deconvolution of the signals.²¹⁵

Table 10 Chemical composition of the poly(SA-pHBA acrylate) brush prodrug (120 min polymerization) on the titanium face and the glass face according to XPS.

poly(SA-pHBA acrylate) brush prodrug			
Element	Theoretical / %	Experimental (Ti) / %	Experimental (Glass) / %
C	69.6	70.0	68.1
O	17.4	21.4	21.2
N	8.7	5.1	6.1
S	4.3	3.5	4.6

The determined composition of the poly(SA-pHBA acrylate) brush layer on the glass face of the substrate was found to be in good agreement with the polymer brush layer on the titanium face, with deviations below 2%. Slightly higher sulfur and nitrogen signals of polymer brushes on the glass face potentially indicate a smaller degree of humidity-related hydrolysis of the imine. Nevertheless, the small differences compared to the more precise values determined on the titanium face prohibit a definite conclusion of the degree of hydrolysis.

By measuring the film thickness on the glass face and on the titanium face, the total film volume was determined. From the total film volume and the estimated density of the monomer, together with the dimensions of the substrates, the amount of SA was estimated and included in the plot of the brush growth kinetics, as can be seen in Figure 35.

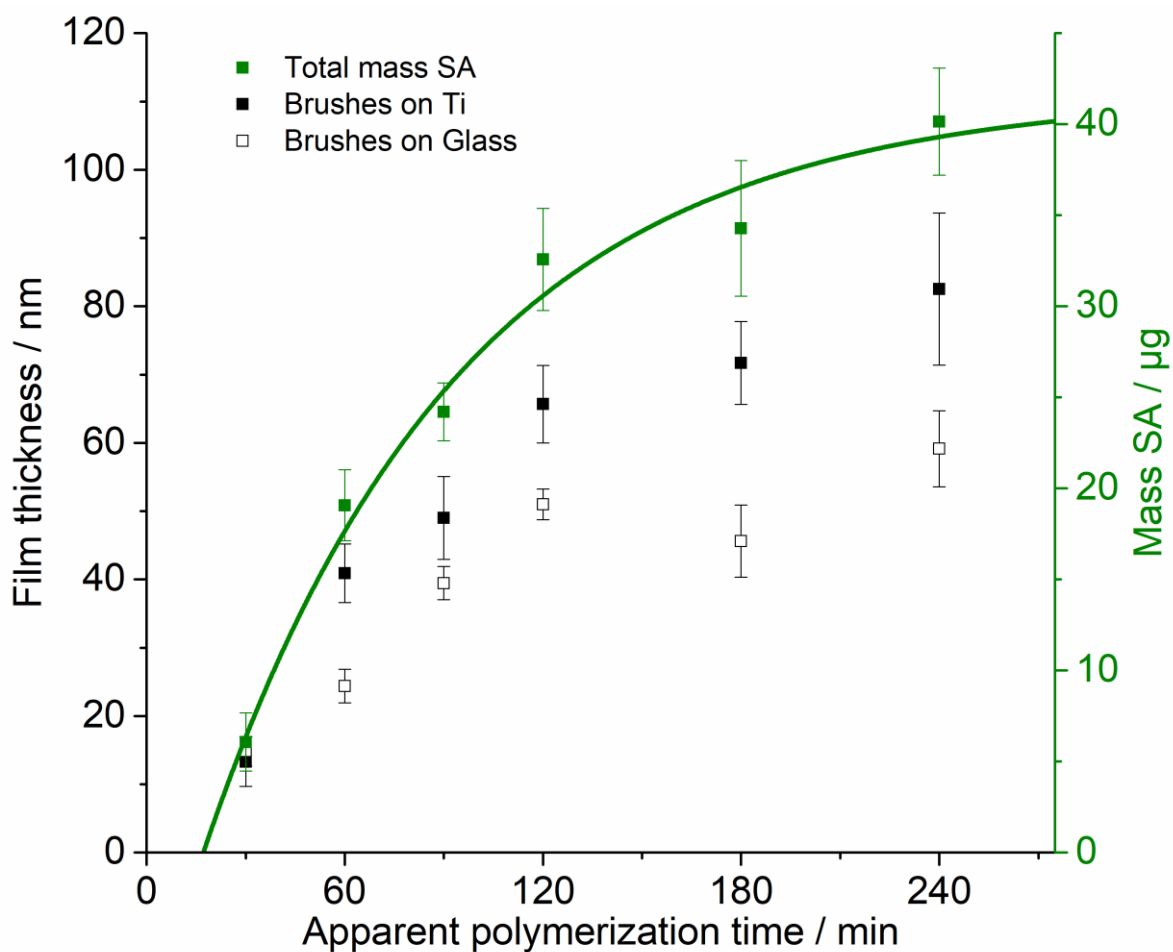


Figure 35 Poly(SA-pHBA acrylate) brush film thickness on the titanium face (black filled squares) and on the glass face (black hollow squares) of the substrates for different polymerization times measured by ellipsometry and AFM, respectively. The total amount of SA stored in substrates polymerized for a given time was calculated based on the dimensions of the substrates and the estimated density of the polymer ($\rho = 1.35 \text{ g/cm}^3$) and plotted with an asymptote, indicating the maximum amount of SA loaded per substrate.

Based on the approximated density of the polymer ($\rho(\text{polymer}) = 1.35 \text{ g/cm}^3$) and the dimensions of the substrate, the mass of SA stored in the brushes ($m(\text{SA})$) was approximated according to Equations 6 and 7, with M being the molar mass. The two y-axes in Figure 35 are therefore not scaling linearly, as the brush-modified areas were assumed to be different for the titanium (3.75 cm^2) and the glass sides ($3.75 \text{ cm}^2 + 0.8 \text{ cm}^2$ (edges of the substrates))).

$$V(\text{polymer}) = \text{area} \cdot \text{thickness}(\text{brush on Ti}) + \text{area} \cdot \text{thickness}(\text{brush on Glass}) \quad \text{Equation 6}$$

$$m(\text{SA}) = \rho(\text{Polymer}) \cdot V \cdot \frac{M(\text{SA})}{M(\text{Monomer})} \quad \text{Equation 7}$$

The growth of poly(SA-pHBA acrylate) brushes on the glass face follows the same trend as on the titanium face, with the obtained polymer layer on the glass face consistently ~70% of the thickness of the polymer film on the titanium face. Therefore, the amount of SA that can be stored in the polymer brush film increases similarly with increasing polymerization time and levels off at around 40 μg of SA stored in the brushes. The polymer brush-coated substrates used by Choi et al. were able to release 3 $\mu\text{g}/\text{cm}^2$ of levofloxacin from 400 nm thick brushes, which corresponds to 8.3 nmol/cm^2 , assuming polymer brushes were only on the titanium face of the used substrates.¹³ If only prodrug brushes on the titanium face are considered with the maximum thickness obtained in the polymerizations described here (80 nm), this would equate to 32 nmol/cm^2 , a 4 $\times$ higher loading capacity. Extrapolating the prodrug brush thickness to 400 nm, the loading capacity would increase to 164 nmol/cm^2 , which is 20 $\times$ higher than that achieved by Choi et al.¹³ As it was not clarified whether both sides of their substrates were modified with polymer brushes, which is highly likely with the described coupling chemistry and would therefore increase the surface area, the observed drug loading capacity is likely overestimated, as the brush-covered glass face was not taken into account.

5.2.3 Drug Release Behavior from Poly(SA-pHBA Acrylate) Prodrugs

To investigate the release of SA from the brush-modified substrates, UV-Vis and HPLC were utilized to detect and quantify the amount of SA in the drug release supernatant. Furthermore, AFM and ellipsometry were utilized to study morphological changes of the remaining polymer brushes and estimate the amount of SA released based on the approximated density of the polymer brush. Additionally, RAIRS and XPS spectra were acquired to investigate the chemical composition of the brushes after defined drug release times.

5.2.3.1 SA Release Studied by UV-Vis and HPLC in PBS

Before the drug release studies, calibration curves for SA in PBS were acquired. A dilution series of SA was prepared and measured by UV-Vis, from which the absorption maximum was determined and plotted against the corresponding concentration of the SA solution. The extinction coefficient was determined as the slope of the calibration

curve and utilized to quantify the release of SA during the drug release experiments. The UV-Vis spectra of SA in PBS with the corresponding calibration curve can be seen in Figure 36.

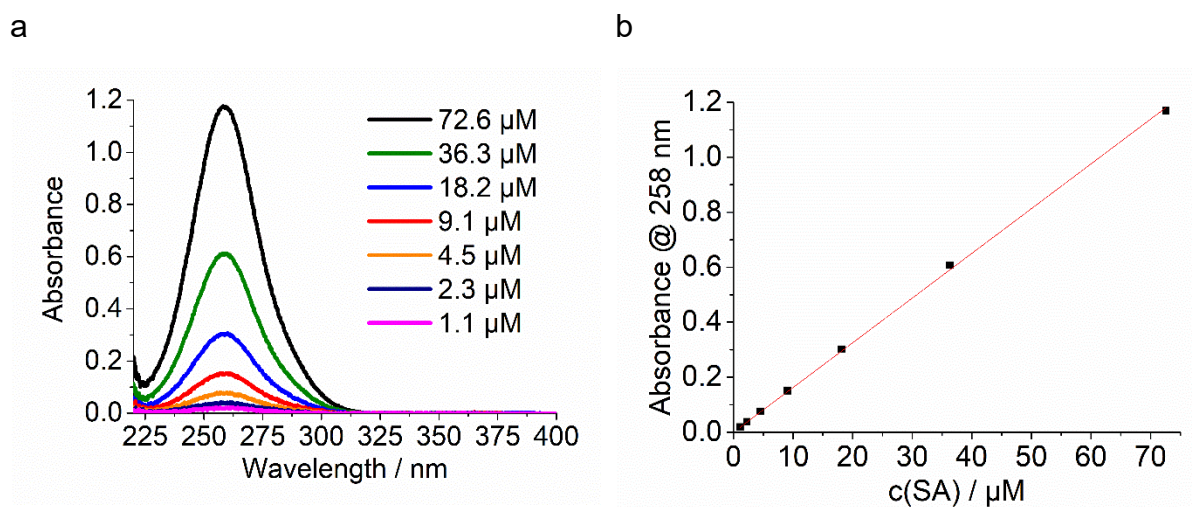


Figure 36 (a) UV-Vis spectra of SA in PBS at different concentrations and (b) the corresponding calibration curve. The extinction coefficient was determined to be $16.3 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ with a regression coefficient $R^2 = 0.9997$.

SA absorbs UV light with an absorption maximum at 258 nm. The absorption increases proportionally with the concentration of SA in PBS, resulting in an extinction coefficient of $16258 \text{ L mol}^{-1} \text{ cm}^{-1}$. Based on the calibration curve, the amount of SA released from the brush-covered substrates can be estimated through the measurement of the optical density at 258 nm. While UV-Vis can be used to observe the change in the optical density, it does not prove the release of chemically intact SA. Therefore, HPLC was employed to not only quantify SA, but also confirm the release of chemically pristine SA based on the retention time of the neat compound. Beforehand, a stock solution of SA in PBS was prepared, which was serially diluted and measured by HPLC to determine the retention time and utilize the peak area at a given concentration to derive a calibration curve. The resulting chromatograms and the calibration curve are given in Figure 37.

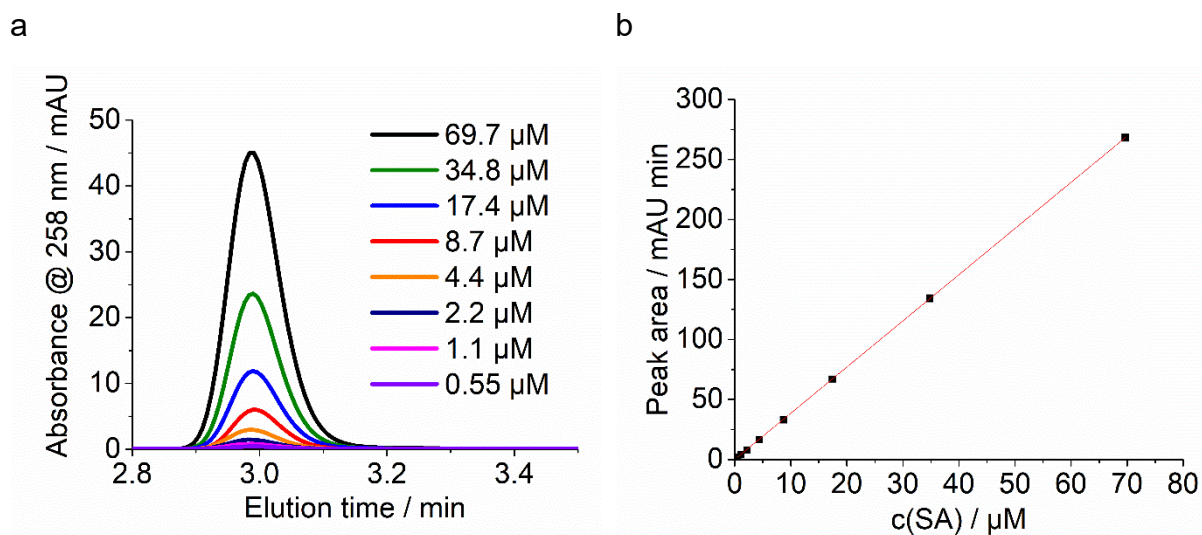


Figure 37 a) HPLC chromatograms and b) the corresponding calibration curve of SA measured in PBS at different concentrations. The slope was determined to be $3.85 \cdot 10^6 \text{ mAU min M}^{-1}$, with a regression coefficient $R^2 = 0.9999$.

SA was found to have a retention time of approximately 3 min, and by measuring the peak area, a calibration curve was obtained which can be used to determine the amount of SA in a PBS solution. The determined calibration curves were used to determine the concentration of SA in the drug release supernatant. The spectra and chromatograms for SA released from poly(SA-pHBA acrylate) brushes of different thicknesses can be seen in Figure 38.

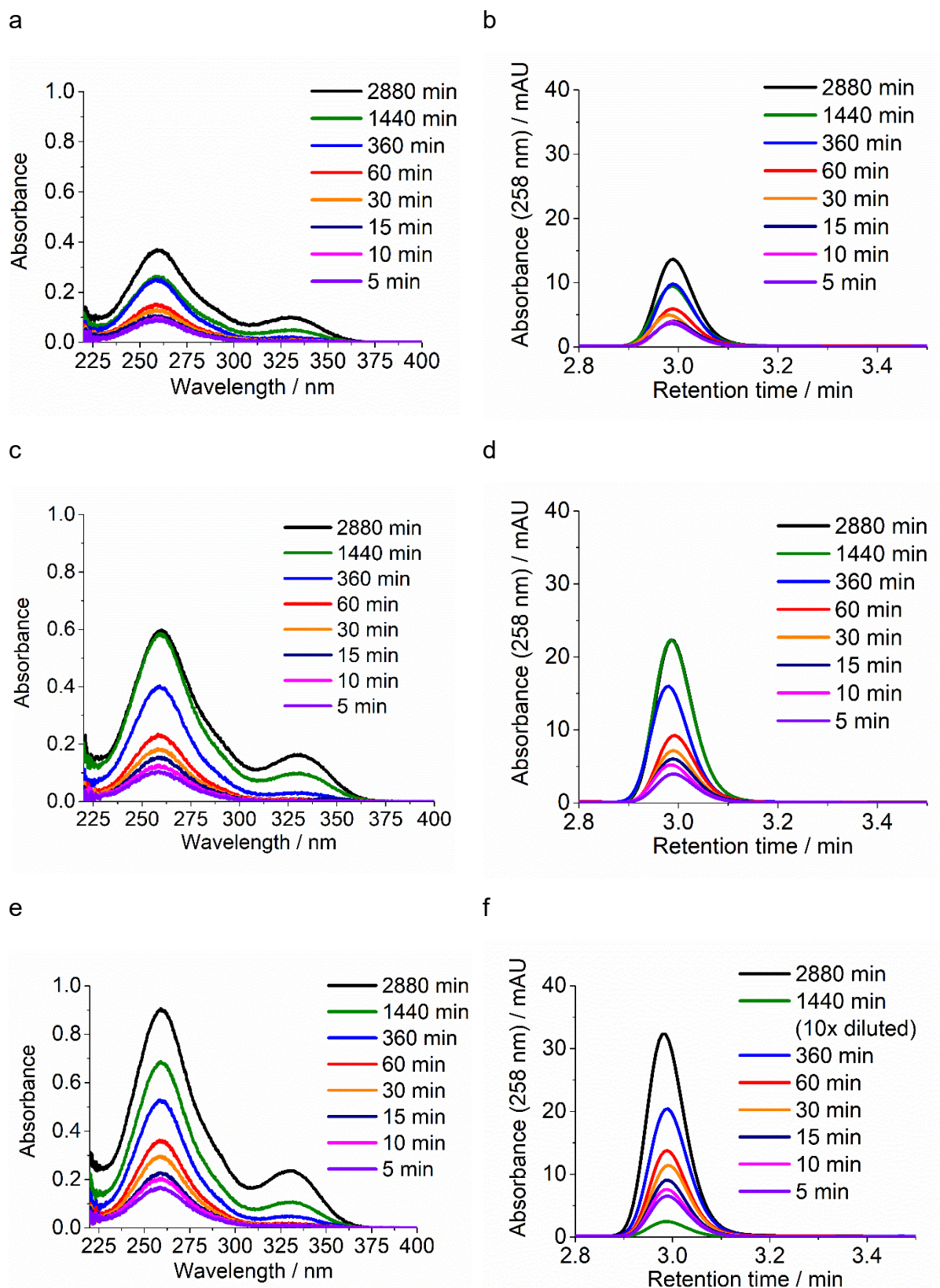


Figure 38 Drug release supernatants studied by a, c, e) UV-Vis and b, d, f) HPLC after incubations up to 48 h in PBS for poly(SA-pHBA acrylate) brush-modified substrates polymerized for a, b) 60 min, c, d) 90 min and e, f) 120 min.

As can be seen in the UV-Vis spectra across samples of different polymerization times and hence different polymer brush thicknesses, a signal emerges at 258 nm after 5 min of incubation, corresponding to the release of SA. With increasing incubation time, the signal intensity increases, indicating a time-dependent release of SA from the brush-modified samples. After approximately 6 h, a further signal emerges at around 330 nm, which also increases over the course of the next two days. As can be seen in Figure 39, the additional signal can be assigned to pHBA released from the brushes through the hydrolytic reaction of the ester bond between the polymeric backbone and the pHBA moiety. The ester is assumed to be less stable in comparison to the previous Chapter due to the electronic structure of the pHBA moiety. The aldehyde functionality with its negative mesomeric effect on the aromatic ring is in the para position to the phenolic ester, therefore destabilizing the ester bond and hence lowering the energetic barrier for hydrolysis of the ester. This effect has been observed before by Jencks et al., where the rate constant of the hydrolysis of *p*-nitrophenyl acetate was more than 20× higher in comparison to unsubstituted phenyl acetate at 25 °C.²⁰⁶ pHBA was also tested for its antibacterial effect by itself, and in combination with TMP; however, no antibacterial effect was observed.

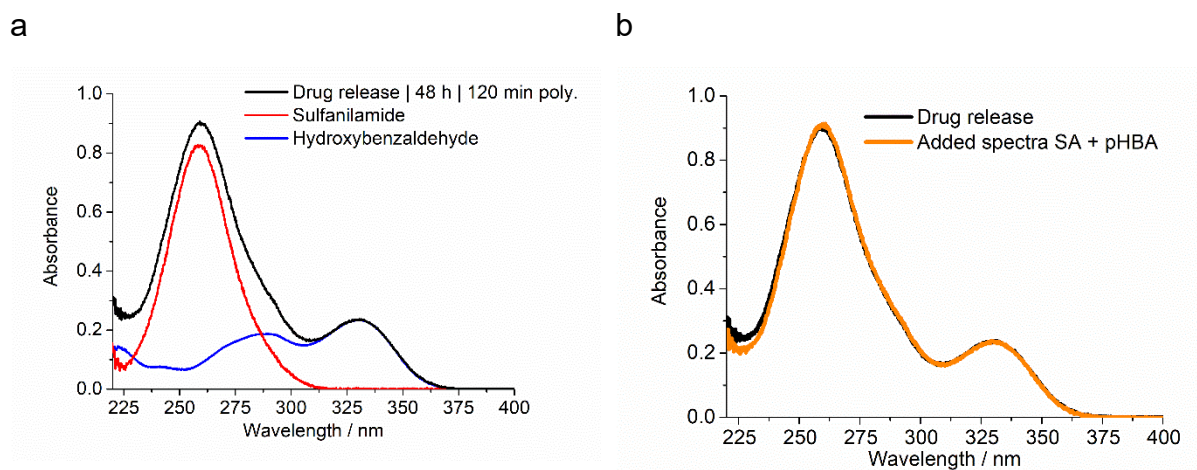


Figure 39 (a) UV-Vis spectra of the drug release from a poly(SA-pHBA acrylate) brush-modified substrate polymerized for 120 min and incubated for 48 h in PBS at 37 °C (black), the absorption spectrum of SA in PBS (red) and the absorption spectrum of pHBA in PBS (blue). (b) The UV-Vis spectrum of the drug release from a poly(SA-pHBA acrylate) brush-modified substrate polymerized for 120 min and incubated for 48 h in PBS at 37 °C (black) overlaid with the combined spectra of SA and pHBA (yellow).

The HPLC experiments confirm the presence of SA, as the same retention time was observed and the peak area increased in the same fashion as observed for the UV-Vis maximum. However, the secondary species released from the polymer brushes could

not be detected in the timeframe studied by the HPLC, hinting at a secondary species with either a longer retention time or a retention time exactly equal to that of SA. Further HPLC experiments were conducted with longer measurement times and detection at multiple wavelengths, but no further signals were observed. The signal corresponding to SA appears weaker by a factor of 4 when measured at 286 nm instead of 258 nm, which agrees with the absorption spectrum of SA (Figure 36). However, in the absorption spectrum of the drug release for the sample polymerized for 120 min and incubated in PBS for 48 h, the absorption ratio at 258 nm to 286 nm observed in the UV-Vis spectrum is approximately 2. This change in ratio was not observed in the HPLC chromatogram, indicating the sole elution of SA in HPLC, as can be seen in Appendix 12. The secondary species could therefore not be detected in the HPLC.

Based on the HPLC and UV-Vis data with the corresponding calibration curves, the release of SA was quantified and plotted to determine the drug release kinetics. The obtained plot can be seen in Figure 40.

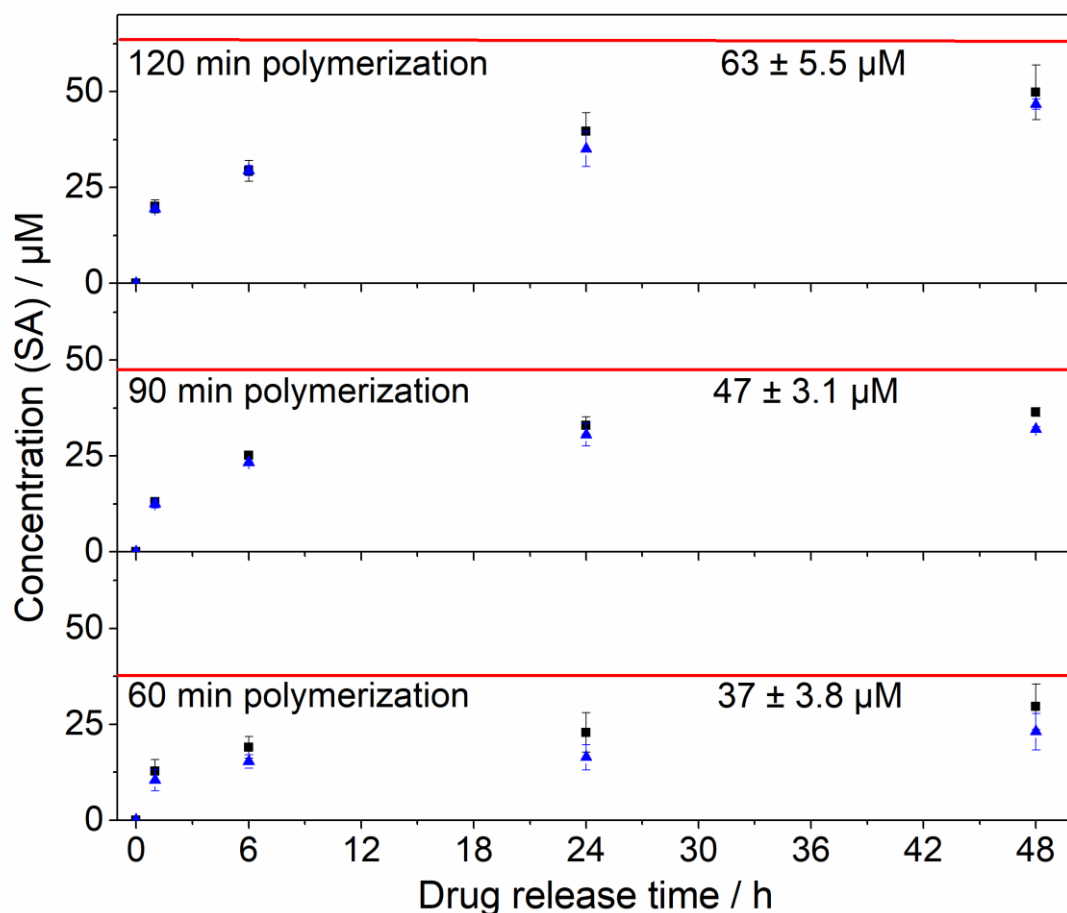


Figure 40 Plot of the concentrations of released SA against the incubation time for poly(SA-pHBA acrylate) brush-modified substrates polymerized for 60 min, 90 min and 120 min. The concentrations were determined based on the UV-Vis absorption maxima at 258 nm with an extinction coefficient of $16258 \text{ M}^{-1} \text{ cm}^{-1}$ (black squares), or the area of the SA signal in the HPLC-UV chromatograms (blue triangles). The concentration of SA in the supernatant of the drug release was observed over the course of 48 h at 37 °C in PBS. The maximum amount of SA that could be released from the brushes was estimated according to the initial brush thickness and is shown for each plot as a red line with the concentration written underneath.

As can be seen in the kinetic plot of the drug release experiments, independent of the film thickness, a burst release of the antibiotic is observed, which levels off after the first hour of incubation. Even after 48 h of incubation, the amount of drug released from the substrates does not reach the estimated maximum of SA, indicating the incomplete release of SA from the polymer film. The UV-Vis signal is consistently higher than the HPLC signal, indicating that the absorption at 258 nm stems not only from SA but also from the secondary species released from the brushes, leading to an overestimation of the drug concentration. This effect can be explained by the weak absorption of released pHBA, effectively increasing the absorbance at 258 nm. The highest achieved

drug concentration released from the thickest brushes is around 50 μM , which corresponds to approximately 0.11% of the maximum reported solubility of SA in water (7.5 g/L or 43.6 mM), indicating that perfect sink conditions ($c \leq 10\% c_{\text{max}}$) are fulfilled and the released SA does not reach saturation.²¹⁶ The early stage of the drug release experiments, as studied by UV-Vis and HPLC, can be seen in Appendix 13.

5.2.3.2 SA Release Studied by AFM and Ellipsometry

The substrates that underwent drug release experiments were investigated for morphological changes after incubation in the release solution. Ellipsometry was utilized to determine changes in thickness on the titanium face of the samples, while for AFM experiments, the glass face of each substrate was scratched, and the resulting scratch was scanned to determine the step height after the drug release. The change in the film thickness was then utilized to estimate the amount of SA released, based on the dimensions of the substrate and the approximated density of the polymer. The observed release of pHBA was not considered for the analysis of film thickness-related changes. The change in film thickness on the titanium face, as measured by ellipsometry, can be seen in Figure 41.

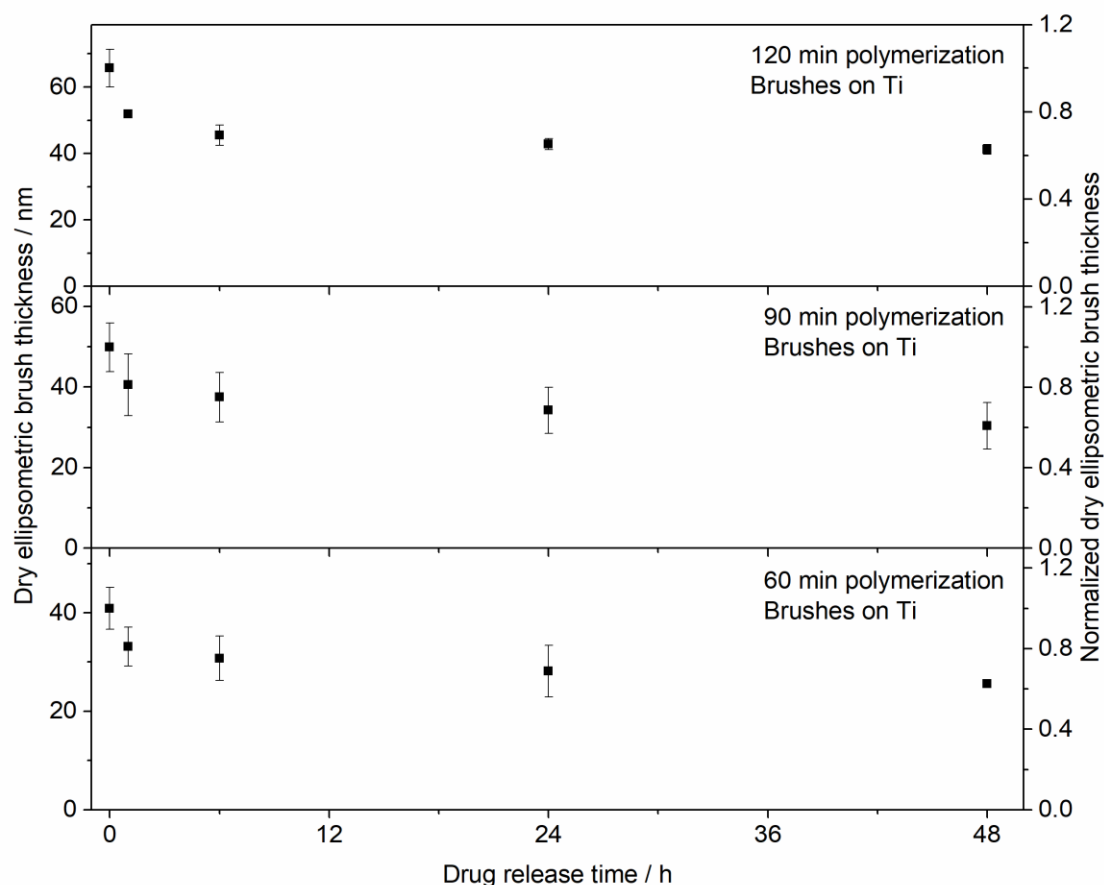
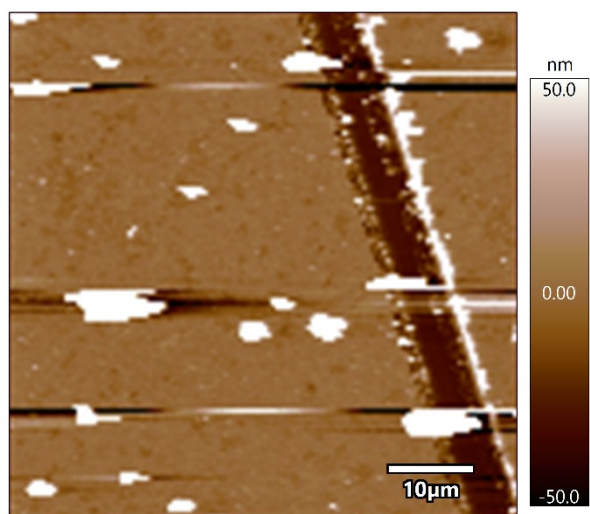


Figure 41 Change in film thickness on the titanium face upon incubation in the drug release supernatant (incubation in PBS at 37 °C) for poly(SA-pHBA acrylate) brush-modified substrates polymerized for 60 min, 90 min and 120 min as measured by ellipsometry. The film thickness is shown together with the change in film thickness normalized to the initial film thickness.

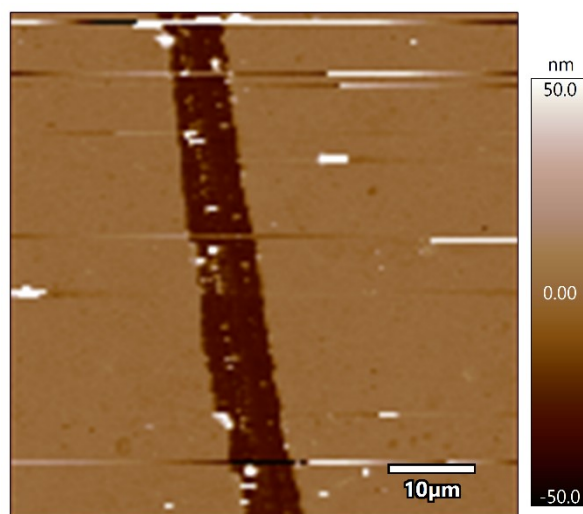
Upon incubating the substrates in PBS, the film thickness decreased over the course of 48 h. For all substrates, a fast initial decrease can be seen during the first hour, followed by a slower, gradual decrease over the next two days. Independent of the polymerization time and therefore also of the film thickness, all samples decrease in thickness to approximately 60% of the initial thickness, as can be seen in the normalized film thickness. The early stage of the drug release experiments, as studied by ellipsometry, can be seen in Appendix 14.

A similar trend is expected to be visible for the polymer brushes, which are present on the glass face of the substrates. The AFM images were recorded for samples polymerized for 90 min and incubated in PBS for up to 48 h. The images can be seen in Figure 42.

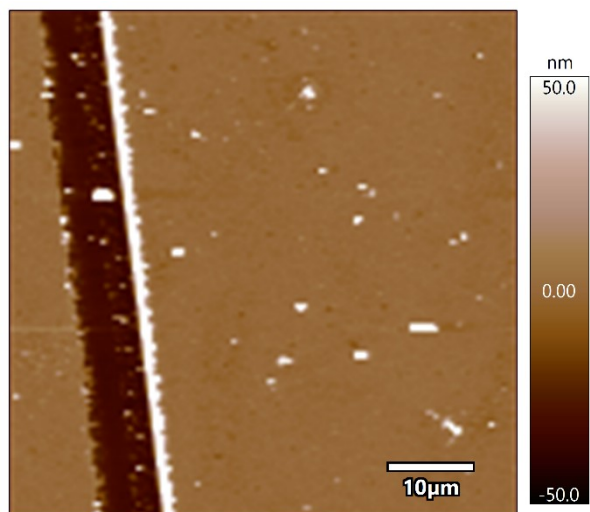
a



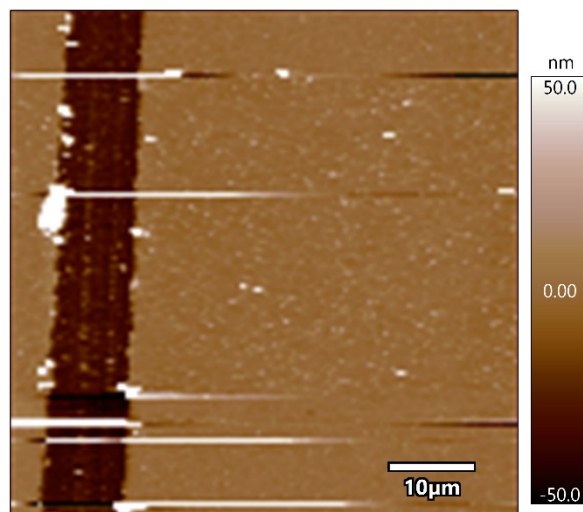
b



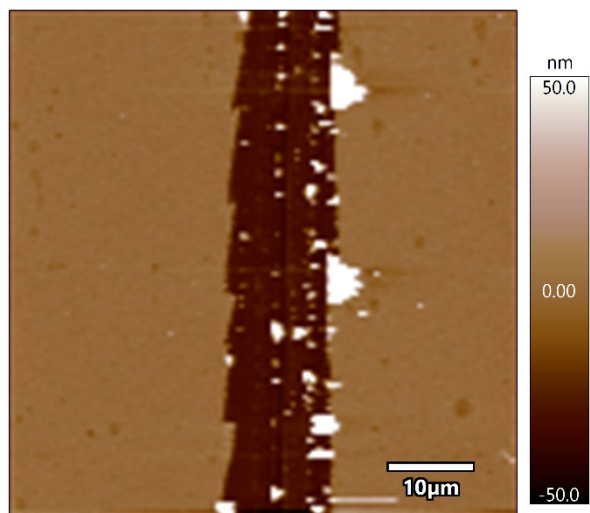
c



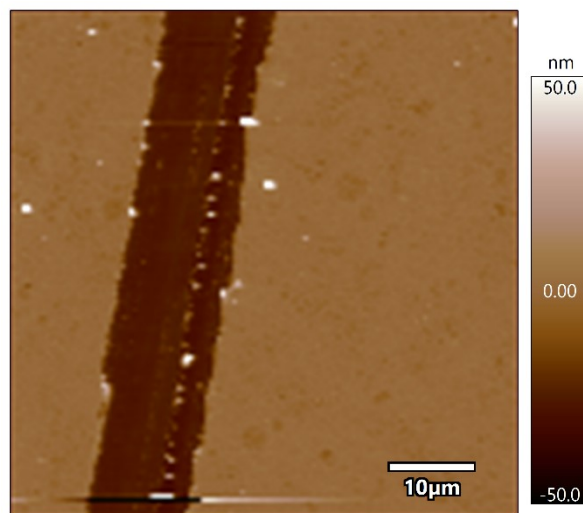
d



e



f



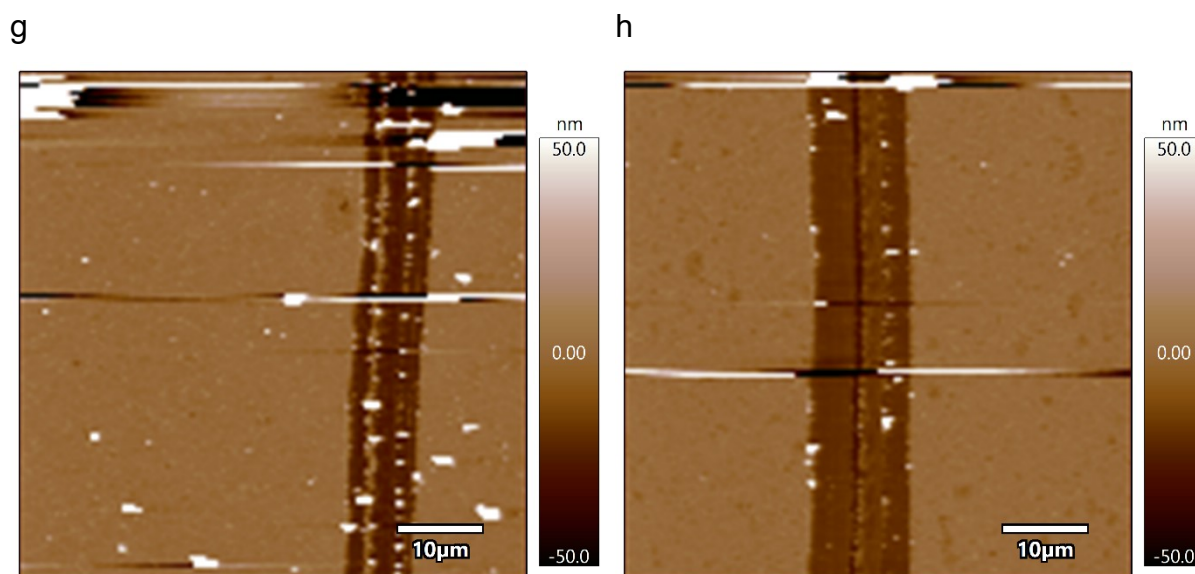


Figure 42 AFM height images of the glass face of poly(SA-pHBA acrylate) brush-modified substrate polymerized for 90 min upon incubation in the drug release supernatant (PBS at 37 °C) for (a) 5 min, (b) 10 min, (c) 15 min, (d) 30 min, (e) 60 min, (f) 360 min, (g) 1440 min or (h) 2880 min.

In the AFM images, a decrease in the film thickness over the course of 48 h can be observed. The measured step height decreases, as can be seen based on the color scale, with a fainting of the color inside the scratch indicating a smaller depth of the scratch and therefore a decrease in the polymer brush layer.

Similar to the observed changes in the polymer film on the samples polymerized for 90 min, the samples that were polymerized for 60 min and 120 min also exhibit a decrease in the film thickness during the drug release, as can be seen in Appendix 15 and Appendix 16. As for the polymer film on the titanium face, during the drug release experiment, the layer thickness on the glass face decreases with increasing incubation time, hinting at the release of a compound from the film into the solution. The change in film thickness for substrates polymerized for different times was plotted against the incubation time and can be seen in Figure 43.

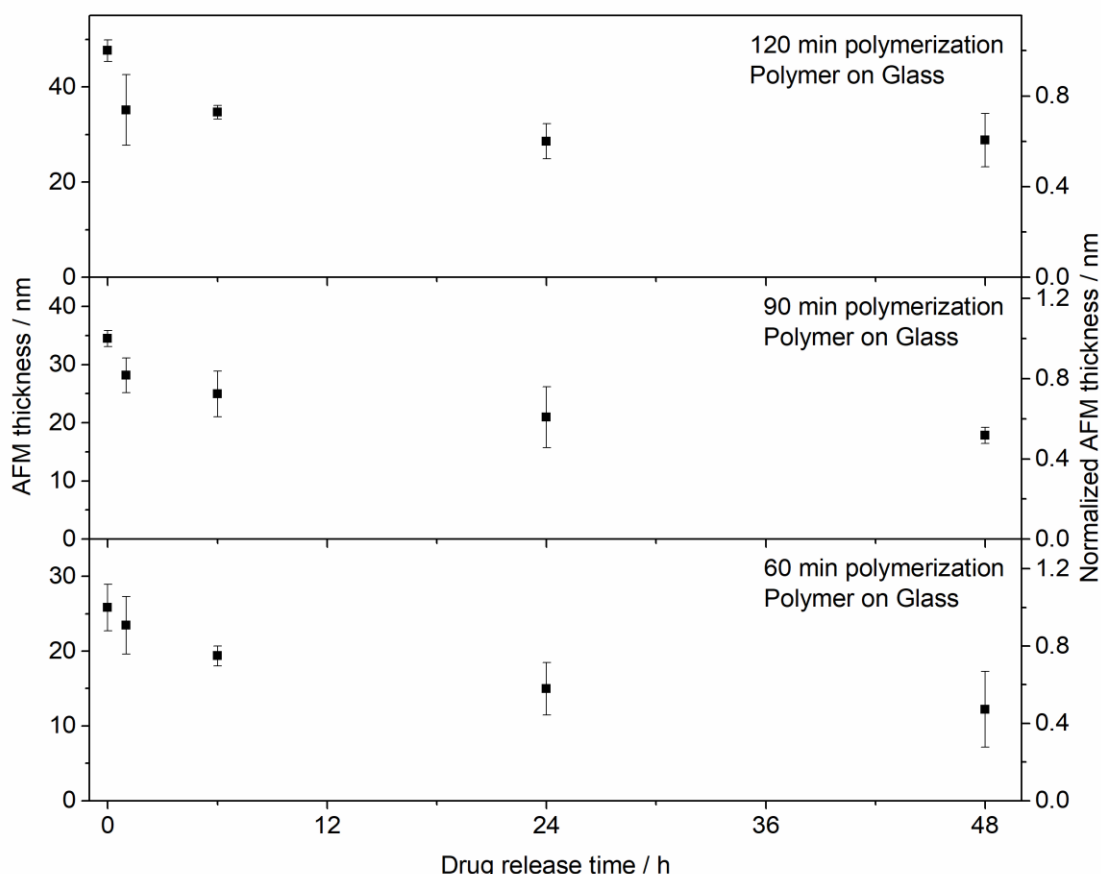


Figure 43 Plot of the measured film thickness on the glass face upon incubation in the drug release supernatant (PBS at 37 °C) for poly(SA-pHBA acrylate) brush-modified substrates polymerized for 60 min, 90 min and 120 min as measured by AFM. The film thickness is shown together with the change in film thickness normalized to the initial film thickness.

The AFM-based plot shows the same trend as in Figure 41. An initial fast decrease in the film thickness on the glass face is observed, which then slows down. The early stage of the drug release experiments, as studied by AFM, can be seen in Appendix 17.

Ellipsometry and AFM were used to study the change in the film thickness, allowing evaluation of the change in the total film volume (ΔV). Assuming that the change in the film volume is caused by the release of SA enabled the quantification of the mass of SA ($m(\text{SA})$) based on the volume change and the known density of SA ($\rho(\text{SA}) = 1.08 \text{ g/cm}^3$) according to Equations 8 & 9.

$$\Delta V(\text{polymer}) = \text{area} \cdot \Delta \text{thickness}(\text{brush on Ti}) + \text{area} \cdot \Delta \text{thickness}(\text{brush on Glass}) \quad \text{Equation 8}$$

$$m(\text{SA}) = \rho(\text{SA}) \cdot \Delta V$$

Equation 9

By translating the measured film thickness changes into a quantity of SA, a kinetic plot of the drug release can be obtained (Figure 44).

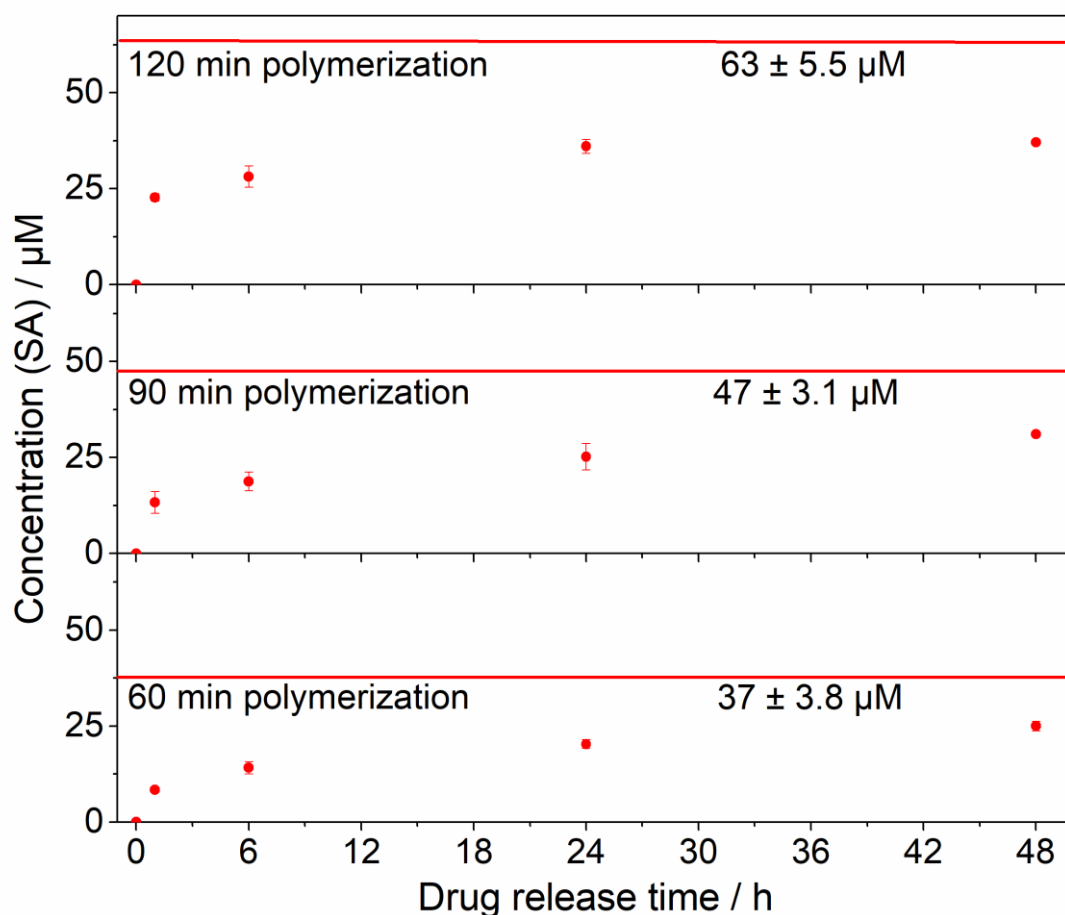


Figure 44 Kinetic plot of the SA released over the course of two days, based on the film thickness changes of poly(SA-pHBA acrylate) brush-modified substrates polymerized for 60, 90 or 120 min measured via ellipsometry and AFM. The solid red line indicates the maximum theoretical amount of SA stored in the synthesized polymer brushes.

The initial fast decrease in the film thickness yields an initial fast release of SA. The early stage of the drug release experiments, as studied by AFM, can be seen in Appendix 18. The joint kinetic plot containing the UV-Vis, HPLC and thickness-derived drug release data can be seen in Figure 45.

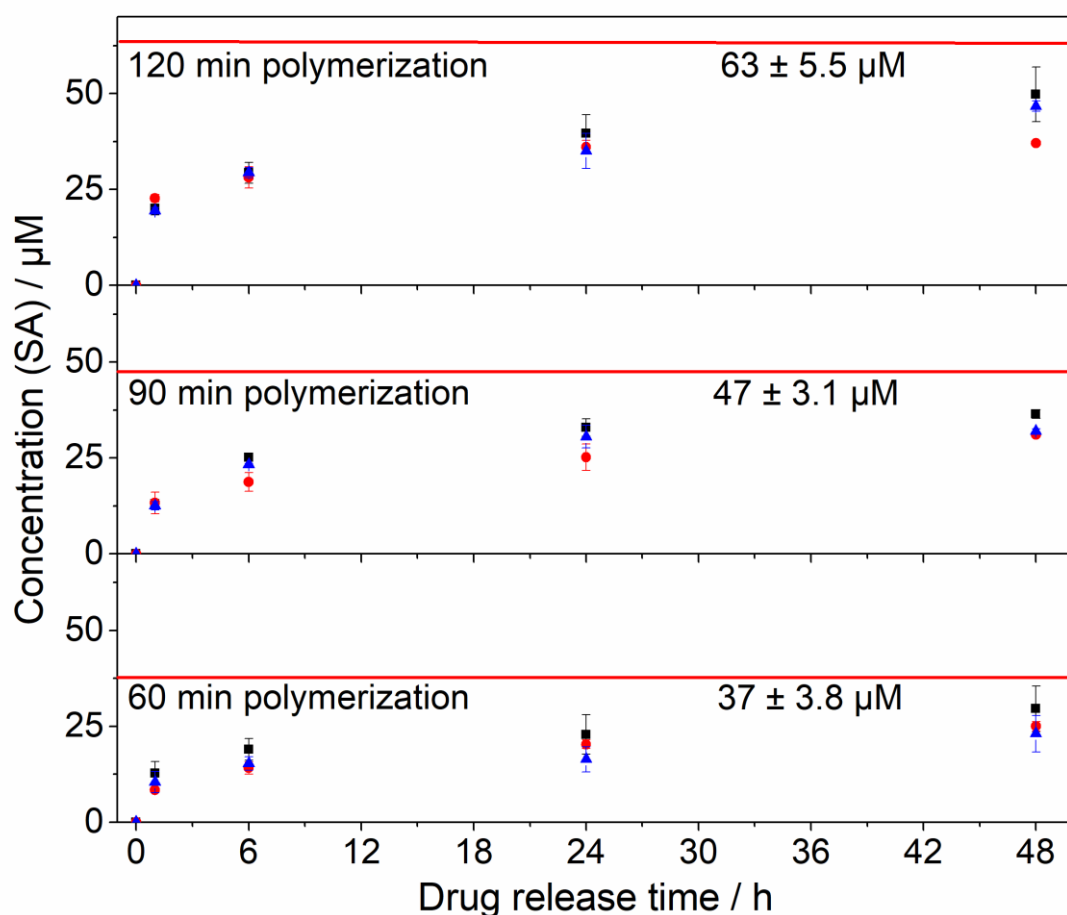


Figure 45 Kinetic plot of the drug release over the course of two days, based on the UV-Vis (black squares) and HPLC (blue triangles) data and the film thickness changes measured via ellipsometry and AFM (red circles). The solid, red line indicates the maximum amount of SA theoretically being stored in the synthesized poly(SA-pHBA acrylate) brushes for different polymerization times.

The data obtained based on the measurement of the drug release supernatant, and the spectroscopic and microscopic investigations of the remaining polymer brush layer, unequivocally prove the release of SA from the polymer brush modified substrates. While UV-Vis can be used as a relatively simple way of observing the drug release kinetics and is useful for a fast determination of the drug concentration, HPLC confirms the release of chemically unaltered SA and can be used to quantify the amount released. AFM and ellipsometry further confirm the mass loss of the polymer film, measurable as a change in the film thickness. From that, the estimation of the amount of drug released from the brushes agrees very well with the concentrations of the drug detected in the supernatant, proving the applicability of AFM and ellipsometry as complementary techniques for the study of the drug release. The early stage of the drug release experiments, as studied by UV-Vis, HPLC and AFM/ellipsometry, can be

seen in Appendix 19. Commonly, drug release from polymeric reservoirs is described according to the Korsmeyer-Peppas model, which connects the fractional drug release (M_t/M_∞) with a release constant (k), the drug release time (t), and a release exponent (n), which is used to describe the release mechanism (Equation 10).²¹⁷

$$\frac{M_t}{M_\infty} = k t^n \quad \text{Equation 10}$$

The drug release data were plotted in a log-log plot, which can be used to determine the release mechanism based on the Korsmeyer-Peppas model, as can be seen in Appendix 20. The linear regression yields the release exponent as the slope, which was found to be around 0.25, independent of the polymer brush thickness, as can be seen in Appendix 21. Although the linear regression fits well to the drug release data, an exponent <0.5 indicates a release mechanism that is not only diffusion-controlled but governed by an additional mechanism.²¹⁸ It is hypothesized that the low release exponents found here are indicative of the easy water penetration into the brush film and hydrolysis of the imine bond, followed by the diffusion of the antibiotic out of the polymer brush film.

The relative amount of drug released from the brushes was found to be independent of the polymer brush thickness. Approximately 70% of the drug was released within 48 h, indicating that SA sitting in deeper layers of the film is released more slowly. This is hypothesized to be due to the steric hindrance of SA diffusing through the polymer layer. The drug-releasing polymer brush platform developed by Choi et al. reached 50% of the drug release within the first 5 min, while the polymer brush prodrug presented here requires ~6 h to reach 50% release.¹³ This corresponds to a release behavior prolonged by ~70 times.

Hydrogel systems like the diclofenac sodium (DS)-loaded PEG-*b*-poly(caprolactone) reported by Patel et al. possess higher absolute capacities for drug delivery; however, the amount of SA per volume of polymer brushes is higher than the corresponding ratio of DS per volume of hydrogel by a factor of 650.¹⁰⁷ This indicates the high drug loading capacity of the prodrug brush and the more sustained release profile in comparison to other drug-releasing polymer brush platforms.

5.2.3.3 SA Release Studied by RAIRS and XPS

The change in the chemical composition of the polymer brush over the course of the incubation was studied by XPS and RAIRS. Figure 46 shows the change in the nitrogen and sulfur high-resolution scans for brushes incubated up to 48 h.

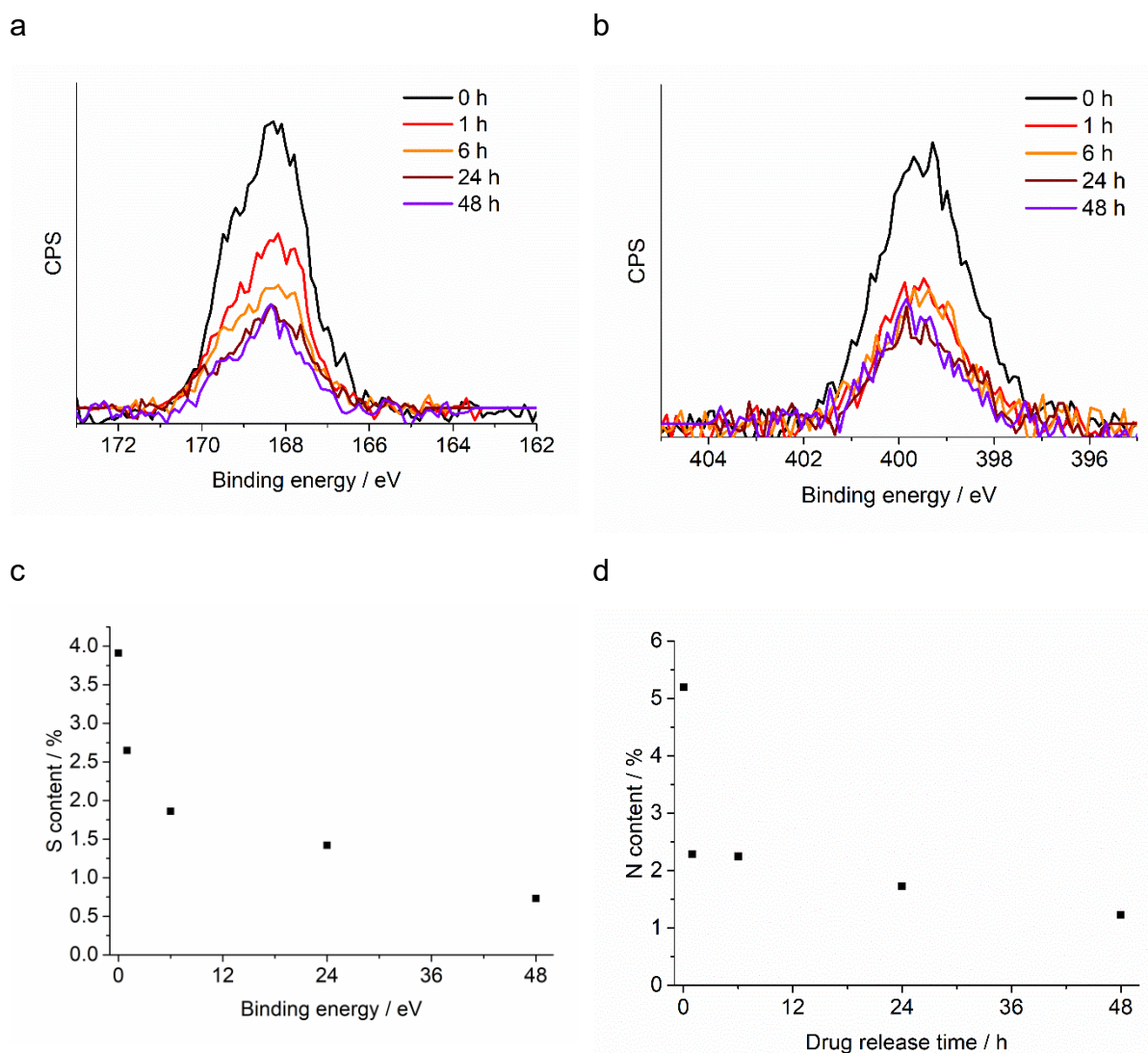


Figure 46 High resolution XPS spectra of (a) sulfur and (b) nitrogen and the content of (c) sulfur and (d) nitrogen in the poly(SA-pHBA acrylate) brush-modified samples (polymerized for 120 min) incubated for up to 48 h in PBS at 37 °C.

Over the course of the drug release, the sulfur and nitrogen signals, which stem solely from SA contained in the brushes, decrease due to the release of SA. Still, after two days of drug release, signals related to sulfur and nitrogen are detectable, indicating that not all SA molecules were liberated from the polymer brush film. The fast initial release of SA from the brushes, as observed by UV-Vis, HPLC, AFM and ellipsometry, is also reflected in the XPS data. This further confirms the release of SA from the polymer brush prodrug. Additionally, the brushes were subjected to RAIRS analysis.

The spectra for the sample polymerized for 90 min and incubated up to 48 h can be seen in Figure 47.

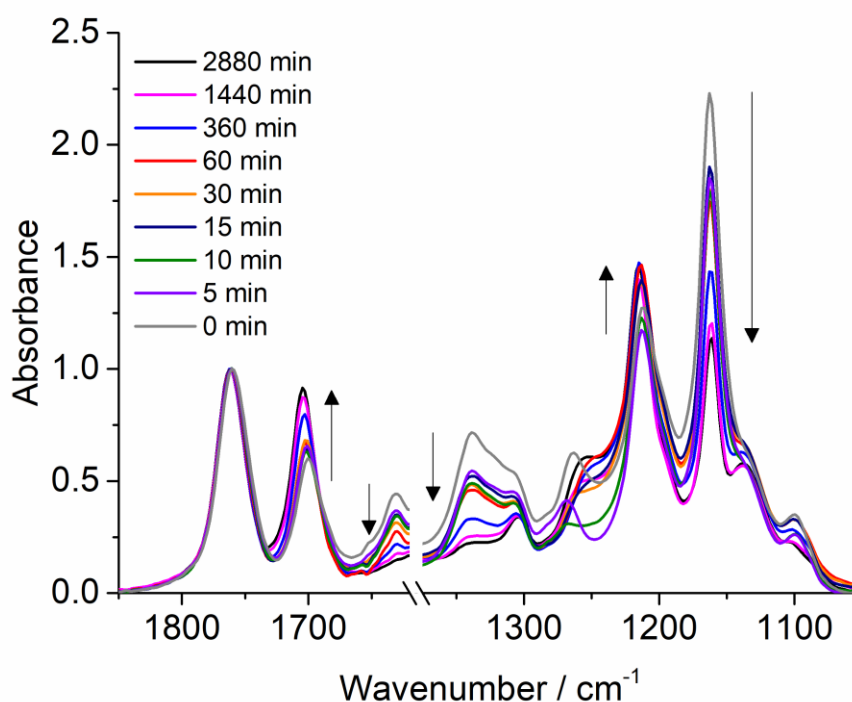


Figure 47 RAIRS spectra of the poly(SA-pHBA acrylate) brush film (polymerized for 90 min) upon incubation in PBS at 37 °C. The spectra were normalized to the carbonyl signal located at 1760 cm^{-1} , corresponding to the ester side chain of the polymer.

As can be seen in the IR spectra, the aldehyde signal positioned at around 1700 cm^{-1} increases while the signal of the imine, located at 1630 cm^{-1} , decreases and disappears completely. The sulfonamide signals at 1340 cm^{-1} and 1160 cm^{-1} decrease during the drug release, highlighting the removal of SA from the film. This indicates the penetration of the polymer film with water, hydrolysis of the imine bond, generation of an aldehyde and liberation of SA, which is then free to leave the polymer brush film via diffusion. The release of SA is expected to be slower than the hydrolytic reaction, as the diffusion of water molecules is expected to be less sterically impeded. By plotting the intensity of the imine signal and the intensity of the sulfonamide signal against the duration of the incubation time, a kinetic plot of the hydrolysis and of the release of SA can be obtained and seen in Figure 48.

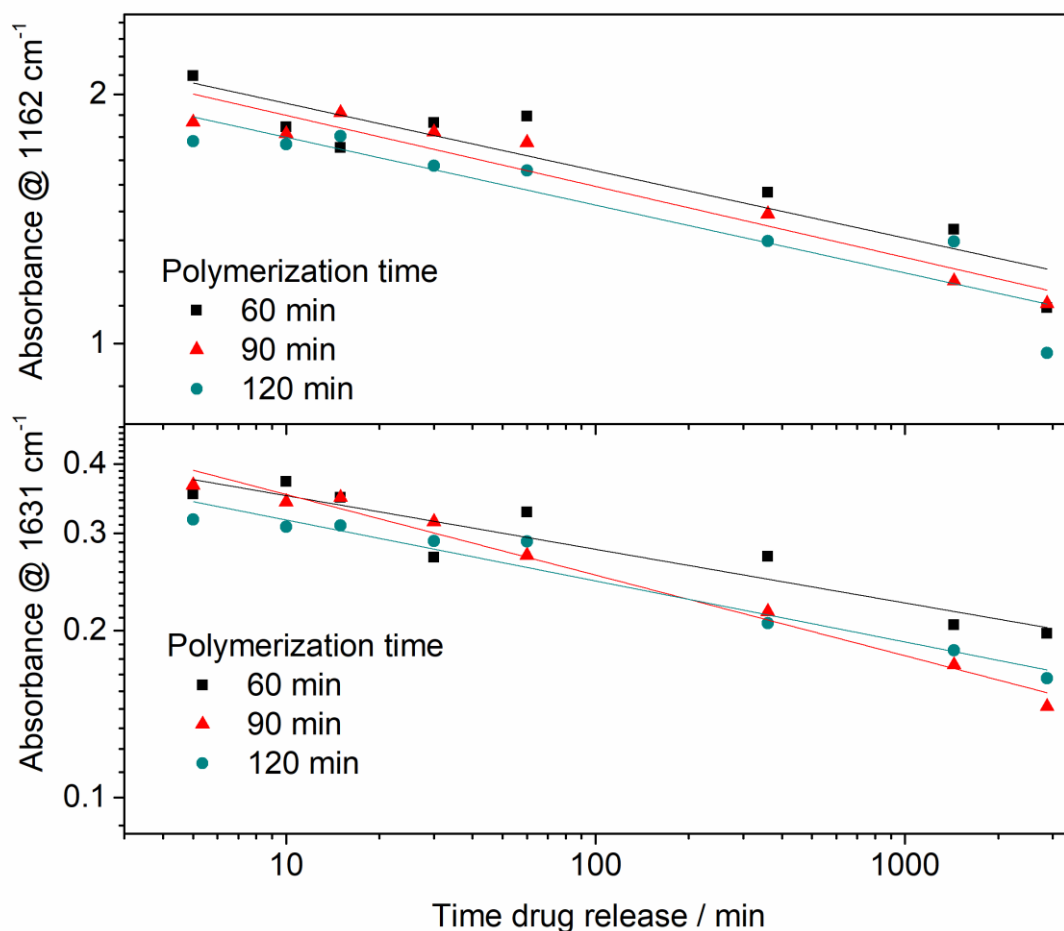


Figure 48 Decay of the sulfonamide signal (1162 cm^{-1}) and the imine signal (1631 cm^{-1}) in the RAIR spectra of poly(SA-pHBA acrylate) brushes upon incubation in PBS at $37\text{ }^{\circ}\text{C}$ over the course of 48 h. Linear regressions were used to plot the data.

The log scale indicates a power-law relationship of the decomposition of the imine bond, as well as for the release of SA into the solution. The determined slopes are in a very narrow range, indicating no dependency of the decomposition or release rate on the polymer brush thickness. However, the less negative slope found consistently for the decay of the sulfonamide signal ($-0.08 \pm 0.001\text{ cm}^{-1}\text{ min}^{-1}$) in comparison to the decay of the imine signal ($-0.12 \pm 0.02\text{ cm}^{-1}\text{ min}^{-1}$) hints at a faster penetration of water molecules into the film and hydrolysis of the imine bond, than the release of the antibiotic. XPS and RAIRS analysis therefore confirm the release of SA from the polymer brush film based on the hydrolysis of the imine linker between SA and the polymer chain.

5.2.4 Temperature- and pH-Dependent SA Release

To investigate the pH dependency of the drug release, stock solutions of SA and pHBA in aqueous hydrochloric acid (HCl) at pH 2 and stock solutions in aqueous sodium hydroxide (NaOH) at pH 13 were prepared and measured by UV-Vis. From the spectra, the extinction coefficients of SA were determined. The spectra can be seen in Figure 49.

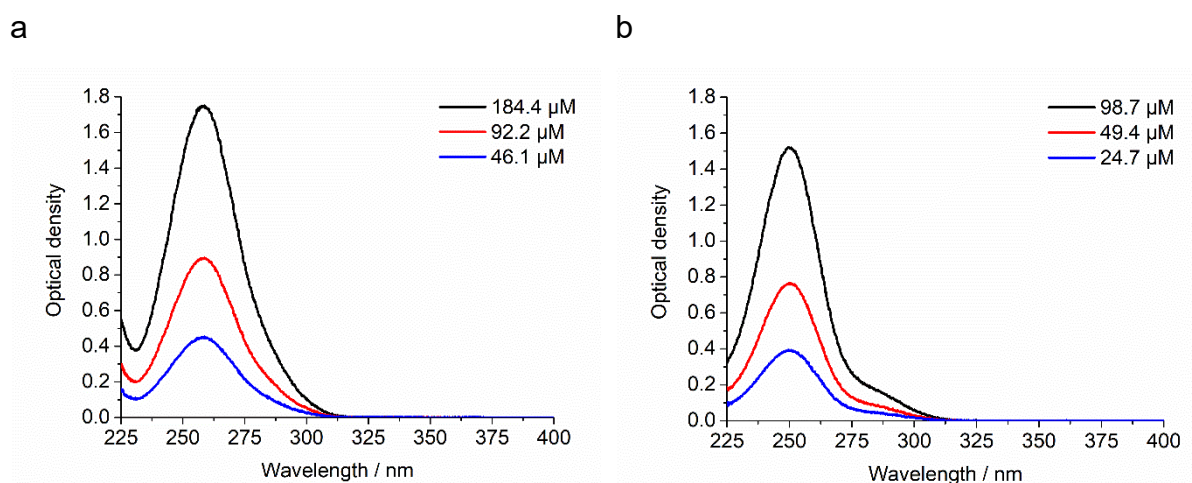


Figure 49 UV-Vis spectra of SA in (a) HCl at pH 2 and (b) NaOH at pH 13. The absorption maxima are at 258 nm (HCl) and 250 nm (NaOH), with extinction coefficients of $9527 \text{ M}^{-1} \text{ cm}^{-1}$ (HCl) and $15392 \text{ M}^{-1} \text{ cm}^{-1}$ (NaOH).

The weak shift in the absorption maximum can be explained by the pH-dependent deprotonation of the sulfonamide group, which has a reported pK_a value of 10.5.²¹⁹ The decrease in the extinction coefficient at low pH can be explained by the protonation of the aromatic amine of SA, effectively inhibiting conjugation of the amine and therefore decreasing the electron density in the aromatic system.²²⁰ Because of the hypothesized release of pHBA in the previous drug-release experiments, the pH-dependent UV-Vis spectra of pHBA were also recorded. The spectra can be seen in Figure 50.

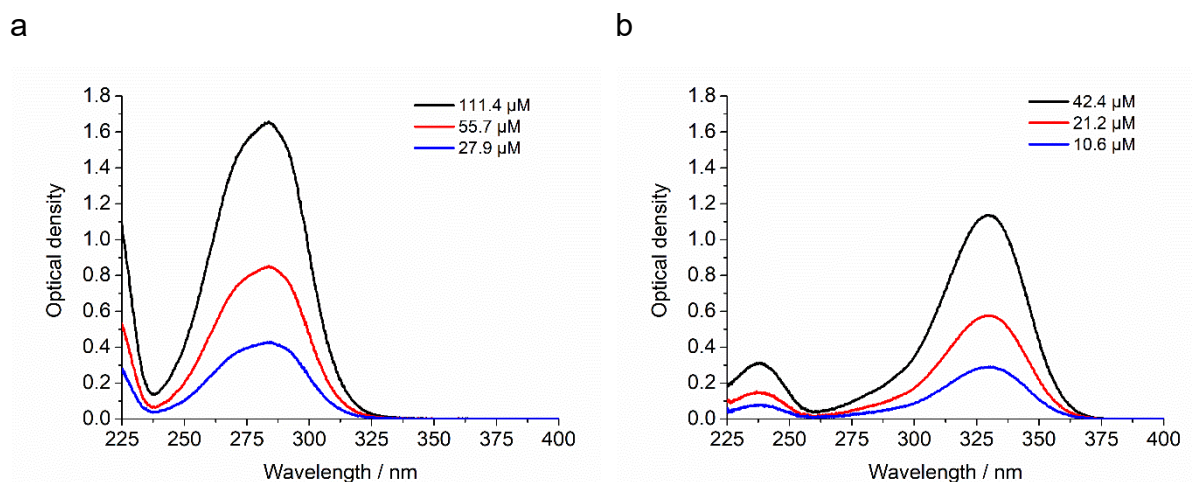


Figure 50 UV-Vis spectra of pHBA in (a) HCl at pH 2 and (b) NaOH at pH 13. The absorption maxima are at 283 nm (HCl) and 330 nm (NaOH). The extinction coefficients were determined to be $14866 \text{ M}^{-1} \text{ cm}^{-1}$ (HCl) and $26858 \text{ M}^{-1} \text{ cm}^{-1}$ (NaOH).

The pH-dependent shift in the absorption spectrum of pHBA is based on the acidity of the phenol group, with a reported pK_a value of 7.66, explaining the presence of both species in the absorption spectrum of pHBA in PBS at pH 7.4.²²¹ In a basic environment, the dominant form is the phenolate with an absorption maximum of 330 nm, while in the acidic environment, the phenol is the dominant form with an absorption maximum of 283 nm. The phenolate has a strong positive mesomeric effect and therefore increases the electron density in the aromatic system, leading to a higher extinction coefficient.²²⁰

Conducting the drug release under different pH and temperature conditions, the change in the layer thickness was investigated. The results for the pH-dependent drug release can be seen in Figure 51.

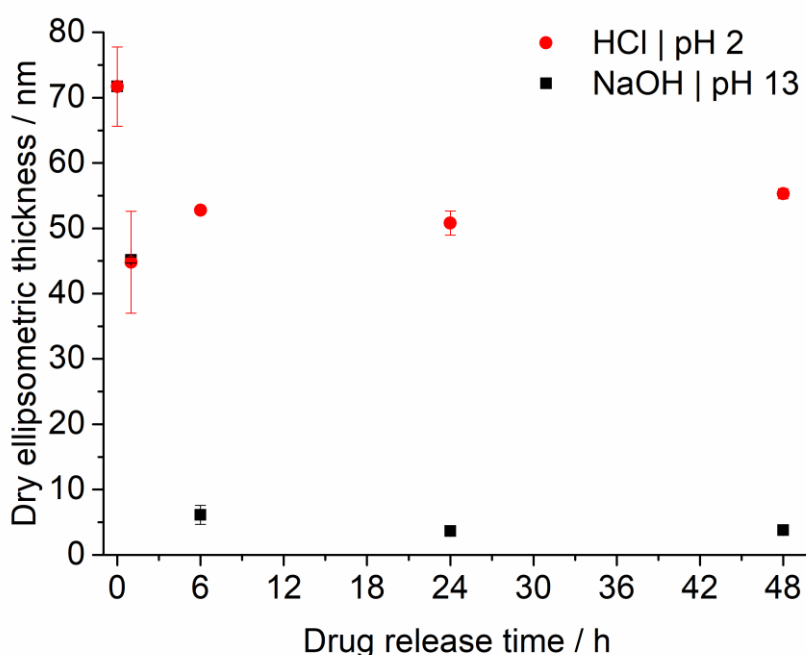


Figure 51 Change in film thickness on the titanium face of poly(SA-pHBA acrylate) brush modified substrates polymerized for 180 min after incubation at 37 °C in HCl at pH 2 (red circles) and NaOH at pH 13 (black squares) as measured by ellipsometry.

Under acidic conditions, the polymer brush layer decreases in thickness and reaches a final brush film thickness of approximately 50 nm after an incubation of 48 h, which corresponds to a decrease of the film thickness to 77% after 48 h. This is contrary to the drug release experiments in PBS, where consistently the layer thickness decreased to approximately 60% as determined by ellipsometry. However, under strong basic conditions, within the first six hours, the polymer brush layer completely detaches from the substrate, very likely due to the hydrolytic decomposition of the anchoring silane layer. The fast hydrolysis of silanes under basic conditions was described before, with a drastic increase in the rate of hydrolysis with increasing pH and temperature.²²²

The drug release under strongly acidic and basic conditions was also investigated by UV-Vis spectroscopy. The results can be seen in Figure 52.

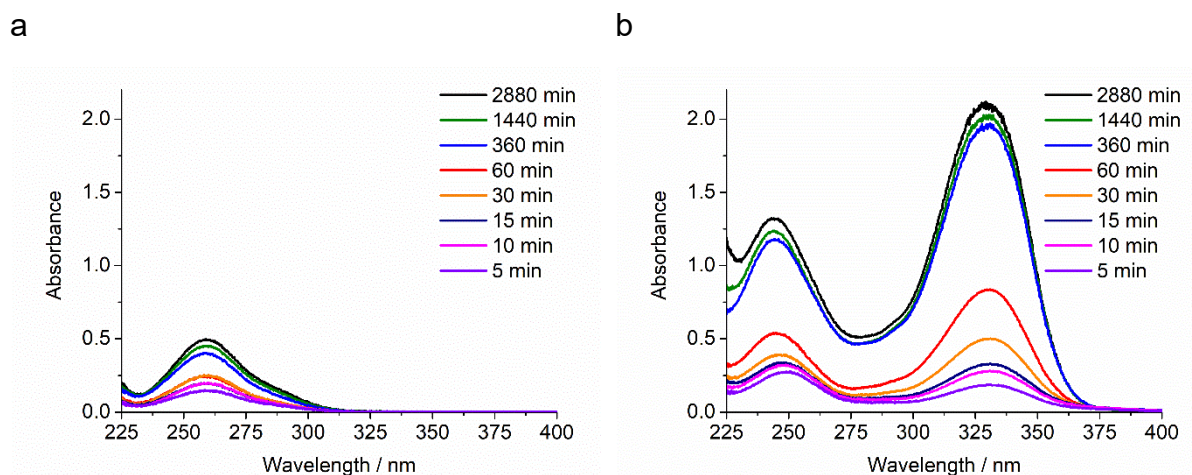


Figure 52 UV-Vis spectra of the drug release supernatant upon incubation of poly(SA-pHBA acrylate) brush-modified substrates polymerized for 180 min under (a) acidic (HCl, pH 2) and (b) basic conditions (NaOH, pH 13) over the course of 48 h at 37 °C.

Conducting the drug release under strongly acidic conditions shows the release of SA as an increase in the absorbance at 258 nm, with a shoulder at 283 nm. This indicates the release of not only SA, but also pHBA in its phenolic form. If the drug release is conducted at high pH values, the release of SA can be seen as an increasing absorption signal at 250 nm. This is assumed to be related to the complete detachment of the polymer brush layer, liberating all available SA molecules into the supernatant. Additionally, the signal of pHBA can be observed at 330 nm. The joint release of SA and pHBA highlights the low hydrolytic stability of the ester bond between the polymeric backbone and the pHBA moiety at elevated pH. The release of both species can be seen again by overlaying the UV-Vis spectra after 48 h for each pH condition with the absorption spectra of the neat components, as can be seen in Figure 53.

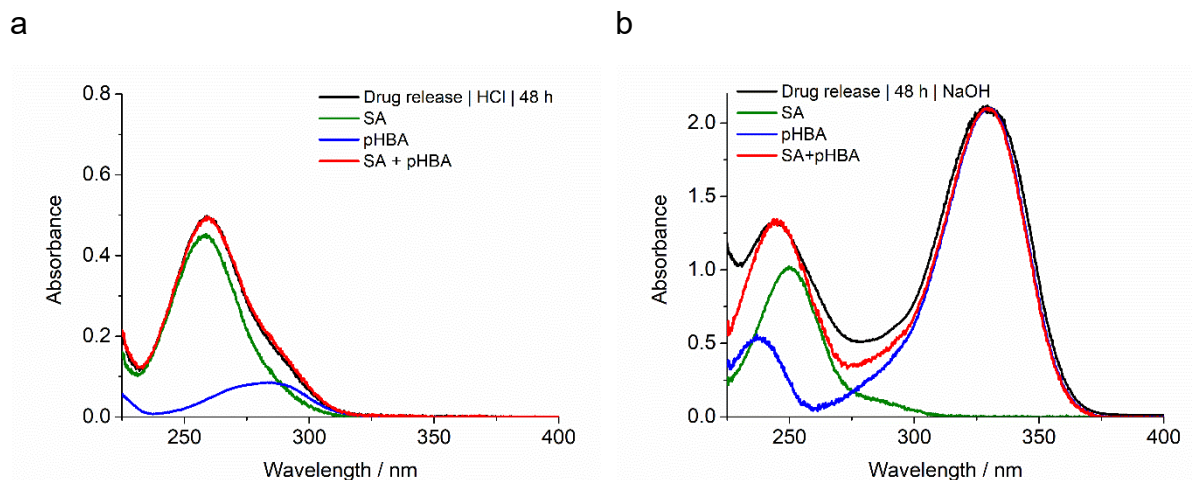


Figure 53 Drug release experiments conducted upon incubation of poly(SA-pHBA acrylate) brush-modified substrates polymerized for 180 min under (a) acidic (HCl, pH 2) and (b) basic (NaOH, pH 13) conditions for 48 h at 37 °C, deconvoluted into SA and pHBA contributions. The addition of the deconvoluted spectra yields the observed drug release spectrum with only minor deviations.

Deconvolution of the drug release spectra of the acidic and basic supernatants reveals the release of SA and pHBA, with a clear overlap of the spectra of the single components with the measured release spectra. In the case of the basic drug release studies, the peak widths of the signals in the drug release supernatant (black curve) are larger than the peak widths of the added contributions of the individual components (red curve), which is hypothesized to be due to light scattering of the detached polymer chains. Furthermore, the drug release from the brush-modified layer was investigated at different temperatures. The results for the change in the film thickness can be seen in Figure 54.

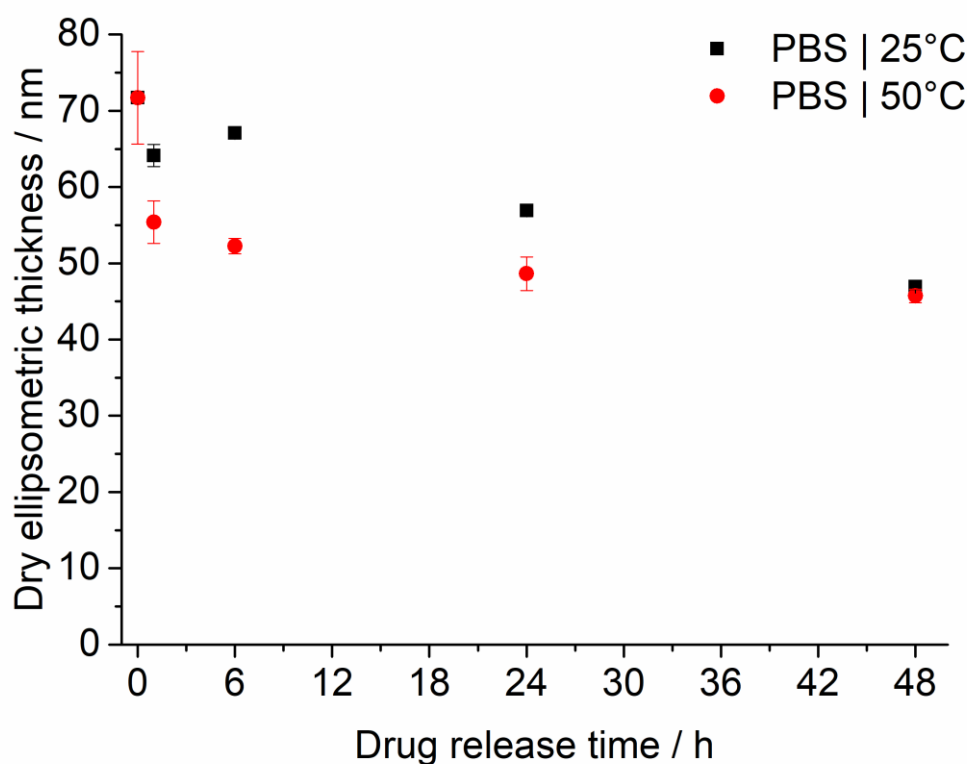


Figure 54 Change in film thickness on the titanium face of poly(SA-pHBA acrylate) brush-modified substrates polymerized for 180 min upon incubation in PBS at 25 °C (black squares) and in PBS at 50 °C (red circles) for substrates.

Conducting the drug release at 25 °C, the layer thickness decreases more slowly, while at elevated temperatures, the film thickness decreases faster and reaches the same thickness faster, which would indicate a limiting factor for the drug release with no significant detachment of polymer chains by increasing the temperature from 25 °C to 50 °C.

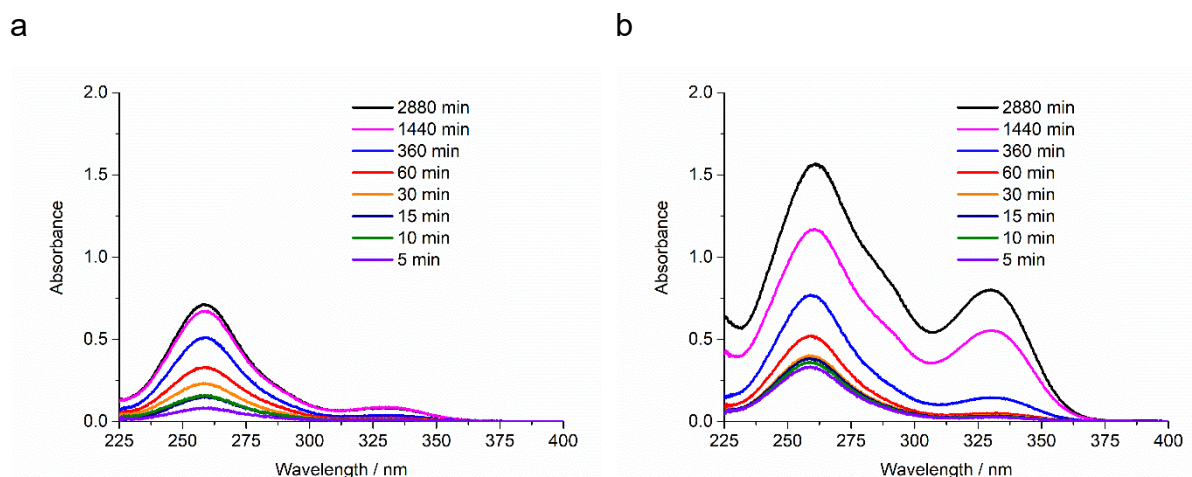


Figure 55 UV-Vis spectra of drug release experiments conducted at (a) 25 °C and (b) 50 °C for up to 48 h from poly(SA-pHBA acrylate) brush-modified substrates polymerized for 180 min in PBS.

Although the remaining brush film was found to be of similar thickness for substrates incubated at different temperatures, which would indicate a comparable release of SA, the UV-Vis spectra show the much higher release of SA at elevated temperatures in comparison to the release conducted at lower temperatures. The reason for this deviation is not clear; however, the substrates with longer polymerization times also have larger standard deviations in the brush film thickness, which could explain the difference between the observed film thickness and the drug release. Furthermore, the experiments conducted at different temperatures and pH levels were performed with one sample per condition and drug release time only, highlighting the need for statistical reproduction of the experiment.

The drug release, observed under acidic and basic conditions and at different temperatures, reveals the pH- and temperature-dependent release characteristics of the developed prodrug system. Due to the decomposition of the polymer brush film under harsh basic conditions, the release kinetics cannot be compared appropriately. Nevertheless, the deconvolution of the UV-Vis spectra allows estimation of the concentration of SA released, based on the predetermined extinction coefficients. By plotting the concentration against the drug release time, a kinetic plot is obtained (Figure 56).

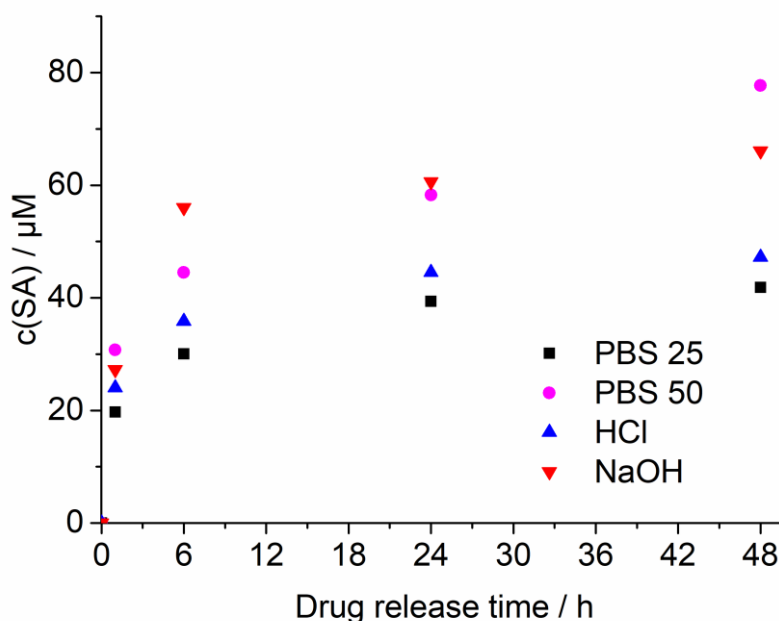


Figure 56 Drug release kinetics for poly(SA-pHBA acrylate) brush-modified samples polymerized for 180 min and incubated in PBS at 25 °C or 50 °C, HCl (pH 2) or NaOH (pH 13) over the course of 48 h at 37 °C.

By determining the drug release kinetics at different temperatures, it becomes clear that the release of SA from the brush-covered substrates can be catalyzed by increasing the temperature. Similarly, more SA is released under basic conditions, which is not necessarily related to the base-catalyzed hydrolysis of the imine but rather to the detachment of the polymer brush layer. The drug release studies conducted at different pH values and temperatures confirm the temperature-dependence of the drug release, while pH only seems to play a minor role in the rate of drug release. However, for a more significant analysis of the pH and temperature-dependence, the conducted experiments need to be reproduced with additional drug release experiments conducted for this sample type (180 min polymerization) under neutral pH conditions and neutral temperatures to establish the baseline release of SA. Furthermore, the applied pH and temperature conditions were used to study the drug release under extreme conditions, far from physiological conditions. Observations in a narrower pH and temperature range are required to give meaningful information on whether the drug release can be catalyzed in the presence of bacteria. Nevertheless, the drug release conducted under physiological conditions hints at a sufficiently fast release to achieve active concentrations of SA.

5.2.5 Antibacterial Activity of SA and SA Released from Poly(SA-pHBA Acrylate) Brush Prodrugs in PBS

As previously discussed, SA is no longer commonly used because of the widespread bacterial resistance to it. However, when combined with TMP, its antibacterial activity is enhanced due to a synergistic effect. SA was therefore tested for its neat antibacterial activity and in combination with TMP, in a fixed mass ratio of 5:1 (SA:TMP). Before the supernatant of the drug release experiments was tested, selection experiments were conducted to find bacterial strains sensitive to SA or the mixture of SA and TMP. For a fast screening of susceptibility of the tested bacteria to the antibiotic, disc diffusion experiments were conducted in which a cellulose disc was soaked in solutions of the antibiotic or the mixture of SA and TMP, followed by drying of the disc and subsequent placing of the disc on bacteria streaked on LB agar plates. The plates were then incubated overnight to allow bacteria to grow and observe potential zones of inhibition around the cellulose discs which appear if bacteria are susceptible to the corresponding antibiotic. In total, 15 strains of bacteria were tested and their susceptibility to the antibiotics are listed in Table 11.

Table 11 List of the tested bacterial strains and the corresponding concentrations of SA, TMP and the combination to which they are susceptible. Highlighted values indicate observation of a synergistic effect.

Bacterial strain	SA	TMP / $\mu\text{g/mL}$	SA:TMP / $\mu\text{g/mL}$
<i>S. aureus</i> H560	X	200	100:20
<i>S. aureus</i> N315	X	200	100:20 (weak)
<i>S. aureus</i> 2569	X	200	1000:200
<i>S. aureus</i> MSSA 69	X	200	100:20
<i>S. aureus</i> 14616	X	200	1000:200
<i>S. aureus</i> MSSA 52	X	200	100:20
<i>S. aureus</i> MSSA 21	X	200	1000:200
<i>S. aureus</i> MSSA 56	X	200	100:20 (weak)
<i>S. aureus</i> MRSA 252	X	200	1000:200
<i>S. aureus</i> RN4220	X	200	100:20
<i>S. aureus</i> eMRSA 9	X	X	X
<i>S. aureus</i> NCTC10788	X	200	100:20
<i>S. aureus</i> MRSA 378	X	200	1000:200
<i>S. aureus</i> MSSA 233	X	200	100:20
<i>S. epidermidis</i> 12228	X	20	100:20
<i>A. baumannii</i> RUH375	X	X	X

X denotes no observed antibacterial effect at concentrations $\leq 1000 \mu\text{g/mL}$.

Based on the disc diffusion experiments, all tested bacterial strains were found to be resistant to neat SA at the tested concentrations. This indicates that SA is not potent

enough to prevent bacterial growth when administered alone. On the other hand, TMP by itself shows large zones of inhibition, indicating the high potency of the drug to prevent bacterial growth. For six of the chosen bacterial strains, the mixture of SA and TMP showed the expected synergistic effect, visible in either the presence of a zone of inhibition at a certain concentration which is not present for the same concentration of neat TMP (*S. aureus* NCTC 10788, RN4220, H560) or a larger zone of inhibition compared to neat TMP (MSSA 69, MSSA 52, MSSA 233).

Three strains were selected for further testing: a lab strain (*S. aureus* RN4220), a reference strain (*S. aureus* NCTC 10788) and a clinical isolate (MSSA 69). The MIC values of SA, TMP and the SA:TMP combination were determined for each of these strains. For the determination of MIC values, bacteria were mixed with solutions of SA and TMP and incubated overnight. If bacterial growth is observed in the solution, the turbidity of the solution will increase, either visible by bare eye or through measurements of the optical density. This method allows estimation of the MIC but will not yield information about whether bacteria are eradicated or not able to proliferate. For detection of viable bacteria, the bacterial solution must be serially diluted, plated and incubated to allow any remaining viable bacteria to grow and be visibly countable through the number of colony-forming units (CFU). Using this method, the MIC values were determined and can be seen in Table 12. The results shown were further substantiated by testing the antibacterial effect of mixtures of SA and TMP at varying ratios in a checkerboard assay, in which the three selected strains were tested. The results can be seen in Appendix 22, Appendix 23 & Appendix 24.

Table 12 Determined MIC values for three strains of *S. aureus*.

Bacterial strain	MIC (SA) / $\mu\text{g/mL}$	MIC (TMP) / $\mu\text{g/mL}$	MIC (SA:TMP) / $\mu\text{g/mL}$
RN4220	> 128	13	16:3.2
NCTC 10788	> 128	3.2	4:0.8
MSSA 69	> 128	3.2	8:1.6

The determined MIC values highlight the synergistic effect between SA and TMP. While SA shows no antibacterial properties at the tested concentrations, in combination with TMP, bacteria were completely eradicated at relatively low concentrations. The concentrations necessary to eradicate bacteria are lower for the combination of the two drugs than for the neat drugs, as, for example, the concentration of TMP needed

to eradicate *S. aureus* RN4220 is approximately 13 µg/mL, but the combination of SA and TMP only requires 3.2 µg/mL of TMP in the presence of 16 µg/mL SA.

The testing of the drug release supernatant was conducted for a poly(SA-pHBA acrylate) brush-modified sample polymerized for 120 min, which was incubated in PBS for 24 h. After one day of incubation, most of the drug is released from the brushes, which allows an assessment of the applicability of the polymer brush prodrugs as systems that release active agents. After 24 h of drug release, the concentration was determined to be 7.2 µg/mL by UV-Vis, which is in the range of the MICs tested for the three strains. After mixing the bacteria with the drug release supernatant and after the addition of TMP, the concentration of SA in the supernatant corresponded to 5.7 µg/mL. After incubation, the bacterial solution was serially diluted and then plated on LB agar to determine the CFU count. The results for *S. aureus* NCTC10788 can be seen in Figure 57.

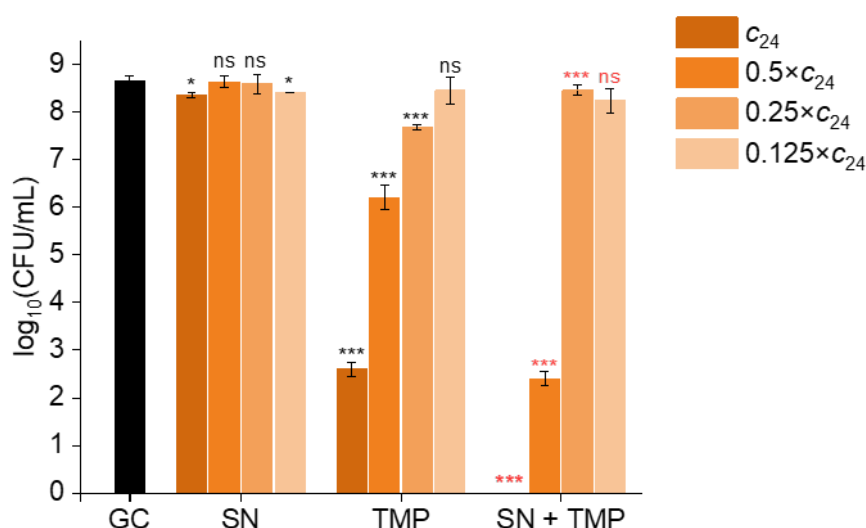


Figure 57 Results of the CFU count for *S. aureus* NCTC10788. The poly(SA-pHBA acrylate) brush prodrug polymerized for 120 min was incubated for 24 h in PBS, resulting in the drug release supernatant (SN). This SN was tested alone, mixed with TMP in a 5:1 mass ratio (SN+TMP) and a TMP solution at the corresponding concentration was also tested. Asterisks represent significant differences from the growth control (black) or the TMP solution at the corresponding concentration (red) (no significance (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)).

The drug release supernatant only showed a minute effect on the bacterial growth at any concentration. TMP on its own results in a significant reduction of bacteria, indicating a strong antibacterial effect, which is also present at half and a quarter of the initial concentration. Only at an eighth of the concentration, the effect of TMP does not significantly influence bacterial growth. The mixture of the drug release supernatant

with TMP in a 5:1 ratio shows a complete eradication of the incubated bacteria, which corresponds to a log reduction factor (LRF) of 1.5 relative to TMP by itself. This highlights the synergy between the released SA and TMP and acts as further proof of the release of chemically neat SA, as the drug release supernatant, which does not contain TMP, only has a minute effect on the bacterial growth. Also, at half the concentration, the mixture significantly reduces bacterial growth in comparison to TMP, with an LRF of 3.5. The lack of an antibacterial effect of the supernatant alone can be explained by the predetermined MIC values of SA, confirming the predicted mechanism of action and highlighting the lack of toxicity of the secondary species detected in the drug release supernatant, which is assumed to be from the release of pHBA. The lack of toxicity of pHBA was tested for all three bacterial strains in checkerboard assays, where the ratio of pHBA to TMP was systematically changed to see if there was any toxicity of pHBA by itself and whether there was a synergistic effect in the presence of TMP. The results can be seen in Appendix 25 (*S. aureus* MSSA69), Appendix 26 (*S. aureus* NCTC10788) and Appendix 27 (*S. aureus* RN4220).

The drug release supernatant was also tested against MSSA69. The results can be seen in Figure 58.

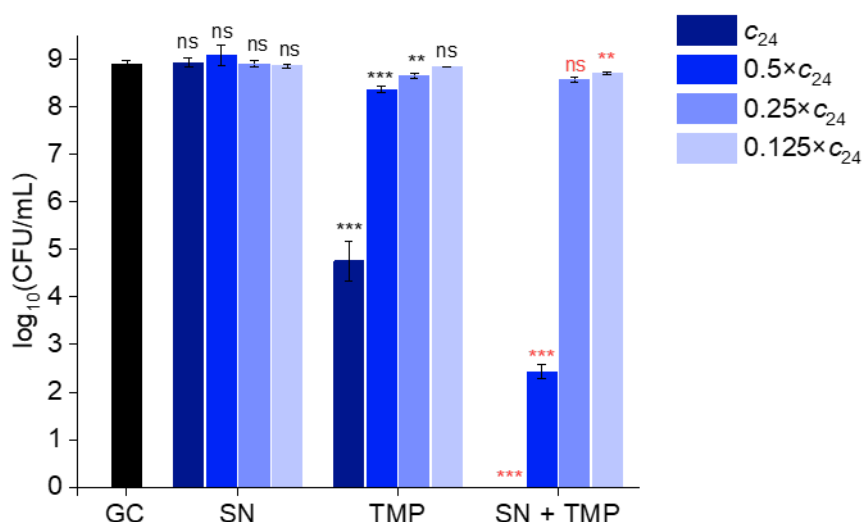


Figure 58 Results of the CFU count for MSSA69. The poly(SA-pHBA acrylate) brush prodrug polymerized for 120 min was incubated for 24 h in PBS, resulting in the drug release supernatant (SN). This SN was tested alone, mixed with TMP in a 5:1 mass ratio (SN+TMP) and a TMP solution at the corresponding concentration was also tested. Asterisks represent significant differences from the growth control (black) or the TMP solution at the corresponding concentration (red) (no significance (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)).

For MSSA69, the neat drug release supernatant shows no effect on the growth of the bacteria, hinting at a lack of cytotoxicity of the released compounds. The concentration of TMP used for the mixture with the drug release supernatant showed a significant reduction in the bacteria when used alone, which is still observable at a half and at a quarter of the initial TMP concentration. Only at an eighth of the initial concentration, TMP has no significant effect on bacterial growth. Similar to the NCTC10788 strain, the mixture of the drug release supernatant with TMP yields the complete eradication of the bacteria (LRF of 3.5 compared to TMP alone), and at half the concentration, the reduction of bacteria is significantly lower than for TMP by itself (LRF of 6). Only at a quarter of the initial concentration, the difference between the mixture and TMP by itself becomes insignificant.

The drug release supernatant was also finally tested against *S. aureus* RN4220. The results can be seen in Figure 59.

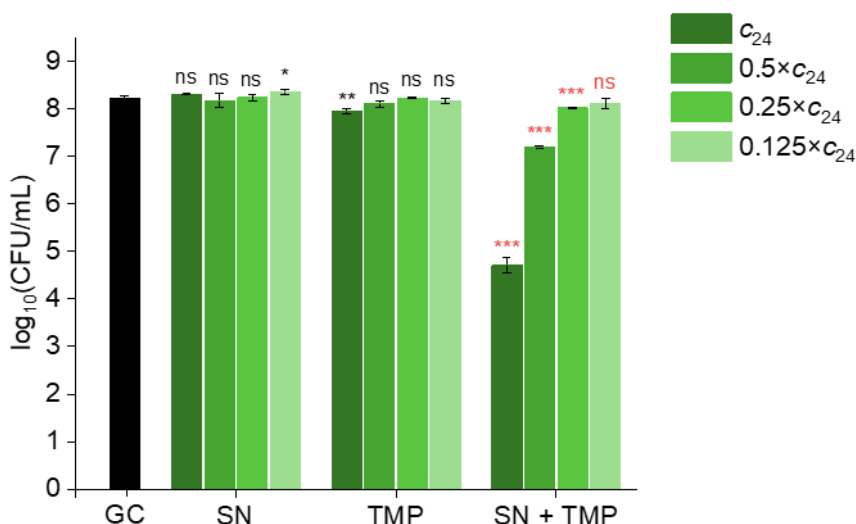


Figure 59 Results of the CFU count for SA RN4220. The poly(SA-pHBA acrylate) brush prodrug polymerized for 120 min was incubated for 24 h in PBS, resulting in the drug release supernatant (SN). This SN was tested alone, mixed with TMP in a 5:1 mass ratio (SN+TMP) and a TMP solution at the corresponding concentration was also tested. Asterisks represent significant differences from the growth control (black) or the TMP solution at the corresponding concentration (red) (no significance (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)).

S. aureus RN4220 showed the highest MIC values determined for the tested strains, which is also reflected in the drug release experiment. While the neat supernatant does not influence the growth of the bacteria, TMP by itself shows only a minute influence on the bacterial CFU count at the highest concentration tested. The mixture of the drug release supernatant with TMP shows a strong antibacterial effect, yielding an LRF of 3 at the highest concentration and still a significant reduction at half the concentration (LRF of 1). This highlights the reduced efficacy of TMP and SA in combination with TMP on this strain, as expected based on the MIC values.

5.3 Summary and Conclusions

In this Chapter, SA was used to generate an imine-based monomeric prodrug by coupling the antibiotic to pHBA, followed by the acrylation of the phenol group. In the subsequent functionalization of titanium-coated glass substrates, polymer brushes of the synthesized monomeric prodrug were fabricated through an SI-RAFT mechanism and then characterized spectroscopically and microscopically. It was found that polymer brushes were generated on both the titanium face and the glass face of the substrates, with brushes on the glass face consistently thinner than brushes on the titanium face.

Brushes of varying thickness were incubated in different release media and were found to release SA in a time- and thickness-dependent fashion from both faces of the substrates. The release of SA was detected by UV-Vis as an increase in the optical density of the drug release supernatant at 258 nm and in the measured HPLC chromatograms as an eluent detected after approximately 3 min of retention time. Furthermore, AFM was utilized in combination with ellipsometry to quantify the change in film thickness on both faces of the substrates, and by estimating the density of the polymer film, the amount of SA released was approximated. All techniques yielded comparable values for the amount of SA released from the substrates, showing a fast initial release that levels off between 1 h and 6 h, and a more sustained release over the subsequent 42 h. The loading capacity relative to the volume indicates a much higher drug content of the generated polymer brushes in comparison to other polymeric carriers, e.g., drug-loaded hydrogels.

Confirmation of the predicted mechanism of drug release was achieved by performing RAIRS analysis, revealing the time-dependent decrease of the imine absorption band, which agrees with the release of SA. Furthermore, the increase of the aldehyde signal confirms the hydrolysis of the imine, leaving behind the aldehyde linker.

The conducted drug release experiments prove the applicability of surface-grafted polymer brushes to act as drug-releasing platforms. Although the utilized prodrug only releases inert SA, the combination with TMP eradicates two of the tested *S. aureus* strains completely and significantly reduces bacterial numbers for the third *S. aureus* strain tested. This shows how the concentrations of released drug achieved in this experiment can be within the range of MIC values necessary to inhibit the growth of bacteria. By using a more potent drug for the synthesis of polymer brush prodrugs, or by copolymerizing the generated monomeric prodrug with a prodrug based on TMP to achieve polymer brushes with the perfect ratio of both compounds, a self-sufficient polymer brush prodrug is predicted to be achievable.

Although unexpected, the release of a secondary species from the brushes, which was detected in UV-Vis spectroscopy but not in HPLC and is assumed to be from the hydrolysis of the ester bond between the polymeric backbone and the pHBA linker, was shown not to interfere with the bacteria experiments. The release of this species might prove beneficial from an applicational point of view, as pHBA was demonstrated to be advantageous in wound healing.²²³ A polymer brush prodrug, which releases an

antibacterial agent and a wound healing accelerator, is a promising system that not only tackles the dangers of early-stage bacterial contamination but also promotes wound healing. Such a bifunctional system based on a polymer brush has not yet been reported. As observed for drug release experiments in a strongly basic environment, the detachment of the polymer brushes based on the hydrolysis of the silane-titanium bond could be used for the gradual decomposition of the protective layer on an implant, yielding an initial protection of the implant surface against bacterial attachment, promoting cell proliferation, followed by detachment of the polymer chain from the implant surface, allowing host cells to attach, facilitating long-term incorporation of the implant.

5.4 Materials and Methods

5.4.1 Materials

Sulfanilamide (SA) ($\geq 99\%$, Avantor, Radnor, USA), acryloyl chloride ($\geq 96.0\%$, Thermo Fisher Scientific, Darmstadt, Germany), triethylamine (TEA) ($\geq 99.5\%$, Sigma-Aldrich, Taufkirchen, Germany), *p*-hydroxybenzaldehyde (pHBA) ($\geq 98\%$, Sigma-Aldrich, Taufkirchen, Germany), dichloromethane (DCM) ($\geq 99.8\%$ (analytical reagent grade), dried over silica gel, Fisher Scientific, Hampton, USA), ethanol ($\geq 99.8\%$, denatured, Carl Roth, Karlsruhe, Germany), acetone (analytical reagent grade, Fisher Scientific, Hampton, USA), 4-((((2-carboxyethyl)-thio)carbonothioyl)thio)-4-cyanopentanoic acid (CTA) (95%, Sigma-Aldrich, Taufkirchen, Germany), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (commercial grade, Sigma-Aldrich, Taufkirchen, Germany), methanol ($\geq 99.9\%$, Carl Roth, Karlsruhe, Germany), (4,4'-azobis(4-cyanovaleric acid) (ACVA) ($\geq 98\%$, Sigma-Aldrich, Taufkirchen, Germany), 1,4-dioxane ($\geq 99\%$ (dried over molecular sieves, stabilized), Carl Roth, Karlsruhe, Germany), aminopropyl triethoxysilane (APTES) ($\geq 98\%$, Sigma-Aldrich, Taufkirchen, Germany), pyridine ($\geq 99\%$, Carl Roth, Karlsruhe, Germany), trimethoprim (TMP) (98%, Avantor, Radnor, USA), toluene (analytical reagent grade, dried over 3 Å molecular sieves, Fisher Scientific, Hampton, USA), acetonitrile ($\geq 99.9\%$, HPLC gradient grade, Fisher Scientific, Loughborough, UK) and trifluoroacetic acid (TFA) ($\geq 99\%$, Carl Roth, Karlsruhe, Germany) were used as received if not mentioned otherwise. Milli-Q water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) was obtained from a

Millipore Direct Q8 device (Millipore, Schwalbach, Germany) equipped with a Millimark Express 40 filter (Merck, Darmstadt, Germany) (hereinafter referred to as water). Phosphate-buffered saline (PBS) solution was prepared by dissolving one tablet of PBS (Sigma-Aldrich, Taufkirchen, Germany) in 200 mL of water.

S. aureus lab strain RN4220 (ATCC 35556) was originally derived from NCTC 8325-4 via UV and chemical mutagenesis and was purchased from Leibniz-Institute DSMZ GmbH (Germany). *S. aureus* reference strain 2569 (ATCC 29213, strain designation: Wichita) is an MSSA strain isolated from an infected wound, purchased from Leibniz-Institute DSMZ GmbH (Germany). The non-pathogenic *E. coli* MACH1TM-T1 (noted as MACH1) was bought from Invitrogen, California, USA, and the non-pathogenic *E. coli* DH5a was purchased from Thermo Fisher Scientific, Germany.

S. aureus clinical isolates MSSA69, MSSA52, MSSA21, MSSA56, eMRSA9, MRSA378 and MSSA 233 were isolated from an invasive *S. aureus* outbreak in John Radcliffe Hospital in Oxfordshire, UK, collected between 1997 and 1998. eMRSA9 has known resistance to penicillin, tetracycline, erythromycin, kanamycin and gentamycin. The pathogenic *E. coli* NCTC 10418, *S. aureus* MRSA252, *S. aureus* NCTC 10788 (ATCC 6538; originally isolated from a human lesion), the bacteriophage K-sensitive *S. aureus* strain H560 and *S. epidermidis* 12228 were kindly provided by Prof. Toby Jenkins, University of Bath, UK. *S. aureus* JAR060131, *S. aureus* MRSA LUH14616 and *A. baumannii* RUH375 were kindly provided by Dr. Bas Zaat, Amsterdam University Medical Centre, The Netherlands. *S. aureus* N315 is a cystic fibrosis clinical isolate with known methicillin and penicillin resistance, kindly provided by Dr. Tony le Gall, University of Brest, France.

5.4.2 Methods

Nuclear magnetic resonance (NMR) spectroscopy. ¹H-NMR spectra were recorded for confirmation of the successful monomer synthesis. ¹H-NMR spectra were measured using a JEOL ECZ 500 device (JEOL Ltd., Tokyo, Japan) in DMSO-d₆ (99.8%, Deutero, Germany) and analyzed using MestReNova software (Version 6.0.2-5475). All chemical shifts (δ) are given in ppm.

Combustion analysis (CHNS analysis). CHNS analysis was performed using a HEKAtech EuroEA combustion analyzer (HEKAtech GmbH, Wegberg, Germany). The dried powders of the precursor and the monomeric prodrug were measured, and the relative composition of carbon, hydrogen, nitrogen and sulfur was compared to the theoretical composition.

Static water contact angle (SWCA) measurements. SWCA measurements were conducted using an OCA-15 contact angle microscope (Dataphysics, Filderstadt, Germany). In the sessile drop conformation, 2 μ L of water was dispensed onto the substrate at room temperature, and the contact angles were determined based on the profile of the drop. For each surface modification, the contact angles were determined in triplicate over at least 3 samples for the determination of the mean value and the standard deviation.

X-ray photoelectron spectroscopy (XPS). XPS spectra were recorded using an ESCA spectrometer (SSX-100 S-probe, Surface Science Instruments, Mountain View, USA). All spectra were calibrated based on the aliphatic carbon signal, which was shifted to 284.8 eV. The instrument is equipped with a 200 W Al K α X-ray source. Survey spectra were obtained from 0 to 1200 eV with an energy resolution of 1 eV. High-resolution spectra were recorded in the energetic range of the respective components, with a window size of 20 eV and a resolution of 0.1 eV. All spectra were analyzed and quantified using the Casa XPS software (version 1.3.19.1).

UV-Vis spectroscopy. The release of SA from the prodrug-modified titanium substrate was quantified via UV-Vis spectroscopy. UV-Vis spectra of the drug release supernatants were recorded at 25 °C in UV-transparent single use cuvettes (Sarstedt AG & Co. KG, Nümbrecht, Germany) using a Varian Cary 50 Bio UV-Visible Spectrophotometer (Varian, Mulgrave, Victoria, Australia) from 200 to 400 nm with a scan rate of 200 nm/min and automatic background subtraction of the PBS spectrum measured beforehand. The concentration of SA was quantified based on a previously acquired calibration curve (serial dilution of SA in PBS), correlating the absorption maximum at 258 nm with the concentration of the measured solution.

High-performance liquid chromatography with UV-Vis detection (HPLC-UV). All HPLC measurements were conducted by M.Sc. Désirée Anna-Maria Schütz. Drug release supernatants were filtered through a syringe filter (0.2 μ m, hydrophilic PTFE,

VWR, Darmstadt, Germany) and measured via HPLC-UV (Agilent 1200 series HPLC system, Agilent Technologies, Santa Clara, USA). For separation, a reversed-phase analytical column (Waters XBridge™ C18, 4.6 × 150 mm, 3.5 μm) was used with an isocratic run profile. Sulfanilamide was detected and quantified at $\lambda = 258$ nm. The mobile phase mixture consisted of 0.01% TFA in water and acetonitrile with a volume ratio of 80:20 at 0.75 mL min⁻¹. The injection volume was set to 5 μL. The concentration of SA was quantified based on a previously acquired calibration curve (serial dilution of SA in PBS), correlating the peak area of eluted SA (retention time of 3 min) with the concentration of the measured solution.

Atomic force microscopy (AFM). AFM measurements were conducted under ambient conditions using an Asylum MFP-3D (Asylum Research Inc., Santa Barbara, USA) equipped with a Tap300AI-G cantilever with a resonance frequency of 300 kHz and a nominal spring constant of 40 N/m (BudgetSensors Innovative Solutions Bulgaria Ltd., Sofia, Bulgaria). For the determination of the film thickness on the glass face of the substrate, a scalpel was used to form a scratch, which was scanned in intermittent contact mode with a scan size of 60 μm and a resolution of 128 points and lines. The pixel height distribution was used to determine the step height, which corresponds to the film thickness. This method was also used to determine the change in film thickness upon incubation in PBS. By measuring the film thickness on 3 substrates from 3 separate polymerizations, incubated for the same time in the drug release solution, the average change in film thickness was determined.

Ellipsometry. The dry thickness of the polymer brushes was measured using an alpha-SE ellipsometer (J.A. Woollam Co., Inc., Lincoln, USA) at different angles of incidence (65, 70, and 75°), with the wavelength ranging from 380 to 900 nm. The Cauchy model with a refractive index of 1.45 at 632.8 nm was used for the determination of the initiating layer thicknesses, while the polymer brush thickness was fitted according to the Sellmeier model with a refractive index of 1.52 at 632.8 nm. For the determination of the polymerization kinetics, 9 substrates per polymerization were utilized with 3 separate polymerizations per polymerization time, yielding 27 samples per polymerization time. The mean values and standard deviations across all samples for a given polymerization time were calculated and used to determine the time-dependent brush growth.

Fourier transform infrared (FTIR) spectroscopy. FTIR spectra were acquired using a Vertex Neo R spectrometer (Bruker Optics GmbH & Co. KG, Ettlingen, Germany) in combination with a VeeMAX-II Variable Angle Specular Reflectance Accessory (PIKE Technologies, Fitchburg WI, USA) for reflection-absorption FTIR (RAIRs) measurements of the brushes, operated at an incidence angle of 80° under vacuum at room temperature using p-polarized light. As the background, a titanium sample was measured immediately after cleaning according to the previously described procedure (including UV/ozone cleaning) with a spectral range from 600 cm⁻¹ to 4500 cm⁻¹ and the aperture set to 6 mm and 1024 scans. To determine changes in the brush films upon hydrolysis, incubated samples were rinsed with water and ethanol and then dried in a nitrogen stream. The spectra were analyzed and corrected using the concave rubber band correction method using 6 iterations per spectrum in the OPUS 9.0 SP1 software. Powdered samples were measured using the included attenuated total reflection (ATR) setup, ranging from 600 cm⁻¹ to 4000 cm⁻¹, with the aperture set to 6 mm and 16 scans.

Electrospray ionization-mass spectrometry (ESI-MS). ESI-MS spectra were acquired by M.Sc. Manuel Heinelt using a Thermo Fisher exactive (Thermo Fisher Scientific Inc., Waltham, USA) device equipped with a high-resolution orbitrap mass spectrometer. For the measurement, the monomeric prodrug was dissolved in methanol (9 µg/mL) and injected into the device at a flow rate of 5 µL/min.

5.4.3 Monomeric Prodrug Synthesis

Synthesis of monomeric prodrug. SA (145 mmol) was mixed with pHBA (162 mmol) in methanol (250 mL) and stirred overnight under ambient conditions. The resulting precipitated imine precursor was filtered, washed with methanol and dried in a vacuum oven (r.t., 5 mbar), resulting in a yield of 70%. The dried imine precursor (1 equivalent (eq.)) was added to a stirring solution of triethylamine (1.2 eq.) in acetone (250 mL). Introduction of the acrylic unit was achieved by dropwise addition of an acryloyl chloride (1.1 eq.) solution in acetone (50 mL) under argon atmosphere and constant ice-cooling of the reaction mixture. After complete addition of acryloyl chloride, the reaction mixture was stirred overnight (r.t.), then filtered (round filter type MN 616, Macherey-Nagel, Carl Roth, Germany). Washing of the filtered product with methanol and vacuum drying

(r.t., 5 mbar) yielded the imine-containing monomeric prodrug. This procedure resulted in a yield of 66%.

5.4.4 Substrate Preparation and SI-RAFT Polymerization of Monomeric Prodrug

Titanium deposition on glass substrates. Glass microscope slides (1 mm thickness, VWR, Darmstadt, Germany) were cut into 1.5 cm × 2.5 cm pieces with a diamond tip glass cutter. The substrates were sonicated in soap water (detergent, Ecolab, Monheim am Rhein, Germany) for 15 min, followed by sonication in water for 15 minutes. Afterwards, the substrates were subjected to the steam of boiling isopropanol, then coated with 100 nm of titanium through electron beam evaporation under high vacuum (Edwards E306 coating system, Moorfield, UK).

Silanization and attachment of the CTA. Titanium-coated substrates were sonicated in acetone, ethanol and water for 5 min each, then dried under a nitrogen stream. Subsequently, the cleaned substrates were placed in a BioForce UV/Ozone Pro Cleaner (Bioforce Nanoscience, Ames, USA) for 20 min to activate the surfaces. The activated substrates were placed in an APTES solution (2 v% in toluene, 50 mL) for 3 h under ambient conditions. The silanized substrates were cleaned by sonication in ethanol for 5 min, then rinsed with ethanol and water before drying under a nitrogen stream. Subsequently, the samples were placed in a solution of CTA (2.3 mg/mL) and EDC·HCl (4 mg/mL) in DCM (30 mL) under an argon atmosphere and stirred overnight at room temperature. The substrates were then thoroughly washed using ethanol, acetone and water, then dried under a nitrogen stream.

Fabrication of polymer brushes via SI-RAFT. The monomeric prodrug was used to form polymer brushes on the CTA-modified substrates. A mixture of the monomeric prodrug (3 mmol) and ACVA (86 μmol) in 1,4-dioxane (20 mL) was added to the reaction chamber. The reaction mixture was sparged with argon, the substrates were immersed and the reactor was closed, maintaining the argon atmosphere. The polymerization was initiated by immersing the reactor in an 80 °C oil bath. To stop the reaction at a defined time point, the samples were removed from the reaction solution,

thoroughly rinsed with acetone, sonicated in pyridine for 5 min, then rinsed with ethanol and dried in a nitrogen stream.

5.4.5 Drug Release Experiments

Antibiotic release from polymer brushes. Brush-covered substrates were immersed in a six-well plate, covered with PBS (3 mL) and incubated at 37 °C for up to 48 hours. Each drug release experiment was performed in triplicate and quantified by UV-Vis spectroscopy, high-performance liquid chromatography with UV-Vis detection, atomic force microscopy, ellipsometry and Fourier transform infrared (FTIR) spectroscopy.

5.4.6 Bacteria Experiments

All bacterial experiments were conducted by M.Sci. Nicola Cusick, except checkerboard assays for pHBA, which were conducted by Dr. Kawaljit Kaur. All bacteria were grown in LB medium at 37 °C. A sterile inoculation loop was used to streak bacterial glycerol stocks onto LB agar plates, which were incubated at 37 °C for 18 h. A single colony was picked from this plate and used to inoculate a tube of LB medium (5 mL). This tube was incubated at 37 °C for 18 h while shaking at 200 rpm to produce an overnight culture.

Disc diffusion assay. To assess the antibacterial activity of SA and TMP, SA/TMP solutions were prepared in PBS at a fixed 5:1 mass ratio. SA concentrations of 1000 µg/mL, 100 µg/mL and 10 µg/mL were evaluated in this combination. Control solutions containing SA alone and TMP alone were also prepared at the corresponding concentrations. Sterile cellulose discs ($\varnothing$ = 6 mm) were impregnated with the antibiotic solutions and allowed to air-dry for ~15 min. Overnight cultures of *S. aureus*, *S. epidermidis*, *E. coli* and *A. baumannii* were grown in LB medium (37 °C, 17 h, 200 rpm). Cultures were diluted in fresh LB medium to an optical density at 600 nm (OD₆₀₀) of 1.2 (*S. aureus*, *S. epidermidis*) or 0.6 (*E. coli*, *A. baumannii*), corresponding to ~10⁸ CFU/mL. Disc diffusion assays were performed by uniformly streaking bacterial suspensions onto LB agar plates using sterile cotton swabs, followed by air-drying for ~5 min. Antibiotic-loaded cellulose discs were then carefully placed onto the inoculated

agar surfaces, and the plates were incubated (37 °C, 18 h). All experiments were conducted in triplicate.

Determination of MIC. To determine the MIC, stock solutions of SA, TMP, and 5:1 SA:TMP were prepared in PBS. Samples were serially diluted (2-fold) in PBS across a 96-well plate. To 100 μ L of antibiotic, 100 μ L of bacteria ($\sim 2 \times 10^6$ CFU/mL in LB) was added. Optical density at 600 nm (OD_{600}) was measured (A_0 , Tecan Infinite 200), the well plate incubated (37 °C, 20 hours), and the OD_{600} measured again (A_{20}). All concentrations were tested in triplicate. The lowest concentration at which the change in absorbance ($A_{20} - A_0 \leq 0.01$) was considered the MIC.

Determination of antibacterial activity of released SA. To determine the antimicrobial activity of the SA released from the polymer brushes, the released supernatant (SN) after 24 h of incubation of the sample polymerized for 120 min was tested alone and with TMP in a 5:1 mass ratio. A control TMP solution was made by mixing with the same volume of PBS. In a 96-well plate, PBS (80 μ L) was added to columns 2-12. The sample to be tested (SN, TMP, or SN+TMP) was added in column 1 (160 μ L) then serially diluted (2-fold) to column 10 (80 μ L/well). To all wells in columns 1-11, bacteria ($\sim 5 \times 10^6$ CFU/mL in LB) were added (20 μ L), while in column 12, LB medium was added (20 μ L, sterile control). Bacteria were incubated in humidity chambers with gentle shaking. After 21 hours, each well was serially diluted ($10\times$) in PBS and 3×10^4 μ L was plated for each dilution step on LB agar plates, resulting in a limit of detection of 34 CFU/mL. Plates were incubated for 16 h at 37 °C, then bacterial colonies were counted. From this count, the $\log_{10}$ (CFU/mL) and the log reduction factor (LRF) were calculated. The arithmetic mean and standard deviation were calculated from 3 independently performed experiments (biological triplicate). Statistical significance was determined by one-way analysis of variance (ANOVA) and the Tukey multiple comparison test using OriginPro 2018. p values < 0.05 were considered statistically significant.

Checkerboard assay. SA or pHBA (50 μ L) and TMP (50 μ L) were added in 2-fold gradient concentrations across and down the 96-well plate, respectively. Bacterial suspension (100 μ L, $\sim 10^6$ CFU/mL in LB) was added to each well. Positive (bacteria with PBS) and negative (LB with PBS) controls were tested in column 12. OD_{600} was measured (A_0), then the 96-well plates were incubated at 37 °C for 20 h and the OD_{600}

was measured again (A_{20}). Values of $A_{20} - A_0 \leq 0.01$ were considered to exhibit 100% inhibition.

6. RAFT Polymerization for Antifouling Polymer Brushes on Polymerized Lipid Membranes

6.1 Introduction and Objectives

While Chapters 4 & 5 focused on approaches to inhibit bacterial growth on implant surfaces, promoting host cell attachment remains essential to win the “race for the surface”. This Chapter introduces a novel polymer brush-lipid membrane platform designed to provide a model system to investigate cell-brush interactions. To generate brush-based cell culture substrates, polymerized and patterned lipid bilayers can act as excellent soft substrates for the synthesis of polymer brushes. Substrate-supported lipid bilayers, immobilized on different solid supports, can be generated based on diacetylene phospholipids through UV photopolymerization, as can be seen in Figure 60.

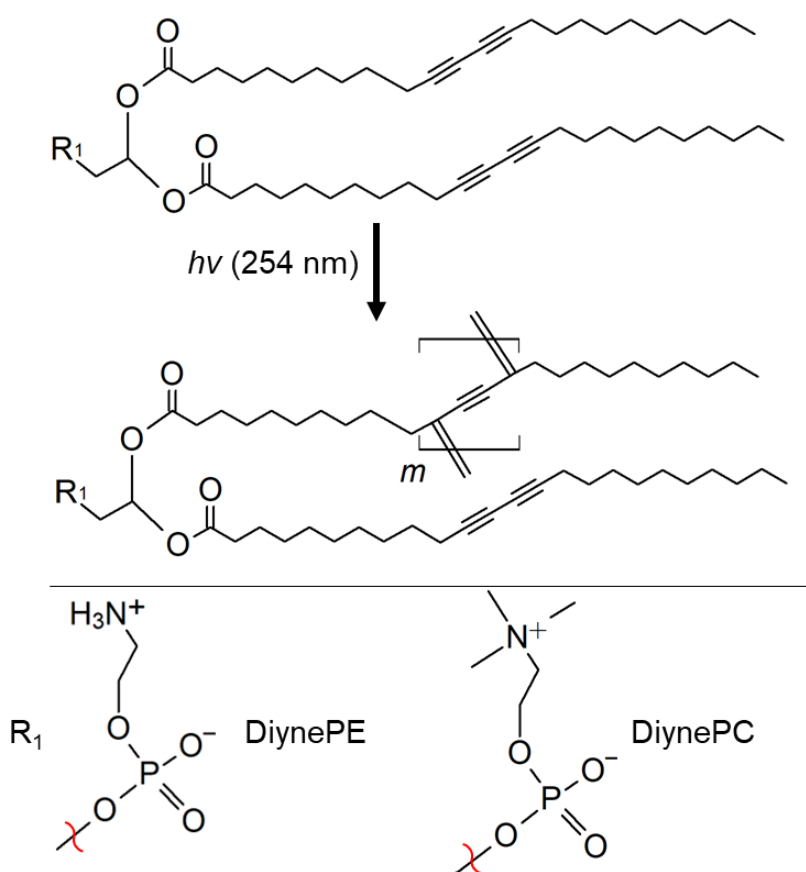


Figure 60 UV photopolymerization of diacetylene phospholipids.

By using the vesicle fusion technique, a bilayer can be deposited on different substrates. The use of a UV photomask allows these bilayers to be crosslinked in

predefined positions. The non-crosslinked areas can be removed using detergents, leaving behind a scaffold of a polymerized, fluorescent bilayer, able to incorporate fluid lipid bilayers in the corrals.⁵³ The spatially confined fluid lipid bilayers can act as micrometer-sized environments for studies of incorporated species.

It has been previously shown that polymer brushes can be synthesized on substrate-supported lipid membranes on either side of the lipid membrane.⁵⁷ The high uniformity, together with the opportunity to detect and investigate the behavior of fluorescent species down to the single-molecule level, opens pathways for the synthesis of novel platforms that can potentially be used for the study of cellular responses to environmental changes or external triggers. Such a system could be realized with a polymerized patterned lipid bilayer functionalized with polymer brushes to spatially control the binding of cells to the surface. By tuning the antifouling properties of the generated polymer brushes, such a polymeric system can be used to immobilize cells across a corral of the patterned bilayer. Upon exposure to chemical or physical triggers, cellular responses, which can manifest as secreted species, are hypothesized to be detectable within the corral. A scheme of the idealized system can be seen in Figure 61.

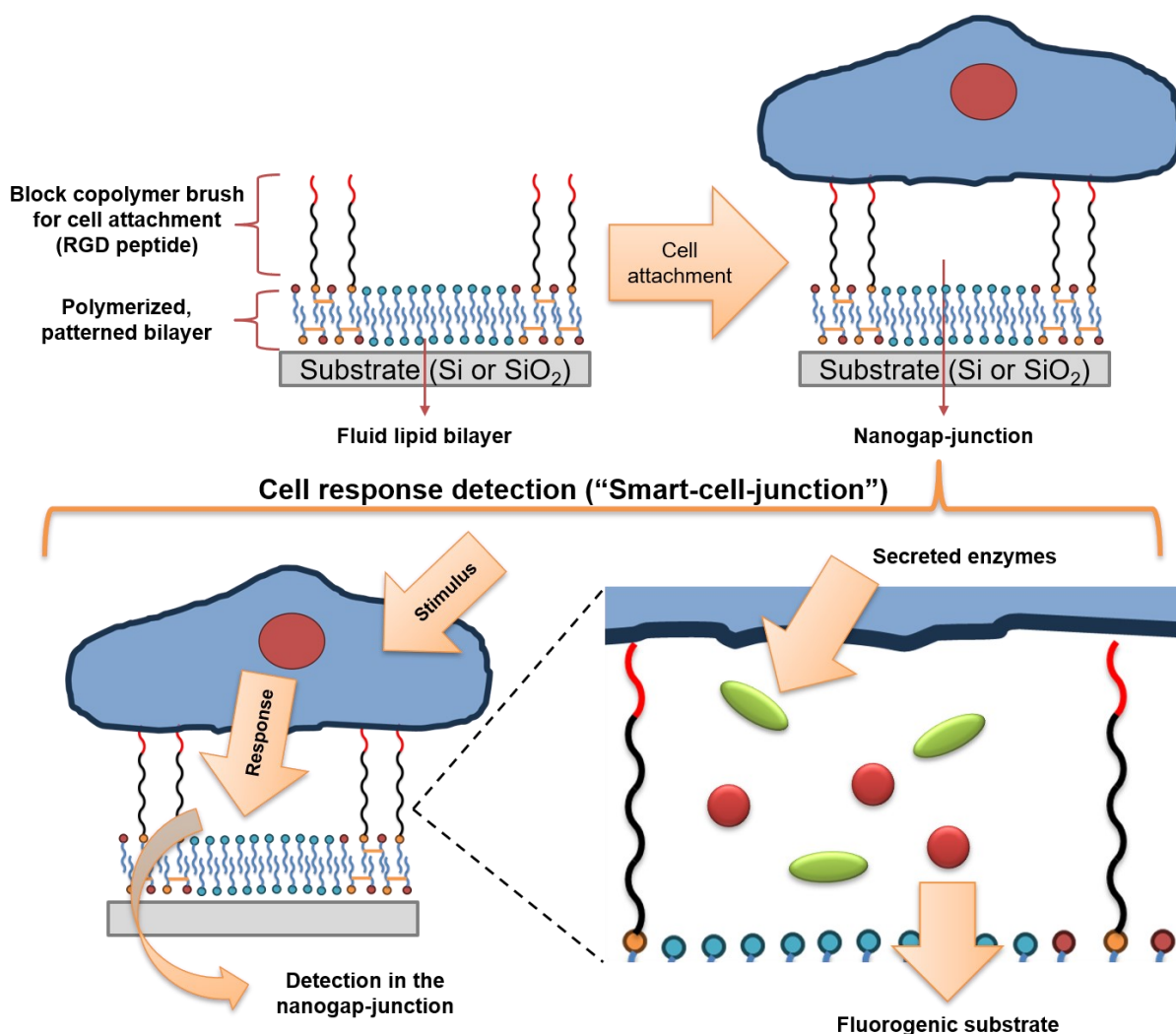


Figure 61 The functionalization of a polymerized lipid bilayer with polymer brushes creates a corral in which cellular responses upon external stimulation of an immobilized cell can be detected.

Due to the aforementioned restrictions and possible cytotoxic side effects of compounds involved in the ATRP mechanism, a novel route for the synthesis of polymer brushes on supported lipid bilayers is investigated. In this Chapter, the chemical functionalization of polymerized lipid membranes with polymer brushes based on a RAFT polymerization technique and subsequently conducted cell-adhesion studies are described. Further experiments to generate a functionalizable block copolymer of POEGMA-*b*-PGMA are also described, which open new routes for the attachment of cell adhesive RGD peptide sequences.

6.2 Results and Discussion

Polymerized and patterned lipid bilayers were received from the group of Prof. Dr. Kenichi Morigaki from the University of Kobe, Kobe, Japan. The lipid membranes were produced by M.Sc. Yu Yoshimura. Cell experiments were conducted by M.Sc. Renée Sophie Hoffmann at the University of Siegen. Although the generated membranes possessed the same nominal chemical composition (ratio of utilized lipids), the membranes that arrived in Siegen were found to be of varying chemical composition between batches. However, within a single batch of samples, the conducted experiments yielded reproducible brush syntheses, therefore all experiments described in this Chapter were performed with samples from a single batch, which arrived in Siegen in October 2025.

The received lipid membranes were characterized by fluorescence microscopy and further analyzed by AFM height imaging, as can be seen in Figure 62.

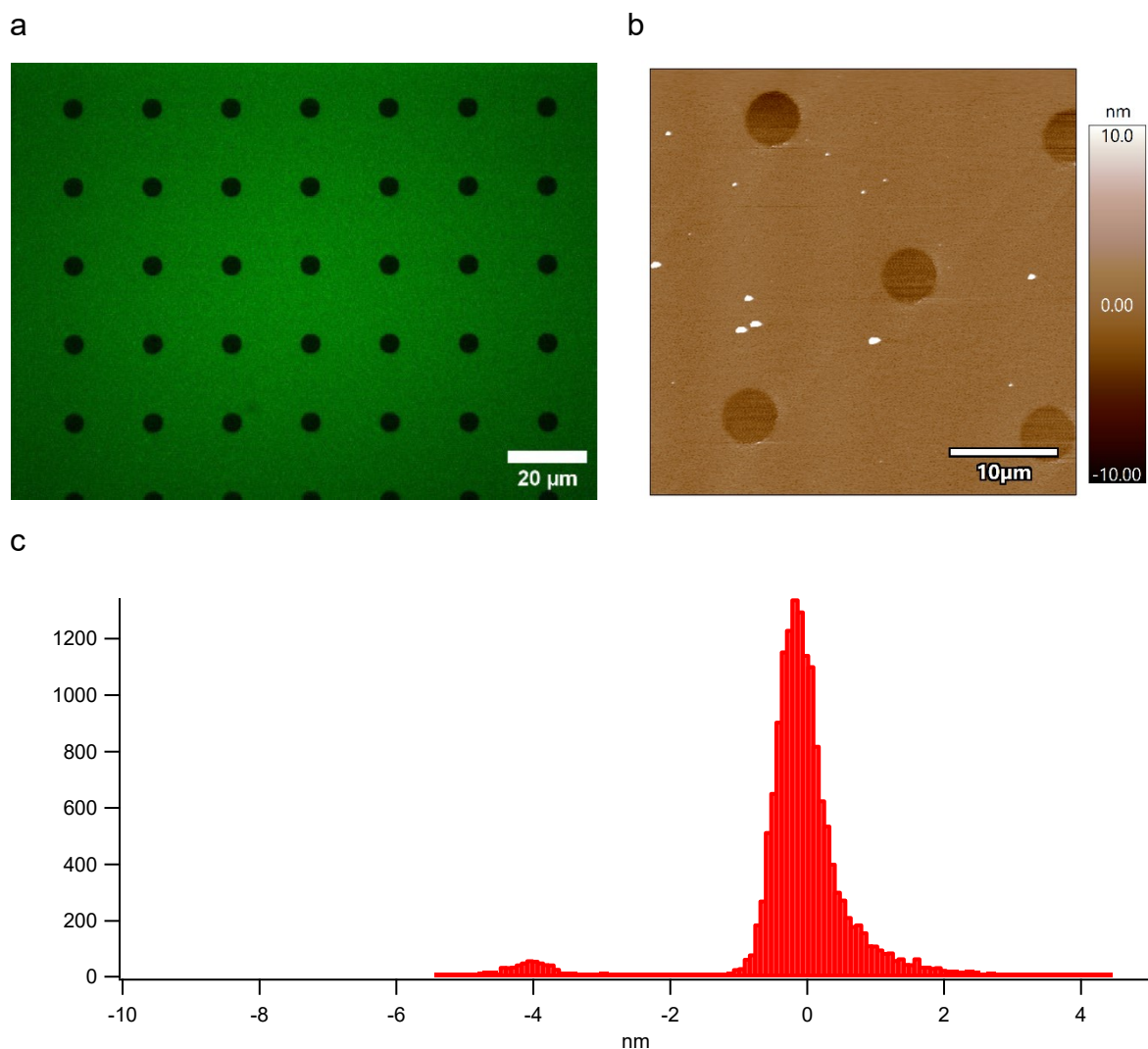


Figure 62 a) Fluorescence microscopy image and (b) AFM height image of a lipid bilayer on glass, recorded in water. c) A histogram was derived from the pixel height in the AFM height image.

The autofluorescence of the membrane can be used to visualize the empty corrals (black spots), while AFM reveals the topology of the membrane. In agreement with previously reported values, the step height determined by AFM is in the range of 4 – 5 nm.¹⁷⁴ The utilized photomask facilitates the synthesis of circular corrals with a diameter of 5 μm and a center-to-center distance of 20 μm , arranged in a P4mm plane group.

6.2.1 Polymer Brush Synthesis on Lipid Membranes by UV-RAFT

The general procedure for the growth of polymer brushes from the neat membrane to facilitate cell attachment is shown in Figure 63.

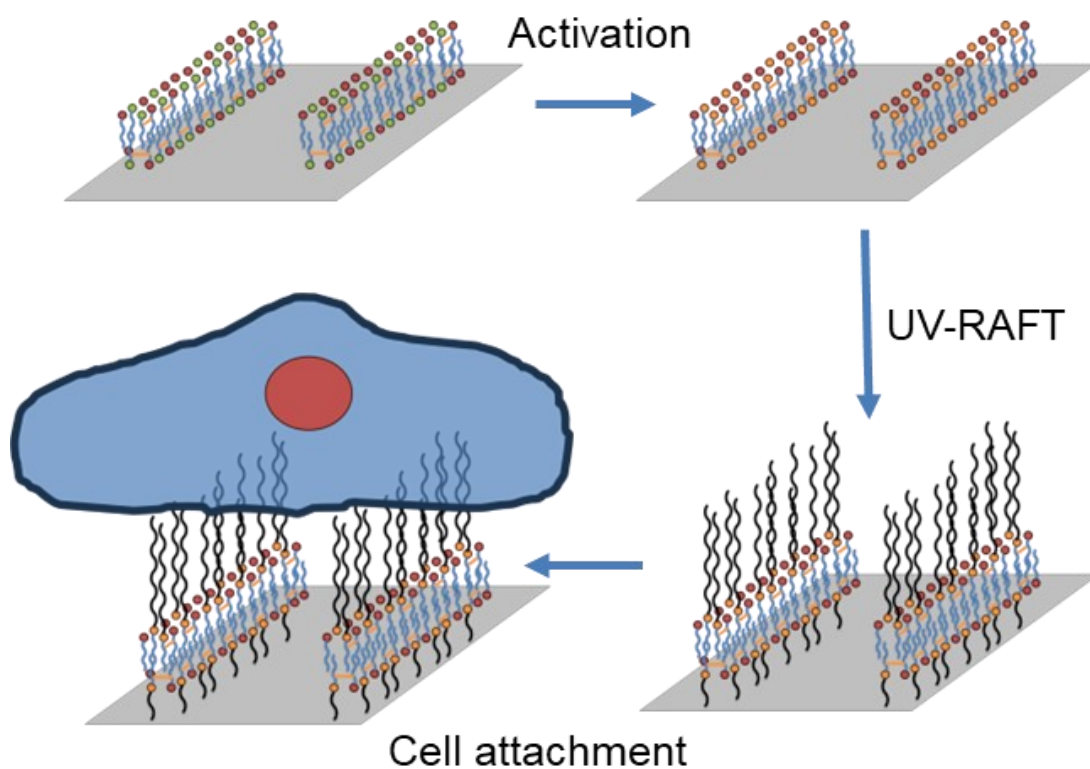
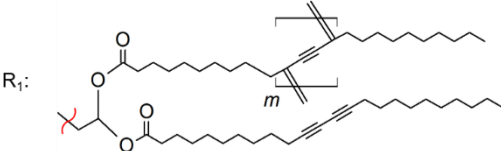


Figure 63 General procedure for the functionalization of polymerized lipid membranes with polymer brushes via UV-RAFT and subsequent cell seeding.

The functionalization of the membrane with PDEGMA or POEGMA polymer brushes via UV-RAFT is illustrated in Figure 64.

R2:NC(=N)NCCCC(N)C(=O)NCC(=O)NC(=O)C(O)C(=O)O

114

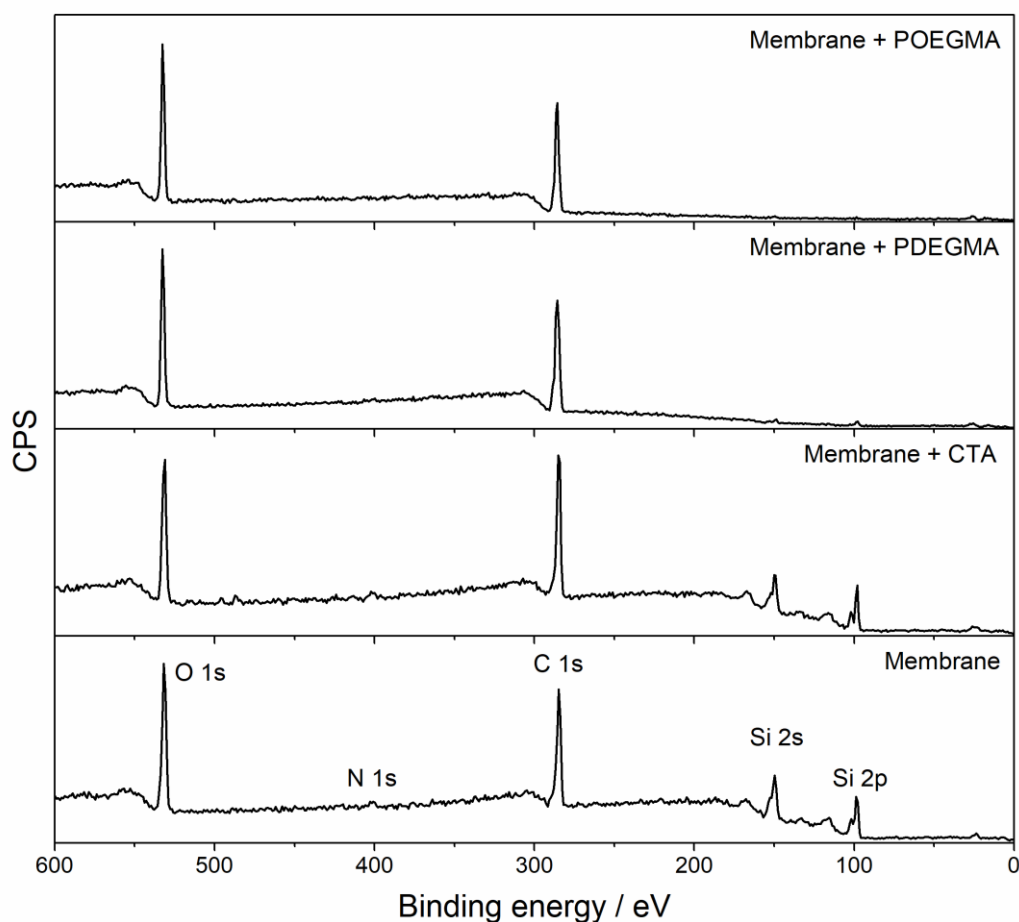


Figure 65 XPS survey spectra of the neat membrane, the CTA-modified membrane, the PDEGMA-modified membrane and the POEGMA-modified membrane. All spectra were calibrated according to the aliphatic carbon signal at 284.8 eV.

In the XPS survey spectra, several signals are visible, either stemming from the underlying silicon substrate (Si 2s, Si 2p) or the polymerized membrane itself (C 1s, O 1s, N 1s). After attachment of the CTA, no obvious changes in the survey spectrum are visible. Nevertheless, after polymerization of either PDEGMA or POEGMA, stark changes are visible. A strong decrease in the intensity of the silicon signals indicates that the silicon signals are only detected through polymer-free corrals. In addition, a complete attenuation of nitrogen photoelectrons through the thick polymer layer on the functionalized membrane is indicated by the lack of a visible nitrogen signal. For further in-depth analysis, high-resolution XPS spectra to determine chemical differences in the composition of the functionalized membranes are shown in Figure 66.

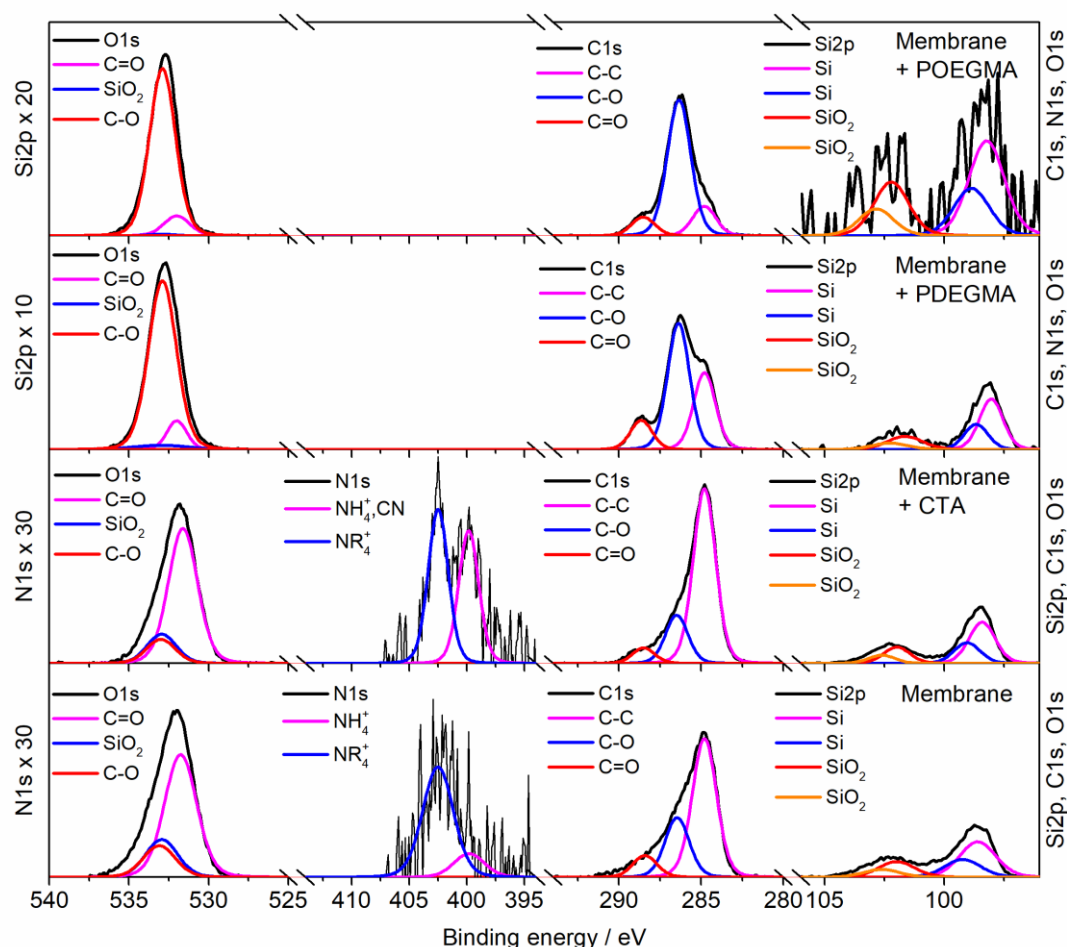


Figure 66 O1s-, N1s-, C1s- and Si2p-high-resolution XPS spectra of the membrane, the CTA-functionalized membrane, the PDEGMA-modified membrane (15 nm of PDEGMA) and the POEGMA-modified membrane (40 nm of POEGMA). All spectra were calibrated according to the aliphatic carbon signal at 284.8 eV.

The neat membrane shows signals for the underlying silicon substrate, a carbon signal which stems from the membrane and partly from adventitious carbon and therefore has a large aliphatic carbon contribution, nitrogen which can be deconvoluted into two species, with the lower energy contribution assigned to the primary ammonium of the DiynePE and the higher energy contribution corresponding to the quaternary ammonium from the DiynePC species. This agrees with the predicted composition of the membrane with a nominal composition of 95% DiynePC and 5% DiynePE. The oxygen signal shows a strong contribution of the underlying oxide and a smaller glycolic oxygen content. The phosphorous high-resolution spectrum only showed a minute signal, possibly reduced due to X-ray damage. The spectrum can be found in Appendix 28.

After the functionalization of the membrane with the CTA, the same signals as for the neat membrane can be observed. The silicon signal decreased in intensity due to the lower photoelectron escape rate via the thicker layer on top of the silicon. In the carbon spectrum, a relative increase of the aliphatic carbon contribution indicates a higher carbon content on the surface, as expected for the coupling of the CTA. The nitrogen spectrum shows a significant change, due to the introduction of the nitrile from the CTA, visible as an increase in the lower energy nitrogen contribution at ~399 eV. In the oxygen spectrum, a decrease of the oxide contribution is apparent, while the organic oxygen contribution increases, due to the introduction of further oxygen-bearing functional groups in the CTA and an overall increased thickness of the layer on the silicon, reducing the photoelectron count related to the substrate. Further sulfur and phosphorus high-resolution spectra were recorded, but only showed very minute signals, possibly due to X-ray damage. These spectra can be found in Appendix 28.

The formation of a thick (~15 nm) layer of PDEGMA on the membrane leads to the disappearance of membrane-based signals, including phosphorus and nitrogen. The substrate-related silicon signal is strongly reduced as the thickness of the polymer layer on the membrane exceeds the photoelectron escape depth. Therefore, the release of photoelectrons is limited to corrals, which are not blocked by the polymer. In the carbon spectrum, the main contribution observed stems from the glycolic carbon species, present in the side chain of the polymer. The aliphatic carbon as well as carbonyl carbon species are observable, coming from the polymeric backbone and the side chain, respectively. In the oxygen spectrum, two contributions can be assigned, according to the chemical structure of the polymer. A carbonylic oxygen species and glycolic oxygen species are observed, in a ratio matching the chemical composition of PDEGMA (0.25). A further deconvolution of the oxygen signal for the assignment of the oxide signal from the substrate is possible due to the known content of oxidic silicon from the silicon high-resolution spectrum and the known composition of silicon dioxide.

The functionalization of the membrane using POEGMA (40 nm thick layer) shows similar effects on the membrane-related spectra as for the PDEGMA-modified membrane. The nitrogen and phosphorus signals disappear, while the silicon-related signals are attenuated. The lower silicon signal intensity observed for the functionalization with POEGMA in comparison to PDEGMA is hypothesized to be related to the larger thickness of the POEGMA layer, potentially shielding the signals

coming from the corrals. Additionally, the polymer that is formed in solution could adsorb to the corrals, forming a thin layer in the corrals. The detection of silicon signals, however, proves that the corrals are not completely blocked. In the carbon spectrum, a relative increase of the glycolic carbon contribution is visible, which agrees with the chemical structure of the polymer, with a longer ethylene glycol side chain than PDEGMA and therefore more glycolic carbons. A similar effect can be seen in the oxygen spectrum, where the contribution related to glycolic oxygen species is increased relative to carbonylic oxygen.

The thickness of the polymer brush films was assessed by measuring AFM height images in water, as can be seen in Figure 67.

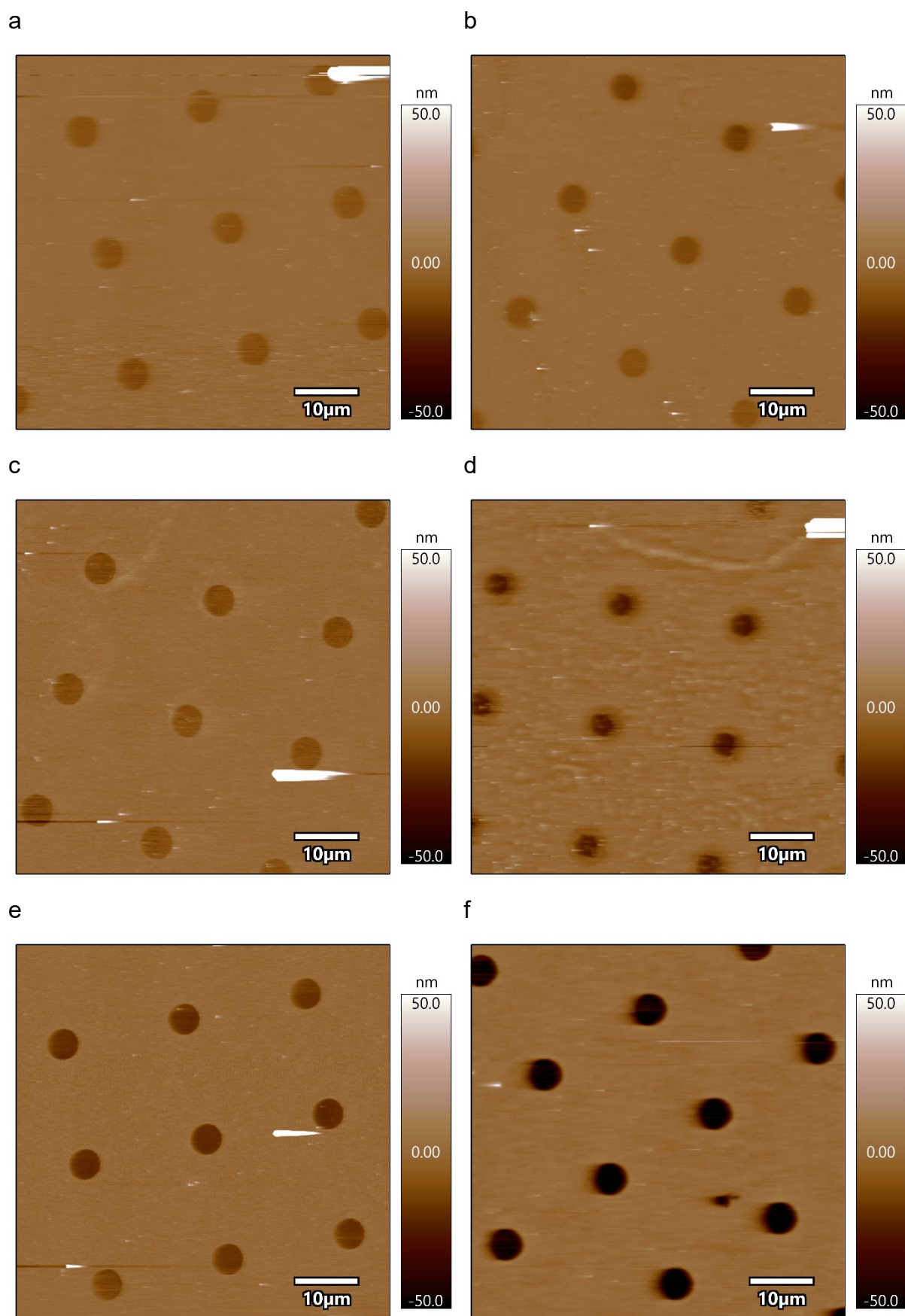


Figure 67 AFM height images of polymer brush-modified lipid membranes. PDEGMA (a, c, e) and (b,d,f) POEGMA were polymerized for (a,b) 30 min, (c,d) 60 min and (e,f) 120 min.

In the height images of the polymer brush-modified membranes, the pattern of the underlying membrane is clearly visible, indicating an SI-polymerization from the membrane only. The generated polymer brush layers are homogeneous in thickness with only minor artifacts found on the polymer films, potentially from adsorbed polymeric particles. Irregularities, as can be seen in Figure 67 (d), are assumed to be coming from irregularities in the membrane, translated into the polymer brush film. From the height images obtained, the brush film thickness was measured as the average step height between the corrals and the brush-modified membranes and plotted against the polymerization time for both polymers. The resulting kinetic plot can be seen in Figure 68.

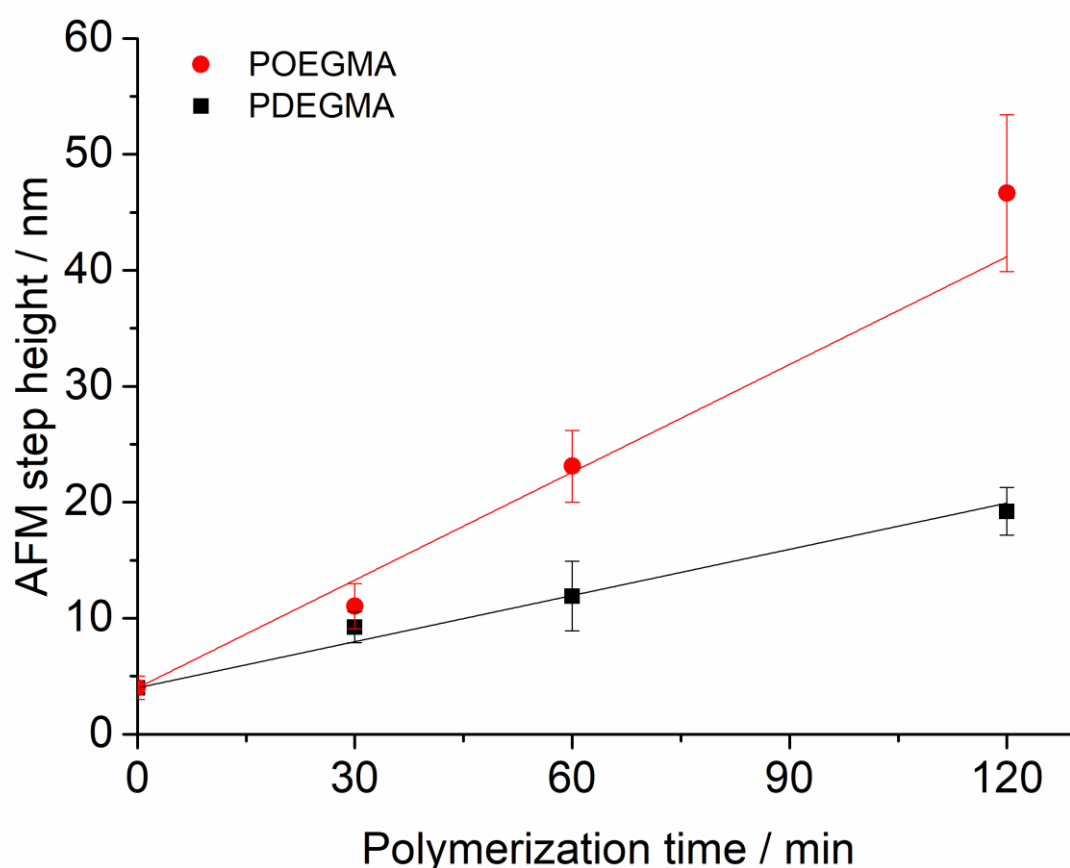


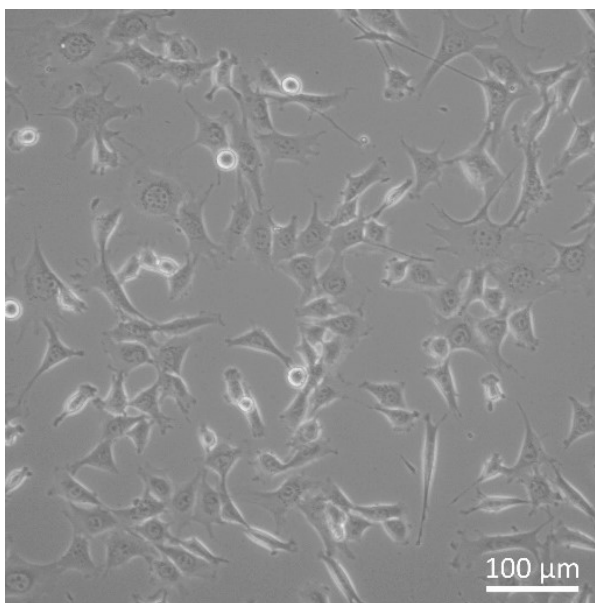
Figure 68 Kinetic plot of the polymer brush growth on lipid membranes for POEGMA (red) and PDEGMA (black). Data points represent the average step height determined by AFM; error bars correspond to the width of the obtained pixel-height distributions. Linear fits were applied to the data, with fixed intercepts at 4 nm according to the neat membrane thickness. PDEGMA: slope = 0.13 nm/min, $R^2 = 0.99$; POEGMA: slope = 0.31 nm/min, $R^2 = 0.92$.

The polymer brush grows linearly with increasing polymerization time, following first-order reaction kinetics typical for controlled radical polymerizations. The larger thickness achieved for the POEGMA brush layer is hypothesized to stem from the longer side chain in OEGMA, yielding steric repulsion along the polymer chain and therefore stretching from the surface. The rates determined for the two polymers are different, at 0.31 nm/min for POEGMA and 0.13 nm/min for PDEGMA. This faster rate of RAFT polymerization of OEGMA vs DEGMA was also observed by Pietsch et al., hinting not only towards a steric but also a kinetic effect, explaining the larger film thickness.²²⁴ In comparison to the ATRP-based system developed by Hebel et al., the RAFT approach offers the distinct advantage of milder conditions and synthesis of thicker polymer brush layers.⁵⁶

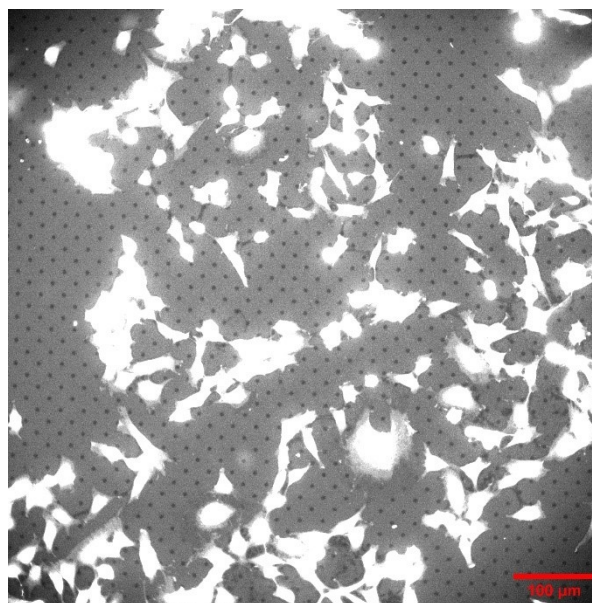
6.2.2 Cell Adhesion Studies

To test the antifouling behavior of the polymer brush-modified membranes, samples were first sterilized in ethanol, then NIH 3T3 cells were seeded and incubated for 24 h. As controls, tissue culture polystyrene (TCPS) Petri dishes were utilized to investigate the cellular response to these surfaces. After the incubation, microscopy images were acquired and can be seen in Figure 69.

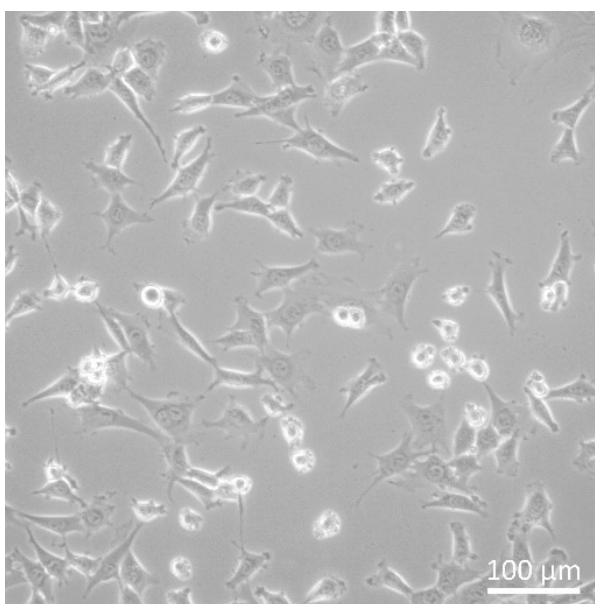
a



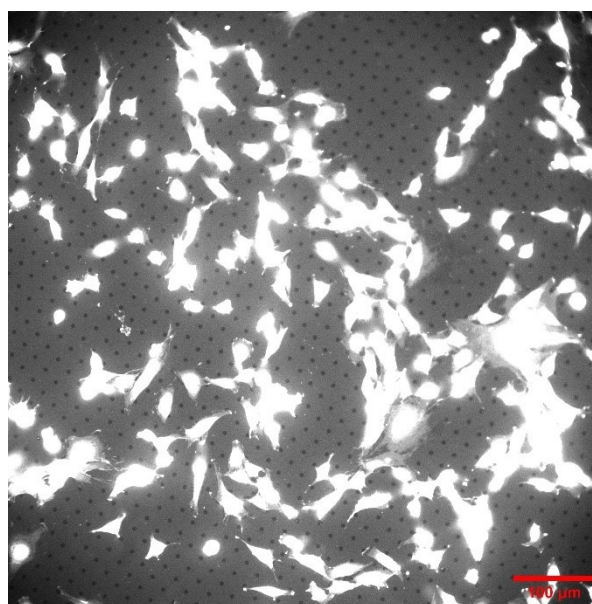
b



c



d



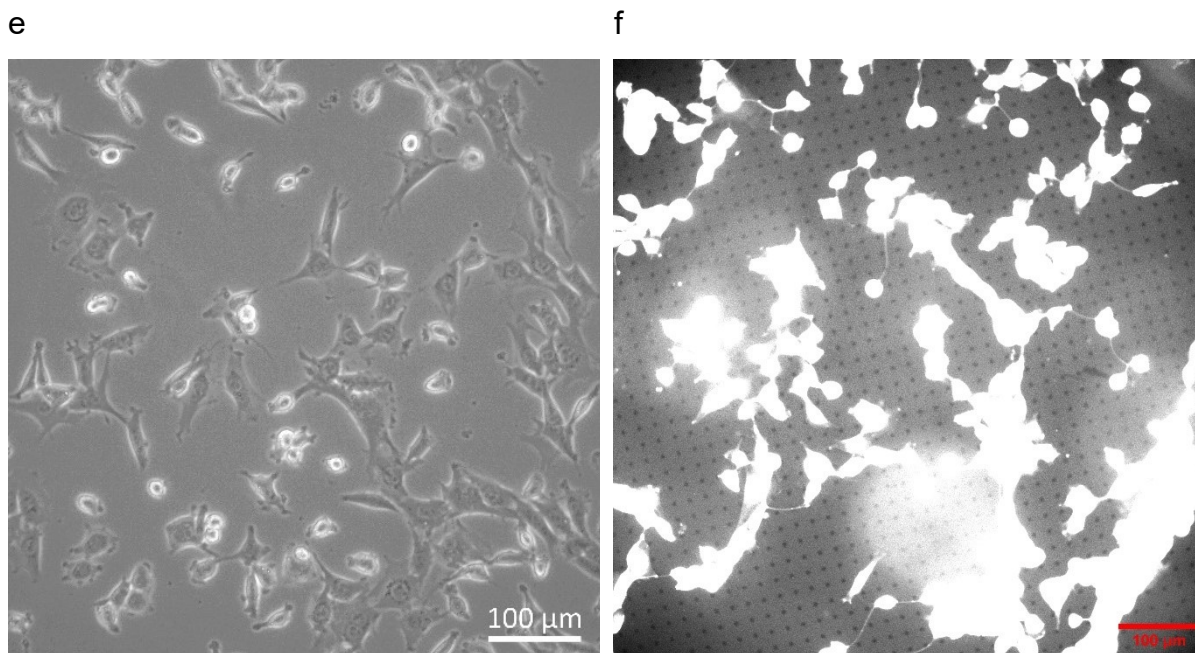
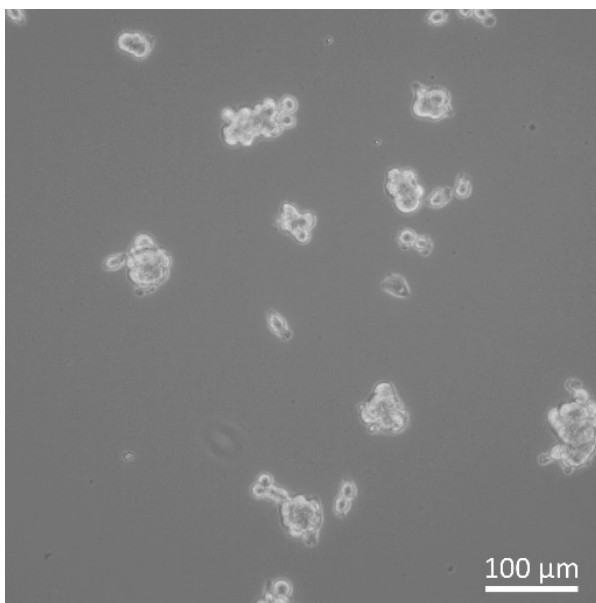


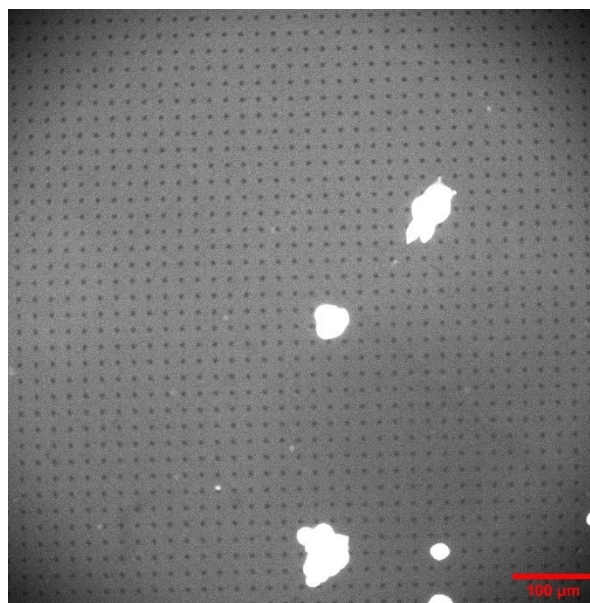
Figure 69 Optical microscopy images (a,c,e) and fluorescence microscopy images (b,d,f) at 10x magnification of NIH 3T3 cells seeded on PDEGMA polymer brush-modified membranes and incubated for 24 h with brush thicknesses of 9 nm (a,b), 11 nm (c,d) and 19 nm (e,f).

As can be seen in the microscopy images, the cells are spreading in what appears to be a non-specific pattern on the brush-modified samples, suggesting non-specific attachment to the underlying substrate. Filopodia, which fulfill among other functions also mechano-sensing, probe the surface and can develop into focal adhesion points, and are typically located in elongated, flattened cells at the forefront of lamellipodia.^{225,226} Non-attached cells appear as rounded cells, indicating no or only a weak adhesion to the underlying substrate. With increasing thickness of the polymer brush layer, no trend in the number of cells attached to the substrate is observed, indicating a lack of antifouling properties. This is in agreement with the observations made by Hebel et al.⁵⁶ Unlike Hebel et al., a clear differentiation between cells attached to the glass-exposed region in the corrals and cells attached to the polymer brush-modified membrane is not possible due to the smaller corral size used in the studies here. The underlying pattern of the lipid bilayer was only visible in the fluorescence microscopy images, confirming the stability of the membrane during the functionalization and cell culturing. Similarly, membranes were functionalized with POEGMA polymer brushes and then subjected to cell-adhesion studies. The results can be seen in Figure 70.

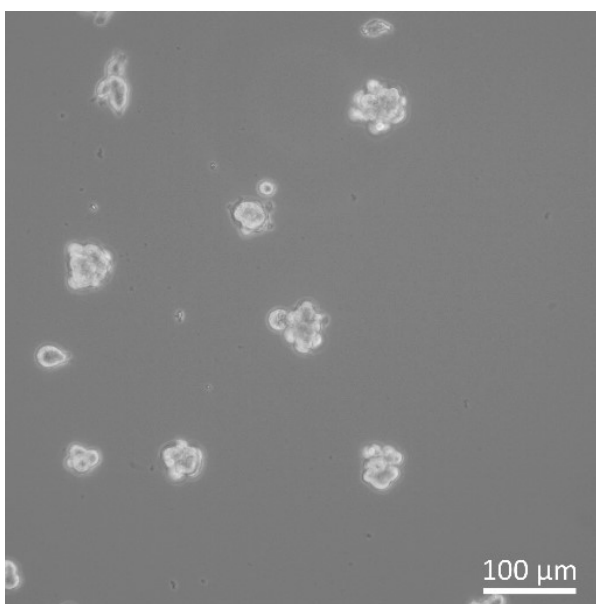
a



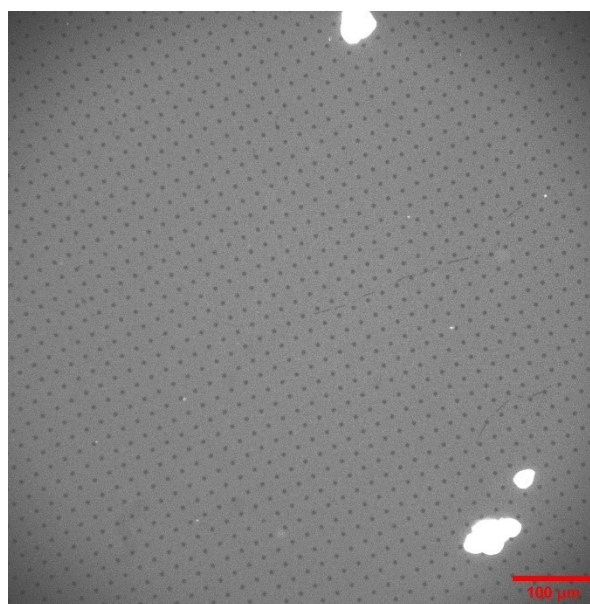
b



c



d



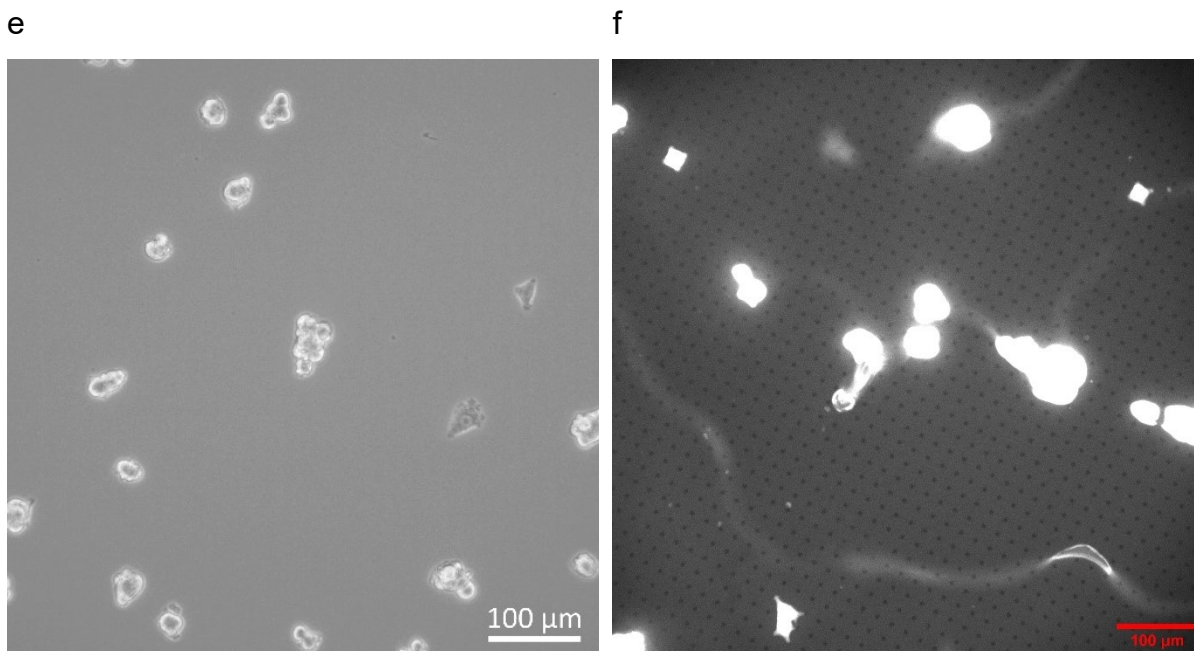


Figure 70 Optical microscopy images (a,c,e) and fluorescence microscopy images (b,d,f) at 10x magnification of NIH 3T3 cells seeded on POEGMA polymer brush-modified membranes and incubated for 24 h with brush thicknesses of 11 nm (a,b), 23 nm (c,d) and 46 nm (e,f).

In contrast with the behavior observed for PDEGMA, POEGMA-modified membranes show pronounced antifouling properties as the seeded cells are all rounded with only few filopodia visible. As for the PDEGMA-modified membranes, the underlying membrane can be visualized in fluorescence microscopy, indicating the stability under the applied conditions. This antifouling effect is observed to be independent of the brush film thickness. By enhancing the contrast of the optical microscopy images of the thickest polymer brush sample, the corrals of the membrane become visible. The resulting image can be seen in Figure 71.

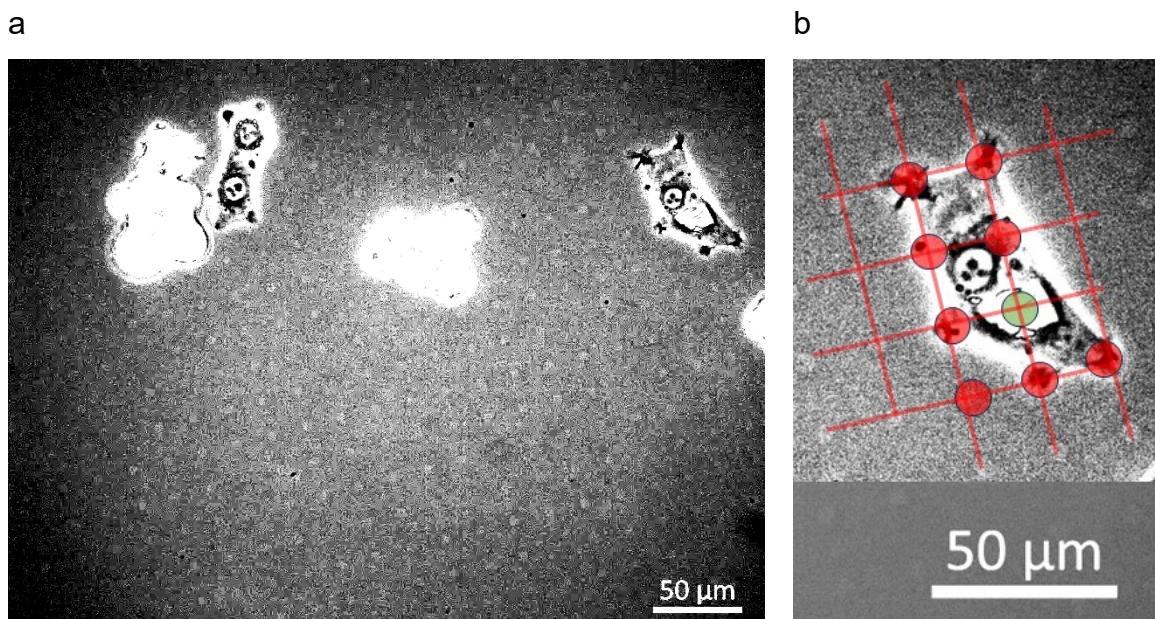


Figure 71 Optical microscopy images at 20x-magnification of NIH 3T3 cells seeded on POEGMA polymer brush (46 nm thick)-modified membranes and incubated for 24 h at 37 °C. (a) The contrast was enhanced by 95% and the brightness increased by 15%. (b) Red lines were added to highlight the grid pattern formed by the corrals, with red dots showing the position of the corrals in the membrane, occupied by a cell. A green dot indicates a corral over which a cell spans.

Only with the thickest POEGMA polymer brush layer could corrals be visualized in optical microscopy. This is hypothesized to be due to the larger difference in the refractive index. Enhancing the contrast enabled visualization of cells spreading on the modified membranes, showing the preferential attachment of cells to the polymer-free corrals. This indicates that the antifouling properties of the polymer prevent cellular attachment; however, due to the corrals acting as structural defects in the membrane, the cells can partly attach to the substrate, and as can be seen, eventually span over a corral. Nevertheless, most cells remain in a rounded state, very likely bound to a single corral only. Similar observations for POEGMA-modified membranes were made by Hebel et al., indicating stronger antifouling properties of POEGMA in comparison to PDEGMA.⁵⁶ The stronger antifouling properties of the POEGMA-modified membranes can also be seen in the number of cells (Figure 72).

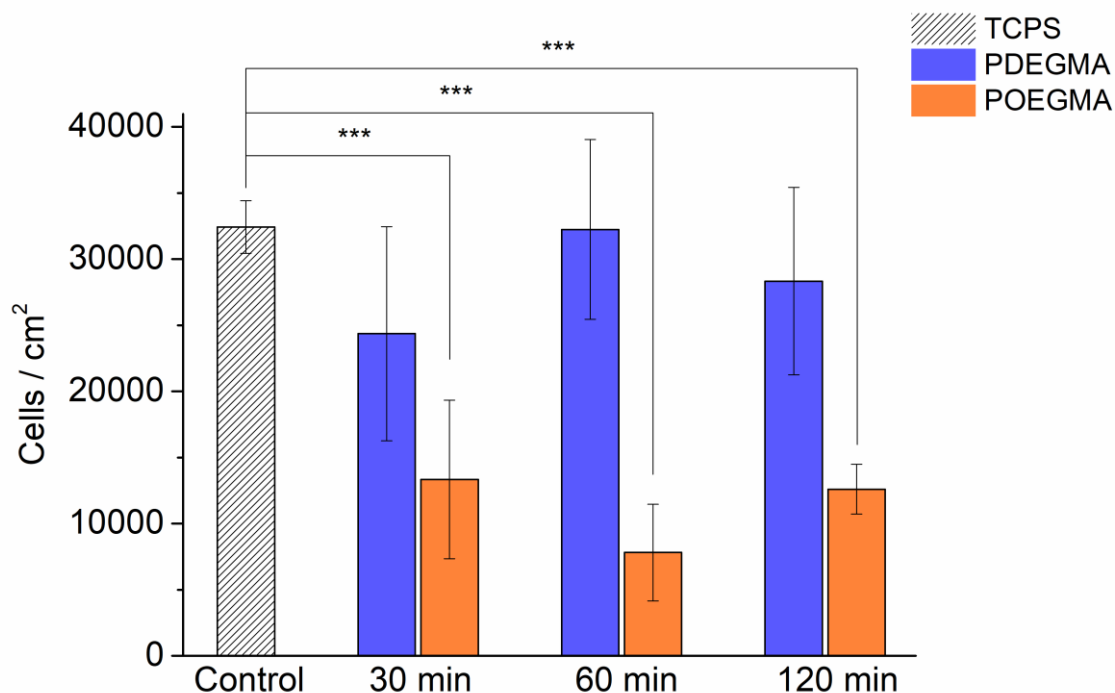


Figure 72 Number of cells for the two polymer brush systems synthesized on the membrane and on TCPS after 24 h incubation. Asterisks represent significant differences from the control (TCPS) ($p < 0.001$ (***)).

As expected, the POEGMA-modified samples have significantly fewer cells attached across all polymer thicknesses compared to the TCPS control, as the cells can only attach to the polymer-free regions inside the corrals. However, for the PDEGMA-modified samples, the number of cells was not found to be significantly different in comparison to the TCPS control. This is in agreement with results found by Hebel et al., who also found a significantly reduced cell number on POEGMA-modified membranes in comparison to PDEGMA and polymer-brush-free membranes.⁵⁶

6.2.3 Block Copolymerization with GMA

To promote cell adhesion to the polymer brush surface, functionalization of the polymer brush with a cell-adhesive peptide such as RGD is necessary. To facilitate this, a functionalizable epoxide group can be introduced by creating a second block of PGMA, as can be seen in Figure 64. The resulting change in the chemical composition can be seen in Figure 73.

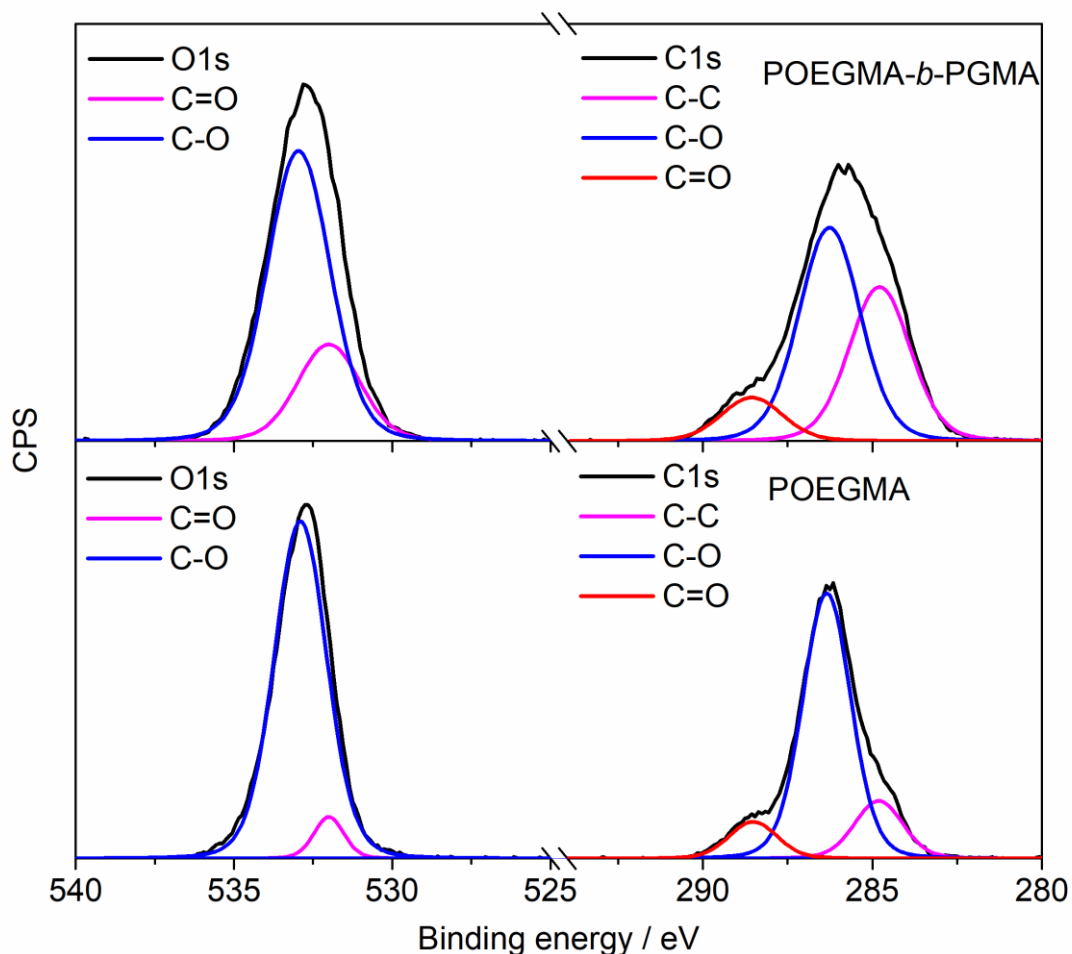


Figure 73 XPS high-resolution spectra of carbon and oxygen of POEGMA and POEGMA-*b*-PGMA.

After the polymerization, the chemical composition of the uppermost polymer brush layer changes upon addition of the PGMA block. In the high-resolution scan of carbon, the aliphatic contribution increases, which agrees with the chemical composition of PGMA brushes observed before.¹⁹⁰ The high-resolution scan of oxygen shows that the contribution corresponding to the carbonyl functionality appears to increase based on the higher width of the oxygen signal, which has a full width half maximum (FWHM) of 2.1 eV for POEGMA and 2.6 eV for POEGMA-*b*-PGMA.

AFM measurements were conducted to investigate the change in the polymer brush film thickness upon co-polymerization. The resulting AFM images can be seen in Figure 74.

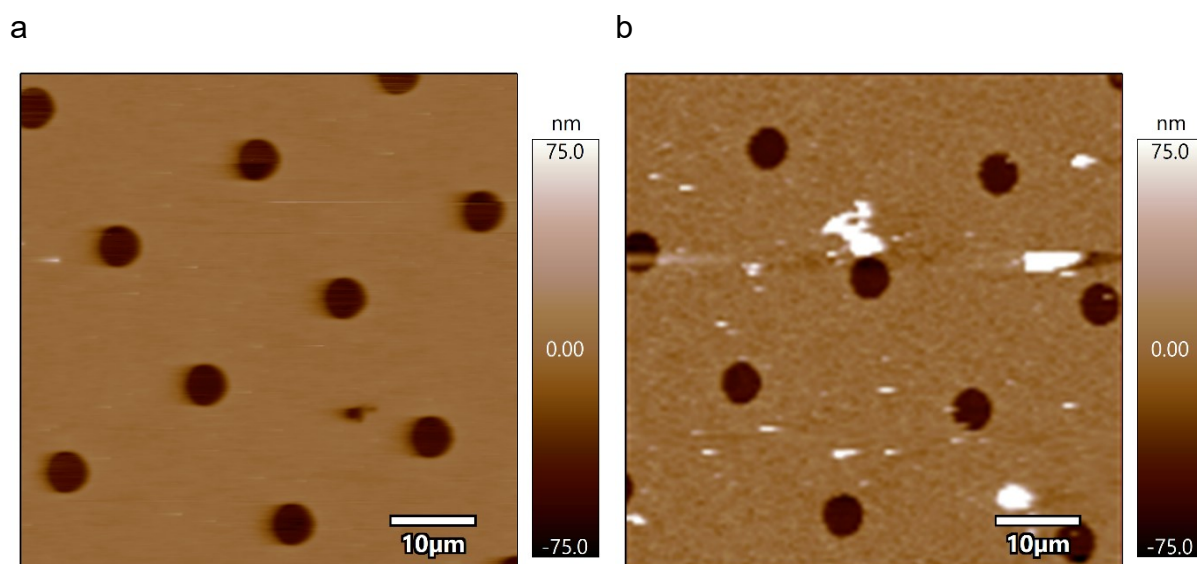


Figure 74 AFM height images of (a) POEGMA and (b) POEGMA-*b*-PGMA polymerized on lipid membranes measured in water.

The successful polymerization of GMA on the POEGMA-modified membrane, forming a block copolymer of POEGMA-*b*-PGMA, can be seen in the AFM images as an increase in the step height. While the step height of the POEGMA-modified membrane reached 46 ± 6.8 nm, after the polymerization of GMA, the step height increased to 52 ± 3.2 nm, indicating an increase of the brush layer of 6 nm. The relatively small increase over the course of 6 h indicates that the spectrum measured in the XPS is also partially influenced by the underlying POEGMA layer. Nevertheless, the information depth of XPS is typically limited to 8 nm, which results in the majority of the observed spectrum stemming from the PGMA layer. From the measured AFM images and recorded XPS spectra, the successful copolymerization was confirmed. The re-initiation potential of the RAFT-synthesized brushes to generate POEGMA-*b*-PGMA block copolymers could facilitate further functionalization of the cell-interacting surface via the nucleophilic ring-opening of the glycidyl methacrylate block.⁵⁶ This could be used to attach peptide sequences which increase cell adhesion, e.g., the well-known RGD peptide sequence, as described by Schulte et al. and Poręba et al.^{183,227} The successful synthesis of POEGMA-*b*-PGMA on the lipid membranes, and previous studies confirming the successful coupling of RGD to PGMA surfaces, indicate that this system would be an interesting starting point to achieve efficient cell-attracting patterned surfaces for the study of cellular responses to stimuli.

6.3 Summary and Conclusions

In this Chapter, a novel synthetic pathway for the generation of polymer brushes on lipid membranes through the attachment of a CTA to a polymerized and patterned lipid membrane and the subsequent RAFT polymerization initiated via the UV-triggered decomposition of a commercially available azo-initiator was developed and validated. The generated hybrid systems consisted of two well-known polymer brushes, namely PDEGMA and POEGMA, and were subjected to cell-adhesion studies, using NIH 3T3 cells. The cells were observed to behave differently depending on the chemical composition of the polymer brush, with cells spreading on PDEGMA and strong antifouling properties of POEGMA. In both cases, cell behavior does not seem to depend on the polymer brush thickness.

For POEGMA-modified membranes, some of the adhered cells stretch over a corral, effectively forming the smart-cell-junction in which secretions of the cell in response to environmental changes could be investigated. However, as this was only observed for a small number of cells, it highlights the need for a functionalizable polymer brush system in which the cell-interacting surface can be tuned to encourage adhesion. Hence, the feasibility of a block copolymer system was investigated, in which the POEGMA brush was extended with a block of PGMA, introducing a functionalizable epoxide group, offering the opportunity to either couple RGD peptide sequences directly or couple PEGylated RGD peptide sequences via click-chemistry to the uppermost polymer brush layer.^{183,227} This promises the increase of the binding affinity of cells to the polymer layer, improving the cell immobilization on top of corrals and therefore forming the smart-cell-junction.

Furthermore, a fluorescently labeled fluid lipid bilayer could be incorporated into the corrals of the brush-modified membrane to prevent adhesion of secreted species to the glass substrate and to potentially incorporate transmembrane species into the model system. Due to the utilized UV-initiation, high temporal and spatial control becomes achievable using UV photomasks, forming asymmetric polymer brush layers on lipid membranes, which could become interesting in further cell-attachment experiments, using e.g., patterned polymer brushes or thickness-gradients.

6.4 Materials and Methods

6.4.1 Materials

Dichloromethane (DCM) ($\geq 99.8\%$ (analytical reagent grade), Fisher scientific, USA) (dried over silica gel), ethanol ($\geq 99.8\%$ (Denatured), Carl Roth, Germany), acetone (analytical reagent grade, Fisher Scientific, USA), 4-((((2-carboxyethyl)-thio)carbonothioyl)thio)-4-cyanopentanoic acid (CTA) (95%, Sigma-Aldrich, Germany), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (commercial grade, Sigma-Aldrich, Germany), 4,4'-azobis(4-cyanovaleric acid) (ACVA) ($\geq 98\%$, Sigma-Aldrich, Germany) and 1,4-dioxane ($\geq 99.8\%$ (unstabilized), Carl Roth, Germany) were used as received if not mentioned otherwise. Di(ethylene glycol) methyl ether methacrylate (95%, Sigma-Aldrich, Germany) and oligo(ethylene glycol) methyl ether methacrylate (95%, $M_w = 300$ g/mol, Sigma-Aldrich, Germany) were filtered through aluminum oxide (90 active neutral, Sigma-Aldrich, Germany) prior the polymerization. Glycidyl methacrylate (GMA) ($>95\%$, TCI Germany, Germany) was used as received. Milli-Q water was obtained from a Millipore Direct Q8 device (Millipore, Schwalbach, Germany) equipped with a Millimark Express 40 filter (Merck, Germany) (referred to as water).

PBS (Dulbecco's Phosphate Buffered Saline 10x, without magnesium, without calcium, Biowest), DMEM (Dulbecco's modified eagle medium, high glucose, without glutamine, Gibco™), FBS (fetal bovine serum, Gibco™), pen/strep (Penicillin-Streptomycin, 5000 U/mL, Gibco), L-glutamine (200 mM, Gibco™), Trypsin-EDTA (0.05%, Gibco™), PFA (paraformaldehyde 4% in PBS, Thermo Scientific Chemicals), Triton® X-100 (Amresco), BSA (albumin fraction V, protease free $M \approx 60000$ g/mol, Carl Roth), NH₄Cl (ammonium chloride, Riedel-de-Haën), Rho-Pha (Rhodamine-Phalloidin, 300 U, Biotium), Hoechst 33258 (10 mg/mL in H₂O, Biotium), ROTI®Mount FluorCare (Carl Roth), Roti®Seal (Carl Roth).

6.4.2 Methods

AFM measurements. All AFM measurements were conducted using an Asylum MFP-3D microscope equipped with a Petri dish heater. A Bruker MLCT-D cantilever was operated in tapping mode at a frequency of 26 kHz and a drive amplitude of 70% of

the free amplitude. To determine the brush thickness, an area of the polymer brush-modified membrane was imaged and the height of the polymerized and not-polymerized regions was plotted as a pixel-height histogram, yielding a bimodal pixel distribution from which the film thickness was determined as the difference between the distribution maxima. All experiments were performed in water with the temperature equilibrated to 27 °C for 15 min before the start of each measurement.

X-ray photoelectron spectroscopy (XPS). XPS spectra were recorded on membranes on silicon wafers using an ESCA spectrometer (SSX-100 S-probe, Surface Science Instruments, Mountain View, USA). The instrument is equipped with a 200 W Al K α X-ray source. Survey spectra were obtained from 0 to 1200 eV with an energy resolution of 1 eV; high-resolution spectra were recorded in the energetic range of the respective component with a window size of 20 eV and a resolution of 0.1 eV.

Fluorescence microscopy. All fluorescence microscopy images were recorded by M.Sc. Yu Yoshimura. Fluorescence microscopy observation of the nanogap junction was performed using an inverted microscope (IX-85, Olympus, Tokyo, Japan) equipped with a LED lamp (X-cite, xylis, Excelitas, Pittsburgh, PA, USA), a 20 $\times$ objective (NA 0.75) or 100 $\times$ objective (NA 1.49) (Olympus, Tokyo, Japan), and a CMOS camera (Orca-Fusion BT, Hamamatsu Photonics, Hamamatsu, Japan). Three types of filter sets were used: (1) excitation 330–385 nm/emission > 420 nm (violet-blue fluorescence), (2) excitation 470–495 nm/emission 510–550 nm (green fluorescence), and (3) excitation 545–580 nm/emission > 610 nm (red fluorescence). Exposure time 100 ms for each filter set, binning 1x1 (2256 pixels x 2256 pixels).

Evaluation software. AFM data was evaluated using the Igor Pro 6.38B01 software. XPS data was evaluated utilizing the CasaXPS 2.1.0.1 software version. Plots were generated using OriginPro version 9.0.

6.4.3 RAFT Polymerization on Membranes

Attachment of the CTA. The CTA (70 mg) and EDC·HCl (120 mg) were stirred in DCM (30 mL) under an argon atmosphere. After placing the nitrogen-stream dried samples in the solution, the mixture was stirred overnight at room temperature. The substrates

were then thoroughly rinsed using ethanol, acetone and water, followed by drying using a stream of nitrogen.

Fabrication of polymer brushes via SI-RAFT. Dioxane (20 mL) was filled in the reaction container, and ACVA (24 mg) was added. Afterwards, the reaction mixture was sparged with argon via a metal needle with a secondary argon flow above the reaction mixture. Following the complete dissolution of ACVA, the filtered DEGMA, OEGMA or GMA (53 mM) was added, and the sparging continued for 5 min. After removal of the metal needle, the substrates sitting in a Teflon holder were immersed in the solution, and the reactor was closed, maintaining the argon atmosphere. The prepared reaction vessel was placed in a water-cooled container situated inside the UV chamber and allowed to acclimatize for 10 min. To keep a constant temperature, the water-cooled container was connected to a home-built cooling circuit, pumping ice-cooled water through tubing, leading to an effective temperature of the reaction vessel of 7 °C during UV illumination. After acclimatization, the UV chamber equipped with UV lamps (365 nm, Bio-Link 365, Vilber Lourmat GmbH, Eberhardzell, Germany) was turned on for the course of the polymerization (up to 120 min for DEGMA and OEGMA, or 6 h for GMA). After the polymerization, the sample holder was removed from the reactor and dipped into fresh dioxane before being thoroughly washed using acetone, ethanol and water. Polymerized samples were stored in ethanol (70%) in the fridge.

6.4.3 Cell Culture

Cell culture and Attachment Experiments. All cell experiments were performed by M.Sc. Renée Hoffmann. NIH 3T3 mouse fibroblasts (provided by Dr. Jürgen Schneckeburger, Biomedical Technology Center of the Medical Faculty Münster, Germany) were cultured in DMEM supplemented with 10% FBS, L-glutamine ($c = 2$ mM) and pen/strep ($c = 100$ µM/mL) at standard conditions ($T = 37$ °C, 5% CO₂). Cell subcultivation was performed by treatment with 0.05% trypsin/EDTA at standard conditions for 3 min. Detached cells were collected and centrifuged (1200 rpm, 4 min) in a falcon tube, counted with a Neubauer improved cell counting chamber (Brand, Wertheim, Germany), seeded at a density of 15 000 cells/cm² on sterilized membranes (70% ethanol for 30 min, washing 3× with PBS 3 mL/well) and subsequently incubated at standard conditions for 24 h.

After the incubation time, the samples were fixated with PFA (preheated, 3 mL/well for 20 min at 22 °C), while the saturation of free aldehyde groups was achieved by incubating the samples in NH_4Cl solution (50 mM, 10 min at 22 °C). Membrane permeabilization was performed with Triton-X 100 (0.2% in PBS, 10 min at 22 °C) while blocking of unspecific binding sites was realized by incubation of the samples in BSA solution (2% in PBS, 45 min at 22 °C). Fluorescent actin staining was realized by incubating the samples with Rho-Pha (1:40 dilution in 1% BSA in PBS, 150 μL /sample in a wet chamber for 30 min at 22 °C). Finally, NIH 3T3 nuclei were fluorescently stained by Hoechst 33258 (1:10000 dilution, 3 mL/well in PBS for 30 min at 22 °C in the dark). Washing steps consisting of PBS (3x, 3 mL/well) were included in between every step. Sample conservation was achieved by ROTI®Mount FluorCare in combination with ROTI®Seal. Light microscopy was performed with a Primovert light microscope (Carl Zeiss, AxioCam, ERc5s, Oberkochen, Germany) equipped with Zen software (Carl Zeiss).

7. Summary and Conclusions

In this Thesis, the potential use of polymer brushes in biomedical applications within the context of the “race for the surface” was investigated. Two fundamental processes of the competitive colonization of implant surfaces were approached by utilizing polymer brush-coatings to minimize bacterial colonization by releasing an antibiotic and understanding cellular attachment to the surface by creating nanogap junctions.

In Chapters 4 & 5, monomeric prodrugs were developed, and polymer brush prodrugs on model titanium surfaces were formed in an SI-RAFT polymerization technique. The first monomer was based on chloramphenicol, which was modified with methacryloyl chloride in an esterification reaction, introducing a polymerizable double bond and a hydrolyzable ester. The monomeric prodrug was characterized thoroughly and its purity confirmed. Afterwards, titanium-coated glass substrates were used and chemically modified with the CTA, facilitating the formation of a poly(chloramphenicol methacrylate) brush prodrug. The brushes were systematically characterized, and spectroscopic analysis confirmed the introduction of the antibiotic into the polymer film. However, drug release studies showed only a small quantity of chloramphenicol was liberated from the brush film over the course of three days, the concentration of which would not have been sufficient to effectively impede bacterial growth. As the XPS analysis after the drug release experiment revealed, the chemical composition of the remaining polymer brush film was similar to the initial brush composition, hence there was only negligible release of chloramphenicol from the film. It can be concluded that under physiological conditions, the hydrolysis of the specific ester generated is too slow for a sufficient release of the antibiotic. In order to utilize chloramphenicol as an antibiotic in a polymer brush prodrug, an alternative bond that is easier to hydrolyze would be necessary, e.g. an acetal or a ketal.

Based on the results of Chapter 4, a different monomeric prodrug was designed, using sulfanilamide as a model drug, linked to pHBA to form a hydrolyzable imine bond. This precursor was then esterified using acryloyl chloride, which introduced the polymerizable moiety of the monomeric prodrug. The monomer was thoroughly characterized and the synthetic approach confirmed. To couple the CTA to the model titanium surface, the substrates were first silanized and then modified with the CTA, which improves the binding of the CTA to the surface, as can be seen in the higher

sulfur content determined by XPS. The polymerization of the monomeric prodrug yielded poly(sulfanilamide-hydroxybenzaldehyde acrylate) brushes with the expected chemical composition on both the titanium and glass faces of the substrates. Ellipsometry and AFM measurements allowed estimation of the amount of SA stored per substrate. Increasing the polymerization time resulted in a higher film thickness and therefore increased loading capacity of the polymer brush film. FTIR analysis confirmed not only the successful introduction of the antibiotic into the polymer brush film but also the coupling of SA to the polymeric backbone via the imine bond.

The release of sulfanilamide from this imine-based system revealed a time-, film thickness-, temperature- and pH-dependent release of the antibiotic from the film, based on the hydrolysis of the imine bond. Drug release was directly observed based on UV-Vis and HPLC analysis, while film thickness measurements allowed indirect estimation of the amount of SA released. As observed in the absorption spectra of the drug release supernatant, not only was the antibiotic released from the polymer brushes, but also pHBA could be detected, indicating hydrolysis of the ester. Although unexpected, pHBA was shown not to possess any antibacterial effect, therefore not interfering with the bacteria experiments conducted with the SA-containing drug release supernatant.

While none of the *S. aureus* strains were inhibited by the sulfanilamide released from the brushes, successful eradication upon co-administration with trimethoprim indicated that the drug is released at sufficient concentrations and chemically intact. While sulfanilamide was shown to be an excellent model drug in the study of polymer brush prodrugs, there is widespread resistance towards this drug class. A self-sufficient polymer brush prodrug based on sulfanilamide would require the incorporation of trimethoprim, either as an adsorbed drug released via diffusion, as shown by Choi et al., or by transforming trimethoprim into a polymerizable monomeric prodrug for copolymerization.

Further improvements to these polymer brush prodrugs could be investigated to improve the hydrolytic stability of the anchoring group, potentially by substituting the silane for a catechol-based anchoring group. Additionally, the aldehyde linker used to form the imine bond and to couple acryloyl chloride could be changed to prevent hydrolysis of the ester, which would prevent the secondary release observed in the drug release studies. Nevertheless, due to the reported beneficial effects of pHBA in

wound healing, a secondary release of pHBA may even prove advantageous in the aimed application of implant coatings. The polyacrylic acid brushes, which remain after hydrolysis, are biocompatible, and due to the slow hydrolytic degradation of the anchoring silane, a slow decomposition of the brush layer could be beneficial for the colonization of the implant with host cells. Further experiments are required to study the effects of the generated polymer brush prodrug with a focus on *in vivo* applications, to not only judge the performance of the antibacterial coating but also assess the biocompatibility and potential cytotoxicity.

Finally, Chapter 6 demonstrated the successful synthesis of polymer brushes of PDEGMA and POEGMA in a novel, UV-RAFT approach onto patterned, substrate-supported lipid bilayers, to understand cellular interactions with polymer brush-modified surfaces. The UV-RAFT approach yielded brushes that were thicker than previously reported systems, providing a larger gap to separate molecules released by immobilized cells. NIH 3T3 cells were seeded on these systems and incubated to investigate the preferential spatial attachment to either the brush-modified membrane or the polymer-free regions where the substrate is exposed. It was found that the cellular attachment depends on the choice of polymer, with no signs of thickness dependence for either polymer.

To tune cell attachment, a second polymerization on POEGMA-brushes was performed, generating a block copolymer brush of POEGMA-*b*-PGMA. The second block of PGMA could serve as a functionalizable block for the attachment of cell-adhesive peptide sequences to encourage selective adhesion at the brush-liquid interface. This would allow immobilization of cells on top of the brush-modified membrane, spanning over the polymer-free regions of the membrane and thereby creating a junction for the detection of cellular secretions upon external stimulation. This, in turn, will deepen the understanding of cell-polymer interactions, crucial in all polymeric surface coatings on implant materials.

Acknowledgements

This Thesis is the product of many years of teaching, inspiration and support from Prof. Dr. Holger Schönherr, who not only transferred knowledge but also motivated me to think outside the box and approach problems from a different angle when the conventional method failed. I want to express my gratitude for the teaching, be it scientifically or non-scientifically, the opportunity to work independently on a project developed by myself, the option to work on an international project with excellent collaborators and the trust I received.

The time as a PhD student is not only a time to develop as a scientist but also as a human. Spending time in the labs of Prof. Dr. Kenichi Morigaki helped me in both aspects, being away from home for an extended period of time and learning about the synthesis and handling of the unique and sophisticated membrane systems. I want to thank Prof. Morigaki for his constant scientific support, the hospitality in his labs, as well as the many activities undertaken during my time in Japan. Particularly, M.Sc. Yu Yoshimura deserves thanks for his constant effort to prepare new batches of samples, always meeting the demands. Furthermore, M.Sc. Koki Takagi and M.Sc. Ayane Sugimachi were not only a constant support during my stays in Japan, but also organized several excursions to show me the Japanese culture and the beauty of the country, for which I will always be thankful and consider them loyal friends. I am grateful to the DAAD (DAAD Projekt Nr. 57610504 (Programm des projektbezogenen Personenaustauschs Japan JSPS 2022-2024)) for the chance to work on such a multidisciplinary project and for the collaboration opportunities.

Also, my colleagues, past and present, deserve acknowledgement. In particular, I would like to thank Dr. Willis Muganda for convincing me to join PCI, his enduring friendship and support during difficult times, and Dr. Hongsuh Choi for introducing me to polymer brushes and casual conversations about football. Furthermore, I would like to thank Dr. Sonu Kumar for sharing his knowledge on RAFT polymerization with me. M.Sc. Renée Sophie Hoffmann and M.Sc. Jonah Leon Decker not only did measurements and experiments for me but also were great company, in particular at conferences and during lunch breaks. M.Sc. Andreas Jagic was of great support in the membrane project. Dipl.-Ing. Gregor Schulte deserves thanks for technical support. I would like to thank M.Sc. Max Müller, M.Sc. Nigar Hajiyeva, Dr. Prasamsha Panta, Dr.

Kawaljit Kaur, M.Sc. Aysha Awan for an always great office atmosphere. Further thanks go to all other members of PCI for creating a productive working environment and nice interactions outside of work.

Not only did people in PCI help me to achieve my goals, but I would also like to thank M.Sc. Désirée Anna-Maria Schütz for performing HPLC analysis of my samples, Dr. Rafał Poręba for introducing me to RAFT polymerization and hosting my visit in Prague, Dr. Thomas Paululat for NMR measurements and Dipl.-Chem. Rochus Breuer for CHNS measurements. Furthermore, I would like to thank M.Sc. Manuel Heinelt for ESI-MS measurements. M.Sc. Gabriel Onyenso and M.Sc. Jakob Derksen ("Dimitri") offered constant emotional support and opportunities for a coffee break.

My friends outside of university offered support constantly throughout my PhD, which I am infinitely grateful for. In particular, Manuel Klassen, Michael Greiter and Dominik Schefczyk were always there to cheer me up after a disappointing day in the lab.

The loving support of my family helped me endure all hardships throughout my life, which ultimately gave me the opportunity to follow my dreams and pursue a PhD. I will always be thankful for that.

The one person who deserves the biggest acknowledgement is my loving girlfriend, the source of constant support, scientifically and emotionally, and the reason I am writing these words today, Nicola Cusick. Without her, I would not have been able to keep on going on, there would not have been the fantastic support during all experiments conducted, in particular during the bacteria experiments, and beyond. The time as a PhD student was only as special as it was due to her, especially looking back at all the fantastic places we have been together, for conferences or on holiday. I could not ask for a better partner, and that is why I want to conclude this Chapter with the most important statement I can consider: I love you, my darling!

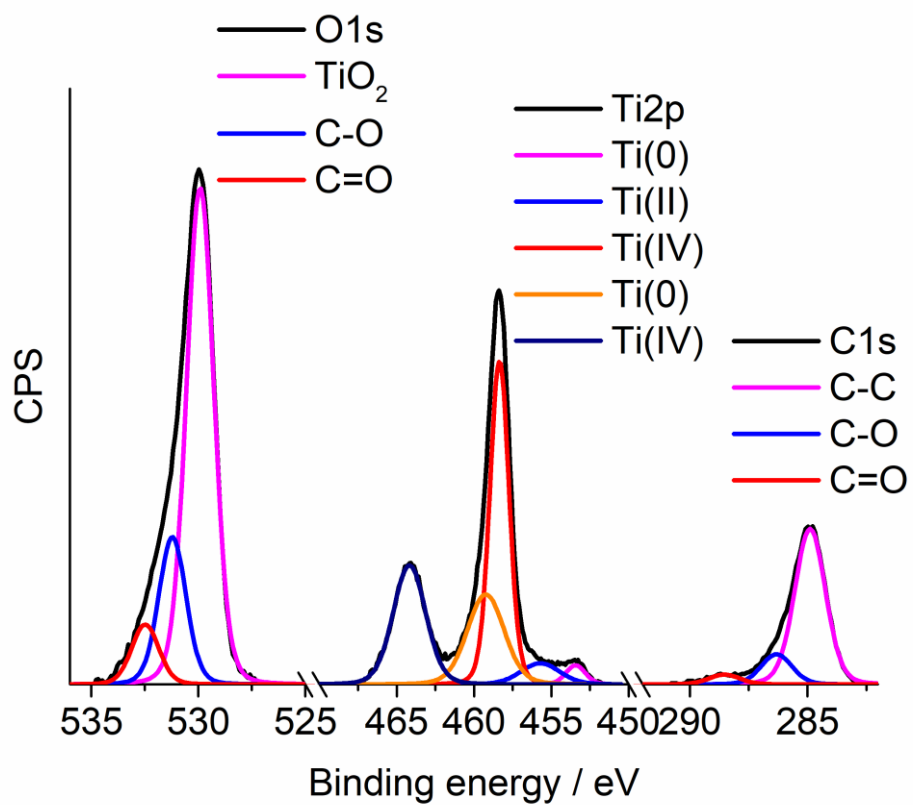
Appendix

Appendix 1 ¹H-NMR signals of CAP observed in DMSO-d₆. The aromatic signals at 7.60 ppm were used as a reference for integration.

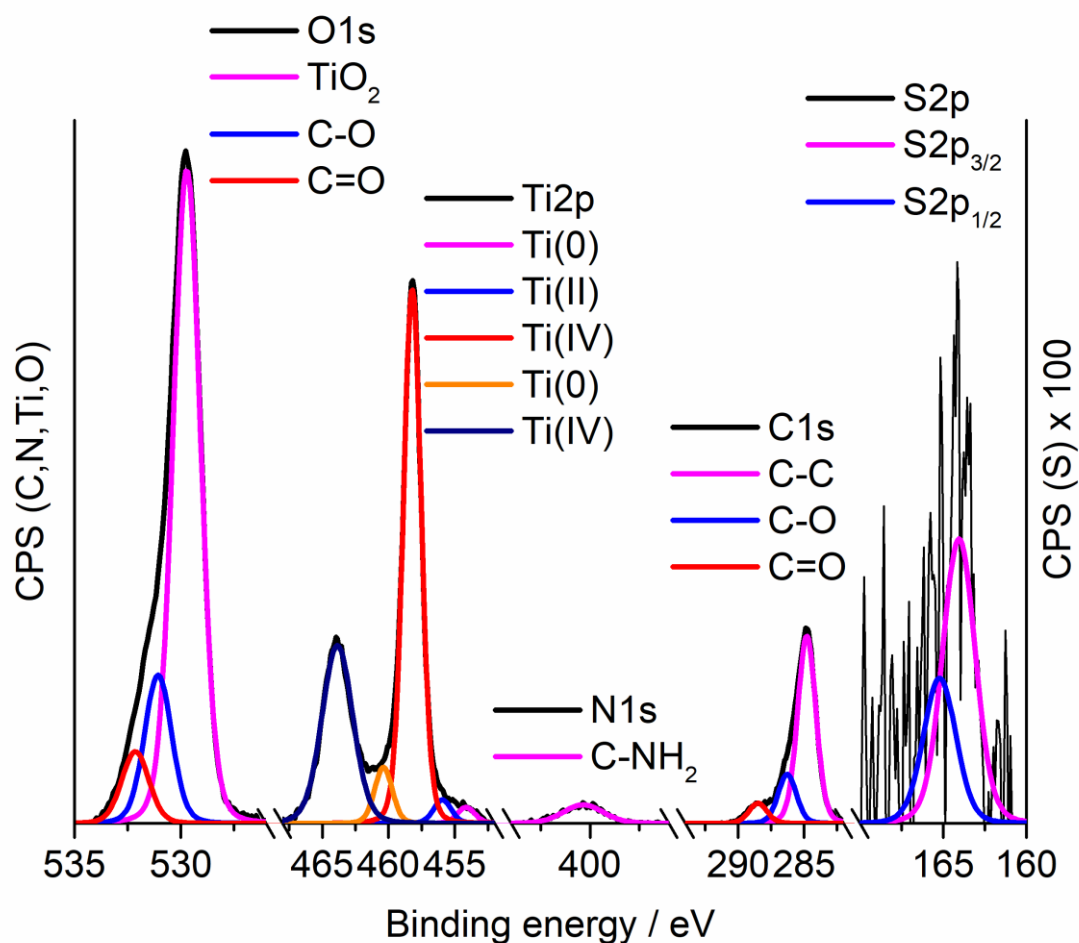
Peak shift / ppm	Multiplicity	<i>J</i> / Hz	Integral	Assignment
2.50	q	1.8	-	DMSO-d ₅
3.33	s	-	-	HDO, H ₂ O
3.37	ddd	10.7/5.9/5.0	1	HO-CHH
3.60	ddd	10.5/7.6/6.4	1	HO-CHH
3.94	td	8.6/2.3	1	NH-CH
4.98	dd	6.2/4.9	1	CH ₂ -OH
5.06	dd	4.4/2.4	1	Ar-C-H
6.04	d	4.3	1	Ar-C-OH
6.48	s	-	1	CCl ₂ H
7.60	m	-	2	Ar-H
8.17	m	-	2	Ar-H
8.31	d	9.2	1	NH

Appendix 2 ¹H-NMR signals of CAP methacrylate observed in DMSO-d₆. The aromatic signals at 7.64 ppm were used as a reference for integration.

Peak shift / ppm	Multiplicity	<i>J</i> / Hz	Integral	Assignment
1.85	s	-	3	CH ₃
2.50	q	1.8	-	DMSO-d ₅
3.33	s	-	-	HDO, H ₂ O
4.18	td	10.6/2.7	1	CHH-OCO
4.33	m	-	2	CHH-OCO, NH-CH
5.05	dd	4.5/2.0	1	Ar-CH
5.66	t	1.6	1	HHC=C
6.01	dd	1.6/1.0	1	HHC=C
6.23	d	4.5	1	Ar-C-OH
6.43	s	-	1	CCl ₂ H
7.64	m	-	2	Ar-H
8.17	m	-	2	Ar-H
8.54	d	9.2	1	NH



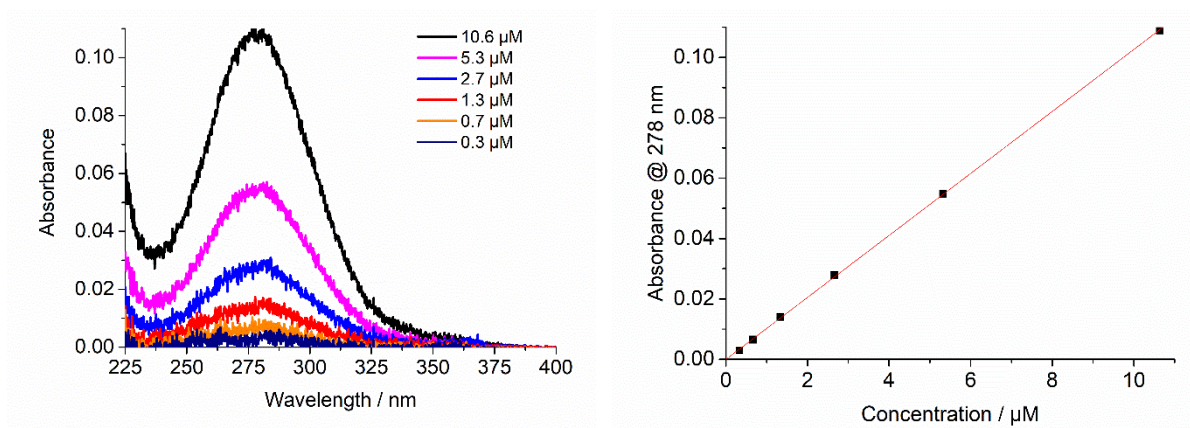
Appendix 3 High-resolution XPS spectra of titanium after UV-ozone cleaning (C1s, Ti2p, O1s; 100 nm on glass). Plotted are the CPS against the binding energy.



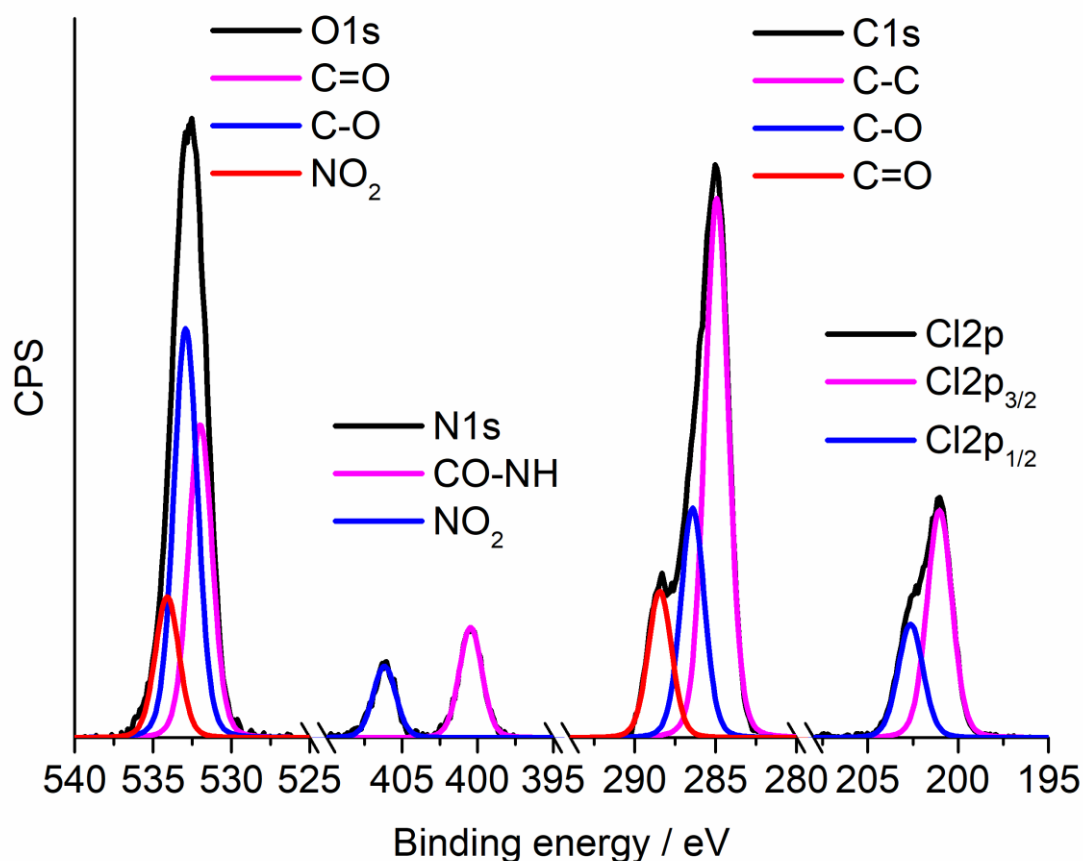
Appendix 4 High-resolution XPS spectra of the CTA coupled to titanium (S2p, C1s, N1s, Ti2p, O1s; ellipsometric thickness of 1.5 ± 0.2 nm). Plotted are the CPS against the binding energy.

Appendix 5 ¹H-NMR signals observed in the reprecipitated polymer of CAP methacrylate in DMSO-d₆. For integration, the aromatic signals corresponding to 4 hydrogen atoms were chosen.

Peak shift / ppm	Integral	Assignment
0.5 – 2.3	5	Backbone
3.3 – 4.7	3	CH ₂ -OCO, NH-CH
4.7 - 5.2	1	Ar-CH-OH
5.8 - 6.2	1	Ar-CH-OH
6.2 – 6.7	1	CHCl ₂
7.2 – 7.7	2	Ar-H
7.7 – 8.2	2	Ar-H
8.3 – 9.0	1	N-H



Appendix 6 UV-Vis absorption spectra of CAP in HCl (a) and the corresponding calibration curve (b). The extinction coefficient was determined to be $10.2 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ with a regression coefficient $R^2 = 0.9999$.



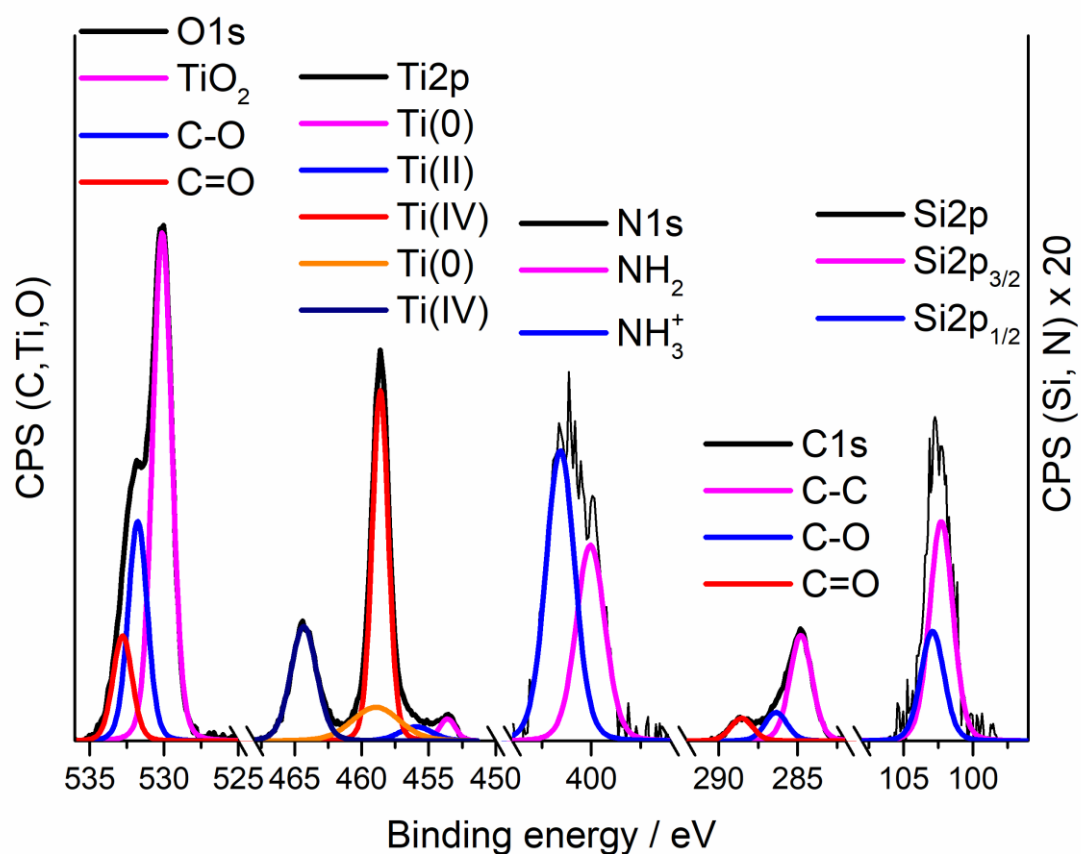
Appendix 7 High-resolution XPS spectra of poly(CAP methacrylate) brushes on titanium (Cl2p, C1s, N1s, O1s; dry ellipsometric thickness 45 nm) after incubation in HCl (pH 4) at 37 °C for 72 h. Plotted are the CPS against the binding energy.

Appendix 8 Chemical composition of poly(CAP methacrylate) brushes on titanium (dry ellipsometric thickness 45 nm) after incubation in HCl (pH 4) at 37 °C for 72 h, derived from the XPS high-resolution spectra.

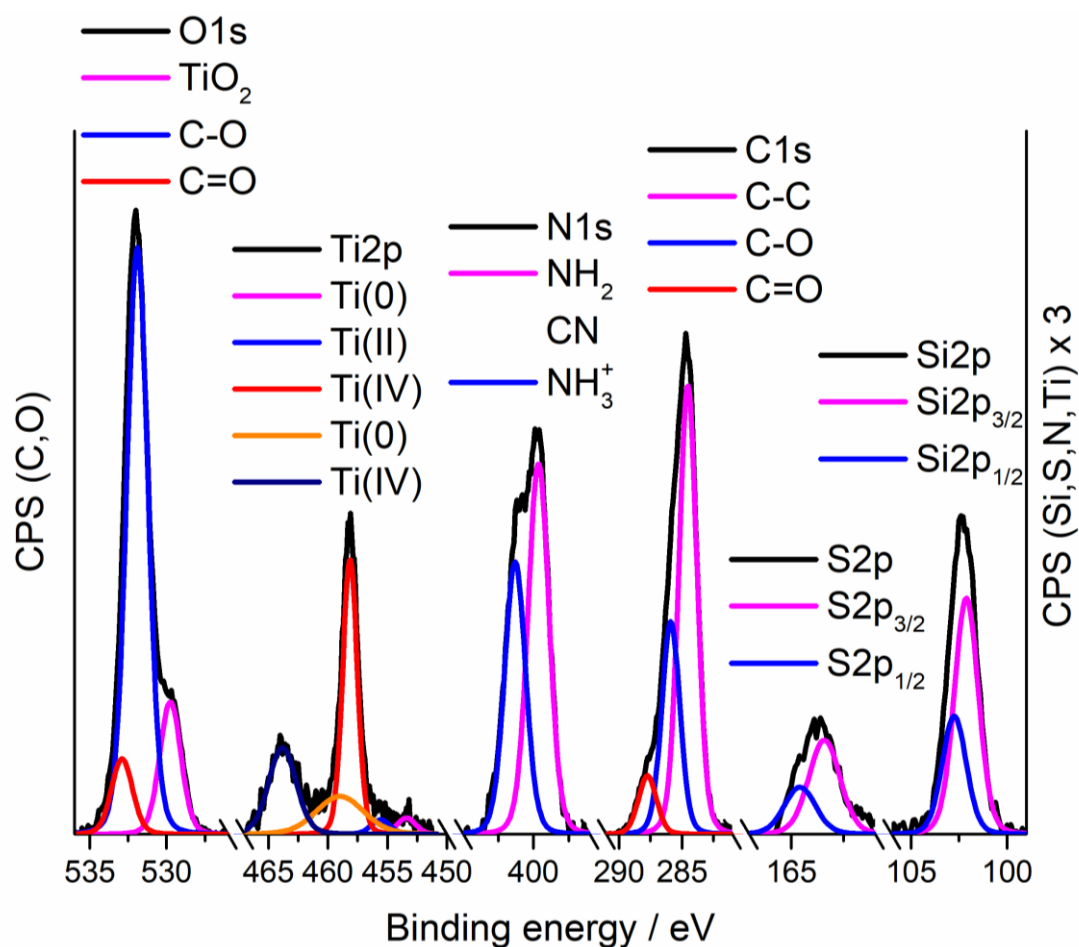
Element	Polymer brushes of CAP methacrylate		
	Experimental	Theoretical	
	After release	Non-Hydrolyzed	Fully-Hydrolyzed
C	60.2	60	66.7
O	23.4	24	32.3
N	7.4	8	0
Cl	9.0	8	0

Appendix 9 ^1H -NMR signals of SA, pHBA, the imine precursor and the monomeric prodrug observed in DMSO- d_6 . The aromatic signals or the aldehyde/imine hydrogen signal were used as a reference for integration.

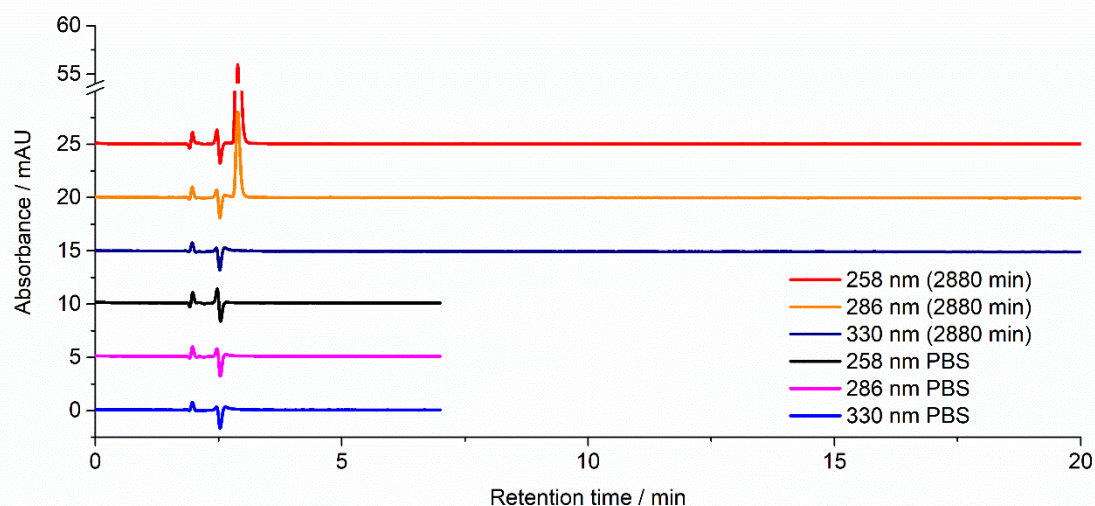
Peak shift / ppm	Multiplicity	J / Hz	Integral	Assignment
SA				
5.79	s	-	2	Ar-NH ₂
6.58	m	-	2	Ar-H
6.87	s	-	2	SO ₂ -NH ₂
7.45	m	-	2	Ar-H
pHBA				
6.93	m	-	2	Ar-H
7.76	m	-	2	Ar-H
9.79	s	-	1	Ar-CHO
10.58	s	-	1	Ar-C-OH
Imine precursor				
6.90	m	-	2	Ar-H
7.33	m	-	4	Ar-H, SO ₂ -NH ₂
7.81	m	-	4	Ar-H
8.48	s	-	1	Ar-C(=NR)H
10.23	s	-	1	Ar-C-OH
Monomeric prodrug				
6.20	dd	10.4/1.2	1	RHC=CH _{cis} H
6.44	dd	17.4/10.3	1	RHC=CHH
6.58	dd	17.4/1.2	1	RHC=CHH _{trans}
7.39	m	-	6	Ar-H, SO ₂ -NH ₂
7.86	m	-	2	Ar-H
8.03	m	-	2	Ar-H
8.67	s	-	1	Ar-C(=NR)H



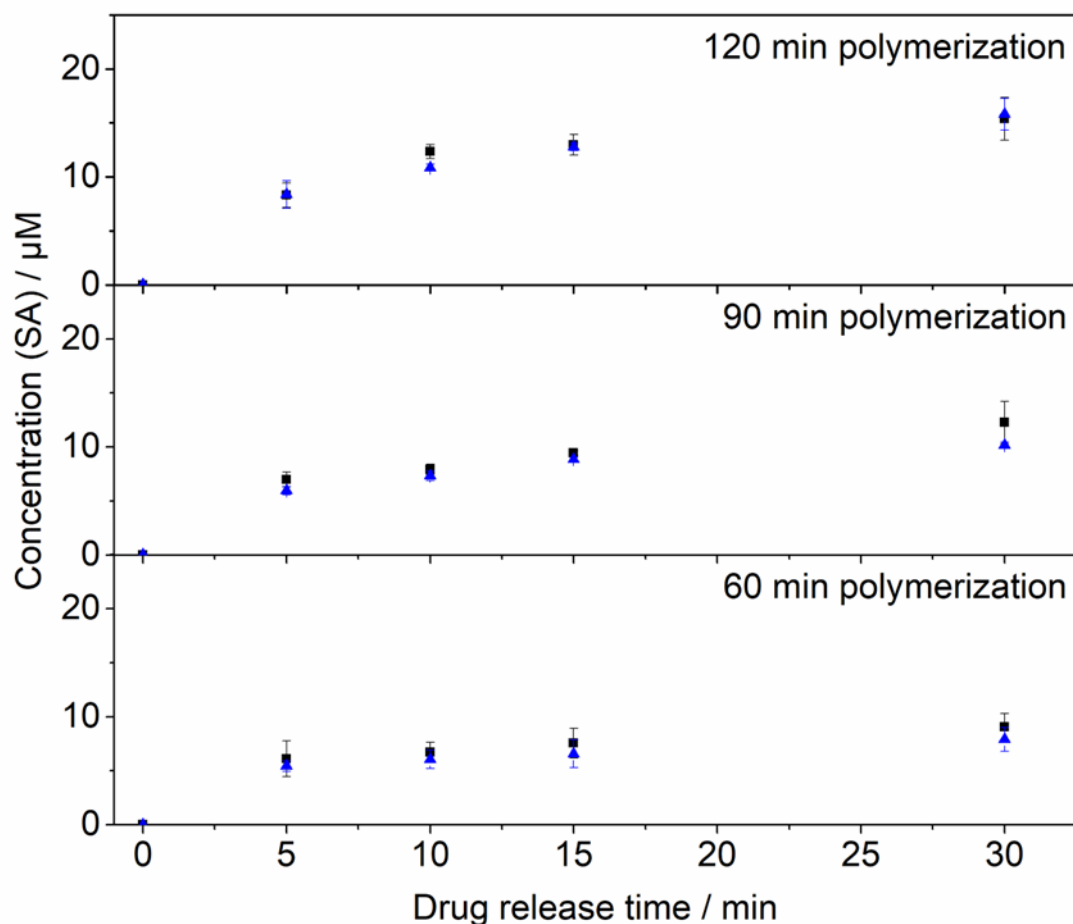
Appendix 10 High-resolution XPS spectra of APTES on titanium (Si2p, C1s, N1s, Ti2p, O1s; ellipsometric thickness 0.9 ± 0.1 nm). Plotted are the CPS against the binding energy.



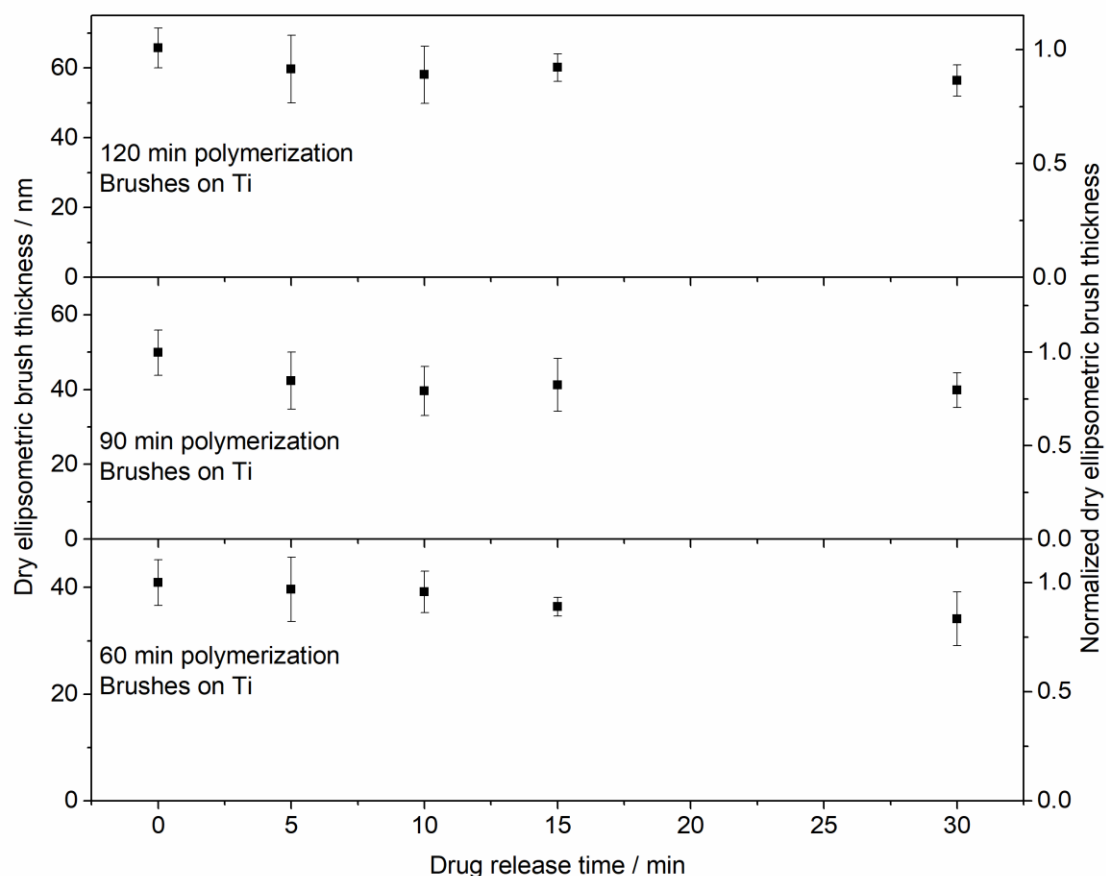
Appendix 11 High-resolution XPS spectra of CTA-modified APTES on titanium (Si2p, S2p, C1s, N1s, Ti2p, O1s, ellipsometric thickness 3.1 ± 0.9 nm). Plotted are the CPS against the binding energy.



Appendix 12 HPLC chromatograms of the supernatant of the drug release from a poly(SA-pHBA acrylate)-modified substrate polymerized for 120 min and incubated in PBS for 48 h at 37 °C, measured at 258 nm, 286 nm and 330 nm. Additionally, neat PBS was measured at the same wavelengths.

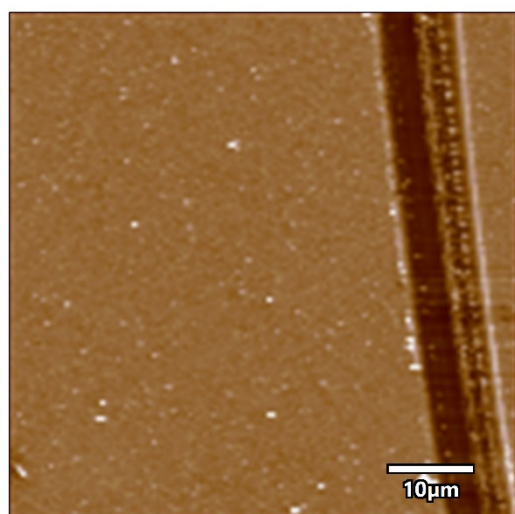


Appendix 13 Plot of the concentrations of SA against the incubation time for poly(SA-pHBA acrylate)-modified substrates polymerized for 60 min, 90 min and 120 min. The concentrations were determined based on the UV-Vis absorption maxima at 258 nm with an extinction coefficient of $16258 \text{ M}^{-1} \text{ cm}^{-1}$ (black squares), or the area of the SA signal in the HPLC chromatograms (blue triangles). The concentration of SA in the supernatant of the drug release was observed over the course of 30 min at 37 °C in PBS.

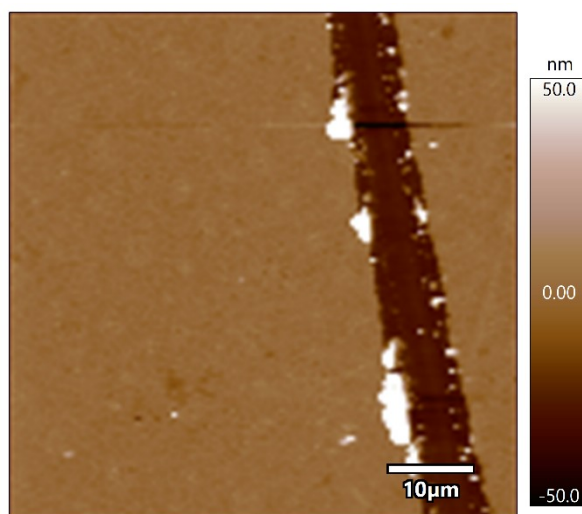


Appendix 14 Plot of the dry ellipsometric brush thickness against the incubation time for poly(SA-pHBA acrylate)-modified substrates polymerized for 60 min, 90 min and 120 min. The samples were incubated in PBS for up to 30 min at 37 °C. After the respective incubation time, the samples were rinsed and dried and then measured by ellipsometry.

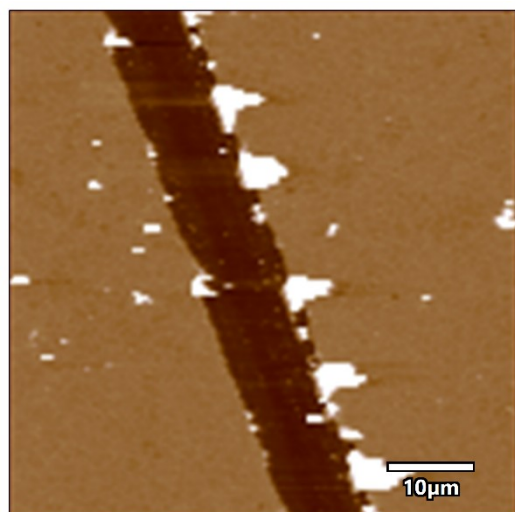
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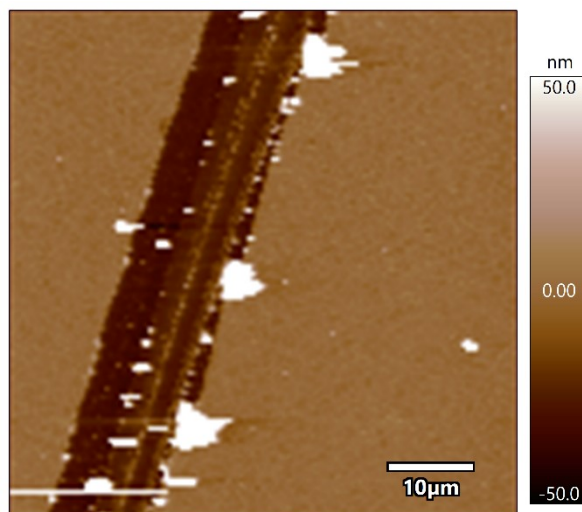
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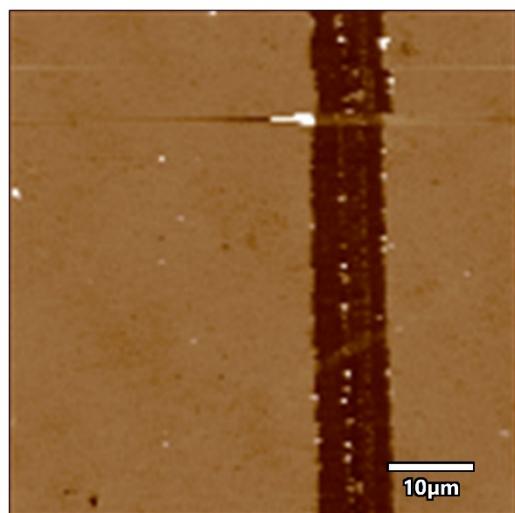
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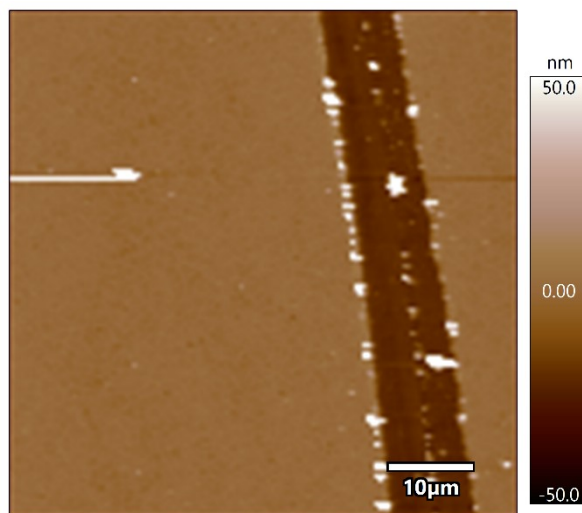
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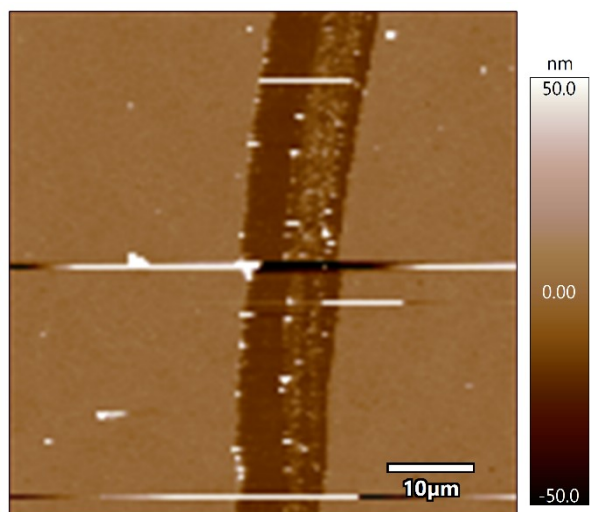
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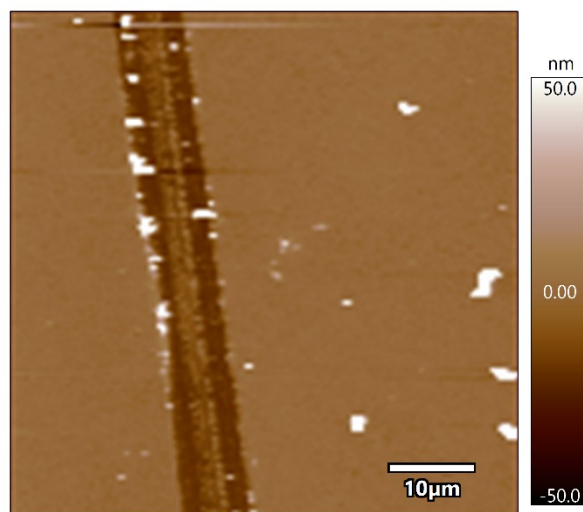
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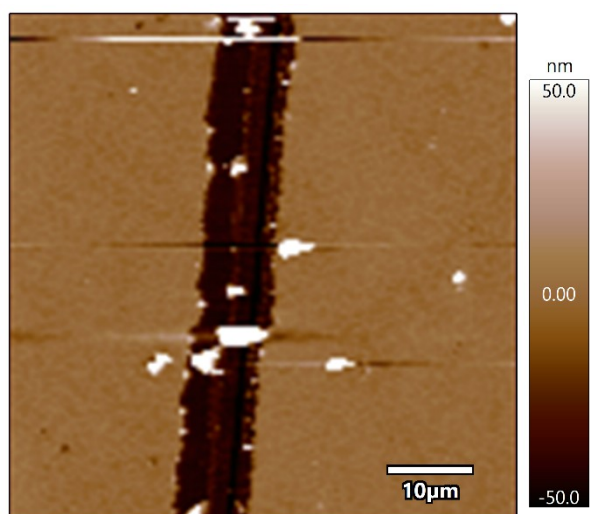


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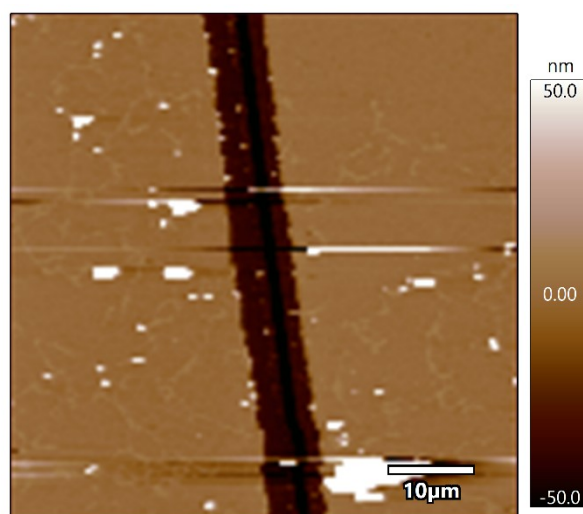


Appendix 15 AFM height images of poly(SA-pHBA acrylate)-modified samples polymerized for 60 min and incubated in PBS at 37° for a) 5 min, b) 10 min, c) 15 min, d) 30 min, e) 60 min, f) 360 min, g) 1440 min, h) 2880 min, measured on the glass face.

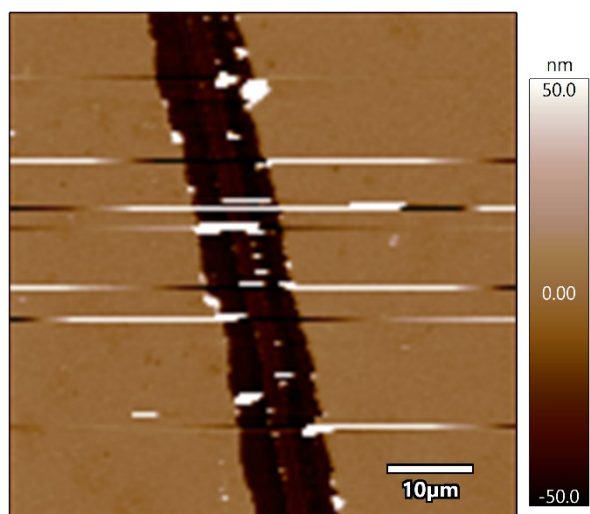
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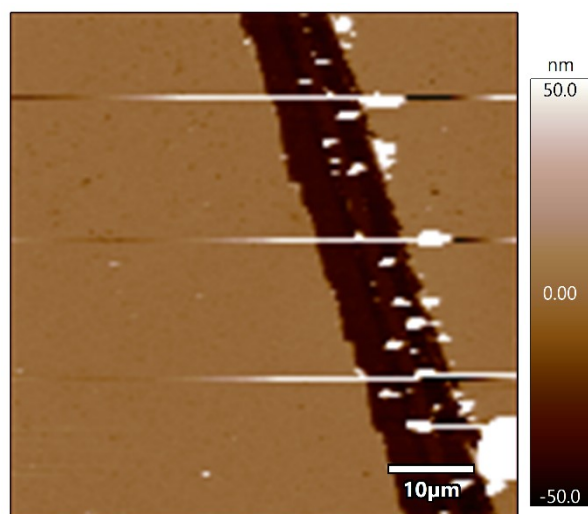
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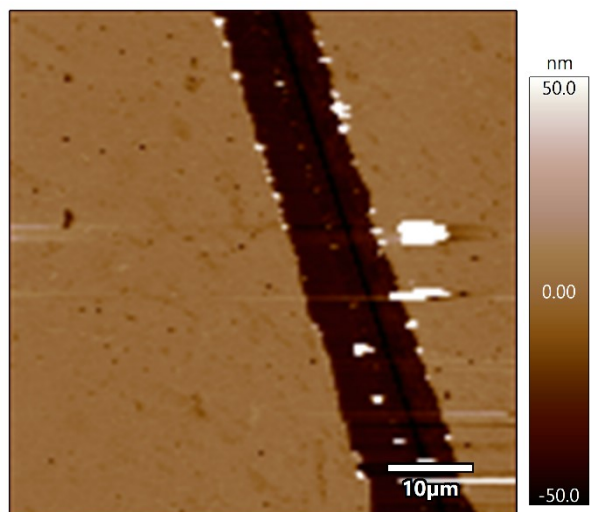
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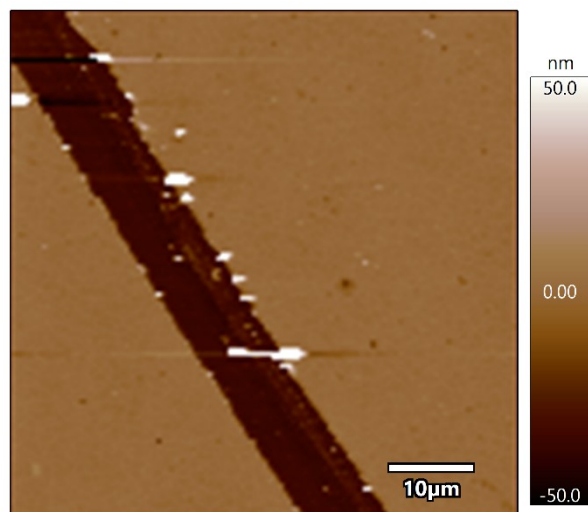
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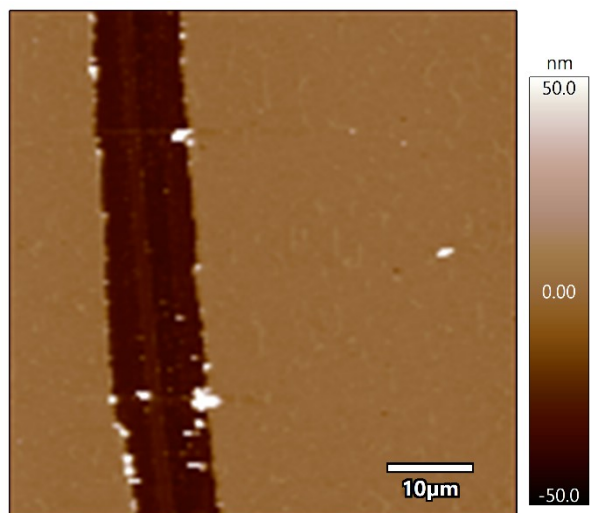
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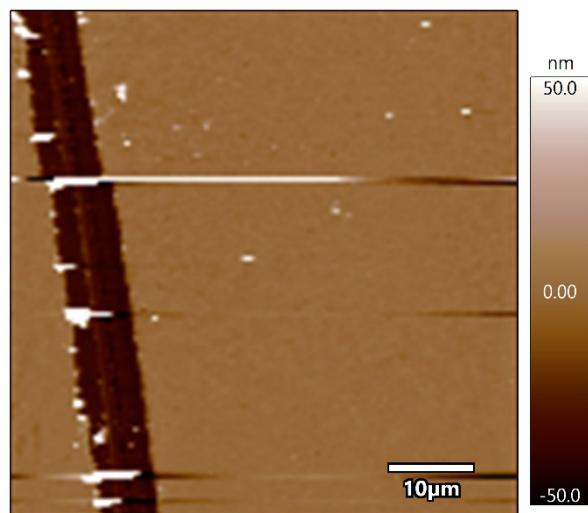
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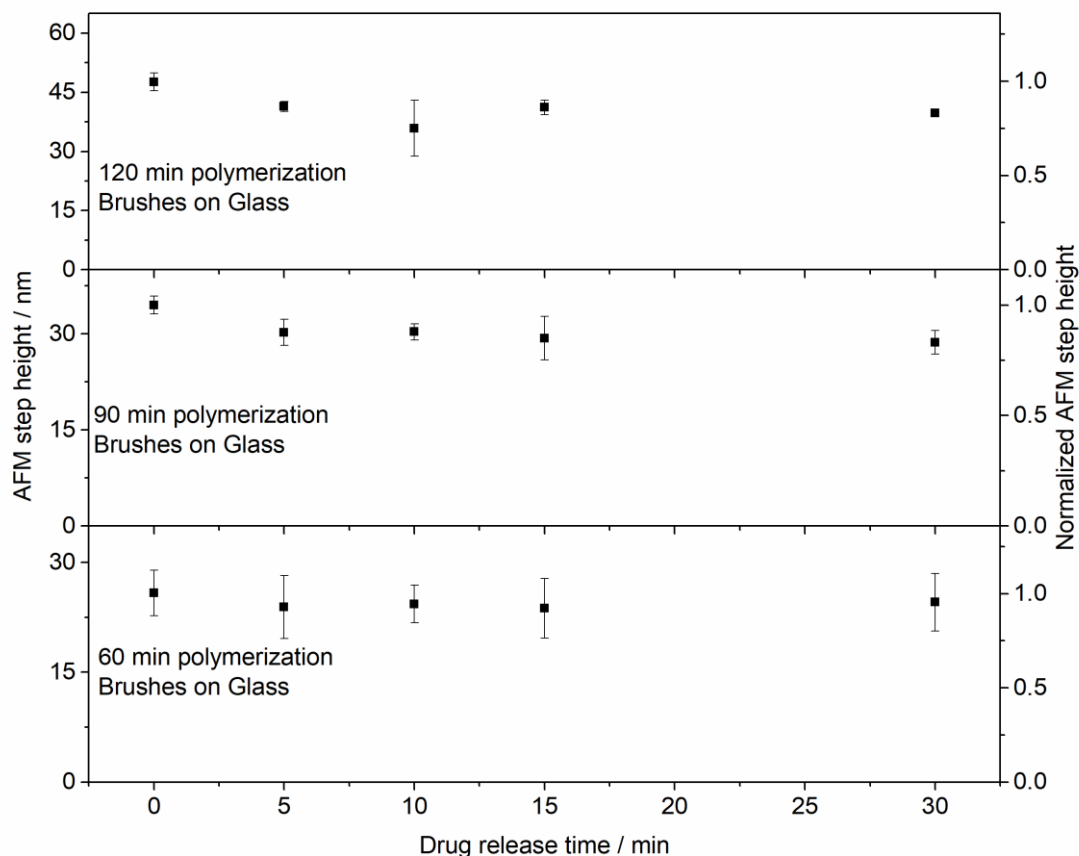
g



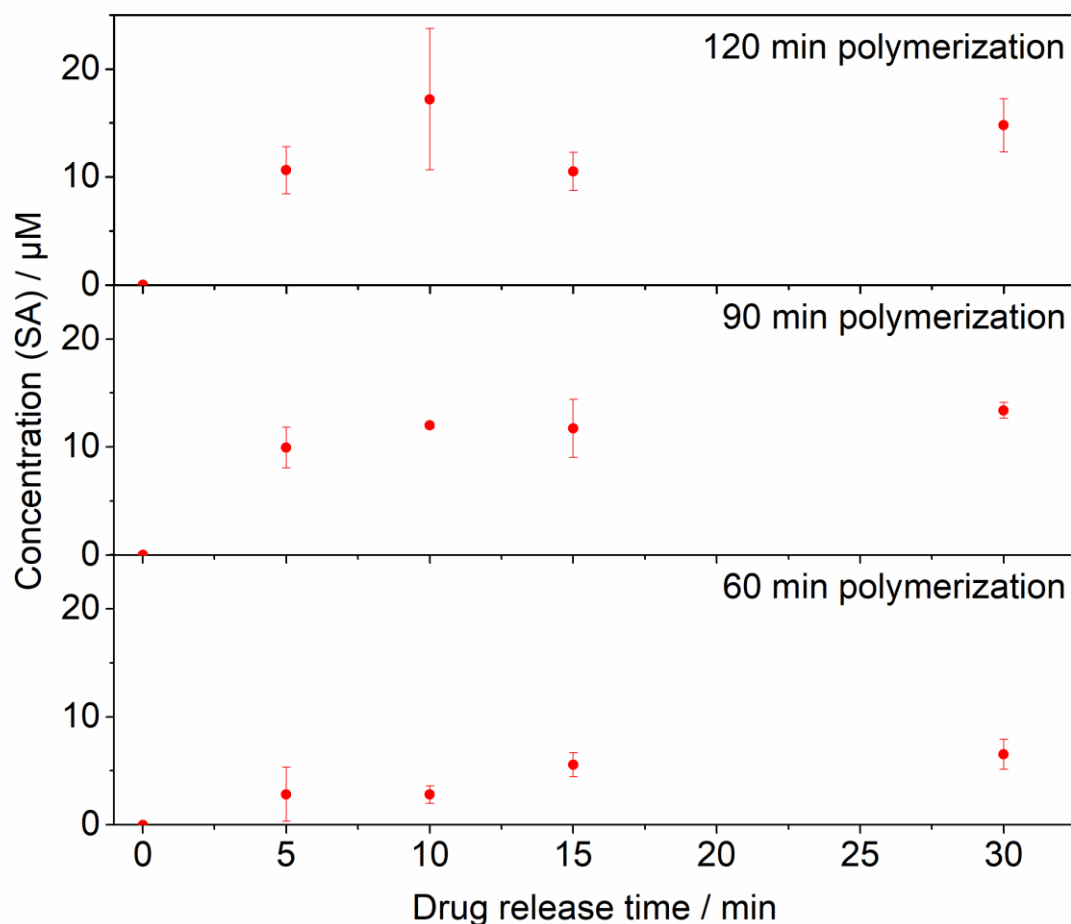
h



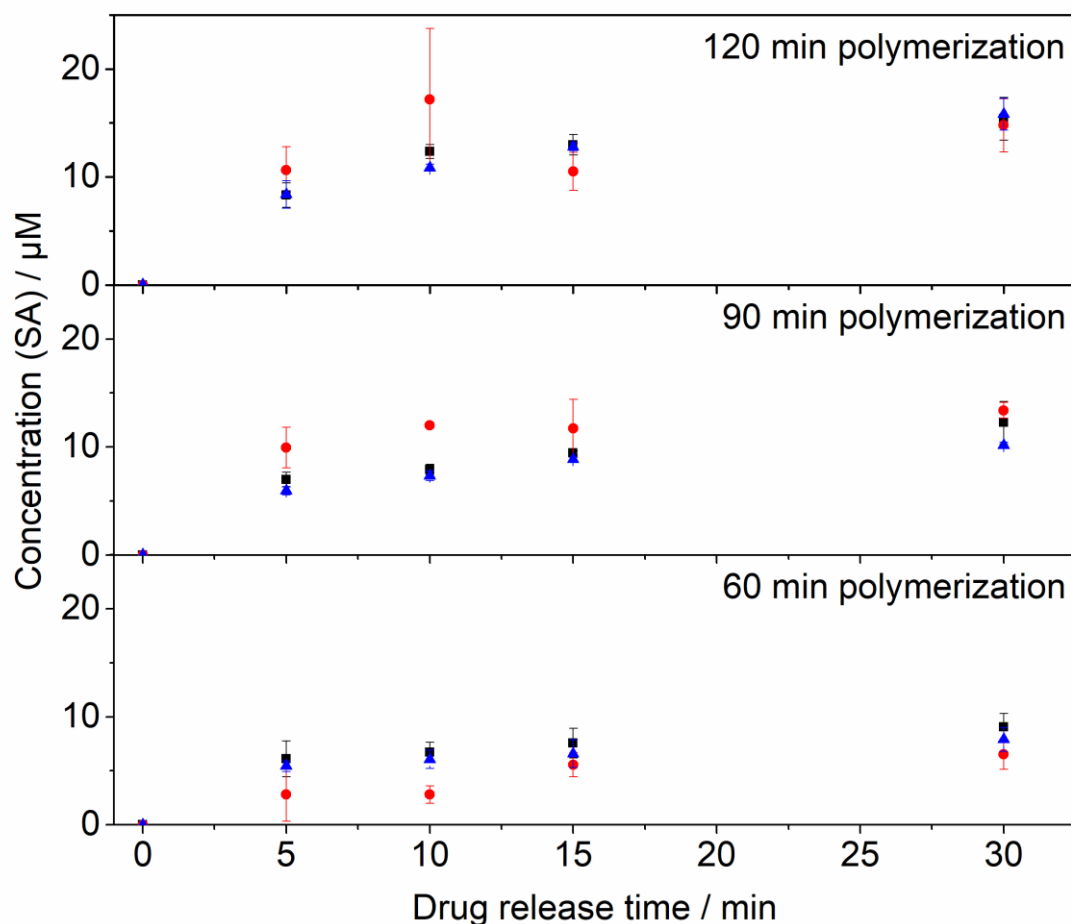
Appendix 16 AFM height images of poly(SA-pHBA acrylate)-modified samples polymerized for 120 min and incubated in PBS at 37° for a) 5 min, b) 10 min, c) 15 min, d) 30 min, e) 60 min, f) 360 min, g) 1440 min, h) 2880 min, measured on the glass face.



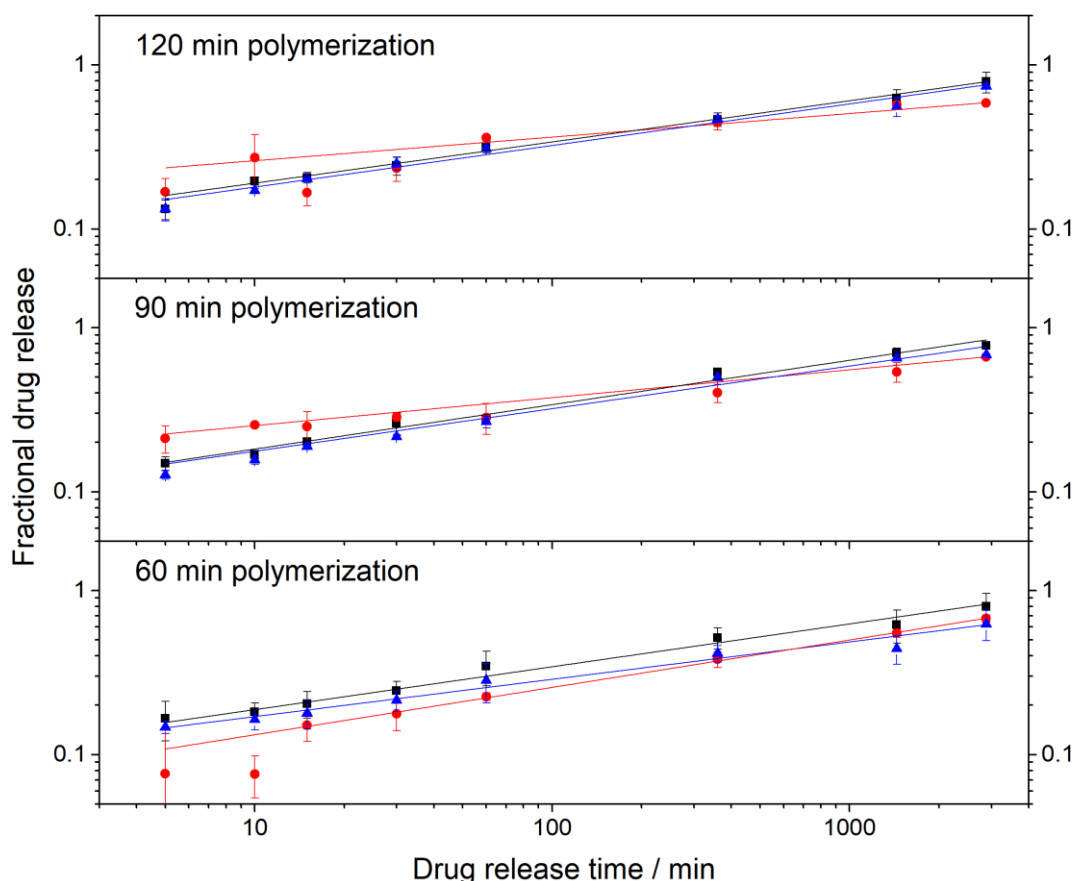
Appendix 17 Plot of the AFM step height against the incubation time for poly(SA-pHBA acrylate)-modified substrates polymerized for 60 min, 90 min and 120 min. The samples were incubated in PBS for up to 30 min at 37 °C. After the respective incubation time, the samples were rinsed and dried and then measured by AFM.



Appendix 18 Plot of the concentrations of SA against the incubation time for poly(SA-pHBA acrylate)-modified substrates polymerized for 60 min, 90 min and 120 min. The concentration of SA in the supernatant of the drug release was observed over the course of 30 min at 37 °C in PBS. The change in the layer thicknesses (determined by ellipsometry (Ti face) and AFM (glass face)) was used to estimate the amount of SA released (red circles).



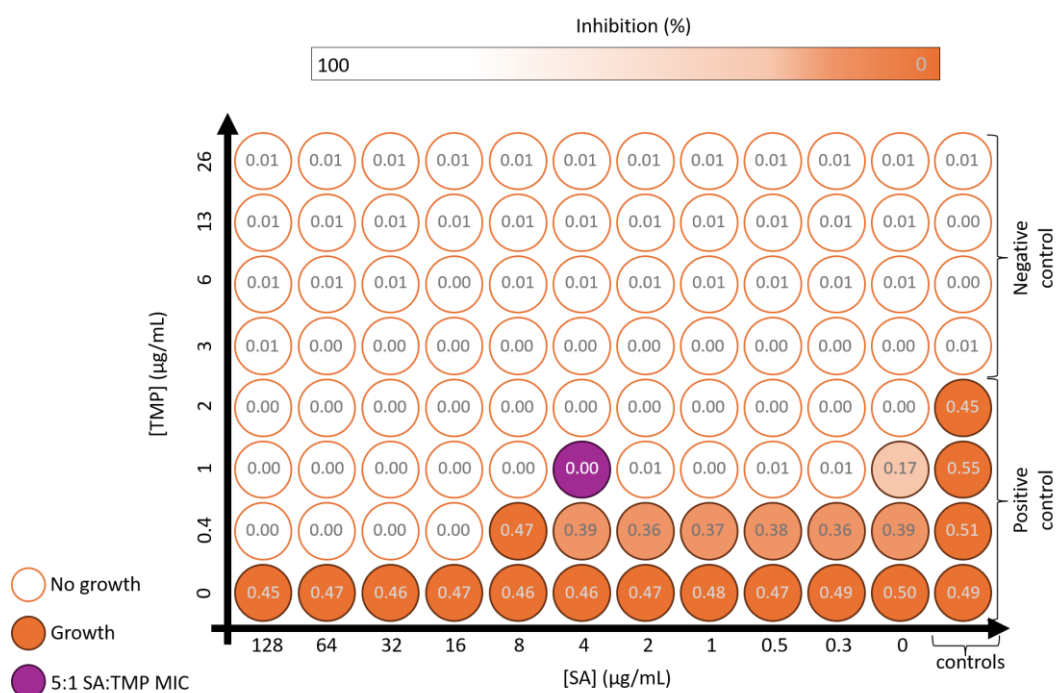
Appendix 19 Plot of the concentrations of SA against the incubation time for poly(SA-pHBA acrylate)-modified substrates polymerized for 60 min, 90 min and 120 min. The concentrations were determined based on the UV-Vis absorption maxima at 258 nm with an extinction coefficient of $16258 \text{ M}^{-1} \text{ cm}^{-1}$ (black squares), or the area of the SA signal in the HPLC chromatograms (blue triangles). The concentration of SA in the supernatant of the drug release was observed over the course of 30 min at 37 °C in PBS. The change in the layer thicknesses (determined by ellipsometry (Ti face) and AFM (glass face)) was also used to estimate the amount of SA released (red circles).



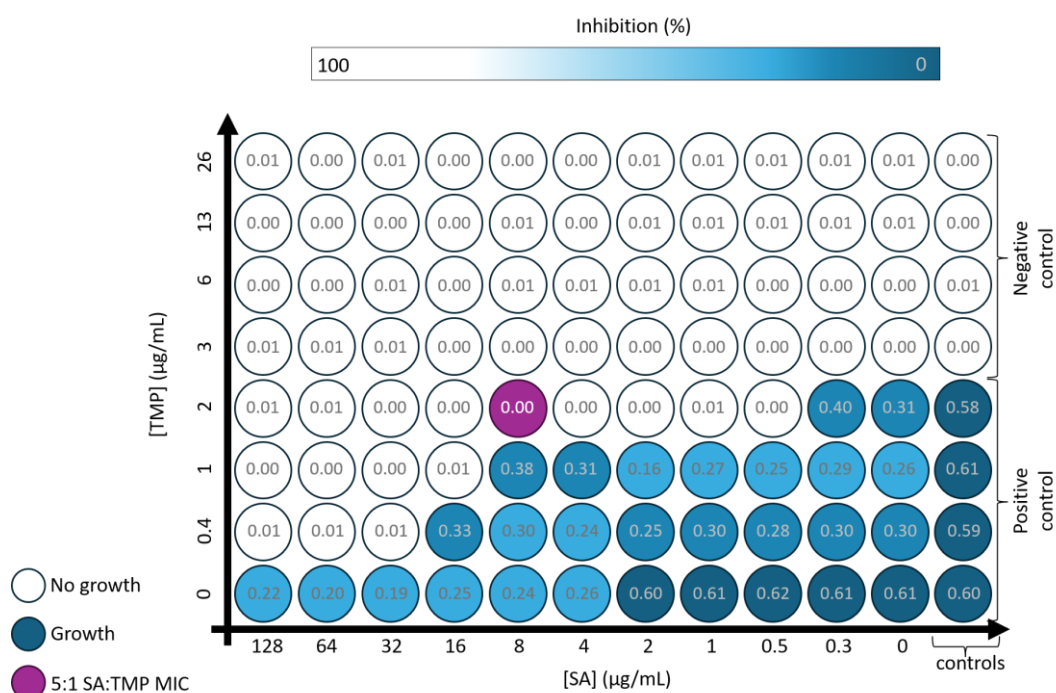
Appendix 20 Plot of the concentrations of SA against the incubation time for poly(SA-pHBA acrylate)-modified substrates polymerized for 60 min, 90 min and 120 min. The concentrations were determined based on the UV-Vis absorption maxima at 258 nm with an extinction coefficient of $16258 \text{ M}^{-1} \text{ cm}^{-1}$ (black squares), or the area of the SA signal in the HPLC chromatograms (blue triangles). The concentration of SA in the supernatant of the drug release was observed over the course of 30 min at 37 °C in PBS. The change in the layer thicknesses (determined by ellipsometry (Ti face) and AFM (glass face)) was also used to estimate the amount of SA released (red circles).

Appendix 21 Slopes and regression coefficients determined from the linear regressions of the log-log plot for the determination of the Korsmeyer-Peppas drug release mechanism.

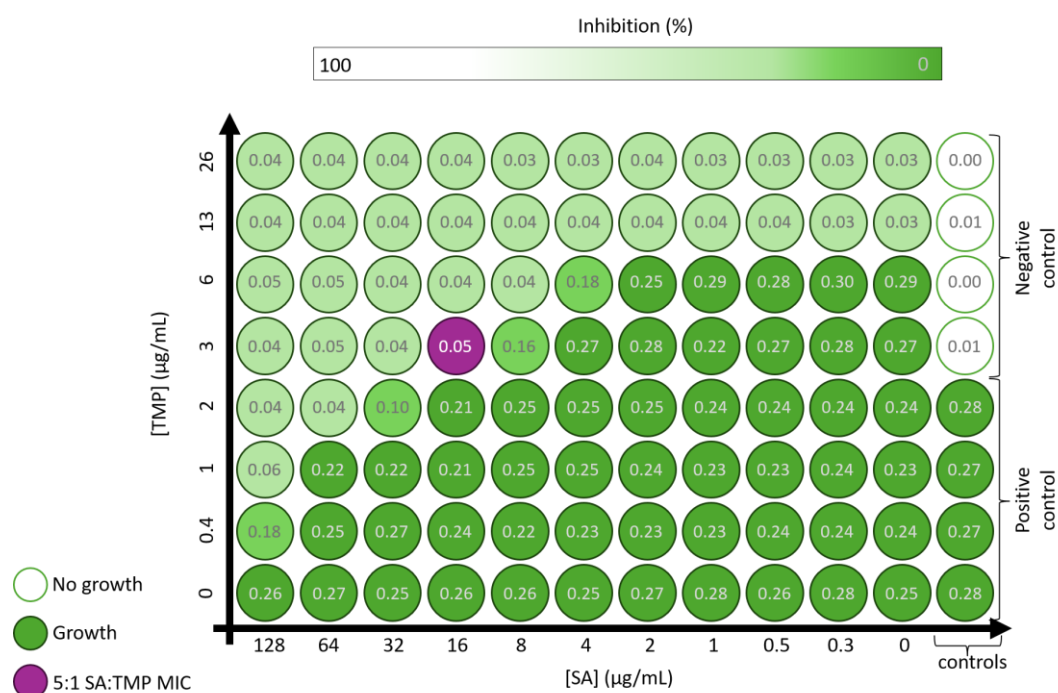
Polymerization time / min	Slope (<i>m</i>)/Regression coefficient (<i>R</i> ²)		
	HPLC	AFM	UV-Vis
60	0.23/0.99	0.29/0.98	0.26/0.99
90	0.26/0.96	0.17/0.99	0.27/0.99
120	0.25/0.99	0.14/0.92	0.25/0.98



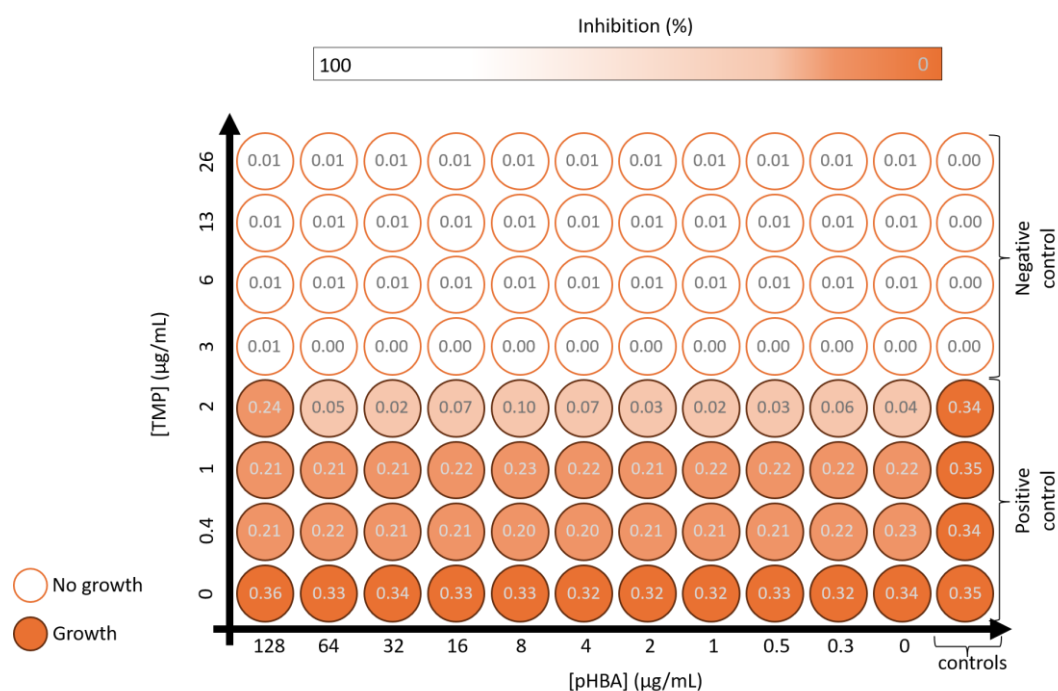
Appendix 22 Checkerboard assay conducted for mixtures of varying concentrations of SA and TMP, tested with *S. aureus* MSSA69. Values in each circle represent the change in OD₆₀₀ after 20 h incubation.



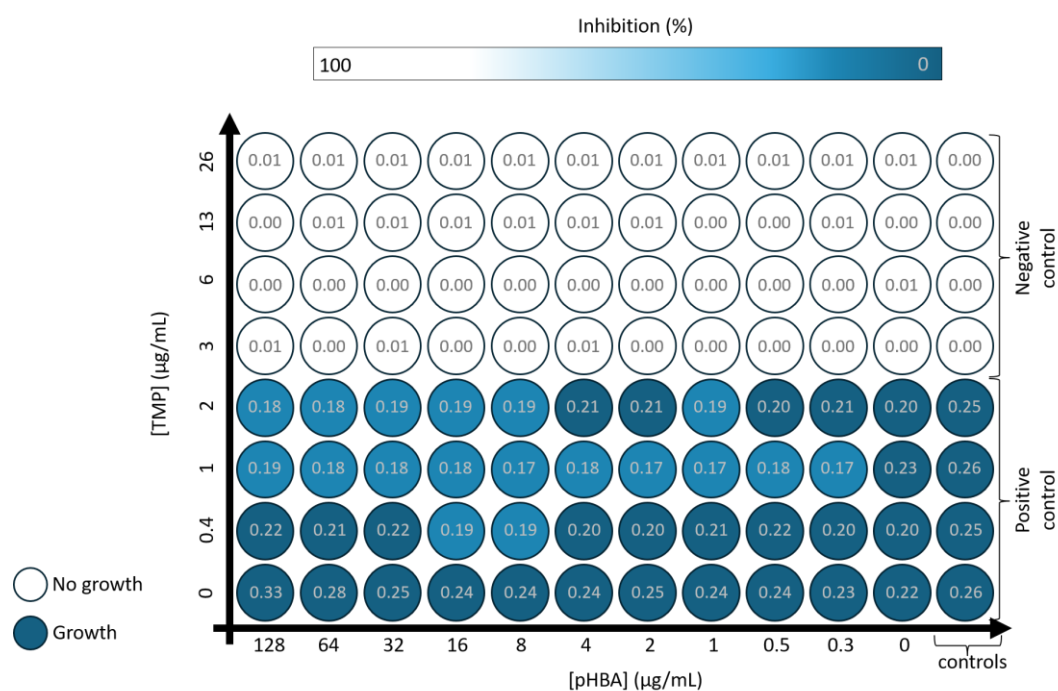
Appendix 23 Checkerboard assay conducted for mixtures of varying concentrations of SA and TMP, tested with *S. aureus* NCTC10788. Values in each circle represent the change in OD₆₀₀ after 20 h incubation.



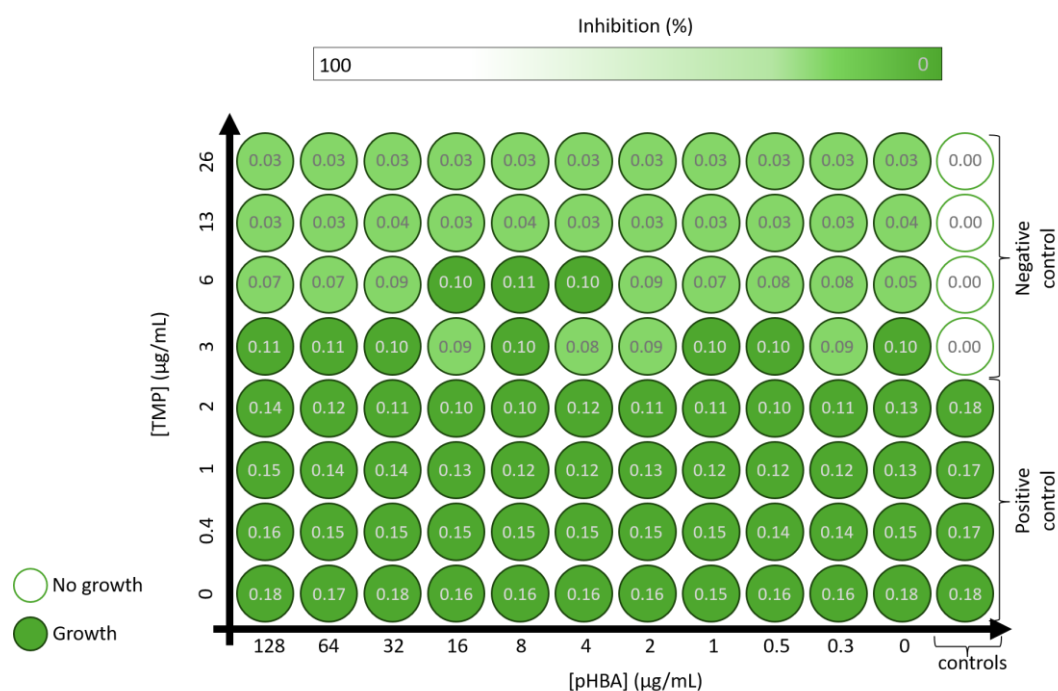
Appendix 24 Checkerboard assay conducted for mixtures of varying concentrations of SA and TMP, tested with *S. aureus* RN4220. Values in each circle represent the change in OD₆₀₀ after 20 h incubation.



Appendix 25 Checkerboard assay conducted for mixtures of varying concentrations of pHBA and TMP, tested with *S. aureus* MSSA69. Values in each circle represent the change in OD₆₀₀ after 20 h incubation.

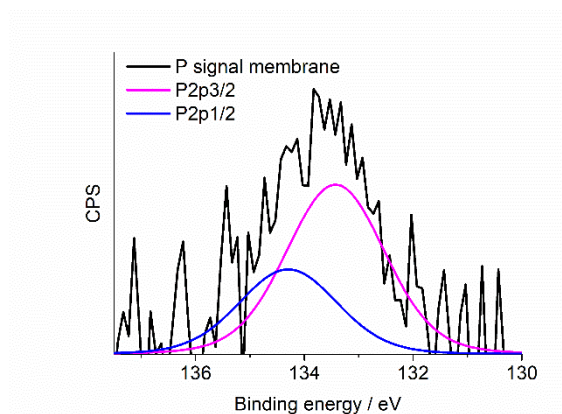


Appendix 26 Checkerboard assay conducted for mixtures of varying concentrations of pHBA and TMP, tested with *S. aureus* NCTC10788. Values in each circle represent the change in OD₆₀₀ after 20 h incubation.

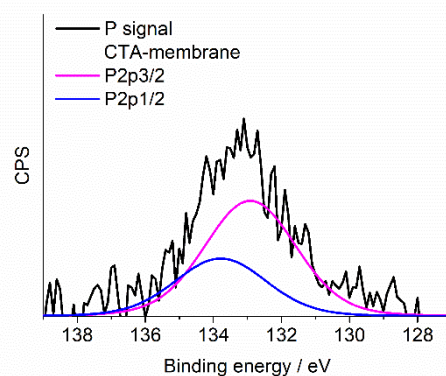


Appendix 27 Checkerboard assay conducted for mixtures of varying concentrations of pHBA and TMP, tested with *S. aureus* RN4220. Values in each circle represent the change in OD₆₀₀ after 20 h incubation.

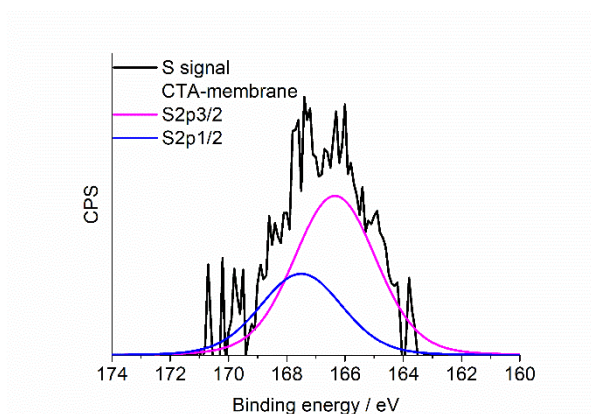
a



b



c



Appendix 28 High-resolution XPS spectra of phosphorous for (a) the neat membrane and (b) the CTA-modified membrane, and the high-resolution spectrum of sulfur (c) of the CTA-modified membrane.

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“Hat der Begrabene

Schon sich nach oben,

Lebend erhabene,

Herrlich erhoben”

Chor der Jünger – Faust. Der Tragödie erster Teil