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Microgels as Carriers for Antimicrobial Peptides

Surface-bound microgels, and factors affecting peptide interactions

LINA NYSTRÖM

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Abstract
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With a growing number of multi-resistant bacteria against conventional antibiotics, there is an urgent need to identify new antimicrobial therapeutics. One example that has gained considerable interest is antimicrobial peptides (AMPs). For AMPs to reach their full potential as therapeutics, as well as for other peptide and protein drugs, the right drug delivery system may overcome reported shortcomings, such as fast clearance in the bloodstream and proteolytic degradation. Microgels are weakly cross-linked polymer colloids, which can be made responsive to various stimuli. In the context of drug delivery, microgels are of particular interest as carriers for biomacromolecular drugs, such as peptides and proteins, as their water-rich environment offers both protection against enzymatic degradation and triggered release possibilities. Combining these, the aim of this thesis was to investigate electrostatically triggered surface-bound microgels as a delivery system for AMPs, as well as evaluate such systems as an antimicrobial and anti-inflammatory coating for biomaterials.

Results presented in this thesis demonstrate effects of microgel charge density, pH, and ionic strength on microgel volume transitions at solid interfaces, surface-induced microgel deformation and nanomechanical properties. In addition, effects of both microgel properties (charge density) and peptide properties (molecular weight, charge density, and posttranslational modifications) on peptide loading and release from surface-bound microgels were investigated. The presented thesis also reports *in vitro* studies of AMP-loaded microgels in dispersion and surface-bound, as either mono- or multilayers. Notably, the interplay between surface- and release-related effects for the antimicrobial properties of AMP-loaded microgels are investigated. In addition, anti-inflammatory properties of AMP-loaded microgels are also reported.

Taken together, microgels prove an interesting and versatile drug delivery system for AMPs. Results obtained in this thesis have demonstrated that several key factors need to be taken into consideration in the development of surface-bound microgels as a carrier for AMPs, and that small changes in microgel and peptide properties can alter peptide loading and release profiles.

Keywords: Antimicrobial peptides, Biomaterial coating, Drug delivery, Host defence peptides, Microgels, pH-responsive, Surface-bound

Lina Nyström, Department of Pharmacy, Box 580, Uppsala University, SE-75123 Uppsala, Sweden.

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Till minne av farfar

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Nyström, L**., Álvarez-Ascencio, R., Frenning, G., Saunders, BR., Rutland, MW., Malmsten, M. (2016) Electrostatic swelling transitions in surface-bound microgels. *ACS Applied material & interfaces*, 8(40):27129-27139

II **Nyström, L**., Nordström, R., Bramhill, J., Saunders, BR., Álvarez-Asenscio, R., Rutland, MW., Malmsten, M. (2016) Factors affecting peptide interactions with surface-bound microgels. *Biomacromolecules,* 17(2):669-678

III Nordström, R., **Nyström, L**., Andrén, OCJ., Malkoch, M., Umerska, A., Davoudi, M., Schmidtchen, A., Malmsten, M. (2018) Membrane interactions of microgels as carriers of antimicrobial peptides. *Journal of Colloid and Interface Science,* 513:141-150

IV **Nyström, L**., Strömstedt, AA., Schmidtchen, A., Malmsten, M. (2018) Peptide-loaded microgels as antimicrobial and anti-inflammatory surface coatings. *Biomacromolecules,* 19(8):3456-3466

V Nordström, R.*, **Nyström, L**.*, Ilyas, H., Atreya, HS., Borro, BC., Bhunia, A., Malmsten, M. Microgels as carriers of antimicrobial peptides - effects of peptide PEGylation. (*In manuscript*)

VI **Nyström, L**., Al-Rammahi, N., Malekkhaiat Häffner, S., Strömstedt, AA., Browning, KL., Malmsten, M. Avidin-biotin cross-linked microgel multilayers as carriers for antimicrobial peptides. (*Submitted manuscript*)

* *These authors contributed equally.*

Additional papers not included in the thesis:

i. **Nyström, L**., Malmsten, M. (2016) Surface-bound microgels – from physicochemical properties to biomedical applications. *Advances in Colloid and Interface Science.* 238:88-104

ii. Malekkhaiat Häffner, S, **Nyström, L**., Nordström, R., Xu, ZP., Davoudi, M., Schmidtchen, A., Malmsten, M. (2017) Membrane interactions and antimicrobial effects of layered double hydroxide nanoparticles. *Physical Chemistry Chemical Physics.* 19(35):23832-23842

iii. **Nyström, L**., Malmsten, M. (2018) Membrane interactions and cell selectivity of amphiphilic anticancer peptides. *Current Opinion in Colloid & Interface Science*, 38:1-17

Contents

Abbreviations

AFM	Atomic force microscopy
AMP	Antimicrobial peptide
BDDA	1,4-butanediol diacrylate
CD	Circular dichroism
CLSM	Confocal laser scanning microscopy
cryoTEM	Cryogenic transmission electron microscopy
D	Dissipation
DLS	Dynamic light scattering
EA	Ethyl acrylate
E. coli	*Escherichia coli*
f	Frequency
FEM	Finite element method
FTIR	Fourier transform infrared spectroscopy
Γ	Adsorbed amount
GOPS	3-glycidoxypropyltrimethoxysilane
HDP	Host defense peptide
LPS	Lipopolysaccharide
MAA	Methacrylic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
NTA	Nanoparticle tracking analysis
PCS	Photon correlation spectroscopy
PEG	Poly(ethylene glycol)
pLys	Poly-L-lysine
ppm	Parts per million
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
QCM-D	Quartz crystal microbalance with dissipation
QNM	Quantitative nanomechanical property mapping

Abbreviations of amino acids

Cationic		
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Anionic		
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Polar, uncharged		
Serine	Ser	S
Threonine	Thr	T
Asparagine	Asn	N
Glutamine	Gln	Q
Cysteine	Cys	C
Hydrophobic		
Alanine	Ala	A
Valine	Val	V
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Proline	Pro	P
Glycine	Gly	G

Introduction

Biomaterials

Through the fast advances in biomaterial research in the last couple of years, we now have the ability to replace and restore body functions. In an ageing population, today's medical healthcare is heavily dependent on the use of implants and biomedical devices. It is estimated that almost everyone will require at least one implant procedure during his/her lifetime.[1] Stents, vascular grafts, heart valves, catheters, stiffer dental and joint replacement implants, and other medical devices, such as pacemakers and insulin pumps, are all examples of biomaterials that have improved the quality of life for many patients suffering from various wear, traumas or diseases.

Biomaterial-associated infections

Unfortunately, the use of biomaterial implants always comes with the risk of an infection. After implantation, the fate of the implant is determined by the competition between tissue integration and bacterial adhesion.[1, 2] If bacterial colonization takes place first, starting with the adhesion of planktonic free-floating bacteria, the biofilm starts to mature and bacteria undergo both genomic and proteomic changes, and form an extra-cellular matrix.[3, 4] The diverse multicellular nature of biofilms and dormancy of persister cells make them less susceptible to antimicrobial treatments than planktonic bacteria, and especially difficult to treat.[5-7] Therefore, the formation of biofilms significantly increases the risk of implant failure and the need for a secondary replacement surgery.[2] Antibiotic prophylaxis can contribute to minimize the risk of biofilm formation, however, most often combinatory antibiotic treatments are needed after surgery. In addition, fibrous capsule formation around biomaterials make systemic delivery hard to reach therapeutic levels at the implant interface.[8]

New therapeutic routes, i.e., immobilization on implant interfaces or antibiotic releasing from the interface, offer promising ways to minimize the risk of biofilm formation.[9-12] However, with the growing number of reports of antibiotic-resistant bacterial strains the future usage of biomaterials and biomedical devices are still at risk.

Antibiotic resistance

According to the World Health Organization (WHO) antibiotic resistance is one of the biggest threats to global health and development today. This affects a vast number of patients and causes an increased pressure on the healthcare system. The use of antibiotics in the 20th century has put an increased selection pressure on pathogens, and new resistance mechanisms against antibiotics are emerging rapidly. The extensive overuse and misuse of antibiotics in medical treatment and agriculture, combined with increased travel habits, accelerates the spread of multidrug-resistant bacterial strains.[13, 14] Additionally, a decreasing number of new antibiotic agents are developed by pharmaceutical companies, at a time where new alternatives are urgently needed to halt the spreading of multi-drug resistant bacteria.[15]

Antibiotic-resistant bacteria strains are commonly found in biomaterial-associated infections.[16, 17] For example, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermis* are the most common microorganisms in orthopaedic implant-associated infections. However, cases of vancomycin-resistant *S. aureus*, multi-drug resistant *Acinetobacter*, extended-spectrum β-lactamase-producing *enterobacteriasceae*, and multi-drug resistant *Pseudomonas aeruginosa* have also been reported.[17] Consequently, antibiotic prophylaxis efficiency has decreased. The decreased efficiency of conventional antibiotics threatens to push medicine back to the pre-antibiotic era.

Novel alternatives

The need of multiple new therapeutic alternatives is evident as microbes are inherently good at evolving new resistance mechanisms. Some examples that have gained interest include, metal nanoparticles (i.e., Ag, Au, ZnO, TiO_2),[18, 19] nitric oxides (NOs),[20] quaternary ammonium compounds (QACs) and antimicrobial peptides (AMPs)[21-24]. The latter is discussed in more detail in the next section. Although new alternatives are also likely to generate resistance eventually, novel alternatives are nevertheless needed. The new resistance may also come at the cost of bacterial fitness.[25] Therefore, the development of several new antibiotic alternatives, combined with smart implant interfaces such as anti-fouling, drug release or drug-grafting alternatives could very well help fight future multi-resistant bacterial strains and biomaterial-associated infections.

Antimicrobial peptides

In the presented thesis, the focus has been placed on antimicrobial peptides (AMPs), also known as host defense peptides (HDPs), as an alternative to conventional antibiotics. AMPs are universal defense molecules, which constitute a part of the first line of defense of our innate immune system. They are an abundant and diverse group of peptides naturally found in many tissues and cells, occurring in mammals, plants, and invertebrates.[21] By learning from nature, this large class of peptides, either endogenous or of synthetic nature, possesses fast antimicrobial actions with a broad spectrum of microorganism targets. In addition to bacteria, some AMPs work against fungi,[26, 27] viruses,[28] parasites,[29] and cancer cells.[30, 31] Other possess immune modulating properties.[32, 33] When it comes to AMPs with antibacterial properties, the peptides are often short (less than 10 kDa) cationic amphiphilic peptides. Although different modes of action have been suggested in literature, most AMPs work by direct membrane rupture, leading to a fast and broad-spectra antibacterial effect, even against tough resistant strains.[23, 34] Efforts have also been made in fine-tuning peptide effectiveness, looking at both structural properties and post-translational modifications, altering peptide charge density, charge placement, amphiphilicity, and hydrophobic end-tagging.[35] A summary of the investigated peptides in this thesis can be found in Table 1.

Table 1. Properties of investigated peptides.

Model peptides

	Sequence	Net charge	Mw (g/mol)	Paper
Poly-L-lysine	(KKKK)n	+32 +81 +1153	4200 10500 149000	II

Antimicrobial peptides

	Sequence	Net charge	Mw (g/mol)	Paper
LL-37	LLGDFFRKSKEKIGKEF-KRIVQRIFLRNLVPRTES	+6	4364	III
DPK060	GKHKNKGKKNGKHNGWK-WWW	+7	2505	III
KYE28	KYEITTIHNLFRKLTH-RLFRRNFGYTLR	+6	3595	IV-VI
KYE28PEG*	KYEITTIHNLFRKLTH-RLFRRNFGYTLR-PEG48	+6	5851	IV-V

* PEGylated KYE28 (48 PEG units), located either PEG-KYE28, PEG-KYE28-PEG or KYE28-PEG

Drug delivery of antimicrobial peptides

The right drug delivery system may overcome shortcomings of AMPs, as well as other peptide and protein drugs, as therapeutic alternatives. For example, administered AMPs risk fast clearance from the bloodstream, serum protein binding and degradation by proteases, together with toxicity concerns.[36, 37] In relation to the number of studies on the effect of AMPs, less focus has been on their drug delivery. Nevertheless, interesting examples of AMP carriers can be found in literature, including both inorganic materials (mesoporous silica,[38, 39] nanoclays,[40] titanium oxides,[41, 42] and metal nanoparticles[43]), surfactant and lipid based formulations,[44] and other polymeric materials[45-48]. Polymeric drug delivery of AMPs span from macroscopic hydrogels and polymeric multilayers down to microscopic polymeric fibers, nanogels, and microgels. The latter is discussed in more detail in the following sections.

Microgels

Microgels are sparsely cross-linked polymer colloids, well studied for their interesting swelling capabilities.[49-51] By incorporating various chemical functional groups in the polymer network during synthesis, microgel swelling can be made responsive to a number of different triggers, such as temperature, pH, ionic strength, redox conditions and specific metabolites. Their fast response times, colloidal stability, ease of synthesis and control of size make them interesting for a wide variety of fields and applications.[52-56]

Microgels in peptide and protein drug delivery

In the context of drug delivery, microgels, as well as macroscopic hydrogels, are especially suitable for biomacromolecular drugs such as peptides and proteins, due to their hydrophilic water-rich environment and triggered release possibilities.[51, 57] Microgels have also been demonstrated to retain native biological activity of released peptides and larger proteins.[58]

For peptide drug delivery, a number of mechanistic studies have been conducted to gain further understanding of factors affecting peptide loading and release from charged pH-responsive dispersed microgels.[57, 59] Peptide interactions depend on microgel properties (cross-linking density[60] and charge density[61]), as well as peptide properties, such as molecular weight,[62] charge density and distribution,[61] hydrophobicity,[63] and secondary structure[64]. Peptide loading to microgels may also affect the degradation of both the peptide[65] and the microgel network[66]. Mechanistic studies of AMP-microgel interactions remain sparse.

Extending from such relatively 'simple' pH-responsiveness, many other interesting examples of specific microgel protein drug delivery exists. Kim and Park investigated glucose-sensitive hydrogels for repeated glucose-dependent release of insulin.[67] Similarly, Tanihara *et al.* and Suzuki *et al.* investigated infection-triggered degradation of hydrogels by incorporating specific peptide sequences in the polymer network, which are sensitive to thrombin-like proteolytic activity of *P. aeruginosa* and other bacteria.[68, 69] In another example, Murthy *et al.* investigated pH-degradable protein-loaded microgels, by incorporating acid-labile acetal cross-linkers, and thereby attained protein release in a pH-dependent manner.[70]

Microgels at interfaces

While most research so far has focused on microgels in dispersion, surface-bound microgels have gained increased attention. Microgels have proved a versatile building block, offering a robust and facile approach for surface modification and functionalization. Microgels have been studied at the air-liquid,[71] liquid-liquid[72, 73] and liquid-solid interfaces.[74, 75] They can be both physisorbed and covalently grafted, and exist in monolayer and layer-by-layer constructs.

Most studies of surface-bound microgels have been of non-ionic temperature-responsive poly(N-isopropylacrylamid) (pNIPAM) and pNIPAM-*co*-monomer mixture microgels. At a solid interface, these types of microgels, or mixtures of microgels, form well-organized adsorbed layers, either by dip coating or spin-coating.[76-78] The packing degree of the latter can be controlled through microgel concentrations and deposition speed.[79] A number of studies have shown that pNIPAM-based microgels volume phase transition temperature is not affected by the confinement of the solid interface, although their swelling capabilities are reduced due to surface pinning effects and their flattened height profile.[80, 81] The addition of co-monomers have been shown to increase the volume phase transition temperature.[82] Similar to microgels in dispersion, the magnitude of swelling/de-swelling capabilities, as well as microgel mechanical properties, depend on a number of factors such as microgel composition and cross-linking degree.[83-85]

For purely charged surface-bound microgels, less is known. However, a few studies exist, showing that cycled pH-dependent swelling of microgels is also possible. FitzGerald *et al.* for example, investigated pH-dependent swelling of adsorbed poly(2-vinylpyridine) microgels using AFM and observed swelling transitions similar to that of microgels in dispersion.[86] Howard *et al.* studied swelling properties of poly[2-(diethylamino)ethyl methacrylate] microgels and found cycled pH-responsive swelling possible, although that swelling of

adsorbed microgels was only of a magnitude of 3-4 times, compared to a factor of 20 for the same microgels in solution.[87]

Multilayers of microgels at a solid interface have also been studied.[88-90] Serpe *et al.* reported the first layer-by-layer deposited microgel multilayers by alternating poly(N-isopropylacrylamide-*co*-acrylic acid) microgels, serving as the anionic polyelectrolyte, and cationic polyallylamine.[88] This created a strongly thermoresponsive film at pH below the pKa of the acrylic acids. Whereas in higher pH, the electrostatic repulsion between the acids and increased osmotic pressure and thereby hindered the thermosresponsive swelling of the film. The system was later further developed by a spin-coating assisted approach and the formed film was shown to keep their pH-dependent swelling over several cycles, where the response time of the film scaled with the number of microgel layers.[89]

Biomedical applications of surface-bound microgels

The responsiveness and large swelling capabilities, together with the use of a wide variety of biocompatible monomers for the production, have made surface-bound microgels interesting for a number of applications that depend on functional interfaces.

Biosensors

One area where surface-bound microgel responsiveness is useful are etalons, in which microgels are placed in between two metal surfaces to form a sensor. As the microgels swell/de-swell the detectable signal of the sensor changes.[91, 92] As another example, Kim *et al.* investigated bioresponsive microgel microlenses able to detect avidin binding to biotin-modified microgel layers. As avidin binds, the number of cross-links increases in the microgel layers, which could be monitored by changes in their optical properties. Enzyme-loaded microgel layers have also been investigated as electrochemistry-based biosensors.[93]

Functional materials

The robust responsive properties of microgel layers are desirable for multiple uses as functional material coatings. As an example of this responsive microgel layers have been investigated in membrane technology.[94] Uhlig *et al.* investigated thermoresponsive microgel coatings for adherent cell cultivation assays, where cell attachment could be controlled by changing the temperature. This offers a noninvasive trypsin-free method, as well as precise control over patterned areas of cell attachment, useful in for example wound healing assays.[95] Another interesting area where multilayered microgels have been investigated are self-healing coatings, where the microgel layers have been shown to repeatably heal micron-sized damages.[96, 97]

Working towards the use of surface-bound microgels as biomaterial coatings, Bridges *et al.* have shown, in a series of works, that surface-bound poly(NIPAM-*co*-PEG) microgels lower fibrinogen adsorption, primary human monocyte, macrophage and leukocyte adhesion, as well as lowering pro-inflammatory cytokines.[98, 99] It was also shown that microgel coatings lower the chronic inflammation of PET disks when implanted subcutaneously in a rat model.[100] Similarly, Wang *et al.* investigated osteoblast adhesion to sparsely spread poly(PEG-*co*-acrylic acid) microgels over an otherwise fibronectin-covered surface and found this to increase osteoblast short-term spreading and cell proliferation.

Drug delivery

Although microgels have shown advantageous properties on their own, the strength of microgel coatings for biomaterials are found in combination with sustained release of therapeutics to tackle both infection and inflammation. Some very intriguing proof of concept works of stimuli-responsive drug delivery of surface-bound microgels exist. For example, Nolan *et al.* showed temperature dependent pulsatile release of insulin from layer-by-layer deposited poly(PNIPAM-*co*-acrylic acid) microgels.[101] In a similar approach Serpe *et al.* showed cycled release of doxorubicin from layer-by-layer microgel constructs.[102] In an example of pH-dependent release FitzGerald *et al.* showed cycled uptake and release of the model hydrophobic probe, pyrene, from adsorbed poly-[2-(diethylamino)ethyl methacrylate] and poly[2-(diisopropylamino)ethyl methacrylate] microgels.[103] In one of few examples of combinations using surface-bound microgels and AMPs, Wang *et al.* showed antimicrobial peptide L5 (PAWRKAFRWAWRMLKKAA) loaded poly(PEG-*co*-acrylic acid) microgels deposited on poly-L-lysine primed silicon to reduce the colonization of *S. epidermis.*[104] Furthermore, by deposition of peptide-loaded microgels on polycaprolactone-chitosan nanofiber, Wang *et al.* showed that the L5 peptide-loaded microgels reduced bacterial colonization also in a 3D environment, while still promoting osteoblast growth.[46] Taken together, these studies demonstrate that responsive drug release and AMP drug delivery from surface-bound microgels are possible.

Motivation

The starting point of this thesis originated from the combination of the above prior knowledge of microgels, microgel coatings, and promising control of peptide-microgel interactions, together with encouraging effect studies of AMPs. The overall idea was to study the use of antimicrobial peptide-loaded responsive microgels as a combined antimicrobial and anti-inflammatory surface coating for biomaterials (*Figure 1*). By finding a simple way of decorating implant surfaces with responsive microgels, regardless of shape, mechanistic investigations of key factors affecting peptide loading and release could be done. Investigations of both microgels and peptide-related properties were performed, in combination with effect studies of any antimicrobial and anti-inflammatory properties of these type of layers and the corresponding experiments of the released peptides.

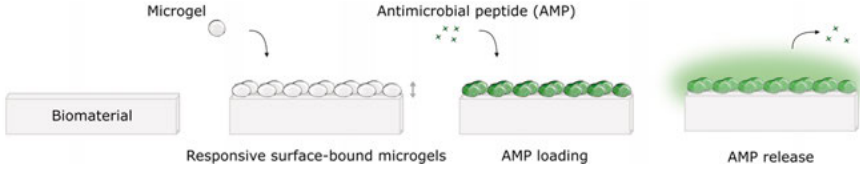


Figure 1. Schematic summarizing the main goal of the thesis. It includes surface-bound microgels, and factors affecting peptide-loading and release from these layers.

Aim of the thesis

The aim of this thesis is to evaluate microgels as a drug delivery system for antimicrobial peptides, with focus on peptide-loaded surface-bound microgels. To achieve this, the following points were addressed during the project:

- Synthesis and characterization of a microgel model system, including comparisons of performance in dispersion and surface-bound to an interface (**paper I**).
- Evaluation of factors affecting peptide loading and release from microgels (**paper II-VI**).
- Evaluation of antimicrobial effects of peptide-loaded microgels, in dispersion (**paper III**) and surface-bound (**paper IV, VI**).
- Evaluation of anti-inflammatory effects, toxicity, and proteolytic stability of the peptide-loaded microgel drug delivery system (**paper III, IV**).

Methods

In this section, a summary of the main techniques used for synthesis, characterization, and visualization of microgels, in dispersion or surface-bound, will be presented. These include methods used for studying peptide-microgel interactions and effect studies of peptide-loaded microgels. In depth theoretical descriptions of the techniques have been published elsewhere, as indicated in the text. For technical details regarding the methodology, please see the respective included papers.

Microgel synthesis and characterization

Emulsion polymerization

The microgels investigated in the thesis were all synthesized by starved-feed emulsion polymerization. For this, monomers and cross-linker are mixed in a water solution containing surfactant. By vigorously mixing the solution using a mechanical stirrer, the solution emulsifies, forming small monomer droplets and micelles. When adding a water-soluble initiator to the reaction mixture this initiates the polymerization inside the small droplets, which are stabilized by the surfactant. Additional monomers and cross-linker are slowly added to the reaction mixture to promote propagation of the polymer network and increase microgel size. When the desired size is reached, the reaction is terminated by cooling the reaction mixture. The remaining monomers, cross-linker and initiator are removed by dialysis. The advantages of this reaction include narrow size distribution due to the emulsion, tight control of the final size and large reaction volumes. Microgel properties can also easily be altered by varying the types and amounts of monomers and cross-linker.

In this thesis, methacrylic acid-based microgels were chosen because of their pH-dependent swelling properties.[105, 106] For the included papers poly(ethyl acrylate/methacrylic acid/ 1,4-butanediol diacrylate) (EA/MAA/BDDA) microgels were therefore synthesized, using a slightly modified protocol than previously reported by Rodriguez *et al.*[105] A schematic of monomers, cross-linker, and the resulting polymer can be found in *Figure 2.*

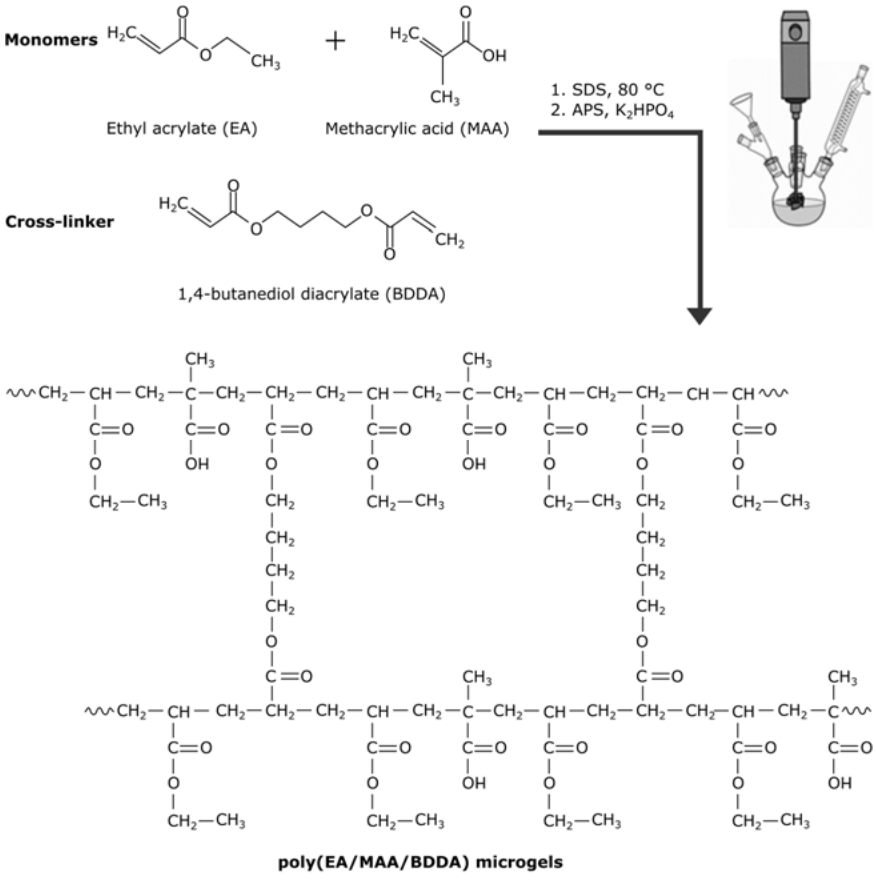


Figure 2. Radical polymerization of monomers (ethyl acrylate and methacrylic acid) and cross-linker (1,4-butanediol diacrylate) into poly(EA/MAA/BDDA) microgels.

The compositions of the monomer solution containing the two monomers (EA and MAA) and cross-linker (BDDA) were varied between 79/20/1 and 39/60/1 (EA/MAA/BDDA) w/w % to vary the charge density of the synthesized microgels while keeping the cross-linking degree at 1 w/w %. Microgels are throughout the thesis abbreviated to the MAA content of this monomer mixture e.g. MAA20 and MAA60. For synthesis, the seed monomer solution (31.5 g of the total 260 g) was added to a nitrogen-purged sodium dodecyl sulfate (SDS)-containing solution, at 80 °C, in a four-necked round-bottom flask equipped with a mechanical stirrer and condenser. This was immediately followed by addition of dipotassium hydrogen phosphate buffer and ammonium persulfate (APS) initiator. After 30 min the additional monomer mixture was added continuously (2.6 mL/min) over 90 min. Microgel size was regularly sampled over time using photon correlation spectroscopy (PCS) to achieve a similar size of the different microgels. In some cases a second, or

third initiator addition could be included to propagate the radical polymerization further and thereby increase microgel size. When the desired size of ~ 100 nm in diameter was reached, the reaction was terminated by cooling the reaction vessel. It was followed by extensive dialyzing (Mw cutoff 12000-14000 Da) of the microgel solution against water to remove unbound fractions. The synthesized MAA-microgels were then titrated to determine the degree of ionization and the methacrylic acid content. The pH-dependent swelling was determined using PCS. Table 2 summarizes the main properties of the investigated microgels.

Table 2. *Properties of investigated microgels*

Abbreviation	%MAA[a]	pK_a[a]	Ionization at pH 7.4	Swelling ratio[b]	d (nm) pH 7.4[b]	Paper
MAA20	22.1±1.1	7.0	0.72	2.4	174	I, II
MAA26.5	34.3±1.1	6.9	0.76	3.1	236	III
MAA33	36.9±0.4	6.4	0.91	3.3	265	I,II,IV-VI
MAA60	63.3±1.5	6.5	0.89	2.7	338	I-III, V

a) Titration from pH 3.5 in 0.01M NaCl, n=2.

b) Hydrodynamic diameter determined by PCS in 0.1 M buffers.

Methacrylic acid-based microgels

The pH-responsiveness of the MAA-microgels arise as pH either protonate or deprotonate the carboxylic acid moieties of MAA monomers, changing the ionization degree of the polymer network (*Figure 3*A). At high pH most acids are deprotonated and the electrostatic repulsion between the polymer chains and osmotic pressure is therefore high, causing the particles to swell. Since acute wounds undergo acidosis[107] and inflamed tissues have up to about 0.5 pH units lower pH than healthy tissue,[108] the pH responsiveness of these MAA-microgels with a pK_a close to physiological pH make them interesting to study as a drug delivery system. Therefore, a library of MAA-microgels with similar size and crosslinking degree was synthesized, see Table 2. Depending on the amount of MAA incorporated into the polymer network during synthesis, microgel swelling properties can be controlled (*Figure 3*). In addition, this library of broad range in microgel charge densities enables further mechanistic studies on how microgel properties affect peptide loading and release.

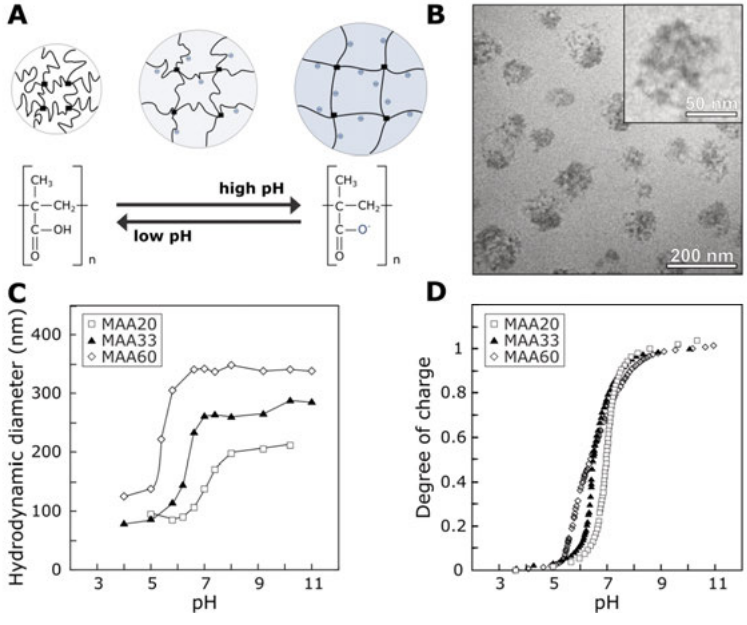


Figure 3. (A) Schematic of microgel swelling due to the protonation and deprotonation of MAA. (B) CryoTEM image of MAA33 microgels in Tris buffer (pH 7.4, 10 mM). (C) pH-dependent swelling measured with PCS and (D) titrated ionization degree (degree of charge) of MAA20, MAA33 and MAA60 microgels.

Covalent coupling of microgels to an interface

To be able to study peptide-loaded microgel as an antimicrobial surface coating, microgels were covalently bound to substrates to ensure a high and stable microgel coverage. In this thesis, silanization of silicon dioxide substrates were chosen. These materials are not typically investigated as actual biomaterials, but for lab-technical reasons this enables the same coupling chemistry to be used for glass slips, which are useful for microscopy techniques, as well as silica substrates and sensors, used for example in ellipsometry, QCM-d and ATR-FTIR experiments.

Covalent coupling of the carboxylic acids in the MAA-microgels to the interface was achieved by silanizing the glass substrates with 3-glycidoxypropyltrimethoxysilane (GOPS).[109] This introduces epoxide functional groups at the interface, which are able to couple to the MAAs of the microgels overnight via an ester bond (*Figure 4*).

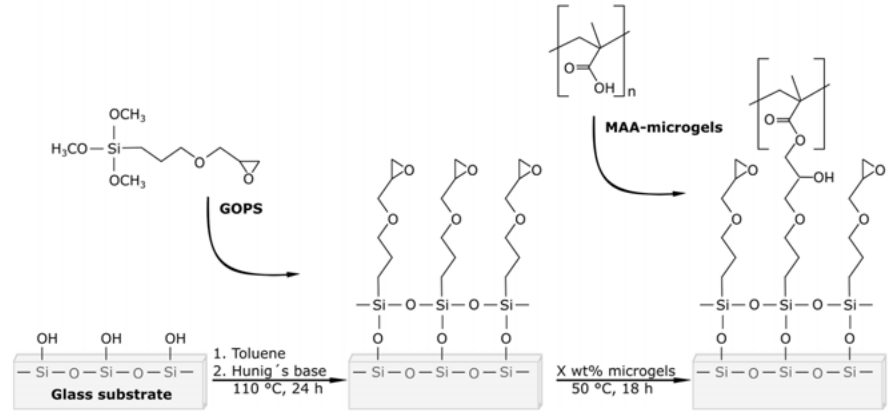


Figure 4. Scheme of GOPS silanization and covalent coupling of the MAAs of the MAA-microgel to the introduced epoxide functional groups at the interface.

Microgel coverage can be controlled by changing the wt % and zeta-potential of the microgels. The different ways used for deposition of microgels on GOPS-treated substrates have been summarized in *Figure 5*. In all included papers, except **paper IV** and **paper VI**, microgels were deposited at a 0.1 wt % concentration reaching a surface fractional coverage of microgels at the interface of ~ 0.1. To increase the surface coverage, the protocol was further developed in **paper IV** and microgels were instead deposited at a 1.0 wt % concentration with an additional 0.9 mM calcium nitrate present. The addition of divalent calcium ions neutralizes the effective zeta-potential of the anionic microgels and thereby lowers the electrostatic repulsion between the particles during deposition. This was shown to increase the average surface fractional coverage up to ~ 0.45. In **paper VI**, cross-linked microgel multilayers were investigated instead, as discussed in more detail in the next section.

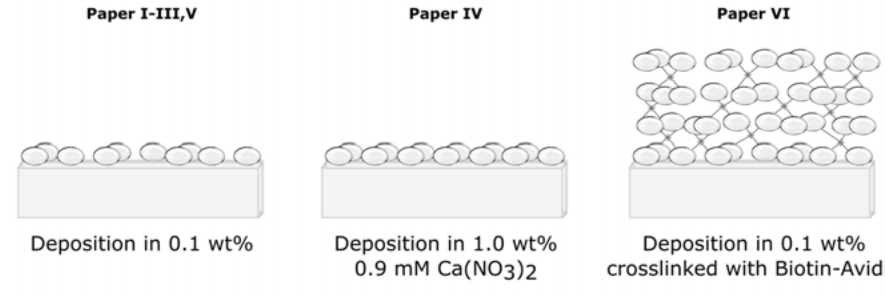


Figure 5. Schematic of the three different microgel depositions used in the thesis.

Microgel multilayers

To increase the peptide-loading capabilities of the MAA-microgel coatings, microgel multilayers were investigated in **paper VI**. The avidin-biotin pair has one of the strongest known non-covalent bond and has been used in numerous applications.[110-112] To form microgel multilayers, MAA-microgels were first functionalized with biotin (*Figure 6*). This allowed avidin, which has four binding sites for biotin, to be used as a cross-linker between the biotinylated MAA-microgel layers. The first monolayer of microgels was prepared as described in the previous section, after which microgel multilayers were achieved by alternating biotinylated MAA-microgels (MAA-Biotin)/avidin or MAA-Biotin/AMP/avidin.

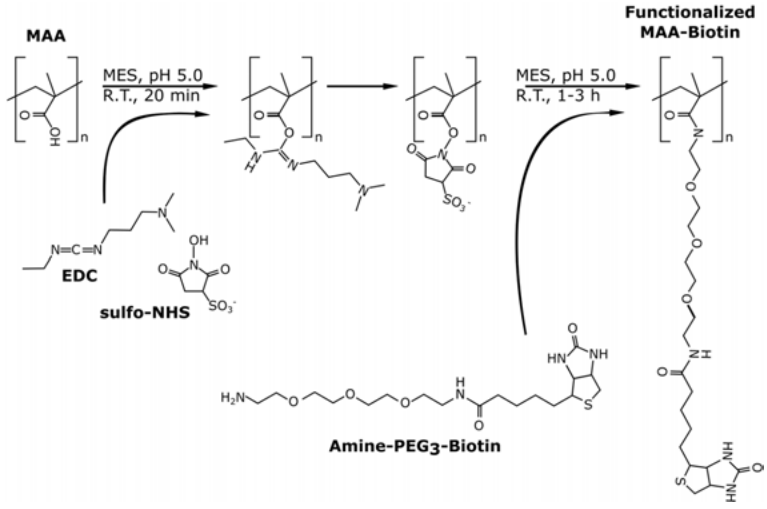


Figure 6. Biotin-functionalization of MAA-microgels via EDC/sulfo-NHS assisted amide-coupling to the carboxylic acid moieties of the MAA-microgels.

Peptide–microgel interactions

Many different techniques have been used to investigate peptide interactions to microgels, and to gain knowledge about peptide loading and release at the submicron length scale. This includes both quantification and visualization techniques, as well as studying peptide and microgel structural changes upon binding. The following sections give a brief description of the main techniques used in this thesis for studying peptide–microgel interactions.

Peptide and microgel structural changes

Upon binding, both peptide and microgel undergo structural changes.[61, 113] Charged microgels de-swell upon binding of oppositely charged molecules, and peptides may restructure in the changed environment upon contact with the charged polymer network. These changes can be studied to gain further knowledge of the peptide–microgel interaction.

Peptide structure

Peptide secondary structure is a good way to monitor peptide–microgel interaction. All peptides investigated in this thesis have a random coil confirmation in solution, unless the concentration is very high and the peptides aggregate. As they are mixed with microgels, the presence of the anionic polymer network may induce conformational changes of the cationic peptides, proving close contact between the two.

Circular dichroism (CD) spectroscopy is an optical technique measuring the ability of optically active chiral molecules to differentially adsorb left- or right-handed circularly polarized light.[114] CD has a wide range of applications, but it is especially useful for determining peptide secondary structures, as α-helices and β-sheets give rise to different characteristic CD-spectra. For short peptides, quantifications of the percentage of α-helices can be calculated using known reference spectra of 100 % random coil and α-helix conformation, respectively at 225 nm.[115, 116]

Nuclear magnetic resonance (NMR) spectroscopy was used for determining the three-dimensional peptide structure when bound to MAA-microgels.[117] The peptide structure was calculated using the inter-proton distances, determined from ^{1}H-^{1}H *transferred* Nuclear Overhauser effect spectroscopy experiments. The refinement of the structure was performed using the CYANA 2.1 software.[118] The ensemble structure was generated from the twenty lowest energy structures and analyzed using the PyMol, MOLMOL, and Chimera softwares. Further, residue-based epitope mapping was performed by saturation transfer difference (STD)-NMR[119] using -4 ppm (on-resonance) and 40 ppm

(off-resonance) frequencies, respectively. A total of 2 s (40 Gaussian-shaped pulses of 49 ms with 1 ms delay in-between) saturation time was used.

Microgel size and zeta-potential

The size of MAA-microgels is pH-dependent, due to the deprotonation of the carboxylic acid moieties and electrostatic repulsion of the polymer chains.[106] In a similar manner, when microgels interact with oppositely charged peptides, the repulsive charges between the polymer chains decrease and microgels de-swell.[62, 113] The small dimensions of the MAA-microgels, rule out direct visualization of single microgels using light- and fluorescent microscopy techniques that previously have been used to investigate larger microgels,[62] however other light based scattering techniques can be used to look at MAA-microgel de-swelling.

Dynamic light scattering (DLS) measures particle-size and zeta-potential. By analyzing fluctuations in intensity of scattered light due to particle Brownian motion, the average hydrodynamic radius of the particles in solution can be calculated using the translational diffusion coefficient and the Stokes-Einstein equation.[120] DLS is a fast and easy tabletop instrument. However, care should be taken as highly concentrated, colored or fluorescent samples, agglomeration, and shape of the nanoparticles affect the results as the equation assumes a hypothetical hard sphere for the hydrodynamic radius calculations.[121] Peak resolution of polydisperse samples are poor. DLS is therefore best suited for monodisperse samples.[121]

Apart from microgel size, DLS can also measure particle *zeta (ζ) potential*. Zeta-potential, i.e. the potential difference of the characteristic slipping plane of electrophoretically mobile particles and the media, is calculated by measuring particle electrophoretic mobility (particle velocity under an applied electric field) and using the Helmholtz-Smoluchowski equation.[121, 122] Here, it should be noted that zeta-potential is less defined for soft diffuse particles, such as microgels,[123] and that the parameter should be considered approximate and hence denoted as "effective zeta-potential". Nevertheless, concentration-dependent measurements of anionic microgel effective zeta-potential in the presence of cationic peptides give information on peptide binding and localization. If peptides are internalized in the microgel core, effective zeta-potential values are unchanged. However, if they are located in the periphery of the polymer network, the cationic peptides make the negative microgel effective zeta-potential less negative, and in some cases cause particle charge reversal.

Nanoparticle tracking analysis (NTA), also measures nanoparticle size (30-1000 nm) from the translational diffusion coefficient and Stokes-Einstein equation. However, NTA tracks Brownian motion by recording the mobility of individual particles from scattered light through a CCD camera to yield size

and concentration.[124] This individual particle tracking offers a better resolution of smaller and more polydisperse samples than DLS, but is not as fast and requires more sample preparation.[125]

Cryogenic transmission electron microscopy (cryoTEM) works on the same basic principles as a light microscope but uses a high-energy beam of electrons instead of visible light. By using an electron beam with a very short wavelength the optical resolution is increased to nm range, suitable for small particles and drug delivery system structures.[126] To visualize individual nanosized microgels, samples were deposited on a grid and vitrified rapidly in liquid ethane. This keeps the polymer structures in a frozen hydrated state close to their native state in solution. Samples are kept below -165 °C in a protected environment as the electron beam is transmitted through the frozen thin-film substrate. The beam interactions with sample atoms are then used to project the image.[127]

Microgels at the solid-liquid interface

To study surface-bound microgels, other techniques have been employed to investigate structural changes due to media (pH, ionic strength) and/or peptide loading and release. The soft, small polymeric surface-bound MAA-microgels are not always straightforward to visualize or analyze, but by using a number of fundamentally different techniques, with their own advantages and drawbacks, the conclusions can be strengthened.

Scanning electron microscopy (SEM) has been used to visualize surface-bound microgels and quantify the surface area coverage. SEM, compared to cryoTEM, visualizes the sample by detecting secondary electrons emitted as the electron beam hits a solid sample to make up the image.[128] A major drawback of conventional SEM for studies of microgels is that samples have to be dried and placed in high vacuum prior to visualization. This collapses the structure prior to the experiment. To detect microgel size in the hydrated state, Environmental SEM has been shown to be able to visualize microgels in a high relative humidity.[129]

Atomic force microscopy (AFM), can be used for both imaging of topographies and force measurements of biological and soft polymer samples in liquid.[130] Using PeakForce Tapping mode, both topography and quantitative mechanical property mapping (QNM) can be extracted at each xy pixel as the cantilever tip scans the samples (*Figure 7*). An important note for these soft particles is that the height profile depends on the instrumental settings and defined force as the ‘contact’ point to the polymer network.[131]

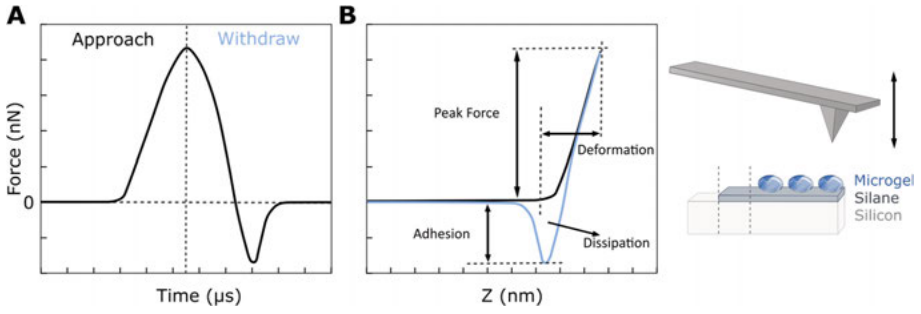


Figure 7. (A) One cycle of a PeakForce Tapping curve, showing the measured forces experienced by the AFM cantilever as it approaches and withdraws from the sample. (B) Resulting quantitative measurements. These graphs are reproduced in each pixel of the image as the sample is analyzed, giving both topography height as well as nanomechanical properties. (Redrawn from ref. 130)

Quartz crystal microbalance with dissipation monitoring (QCM-D), is an acoustic technique able to study nanoscale surface thin films by measuring changes in resonance frequency (*f*) and dissipation (D) of a quartz disc sensor.[132, 133] This yields information, not only of mass changes, but also about structural changes as the dissipation of the sensor depends on both the mass and rigidity of the sample. QCM-D measures the hydrated mass at the interface. This enables investigations of both peptide binding and release to surface-bound microgels, as well as changes in bound water and counterions coupled to the polymer chains as the microgels swell/de-swell due to changes of the ambient surroundings (*Figure 8*).

Ellipsometry is a sensitive optical technique which can be used to analyze *in situ* changes at the solid–liquid interface by measuring changes in the polarization state of a polarized light beam as it reflects from the sample.[134] The changes in polarization are expressed as an amplitude ratio (ψ) and phase difference (Δ). The numerically determined mean refractive index and average thickness can then be used to model the adsorbed amount per unit area (Γ, mg/m^2) according to de Feijter.[135] Ellipsometry measures the dry mass of the adsorbed sample, and can therefore not detect microgel swelling and changes in hydration state as QCM-D does.

An important note is that modeling of both ellipsometry and QCM-D data assumes a homogenous thin layer. From AFM images of the surface-bound MAA-microgels (see *Figure 10* for example) it is obvious that these type of layers do not fulfill this criteria. Still, results show that the modeling of these data are good enough for comparing the different MAA-microgels and conditions, although absolute values should be seen as approximate.

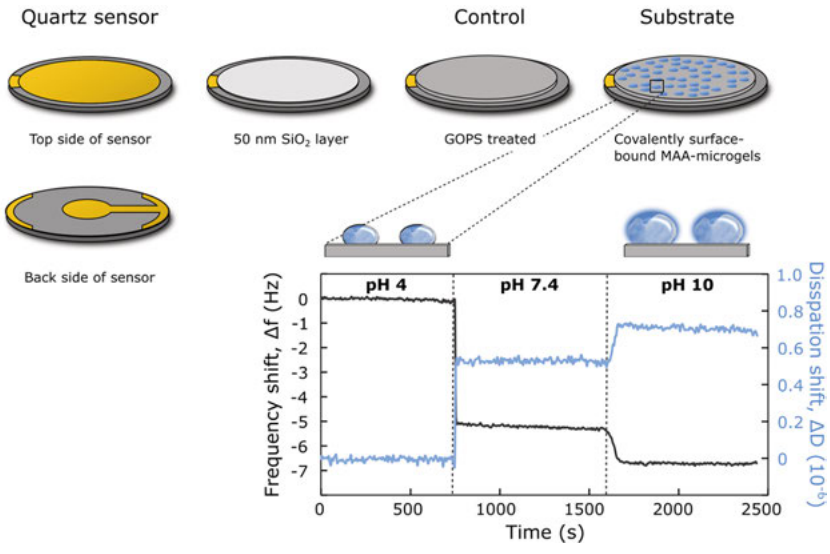


Figure 8. Schematic of the piezoelectric quartz sensor with gold electrodes, modified with SiO_2 and GOPS, enabling covalent coupling of microgels. The coupled microgels are used as the starting value to investigate microgels swelling due to changes in the media, and/or peptide incorporation. As an example, if pH increases microgels swell due to electrostatic repulsion and osmotic pressure increase. As a result the amount of coupled water and counterions increases, to the constant polymer mass at the sensor interface, causing the resonance frequency of the sensor decrease while the dissipation increases.

Confocal laser scanning microscopy (CLSM) has been used in the thesis to quantify peptide-loading to surface-bound microgels. By fluorescently marking the peptides with a fluorophore, the fluorescence intensity of a confocal plane at the interface can be quantified.[136] Low labeling densities (0.01-0.1 fluorophore/peptide) were used to assure that the coupled fluorophores do not affect the peptide interactions, although previous studies have shown that labeled and unlabeled pLys have similar binding to poly(acrylic acid)-based-microgels.[113] Here it is important to note that the results are based on an average fluorescence intensity over a quite large area of 150 μm^2, and the resolution is far from being able to visualize peptide loading on a single microgel level. CLSM is mainly a qualitative technique, since fluorescence intensity depends on multiple settings of the microscope. However, by carefully choosing appropriate settings and keeping them fixed, quantification of the fluorescence intensity differences between samples is possible.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) is an adsorption spectroscopy technique measuring characteristic vibrations of chemical functional groups *in situ* at the solid-liquid interface.[137, 138]

Infrared light is emitted at an angle to a crystal, taking advantage of the property of total internal reflection, resulting in an evanescent wave at the crystal interface. After multiple internal reflections, the resulting light is analyzed, and determination of strong adsorption bands can be directly related to specific compounds at the interface.

Effect studies

To evaluate the performance of microgels as a drug delivery system for antimicrobial peptides, microgel performance as well as the antimicrobial and anti-inflammatory properties of peptide-loaded microgels have been investigated.

Microgel performance was assessed by looking at the toxicity of microgels either by hemolysis of erythrocytes[39] or disruption of human cell-like liposomes, together with assessing microgel ability to protect peptides against proteolytic degradation by bacterial elastase[139].

Antimicrobial effects of peptide-loaded microgels

Bacterial viability

In this thesis, several assays have been used to investigate peptide-loaded microgels antimicrobial abilities. For microgels in dispersion, minimal inhibitory concentration (MIC) of free peptides and peptide-loaded microgels was assessed in 96 well plates.[140] MIC was defined as the lowest concentration that completely inhibits the growth of bacteria (herein MRSA, *Escherichia coli* or *P. aeruginosa*) when mixed with the sample 1:1. These results were correlated to membrane disruptive activity of peptide and peptide-loaded microgels, measured either by using model lipid bilayers and ellipsometry[141] or by studying leakage of model membrane liposomes[35].

For surface-bound microgel mono- and multilayers, bacterial viability of *E. coli* was assessed in either bulk or at the interface. Bulk viability was assessed by the PrestoBlue Cell Viability Reagent assay (Thermo Fisher Scientific, Waltham, MA, USA). The assay is based on metabolic activity of live cells and their ability to reduce the blue reazurin dye to the pink highly fluorescent resorufin.[142] The viability of the bacteria can then easily be assessed by measuring the fluorescence intensity of resorufin with a plate reader. At the interface, distance-dependent antimicrobial properties of the peptide-loaded surface-bound microgels were analyzed by quantification of fluorescence intensity of confocal planes perpendicular to the interface. For this, bacteria were

stained using the LIVE/DEAD Bacterial Viability Kit (*Bac*Light; Sigma Aldrich, Schnelldorf, Germany). The LIVE/DEAD stain contains two components; the green-fluorescent nucleic stain SYTO9 that stains all bacterial membranes and the red-fluorescent propidium iodide (PI) nucleic stain that only stains bacteria with impaired membranes, see schematic in *Figure 9*.[143] This allows for quantification of bacterial viability by comparing the green-to-red fluorescence intensity ratio.

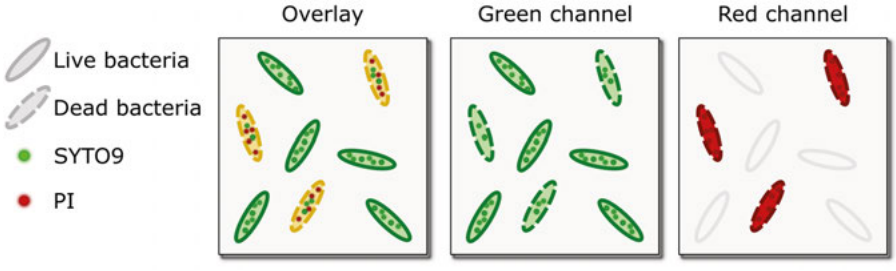


Figure 9. The LIVE/DEAD stain contains two fluorescent probes. The green SYTO9 stains all bacterial membranes, whereas the red PI only stains bacteria with impaired membranes. By comparing the fluorescent intensity of the green channel and red channel, quantification of bacterial viability can be assessed.

Bacterial Adhesion

A lot of research efforts have been made on polymer-based coatings for anti-adhesive properties to lower the risk of biofilm formation.[144] The anti-adhesive properties of surface-bound microgels were assessed by quantifying bacterial adhesion after 4 hours using CLSM and LIVE/DEAD stained bacteria. *E. coli* bacteria were incubated on the samples to allow for bacterial adhesion to the interface before any loosely attached bacteria were gently rinsed off and the amounts of adhered bacteria were visualized and quantified.

Anti-inflammatory effects of peptide-loaded microgels

Some AMPs and host defense peptides (HDPs) are not only antimicrobial, but also possess other effects such as immunomodulation.[145] Investigated KYE28 and KYE28PEG peptides have previously been shown to have anti-inflammatory effects by lowering NF-κB activation of RAW-Blue cells and THP1-XBlue-CD14 cells.[146, 147] The anti-inflammatory effects of peptide-loaded microgels were assessed in a similar manner as in the previously published papers and the decreasing of NF-κB activation and subsequent production of secreted embryonic alkaline phosphatase (SEAP) of bacterial lipopolysaccharide (LPS)-triggered THP1-XBlue-CD14 cells were analyzed.

Results

Characterization of surface-bound microgels (paper I)

While most published work on microgels involves microgels in dispersion, surface-bound microgels have gained considerable interest. In **paper I** the mechanical properties of covalently surface-bound MAA-microgels as a function of charge density, pH and ionic strength were investigated.

pH and ionic strength dependence

Covalently surface-bound MAA-microgels keep their pH-dependent swelling although their height-to-width ratio is decreased, as evidenced by AFM measurements (*Figure 10A*). These results are in line with previously published data of similar systems.[81, 148] The pH-dependent swellings were also captured by theoretical finite element method (FEM) modeling, showing that due to the covalent attachment it is mainly the outer rim of the polymer network that is affected by the increased internal electrostatic repulsion as pH is increased (*Figure 10B*). In addition, QCM-D measurements of surface-bound microgel pH-dependence show that the increase in mass is due to coupled water and/or counterions, quantitatively similar to the volume changes from AFM topographies. QCM-D was also used to examine microgel response to changes in ionic strength. Results show that microgel responses are fully reversible, and, somewhat unexpected, that the resonance frequency dropped with increasing ionic strength. This was attributed to an increased accumulation of counterions inside the microgels, dominating over any osmotic de-swelling due to ionic screening, in contrast to what is usually found for larger microgels in dispersion.[149]

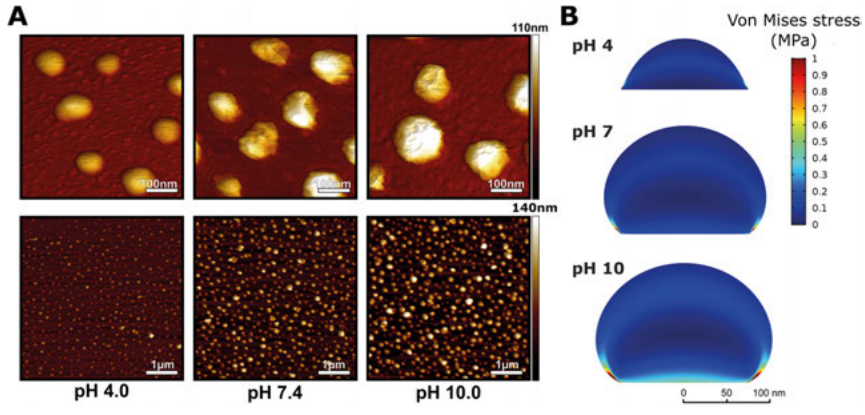


Figure 10. (A) AFM images and (B) FEM modeling of pH-dependent swelling of covalently bound MAA33 microgels.

Microgel deformation

PeakForce-QNM enables *in situ* measurements of single surface-bound microgel particles in solution. By tracking the forces experienced by the cantilever tip during approach and retraction from the sample, force-distance curves are extracted and thereby various mechanical properties within each xy pixel of the AFM image can be calculated (*Figure 7*). One example of this is shown in *Figure 11*, where the deformability of single microgel particles as a function of microgel charge density is plotted, together with representative PeakForce-QNM images. This non-linear response in deformability could be attributed to the strength of the electrostatic repulsion between the anionic charges of the polymer chains. With a charge density of between 20-35 % the repulsion forces are large enough to stretch the polymer network. However, under a pressure of 800 pN applied by the cantilever tip the structure collapses and deforms up to 60 % of its original height. Only with higher charge densities repulsion makes the polymer structure rigid enough to withstand the applied pressure, and therefore low deformability is found. Moreover, two different modes of deformation, one elastic fully reversible and one viscoelastic, were found by varying the applied pressure. The latter, shown as hysteresis between the trace and retrace in the force-distant curve, suggest the loss of work, presumably by pushing out water from the microgel particle.

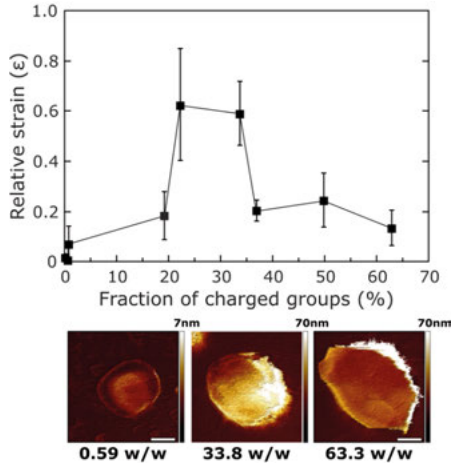


Figure 11. Microgel deformability as a function of microgel charge density, plotted as the relative strain vs. number of charged groups. Below, representative PeakForce-QNM deformation images of microgels of different charge density. (Scale bars 50 nm)

Polymer chain dynamics

Finally, AFM was used to further examine polymer chain dynamics. The number of contact points between the cantilever tip and polymer chains as a function of time was quantified. The same experimental set up as for the deformation measurements described above was used, but the contact time under constant applied pressure was increased before the cantilever tip was retracted from the sample. Results show an increased number of contact points (defined as forces above a 0.05 nN cutoff value) with increased contact time. This demonstrates the high flexibility of the polymer chains and that equilibration within single microgels takes place in the time scale of seconds.

FEM modeling was again used to capture propagation effects in single microgel particles, this time to study cantilever tip indentation under similar conditions as the AFM microgel deformation and polymer chain dynamic experiments. Results show that the microgel polymer network is only affected locally at the indentation site, and that only < 5 % of the total microgel volumes are probed during this type of experiment.

Factors affecting peptide–microgel interactions

A significant part of this thesis has been focusing on factors affecting peptide–microgel interactions, including peptide loading and release studies. Systematic investigations of basic factors affecting the interactions was performed to clarify key parameters of importance for high peptide loading, together with controlled release profiles to reach prolonged therapeutic levels.

Loading and release of model peptides (paper II)

As a first step, to evaluate any differences compared to previous literature of microgels in dispersion, the loading and release of the model peptide poly-L-lysine (pLys) to surface-bound MAA-microgels was investigated. For this, three different peptides with varying length and charge densities (see Table 1) and three different microgels of varying charge densities (see Table 2) were studied as a function of media pH and ionic strength.

Peptide properties

Results show that the pLys molecular weight controls binding to surface-bound microgels. Peptides bind in a concentration-dependent manner up to a concentration of about 15 μM. The shorter the peptide the higher molar adsorption. In terms of electrostatics, previously demonstrated to be a key factor for incorporation of peptides in acrylic acid-based microgels in dispersion,[62, 113, 150] binding of the shortest cationic peptide comes close to a charge ratio of 1:1 to the number of negative charges in the microgels. The larger peptides are excluded due to their size and most likely located in the periphery of the microgel network. Evidenced by the fact that microgels in the presence of the higher molecular weight peptides reverse their effective zeta-potentials from – 30 mV to + 40 mV, and visual particle flocculation observed in cryoTEM images. In terms of peptide release, the shorter the peptide the higher the percental release after 2h.

Microgel properties

Microgel charge density also controls peptide binding. The higher the charge density, i.e., the more carboxylic acid moieties, the higher the molar adsorption of pLys to surface-bound MAA-microgels. These findings are in line with previous results for larger acrylic acid-based microgels in dispersion.[61, 151] Microgel charge density also controls peptide release. A higher charge contrast between peptide and microgel causes a stronger binding and thereby a lower release of the peptides over time.

Incorporation of peptides affects both microgel sizes and mechanical properties. PeakForce-QNM topographies of surface-bound MAA33 microgels as a function of peptide molecular weight (at 7.5 μM peptide concentration)

showed that microgel volume decreases and the microgel structure flattened, i.e., the height/diameter decreased (*Figure 12A,B*). In agreement with previously discussed charge ratios, the shortest peptide is the most effective at causing volume decrease with close to full charge neutralization, whereas the longer peptides are not as efficient. Interestingly, this trend differs from microgels in dispersion where microgel volumes decrease with increasing peptide length, indicating that the largest 150 kDa peptide is not completely excluded from the microgel network. In addition, the deformability of the polymer network also decreases as peptides are incorporated, meaning that under the same (800 pN) pressure applied the bound peptides rigidify the polymer network (*Figure 12C*).

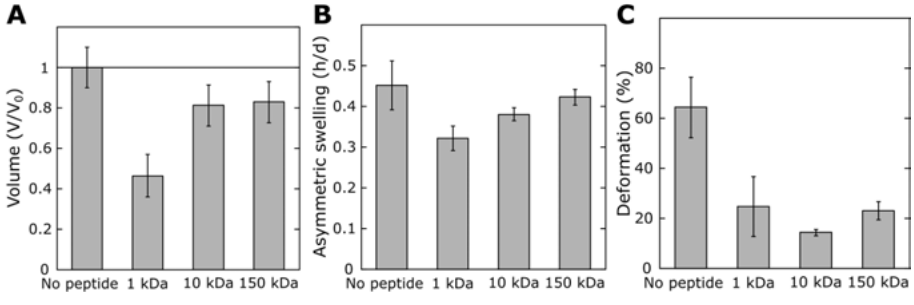


Figure 12. Surface-bound MAA33 microgel topography and deformation changes after peptide incorporation (7.5 μM). Peptide incorporation (A) de-swells microgel volume and (B) flattens the structure (height/diameter ratio decreases), as well as (C) decreases the deformability under an 800 pN applied pressure.

Ambient condition - pH and ionic strength

All peptides were loaded in Tris buffer (10 mM, pH 7.4), but peptide release was investigated as a function of both pH and ionic strength. Results show that the higher the charge contrast between peptide and microgel the lower the peptide release. This results in a higher peptide release when the charge contrast is lowered, i.e., both in lower and higher pH. pK_a of the investigated MAA-microgels vary between 6.4-7.0 (see Table 2) and the pK_a of pLys has been reported to be around 9 (10.5 for isolated lysine monomers[62]). At low pH MAA-microgels are close to charge neutral (see (*Figure 3*D) which results in a high release. At high pH, above pK_a of pLys, the peptide starts to neutralize due to lysine protonation, and therefore the peptide release increases. It was also found that an increased salt concentration (ionic strength) screens the electrostatic binding of peptides, which causes the peptides to release.

Loading and release of AMPs (papers III-VI)

The following papers all investigated the loading and release of AMPs, again looking at both peptide and microgel properties, in dispersion or as mono- and multilayers.

Comparison of two AMPs (paper III)

In **paper III** two AMPs, LL-37 and DPK060 (currently in clinical trials[152]), were investigated. The loading and release of these two peptides was studied from MAA-microgels of different charge densities. When comparing two AMPs instead of carefully chosen model peptides, as in **paper II**, the smaller difference in length, charge density and composition (see Table 1) make the results less conclusive.

Nevertheless, surface-bound microgels can bind considerate amounts of both of the studied AMPs. A slightly higher, but not significant, binding to the MAA60 microgels of higher charge density than the MAA26.5 microgels was found. As peptides bind to the microgels, LL-37 forms considerable α-helix conformation, as has previously been shown after binding to both anionic phospholipid membranes and anionic polysaccharides.[153] However, DPK060 only goes through minor conformational changes and keeps a random coil structure.[154] Just as for the pLys model peptides of **paper II**, both LL-37 and DPK060, induce drastic de-swelling of the MAA-microgels. However, the lower charge density of the AMPs (+6 and +7 for LL-37 and DPK060, respectively) are not able to reverse the negative effective zeta-potential of the microgels. At very high (100 μM) peptide concentrations, only a slight neutralization was observed. NTA measurements show that peptide loading causes microgels to form oligomers. These results indicate that microgel effective zeta-potential is dictated by dilute anionic polymer chains since the repulsive barrier between the microgels is partially overcome already at very low peptide concentrations. Ellipsometry measurements showed a minor released amount of both peptides at low ionic strength. However, in high salt concentration (additional 150 mM NaCl), up to 40 % of LL-37 and 70 % of DPK060 are released after 2 h.

Membrane interactions

As a drug delivery system, empty MAA-microgels display no binding to bacteria-mimicking DOPE/DOPG-supported bilayers in the concentration range of 1-1000 ppm, nor do they cause any leakage of liposome model membranes up to 100 ppm concentrations. At low ionic strength, the potent membrane disruptive capabilities of the AMPs are lost if bound to microgels. Most likely, this is an effect of peptide internal placement and the still negative effective zeta-potential of the microgels. At high, physiological ionic strength, however, some of the peptide membrane disruption is restored as the peptide is

released from the microgels. An effect, most perceptible for the lower charged MAA26.5 microgels. Therefore, it is evidenced that it is the released peptides, not the peptide-loaded microgels, that bind to the bacteria-mimicking membranes.

Hemolysis & proteolytic stabilization

Reflecting the low membrane affinity of the MAA-microgels, microgels also display low hemolysis of erythrocytes up to 100 ppm concentrations (*Figure 13A*). For DPK060 the hemolysis of peptide-loaded microgels is similar to that of the free peptide, whereas incorporation of LL-37 into microgels significantly lowers the hemolysis compared to the free peptide (*Figure 13B*). This effect is more pronounced at higher microgel charge densities, i.e. for MAA60-microgels.

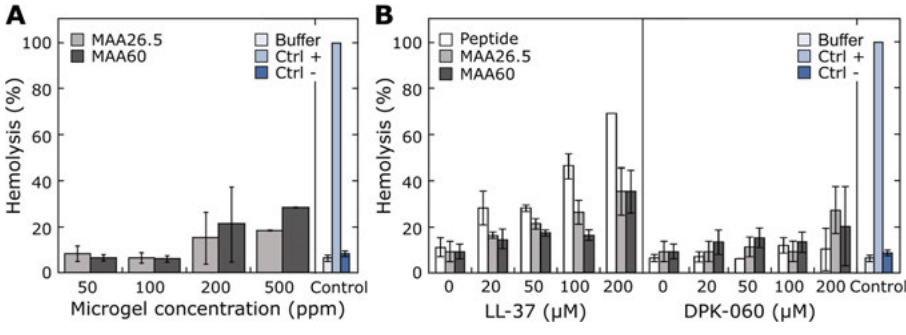


Figure 13. (A) Microgel hemolysis at increasing concentrations and (B) comparison of free AMP hemolysis (white) compared to AMP-loaded MAA-microgels (light and dark grey) at a 100 ppm concentration of microgels.

The ability of microgels to protect AMPs from proteolytic degradation by infection-related enzymes was also investigated. Results show that the higher charge density microgels (MAA60) can reduce proteolytic degradation of LL-37 by *P. aeruginosa* elastase (*Figure 14*). This could be explained by the internal localization of LL-37, tight binding to MAA60 microgels, and therefore lower degree of released peptide during the duration of the experiment than from corresponding MAA26.5. Another contributing factor could be that the larger enzyme is not able to penetrate the small pores of the microgel network.

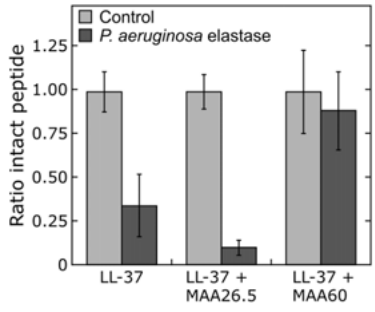


Figure 14. Proteolytic degradation of free LL-37 compared to peptide-loaded MAA-microgels in the presence of *P. aeruginosa* elastase.

Effect of peptide PEGylation (papers IV-V)

In **paper IV** and **paper V** the binding of host defense peptide KYE28 and poly(ethylene glycol)-modified (PEGylated) variants were compared. KYE28, a 28-mer peptide from the D-helix of heparin cofactor II, has previously been shown to possess antimicrobial properties against gram-negative *E. coli* and *P. aeruginosa,* gram-positive *S. aureus* and *Bacillus subtilis,* as well as the fungus *Candida albicans*.[146] KYE28 also possesses anti-inflammatory properties, shown *in vitro* by binding to bacterial LPS and decreasing the NF-κB activation of human monocytic reporter cells, and *in vivo* by significantly enhancing the survival rate of a mouse model with LPS-induced shock.[146]

PEGylating drugs is a common approach to reduce serum protein binding and thereby increase circulation half-time in the blood stream. This has also been investigated for AMPs.[155, 156] PEGylation of the KYE28 peptide has been shown to decrease its antimicrobial effect, while anti-inflammatory effects are still maintained.[147] However, conditions could be found where the peptides are still able to kill bacteria in blood.[147] PEGylation did however not prove to be enough to protect the peptide from proteolytic degradation; therefore the combination of PEGylated peptides and the MAA-microgels, which have previously been shown to offer protection of AMPs, were investigated. Two papers in the presented thesis focused on comparing the loading and release of KYE28 and PEGylated versions of KYE28 (KYE28PEG). Peptides were PEGylated with 48 PEG units located either fully in the N- or C- terminal or distributed half on each side, see Table 1.

In **paper IV**, results show that the loading of KYE28 and KYE28PEG, as for the previous discussed papers, are also dependent on microgel charge density. A higher binding to MAA60 microgels than the corresponding MAA33 mi-

crogels was found, while PEGylation of KYE28 decreases the molar adsorption to surface-bound MAA-microgels. Zeta-potential and CD measurements confirm that the PEGylated peptides are located more profoundly in the outer periphery of the microgel network compared to non-modified KYE28. This is shown by the increased neutralization of microgel effective zeta-potential, and the larger α-helical induction. The latter is probably due to more flexible polymer chains in the outer region of the microgel, and the fact that PEGylation decreases peptide charge density, thereby decreasing the binding strength. In line with the previous results, the release of PEGylated KYE28 located in the outer periphery is promoted compared to KYE28, and considerably so at high ionic strength (150 mM NaCl).

In **paper V**, KYE28 and four PEGylated KYE28-variants were investigated together with MAA60 microgels (Table 1). Results show that PEGylation, but interestingly, not the location of PEGylation, significantly affects the loading or release profile of the peptides as evidenced by ellipsometry (*Figure 15*). Alpha helix induction is slightly increased if PEG is located at the N-terminus of the peptide, i.e., PEG48-KYE28 and PEG24-KYE28-PEG24.

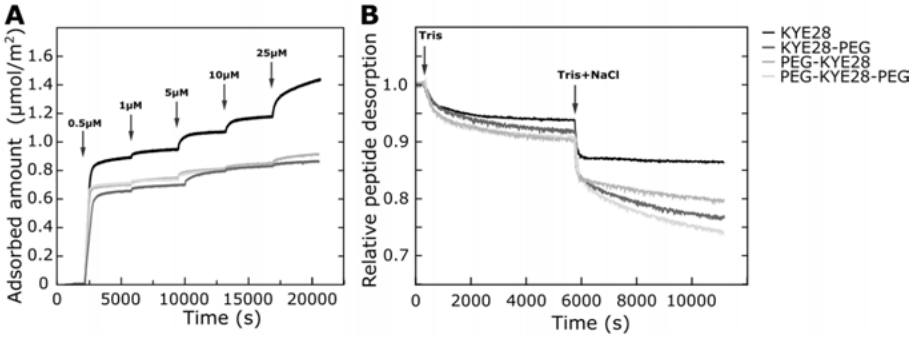


Figure 15. (A) Representative curves of sequential loading and saturation binding of KYE28 and PEGylated KYE28 peptides to surface-bound MAA60 microgels measured with ellipsometry, followed by (B) relative desorption in Tris buffer and Tris buffer with additional 150 mM NaCl, 1.5 h, respectively.

Furthermore, NMR measurements in **paper V** revealed that the amphiphilic nature of KYE28-PEG48 depends on both hydrophobic and electrostatic interactions as the peptide binds to MAA60 microgels. The peptide is primarily stabilized by an amphipathic helical structure located in the C-terminus (H16-L27) when bound to MAA60 microgels. The inside of the helical structure is stabilized by hydrophobic interactions of Phenylalanine F11, F19 and F23, while cationic and polar uncharged residues remain flexible and exposed to-

wards the solvent even when bound to the microgel (*Figure 16*). CD measurement also confirmed a high α-helical structure when bound to MAA60 microgels, in contrast to KYE28-PEG48 in solution, which have a random coil conformation.

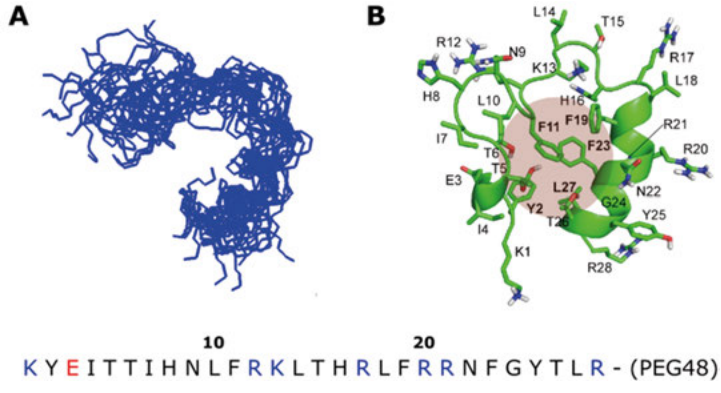


Figure 16. (A) Ensemble structure and (B) cartoon representation of KYE28-PEG48 conformation when bound to MAA60 microgels showing the hydrophobic F11, F19, F23 triad.

AMP loading in microgel multilayers (paper VI)

In the final paper of the presented thesis (**paper VI**), the buildup of four avidin–biotin cross-linked microgel multilayers was explored. Peptide loading was investigated by incorporating KYE28, either during buildup after each microgel layer, or in one shot after microgel multilayer formation. Results show that biotin-functionalization of MAA-microgels enables microgel multilayer buildup by cross-linking with avidin, which is known to strongly bind up to four biotin molecules.[110, 112] However, due to the cationic nature of avidin, the protein also binds electrostatically to the anionic microgels, similar to the more often studied polyelectrolyte layer-by-layer buildup of microgel multilayers.[88, 89, 157] Results show that if KYE28 is added after multilayer formation, the peptide only partially diffuses through the four layers. This effect is masked by the probable release of the electrostatically bound avidin, making quantification using ellipsometry and QCM-D difficult since neither of the two techniques distinguish between the peptide or avidin. However, by sequentially adding the peptide between each layer, the unwanted electrostatic binding of avidin was lowered and the peptide load increased for each microgel layer of the coating (*Figure 17*).

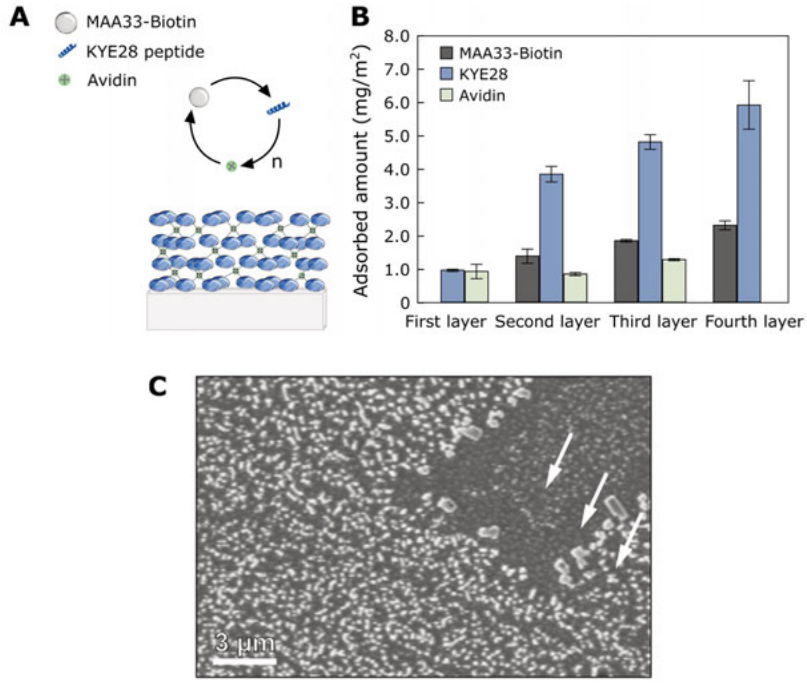


Figure 17. Incorporation of KYE28 during build-up of four MAA33-Biotin microgel multilayers. (A) Schematic and (B) adsorbed amount of each addition measured with ellipsometry. First microgel layer covalently attached prior the experiment. (C) SEM image of four MAA33-Biotin/KYE28/Avidin microgel multilayers. In this defect, three of the four microgel layers are visible, each layer marked with a white arrow.

Effect studies

As been already discussed in **paper III**, microgels offer a non-toxic drug delivery system that can be synthesized in large quantities. Microgels do not induce hemolysis of erythrocytes up to 100 ppm concentrations. Under the right conditions they are able to reduce hemolysing properties of LL-37 and protect peptides from proteolytic degradation. Next, antimicrobial effects of peptide-loaded microgels in solution, peptide-loaded microgel monolayers, and peptide-loaded microgel multilayers will be discussed.

Antimicrobial effects of peptide-loaded microgels

Microgels in dispersion (paper III)

In line with MAA-microgels low affinity to supported lipid bilayers and liposomes, microgels possess no antimicrobial properties towards either, gram-negative *E. coli* and *P. aeruginosa* or, gram-positive MRSA bacteria.

When comparing MIC values of free AMPs to those of AMP-loaded MAA-microgels (Table 3), results showed that by incorporating LL-37 in MAA-microgels MIC values are increased due to peptide internal placement and slow release. DPK060, however, keep comparable MIC values similar to those of the free peptide against *E. coli* and *MRSA*. Promoted at lower charge densities (MAA26.5), reflecting the faster release of DPK060. Interestingly, against the two *P. aeruginosa* strains, DPK060-loaded MAA26.5 microgels decreased MIC values.

Table 3. *Minimal inhibitory concentrations (MIC; μM)*

Peptide	Microgel (100 ppm)	MRSA	*E. coli*	*P. aeruginosa* (clinical)	*P. aeruginosa* (ATCC)
LL-37		1.8-3.6	3.6	1.8-3.6	1.8-3.6
LL-37	MAA26.5	>7.1	>7.1	>7.1	7.1
LL-37	MAA60	>7.1	>7.1	>7.1	>7.1
DPK060		1.6	3.2	6.4	3.2
DPK060	MAA26.5	0.8-1.6	1.6-3.2	0.8	0.8
DPK060	MAA60	3.2	6.4	6.4	3.2

Surface-bound microgel monolayers (Paper IV)

In **paper IV** a more mechanistic approach to study the antimicrobial effects of peptide-loaded surface-bound microgels was performed. This was done by investigating anti-adhesive and antimicrobial effects on *E. coli*, of KYE28-loaded surface-bound MAA-microgels, both close to the interface and in bulk, as a function of both ionic strength and peptide PEGylation.

Anti-adhesive effects

Surface-bound MAA-microgels exposed to a high concentration of *E. coli* bacteria (10^8 colony forming units (CFU)/mL) for 4 h exhibit anti-adhesive properties compared to controls of un-treated glass slips (*Figure 18*). Results also show that GOPS-treated samples, i.e., epoxide-containing interfaces without microgels, also reduce the bacterial adhesion. The largest difference in bacterial adhesion is found in low salt concentrations (10 mM), where the electrostatic repulsion between the negative surface and negative bacterial membranes is the greatest. The effect is screened with increasing salt concentrations (150 mM). In addition, surface-bound MAA33 microgels keep their bacteria repelling properties even after loading of KYE28 and KYE28-PEG48 peptide (100 µM). Bacteria adhesion was quantified using LIVE/DEAD staining and CLSM.

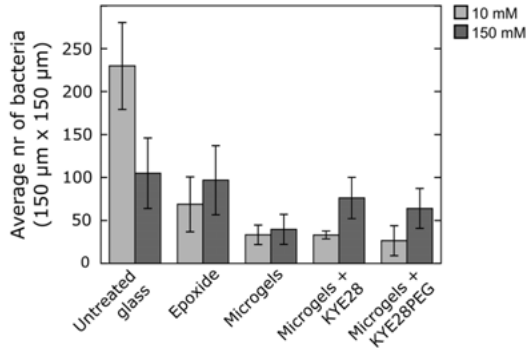


Figure 18. Bacterial adhesion of *E. coli* (10^8 CFU/mL in Tris buffer with or without additional 150 mM NaCl after 4 h incubation) to surface-bound MAA33 microgels.

Antimicrobial effects

Bacterial viability close to the interface could be quantified using the same methodology as described above, but instead of only counting the number of bacteria present at the interface, the green-to-red fluorescence intensity of the individual green (SYTO9) and red (PI) channels of the LIVE/DEAD stained bacteria were quantified. By imaging at increasing distance perpendicular from the interface, any antimicrobial effects could be quantified at a known distance from the interface (*Figure 19A*). Results show that in low ionic strengths KYE28 and KYE28PEG peptide-loaded MAA33 monolayers exhibit strong antimicrobial effects close to the interface with decreasing antimicrobial properties up to a distance of about 5 µm from the interface. However, a substantial fraction of the peptides is released with additional 150 mM NaCl and therefore only a low number of killed bacteria are found in close proximity to the interface (*Figure 19B,C*).

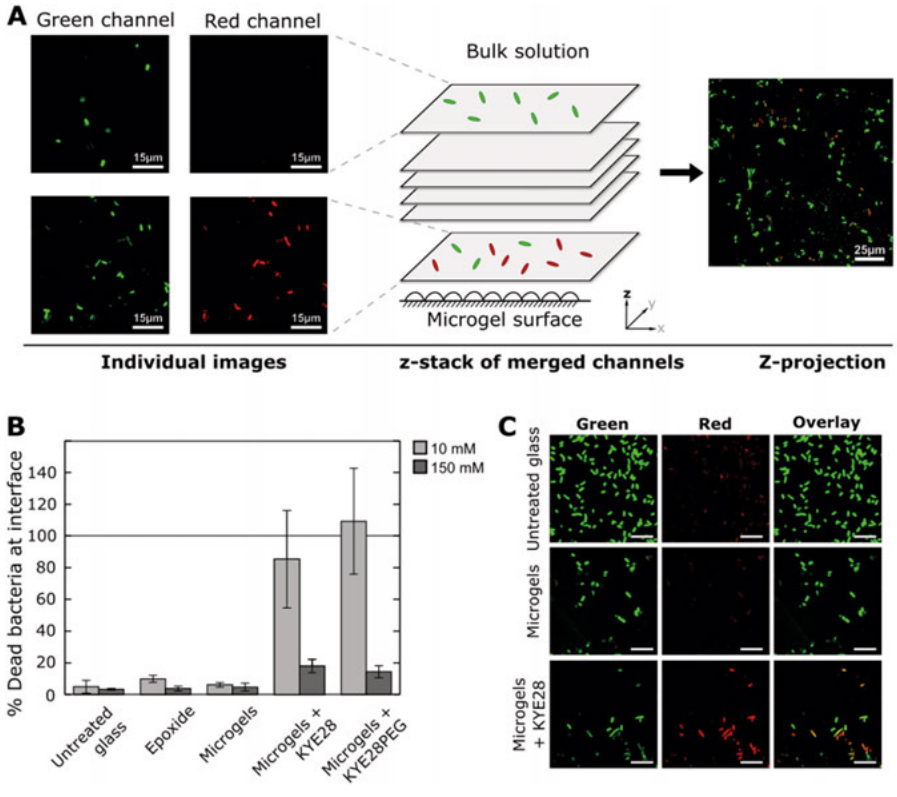


Figure 19. (A) Schematic representation of CLSM measurements of LIVE/DEAD stained *E. coli* at increasing distance in the z-direction from the interface. (B) Quantified bacterial viability from LIVE/DEAD stained *E.coli* close to the interface and (C) representative CLSM images of z-projections of merged z-stacks. (Scale bars 15 µm)

In **paper IV** bacterial viability was also investigated by measuring bacterial metabolic activity in bulk solution. Results show that peptide-loaded microgel monolayer modified glass slips are not enough, to any large extent, to kill bacteria in the bulk. By increasing the area-to-volume ratio by using modified glass beads (2 mm in diameter) instead, the antimicrobial effect in physiological ionic strength was increased and the peptide-loaded surface-bound microgels were able to kill about 40 % of the high bacterial concentration tested. These results indicate that the microgel monolayers are not binding enough peptide, or are too thin to reach high enough concentrations of the released peptide, to have any antimicrobial effects in physiological ionic strength.

Surface-bound microgel multilayers (paper VI)

To overcome the problem with too low peptide loading and release from the MAA-microgel monolayers in order to reach sufficient antimicrobial effects in high salt (**paper IV**), **paper VI** instead focused on peptide-loaded cross-linked microgel multilayers. This time only the loading of KYE28 and antimicrobial effects in physiological pH and ionic strength were investigated. Four biotin–avidin cross-linked MAA33 microgel layers were formed, and KYE28 (25 μM) was sequentially added after each microgel layer addition. As previously discussed, this resulted in a 15 times increase of the adsorbed amount of peptide per area unit, compared to a single surface-bound microgel monolayer (deposited in a 0.1 w/w microgel concentration, 25 μM peptide). Antimicrobial testing of the obtained microgel multilayer showed that the increased amount of peptide in the layer was able to both lower the bacterial viability of *E. coli* in bulk (*Figure 20A*) and significantly increase the amount of dead bacteria found at the interface at physiological pH and ionic strength (*Figure 20B,C*).

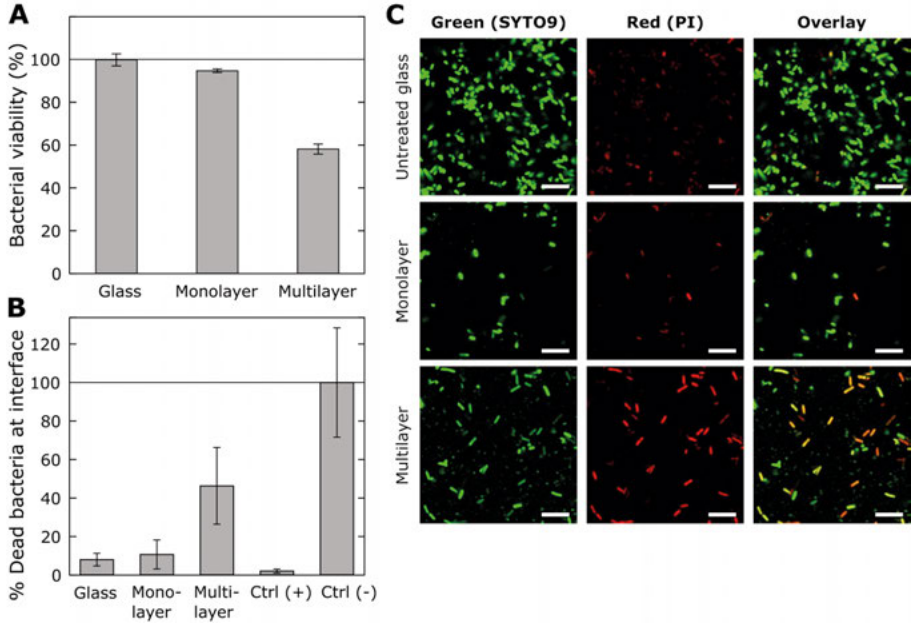


Figure 20. (A) Bulk viability of *E. coli* in the presence of a control glass slip, KYE28-loaded MAA33 monolayer or KYE28/MAA33-Biotin/avidin multilayer in Tris buffer (pH 7.4, 150 mM NaCl), quantified using PrestoBlue. (B) Bacterial viability at the interface quantified using LIVE/DEAD stained bacteria and CLSM, and (C) representative CLSM images. (Scale bars 10 μm)

Anti-inflammatory effects of peptide-loaded microgels (Paper IV)

As a final point, anti-inflammatory effects of AMP-loaded MAA-microgels will be mentioned. KYE28 has previously been shown to possess anti-inflammatory properties.[146, 147] In **paper IV**, the NF-κB activation of LPS triggered human monocytic reporter cells in the presence of free peptide and peptide-loaded microgels was investigated. Results show MAA-microgels do not cause any inflammatory response on their own, and that peptide-loaded MAA33 microgels keep the anti-inflammatory properties to that of the free KYE28 and KYE28PEG peptides (*Figure 21*).

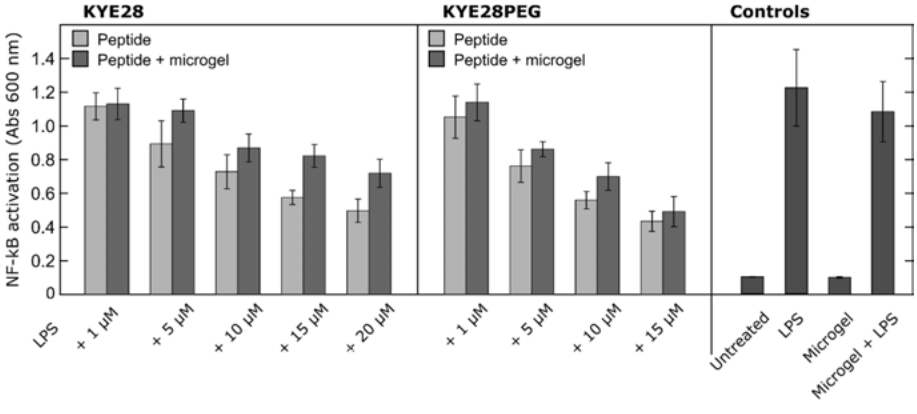


Figure 21. Free KYE28 and KYE28PEG or peptide-loaded MAA33-microgels (10 ppm) inhibition of NF-kB activation of LPS-triggered THP1-XBlue-CD14 cells.

Conclusions

In the presented thesis, a library of methacrylic acid-based MAA-microgels with varying charge densities have been synthesized and characterized. The MAA-microgels are pH-dependent and do not trigger inflammatory responses of monocytes or cause hemolysis of erythrocytes up to high (100 ppm) microgel concentrations. Results show that they are able to incorporate substantial amounts of antimicrobial peptides. Incorporation of peptides is mainly driven by electrostatic interactions between the anionic microgels and cationic peptides, although NMR studies did provide proof that hydrophobic interactions of the amphiphilic peptides also play a role. Peptide–microgel interactions have been shown to depend on properties of the peptide (length, molecular weight, charge density, PEGylation), microgel (charge density), and media (pH, ionic strength) properties. These results demonstrate the delicate interplay that needs to be controlled to achieve a well-functioning drug delivery system with sustained release of peptides over time.

The incorporation of AMPs into microgels offers beneficial advantages as a drug delivery system. By incorporating LL-37 into MAA-microgels, peptide hemolysis was lowered, and microgels (of high enough charge density) offer protection from proteolytic degradation by an infection-relevant enzyme. This is an important point because one of the main problems with the use of AMPs as therapeutics is their fast degradation and clearance in the body. KYE28 and KYE28PEG peptides bound to MAA-microgels were also shown to keep their anti-inflammatory properties.

Surface-bound microgels are anti-adhesive, with or without incorporated peptides. As an antimicrobial coating, the amount of AMPs loaded in the surface-bound microgel layer determines the effect. Contact killing of bacteria was observed in low ionic strength, but in high (physiological) ionic strength the concentration of released peptides is only able to reach therapeutic levels if the peptide loading is very high. Increased peptide loading could be achieved either by increasing the peptide-loaded microgel monolayer surface modified area-to-volume ratio or to incorporate AMPs in microgel multilayers. Both are interesting approaches for increasing coating efficiency, as long peptide diffusion is not compromised by the layer thickness of microgel multilayers and peptides are therefore trapped in the microgel film.

Taken together, microgels have proved to be an interesting, versatile drug delivery system, suitable for AMPs. However, results show that small changes in peptide and microgel properties significantly change peptide loading and release profiles. Therefore, more clarifying work is needed to investigate factors affecting peptide loading and release, as well as their correlation to biological effects.

Future perspective

Today's wide use of implants and biomaterials depends on the efficiency of broad spectra antibiotics. Therefore, it is alarming with the growing number of reports of multi-resistant bacterial strains. Furthermore, since bacteria is evolving rapidly and developing new resistance mechanisms, it is a constant battle to stay one step ahead of the bacteria with new, efficient antimicrobial treatments. AMPs have shown great potential with their fast broad spectra activity and may play an important role in the fight against resistance. However, microbial resistance towards AMPs has also been found.[158] Future research efforts is therefore important to find additional antimicrobial therapeutic alternatives to combat the problem with antimicrobial resistance.

Biomaterial research has come a long way, but since biology involves many complex regulatory systems, the future challenges of biomaterial interfaces need to meet multiple functions and requirements. This includes promoting tissue integrations and angiogenesis, modulating the immune system, lowering the foreign body response and fibrous capsule formations, as well as lowering bacterial adhesion and biofilm growth. A lot of research efforts have been made on these individual issues and many have shown great potential. However, recent reviews on the subject agree that to increase the success rate of implants, the next step of 'smart' biomaterial coatings will involve combinatory multifunctional coatings to address more of these issues simultaneously.[159, 160] The latest developments of new therapeutic routes, taking advantage of surface structural properties in combination with immobilized and/or releasing therapeutics are the ones most likely to revolutionize biomaterial uses.

Peptide-loaded surface-bound microgels have shown an interesting approach for combined anti-adhesive, anti-microbial and anti-inflammatory effects. However, to reach its full potential in physiological pH and ionic strength, higher peptide loads are required to reach prolonged therapeutic levels. Therefore, thicker microgel multilayers with an increased volume and number of binding sites for AMPs have higher potential of succeeding to meet the requirements of implant coatings. Questions regarding peptide mobility within these thicker microgel-constructs still need to be further addressed, verifying that peptides from the inner layers also play a role in the antimicrobial effects. Nevertheless, interesting new degradable microgels[161, 162] and labile chemical

cross-links[163] could prove the next step for this project. In this way, incorporating degradable crosslinks in between the microgel multilayers would allow the release of peptide-loaded drug delivery systems, at site when needed. Combining this idea with previously investigated infection-triggered cross-links[68, 69] to only get released peptide-loaded microgels during infection could prove a very successful approach.

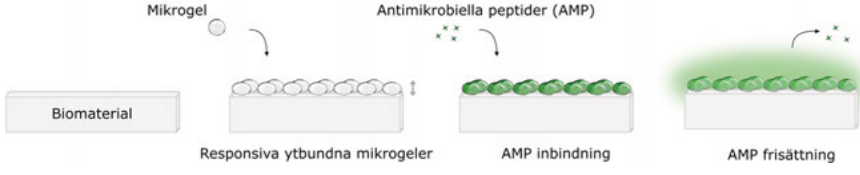
Populärvetenskaplig sammanfattning

De senaste årtiondenas stora forskningsframsteg gör att vi idag har möjlighet att restaurera en rad olika kroppsfunktioner med hjälp av implantat och biomaterial. Detta inkluderar allt ifrån enklare kontaktlinser, slangar för dränage, till mer avancerade stents för att vidga blodkärl, titanskruvar för tandimplantat och fixering av benbrott, samt pacemakers. För att ett implantat i kroppen ska lyckas krävs att implantatet både kan ersätta den förlorade kroppsfunktionen, samtidigt som materialet ska kunna integreras i den kringliggande vävnaden och orsaka så lite inflammation hos kroppens immunförsvar som möjligt. Tyvärr medför användandet av implantat och biomaterial alltid en risk för bakteriella infektioner efter operation, då bakterier lyckas växa och bilda biofilm på implantatets yta. Detta bekämpas idag med höga doser antibiotika, ofta både före och efter operation, för att tillräckligt hög koncentration av antibiotikan ska ta sig till implantatytan och kunna bekämpa bakterietillväxten. Tyvärr har det senaste århundradets överanvändning av antibiotika i både medicin och djurhållning gjort att vi idag ser en stor ökad mängd av antibiotikaresistenta bakteriestammar, som inte kan behandlas med dagens läkemedel. Detta gör att vi idag har ett stort behov av att snabbt ta fram nya antibakteriella läkemedel för att kunna bekämpa infektioner som vi tidigare kunnat behandla med antibiotika.

För att lösa detta, och kunna fortsätta använda implantat och biomaterial på det sätt vi gör idag, forskar man idag på olika nya läkemedel och smarta beläggningar på implantaten för att kunna bekämpa bakterier och minska risken för infektion och inflammation. I den här avhandlingen har vi valt att fokusera på antimikrobiella peptider som ett nytt alternativ till antibiotika. Antimikrobiella peptider är en del av vårt immunförsvar, och liknande strukturer finns även hos andra däggdjur, ryggradslösa djur och växter. Beroende på struktur har dessa små peptider visat sig ha effekt mot både bakterie- och svampinfektioner, samt kunna påverka kroppens inflammationsrespons och cancerceller. Det som dock är nackdelen med denna typ av läkemedel är att vårt immunförsvar är bra på att känna igen icke-kroppsegna peptider och har många försvar för att snabbt bryta ner och rena kroppen från dessa strukturer. Det krävs därför ny forskning på läkemedelsformuleringar som dels skyddar peptiden mot dessa försvar och ser till att rätt mängd läkemedel släpps ut på rätt plats i kroppen.

Mikrogeler är små polymera material som tidigare visat sig lämpade som läkemedelsbärare för peptid- och proteinläkemedel i lösning, då de kan binda upp stora mängder av dem och skydda emot nedbrytning i kroppen. Beroende på vilka kemiska strukturer som man inkluderar i mikrogelernas struktur går dessa att göra responsiva på en rad olika faktorer så som temperatur, pH, salthalt och andra specifika ämnen vilket gör att mikrogelernas storlek påverkas och därigenom läkemedelsfrisättningen.

Fokus på denna avhandling har därför varit att studera kombinationen av ytbundna mikrogeler och antimikrobiella peptider för att få en ökad kunskap hur dessa skulle kunna användas som en kombinerat antimikrobiell och anti-inflammatorisk beläggning för implantat. Se schematisk representation i *Figur 22*.



Figur 22. Denna avhandling har till stor del handlat om att hitta enkla sätt att göra responsiva mikrogel-beläggningar, samt studera faktorer som påverkar läkemedelsinbindning och frisättning från dessa.

För att kunna studera detta har tid lagts på att utveckla sätt att dekorera ytor med mikrogeler, samt studera hur dessa påverkas av ytan (storlek, form, samt peptidinbindning och frisättning) genom en rad olika mätmetoder. Detta i kombination med vidare bakterie- och cellstudier för att analysera eventuell antimikrobiell och anti-inflammatorisk effekt av dessa typer av beläggningar och frisatt antimikrobiell peptid.

Resultat från denna avhandling visar att det är kemiskt möjligt att binda mikrogeler till solida ytor, samt att deras responsiva svällningsegenskaper inte påverkas märkbart mer än att deras form plattas till. Vidare kan ytbundna mikrogeler binda upp stora mängder antimikrobiell peptid. Peptidinbindning och frisättning kan kontrolleras både genom mikrogelernas egenskaper (laddning) och peptidernas egenskaper (storlek, laddning, struktur och andra modifikationer), samt lösningsegenskaper så som pH och salthalt. Genom att binda in antimikrobiella peptider till mikrogeler kan dessa, under rätt förhållanden, dels sänka peptidernas toxicitet mot celler samt skydda peptiden från nedbrytning. Resultat från denna avhandling visar också att peptid-laddade ytbundna mikrogeler är bakterieavvisande och antibakteriella, men att antibakteriell effekt endast fås om tillräckligt mycket peptid är inbunden till mikrogel-lagret.

Detta kunde uppnås genom att antingen öka mängden ytmodifierad area eller genom att bilda multilager av ytbundna mikrogeler. Vidare kunde visas att antimikrobiella peptider behöll sina anti-inflammatoriska egenskaper trots att de var inbundna till mikrogeler.

Framtidens implantatbeläggningar måste möta en mängd olika kravspecifikationer för att kunna styra vävnadsintegrering, kontrollera kroppens immunförsvar och samtidigt bekämpa bakterietillväxt. Som slutsats av resultaten från denna avhandling konstateras att peptid-laddade ytbundna mikrogeler verkar lovande som en multifunktionell implantatbeläggning då flera viktiga egenskaper så som bakterieavvisande, bakteriedödande och anti-inflammatorisk effekt kunde påvisas. Genom att bygga multilager av mikrogeler kan en större mängd peptid bindas in vilket ger en bättre bakterieavdödning även på ett större avstånd från ytan. Dock, då antimikrobiella peptiders inbindning och frisättning påverkas av så många faktorer, beroende av både material- och peptidegenskaper, kommer liknande tester behöva göras i framtiden för att optimera användningen av dessa system som implantatbeläggningar. Nästa steg i utvecklingen, för att möta framtida krav på implantatbeläggningar, är att fokusera på nedbrytbara mikrogeler eller nedbrytbara länkar mellan multilager av mikrogeler för att komma åt en infektionstriggad frisättning av kombinationer av både antimikrobiella och immunomodulerande substanser.

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I addition, I would like to express my sincere gratitude to all the people involved that made this work possible.

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To my co-supervisor professor *Per Hansson* for discussions regarding physical chemistry.

A special thanks to all collaborators of the included papers, without your expertise these projects would not have been the same.

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