COLLOIDAL NANOMATERIALS FOR LIFE SCIENCE APPLICATIONS – FABRICATION AND PHYSICOCHEMICAL STUDIES

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Declaration

I hereby declare that the thesis is my original work and that it has been written by me in its entirety. To the best of my knowledge, I have duly referenced the sources of information and duly acknowledged the origin of other materials used in this thesis.

This thesis has not been submitted for any degree in any university previously.

Sebastian Beyer

29 November 2012

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List of Publications & Conferences

PUBLICATIONS RELATED TO THE SCOPE OF THIS THESIS:

PATENTS:

Sebastian Beyer, Wing Cheung Mak and Dieter Trau. Reverse Phase Layer-by-Layer Encapsulation of Highly Water-soluble Materials. PCT Int. Appl. (2008), 20pp. WO2008091228; A220080731 **(Prior to PhD candidature).**

BOOK CHAPTER:

Sebastian Beyer, Jianhao Bai & Dieter Trau. Assembly of Polymer Multilayers from Organic Solvents for Biomolecule Encapsulation; Multilayer Thin Films - Sequential Assembly of Nanocomposite Materials, second edition. Editors: Gero Decher, Joseph B. Schlenoff. (2012) Wiley-VCH Verlag GmbH & Co. KGaA.

PRIMARY RESEARCH ARTICLES:

Sebastian Beyer, Jianhao Bai, Anna M. Blocki, Chaitanya Kantak, Xue Qianru, Michael Raghunath and Dieter Trau. Assembly of Biomacromolecule Loaded Polyelectrolyte Multilayer Capsules by Using Water-soluble Sacrificial Templates. Soft Matter. (2012), 8, 2760-2768.

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PUBLICATIONS OUTSIDE THE DIRECT SCOPE OF THIS THESIS:

BOOK CHAPTER:

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PRIMARY RESEARCH ARTICLES:

Chaitanya Kantak, Qingdi Zhu, **Sebastian Beyer**, Tushar Bansal and Dieter Trau. Multiphase Microfluidics: Synthesis of PEG Microbeads for Fluorescence Based Glucose Sensing, Biomicrofluidics (2012), 6, 022006.

Chaitanya Kantak, **Sebastian Beyer**, Levent Yobas, Tushar Bansal, Dieter Trau. A 'Microfluidic Pinball' for on-chip Generation of Layer-by-Layer Polyelectrolyte Microcapsules. Lab on a Chip (2011), 11(6), 1030-1035.

Jianhao Bai, **Sebastian Beyer,** Wing Cheung Mak, Raj Rajagopalan, Dieter Trau. Inwards Buildup of Concentric Polymer Layers: A Method for Biomolecule Encapsulation and Microcapsule Encoding. Angewandte Chemie - International Edition (2010), 49(30), 5189-5193.

Jianhao Bai, **Sebastian Beyer**, Wing Cheung Mak, Raj Rajagopalan und Dieter Trau. Nach innen gerichteter Aufbau konzentrischer Polymerschichten: eine Methode zur Verkapselung von Biomolekülen mit simultaner Kodierung Angewandte Chemie (2010) 122, (30), 5316–5320 [entire manuscript in German language].

SELECTED CONFERENCE ATTENDANCE & PRESENTATIONS

2011 20 Years Layer-by-Layer Assembly: New Frontiers for Fundamental Science and for Applications, Strasburg, France (Poster, invited)
International Conference on Materials for Advanced Technologies (ICMAT), Singapore, (Poster)
2010 TERMIS EU Conference, Galway Ireland (Poster)
2009 International Conference on Materials for Advanced Technologies (ICMAT), Singapore, (Poster)

Contribution to conference proceedings, abstracts and presentation of collaborators are numerous and not listed.

Summary

Layer by Layer (LbL) polyelectrolyte self-assembly has long been used for the fabrication of polyelectrolyte microcapsules that are used for various applications. However, the encapsulation of water-soluble materials has been a challenge due to the media from which the polyelectrolytes are assembled into microcapsules being water. A process that allows the encapsulation of highly water-soluble materials with the same polymers conventionally used for aqueous LbL methods was developed. Instead of aqueous media, organic solvents are used for the self-assembly of polyelectrolyte membranes which led to the term of reverse phases (RP) Layer by Layer self-assembly (RP-LbL). Due to the insolubility of polyelectrolyte salts in organic solvents, their free amine or free acid counterparts are used that form likewise polyelectrolyte multilayer upon interaction albeit by a different mechanism.

This thesis addresses two aspects of Layer by Layer polyelectrolyte assemblies. The first part deals with the applications of the developed technology in terms of microcapsule formation and its potential use for life sciences. The second part addresses the physico-chemical peculiarities that distinguish the RP-LbL process from conventional approaches.

The literature on polyelectrolyte microcapsule formation especially by using organic solvents as deposition media is reviewed. A protocol for the encapsulation of highly water-soluble substances with polyelectrolyte membranes (e.g. composing of Polystyrenesulfonate and polyallylammonium salts) is established. Procedures to

control the concentration of biomacromolecules accurately within such capsules have been developed. The semi permeability of the resulting polyelectrolyte membranes has been studied. Potential applications in life sciences are outlined and related to current applications of polyelectrolyte microcapsules prepared by conventional approaches.

The properties of polymers in aqueous solution and aliphatic alcohols are studied by potentiometric titration. A procedure to estimate the charge density of polyacids in organic solvent has been established. The nature of polyelectrolyte complex formation of polyacids and polyamines dissolved in aliphatic alcohols has been investigated in bulk phase and at interfaces. Theoretical considerations are applied to polymer solutions used and general conditions encountered during RP-LbL assembly and related technologies.

Significance of this thesis in terms of applications is marked by extending the spectrum of templates that can be used for encapsulation by Layer by Layer self-assembly of polyelectrolyte membranes to that of highly water-soluble materials. Scientific significance is marked by the investigation of the physicochemical peculiarities of this process that starts to become more important in the field. Beyond that, basic research is addressed to a general procedure that can be employed to estimate the charge densities on polyacids in organic solvents. This is of special interest since earlier studies in the field are marked by inaccuracy neglecting important factors.

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List of Important Abbreviations

LbL	Layer by Layer
PSS	Polystyrenesulfonate (ionic moiety, ion or salt)
PAH	Polyallylammonium (ionic moiety, ion or salt e.g. hydrochloride)
PSA	Polystyrenesulfonic acid
PA	Polyallylamine
MF	Melaminformaldehyde
RP-LbL	Reverse Phase Layer by Layer
GRAS	Generally Recognized As Safe (acronym by FDA for safe to consume substances)
FDA	Food and Drug Administration of USA
USA	United States of America
BSA	Bovine Serum Albumin
FITC	Fluoresceineisothiocyanate
SBME	Surface Bound Microenclosures
DNase	DNA hydrolase (DNA degrading enzyme)
DNA	Deoxyribonucleic acid
HRP	Horse Radish Peroxidase
GOX	Glucose Oxidase
TRITC	Tetramethylrhodamineisothiocyanate
AFM	Atomic Force Microscopy
NSOM	Near Field Scanning Optical Microscopy
Da	Dalton (mass unit)
kDa	kilo Dalton
w/w	weight per weight usually expressed as per cent
pKa	negative logarithm of Ka
Ka	acid constant
C_0	total concentration of a substance in the system
R	universal gas constant
T	temperature
F	Faraday constant
E	potential
EMF	Electro Motive Force
E^0	standard potential
pH	negative logarithm of proton concentration (Power of H)
pH(x)	pH of unknown sample
pH(s)	pH of standard reference sample
E_x	potential of sample solution
E_s	potential of standard reference solution

a_H	proton activity
γ_s	activity coefficient of solute species
γ_w	activity coefficient of standard state aqueous solution
γ_t	transfer activity coefficient also known as medium effect
ΔG_t^0	Gibbs energy of transfer
D	Debye-length
L_b	Bjerrum Length
c*	overlap concentration, the concentration of polymers where the average distance between polymers becomes smaller then their persistence length in solution
b	bond length
u	Bjerrum length devided by bond length
f	fraction of charged monomers in a polymer molecule
g_e	number of monomers in a electrostatic blob
α	percentage of dissociated acid groups in a system
ξ	Oosawa-Manning parameter (ξ>1, counter ion condensation occurs)
e	elemental charge
ε	dielectric constant of the medium
k_b	Boltzmann's constant
L	total polymer length (maximally stretched)
PTSA	Paratoluenesulfonic acid
pKa(max_{OM})	the maximally permitted pKA of a poly acid before counterion condensation occurs according to the Oosawa-Manning theory
IUPAC	International Union for Pure and Applied Chemistry
MeCN	Acetonitrile
MeOH	Methanol
NMP	N-methylpyrrolidone
DCM	Dichloromethane
DMF	Dimethylformamide
FA	Formamide
HB-LbL	hydrogen bonding LbL

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Chapter 1: Introduction and Literature Review on Self Assembled Polymeric Core Shell Materials and the Use of Organic Solvents for their Fabrication

Scope & Specific Aims of Chapter 1

This chapter aims to review the fabrication of core shell materials with LbL methods and resulting possibilities to encapsulate biomolecules. The LbL technology as well as its application for the fabrication of core shell materials, coatings and general engineering of interfaces has been reviewed extensively. All aspects of LbL technology and associated fields such as polyelectrolyte chemistry and physical chemistry have been reviewed in *"Multilayer Thin Films"* first edition in 2002[1] and the very recent second edition[2]. Excellent references to get familiar with the development within this field are given by pertinent reviews that have been written by the groups of Caruso[3-12], Hammond[13-16] and Schaaf[17-19]. An application of polyelectrolyte microcapsules that emerged during the past few years is that in life sciences for internalization into cells or in general for drug delivery. Those recently developed application have been reviewed by pioneers in the fields such as, De Geest [20]& De Schmedt[21-23], Skirtach[24-26] and Sukhorukov[27-29] Due to the abundance of general reviews of LbL technology and its application, this chapter aims to highlight

possibilities to increase the encapsulation efficiency for biomolecules and to compare different methods of LbL polymer assemblies from organic phase.

The first chapter of this thesis deals primarily with two fields of polymer self assemblies, my work related to polymer self assemblies (in particular RP-LbL) and that of Dr. Jianhao Bai (in particular using agarose microbeads as templates and the inwards build up technique).[i]

Specific aims of this chapter include:

1. To review possibilities of biomolecule encapsulation in Layer by Layer assembled polyelectrolyte microcapsules.

2. To identify short comings of state of the art technology in particular the encapsulation efficiencies and loss of biomolecules of different encapsulation protocols and to devise a solution for such problems.

3. To review methods that use organic solvents for the fabrication of polyelectrolyte microcapsules and to distinguish the novel developed RP-LbL process primarily due to differences in the process.

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[i] This chapter has been published in parts as a chapter in the second edition of *"Multilayer Thin Films"* and was co–authored by Dr. Bai Jianhao and myself with equal contributions, the complete bibliographic data are given in the following.

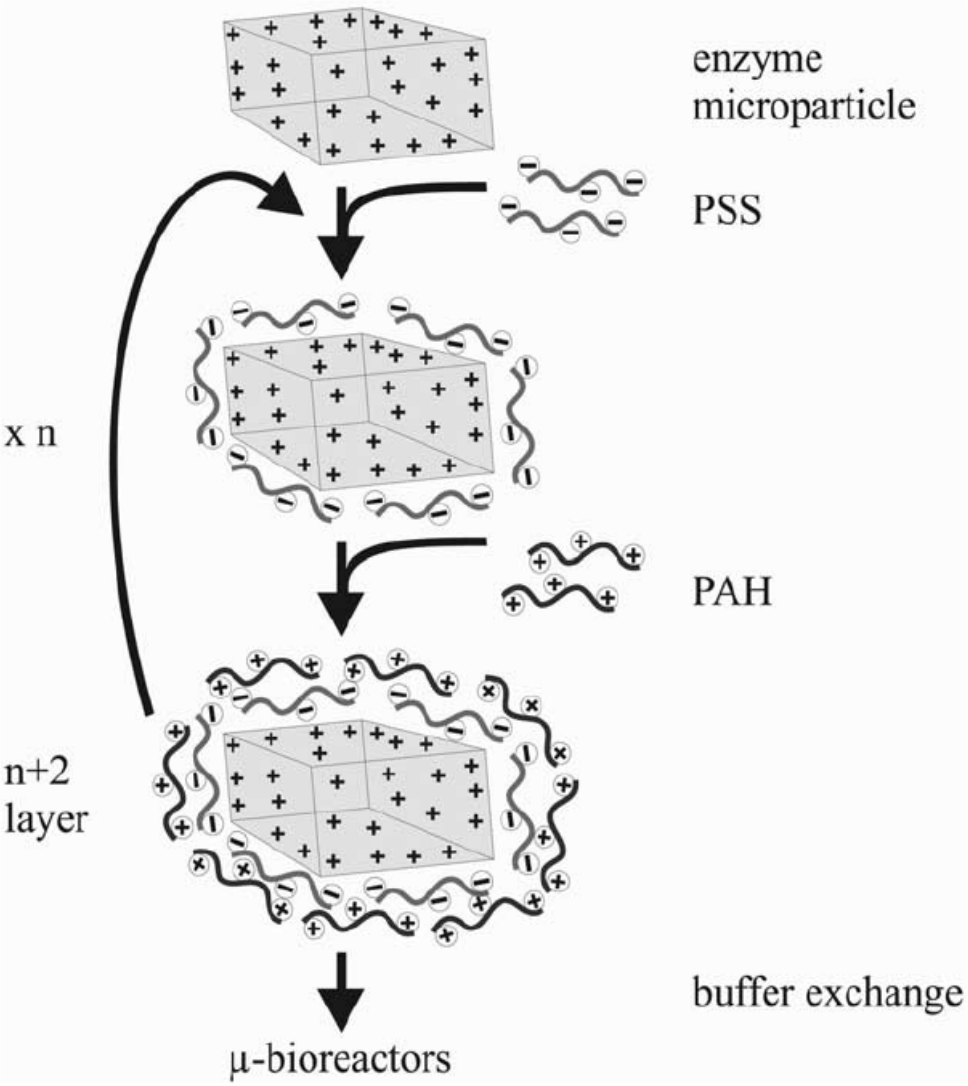
Sebastian Beyer, Jianhao Bai & Dieter Trau. Assembly of Polymer Multilayers from Organic Solvents for Biomolecule Encapsulation; Multilayer Thin Films - Sequential Assembly of Nanocomposite Materials, second edition. Editors: Gero Decher, Joseph B. Schlenoff. (2012) Wiley-VCH Verlag GmbH & Co. KGaA.

This chapter largely resembles the book chapter although altered partially. No further references to the above named book chapter are made within the following text.

Introduction

The purpose of microencapsulation is to separate or concentrate a particular substance in a confined space from its environment. Through the introduction of microencapsulation many life-science and biomedical engineering fields made tremendous progress such as cellular[30, 31] or biomolecular[9] therapy, drug delivery[28, 32], enzymatic bioreactors[33] and biosensors[34]. The popularity of microencapsulation in these fields stems from many reasons including: 1) The minute nature of microcapsules allows for efficient exchange of materials between the microcapsules and its environment, 2) the prevention of infinite dilution and 3) the protection of encapsulated materials (e.g. proteins and DNA) from undesired external agents (e.g. proteases and nucleases). Many microcapsule fabrication techniques have been developed including the self-assembly of micro particles[35, 36] or polymers[37], solvent evaporation[38] and spray drying[39, 40]. The common parameter that is equally crucial for all methods is the efficiency with which the cargo can be encapsulated. Ideally, the loss of cargo during the encapsulation process should be negligible, which is however seldom achieved. The Layer-by-Layer (LbL) polymer self-assembly technique has proven to be a very reproducible and versatile approach for microcapsule fabrication and biomolecule encapsulation. Although state of the art methods in this field are marked by low encapsulation efficiencies. Initial approaches of biomolecule encapsulation, via the LbL technique, were conventionally conducted in an aqueous phase and we have generalized these approaches into three main categories: 1) Bio-template based[41, 42], 2) Loading based[43-49] and 3) Diffusion based encapsulation[50-56].

Bio-Template Based LbL Encapsulation in Aqueous Phase



Scheme 1-1: Schematic illustration of the bio-template based aqueous LbL encapsulation. From top to bottom: A micro particle composed of amorphous or crystalline biomolecules is used as a template dispersed in aqueous phase under conditions that render the biomolecule water insoluble. The polyelectrolytes polystyrenesulfonate sodium salt (PSS) and polyallylamine hydrochloride (PAH) are deposited sequentially to form a polymeric capsule around the solid bio-template (image taken from reference[42]).

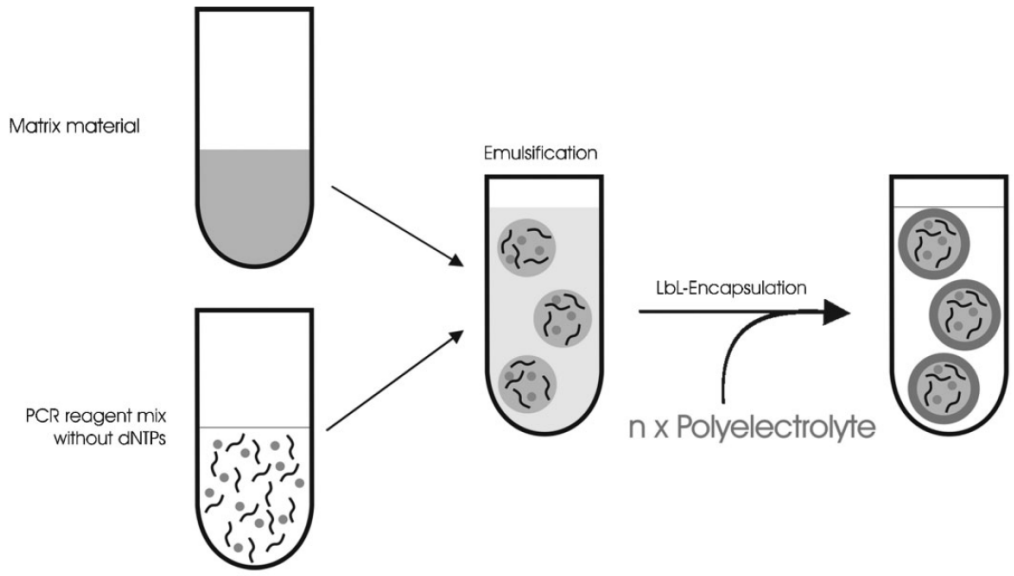
Biomolecule encapsulation within LbL microcapsules can be achieved via the LbL self-assembly of polymers onto biomolecule micro crystals (bio-template) as depicted in Scheme 1-1. The solubility of biomolecule micro crystals needs to be reduced

through the use of chilled aqueous solution with a high salt content[41, 42]. Significant advantages of this approach are the very high biomolecule loading and encapsulation efficiency. By using e.g. enzyme crystals with the highest possible packing density of molecules per volume element, the probably highest encapsulation densities can be achieved. However, encapsulating mixtures of biomolecules with defined concentration using this approach is limited by difficult fabrication of suitable template micro-particles. The fabrication of highly packed solids of more then one biomolecule is difficult due to the tendency of proteins to crystallize. Water-soluble bulk material needs to be crushed in a mortar or milled when larger quantities are needed. The shape of the resulting template particles will be anisotropic and with a relatively high size distribution. In addition most biomolecules such as carbohydrates, oligo or polynucleotides as well as many proteins will still dissolve in chilled aqueous solution with a high salt content. This prevents a general route for the fabrication of solid bio-templates and thereby limits this approach to a certain class of biomolecules that are insoluble in aqueous solutions under these conditions.

Loading Based LbL Biomolecule Encapsulation in Aqueous Phase

Another approach involves the loading of biomolecules into a template matrix material prior to fabrication of an LbL multilayer polymer shell around the template (Scheme 1-2). Examples of such loading techniques include the adsorption of biomolecules within silica[43, 44] or carbonate[45] micro particles, or pre-mixing of

biomolecules during template formation. The latter found application in the fabrication of agarose microbeads[46, 47] or carbonate[48, 49] micro particles. In the case of agarose microbeads, solutions of the biomolecules of interest are mixed with molten agarose solution and the biomolecules will be entrapped upon solidification. The template is generated in an emulsion process, which results in microbeads of solidified agarose biomolecule solutions. In a similar way, biomolecules of interest might be mixed with aqueous solutions of metal ions that precipitate and thereby entrap biomolecules upon addition of carbonate ions. Although loading of biomolecules via adsorption is a simple procedure, the quantity of biomolecules to be loaded cannot be controlled easily since the loading density is primarily due to the inner surface of the precipitates, which varies strongly under different precipitation conditions. In addition, not all biomolecules will be loaded since the bulk phase contains biomolecules in excess. This results in low initial loading efficiencies and a loss of potentially expensive biomolecules if they are not recovered from the bulk solution. By using a 'water in oil' emulsification approach to load biomolecules into emulsified agarose microbead templates, quantitative biomolecule loading can be achieved. In this approach, the agarose acts as a matrix material in which any desired biomolecules or mixture thereof can be loaded. However, after transfer of the biomolecule loaded agarose templates into an aqueous phase for LbL encapsulation a considerable amount of biomolecules is leaching out prior and during LbL encapsulation, resulting in relative low (~50%) encapsulation efficiency for biomolecules [46].

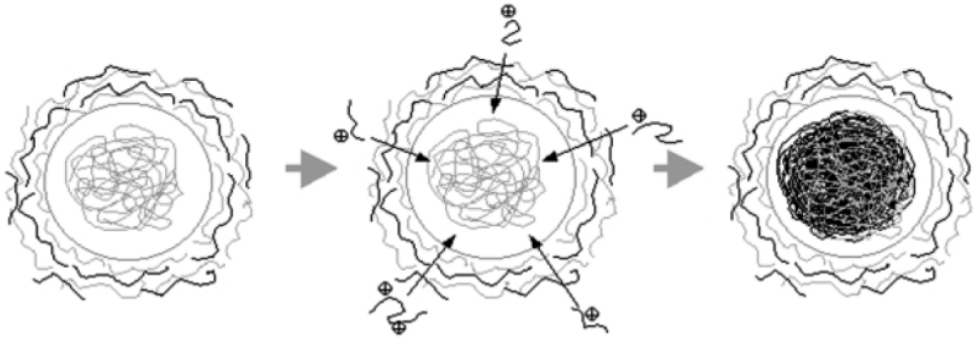


Scheme 1-2: Illustration of the "Matrix-assisted LbL encapsulation" technique; an example of aqueous LbL preloading biomolecule encapsulation approach.
From left to right: Mixing of matrix material (e.g. agarose) and biomolecules (e.g. polymerases and primers) followed by emulsification in an oil phase to form water-in-oil emulsion at elevated temperature. Solidification by cooling of the matrix material forms micro bead templates. Fabrication of LbL multilayer polymer shells for the encapsulation and retention of the biomolecules within the micro bead templates (image taken from reference[46]).

Diffusion Based LbL Biomolecule Encapsulation in Aqueous Phase

Another approach to encapsulate biomolecules within LbL microcapsules is by diffusion of biomolecules from an external solution into hollow LbL microcapsules. This diffusion process is driven by the biomolecule concentration gradient between the external solution and the interior of the hollow LbL microcapsules. Retention of biomolecules within the hollow LbL microcapsules can be achieved through a number of methods. For example, by changing (reducing) the permeability of the LbL "semi-permeable membrane" shells through pH[50], solvent[51], drying[52], ionic strength[53] or ultraviolet irradiation[54] means after the biomolecules had diffused into the core. Unfortunately, for this diffusion driven loading approach, the concentration of loaded

biomolecules is limited to the concentration of biomolecules in the external solution and is difficult to control. Therefore, only a fraction of biomolecules will be encapsulated and this method results in low encapsulation efficiency. In a variation of this approach as illustrated in Scheme 1-3, a complex is formed within the microcapsules and a higher loading and better encapsulation efficiency can be achieved [55, 56]



Scheme 1-3: Schematic illustration of protein encapsulation into hollow LbL microcapsules. From left to right: LbL microcapsule made from melamine-formaldehyde (MF) template and containing polystyrenesulfonate (PSS)/MF complex. Positively charged proteins diffusing into the microcapsules. Due to the negative charge of the PSS complex within the microcapsule, an insoluble protein/PSS complex is further formed and which is retained within the microcapsule (image taken from reference[56]).

Limitations of LbL Based Biomolecule Encapsulation in Aqueous Phase

Performing LbL in aqueous phase has some limitations: Encapsulation efficiency for biomolecules is relative low and water-soluble biomolecules cannot be used in a direct templating approach. In addition, any water-sensitive materials cannot be encapsulated (e.g., biomolecules hydrolyzing fast in water or reactive materials such as sodium borohydride). Furthermore, it has been recently pointed out that common entrapment mechanism such as reversibly changing membrane permeability, heat induced membrane densification and spontaneous accumulation due to electrostatic interaction between the molecule of interest and matrix material inside microcapsules have one great common limitation. It was stated by J. De Cock and coworkers that the most prevailing limitation of this method is that, *"it suffers from very low encapsulation efficiency and possible loss of bioactivity and low integrity of therapeutic macromolecules because of the harsh conditions required to make the PMLC* (polymeric multilayer capsules) *membrane permeable."*[28]*"*. Encapsulation efficiency here refers to that is the amount of protein that becomes encapsulated within the capsules, relative to the amount of protein that was initially added. A recent study by De Smedt[57] and coworkers stressed that understanding parameters which influence encapsulation yield for aqueous LbL using the calcium carbonate sacrificial template method is the essential requirement to pave the way for pharmaceutical application of protein filled polyelectrolyte microcapsules. The same study revealed

that entrapment efficiencies of proteins within calcium carbonate particles is highly dependent on their isoelectric point and that only highly negatively charged proteins can be entrapped with suitable yield of around 90% and that positively charged proteins are excluded from this method by very low encapsulation efficiency. Neutral biomacromolecules such as polysaccharides were not included in this study; it might be assumed that entrapment of those molecules within calcium carbonate particles is relatively low due to absence of charged groups. In addition, calcium carbonate as template material for the encapsulation of biomolecules is limited to cargo that is not pH sensitive and which might not loose function upon the interaction with EDTA[28]. The later applies especially to di- or trivalent cations that might be necessary as cofactor for enzymes.

Given this limitation of the state of the art technologies, it was one of the specific aims of this PhD work to devise a novel process, which allows the accurate control over the resulting biomolecule concentration within the resulting polyelectrolyte multilayer capsules that achieves 100% encapsulation efficiencies on the same time.

Two processes that were developed in the NanoBioanalytics laboratory RP-LbL (developed by myself) or the "inwards build up technique" (developed by Dr. Jianhao Bai) overcome most of those limitations. Both methods use polymers that are also frequently used in aqueous based LbL. For direct encapsulation of protein crystals with polyelectrolyte multilayer using the RP-LbL technique an initial entrapment of

100% can be achieved due to the insolubility of proteins in organic solvents. In cases were hydro gel microbead template materials are used for RP-LbL or the inwards build up method, the initial biomolecule concentration within the aqueous phase can be precisely controlled. The template material for microbeads can be prepared by an emulsion approaches and subsequent coated with the RP-LbL protocol or the inwards build up of polymer layer might be performed. This procedure leads to very high encapsulation yields. This should be valid for all water-soluble biomolecules due to their insolubility in the organic phase that surrounds the template and later the core shell material during the entire manufacturing process. The retention (upon transfer to aqueous phase) of biomolecules within core shell structures after completion of the manufacturing process (RP-LbL or the inwards build up technique) was demonstrated to be similar to that of core shell materials prepared by conventional methods. Retention of biofunctionality for various enzymes was as well demonstrated within the same studies.

LbL Biomolecule Encapsulation in Organic Phase

Performing LbL in organic phase opens some interesting new avenues; a variety of new templates and polymers can be used in organic solvents to create an even larger pool of LbL derived core shell materials or surface modifications by LbL coatings. LbL templates can be extended into biomolecule based templates such as amorphous or microcrystalline proteins (e.g. enzyme crystals) or organic materials (e.g. sodium

ascorbate). The "Reverse-Phase LbL" (RP-LbL)[58] and the "Inwards Buildup Self-Assembly Method"[59], both are performed in organic solvents to minimize loss of biomolecules during template formation and microencapsulation by polymer multilayer buildup. In addition, the use of organic solvents allows the encapsulation of water-sensitive materials. The following paragraphs of this chapter describes the biomolecule encapsulation via the RP-LbL method as well as the "Inwards buildup self-assembly" method and its applications. The physico-chemical similarities and differences between polymers in aqueous and organic solvents; different mechanisms for aqueous LbL, hydrogen-bonded LbL (HB-LbL) and the newly established RP-LbL are discussed in Chapter 4 of this thesis.

Reverse-Phase LbL (RP-LbL) Technique

The advantage for biomolecule encapsulation is the relatively strong electrostatic interaction and thus a stable resulting microcapsule combined with high encapsulation efficiency as no biomolecules can dissolve in the organic phase. The RP-LbL technique works similar to other techniques to prepare microcapsules employing sequential absorption of polymer layer followed by washing off excess of non-absorbed polymer after each deposition step. Except that the polymers are in their free respective amine or acid form dissolved in organic solvents while other methods utilize polyelectrolyte salts in aqueous solution.

Polymers: Polymers used for RP-LbL technique are usually polyamines or polyacids. Many suitable polymers are commercially available but may also be prepared from their polyelectrolyte form by acid/base chemistry in which small molecular weight counter ions are removed in analogy to ion exchange resin chemistry. Polymers prepared in that way were termed non-ionized or non-ionic to indicate their polyelectrolyte origin. Non-ionic polymers are preferred for the RP-LbL technique. This allows for polymer dissolution in organic solvents. It needs to be mentioned here that only polyamines can be considered truly non-ionic while polyacids dissolved in organic solvents exhibit a certain degree of auto protolysis that induces a related charge density on the polymer backbone. Nonetheless, the autoprotolysis rate of polyacids creates relatively little charge when compared to aqueous polyelectrolyte solution. Thus, both polyamines and polyacids are referred to as non-ionized polymers. This terminology and its derivation is in accordance with literature that addresses the behavior of salt free polyacids in aqueous solution[60]. A summary of polymers that can be used for RP-LbL and their employed concentration in organic solvents can be found in Table 1.

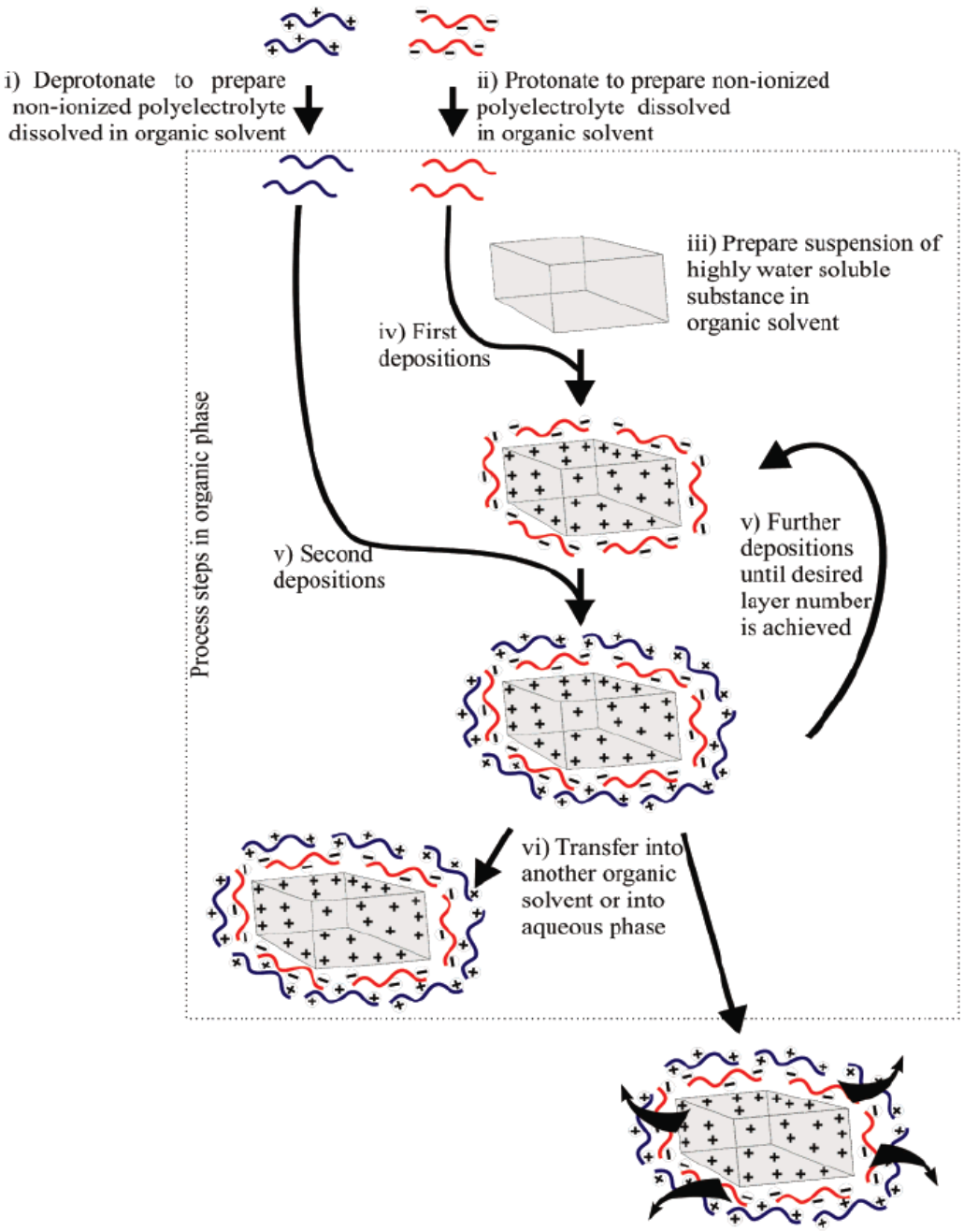
Solvents: Solvents for RP-LbL range over various classes of solvents, the main criteria is sufficient solubility for polyamines and polyacids. Although solubility of polymers has been shown for various solvents such as dimethylformamide, dimethylsulfoxide, formamides and dichloromethane, the use of aliphatic alcohols are favored in this work as they are less toxic, bio friendly and some are even considered Generally Recognized as Safe (GRAS) by the US Food and Drug Administration

(FDA). In addition, solvents such as dimethyl formamide (DMF), dimethylsulfoxide (DMSO), formamides (FA) are very polar and known to dissolve salts or sugars which prevents the use of those materials as templates.

Polymer	Solvent	M_W in kDa	Concentration used	References
Amine functional polymers				
Polydiallyldimethylammonium chloride	Ethanol	100-200	10 mg mL^{-1}	58
Polyethyleneimine, linear	Chloroform	250	Saturation	58
Polyallylamine	1-butanol	65	1 mg mL^{-1}	61, 62
Polyethyleneimine, branched	Dichloromethane	10-25	4 mg mL^{-1}	63
Acid functional polymers				
Polymethacrylic acid	Ethanol	100	10 mg mL^{-1}	58
Polyacrylic acid	1-butanol	450	1 mg mL^{-1}	61
Polyacrylonitrile-co-butadiene-co-acrylic acid	Dichloromethane	3.6	4 mg mL^{-1}	63
Polystyrenesulfonic acid	1-butanol	70	1 mg mL^{-1}	45
Polystyrenesulfonic acid	1-butanol	70	5 mg mL^{-1}	64

Table 1-1: Examples of polymers used for the RP-LbL technique.

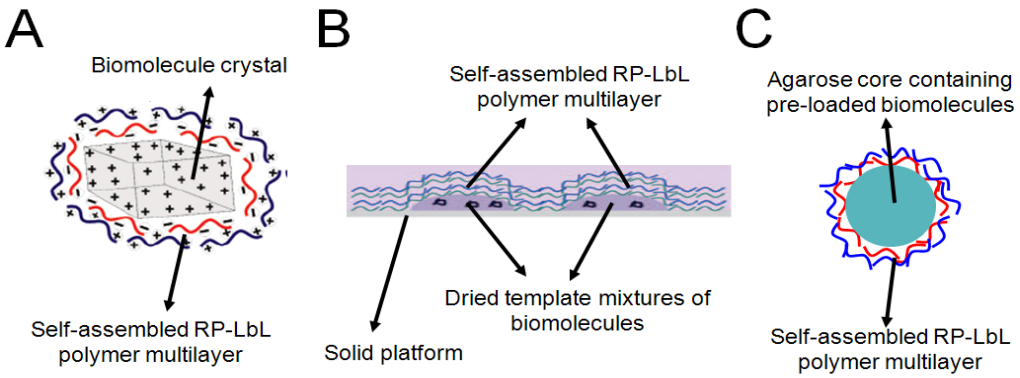
Template materials: Template materials can be chosen from a broad range of materials. Special attention has been attributed to highly water-soluble materials that cannot be encapsulated by "direct bio-templating" using common aqueous LbL methods such as saccharides, poly saccharides, proteins, nucleic acids, organic or inorganic salts, drugs and hydrophilic vitamins.



Scheme 1-4: Diagram of the Reverse-Phase LbL (RP-LbL) technique.
Significant differences, as highlighted in this figure, is the use of an organic phase and Brønsted acid base reactions taking place between polymers or polymers with the template (image taken from reference[58]). The entire process of polyelectrolyte multilayer formation is carried out in organic solvents.

Encapsulation of Biomolecules using the Reverse-Phase LbL (RP-LbL) Technique

Demonstrated methods of encapsulating biomolecules via the RP-LbL technique include the direct encapsulation of biomolecule crystals[58] (Scheme 1-5 A), self-assembly of polymers onto biomolecules dried onto a flat solid substrate to form surface-bound microenclosures[65] (Scheme 1-5 B) and encapsulation of biomolecules loaded into agarose microbeads[61, 66] (Scheme 1-5 C). Encapsulation is achieved by dispersing the colloidal template material or surface bound template in an organic solvent such as aliphatic alcohols containing the polymer. Next, polymers with good absorption properties at the template interface will be absorbed until the surface is covered with the first polymer layer. Excess polymer is washed off followed by incubation of the polymer coated template into a complimentary polymer solution followed by washing of all excess non-absorbed polymers. This process can be repeated to achieve the desired number of RP-LbL polymer multilayer.



Scheme 1-5: Diagram illustrating methods for encapsulation of biomolecules via RP-LbL. (A) Direct encapsulation of biomolecule crystals, (B) self-assembly of polymers onto dried template mixtures of biomolecules on a flat solid substrate to form surface-bound microenclosuress and (C) encapsulation of biomolecules loaded into agarose microbeads (Images taken and modified from references[58, 65]).

Direct encapsulation of water-soluble molecules has the advantage that no intermediate steps, such as biomolecule loading or removal of a template is necessary. However, upon transfer into aqueous phase encapsulated macromolecular biomolecules might build up sufficient osmotic pressure to rupture the capsule[58] while small molecular weight substances permeate through the membrane, indicating their size dependent semi permeability (Figure 1-1).

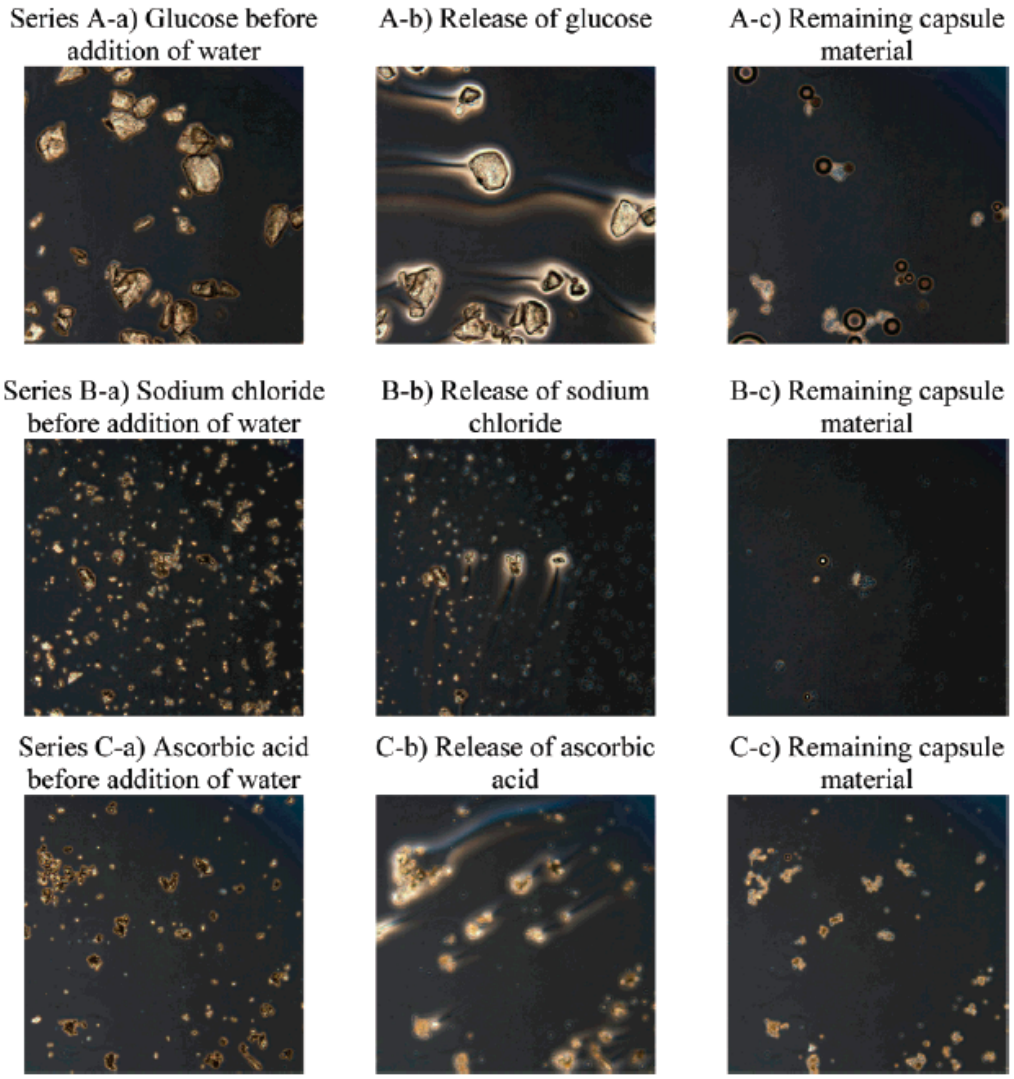


Figure 1-1: Light micrographs demonstrating the RP-LbL encapsulation.
A) glucose, B) sodium chloride and C) ascorbic acid. Images "a" shows RP-LbL encapsulated crystals in ethanol before addition of water; images "b" after addition of water and "c" the empty capsule material after completed release. A fluorescein labeled non-ionized polyelectrolyte was used in the RP-LbL process to demonstrate capsule formation (images not shown) (image taken from reference[58])

Figure 1-2 depicts the release of encapsulated BSA-FITC (M_W 65 kDa), obtained via the RP-LbL technique, when transferred into an aqueous phase by a burst release.

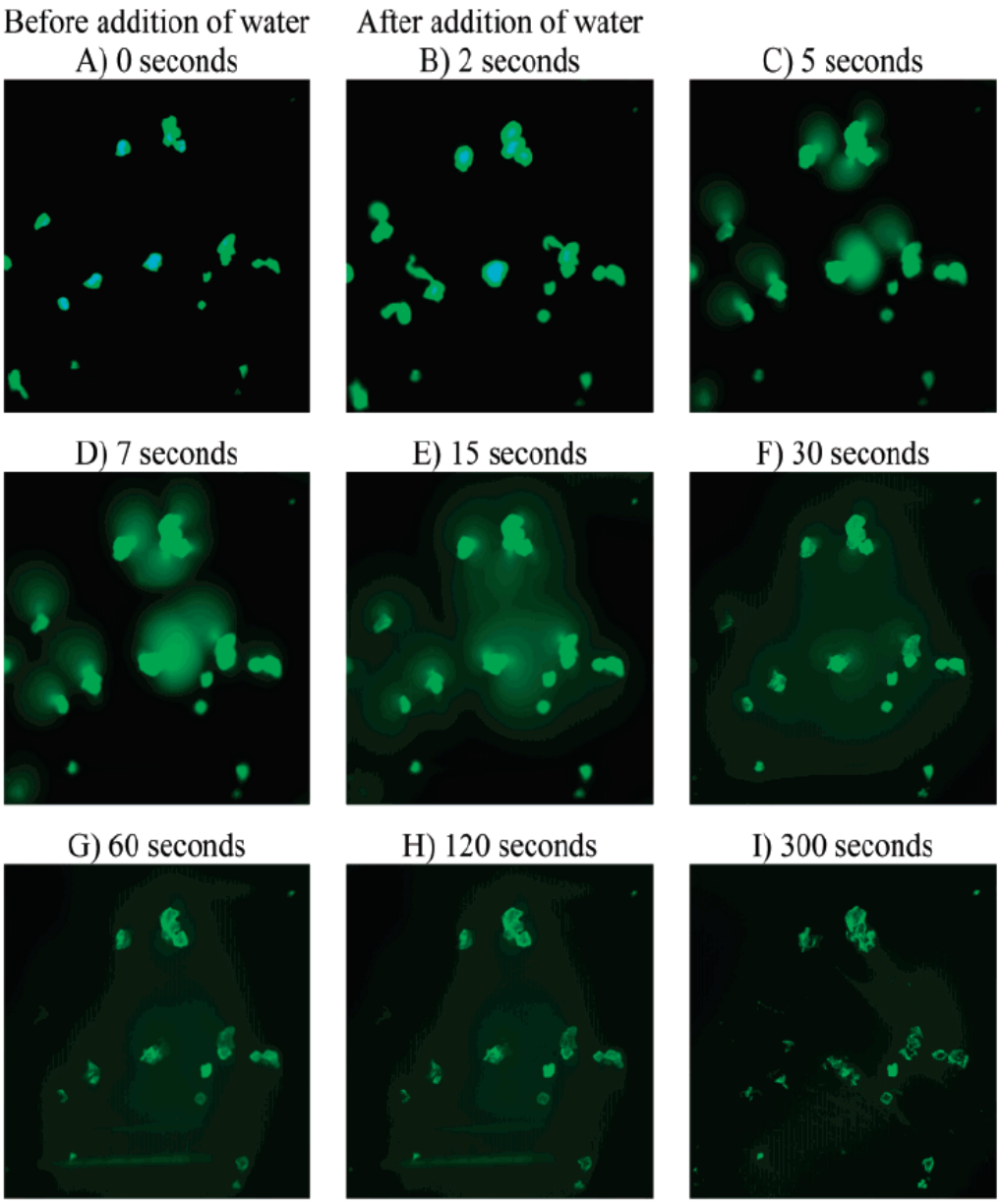


Figure 1-2: Fluorescent micrographs demonstrating the Reverse-Phase LbL encapsulation of the highly water-soluble protein bovine serum albumin (BSA):
(A) Before addition of water. (B) Capsule volume increases due to buildup of osmotic pressure in capsules. Some capsules show "small jets" releasing BSA. (C to H) Further release of BSA forming "clouds" and diffusion of BSA away from capsules. (I) Remaining capsule material. BSA-FITC was used to demonstrate release. The particle size was 5-20 µm. Non-encapsulated BSA dissolves immediately in the first 2 seconds (image taken from reference[58]).

Fortunately, the problem of bursting microcapsules comprising of encapsulated pure protein crystals can easily be circumvented through the simultaneous encapsulation of saccharides[65]. Certain organic solvents (e.g. ethanol) are inherently much more volatile than water. By using such solvents to encapsulate biomolecules via the RP-LbL technique powders are easily prepared by evaporation of the solvent (Figure 1-3).

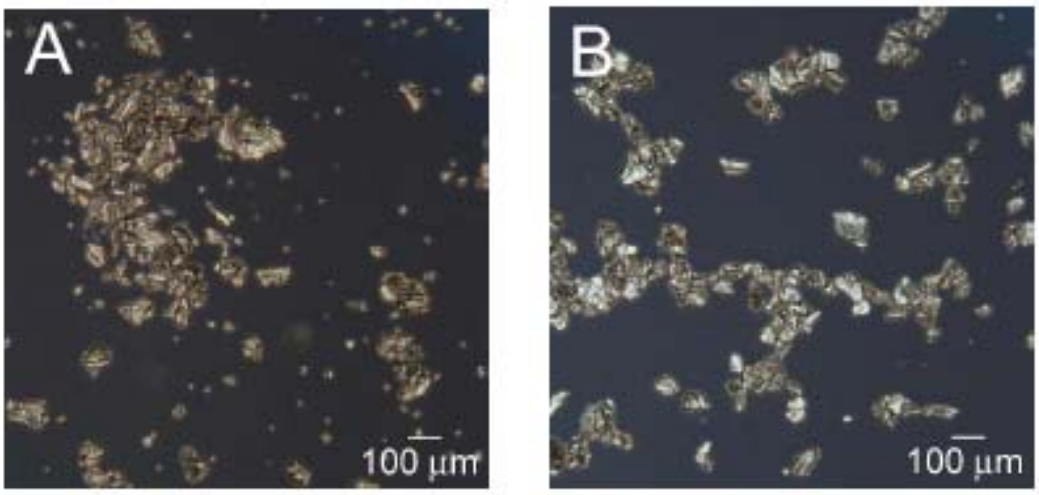


Figure 1-3: Particle morphology and size of encapsulated BSA before and after drying. A) Before evaporation; B) after evaporation of ethanol to manufacture a powder. No significant aggregation or increase in particle size was observed (image taken from supporting information of reference[58]).

Preferentially, the solvent is evaporated under vacuum at room temperature at very mild conditions to prepare powders of biomolecule filled microcapsules. It was demonstrated that the powder could be reconstituted in water or buffer to form a microcapsule suspension. Powders have many favorable properties, which include often longer shelf life compared to liquid suspensions. Another application to encapsulate biomolecules by RP-LbL was demonstrated by forming "surface bound microenclosures" (SBMEs)[65] onto flat substrate surfaces. Instead of directly encapsulating biomolecule crystals, a solution of biomolecules and glucose was dried

on a solid substrate forming a solid bound template. Then, sequential immersion of the substrate with the dried template into ethanol with dissolved polymers caused the deposition of RP-LbL membranes and formation of SBMEs. By encapsulating the desired biomolecules within the SBMEs, various physical properties and biological reactions could be studied in a closed confinement. This is especially useful for parallel and multiplexed observations of bio-chemical reactions and bio-physical processes by an "easy to handle" protocol. By using this method with encapsulated NeutrAvidin, membrane permeability of fluorescence labeled biotin and its interaction with NeutrAvidin was studied. In addition, the control of enzymatic reaction within the SBMEs was demonstrated by DNase activity that could be switched on and off by addition or removal of Mg^{2+} ions to the external solution, indicating selective permeability of the RP-LbL membrane for small molecular weight materials (Figure 1-4).

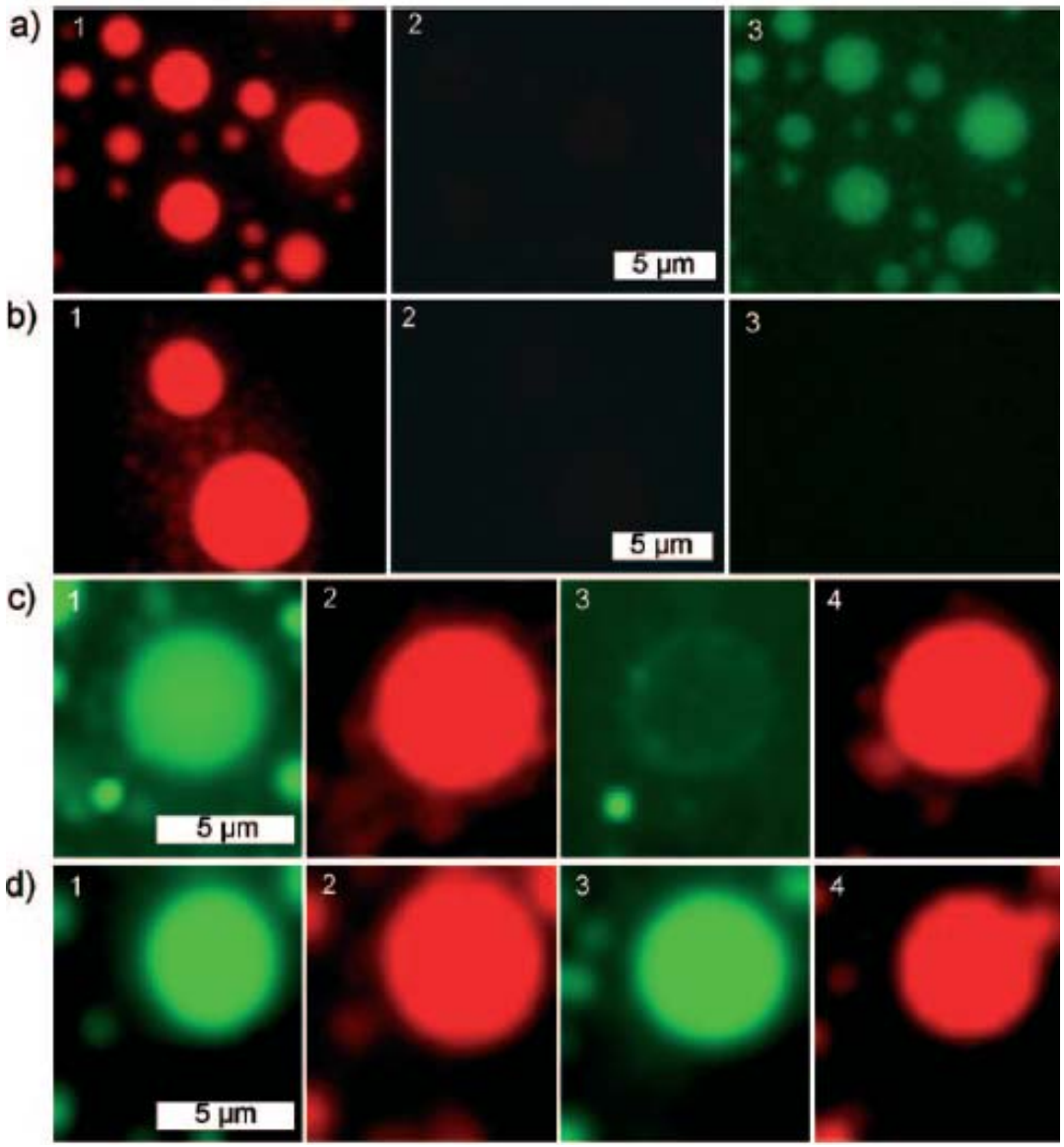


Figure 1-4: Applications of SBMEs observed with fluorescence microscopy.

a) Interaction of enclosed NeutrAvidin molecules with FITC-biotin diffusing in from outside the SBMEs. 1) SBMEs filled with Texas Red labeled BSA and NeutrAvidin (red channel); 2) Image of the same area before adding FITC-biotin (green channel); 3) same area 3 min after adding FITC-biotin (green channel). b) Control experiment without NeutrAvidin. Same sequence of events as in (a). c) Enzymatic DNA digestion. 1) SBME filled with Alexa Fluor 488 labeled oligonucleotide, DNase I, and Texas Red/BSA (green channel); 2) same area as in (1) (red channel); 3) same area 10 min after adding buffer containing Mg^{2+} and Ca^{2+} ions to start the enzymatic reaction (green channel) 4) same area as in (3) (red channel). d) Control experiment of enzymatic DNA digestion with SBMEs containing no DNase. Same sequence as in (c) (Image taken from reference[65]).

Core-shell materials employing an agarose core are particularly useful for capsule-based bio-reactor and biosensor applications. The high water content of the agarose core provides a good environment for biochemical reactions to take place. Mixtures of biomolecules can be loaded within the agarose with high control over absolute concentration and ratio of biomolecules for encapsulation. However, before polymers can be self-assembled from organic phase onto agarose templates to form core-shell materials it is necessary to prevent aggregation and shrinking of the agarose microbead core. Shrinking could occur due to loss of water into the organic phase and aggregation might be caused by the relative hydrophobic organic phase. Two approaches to prevent shrinking and aggregation have been demonstrated. The first approach entails the use of polystyrene micro particles to form colloidosomes[66] and the second approach is based on ADOGEN® 464 detergent [59, 61]. After stabilization of the agarose bead, polymers can be self-assembled to form biomolecule filled agarose core based core-shell materials.

By using the RP-LbL self-assembly of polymers in organic solvents onto stabilized agarose core templates containing biomolecules, (Figure 1-5) the encapsulation yields for biomolecules were at least doubled compared to fabrication via the conventional aqueous LbL approach. It was also shown that these core-shell materials have high retention stability (~100%) of encapsulated BSA when dispersed in an aqueous phase for a period of seven days[66]. This ability to achieve a high encapsulation efficiency and retention stability for biomolecules with the RP-LbL technique allows the fabrication of better performing core-shell materials for various applications.

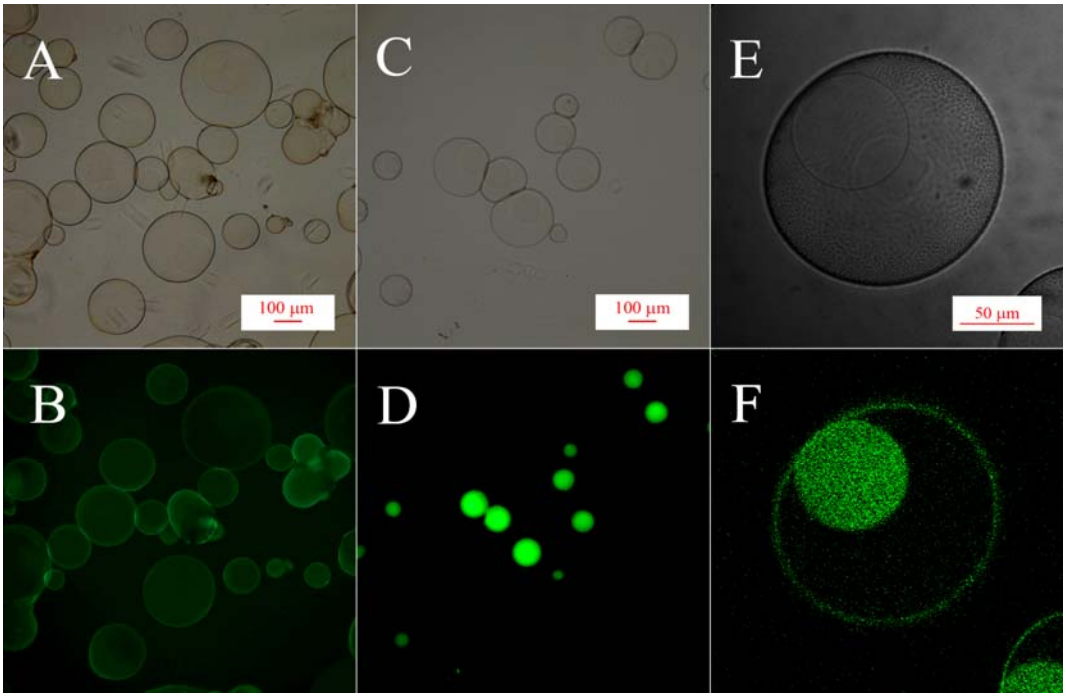


Figure 1-5: Inflated microcapsules.
(A) Optical and corresponding (B) fluorescence micrograph of inflated microcapsules fabricated with polyacrylic acid – Rhodamine 123 conjugate and by the RP-LbL method. Fluorescence is observed from the RP-LbL polymeric shell. (C) Optical and corresponding (D) fluorescence micrograph of inflated microcapsules fabricated with agarose–Rhodamine 123. The agarose microbeads are clearly fluorescent. (E) Confocal optical and corresponding (F) fluorescence micrograph of inflated microcapsules fabricated with both Polyacrylic acid – Rhodamine 123 and agarose–Rhodamine 123. The agarose microbead is observed to be partially attached to the LbL capsular wall (image taken from reference[61]).

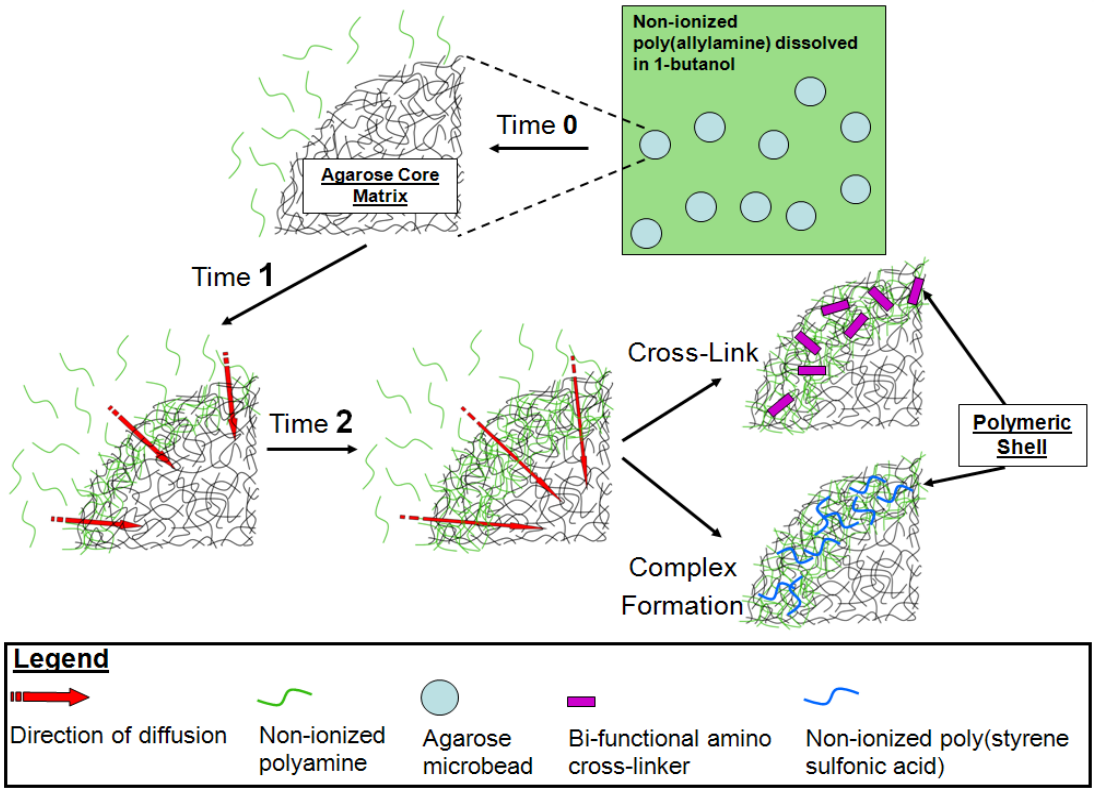
Interestingly, additional loading of small molecules such as tris(hydroxymethyl)aminomethane (TRIS) and sucrose within the agarose-polymer core-shell materials resulted in the fabrication of "inflated" microcapsules with a unique "bead-in-a-capsule" morphology (Figure 1-5)[61]. The inflation of the RP-LbL shell is driven by a difference in osmotic pressure between the core-shell materials' interior and exterior environment when the agarose-polymer core-shell materials were transferred from an organic phase into an aqueous phase. The different distribution of materials within the multi phase interior of "inflated" microcapsules was

demonstrated. Small molecules could diffuse out of the bead occupying the entire interior whereas entrapped particles remained in the agarose bead creating a distinct localization. This demonstrates that "inflated" microcapsules could permit control over localized chemical or enzymatic reactions for future studies.

Inwards Buildup Self-assembly of Polymers for Biomolecule Encapsulation in Organic Phase

The inwards build up represents a complimentary technology for the encapsulation of biomolecules with high efficiency due to the insolubility of biomolecules in the organic phase that is employed during the assembly process. If agarose microbeads are used as template material it is in general possible to chose the assembly conditions in a way to either yield RP-LbL based core shell materials or "Inwards build up" core shell materials. However the exact boundaries of the conditions to yield either RP-LbL based or "Inwards build up" based core shell materials are not yet exactly understood and scope of current investigations. The "inwards build up" method is presented shortly in the following; the interested reader can refer to the PhD Thesis submitted by Dr. Jianhao Bai in 2010 to the National University of Singapore. An alternative and recently developed novel method for polymer self-assembly from organic solvents to form polymer multilayer is termed "inwards buildup self-assembly" technique [67].

"Inwards Buildup Self-assembly" Mechanism and Technique



Scheme 1-6: Inwards build up mechanism

Diagram illustrating the self-assembly of polymers from an organic solvent, via the "inwards buildup self-assembly" technique, into porous agarose core templates for the formation of agarose-polymer core-shell materials (This figure is originally part of the PhD thesis by Dr. Bai).

Unlike conventional LbL techniques where polymers are buildup outwards from the template, the "inwards buildup self-assembly" technique generates well-defined polymer multilayer through the deposition of polyamines into and within the porous matrices of agarose microbeads that "grow inwards". This novel process is driven by a polyamine (e.g. polyallylamine and branched polyethyleneimine) concentration gradient between the exterior and interior environments of agarose microbeads

causing the polymers to diffuse into the microbeads. The details of the interaction between the polyamines and the agarose polymers are not fully known and still under investigation. However, an initial assessment is that certain distribution coefficients for the polymer exist between the organic solvent and the aqueous agarose matrix causing the deposition of the well-defined shells on the agarose matrix. After a first shell is formed, further addition of polymer will again result in in-diffusion of polyamines and the formation of another inner shell within the agarose microbeads. It should be noted that the shells are formed by the same polymer and not as in LbL from complementary polymers in charge or HB-bonding capabilities. Finally, these self-assembled polyamines can be "immobilized" within agarose microbeads through the use of bi-functional amino cross-linkers or polymers of opposite charge (e.g. polystyrenesulfonic acid) (Scheme 1-6)[64]. Although it appears that the "inwards buildup self-assembly" technique is limited to hydro gel type templates such as agarose, this alternative technique can however fabricate unique core-shell materials and perform biomolecular encapsulation tasks that are not feasible with the conventional LbL technology. Typically a self-assembled LbL polymer layer is ~3–5 nm thick due to its self-limiting characteristic; in contrast, polymer layers assembled by the "inwards buildup self-assembly" technique are limited by the amount of polyamines available or the incubation time. As a consequence, the thickness of self-assembled polymer layers of the "inwards buildup self-assembly" type is easily tunable by varying the incubation time and polyamine concentration and micrometer thickness of polymer layers was demonstrated to be easily achievable within minutes

and in a single step. In addition, the density of the self-assembled polymer layers obtained via the "inwards buildup self-assembly" technique is tunable by the density of the hydrogel material forming the microbead template[64].

Encapsulation of Biomolecules Using the "Inwards Buildup Self-assembly" Technique

LbL encapsulation and retention of macromolecules of relatively low M_W is a challenge due to their relative good permeability through self-assembled polymeric shells. Reduction of permeability for successful biomolecule encapsulation was demonstrated by a post-treatment of the LbL layers [68]. Advantageously, by employing the "inwards buildup self-assembly" technique, polyallylamine shells of micrometer thickness could be easily achieved within 2 hours to encapsulate and retain relatively low M_W dextran (M_W 4 kDa)[64]. Interestingly, by using branched polyethyleneimine a decrease in shell thickness and by using lower agarose % a decrease in shell density was observed, in both cases permeability is increased and thereby providing means to easily control permeability. Core-shell materials obtained by this method are good candidates for passive release applications such as drug delivery. A "redox responsive" core-shell material was obtained by using cleavable disulfide cross-linkers (-SS-) for polyamine fixation during fabrication. An immediate release of biomolecules was demonstrated after addition of a reductive agent (dithiothreitol) to emulate reductive physiological conditions.

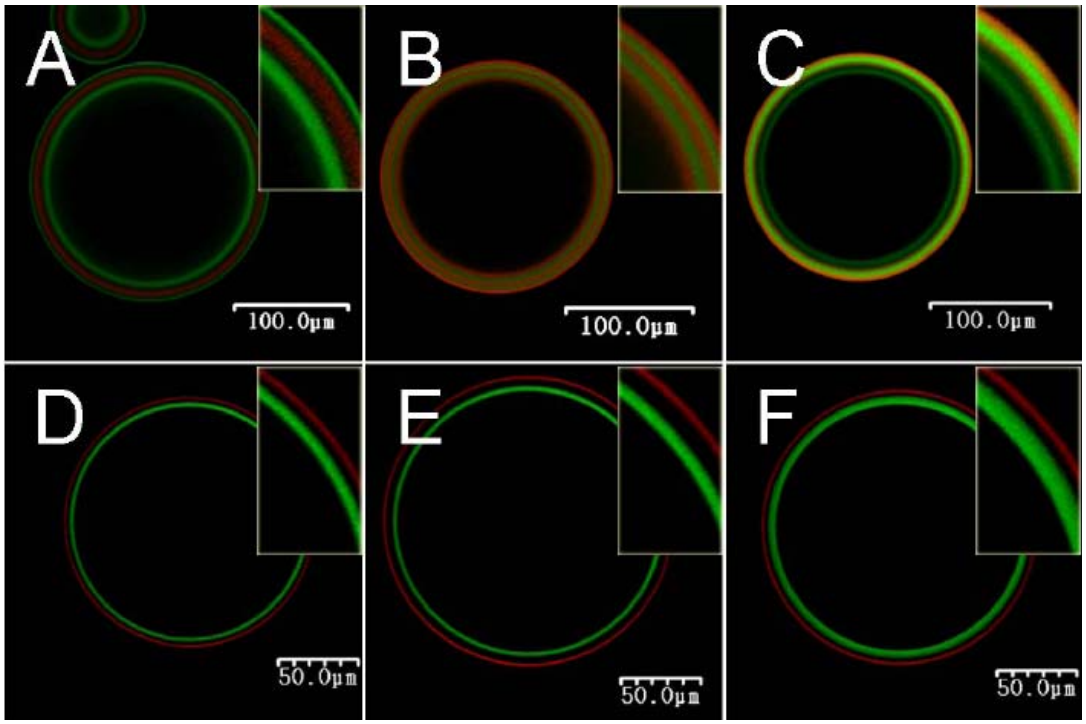


Figure 1-6: Confocal images of agarose microbeads demonstrating color permutation of layers generated by the inwards build up technique.
(A-C) Confocal images of agarose microbeads in 0.01x PBS with 5 concentric layers of different color coding permutations (R – RED, G – GREEN, B – BLANK). Fabrication was done in the following order: Layers 1/2/3/4/5 (A) G/B/R/B/G (B) R/G/R/G/R (C) R/G/R/B/G. (D-F) Confocal images of agarose microbeads in 0.01x PBS with 3 concentric layers (Layer 1/2/3) of the same color encoding permutation (R/B/G) but with different thickness permutations due to the variation of the polymer amount by using different volumes. (D) 500 µl /500 µl /500 µl (E) 500 µl /1 ml /500 µl (F) 500 µl /500 µl /1 ml. The insets in the confocal images are magnified images of the fluorescence layers (image taken from reference[59]).

Encoding of polymer self-assembled capsules has been demonstrated using encapsulated CdTe nanocrystals of different fluorescence emission wavelengths in previous studies[69]. Advantageously, the "inwards buildup self-assembly" technique provides two modes for encoding: By permutation of the layer color and/or the layer thickness (Figure 1-6). Permutation of layer color was achieved through incubation of polyamines, conjugated with different dyes in a desired sequence while the

permutation of layer thickness was achieved by tuning the incubation time and polyamine amount (by volume or concentration variations).

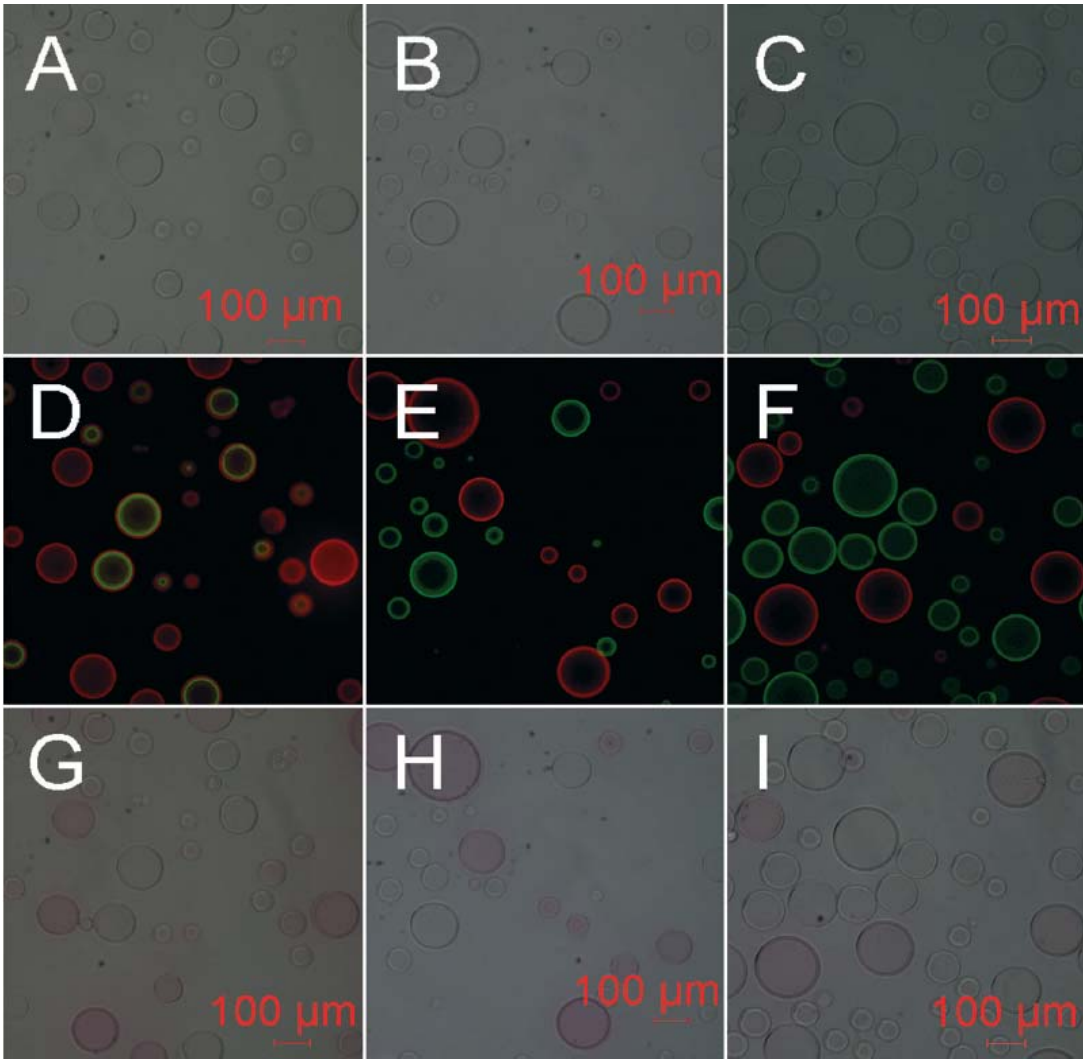


Figure 1-7: Demonstration of enzymatic viability in core-shell materials encapsulating HRP (labeled red only), encapsulating GOx (labeled green only) and encapsulating BSA (labeled green and red) used as a control.

Optical transmission images of A) HRP and BSA filled capsules, B, C) HRP and GOx filled capsules, and corresponding overlapping FITC and TRITC fluorescence images of D) HRP and BSA filled capsules and E, F) HRP and GOx filled capsules before addition of substrates. G, H) Addition of H_2O_2 and Ampliflu Red (AR) to the HRP and BSA filled capsules (G) and HRP and GOx filled capsules (H). After 10 seconds, only the HRP filled capsules were observed to turn purple. I) Addition of glucose and AR to the HRP and GOx filled capsules. After two minutes, only the HRP filled capsules turned purple. A longer time was required for the H_2O_2 produced by the GOx filled capsules to diffuse to the HRP filled capsules to cause a color change (Image taken from Ref [59]).

Core-shell materials with multiplexing analytical capabilities based on encapsulated enzymes were fabricated using the "inwards build up "technique. Encapsulation of three different biomolecules (GOx, HRP and BSA) was performed within three different color-coded (green, red and green/red fluorescence respectively) core-shell materials. Then, the three capsule batches were mixed and split into three samples (Figure 1-7 A, B and C) in which the capsules look similar in white light microscopy. Through de-coding of the stained fluorescence dyes (Figure 1-7 D, E and F) by fluorescence microscopy, it was possible to distinguish the different types of core-shell materials in the samples. To confirm the de-coding HRP and GOx specific substrates where added causing typical staining of capsules (Figure 1-7 G, H and I). Results obtained in the work by Dr. Bai point out the potential and capabilities of the "inwards buildup self-assembly" technique to create multifunctional layer assemblies for encapsulation and decoding.

Conclusion & Outlook

The encapsulation of biomolecules within core shell materials prepared by RP-LbL or the inwards build was demonstrated to be superior for neutrally charged macromolecules such as dextrans compared to aqueous LbL in terms of biomolecule loading and encapsulation efficiency due to insolubility of biomolecules in organic phase. Yet, methods to achieve accurate control over the resulting biomolecule concentration within core shell materials and especially polyelectrolyte microcapsules are not reported within this chapter. A method that yields 100% encapsulation

efficiencies as well as accurate control over the resulting concentration will be explained in the second chapter of this thesis.

Organic solvents have often been claimed to be unfavorable for biomedical applications or industrial processes due to their toxicity or for economic reasons. In the case of aliphatic alcohols that are mainly used for RP-LbL and inwards build up, toxicity is not an issue except for methanol and to limited extends for 1-propanol, which can be easily avoided. Some aliphatic alcohols might even be consumed and are “Generally Recognized as Save” (GRAS) by FDA. The use of organic solvents is economically favorable when one takes into consideration that biomolecule loss using water based LbL encapsulation can be up to 90%. Biomolecules especially those that might be used as food supplements or for pharmaceutical purposes are generally costly. Encapsulation efficiency is close to 100% when employing organic phase based RP-LbL, which leads to the assumption that organic solvents are economically favorable when compared to water as deposition media. Furthermore, in the case of lower aliphatic alcohols, evaporation has inherently less energy requirement, fostering easier recycling processes of excess polymer or washing solutions when compared to water as solvent. Denaturation of biomacromolecules by organic solvents might be another concern in the biomedical or diagnostic field. This, however, only applies to proteins for which the tertiary structure is crucial, macromolecules such as DNA, RNA, saccharides, peptides, hormones (even insulin) and macromolecular drugs such as paclitaxel and it derivatives are generally not affected by organic solvents. The encapsulation of model enzymes (glucose oxidase (GOx) and horseradish peroxidase

(HRP)) with retained biological activity [67] further highlights the potential of using the RP-LbL technique to fabricate core-shell materials for biomedical or diagnostic applications. Much groundwork has been done to establish the RP-LbL and inwards build up method as alternative methods to aqueous based LbL and other LbL encapsulation methods. The focus of this work was to extend the spectrum of template materials to generate LbL microcapsules to those that are not feasible for other methods (e.g. small water-soluble molecules and water-soluble biomacromolecules).

Challenges of the future will be to create uniform, ideally spherical, microcapsules by the RP-LbL method from water-soluble template materials. Another interesting development is to perform the LbL process in microfluidic devices; up to date only water based microfluidics LbL is demonstrated[67]. Microfluidics based LbL in water or organic solvents is potentially very useful due to the high degree of control over the LbL process, microcapsule morphology and its speed. The relatively young RP-LbL technique has yet to prove its applicability in biomedicine and other fields as the 20 years old aqueous LbL technique did in recent years. Since RP-LbL and conventional aqueous LbL can be often used interchangeably for many applications, the RP-LbL might proof more suitable for applications in which minimum loss of often expensive biomacromolecules is crucial.

Chapter 2: Water-soluble Sacrificial Template Materials for Biomolecule Loaded RP-LbL Polyelectrolyte Microcapsules

Scope and Specific Aims of this Chapter

The first introductory chapter of this thesis reported on general possibilities to encapsulate biomolecules by Layer by Layer (LbL) self assemblies and especially on methods developed by the NanoBioanalytics laboratory using organic solvent as assembly media. This second chapter reports on a novel approach employing water-soluble sacrificial templates to address increasing interest to achieve greater control over biomacromolecular loading and higher encapsulation efficiencies for biomacromolecule-loaded microcapsules. [ii] In traditional LbL methods, aqueous solutions of polyelectrolyte salts in combination with water insoluble sacrificial template materials are used to prepare polyelectrolyte microcapsules that can be loaded with biomacromolecules.

[ii]This chapter has been published in part as a primary research article, text passages and figures are partially identical and no further reference are made to the article in the following.

Sebastian Beyer, Jianhao Bai, Anna M. Blocki, Chaitanya Kantak, Xue Qianru, Michael Raghunath and Dieter Trau. Assembly of Biomacromolecule Loaded Polyelectrolyte Multilayer Capsules by Using Water-soluble Sacrificial Templates. Soft Matter, (2012), 8, 2760-2768.

In the newly devised method, the aqueous phase was replaced with pure aliphatic alcohols (Reversed-Phase) to greatly enhance the retention of biomacromolecular cargo close to 100% during microcapsule preparation in this Reverse Phase Layer by Layer (RP-LbL) process.

Although the use of polystyrenesulfonic acid (PSS) and polyallylamine (PA) has been reported in chapter 1 for the fabrication of core shell materials, the formation of stable multilayered polyelectrolyte membranes onto water-soluble template materials by their sequential deposition from 1-butanol has neither been reported in the literature nor in this thesis before. The challenge to exert control over the biomacromolecule concentration within the template material and the resulting microcapsules was addressed by sacrificial template materials that are water-soluble and comprise of biomacromolecules embedded into a matrix of small molecular weight molecules such as glucose. Control over the concentration of biomacromolecules in the template material and microcapsules are conveniently exerted by adjusting weight ratios of the two template material components. This approach is envisioned to be applied alternatively to traditional polyelectrolyte microcapsule preparation techniques in cases were minute losses of expensive biomacromolecules is unfavorable or when accurate control over biomacromolecule concentration is important.

Specific aims of this chapter are:

1. To demonstrate that nm thin layer of polystyrenesulfonic acid and polyallylamine can be assembled multilayer wise from organic solvent such as 1-butanol.

2. To devise a protocol that allows the encapsulation of biomacromolecules into PSS/PAH polyelectrolyte multilayer capsules with efficiency close to 100%.

3. To demonstrate possibilities to control the concentration of biomacromolecules within polyelectrolyte multilayer capsules.

4. To compare the membrane permeability of the polyelectrolyte capsules obtained by the novel RP-LbL approach with that of capsules obtained from fabrication using aqueous solution of polyelectrolytes.

Introduction

The loss of biomacromolecules during fabrication of loaded polyelectrolyte microcapsules was briefly discussed in Chapter 1 and is as well a often reported problem in recent literature[28, 57, 70] by others. The loss of often expensive biomacromolecules during the fabrication of microcapsules with applications as micro or bioreactors[46, 71], biosensors[42], host for catalytic systems and lately as carrier for therapeutic agents for various potential biomedical applications[21, 28, 72], limits the success of those technologies which creates need for the advancement of encapsulation technologies.

High encapsulation efficiency and control over cargo concentration are pre-requirements for most commercial applications in pharmaceutical and other industries. Although polyelectrolyte microcapsules and the associated LbL technologies are envisioned for those applications, they often do not match this requirement. The root of the problems are the encapsulation procedures using the Layer by Layer (LbL) method in which alternating deposition of oppositely charged polyelectrolyte salts in aqueous solution is followed by template dissolution[73-75]. Three stages of that process were reported to be in particular responsible for low encapsulation efficiencies.

1. Incomplete incorporation or adsorption into template materials, especially when calcium carbonate or silica particles are used as templates[45, 57].

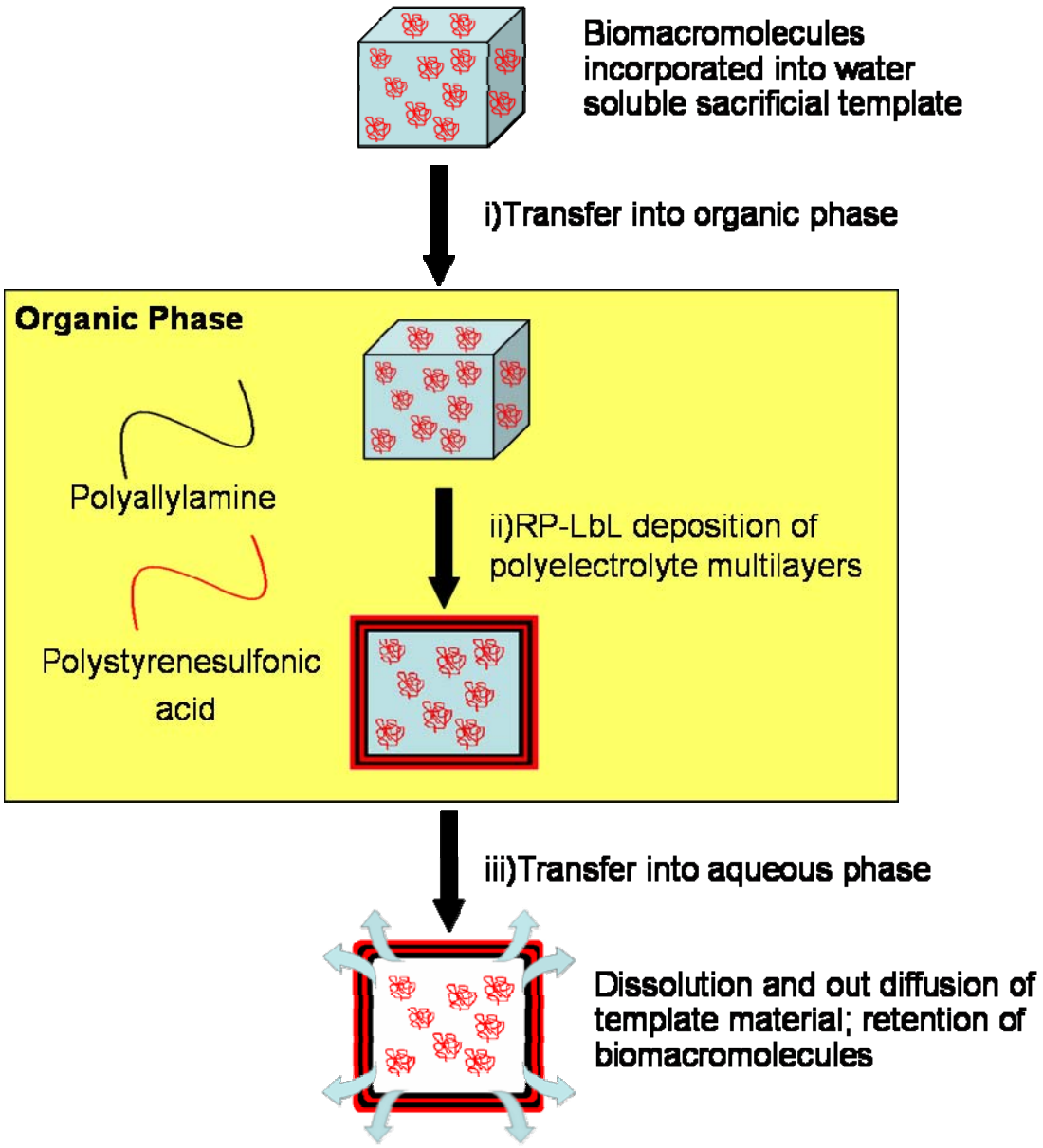
2. Out diffusion of biomacromolecules during encapsulation until a certain number of layers is established. This is most commonly observed when colloidal agarose hydro gel particles are used as template material[46].

3. A burst release of template material and biomacromolecules when the sacrificial template is dissolved after encapsulation, partially driven by temporary increase of osmotic pressure inside the capsule[70, 76].

Strikingly, as reported in Chapter 1 neither co-precipitation of calcium and carbonate ions with biomacromolecules, nor the absorption of the same onto silica particles yields the desired accurate control over the initial biomacromolecule concentration within the template material.

Alternatively, hollow polyelectrolyte microcapsules prepared by the sacrificial template method that initially does not contain any biomacromolecules might be loaded after fabrication through diffusion. This can be achieved by reversibly increasing the membrane permeability. Obviously, this loading method depends on and is limited by the concentration gradient between bulk phase and microcapsule interior[51, 77]. Only a fraction of biomacromolecules is encapsulated and much remains in the bulk solution. In this chapter, the question how accurate control of biomacromolecule concentration could be combined with high encapsulation efficiency into one feasible method is addressed. Since low encapsulation efficiency is

strongly associated with the template material – cargo interaction, the need for novel template materials is evident.



Scheme 2-1: Schematic of the newly devised encapsulation process.
From top to bottom: i) Transfer of solid water-soluble sacrificial template materials with incorporated biomacromolecules into an organic phase (yellow box). ii) RP-LbL encapsulation carried out in organic phase (yellow box). iii) Transfer to aqueous phase to dissolve the sacrificial template material.

High encapsulation efficiencies for biomacromolecules within polyelectrolyte microcapsules were previously reported using the so called RP-LbL method[58, 61, 66, 78] for which the possibility to use polystyrenesulfonic acid and polyallylamine are as well discussed in this chapter. In general, most accurate control over the biomacromolecule concentration can be achieved by preparing a template material that comprises a homogeneous mixture of biomacromolecules, as it is usually the case for aqueous hydrogels. However, accurate control over the concentration of biomacromolecules after the RP-LbL encapsulation of solidified homogeneous agarose solutions is limited by the dehydration that occurs upon interaction with organic solvents such as aliphatic alcohols[79]. Dehydration causes the agarose microbeads to shrink and thus leads to significant volume change. Furthermore, agarose hydrogel particles tend to aggregate in organic solvents. This needs to be overcome with colloidosomes[66] or surface-active agents[79] as reported in Chapter 1. Surface-active agents in combination with RP-LbL result in a weakened interaction of the LbL membrane with the agarose core and might cause membrane detachment upon transfer into aqueous phase[61]. A stable membrane assembled from organic phase for agarose-based core shell materials was reported for electrostatic or covalent cross-linking of 'inwards build up" polyamine layers[80]. This cross-linked multilayer within an agarose microbead enables good encapsulation efficiency and additional encoding capability[59]. However, unlike the RP-LbL method the 'inwards build up' of polyamine layers is not self-limited which impairs its application to templates in the size range of a few microns.

Thus, it is aim of this chapter to evaluate alternative template materials that allow for accurate control of biomacromolecule concentration in combination with high encapsulation efficiency to yield microcapsules with a low micron size range. The pivotal concept of this chapter is the use of water-soluble sacrificial template materials. Those water-soluble sacrificial templates comprise of a solid homogeneous mixture of biomacromolecules in a low molecular weight template material such as glucose. In this novel approach, solutions of polystyrenesulfonic acid (PSS) and polyallylamine (PA) in pure aliphatic alcohols are used with the RP-LbL method to create polyelectrolyte microcapsules with nm thin membranes. Polyelectrolyte hollow capsules are simply prepared by transfer into an aqueous environment. Upon transfer into an aqueous environment, the template constituents are simply dissolved and diffuse out, leaving biomacromolecules behind which are entrapped within the now hollow polyelectrolyte capsule. Concerns over the use of organic solvents and possible aggregation of polyelectrolyte microcapsules prepared by RP-LbL were overcome by the implementation of an aggregation-free phase transfer protocol.

Experimental Section

Materials

Polyallylamine 20% aqueous solution (Sigma-Aldrich) and polystyrenesulfonic acid 30% aqueous solution (Polysciences, US), both 70 kDa. Polystyrene particles with a diameter of 5.01 μm were purchased from Micro particles GmbH (Germany). Methanol and ethanol were purchased from Fisher Scientific (USA), 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol and 1-octanol were purchased from Sigma-Aldrich. All alcohols were anhydrous and of ACS reagent grade or of higher purity. Fluoresceineisothiocyanate, Dextran-FITC 4 kDa, Dextran-FITC 20 kDa, Dextran-TRITC 70 kDa, Dextran-TRITC 155 kDa, Dextran-FITC 500 kDa, glucose, sodium chloride and branched polyethyleneimine were purchased from Sigma-Aldrich. AFM equipment was purchased from Nanonics Israel.

Polyelectrolyte Hollow Shell Formation, Loading and Membrane Permeability Studies

Glucose particles for the formation of polyelectrolyte hollow shells were suspended in 1-butanol and directly ground in a mortar. The resulting suspension was filled in a 2 ml Eppendorf vial and the suspended particles were roughly separated by sedimentation in 1-butanol. By using only the slower sedimenting smaller particle fraction for experiments capsules with a similar size could be obtained. Solid mixtures

of glucose and fluorophore labeled dextran were prepared by first mixing solutions of 100 mg/mL glucose and 100 mg/mL dextran in ratios yielding different concentrations in the range from 0.5 to 5% w/w of dextran in glucose after drying completely. Sodium chloride and dextran-TRITC were mixed in a ratio of 98:2 w/w, dissolved in distilled water to a concentration of 100 mg/ml followed by drying and grinding in a mortar. Loading efficiency was assessed by change in fluorescence intensity of solid homogeneous mixture particles comprising of glucose and a varying degree of dextran-TRITC (155 kDa) suspended in 1-butanol. Fluorescence intensities were normalized with the intensity of the lowest concentration and expressed as an x-fold change and depicted as average values with indication of their standard errors. Encapsulation efficiency was assessed from microcapsules 5 minutes after transfer to water in the same way as the loading efficiency from micro particles. Membrane permeability was assessed by addition of water to microcapsule suspensions in ethanol. Careful attention was attributed to keep the final ethanol concentration below 10% to ensure dextran solubility. Template materials comprised of homogeneous solid mixtures of glucose and fluorescent labeled dextran of different molecular weight (4 kDa, 20 kDa, 70 kDa, 155 kDa and 500 kDa) at a concentration of 2.5% w/w coated with a total of 7 alternating polyelectrolyte layers with PA as first absorbed layer. Normalization of fluorescence intensities to the value obtained directly after addition (<5 s) of water allowed comparison between average values and their standard errors between different batches of microcapsule with different fluorophores.

Phase Transfer Protocol for RP-LbL Microcapsules

RP-LbL coated glucose particles that might optionally contain biomacromolecules, were transferred from 1-butanol to ethanol to avoid phase separation and microcapsule aggregation during the addition of water. Typically 1.5 ml suspension of coated particles in 1-butanol was centrifuged and the supernatant discarded. 200 µl of 1-butanol was added to suspend the particles. It was empirically determined that addition of 1.3 ml pure ethanol yields aggregation free suspensions. Centrifugation of the ethanolic suspension, removal of the supernatant and re-suspension in 200 µl ethanol allowed the addition of water without phase separation or microcapsule aggregation. Washing the microcapsules with pure water with at least 4 repeated steps of centrifugation and supernatant removal leads to sufficiently low levels of ethanol for most cell culture related application.

Results & Discussion

Template Materials and Hollow Polyelectrolyte Microcapsule Formation

The advantage of the RP-LbL based polyelectrolyte microcapsule formation is the utilization of water-soluble template materials in which biomacromolecules of interest might be incorporated. Elegant, yet simple is this approach to generate hollow polyelectrolyte microcapsules by transfer into aqueous phase (which is the medium of choice for most biological applications). It is demonstrated in a simple set-up that the diffusion of water into an organic phase, is already sufficient to affect a gradual solvent exchange that allows the visualization of template dissolution, out diffusion and subsequent formation of hollow polyelectrolyte microcapsules (Fig. 2-1). A suspension of encapsulated glucose particles is placed on a glass slide covered by a thin microscopy glass cover slip. Water that is placed on the edge of the thin microscopic glass cover slip diffuses slowly into the space between the glass slide and the microscopy cover slip. The in diffusion of water into this system leads to a gradual dissolution of the template material and leaves hollow polyelectrolyte capsules behind. In contrast to previous studies in which polymers with a significantly higher cationic charge density were assembled on similar template materials from ethanol no shrinkage of capsules after core dissolution was observed[58]. Potential template materials besides glucose and other saccharides could be small molecular weight salts.

Fluorescent labeled dextrans were used as cargo material in this study because of their convenient availability in different molecular weights that allow assessing initial membrane permeability. We found that aqueous glucose solutions yielded a homogeneous distribution of fluorescent labeled dextran upon drying. In contrast, sodium chloride appeared to entrap fluorescent labeled dextran less homogeneously. Yet, sodium chloride might be preferable over glucose as template material where high encapsulation efficiency is more important than exact control over loading concentration. Although salt based template materials would exclude proteins as cargos that are salt sensitive, they could be useful for other macromolecules. Spherical sodium chloride particles in colloidal dimension might be especially useful for microcapsule fabrication[81]. The fabrication of spherical colloidal glucose particles with controlled size remains a challenge to be solved. Although spherical template materials are often desired, anisotropic capsules[82] have proven useful for biomedical application[25] for which this approach provides a good fabrication strategy. No dissolution of glucose, sodium chloride or dextran was observed in 1-butanol. While simple sugar molecules such as glucose or fructose can be dissolved in lower alcohols (methanol or ethanol), the solubility of sugars decreases strongly with increasing complexity or with decrease of the polarity of the alcohols [83]. In contrast to traditional LbL approaches[46, 57, 70], no loss of biomacromolecules during RP-LbL membrane assembly was observed.

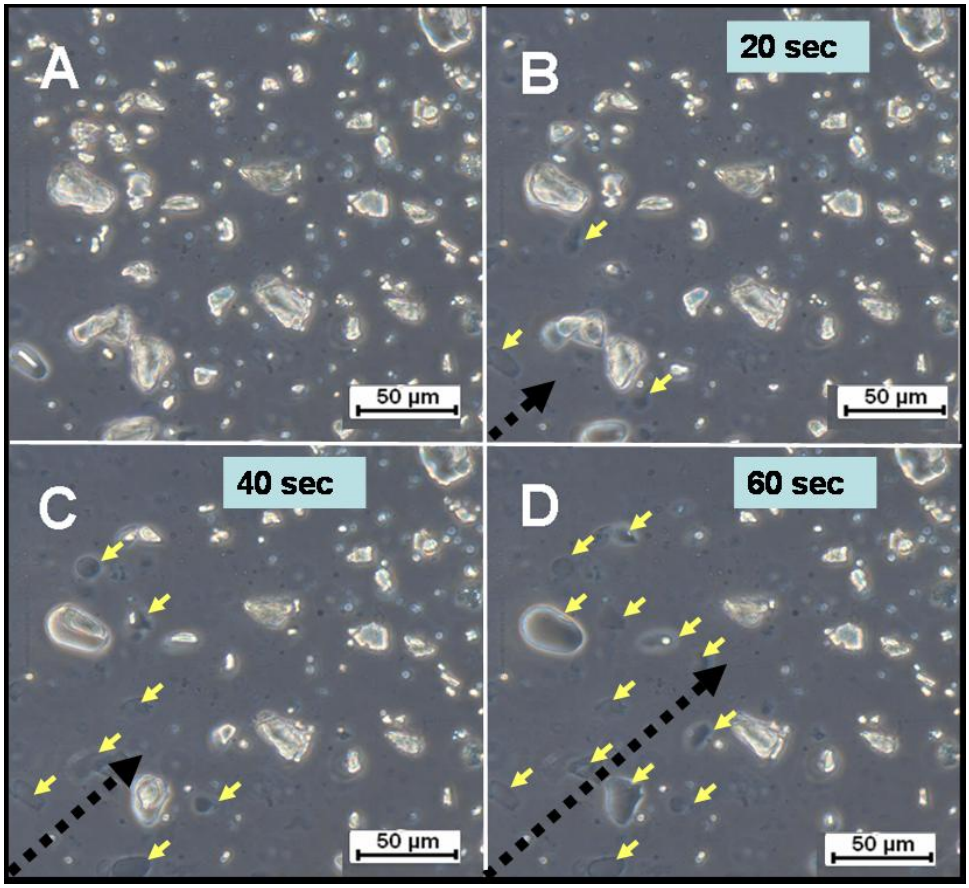


Figure 2-1: Phase contrast microscopy demonstrating gradual dissolution of water-soluble sacrificial template material and hollow polyelectrolyte capsule formation within 60 seconds upon contact of RP-LbL coated particles with aqueous phase.
Suspension of encapsulated glucose crystals in ethanol (A). A drop of water was placed on the left bottom corner of the cover slip, upon which water diffused (indicated by black dotted arrows) towards the center (B). The solubility of glucose in the resulting ethanol/water mixture is sufficient to cause rapid core dissolution (C&D) and hollow shell formation (small yellow arrows).

Biomacromolecule Loading

Capsules comprising of (PA-FITC/PSS)$_2$PA were loaded with TRITC-labeled dextran (Figure 2-2) with a molecular weight of 155 kDa. Capsule loading was achieved by encapsulating crystals comprising of sodium chloride or glucose and dextran-TRITC with an RP-LbL protocol followed by dissolution of the template by addition of water. Salt or glucose is released from the polyelectrolyte capsule whereas the macromolecular weight dextran-TRITC was retained. The entanglement of TRITC

labeled dextran within the polyelectrolyte membrane (Figure 2-2 C, red channel) upon core dissolution was reported earlier by other groups [84, 85] for aqueous LbL and is attributed to the dissolution process. In contrast to previous reports where crystals of pure macromolecular biomolecules were encapsulated[58] membranes did not burst upon transfer to aqueous environment. The biomacromolecule concentration can be controlled by this method and thereby ensuring stable osmotic pressure. Osmotic pressure within polyelectrolyte microcapsules is a important parameter and its control allows accurate design of capsule morphology, even their fusion[86]. Sodium chloride might be used as water-soluble sacrificial template material due to the ease of drying compared to saccharides, but especially proteins are generally sensitive to higher salt concentrations. Two parameters are crucial for water-soluble sacrificial template materials in order to achieve good control over biomacromolecule concentration within polyelectrolyte microcapsules.

1. The template material must allow homogeneous distribution of biomacromolecules at defined concentrations.

Accurate control over biomacromolecule concentration within template materials can be achieved by adjusting the weight ratios of small molecular weight template materials (e.g. glucose) and biomacromolecules.

2. Minute amount of moisture have to be removed by vacuum drying to allow the glucose based template material to be crushed into micron sized fragments.

To avoid denaturation of proteins the temperatures should be kept below 40 °C. Interestingly, proteins tend to be more stable against denaturation with increasing

concentration of small molecular weight sugars, since the activation energy of unfolding increases likewise[87].

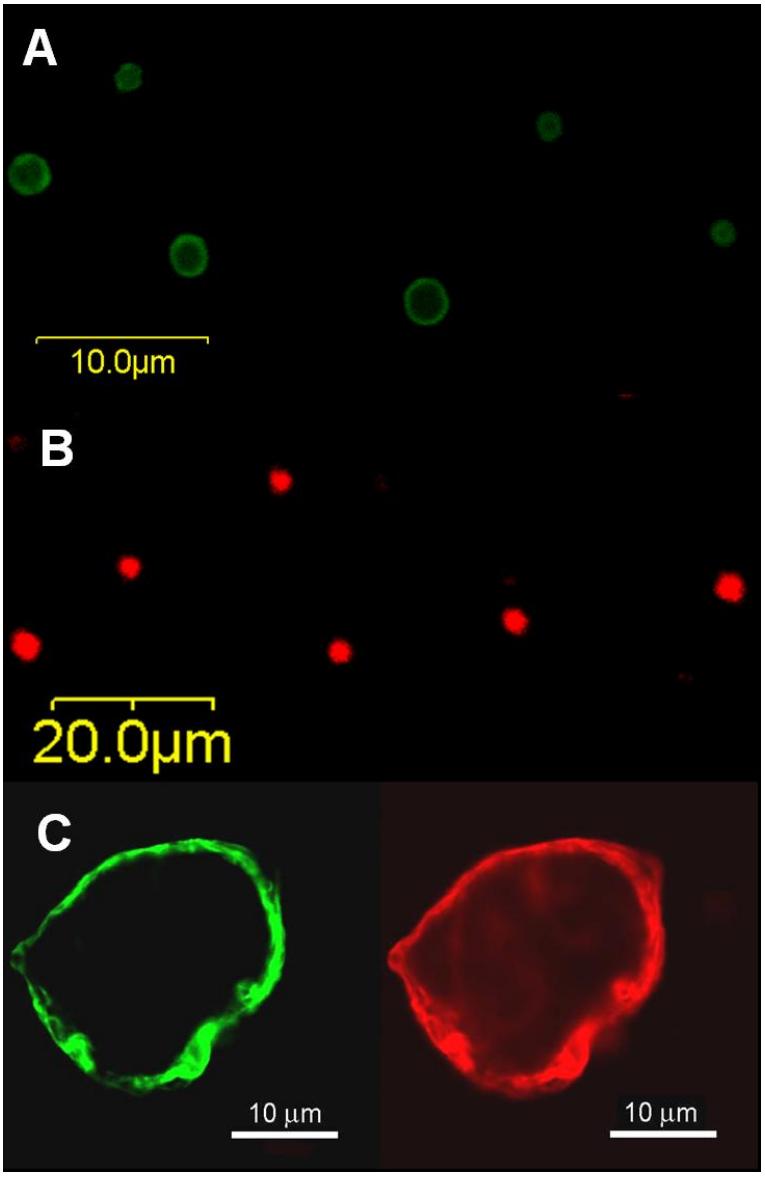


Figure 2-2 Confocal laser scanning micrographs of different polyelectrolyte capsules dispersed in water.
Microcapsules were produced by means of RP-LbL and Glucose (A&B) or sodium chloride (C) as water-soluble sacrificial template. Pictures A and B depict different sets of microcapsules. Micrographs with FITC excitation visualize only the microcapsules membrane (A). TRITC-excitation (B), gives a signal that spans the entire lumen and membrane of the capsule, demonstrating overall distribution and retention of dextran-TRITC (155 kDa) within the hollow capsules. Picture C shows a single capsule of which the membrane was fluorescent labeled with FITC and that contained Dextran-TRITC simultaneously.

Biomacromolecule Retention and Encapsulation Efficiency

A clear relationship between the initial biomacromolecule concentration in the template and fluorescence intensities of resulting polyelectrolyte microcapsules was observed with fluorescence microscopy (Figure 2-3) for a relatively large dextran-FITC conjugate of 155 kDa used as biomacromolecule.

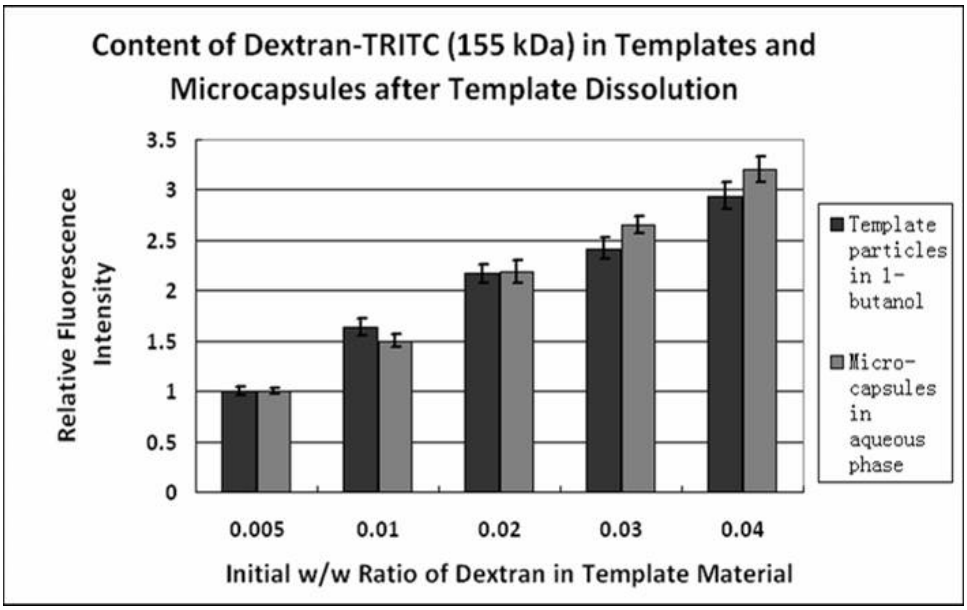


Figure 2-3: Fluorescence signal of RP-LbL coated $(PA/PSS)_6PA$ particles of dextran-TRITC homogeneously distributed within glucose.
The capsules are dispersed in 1-butanol (black) and polyelectrolyte microcapsules resulting from the same batch of particles upon transfer to aqueous phase and subsequent core dissolution (grey).

Apparently, the nature of common fluorescence microscopy limits the accuracy of information that can be obtained from multi shaped and polydisperse particles. Furthermore, very high concentration of fluorescently labeled biomacromolecules might quench the fluorescent signal obscuring the expected direct proportional dependency between signal and initial concentration. Yet, no escape of fluorescent labeled biomacromolecules into the surrounding solvent was observed during

membrane assembly, thus, suggesting that encapsulation of the biomolecular cargo occurred quantitatively. However, the current results are more of qualitative nature and the supernatant that evolves from various steps of the process should be carefully checked for fluorescence, which would indicate membrane permeability towards the macromolecules or membrane rupture. Most crucial for yielding high encapsulation efficiency is the time span of template dissolution[76]. Fast template dissolution leads to a temporarily increased osmotic pressure resulting in a slightly swollen capsule with a membrane of higher permeability. Such swollen capsules often facilitate out diffusion of the biomacromolecular cargo until osmotic pressure decreases and the membrane becomes impermeable. This phenomenon has been described as burst release especially for traditional aqueous based LbL approaches using calcium carbonate template materials in which a high ion concentration decreases the strength of polyelectrolyte interactions and thereby additionally increases the membrane permeability.[70] Even relatively large proteins such as BSA-TRITC with a molecular weight of 68 kDa have been observed to leave the otherwise impermeable capsule during the burst release phenomenon[57, 70]. Thus, we paid particular attention to potential occurrence of burst release of the biomacromolecular cargo occurs during transfer of RP-LbL coated particles to aqueous phase with subsequent template material dissolution. The following cargo release study only addresses the question whether burst release of macromolecules might occur or not and longer release studies would be necessary to study the release profile of macromolecules for applications such as drug delivery. For this purpose we monitored the out diffusion of

fluorescently tagged dextrans over a wide range of molecular weights (4 kDa to 155 kDa). Interestingly, we did not find evidence for such a sudden and early cargo release from RP-LbL coated template for dextran-FITC conjugates (4 kDa to 155 kDa). Although, release of smaller dextrans (20 kDa & 4 kDa) was observed to occur over a longer time frame and is attributed to the membranes semi permeability. This release of smaller molecular weight dextrans occurs fairly fast within a few minutes (Figure 2-4) to a certain extend until an osmotic equilibrium between the capsule surrounding and their interior is established followed by a slow release based on diffusion through the membrane. The fabrication protocol allows for easy preparation of LbL coated microparticle suspension in edible oils that provides avenues for drug delivery if capsule material and solvents are chosen accordingly. A increase of ionic strength is known to increase polyelectrolyte permeability towards a higher cut off weight[88]. In contrast to aqueous LbL that often uses calcium carbonate or silica particles as template, no increased salt ion concentration occurs upon dissolution of glucose based template materials in RP-LbL. On the other hand, the transfer of polyelectrolyte microcapsules from aqueous solutions that contain organic solvents to pure water, is known to decrease the polyelectrolyte membrane permeability[51]. Those reported polyelectrolyte membrane responses to salt or solvents thus support the idea that circumstances during hollow capsule formation and template dissolution contribute to a high retention of biomacromolecular cargo and prevent a burst release. The release during the template material dissolution was studied by observing the change in ratio of fluorescence intensities between the microcapsules and their

immediate vicinity over a time of 3 minutes (Figure 2-4). A time of 3 minutes was empirically determined to guarantee complete template material dissolution. The data indicate that the resulting polyelectrolyte microcapsules are impermeable for macromolecules with a molecular weight >70 kDa during core dissolution and while dispersed in aqueous phase at least for the investigated time frame. Most important for many applications of polyelectrolyte microcapsules such as in diagnostic applications is the semi permeability of the membrane that allows interaction of small molecular weight components with the retained biomacromolecules.

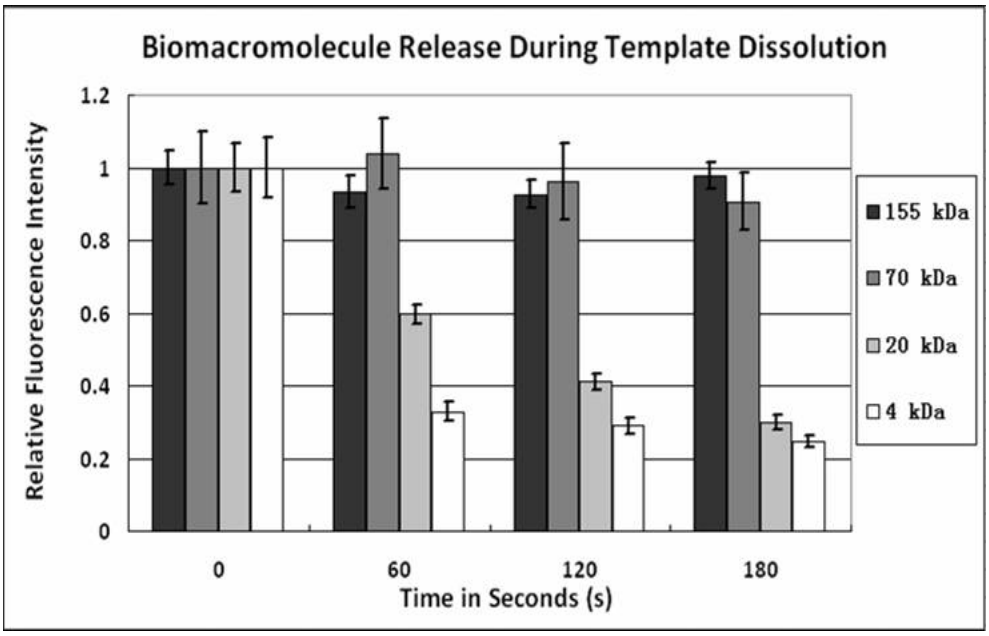


Figure 2-4: Change of fluorescence intensity (normalized) from polyelectrolyte microcapsules containing fluorescent labeled dextran of various molecular weights plotted against time.
No significant decrease of fluorescence intensity can be observed from microcapsules containing dextran greater than 70 kDa indicating the membranes cut of weight to be between 20 kDa and 70 kDa.

Indeed, the membranes of the polyelectrolyte microcapsules presented here demonstrate this feature. This semi permeability of the capsule membrane is in accordance with earlier investigations on membrane permeability for microcapsules

comprising of the same polymers prepared by the traditional aqueous approaches[89]. Although similarities of membrane properties between the traditional aqueous approach and the newly devised RP-LbL approach using water-soluble sacrificial template materials have been pointed out, more detailed investigation on long term retention stability and cut of weight might be worth to be addressed in dedicated studies.

Concerns over the use of organic solvents might apply for biomedical application that requires the interaction with cells or tissue. For this reason, aliphatic alcohols were chosen as solvent. Many aliphatic alcohols are reported to be non toxic or are even considered to be Generally Recognized as Safe (GRAS) by the Food and Drug Administration of the United States of America[90]. Furthermore, microcapsule aggregation did not even occur in cell culture media by using the phase transfer protocol as described in the materials section.

Conclusion

A newly devised approach to fabricate biomacromolecule loaded polyelectrolyte microcapsules via RP-LbL using water-soluble sacrificial template materials is presented in this chapter. Besides the very high encapsulation efficiency that is generally reported for RP-LbL due to the insolubility of biomacromolecules in organic solvents, the novel class of template materials offers alternative approaches to fabricate PSS/PAH polyelectrolyte multilayer capsules. From the perspective of

potential applications, the use of aliphatic alcohols for the deposition of PSS/PAH polyelectrolyte multilayer provides new possibilities to use macroscopic, planar, colloidal or other template materials that were previously excluded from LbL applications due to their solubility in very polar solvents (e.g. water, dimethylformamide, ionic liquids) that are traditionally used. As final conclusion, a new class of templates was introduced leading to substantial improvements of encapsulation efficiency and concentration control of biomacromolecule loaded polyelectrolyte microcapsules.

Specific aims of this chapter are:

1. To demonstrate that nm thin layer of polystyrenesulfonic acid and polyallylamine can be assembled multilayer wise from organic solvent such as 1-butanol.

This specific aim was fulfilled by demonstrating hollow polyelectrolyte multilayer capsules remain after the water-soluble glucose core was dissolved.

2. To devise a protocol that allows the encapsulation of biomacromolecules into PSS/PAH polyelectrolyte multilayer capsules with efficiency close to 100%.

The RP-LbL technique allows the encapsulation of biomacromolecules due to their insolubility in organic solvents in addition PAH and PSS in their respective free acid and free base form exhibit good solubility in lower aliphatic alcohol. No dissolution

of biomacromolecules was observed during the encapsulation process. This specific aim has thus been fulfilled.

3. To demonstrate possibilities to control the concentration of biomacromolecules within polyelectrolyte multilayer capsules.

The initial loading concentration can be adjusted by varying the ratio of biomacromolecular cargo to water-soluble sacrificial template material. No cargo material was observed to dissolve into the organic phase during encapsulation. Fluorescence microscopy studies revealed that the initial ratio is maintained upon transfer to aqueous solution and after template material dissolution.

4. To compare the membrane permeability of the polyelectrolyte capsules obtained by the novel RP-LbL approach with that of capsules obtained from fabrication using aqueous solution of polyelectrolytes

The membranes of polyelectrolyte micro capsules prepared by the RP-LbL technique exhibit a similar semi permeability compared to polyelectrolyte microcapsules prepared by conventional approaches, as demonstrated by comparing the retention of fluorescent labeled dextrans with different molecular weight.

Chapter 3: Properties of Polymers in Solution and Characterization of Polyacids

Scope and Specific Aims of Chapter Three

In order to understand the physico-chemical peculiarities during the RP-LbL assembly process better, it is scope of this section to characterize solutions of polyacids in organic solvents. In addition, it is the aim of this chapter to provide a rule of thumb to assess whether potentiometric measurements directly yield accurate parameters of the polyacid in organic solvents such as the autoprotolysis rate or other acid constants. It will be established that potentiometric measurements yields basic information about the polyacids in organic solvents under certain conditions where no counter ions or protons condense at the polymer backbone but are freely distributed in the entire solution volume. The advantage of potentiometric measurements over other methods currently used to determine properties of polyacids is that they are easily conducted with minor equipment usually present in every scientific laboratory.

Specific aims of this chapter include:

1. Development of an experimental setup that allows potentiometric measurements on polyacids in non-aqueous solvents.
2. Theoretical consideration whereas auto-protolysis of polyacids can be measured straight forward in organic solvents or not.

3. Establishment of a simple relation between the pKa value of an acid in an organic solvent that describes the monomer unit best and the applicability of potentiometric measurements to the related polyacid dissolved organic solvents.
4. Determination of pKa values for paratoluenesulfonic acid in linear aliphatic alcohols.
5. Determination of apparent pKa values of polystyrenesulfonic acid in linear aliphatic alcohols.

Introduction

One of the limiting problems in nowadays research is that a vast amount of data is produced and theoretical models are dramatically refined separately within their respective sub-research field to an extend where less and less synergistic effects are generated by the whole field. One example of this problematic is polyelectrolyte and polyelectrolyte self-assembly research. Physicist and theoretical chemists refine models to describe the accurate behavior of polyelectrolytes in solution[91] while practical chemists, nanotechnologists and materials scientists produce self-assembled materials by "gut-feeling" neglecting theoretical approaches that are sometimes tough to translate into a practical context. Yet, advancement in self-assembly technologies will come to a halt when the gut feeling is not sufficient to further enhance the properties or function of self assembled materials. Much effort has been attributed to characterize the theoretical aspects of polyacid dissociation mainly on polycarboxylic

acids[92-98]. Polystyrenesulfonic acid is of special interest in the scope of this work and in general due to its use for doping conjugated polymers[99, 100] for semiconductor or organic solar cell applications. Parameters, such as its acidity in terms of ability to interact with conjugated polymers is a crucial parameter especially when the polymer is doped by acid base chemistry as it is the case for polyaniline[101]. Polystyrenesulfonic acid might also be used as a catalyst[102] or as component in ion exchange resins where acidity is a crucial parameter[103]. The aforementioned applications have in common that they often employ organic solvents to facilitate the utilization of water insoluble components.

Particular interests of this thesis lies within the self-assembly of polyacids and polyamines from organic solvents to form polyelectrolyte multi layer structures. For the latter application, it has been shown for aqueous phase that polymer conformation, and solution properties are crucial parameters for the rational design of polyelectrolyte multilayer structures[104, 105]. One of the most crucial underlying parameter that is used to describe polymers by various theories is the charge density or fraction of charged monomers on the polymer backbone[91]. The charge density on a polyacid is directly linked to its autoprotolysis in solution. However, the determination of the autoprotolysis constant is not as straight forward as for mono protic acid in aqueous solutions for various reasons that will be explained in the course of this chapter. The determination of polyacid dissociation constants especially of PSS has been attempted by conductivity and spectroscopic measurements[106, 107].

Conductometric measurements are however difficult due to the low ion concentration and low permittivity within organic solvents. Spectroscopic measurements might be time consuming and require special instrumentation such as a Raman spectrometer.

Thus, this section aims to give a guide based on the Oosawa-Manning and refined pH theories to which extend potentiometric measurements are easily applicable to polyacids in organic solvents.

Furthermore it is scope of this section to provide a rule of thumb to assess whether potentiometric measurements directly yield the desired parameters of the polyacid in organic solvents.

Theoretical Consideration and Experimental Setup

Solvent Properties

The RP-LbL method mainly uses solutions of polyacids and polyamines in aliphatic alcohols. Many theories of polyelectrolytes and polymers in solution require the assumption to be true that the solvent constitutes of a Θ (Theta) solvent. The theta condition is a special case of solvent quality. In general solvent quality refers to the nature of interaction between the polymer and its coordinated solvent molecules. A good solvent will cause the polymer coils to expand in solution whereas a poor solvent causes a stronger polymer-polymer self interaction which results the coils to contract in solution. The quality of the solvent is a property that depends on both, the nature of the polymer and the solvent molecule as well as the temperature[108]. An

example that illustrates these properties is that of a hydrophobic polyelectrolyte in solution. In pure water the solubility is given due to the high charge density, yet a dense coil can be observed due to the hydrophobic-hydrophobic interactions of the polymer chains[109]. The dense coil starts to expand once organic solvents are added in small quantities which decrease the polarity of the solvent to a slightly more hydrophobic environment which constitutes a solvent of better quality[110]. Another temperature dependent example can be found in the conformational transition of proteins upon increase of temperature[111]. The protein changes its conformation to achieve an energetically more favorable conformation at increased temperature and might become insoluble.

A theta solvent, which is assumed for many theories that describe the conformation of polyelectrolyte in solution, constitutes of the following special case. The theta solvent condition is fulfilled for a solvent –polymer pair at a specific temperature when in addition to the above named properties, the polymer (non charged) adopts the same conformation in solution as it has in the bulk phase[112]. On example that visualizes this condition in general is the dissolution of proteins in water and their structure determination from crystal by x-ray diffraction analysis. The proteins are assumed to have the same conformation in water as a theta solvent as they appear to have in crystal form. In the case of a protein, water might not necessarily be theta solvent. This is due to the fact that under biological conditions no pure water but aqueous buffers are present. The aqueous buffer systems, which are native for all natural proteins, comprise of various organic and inorganic salts that largely determine

solvent properties. Crystallization of proteins might thus be attempted from aqueous buffers for which the same assumptions should be true.

RP-LbL uses mainly aliphatic alcohols which are considered good solvents for polystrenesulfonic acid and polyallylamine. The amphoteric nature of aliphatic alcohols allows interaction with the polar and unpolar groups of the polymers. In the case of polystyrenesulfonic acid, aliphatic alcohols provide good conditions for proton dissociation since alcohols might be considered homologues of water in this case. Thus, lower aliphatic alcohols (C_1-C_4) and water are assumed to be Θ solvents for polyallylamine and polystrenesulfonic acid over a broad temperature range.

Polymers in Solution

Properties of Polyelectrolytes in Aqueous Solution:

Polyelectrolytes used in aqueous based LbL are most commonly alkali salts of poly carboxylic acids, polysulfonic acids and polyammonium or quaternary polyalkylammonium salts with a halogenide counter ion. The nature of those polymers implies a high charge density per polymer molecule in aqueous solution. Since the charge is the same between the monomer units[113], charge – charge interaction within in a polyelectrolyte are repulsive. It is easy to visualize that a polyelectrolyte thus appears to be in a stretched form when dissolved in pure water. Shielding of the charges around each charged group is possible by addition of salts, gradually changing the polyelectrolyte conformation from a stretched to a coiled form[113]. The addition of water miscible organic solvents leads to a decrease of solvent

quality, leading to a more coiled polymer conformation. So far only polyelectrolyte salts, able to dissociate to a large extend in aqueous based solutions with a significantly decreased ionic bond dissociation causing polyelectrolyte precipitation at high solvent concentration were reviewed. In the case of the "RP-LbL" and the "Inwards build-up" techniques, polyamines in free base form and polyacids as free acid are dissolved in organic solvents. Those polymers exhibit excellent solubility in solvent mixtures and in pure aliphatic alcohols with various polarities. The solvent quality might thus not decrease that dramatically with decreasing permittivity, as it is the case for polyelectrolytes used in aqueous LbL. Furthermore, amines do not have a significant charge in organic solvents and can be considered neutral. The spatial conformation for non charged polymers in solvents is well explained and can be considered a sphere or a coil in a Θ solvent. If the solvent quality decreases the sphere might appear more dense and of smaller radii [114]. Furthermore, especially in the case of polyamines, inter and intra molecular hydrogen bonding might play an important role for solution behavior of polymers, as hydrogen bonding is not possible between poly ammonium cations in aqueous phase. The most crucial property for the RP-LbL assembly process is that the polymer molecules do not 'feel' or at least a much reduced electrostatic force between them in solution due to the absence of charges. The absorption at neutral or weakly charged interfaces is as well governed by different parameters than that of polyelectrolytes. Neutral polyamines that have absorbed on an interface will exhibit no electrostatic repulsion to polyamines in solution, as it would be the case for their respective polyelectrolyte salts. The

absorption of neutral polymers at interfaces and the formation of coherent nm thin polymer layer has been extensively investigated and reviewed[115] and will not be reported in detail in this thesis. A self-assembly process based on neutral polymers in organic solvents or water is known as hydrogen bonding LbL which has been extensively reviewed as well[12].

Properties of Polyacids in Solution:

Polyacids are a much more complex system in solution when compared with neutral polyamines. Their charge density and thus their conformation varies strongly due to several parameters such as inherent acidic strength of the monomer units, the solvent properties in terms of stabilization of dissociated species, solvent properties in terms of electrostatic interaction and other environmental parameters such as temperature.

Determination of the Degree of Dissociation of Polyacids in Organic Solvents

The determination of the correct degrees of dissociation of polyacids in organic solvents is not a problem that can be solved straight forward.

1. The degree of dissociation is often determined via the apparent pKa value which is itself a function of the degree of neutralization[92]. For nanotechnology self-assembly systems, the intrinsic apparent pKa value where no base is added to the solution is often considered most important. Determination of polyacid properties by titration with bases is difficult since it requires the

assumption to be fulfilled that neutralized acid groups dissociate completely into ionic species. This, however, can only be sustained in water since organic solvents are known to prevent complete salt bond dissociation[116].

This problem is overcome by choosing not to draw information on the dissociation constant of the polyacid through titration. The titration curve of polystrenesulfonic acid appears to be linear if the degree of titration is less then 30%. A linear regression function through this linear titration range is extrapolated to 0% degree of titration and the calculated pH value is considered the bulk pH of the solution. The reason to adopt this method rather than performing a one-point measurement to determine the pH of the polyacid solution is because of the reading of the pH electrode that is relatively unstable at 0% titration which might be attributed to the low ionic strength of the media. Nonetheless, the first reading coincides well with the extrapolated value and was empirically found only to differ by less then 0.05 pH units.

2. The intrinsic polyacid properties might easily be investigated by potentiometric measurements or pH titration in aqueous media but not in organic solvents.

In organic solvents, liquid junction potentials at the pH electrode and other solvents effect on the proton activity have to be taken into account in order to deduct the true proton concentration from the measured (working) pH value.

The following paragraph “pH measurement in organic solvents” explains how a correction factor can de derived that corrects the working pH to the real pH that reflects the true proton concentration. The working pH is the value that is obtained with a standard pH electrode that uses an Ag/AgCl reference electrode and that was calibrated against aqueous buffers.

3. pH measurement of polyacids does not necessarily reveals accurate information on the degree of dissociation directly. This is due to the counter ion (protons in the case of polyacids) condensation in close proximity to the polymer backbone. pH measurements only reveal the bulk pH but not the local pH at the polymers backbone if counter ion condensation occurs.

Direct potentiometric measurements only leads to dissociation constant in cases where no counter ion condensation is to be expected according to Oosawa-Manning theory.

A more detailed explanation of the Oosawa-Manning theory and its application to polyacids will be given later in this chapter.

The accurate procedure to determine the degrees of dissociation of polyacids in non-aqueous media that was developed during the scope of this thesis is thus to divide the concentration of dissociated acid groups that can be obtained from pH measurements using the extrapolation method by the theoretical concentration of acid groups. The theoretical concentration of all acid groups (C_0) can be calculated from volume of a base solution that is needed to titrate the polyacid completely (determine moles of acid groups).

The degree of dissociation is a crucial parameter of polyacids in organic solvents since it leads easily to the fraction of charged monomers that is needed for theoretical considerations in polyelectrolyte theory. The theoretical considerations that become possible with the fraction of charged monomers of a polyacid and the polyacid concentration allows calculating the polymer conformation and other basic solution properties (e.g. if the solution is within the diluted or semi diluted regime). Those parameters allow to explain polymer absorption phenomena that were observed during the RP-LbL process or the '*a priory'* design of RP-LbL self-assembly conditions.

pH Measurements in Organic Solvents

The pH measurement is conveniently adopted to describe the acidity of aqueous solvents and is suitable for most applications in which a certain acidity needs to be maintained e.g. in biological buffers. However, when pH measurements are used to determine the actual proton concentration, it is far from being straight forward and many parameters have to be considered in order to allow assumptions about the proton concentration in solutions[117]. This is especially the case for non-aqueous solutions or mixtures of aqueous and non aqueous solution. This topic has been of intense discussion within the physico-chemical research community and several IUPAC commissions[118, 119]. The result of these discussions is the IUPAC recommendation to use a pH electrode with a common Ag/AgCl or calomel reference electrode in aqueous KCl solution that is calibrated with an aqueous buffer concentration for titration in non aqueous solvents. The obtained pH values during

titration need to be corrected by a certain factor in order yield the true pH value that can be used for calculating proton concentrations.

It was initially recommended to obtain the correction factor by determining the accurate value for the liquid junction potential and to use the established solvent transfer coefficients for protons from one media into another in order to account for the proton activity. The general characteristic of this correction factor is that it is a constant that is added to the working pH (the value that is given by a Ag/AgCl electrode using saturated KCL as a electrolyte and that was calibrated against a aqueous buffer). The addition of the correction factor is without any dependency on environmental conditions such as degree of titration or initial acid concentration as long as the same standard conditions (temperature, pressure, solvent purity) are maintained during which the constant was initially determined. This however seems to be a complicated procedure of little practical value when it is attempted to characterize an acid and not the liquid junction potential itself. It is important to recall how the pH electrode design and principles of measuring the pH are related to the above described procedure in order to judge the validity of a more practical solution that was chosen in this work.

The following paragraph aims to explain how pH was initially measured and related to proton concentrations, which implication the design of the pH electrode imposes on reliability of proton concentration measurements and which considerations need to be made when the measurement of potentials in organic solvents with a pH electrode

should lead to the actual proton concentration[iii]. The latter is crucial to determine parameters of polyacids in aliphatic alcohols such as degree of dissociation. Only with an accurate determination of those parameters, theoretical consideration of polymer conformation in solution and their absorption at interfaces becomes possible.

The pH was initially related to the change in electromotive force (EMF) of a cell of the following construction:

Pt; H_2(g) | reference solution || KCl solution 3.5 Molar || unknown solution | H_2(g); Pt

The change in electromotive force was then related by an ideal equation

$$E = \frac{R*T}{F} * \ln\left(\frac{C_{Hunknown}}{C_{H\text{Reference}}}\right)$$

The above cell architecture has is inherent property, that the half cell with the reference solution remains unchanged throughout a series of measurements. For this reason, the cell with a reference solution was replaced by a reference electrode (e.g. mercury-calomel or more practically silver-silver chloride) that is connected with the

[iii] The theoretical basics of pH measurement as well as all equations related to pH measurements in the following are largely drawn from the much respected review by R. G. Bates117. R. G. Bates, *Crc Cr Rev Anal Chem*, 1981, **10**, 247-278.. The author of this thesis would like to point out that the derivation of the equations as well as the related physicochemical circumstances and relationships are produced in the review by R.G Bates. The material by R. G. Bates is included into this introductory paragraph of the thesis in a shortened and altered way for the reason of a better understanding for the potential readership without further explicit referencing to the original work. However, the actual work by R.G Bates is supplemented with details that tailor the basics to the current problems or circumstances.

concentrated KCl solution of the salt bridge. The resulting cell architecture is still the same in most commonly found pH electrodes nowadays. The above stated relation can be rewritten as follows under consideration that the proton concentration is expressed as pH and that a reference electrode is introduced. The measurements of the change in EMF where then related by the equation

$$pH = \frac{E^{0'} - E}{\frac{RT * \ln 10}{F}}$$

The only parameter that has to be found in the above equation is $E^{0'}$ since E is measured in the solution of which the pH needs to be determined. $E^{0'}$ is determined by measuring solutions with known E or more precisely with known pH. Determination of E^0 is exactly the step that is most commonly performed through the calibration of a common pH meter. The equation that is used in today pH meters to relate the known pH of reference solutions to the unknown pH of a sample is:

$$pH(x) = pH(s) - \frac{Ex - Es}{\frac{RT * \ln 10}{F}}$$

pH(X) = unknown sample
pH (S) = Standard Reference
Ex = potential of sample solution
Es = potential of standard reference solution

The reference solutions are defined composition of salts with exact pH at a certain temperature. By convention, the buffer solutions have to be measured at 25 degree Celsius to allow an accurate calibration. Under a strict point of view the aqueous

buffers only have the correct concentration of protons as indicated by their pH if the correct temperature is maintained, since the proton concentration is the temperature dependent product of dissociation constants of various buffer systems. In practical point of view it should be at least attempted to do the measurement of sample pH at the same temperature as the calibration was done.

The above stated relationship was further refined by correct thermodynamical analysis of the reference cell that led to the following equation when the liquid junction potential at the bridge solution was ignored or eliminated and in which proton concentration (C_H) was replaced by the proton activity (a_H)

$$pH = -\log a_H = paH$$

Where a_H is the activity coefficient of protons in solution.

Practical Implications for pH Measurements

The above definitions (if strictly interpreted) are only valid if the standard Pt, H2 reference electrode, measures the change in EMF of an unknown solution. However, for practical reasons most commonly glass electrodes are utilized for pH measurements. The reason for this is that the membranes of glass electrodes behave very similar to the changes in hydrogen ion activity when compared to standard H_2 reference electrodes as long as they are in water. If the solvent changes from water to another medium, the glass of the electrode needs to be designed in an ambient way in

order to approximate the properties of the Pt, H_2 electrode as good as possible. For this and other reasons the ETOH-Trode from Metrohm AG, Switzerland was used in the scope of this thesis. The potential response of a glass electrode simulates very well that for the electron transfer reaction $2H^+ + 2e = H_2(g)$. Its potential changes with hydrogen ion activity (a_H) in accordance with the theoretical Nernst-slope

$$E_H = E^0 - \frac{RT}{F} \ln \frac{1}{a_H}$$

By convention, the E^0 is 0 for the standard hydrogen reference electrode. However, this is not applicable for other electrodes such as the commonly utilized glass electrode which is represented by $E^{0'}$value.

There are three possible sources of potential differences in the pH measuring cell, the potentials across the metal/solution phase boundaries at the indicator electrode (E_H), and the reference electrode (E_R) and the liquid-junction potentials (E_j) across the liquid-liquid boundary. The latter becomes especially important when not only the difference of concentration between aqueous solutions exists but also two different solutions build an ion impermeable interface. The potential can be imagined to be especially strong when one of the solutions does not allow the presence of ions (or only in much reduced concentration) that can be found in the other solution (e.g. K^+ or Cl^- at the water/methanol or water/ethanol junction). The total cell EMF is thus given by:

$$E = E_H - E_R + E_j$$

For practical reason it can be assumed that the standard potential of the indicator electrode and the reference electrode remain constant at constant temperature and pressure.

This allows the following expression:

$$E^0 - E_R = const = E^{0'}$$

Combining those equations yields

$$E = E^{0'} - \frac{RT}{F} \ln \frac{1}{a_H} + E_j$$

This fact allows writing the following equation:

$$paH = \frac{(E^{0'} + E_j) - E}{\frac{2.3026RT}{F}}$$

In praxis, when measuring the pH of aqueous solution the liquid junction potential is known to be negligible e or limited to minimum when saturated KCl solution is used. This represents a practical solution to ease pH measurements. This however is not applicable when different solvents are used, e.g. the reference electrode is immersed in saturated aqueous KCl solution while the indicator electrode is immersed in an alcoholic solution containing an acid.

In this case the pH measured paH(X) in relation the pH(S) of a standard reference is:

$$paH(x) - paH(s) = \frac{Es - Ex}{\frac{2.3026 * RT}{F}} + \frac{Ej(x) - Ej(s)}{\frac{2.3026 * RT}{F}}$$

This however does not respect the fact, that the activity coefficient of hydrogen changes dramatically when other solvents then water are used. For this reason, a closer look at the influence of the activity coefficient of hydrogen on the measured pH is necessary.

The changed activity coefficient and the liquid junction potentials have to be considered in order to allow the accurate determination of proton concentration from measuring the pH with a glass electrode that uses a Ag/AgCl reference electrode in saturated KCL solution. According to Bates Review from 1981 it is most *"convenient to shift the standard state in such a way that the activity coefficient (γ_s) of the solute species approaches unity at low ionic strength in the medium rather then in pure water*".

This leads to the following expression:

$$\gamma_s = \frac{\gamma_w}{\gamma_t}$$

In the above equation, γ_w represents the activity coefficient of the standard state of aqueous solution and γ_t is the transfer activity coefficient or also known as the medium effect. The medium effect is directly linked to the Gibbs energy of transfer

ΔG_t° from one solvent into another. The determination of those transfer activity coefficient into various media have been a intense research topic for decades and reference values have been collected, analyzed and published by IUPAC[120]. The transfer activity coefficient of species (i) from the standard state in water to the new standard state is related by:

$$\Delta G_t^0 = \upsilon RT \ln \gamma_t(i)$$

n represents the number of ions from one molecule of the solute species. A pH scale that is based on the proton activity might thus be defined in two ways

$$paH = -\log m_H \gamma_w(H) = -\log m_H * \gamma_s(H) * \gamma_t(H)$$
$$paH* = -\log m_H \gamma_s(H) P = paH + \log \gamma_t(H)$$

m_H = molality of H

Thus when a pH scale defined with aqueous buffers is used to determine the actual proton concentration in organic solvents, two parameters (the changed proton activity and the liquid junction potentials) need to be accounted for since the resulting error would be too large to be neglected. For this purpose a correction factor can be used that relates the operational pH to the real proton activity.

$$paH* = pH - \delta$$

δ is evident from the above stated equations to be:

$$\delta = \frac{E_j(s) - E_j(w)}{\frac{2.3026RT}{F}} - \log \gamma_t(H)$$

Or more simple

$$\delta = \Delta E_j - \log \gamma_t(H)$$

ΔEj is the difference of liquid junction potentials defined in pH units.

To measure pH in organic solvents is thus possible in order to obtain accurate proton concentrations and thermodynamic values such as equilibrium constants. However, while log γ_t(H) can be calculated from reference values provided by much respected sources as for example IUPAC[121], the liquid junction potentials would need to be determined every time as initially demonstrated by Deligny et al.[122].

One example of this approach to determine the apparent pKa of poly acrylic acid by titration in methanol was given by Klooster et al.[123]. Klooster et al used reference values for δ and liquid junction potentials that were previously determined and neglected the fact that dissociation of alkaline earth metal ions from polymer backbones might be significantly inhibited in organic solvents and thus infringing with the basic definition of the acid dissociation constant. An accurate method to establish various liquid junction potential differences between various solvents does not exist.

For this reason a different, more practical but equally valid method to link the working pH to he true proton concentration was adopted in this work. A solution of a

mono protic acid of which the true pKa in the solvent of interest is known needs to be titrated completely with an organic phase soluble base. From practical point of view benzoic acid is chosen due to its solid and stable appearance that is as well responsible for its use as standard reference material for the titrimetric adjustment of non-aqueous base solutions. Tetramethylammoniumhydroxide is chosen due to its good solubility in organic solvents and due to the presumed ability of its salts to be more easily dissociable in organic solvents compared to alkaline bases. The difference between the working pKa value that is obtained by the half equivalence point method and the true known pKa value is used as the correction factor to link the working pH to the true proton concentration. The major difference of this correction value when compared to the suggested way of obtaining this correction value is that the current method does not resolve the contribution by the liquid junction potentials and the medium effect. This however, might not be important for practical considerations since the current method still leads to a correction factor that allows the determination of the accurate proton concentration in organic solvents. The accuracy of this method depends however on the accuracy and care with which the reference pKa value was determined or selected from literature.

The Oosawa-Manning Theory Applied to Polyacids used in RP-LbL

The above-described procedure is valid for mono protic acid in organic solvents. When polyacid properties shall be determined, a restriction of the above procedure has to be considered. potentiometric measurements only yield the accurate proton

concentration if no proton condensation in close proximity to the polymer but a free distribution of protons in the solution is to be expected.

A theoretical approach to assess whether proton condensation occurs is given by the Oosawa-Manning theory. Although the Oosawa-Manning theory is a much simplified model, it is frequently employed to address practical questions and has proven to be of sufficient accuracy for many practical aspects on the determination of counter ion distribution. The interaction of charged polymer backbones with their counter ions in solution might result in a condensation of counter ions on the polymer backbone. Basic considerations whether counter ion condensation are expected or not are based on a fine equilibrium between an unfavorable entropic effect that is inherent to counter ion condensation and the attractive electrostatic force that is felt by the counter ion from the polymer backbone. In cases where no proton condensation occurs, the protons are expected to be freely distributed in solution. Another approach that supports this assumption is given by Tsuneo Okubo[60] who has investigated the proton distribution around single polyacid chains in finite dilution in cases where no countering condensation occurs. Okubo found that proton distribution can be explained by Debye-screening and that the Debye-length reaches easily a magnitude of 100nm in salt free aqueous solutions.

Noteworthy the Debye-length in the case where the screening is only facilitated by the dissociated protons and no foreign salt is added reduces to[60]:

$$D = \frac{1}{\sqrt{4\pi L_b n}}$$

Where L_b is the Bjerrum length and n is the concentration of protons per ml, which might be calculated from the apparent pKa value of the polyacid and the initial concentration.

Solutions of polyacids that will be discussed in this section are different by two properties in comparison the special case that was considered by Tsuneo Okubo. First the solution is in a dilute regime but not in infinite dilution, second the Debye-length is expected to be longer due to the dramatically decreased permittivity of organic solvents. The implication is that the Debye-length is much greater then the average distance between polyelectrolyte chains. This leads in turn to the assumption that the proton distribution is sufficiently homogeneous in order to be determined by measuring the bulk pH of the polyacid solution under the condition that no counter ion condensation occurs.

According the Oosawa-Manning theory, the entropic penalty for the condensation of counter ions is very high within the dilute polyelectrolyte regime[124]. This causes almost all counter ions to be freely dispersed in solution if the polyelectrolyte solution can be considered within the dilute regime[91]. Different regimes of polyelectrolyte solutions are distinguished of which three are of special consideration in this case, the dilute regime, the semi dilute regime and the concentrated regime. The criteria for the concentrated regime is fulfilled when the total volume of the solution is smaller then

the volume that would be occupied by all polymers in solution if they would exist in their ideal stretched cylindrical shape. Within the concentrated regime the polyelectrolyte 'crowd' each other, they adopt a more coiled conformation compared to their cylindrical shape in more diluted regimes.

The semi dilute regime is defined to start at a polyelectrolyte concentration that causes the average length between single polymer molecules in solution to be smaller then the major axis length of the polymers[91]. This definition leads to the definition for the dilute solution regime that is given when the average distance between the polyelectrolyte molecules in solution is greater then the major axis length of a single polymer.

The first condition that needs to be fulfilled in order to assume that counter ion do not condense according to the Oosawa-Manning theory is that the solution is in the dilute concentration regime. The magnitude of interest is thus the concentration of polymers at which the solution transits from the dilute into the semi dilute regime. This concentration is also known as the 'overlap concentration' and is given by the following equation[91]:

$$c^* = \frac{N}{\left(\mathrm{R}_e^F\right)^3} \approx \frac{1}{b^3 * u * f^2 * N * \ln\left(\frac{N}{ge}\right)}$$

b = bond length

u = L_B/b

f = fraction of charged monomers

N = number of monomers

g_e = number of monomers in a electrostatic blob

$$g_e^{\circ} = (uf)^{-2/3}$$

The term ‘electrostatic blob’ originates from polyelectrolyte theories, where polyelectrolytes are considered to be a line of spheres. One electrostatic blob is considered to be one of the spheres in the line that contains a certain number of monomers depending on electrostatic and thermodynamic parameters.

Note worthy is that the overlap concentration only depends on the charge fraction for polymers of the same degree of polymerization with the same kind of monomer (e.g. all vinyl based polymers). Since the charge fraction is relatively low (<20%) an equation from the scaling theory that describes polymer conformation of partially charged polymers is used. This theory is assumed to be applicable to the real situation during the RP-LbL process. However it might be relatively inaccurate for very high or very low charge densities as depicted in Figure 3-1.

The total polyacid concentration where the polyacid has a certain degree of dissociation must thus be smaller then the overlap concentration in order to exhibit no counter ion condensation.

The calculations in this section are given for a polystyrenesulfonic acid polymer with an average molecular weight of 70000 g/mol and a polydispersity index of 1.07, which can be fairly considered as monodisperse. It is important to note that the overlap concentration would change for other polymers of the same molecular weight or other polystyrenesulfonic acid polymers with different molecular weight, since the

major axis length of those polymers is expected to change likewise. The following graph depicts the calculated overlap concentrations for polystyrenesulfonic acid (70000 g/mol) in different solvents as a function of the degree of dissociation alpha.

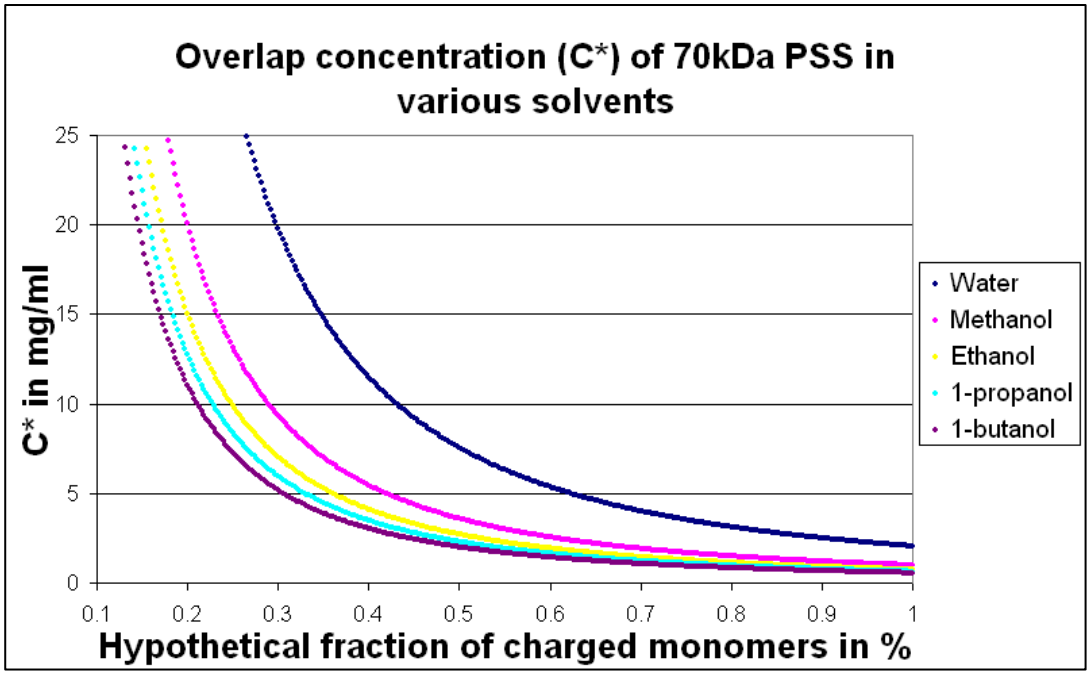


Figure 3-1: Overlap concentration of polystyrenesulfonic acid (70 kDa) as function of hypothetical degree of dissociation.

The calculations are in line with literature reports that describe a medium strongly charged polyacrylic acid to be in the dilute regime at concentrations smaller then 0.5 mg/ml and a completely charged polystyrenesulfonic acid (sodiumpolystyrenesulfonate) to be within the semi dilute regime at a 5 mg/ml concentration[125].

In addition to the polymer concentration, the charge density on the polymer backbone is a crucial factor that governs counter ion condensation. Long-range collective electrostatic forces determine the number of counter ions condensed in close

proximity to the polymer backbone[91]. The magnitude of the collective electrostatic force that can be 'felt' by the counter ions depends strongly on the charge density at the polymer backbone. Highly charged polymer backbones have a stronger collective electrostatic force compared to weakly charged polymers. The number of charges is directly governed by the autoprotolysis in case of polyacids. Autoprotolysis of an acid can be expressed as percentage of dissociated groups compared to the total acid groups within the system.

$$\alpha = \frac{\left[A^-\right]}{\left[HA_{total}\right]}$$

A similar parameters for polyacids is the charge fraction or fraction of charged monomers.

Since the magnitude of the collective electrostatic force needs to be big enough to cause the counter ions to condense, no counter ion condensation is expected to occur if the auto protolysis in the solution of interest is relatively weak. A threshold value for the possibility of counter ion condensation is given by the Oosawa-Manning parameter[126].

$$\xi = \frac{e^2}{\varepsilon k_b T b}$$

Where:
e = Elemental charge
ε = Dielectric constant of the medium
k_b = Boltzmann's constant

T = Temperature
b = Distance of charges

$$b = \frac{L}{fN}$$

Where:
L = total polymer length
f = Fraction of charged monomer
n = Number total monomers per molecule

Since the Bjerrum length is given by:

$$l_B = \frac{e^2}{\varepsilon k_B T}$$

The above equation can be simplified to

$$\xi = \frac{l_B f N}{L}$$

Based on considerations that addresses the equilibrium between the magnitude of the electrostatic force that is felt by the counter ion and the negative entropic effect that is associated with counter ions condensation, Oosawa and Manning found that counter ion condensation only occurs if $\xi > 1$ and that at conditions where $\xi < 1$ no counter ion condensation is expected to occur. The following graph shows the Oosawa-Manning parameter as function of the degree of dissociation (α) of polyacids in water and linear aliphatic alcohols from methanol to 1-butanol.

It is worth to be noted that unlike the overlap concentration, the Oosawa-Manning parameter is independent of the degree of polymerization since only the average charge to charge distance is the governing magnitude for this parameter.

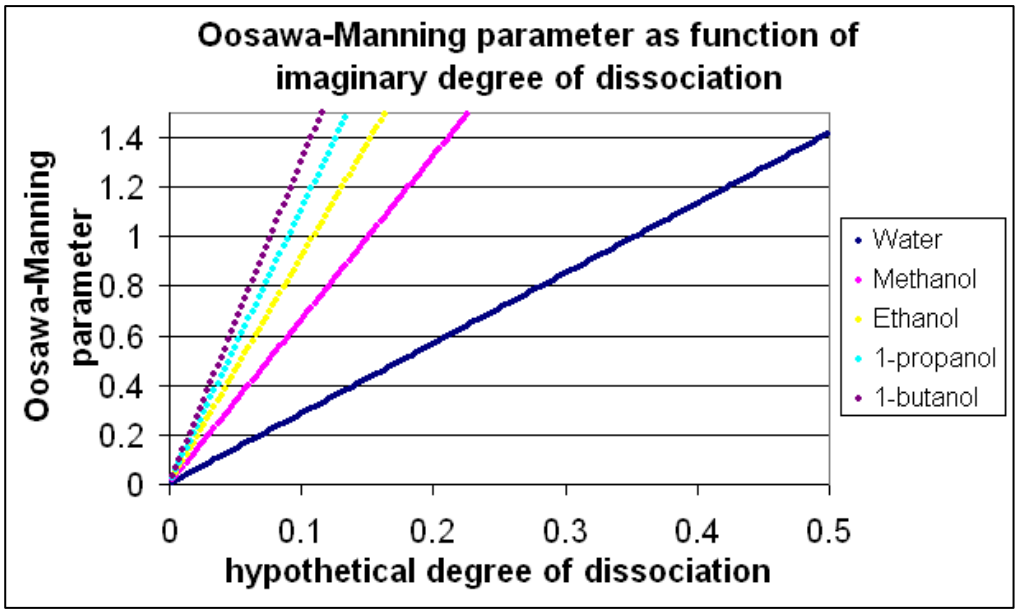


Figure 3-2: Oosawa-Manning parameter as function of imaginary degree of dissociation.

The above graphs allow easily to determine the maximum degree of dissociation for a polyacid in the respective solvent before counter ion condensation is expected to occur or might be calculated by the following equation.

At the special case where $\xi = 1$, the maximally permitted charge fraction can be calculated as follows $f = \frac{L}{l_B N}$

Solvent	Dielectric constant	Maximum permitted charge fraction ($\xi = 1$)
Water	78.54	0.35
Methanol	33	0.15
Ethanol	24.3	0.11
1-propanol	20.1	0.090
1-butanol	17.1	0.076

Table 3-1: Maximally permitted charge fraction of polymers without counter ion condensation for various solvents.

Given the two aforementioned parameter, counter ion condensation does not occur as long as the polyacid solution has a concentration within the dilute regime and the acid

strength leads to a degree of dissociation that is smaller then the maximum theoretical degree of dissociation at $\xi = 1$.

The Oosawa-Manning Parameter and Overlap Concentration in a Practical Context

In a practical context, e.g. if a material scientist or a nanotechnologists attempts a rational design of a self-assembly based process or material, the Oosawa-Manning parameter is hard to calculate since the degree of dissociation is dependent on other parameters (e.g. total acid concentration) and usually not tabulated. Although the same is true for apparent pKa values of polyacids, pKa values of acids that describe the monomer unit best are often available for various solvents.

The main difference between pKa values of acids that describe best the monomer unit and the apparent pKa value of polyacids is that the dissociation of single monomer units is not independent of each other but have a coupled protolysis equilibrium. This is best to be visualized with a common inorganic acid such as H_3PO_4. The first proton is very easily dissociated and thus has a high pKa value (2.16)[127], the second proton is more difficult to dissociate which is reflected in a pKa of 7.21[127] while the last proton is very difficult to dissociate if the previous protons are already dissociated, the pKa value of the third proton is 12.32[127]. In case of a polyacid, it is not possible to distinguish between single pKa values and the apparent pKa that is stated. The apparent pKa is the average pKa of all monomer units and describes the real proton dissociation equilibrium well for a high degree of polymerization.

Not surprisingly, the apparent pKa values of a polyacid lies about 1-2 units above that of the monoprotic acid that describes the monomer unit best as indicated by studies addressing the solutions of poly carboxylic acids in aqueous solutions[123]. An acid that describes the monomeric unit best is different from the monomeric acid. In case of polystyrenesulfonic acid, paramethylbenzensulfonic acid (PTSA) is considered to be a better reference then paravinylbenzenesulfonic acid. Two different values that describe the proton dissociation of polyacids are used for theoretical considerations and practical applications. In theory, most assumptions about polymer conformation and solution properties are based on the charge fraction or the degree of dissociation, while practically the apparent pKa value is stated to assess the strength of a polyacid. It seems thus to be favorable to develop a simple relation between the acid strength (in terms of pKa value) and counter ion condensation in order to be able to judge if accurate determination of polyacid properties is possibly straight forward with potentiometric measurements or not. For this reason, the maximal allowed acid strength in terms of the degree of dissociation that is given by the Oosawa-Manning parameter and the overlap concentration can be used to calculate the acidic strength (expressed as pKa_{MaxOM}) that is maximally allowed without counter ion condensation to occur. In praxis, this would mean that accurate parameters of any polyacid with an apparent pKa value greater or equal to pKa_{MaxOM} at a concentration smaller then the overlap concentration can be determined straight forward with potentiometric measurements since no counter ion condensation is expected to occur. A rough estimation if direct potentiometric determination of polyacids properties is possible

might be obtained by using the pKa values of acids that describe the monomer unit best by the following relation:

$pKa(monomer) + x > pKa_{MaxOM}$

Where x is depending on the kind of acid and is usually in the order of magnitude of 0.5 to 1.5 pKa units.

Relation of Apparent pKa to the Overlap Concentration and Degree of Dissociation

$$pH = -Lg\left[H^+\right]$$

For very strong acids (pKa<0) it is assumed that $\left[H^+\right] = C_0 = \left[A^-\right]$ however, this is most likely not the case for solutions of polyacids in organic solvents.

The relation of proton concentration to the dissociation constant for strong acids (4.5>pKa>0) is given by[128]:

$$\left[H^+\right] = -\frac{K_a}{2} + \sqrt{\frac{K_a^2}{4} + K_a * C_0}$$

Where
K_a = Acid constant
C_0 = Overlap concentration

It is well established that the pH of a solution is related to the degree of dissociation of a polyacid be the following equation:

$$pH = pKa + \lg\left(\frac{\alpha}{1-\alpha}\right) \text{ Thus } \left[H^+\right] = 10^{-\left(pKa + \lg\left(\frac{\alpha}{1-\alpha}\right)\right)}$$

Here, alpha is the maximum degree of dissociation that is allowed according to the Oosawa-Manning theory before counter ion condensation occurs in a certain solvent.

One might thus write:

$$-\frac{K_a}{2}+\sqrt{\frac{K_a^2}{4}+K_a * C_0}=10^{-\left(pKa+\lg\left(\frac{\alpha}{1-\alpha}\right)\right)}$$

Solving this equation for Ka yields:

$$K_a=\frac{C_0}{\frac{1-2\alpha+\alpha^2}{\alpha^2}-\frac{1}{4}}$$

Or

$$pK_a=-\lg\left(\frac{C_0}{\frac{1-2\alpha+\alpha^2}{\alpha^2}-\frac{1}{4}}\right)$$

A similar relation might be obtained by using the equation for the dependency of proton concentration to the dissociation constant for weak acids ($4.5 < pKa < 9.5$)[129]

$$\left[H^+\right]=\sqrt{K_a * C_0} \text{ Or accordingly } pH=\frac{1}{2}\left(pK_a-\lg C_0\right)$$

$$\frac{1}{2}\left(pK_a-\lg C_0\right)=pKa+\lg\left(\frac{\alpha}{1-\alpha}\right)$$

Yields:

$$pK_a=-2\lg\left(\frac{\alpha}{1-\alpha}\right)-\lg C_0$$

Results & Discussion

Potentiometric Determination of the Correction Factor δ for Measurements in Aliphatic Alcohols

The section on the accurate and correct determination of the pH in organic solvents describes that the reading of the pH meter needs to be corrected by a factor in order to yield the correct negative decadic logarithm of the proton concentration. The correction factor incorporates the liquid junction potential and the phase transfer coefficient of protons from one media into another, albeit not explicitly determined by the method that was adopted in this work.

$$paH^* = pH - \delta$$

It needs to be remembered, that the phase transfer coefficient is a value that is commonly available in data collections, whereas the difference in liquid junction potential is dependent on electrode parameters, temperature and other environmental parameters and would thus have to be determined for every measurement. An alternative that has been suggested by IUPAC is to determine the pKa value of acids in organic solvents with a common Ag/AgCl pH electrode that is calibrated against an aqueous buffer system and to take the difference between the obtained value and the literature value as the correction factor δ. This method has been adopted in this work and benzoic acid was chosen as reference materials since it is conventionally used as titrimetric reference standard for non aqueous solutions of strong bases such as

tetramethylammoniumhydroxide. Titration curves for benzoic acid in water and aliphatic alcohols with tetramethalammoniumhydroxide solutions of the respective solvent are shown in the following figure.

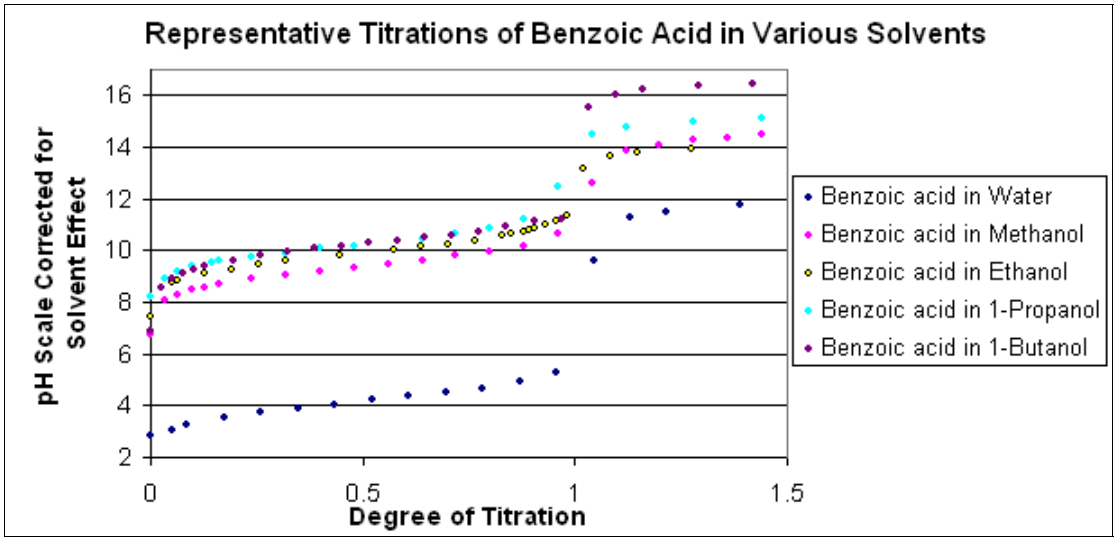


Figure 3-3: Titration of benzoic acid in various solvents.

The pKa values of benzoic acid were determined by titration with a solution of tetramethylammoniumhydroxide solution in the respective solvent using the half equivalent point method. The pH was recorded with an Ag/AgCl pH electrode that was calibrated against an aqueous buffer solution. The observed pH values were corrected by a factor that represents the difference between apparent pKa values and literature values as demonstrated in the table below.

Potentiometric determined parameters of benzoic acid				
Solvent	Averaged working pKa value	Standard deviation	Literature value	Correction factor to yield correct proton concentration
Water	4.152	0.0201	4.21[130]	-
Methanol	6.531	0.0246	9.4[130]	2.87
Ethanol	7.502	0.1154	10.1[130]	2.597
1-propanol	7.298	0.0780	10.17[iv]	2.871
1-butanol	7.545	0.0386	10.24[131]	2.694

Table 3-2: Determination of potentiometric correction factor (δ) for aliphatic alcohols.

[iv] The pKa value of benzoic acid in 1-propanol is the arithmetic middle of that in ethanol and 1-butanol in lieu of a reference.

It needs to be mentioned here that a special electrode was used for the determination of potentials in non-aqueous solution. The ETOHtrode is an electrode that was especially designed for pH measurements in ethanol by the Metrohm AG. The characteristic of this electrode is the special glass membrane that yields a fast stabilization of the measured potential and the fact that no direct salt bridge exist to the sample solution. Although it would be theoretically possible to exchange the saturated KCl solution around the reference electrode with a saturated solution of lithium chloride in the respective solvent in order to reduce the liquid junction potential to a negligible value, this does not represent a standardized procedure and would require the exchange for every different solvent used. Therefore an aqueous saturated KCl solution was chosen as electrolyte and the method for determining δ as described above was adopted. For all further titrations of PTSA or PSA the observed pH values were treated with the correction factors obtained by this procedure.

Potentiometric Determination of the pKa Values of PTSA and PSS in Water and Aliphatic Alcohols

PTSA at a concentration of circa 1.5 mg/ml in the respective solvent was titrated with tetramethylammoniumhydroxide solution in the same solvent. The pH was measured as described above.

Solvent	pKa value of PTSA
Water	0.7
Methanol	3.024
Ethanol	3.035
1-propanol	2.984
1-butanol	2.867

Table 3-3: Determined pKa values of Paratoluenesulfonic (PTSA) acid in aliphatic alcohols.

The pKa values of PTSA in different aliphatic alcohols appear to have almost the same strength. It is well established that various media effects are responsible for the ability of protons to dissociate. The ability of the solvent to stabilize dissociated protons and the anion as well as the ability of the solvent to inhibit acid-acid molecule interaction (e.g. dimer formation, aromatic stacking and others) is responsible for the overall acid dissociation. The exact effect of different aliphatic alcohols on PTSA remains elusive. Yet, the slight differences are in accordance with findings by others for benzoic acid as mentioned above.

In the preceding sections it was established that the properties of polyacids might be determined straight forward under the condition that no counter ion condensation occurs. For this the Oosawa-Manning parameter needs to be smaller then unity and gives the maximum degree of dissociation possible before counter ion condensation

occurs at ξ =1. On the same time the concentration of polymers needs to be in the dilute regime, which in turn depends on the charge fraction on the polymer backbone as governing parameter for the persistence length of polymers in solution. Subsequently the pKa value of acids was related to the degree of dissociation (alpha) and the concentration of all dissociable groups. Using the maximum degree of dissociation possible (ξ=1) in a certain solvent together with the overlap concentration at that degree of dissociation in those equations yields the maximum apparent pKa value of a polyacid before counter ion condensation occurs. Any polyacid that has a greater apparent pKa value then the calculated pKa value at any concentration smaller then the overlap concentration might thus be subjected straight forward to potentiometric measurements in order to determine the accurate degree of dissociation. It was furthermore established that a rough estimation if direct potentiometric determination of polyacids properties is possible might be obtained by using the pKa values of acids that describe the monomer unit best by the following relation.

pKa(monomer) + x > pKa_{MaxOM}

Where x is depending on the kind of acid and usually is in the order of magnitude of 0.5 to 1.5 pKa units. The following table uses this relation to estimate whether the accurate characterization of polystyrenesulfonic acid in different solvents is possible or not. Since the x value is hard to estimate, as it appears to be a function of the acidic strength of the monomer units itself, it seems best to assume it to be 0 for a careful initial approximation.

Alpha max is determined from Figure 3-2 and C_0 might be easily calculated from the overlap concentration. PKa PTSA refers to the pKa value that was potentiometrically determined as described before.

Solvent	**α max**	**C_0**	**pKa_{maxOM}**	**pKa PTSA**	**Potentiometric determination of polyacid properties possible?**
Water	0.3522	0.083	1.609	0.700	No, counter ion condensation is to be expected
Methanol	0.15	0.195	2.217	3.024	Yes, counter ion condensation is not to be expected
Ethanol	0.11	0.263	2.395	3.035	Yes, counter ion condensation is not to be expected
1-propanol	0.089	0.332	2.498	2.984	Yes, counter ion condensation is not to be expected
1-butanol	0.0761	0.3895	2.578	2.867	Yes, counter ion condensation is not to be expected

Table 3-4: Assessment of direct applicability of potentiometric measurements to polystyrenesulfonic acid in various solvents.

The above table indicates that the accurate determination of acid dissociation properties is possible for polystyrenesulfonic acid in the organic solvents stated. It needs to be remembered that during the RP-LbL process, polymer concentration are between 1 mg/ml and 10 mg/ml which appear to be lesser then the overlap concentration in the case of polystyrenesulfonic acid with a molecular weight of 70000 g/mol.

It is furthermore important to know, that the pKa of polyacids is dependent of the degree of dissociation, which has been extensively investigated. In addition the determination of polyacid properties by titration with bases is difficult since it requires the assumption to be fulfilled that neutralized acid groups dissociate completely into ionic species. This, however, can only be sustained in water since

organic solvents are known to prevent complete salt bond dissociation. The pKa that might be obtained at the half equivalent point of the titration is thus of little value for a material scientists or nanotechnologists who attempts to design a self-assembly based process or material. Instead this problem is overcome by choosing not to draw information about the pKa of the polyacid directly from the titration. The titration curve of polystyrenesulfonic acid appears to be linear if the degree of titration is less then 30%. A linear regression function through this linear titration range is extrapolated to 0% degree of titration and the calculated pH value is considered to be the bulk pH of the solution. The reason to adopt this method rather than performing a one point measurement to determine the pH of the polyacid solution is due to the fact that the reading of the pH electrode is relatively unstable at 0% titration with might be attributed to the low ionic strength of the media. Nonetheless, the first reading coincides well with the extrapolated value and was empirically found only to differ by less then 0.05 pH units. However, due to the strong acidity of polystyrenesulfonic acid, the titration curve shows a strong shift in the pH value at the point of neutralization. This is important since the total concentration of acid groups is revealed if a tetramethylammoniumhydroxide solution with known concentration is used. The exact concentration of tetramethylammoniumhydroxide solution is accurately determined through the titration of benzoic acid that is considered a standard reference material to norm non aqueous base solutions. This leads to an easy determination of the degree of dissociation

$$\alpha = \frac{C_0}{\left[H^+\right]}$$

While the volume used for titration determines C_0, $[H^+]$ is given by the pH value of the solution in cases where no counter ion condensation occurs.

The following relation can thus be conveniently determined by the apparent pKa value:

$$pH = pKa + \lg\left(\frac{\alpha}{1-\alpha}\right)$$

The pKa values of paramethylbenzenesulfonic acid and polystyrenesulfonic acid are given in the following table.

Solvent	PTSA	PSS
Water	0.7	-
Methanol	3.024	5.049
Ethanol	3.035	5.020
1-propanol	2.984	5.852
1-butanol	2.867	4.998

Table 3-5: Comparison pKa values of PTSA and PSS.

Conclusion

This chapter provides a guide for practical applications to judge whether properties of polyacids that are dissolved in organic solvents can be directly determined by potentiometric measurements. This guide is based on the Oosawa-Manning theory of counter ion condensation. Particular attention was attributed to find a possibility to link parameters that are commonly used in a practical context to that of polyelectrolyte theories. A most commonly practically used parameter to characterize acids and polyacids is the apparent pKa value while the fraction of charged monomers is the fundamental parameter on which most polyelectrolyte theories are based. Traditionally the pKa values and apparent pKa values of polyacids are determined potentiometrically. Potentiometric measurements are possible in organic solvents if correction parameters for the occurring liquid junction potentials and for the different proton activity in non aqueous solvents are used. In order to assess whether potentiometric determination of polyacid properties is possible by measuring the bulk pH of a solution in organic solvents, it needs to be ruled out that counter ion condensation occurs which would give a false impression on the total proton dissociation. In order to facilitate this judgment in a practical context the apparent acidity (in terms of pKa) of a polyacid was linked to the limiting parameters of the Oosawa-Manning theory by defining a maximum acidic strength (pKa_{MaxOM}) where no counter ion condensation is to be expected while the polymer concentration is in the diluted regime. The result that is considered most valuable in a practical context is

that this judgment is possible on the basis of pKa values of monoprotic acid that describe the monomeric unit of the polymers in the best way. In case of polystyrenesulfonic acid, paramethylbenzenesulfonic acid (PTSA) is considered to describe the monomer unit in the best way. The advantage is that literature values for the pKa of paramethylbenzenesulfonic acid are available for various solvents. If a polyacid might be characterized by potentiometric measurements might thus be judged based on the following relation as long as the polymer concentration is smaller then the overlap concentration.

$$pKa(monomer) + x > pKa_{MaxOM}$$

Theoretical consideration established that characteristics of polystyrenesulfonic acids might well be determined by potentiometric measurements in lower aliphatic alcohols but not in water. This finding is given by the inherent charge density that results from autoprotolysis in the respective solvent. Autoprotolysis of polystyrenesulfonic acid in water is strong and results in counter ion condensation, whereas the autoprotolysis in aliphatic alcohols is too weak for counter ion condensation to occur.

Although apparent pKa values for polystyrenesulfonic acid have been produced, more accurate methods such as automated titrations need to be employed to verify those values.

The significance of this research is given by various applications of polystyrenesulfonic acids solutions in organic solvents that are used in cutting edge technology. Polystyrenesulfonic acid is frequently used to dope conjugated polymers for photovoltaic and other organic semiconductor applications as described in the introduction of this section. The approach that is described in this section is envisioned to be valuable for material scientists and nanotechnologists if fine tuning of self-assembly based processes or materials is attempted or when a new process or material employing polystyrenesulfonic acid solutions shall be devised.

The data stated in this chapter might provide an initial reference whether potentiometric measurements of polyacids in aliphatic alcohols are feasible but are not necessarily accurate for other solvents with a similar dielectric constant. This is due to the inherent property of the pKa value to depend on the inherent acidic strength and environmental parameters such as the temperature, the solvent ability to stabilize the dissociated acid species and others.

Acetonitrile (dielectric constant 37.5), Methanol (dielectric constant 33) and N-methylpyrrolidone (dielectric constant 33) are solvents in which PTSA is well soluble and all have a very similar dielectric constant. The pKa value of PTSA, however changes by several units which is due to the different proton affinities of the solvent molecules (MeCN<MeOH<NMP), which marks the importance for accurately determined pKa values of the monoprotic acids in the respective solvents.

This fact adds further significance on this section since initial references for pKa values of PTSA in aliphatic alcohols are provided and produced. Despite intensive search, no such reference values were found in literature. The literature seems not to be very specific on the pKa values of PTSA in water neither. PTSA is generally referred to be a strong acid and pKa value range from -2.8 to +1.7. In this work the reference value that is given in the Handbook of chemistry and physics for benzenesulfonic acid (pKa = 0.7) and in the Römpp encyclopedia for paramethylbenzenesulfonic acid (pKa = 0.7) is used.

Special significance of the here described investigation are given for the RP-LbL since it is the aim of this thesis to verify or change the paradigm that the multilayer absorption of polyamines and polyacids from organic solvents is based on acid base reaction in close proximity to the interface. The degree to which polyacids dissociate and to which degree the dissociated protons are confined in close proximity to polymer backbones or interfaces is crucial for colloidal interactions and multilayer build up. Indeed this section allows the assumption that protons are freely distributed within a system during RP-LbL polymer self-assembly and that the multilayer build up is much more governed by electrostatic interactions as previously assumed. This however is subject of the next sections of this Thesis.

Given the outcome that is presented in this chapter, the specific aims that were defined at the beginning of this chapter might be considered well achieved.

Specific aims defined at the beginning of this chapter:

1. To develop an experimental setup that allows potentiometric measurements on polyacids in non-aqueous solvents.
2. Theoretical consideration whereas autoprotolysis of polyacids can be measured straight forward in organic solvents or not.
3. To establish a simple relation between the pKa value of an acid in an organic solvent that describes the monomer unit best and the applicability of potentiometric measurements to organic solvents.
4. Determination of pKa values for paratoluenesulfonic acid in linear aliphatic alcohols.
5. Determination of apparent pKa values of polystyrenesulfonic acid in linear aliphatic alcohols.

Chapter 4: Nature of Polymer-Polymer Interaction and RP-LbL Multilayer Build Up

Scope and Specific Aims of this Chapter

This chapter [v] aims to describe the physico-chemical differences between the established Layer by Layer self-assembly of polyelectrolyte multilayer and the novel Reverse-Phase Layer by Layer self-assembly method which is scope of this thesis.

Specific aims: of Chapter 4

1. To investigate the nature of polymer-polymer interactions by IR spectroscopic investigation, in particular to establish whether hydrogen bonds or ionic linkages exist between polyallylamine and polystyrenesulfonic acid when or mixed in 1-butanol.

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[v] Parts of this chapter have been published in the following research article and book chapter. The text has been altered and complemented in the scope of this thesis and no further referencing of text passages that are similar or identical to the earlier publications have been made.

1. **Sebastian Beyer**, Jianhao Bai, Anna M. Blocki, Chaitanya Kantak, Xue Qianru, Michael Raghunath and Dieter Trau. Assembly of Biomacromolecule Loaded Polyelectrolyte Multilayer Capsules by Using Water-soluble Sacrificial Templates. Soft Matter. (2012), 8, 2760-2768.

2. **Sebastian Beyer,** Jianhao Bai & Dieter Trau. Assembly of Polymer Multilayers from Organic Solvents for Biomolecule Encapsulation; Multilayer Thin Films - Sequential Assembly of Nanocomposite Materials, second edition. Editors: Gero Decher, Joseph B. Schlenoff. (2012) Wiley-VCH Verlag GmbH & Co. KGaA.

2. To address the mechanism of multilayer build up by measuring of surface properties such as the zeta potential or by fluorescence based methods.
3. To investigate the peculiarities of polymer adsorption at interfaces during the RP-LbL process.
4. To confirm or refute the hypothesis that RP-LbL multilayer build up and polymer interaction is due to an acid base reaction in close proximity to the interface.

Introduction

An excellent introduction into the theory of polyelectrolyte conformation in solution and their adsorption at interfaces as well as multilayer formation is available from earlier sources. This chapter shall only provide a limited theoretical aspect in order to distinguish the RP-LbL from other established methods. In particular the polymer adsorption at interfaces shall be investigated.

In classical LbL approaches one of the most studied polyelectrolyte pair is polysodiumstyrenesulfonate and polyallylamine hydrochloride dissolved in aqueous solutions. RP-LbL uses the free acid and the amine form of the same polymers dissolved in aliphatic alcohols.

The charge densities of polystrenesulfonic acid were estimated with potentiometric titration in various non-aqueous media in the previous chapter. The determined charge densities allow conclusion about their adsorption behavior at interfaces that were

practically confirmed with AFM measurements. The process of polyelectrolyte absorption at interfaces from aqueous solutions in classical LbL approaches is compared to polymer absorption at interfaces during the RP-LbL process in organic solvents. The proposed mechanism of RP-LbL multilayer formation to be based on an acid base reaction in close proximity to the interface could be confirmed by theoretical considerations, infrared spectroscopy and atomic force microscopy. Although the mechanism needs to be refined in order to account for the electrostatic interactions that are likely to occur during RP-LbL due to autoprotolysis of the polyacid and associated phenomena of acid base and colloidal chemistry.

Experimental Setup

Polymer Solutions and Complex Formation Ability

Aqueous polymer solutions and all other solution derived materials were usually vacuum dried at 55 °C overnight, followed be dissolution in the solvent of interest. To test polymer solubility and polyelectrolyte complex formation, solutions of 100 mg/ml polystyrenesulfonic acid (PSS) and polyallylamine (PA) in ethanol were mixed with the alcohols (methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol and 1-octanol) in a ratio of 1 to 99, resulting in solutions of 1 mg/ml PSS or PA in the respective alcohols containing 1% ethanol. Sufficient polymer solubility could be observed in all alcohols and complex formation was demonstrated by mixing equal volumes of PSS and PA solution, both of the same respective alcohol. A PA-

FITC conjugate with a conjugation ratio of 1:125 (dye molecule to monomer unit) was used for fluorescence-; confocal- and atomic force microscopy studies. IR spectra of all polymers were recorded from dried solutions comprising of 10 mg/ml polymer in 5% sodium chloride solution. For aqueous LbL complexes, polyelectrolytes were dissolved at 5 mg/ml in 0.5 mol/l sodium chloride solutions. RP-LbL complexes were prepared from polymer solutions with arbitrarily chosen concentration of 2 mg/ml PA and 3 mg/ml PSS in 1-butanol. The dry materials were ground in a mortar and mixed with KBr at a concentration of 2% w/w. Spectra were recorded with the KBr cake method under a nitrogen atmosphere.

Adsorption of Polymers on Silica Interfaces

A silicon wafer with a 100 nm layer of thermal silicon oxide was cut into small chips of circa 1.5 cm x 2 cm. The silicon chips were cleaned with piranha solution overnight (concentrated sulphuric acid: 20% hydrogen peroxide 1:3). Followed by rinsing with double distilled water and subsequently with pure ethanol. Cleaned chips were dried under nitrogen flow. The silicon chips were coated with an automated dip-coater machine (KSV NIMA dip-coater, Finland). Samples Dipping speed: 14.26 millimeter per minute coating time: 15 minutes coating concentrations 1 mg/ml polymer (PA and PSS) dissolved in pure 1-butanol (fresh 5 ml volume was used in a 10 ml beaker for each coating) Washing: 1x with pure 1-butanol (dip into 1-butanol, then withdrawing is considered as 1 washing step).

Proof of Multilayer Build Up and Polymer Interaction.

A RP-LbL encapsulation protocol was used as described previously[58] except that PA and PSS were separately dissolved in 1-butanol. Polystyrene beads were used as templates for RP-LbL assembly to prove multilayer deposition by fluorescent microscopy and zeta-potential measurements. The zeta-potential was measured in 1-butanol (ZetaSizer Nano, Malvern, UK) with parameters set to 1.39931 (20 ºC) for refractive index, 3cP (25 ºC) for viscosity and 17.1 (25 ºC) for permittivity to obtain a valid zeta-potential by the Smoluchowski equation.

AFM Study to Investigate Polyelectrolyte Layer Thickness Growth

AFM measurements were conducted with an AFM/NSOM system (Nanonics, Israel). Polymer assemblies on silicon surfaces were recorded after washing of excess non-adsorbed polymers after dip coating followed by drying on air. Encapsulated glucose crystals were prepared in a batch process and samples were taken of each second layer with PSS as outermost layer. The encapsulated glucose crystals in 1-butanol suspension were first transferred to ethanol and then diluted with water to a final concentration of 50% v/v. Glucose has moderate solubility in ethanol and was observed to dissolve quickly under those conditions. Hollow capsules were gently centrifuged to form a pellet and deposited onto freshly cleaved mica surfaces that were coated with a layer of branched polyethyleneimine as reported earlier[132]. After air drying the remaining (but now collapsed) capsules were imaged in tapping mode. The cross sectioning function of the WSMX software[133] was used to determine the

thickness of the collapsed microcapsule at its lowest fold.

Results & Discussion

Solubility of PSS and PA in Organic Solvents and Polymer Complex Formation

Irrespective of the exact mechanism of polymer complex formation or layer wise adsorption of polymers, their solubility in solvents is the crucial parameter that facilitates all self-assembly processes. It is well established that addition of organic solvents to aqueous PSS or PAH polyelectrolyte solution beyond a certain threshold, causes precipitation[134, 135]. Polyelectrolyte solubility in water largely depends on the dissociation ability of the small molecular weight counter ions, which diminishes with increasing concentration of organic solvents[136]. Formamides[137] and ionic liquids[138] represent two exceptions of organic solvents since they are able to dissolve polystyrenesulfonic acid sodium salts and polyallylamine hydrochlorides. This is attributed to the very high polarity and dielectric constant of the solvents that facilitates ionic bond dissociation. Although not specifically reported, propylenecarbonate and N-methylpyrrolidone (NMP) might be good candidates for organic solvents that might facilitate ionic bond dissociation and thus polyelectrolyte dissolution. In the case of NMP, polystrenesulfonic acid likely forms a salt that might exhibit interesting solubility parameters in NMP or other organic solvents. It might be furthermore an option to form a salt of polyallylamine with organic acids rather then

with hydrochloric acid in order to facilitate the solubility in organic solvents. One of the most bio friendly and safe solvent classes that in addition are of relatively low cost are aliphatic alcohols. Aliphatic alcohols have relatively low dielectric constants that make ionic bond dissociation difficult. Aliphatic alcohols however have the ability to interact and solvate hydrophobic and hydrophilic parts of polymers through their amphiphilic nature. In the case of RP-LbL the polymers are dissolved as free amine or free acid and usually exhibit good solubility in aliphatic alcohols. This is believed to be due to the absence of small molecular weight counter ions and the solvent properties of aliphatic alcohols. Sufficient solubility of polyallylamine and polystyrenesulfonic acid to form polyelectrolyte complexes was observed for all aliphatic n-alcohols from methanol up to 1-octanol, that were tested. Other studies have in addition shown the solubility of various poly-carboxylic acids and other polyamines in organic solvents as summarized in Table 1-1. The RP-LbL process is specified to use polyacids and polyamines although various solvents are theoretically possible since they exhibit sufficient polymer solubility, special consideration needs to be attributed to the template material. In cases where polyelectrolyte microcapsules shall be fabricated from water-soluble sacrificial templates as described in chapter two, solvents need to be chosen that do not dissolve the template material. For this reason 1-butanol was chosen as an ideal solvent for most experiments, since higher alcohols are costly and less easy to process and lower alcohols might partially dissolve the sacrificial template.

For similar reasons, earlier approaches to use organic solvents that allow ionic

dissociation are commonly known to dissolve possible template materials such as glucose or salts as well. Besides their unfavorable solubility properties towards possible templates materials within the RP-LbL process to fabricate polyelectrolyte microcapsules based on water-soluble template material, those solvents often exhibit a considerable toxicity.

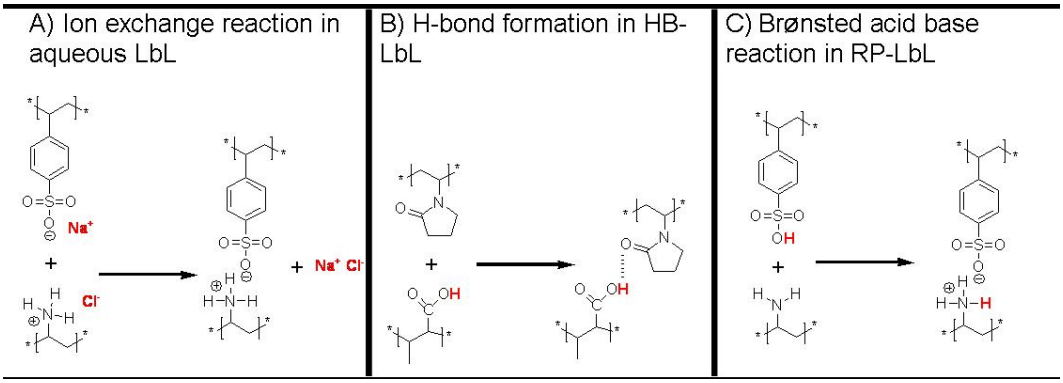
All solvents that exhibit sufficient polymer solubility might however be used in less sensitive applications such as the fabrication of coatings and surface modifications.

After mixing solutions of PA and PSS separately dissolved in the respective alcohol, increasing turbidity of the mixture and precipitation of the insoluble polyelectrolyte complex was observed.

Investigation of the Chemistry that is Responsible for Polymer Complex Formation.

It was postulated that, once the non charged polymers come in close proximity to each other or to an interface, a Brønsted acid base reaction (e.g. the non dissociated proton of the polyacid protonates the amine to form the respective ammonium salt) takes place. This mechanism was initially postulated to be responsible for polyelectrolyte complex formation within organic solvents and for the multilayer to hold together due to electrostatic interactions. This mechanism is significantly distinct from previously introduced mechanism such as aqueous LbL based on electrostatic interaction and HB-LbL as depicted in Scheme 4-1. To which extend polymer complex formation in solution and multilayer adsorption at interfaces is based on

electrostatic interaction, diffusional motion and other types of interaction is focus of this section and a refined mechanism will be proposed based on experimental results.



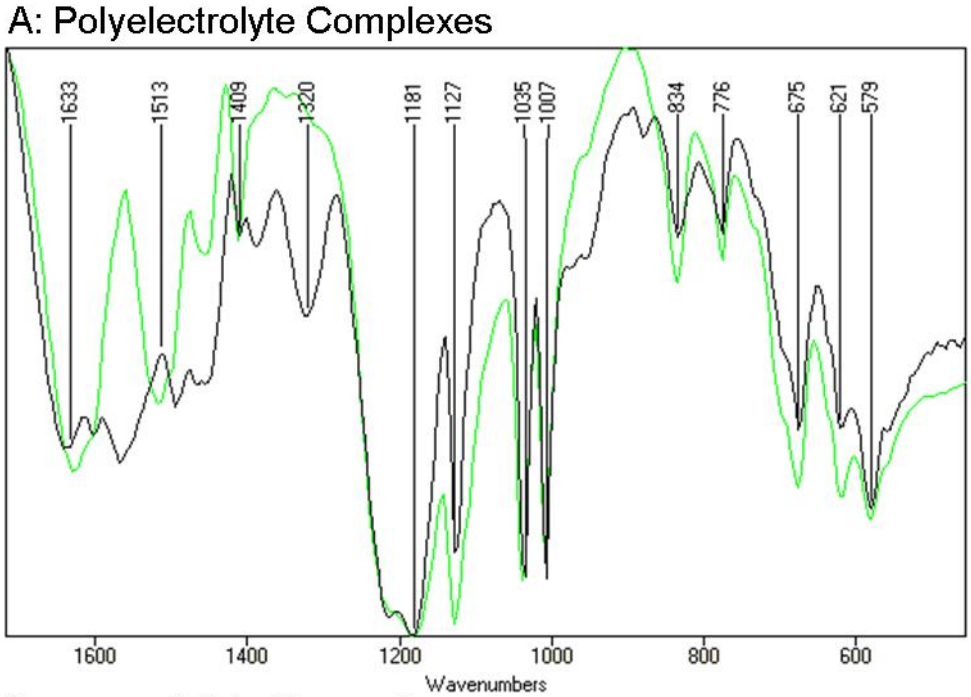
Scheme 4-1: Molecular interactions between polymers in aqueous LbL, HB-LbL and RP-LbL techniques.

Strikingly, the usage of aqueous solutions of polyelectrolyte salts or solution of polymer in organic solvents, leads to the same nature of bonds in the final polyelectrolyte complex. The bonds are salt linkages that are formed by polystyrenesulfonate and polyallylammonium salts as supported by IR-Spectroscopic investigations as described in the course of this section in detail.

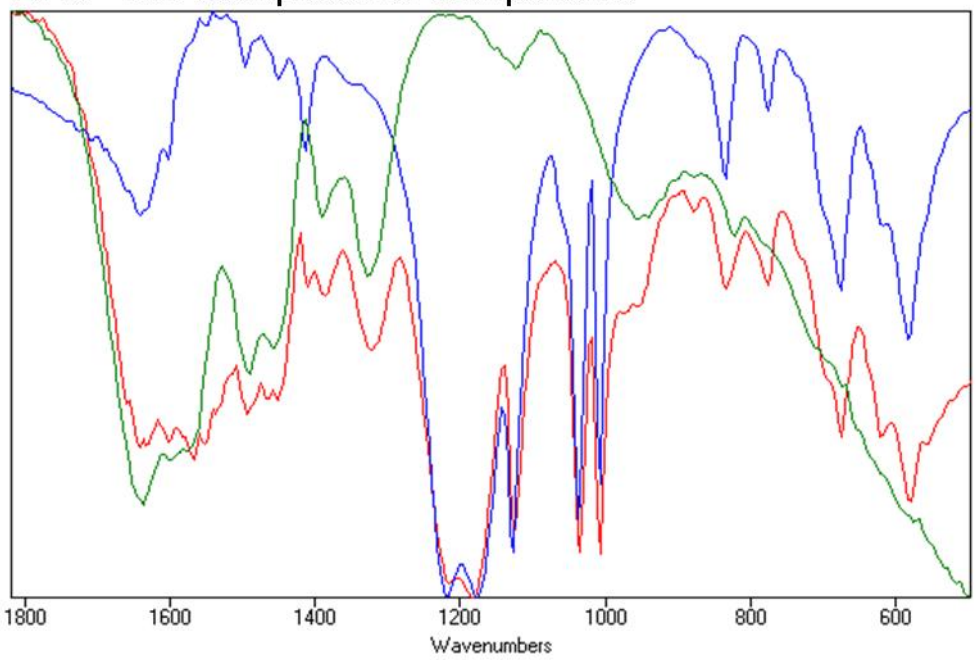
Earlier investigations have revealed that in aqueous LbL, the driving force to form water insoluble polyelectrolyte complexes is entropic due to the release of small molecular weight counter ions[139]. This might also be the case for methods in which common polyelectrolyte salts are dissolved in very polar organic solvents such as formamide[140] or ionic liquids[141] in which ionic bond dissociation is possible. In this case the interaction between a polymer pair is electrostatic and far reaching and layer formation is via an ionic bond. In contrast to electrostatic interaction, HB-LbL relies on hydrogen bonding interaction. Occasionally the use of organic solvents has been reported to facilitate HB-LbL. The main reasons for using organic solvents in HB-

LbL was water insolubility of certain polymers and the fact hat hydrogen bonds have a stronger interaction in non polar solvents. RP-LbL was postulated to be based on an acid base reaction as driving force that differs in nature from those of aqueous LbL and HB-LbL. In RP-LbL no small molecular weight counter ions exist that could be released upon polyelectrolyte complex formation. The acidic strength of the polyacids used in combination with the strong proton accepting ability of primary amines resulting in the formation of ionic bonds by acid base reaction. In contrast to HB-LbL the nature of polymer complex interaction is electrostatic. This hypothesis is supported by previous studies in the field of ion exchange resins in which thermodynamic studies have been performed to proof that monomolecular amine bases absorb onto ion exchange resins in acid form to produce a salt[142, 143]. Another study of polymer interaction using spectroscopic methods has been demonstrated by Borodina et al. for RP-LbL[63]. In this study it was clearly shown that a polycarboxylic acid forms ionic bonds to some extend upon interaction with primary polyamines (good proton acceptors) when both polymers are dissolved and mixed in dichloromethane (DCM). For relatively weak poly carboxylic acids IR-spectroscopy revealed that hydrogen bonding interaction occur between polyacids and relatively weak proton acceptors[144, 145]. Hydration of sulfonic acids makes it difficult to investigate proton dissociation from the acid group, as they resemble sulfonates in hydrated form. However, it might be reasonable to assume a higher degree of salt formation with increasing acidic strength.

IR spectra of sulfonic acids often resemble those of sulfonic acid salts due to hydration to hydronium salts under normal working conditions. However, distinct features in IR spectra were observed allowing conclusion of polymer interactions. In summary the components of the spectra for the polyelectrolyte complex assembled in water resembles more those of PA hydrochloride and that of PSS sodium and components of the spectra for the polyelectrolyte complex assembled from 1-butanol resembles more those from PA and PSS.

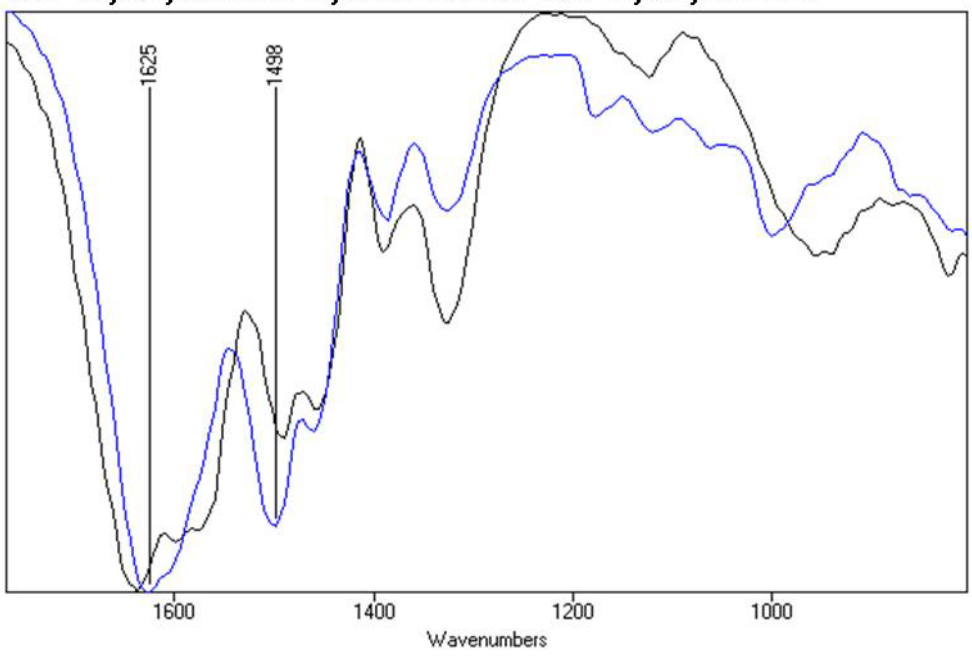


B: RP-LbL Complex and Components

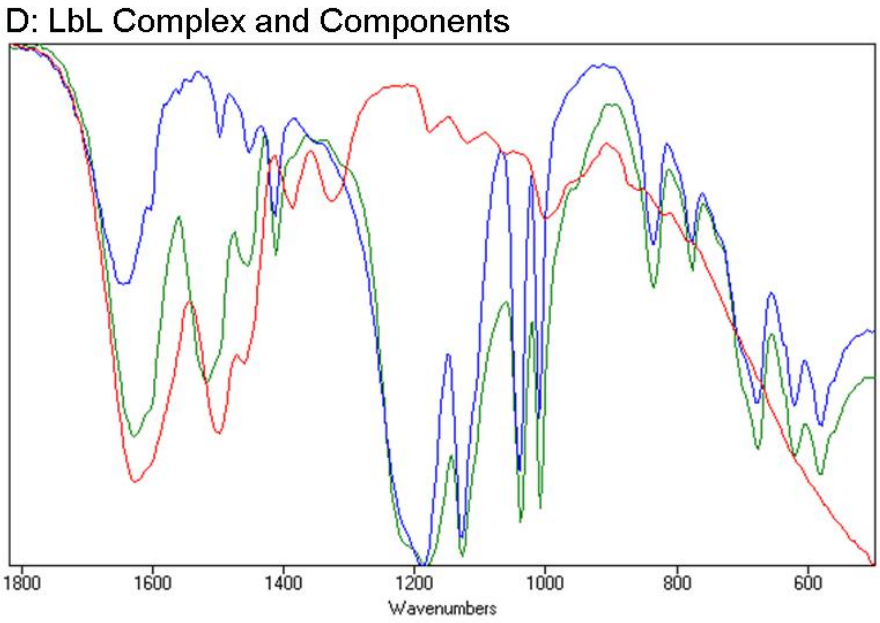


Red: RP-LbL complex (formed in 1-butanol)
Green: Polyallylamine
Blue: Polystyrenesulfonic acid

C: Polyallylamine Hydrochloride & Polyallylamine



Blue: Polyallylamine hydrochloride
Black: Polyallylamine



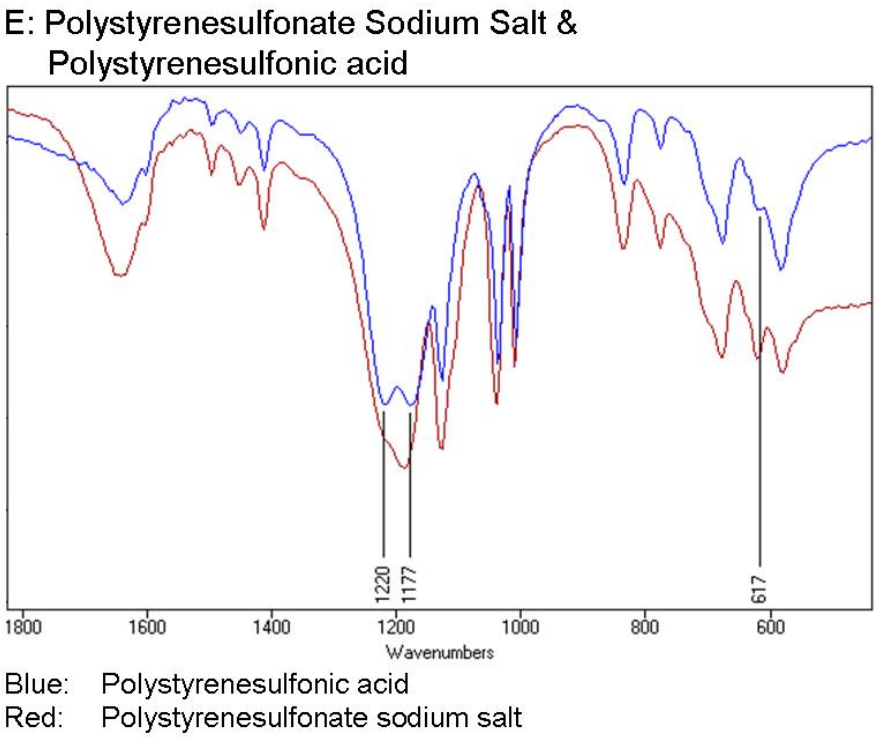


Figure 4-1: Infrared spectra depicting characteristics of polyelectrolytes, polyallylamine, polystyrenesulfonic acids and their complexes.

It is remarkable that the spectra of the PSS/PA complex formed in Butanol still resemble properties of free polystyrenesulfonic acid even though amine groups are roughly in 2.4 times molar excess. Comparing the spectra from PSS/PA complex assembled from water and 1-butanol (Spectra A) it can be seen that the spectra for aqueous LBL in the region of 1800 cm^{-1} to 1400 cm^{-1} is similar to that of polyallylamine hydrochloride and that of RP-LbL similar to that of polyallylamine. From Spectra B, it can bee seen that the peak at 1500 cm^{-1} becomes more defined upon PSS and PA complex formation in 1-butanol compared to the spectra of the free base polyallylamine, indicating ammonium salt formation. Differences between polyallylamine and polyallylamine hydrochloride can be observed at 1630 cm^{-1} and 1500 cm^{-1} which are bend and deformation vibration for free base primary amines and primary ammonium salts, respectively (Spectra C). The bend vibration peak of ammonium salts is significantly more defined for the spectra of the polyelectrolyte complex assembled from water (Spectra C). The peak at 1200 cm^{-1} is sharp for polystyrenesulfonate sodium salt but a symmetric shoulder for polystyrenesulfonic acid with maxima at 1220 cm^{-1} and 1177 cm^{-1}. Furthermore the C-S stretch peak at 620 cm^{-1} is more defined for the salt form compared to that of the free acid. Comparing the spectra of both polystyrenesulfonate sodium salt and PSS/PA polyelectrolyte complex from water and polystyrenesulfonic acid and PSS/PA polyelectrolyte complex from 1-butanol in the area from 1800 cm^{-1} - 1400cm^{-1}, it is clear in both cases that the peaks of the polyelectrolyte complex are N-H bend or deformation vibration and not found in polystyrenesulfonic acid or

polystyrenesulfonate sodium salt (Spectra B and D). Comparing the spectra of polystyrenesulfonic acid and polystyrenesulfonate sodium salt, (Spectra E) only a few differences can be seen. When comparing the spectra of PSS/PA polyelectrolyte complex from Butanol and PSS/PA polyelectrolyte complex from water, it becomes clear that the peak at 620 cm^{-1} becomes more defined in the spectra of PSS/PA polyelectrolyte complex from 1-butanol compared to the spectra of the polystyrenesulfonic acid but less defined compared to the spectra of sodium polystyrenesulfonate or that of PSS/PA polyelectrolyte complex from water. When comparing the peak at 1200 cm^{-1} it can be seen that the peak has still a slight shoulder form for PSS/PA polyelectrolyte complex from 1-butanol but not in the spectra of PSS/PAH complex assembled from water.

This leads to the conclusion that polymer complex formation of polyacids and polyamines in organic solvents is primarily due to acid base reactions. However, free acid groups and non protonated amine groups might still exist. The ratio to which acid and amine groups exist in their neutral form or their charged species is dependent on the acidic strength and solvent properties. In solvents which approach the polarity of water, the acid base interaction might be assumed to be in analogy of buffer system where single groups might alternatively be present as charged or uncharged species in an equilibrium based manner. In organic solvents, the ratio between neutral and charged species might depend much more on spatial confinements that restrict the stoichiometric interactions of monomer units.

Interfaces in Organic Solvents

Interfaces in organic solvents can be interpreted very much like interfaces in aqueous phase especially if the interface is electrostatically neutral or only weakly charged. Interfaces with a higher charge density need to be interpreted with the following considerations. The surface chemistry of interfaces might be altered in a way that decreases the charge density. This is primarily due to the inhibition of ionic dissociation. Alkaline earth salts of acid groups on the interface will dissociate to a much lesser extend since ionic bond dissociation is less likely and in addition alkaline earth ions are more difficult to be solvated. Acid groups by themselves might still dissociate in many organic solvents such as aliphatic alcohols although, their acid dissociation constant is significantly decreased as described in previous sections. On the other hand, electrostatic interactions are more far reaching due to the decreased permittivity of the organic solvent in comparison to water. This is marked by the decrease of the dielectric constant as shown in the following table.

Solvent	Dielectric constant	Bjerrum length in nm
Vacuum	1.00	55.91
Water	78.54	0.72
Methanol	33.63	1.65
Ethanol	24.3	2.30
1-propanol	20.1	2.77
1-butanol	17.1	3.27
1-pentanol	15.13	3.69
1-hexanol	13.03	4.28
1-heptanol	11.75	4.76
1-octanol	10.3	5.43

Table 4-1: Bjerrum length in dependence of solvent polarities for aliphatic alcohols and water.

The Bjerrum length is a parameter that is defined by the distance in a certain media at which the electrostatic attraction is of the same magnitude as the thermal energy. Or in other words the Bjerrum length is the distance between two colloidal particles or polymers in solution or at interfaces at which they can 'feel' a repulsive force. In this case the Bjerrum length has been calculated from tabulated values for the permittivity of the respective solvent.

$$L_b = \frac{e^2}{4\pi\varepsilon_0\varepsilon_r k_b T}$$

The equation above only comprises of constants previously named and a solvent specific parameter ε_r which is the permittivity of the solvent that is tabulated for most solvents. The Bjerrum length is a measure that describes the solvent in terms of its ability to shield electrostatic charges. Relating the Bjerrum length to polyacids, one can imagine a hypothetical case where two polyacid have the same fraction of charged monomer but are dissolved in two different solvents. The polyacid in the solvent with a longer Bjerrum length will appear to be more stretched. Another important consideration addresses the adsorption of polyacids at interfaces. The greater the Bjerrum length of the medium, the greater is the surface are that is occupied by one polyacid molecule in which no further adsorption can take place.

Adsorption of the First Polymer Layer

A silicon oxide surface that is treated with an acidic and oxidizing solution as described in the experimental section of this chapter might be considered to comprise of hydroxyl (silol) groups on the surface, which carry almost no charge in organic

solvents. Indeed, it has been shown previously that such prepared silicon samples need to be treated with strong bases in order to induce a significant surface charge. Adsorption of polymers on a neutral or slightly positive (due to acid treatment) silicon interface might be due to hydrogen bonding. Polymers such as polyalcohols, neutral polysaccharides or polyamines thus adsorb on the interface as a coherent layer without a significant electrostatic repulsion between single polymer molecules. This fact is most commonly utilized when silicon samples are pretreated with highly branched polyethyleneimine prior to further linear and potentially charged polymers. Polyethyleneimine anchors well on the surface and gives a coherent adsorbed layer due to its branched form and multiple hydrogen bonding interactions. An interface that is created by a carbohydrate crystal and an organic solvent is very much similar in those properties since charge density and surface chemistry are similar. This is of significance for the RP-LbL process in which water-soluble colloidal templates based on carbohydrates might be used to fabricate biomolecule loaded polyelectrolyte microcapsules as described in the preceding section of this thesis. These theoretical considerations provide the basis for the practical RP-LbL encapsulation protocol to start the first layer with a polyamine.

Polyacids in general are capable of hydrogen bond formation and would thus most likely adsorb at neutral interfaces in a similar manner to the above described polymers under the condition that autoprotolysis is limited to a negligible extend. Earlier section of this work however describe polyacids to have a significant charge density in aliphatic alcohols especially in the case of polystyrenesulfonic acid of which the

apparent pKa was determined to be ~ 5 pKa units in various aliphatic alcohols. Poly acrylic acid on the other hand was reported to have an apparent pKa equal to approximately 6 in water. Polystyrenesulfonic acid in 1-butanol should thus not adsorb in a coherent layer on a neutral interface.

Polyacids have been reported to adsorb sparingly from dilute solution regimes on neutral interfaces and to create a surface area cell by its negative charge in which subsequent polyacid adsorption is prevented[91]. This can only be overcome by using semi dilute or concentrated polyacid solutions for the deposition of multilayer or by screening electrostatic interactions through very high salt contents.

An experimental confirmation of this theoretical assumption is given by AFM imaging of adsorbed polystyrenesulfonic acid molecules from a dilute solution regime (1 mg/ml) in 1-butanol in the absence of any foreign salt. The adsorbed polymer layers were scratched with a plastic sharp in order to partially remove the coating.

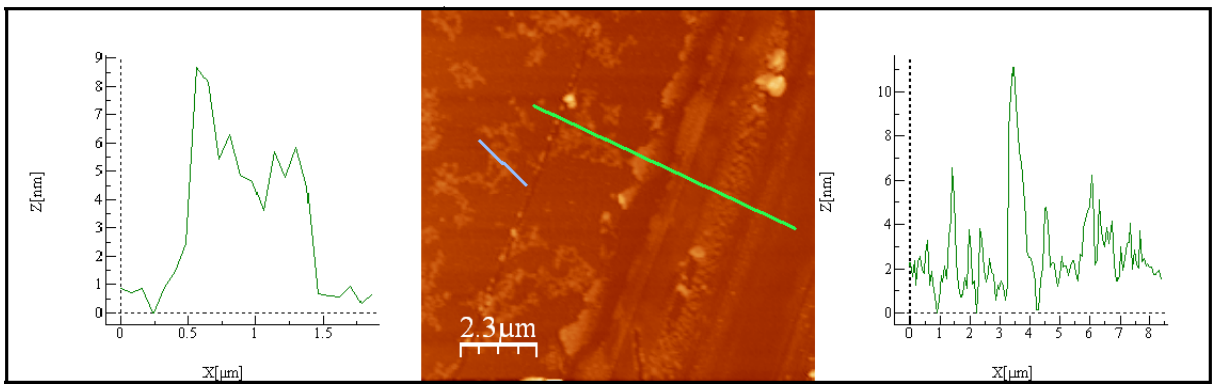


Figure 4-2: Adsorption of polystyrenesulfonic acid on silicon/silicon oxide interface from dilute solution.

Adsorption of polystyrenesulfonic acid from a dilute concentration results in a patchy incoherent adsorption profile which is in line with earlier reports and theoretical

aspects of polyelectrolyte adsorption at interfaces. It needs to be noted that the resolution is not strong enough to visualize both the polymer conformation on single polymer molecule level and the morphology that results from scratches. The patchy structure that is visualized here resembles rather a macroscopic structure than single molecule conformation. Nonetheless, incoherent layer formation is shown for a polystyrenesulfonic acid that is adsorbed from a dilute solution in 1-butanol. The height of adsorbed polystyrenesulfonic acid patches amounts to a thickness of 4 nm as indicated by the line profile on the left. The scratch morphology that is seen in Figure 4-3 resembles rather a smear of polymers than a scratch within a layer as indicated by the line profile (right).

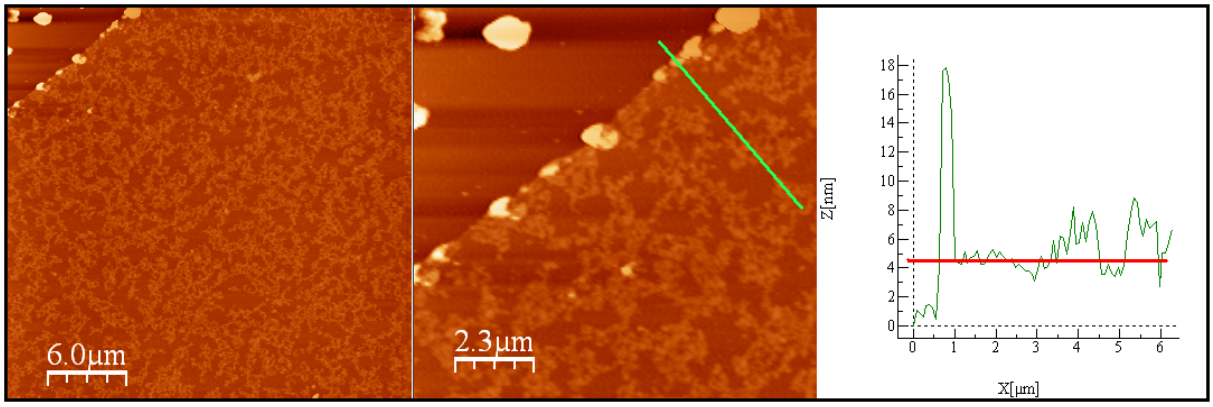


Figure 4-3: Adsorption of a coherent polyallylamine layer on top of polystyrenesulfonic acid adsorbed at the silicon/silicon oxide interface.

Figure 4-4 shows a sample that has a first patchy and incoherent layer of polystrenesulfonic acidand a second coherent layer of polyallylamine on top. In contrast to Figure 4-3 it can be seen that a scratch clearly results in the removal of a part of the coating (top left corner). The line profile reveals an average height of about 3.5 nm for regions where no underlying patchy polystyrenesulfonic acid adsorption

can be observed. The coherent polyallylamine layer thus constitutes of an average thickness of about 3.5 nm. Not surprisingly, the average thickness of regions where polystyrenesulfonic acid is adsorbed followed by a coating of polyallylamine amounts to roughly 7 nm.

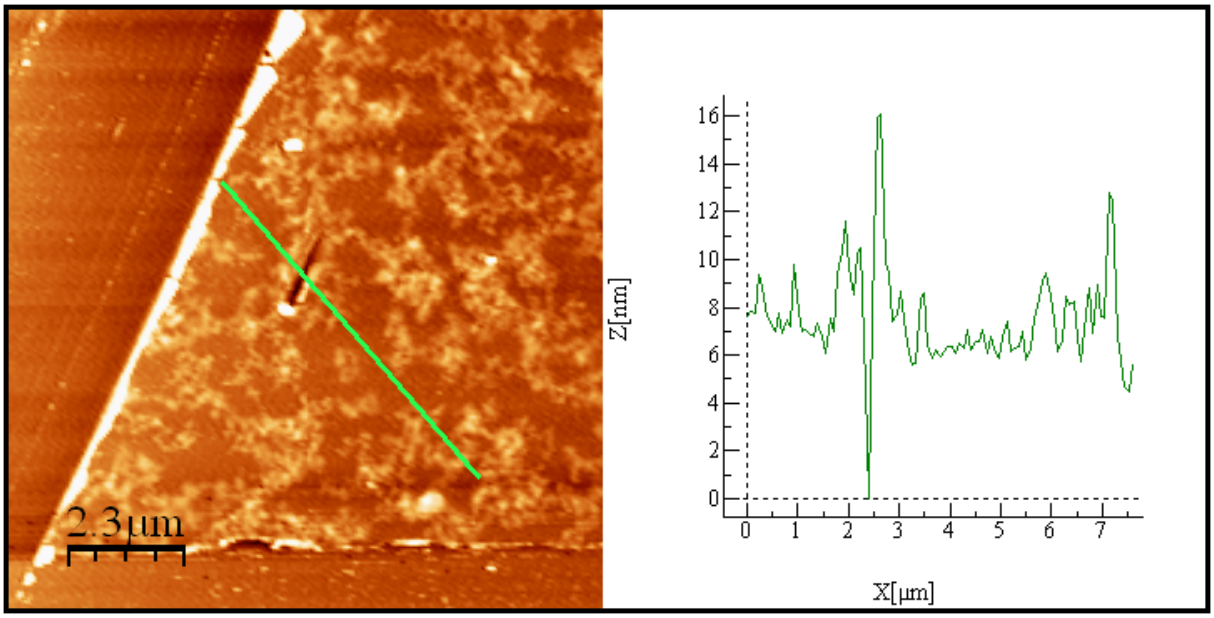


Figure 4-4: Adsorption of a coherent polystyrenesulfonic acid layer on top of a polyallylamine layer.

Figure 4-5 yields the most interesting result, being that the adsorption of a polystyrenesulfonic acid layer on top of a polyallylamine layer must be governed by different parameters compared to the adsorption of polystyrenesulfonic acid onto the silicon oxide template. This is marked by the fact that the third layer (polystyrenesulfonic acid) appears to be coherent and uniform. The average thickness of the entire polymer layer in areas where no patchy adsorbed polystyrenesulfonic acid is present, amounts to approximately 7 nm. This resembles the average thickness for polystyrenesulfonic acid plus polyallylamine as can be seen in Figure 4-4. The fact that areas that appear to have patches of polystyrenesulfonic acid adsorbed as first

layer reach a thickness of about 10 nm furthermore indicate that the third layer comprises of polystyrenesulfonic acid is adsorbed as a coherent polymer layer. If the third polystyrenesulfonic acid layer would not adsorb coherently on top of the polyallylamine layer, an increase in patchiness would be observed instead. In addition the line profile would show regions in which only one polymer layer is adsorbed on the silicon interface that would amount to roughly 3-4nm.The absence of features with a height of 3-4nm in Figure 4-5 supports this interpretation of the results.

This observation on silicon oxide interfaces is in line with images that were obtained from coated glucose crystals were polyallylamine was used as the first deposition layer during the RP-LbL encapsulation protocol and that show an absolutely uniform polymer layer (Figure 4-6).

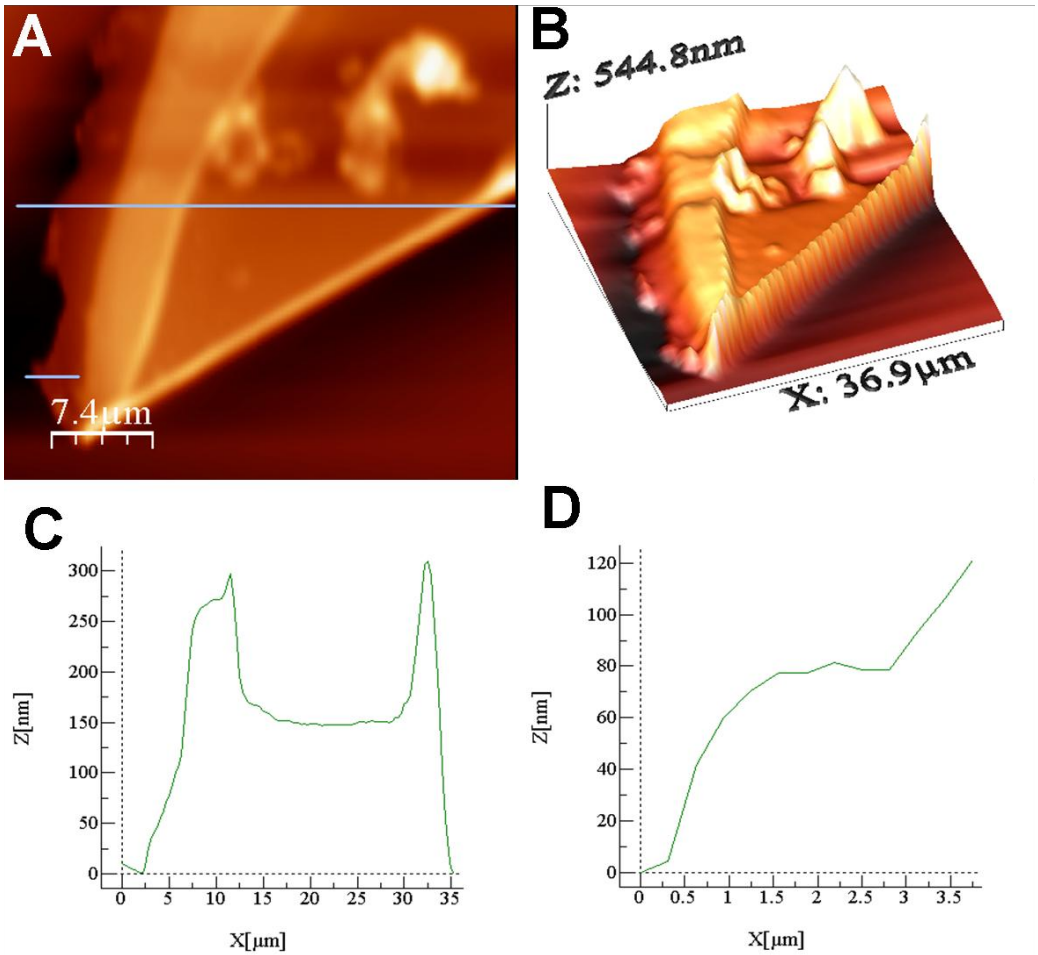


Figure 4-5: AFM studies to determine average layer thickness of RP-LbL multi layer.
AFM image of a collapsed air dried RP-LBL multilayer $(PA/PSS)_3PA$) shell previously coated on a glucose crystal that was removed by rinsing with water (A) and its three dimensional visualization (B). Cross sections of the collapsed shell results in an overall thickness of ~150 nm at the lowest area for 14 polymer layer (C). A second shorter cross section at an area believed to be a single wall of the shell results in ~77 nm for 7 polymer layer.

First, it needs to be mentioned that the difference in average layer thickness between the layers on silicon oxide and the layers of the collapsed hollow shell that was previously around a glucose crystal might be attributed to the different post assembly protocol. All samples were prepared by polymer adsorption from 1-butanol and silicon samples were air-dried. In contrast the samples with collapsed hollow capsules were first immersed in water and then air dried. It has been shown earlier for aqueous based assembly protocols that post assembly processing might significantly change the layer thickness and morphology. The reason why the adsorption of

polystyrenesulfonic acid onto polyamine coated surfaces is different to that of adsorption on more inert surfaces might be attributed to the possible surface chemistry. Primary amine groups are known to absorb protons. When a polystyrenesulfonic acid molecule adsorbs on the poly amine surface the negative charges might be neutralized by the positively charged protonated primary amine groups. In addition protons that roam freely in the solution as is indicated by the Oosawa-Manning theory (described in preceding sections of this thesis) might induce a positive charge on the interface by protonating primary amine groups. This would result in a strong interaction between the outermost positively charged polyamine layer that would obtain a positive charge with the polystyrenesulfonic acid molecules that have then a negative charge. Compensation of those un-equilibrated charges would then lead to coherent layer adsorption.

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Multilayer Assembly

Crucial for stable LbL polyelectrolyte multilayer formation is the repeatable alternating absorption of adhering polymers. Absorption of a charged polyelectrolyte reportedly leads to an over compensation of charge resulting in a reversal of the zeta-potential. This allows the absorption of the next oppositely charged polyelectrolyte in classical LbL approaches[37, 63, 146]. It was demonstrated that polystrenesulfonic acid is the only polymer that could dissociate into a charged species in aliphatic alcohols to a certain extent. A reversal of zeta-potential is observed in accordance with the

sequential absorption of PSS and PA from 1-butanol (Figure 4-7) onto colloidal particles.

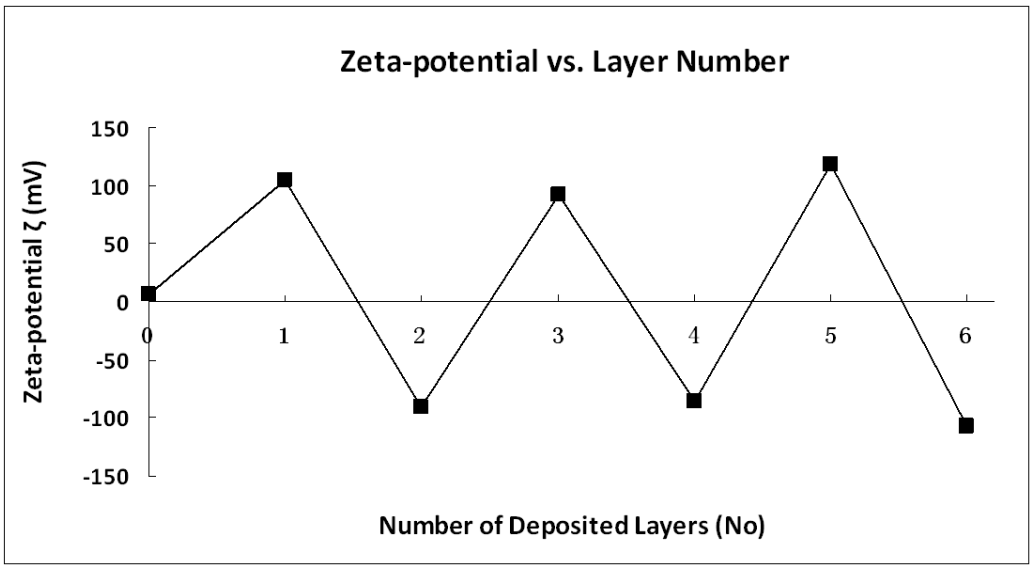

Figure 4-6: Zeta potential versus layer number of colloidal particles coated with PSS and PA from 1-butanol.

Thus, polyallylamine layers have to acquire charge during the assembly process by forming positively charged ammonium salt functionalities. Measurements suggest that the negative zeta-potential originates from the fraction of protons that dissociate from the polystyrenesulfonic acid and act as counter ions to the outermost polystrenesulfonic acid layer. The protons thus compensate the negative potential that is incurred by the polystyrenesulfonate surface. The distribution of the protons around the surface towards the medium might be explained by Debye-screening that has a Debye-length in the magnitude of 0.1 micrometer due to the organic solvent used and absence of any foreign salt as explained in the third chapter of this thesis. In addition to the Debye-screening, counter ion condensation is believed to occur since the interface with an outermost PSS layer represents a very high concentration of acid groups, which can not be considered to be in the dilute regime anymore.

In addition, the presence of PA in polyelectrolyte form as outermost layer with a positive zeta-potential, suggests protonation of the amine functionality. Unlike polyelectrolyte multilayer assemblies from aqueous phase that use polyallylamine hydrochloride, no counter ions are expected within the medium outside the polymer multilayer structure. The charge compensation is believed to be accomplished by the preceding polystyrenesulfonate layer. The exact mechanism how the polyallylamine layer acquires charges and how those are compensated needs yet to be investigated in detail. However, PA in its polyelectrolyte form is insoluble in 1-butanol and no precipitates are observed in the supernatant during multilayer preparation. In combination, these findings support the assumption of a Brønsted acid base reaction in close proximity to the layer surface as main driving force. Although it might be envisioned that polyamines acquire charges due to the absorption of freely diffusing protons from dissociated polyacids. This would cause a very strong electrostatic attraction, which plays a role in multilayer formation, too.

Confirmation of multilayer deposition can only be obtained by combination of zeta-potential measurements with another experiment, indicating increased material deposition with each polymer deposition cycle. Although this was sufficiently demonstrated by atomic force microscopy studies, it seems to be worth to address this question with a further experiment that might yield more information on the internal layer structure.

Increased material deposition during the LbL assembly process can as well be observed by fluorescence microscopy when fluorescent labeled polymers are used. The fluorescence intensity increases with each deposition of fluorescent labeled PAH (PAH-FITC). Subsequent incubation with non fluorescent PSS causes a slight decrease in fluorescence intensity (Figure 4-8).

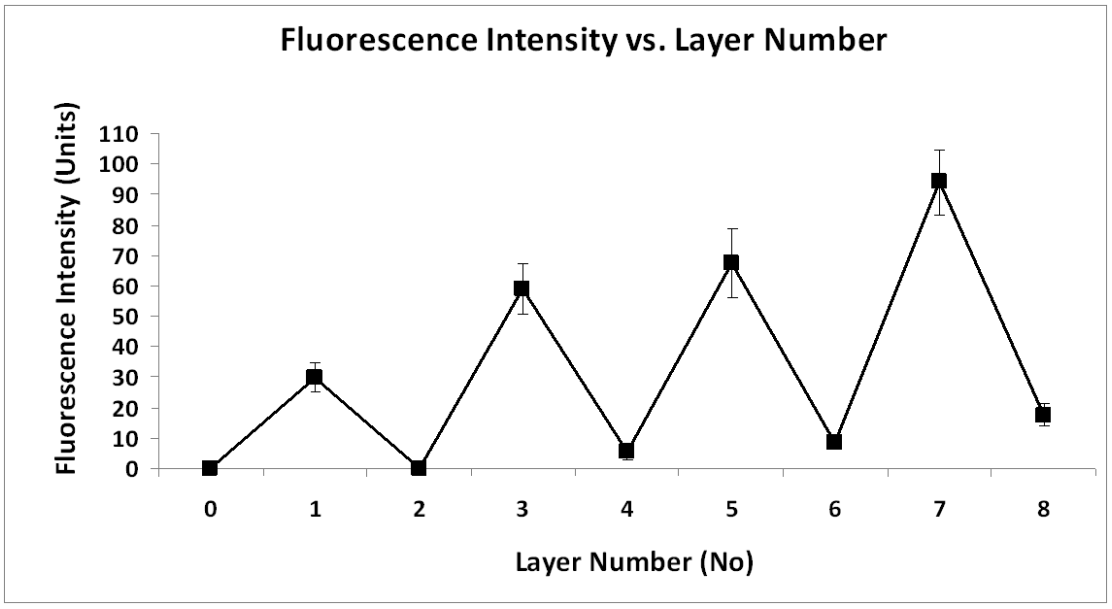

Figure 4-7: Fluorescence intensity versus layer number of colloidal particles subsequently coated with polyallylamine-FITC and polystyrenesulfonic acid using 1-butanol as deposition medium.

The continuously increasing fluorescence intensity with each deposition of a fluorescent labeled polymer indicates multilayer deposition[147].

Interestingly, the decrease of fluorescence intensity after deposition of non-fluorescent PSS close to zero, is much stronger compared to earlier reports in which polystyrenesulfonate sodium salts are used[147]. This decrease in fluorescence intensity might be attributed to the fluorophore's sensitivity to free protons and the ability of PSS to dissociate in 1-butanol. This assumption is derived from the well known pH sensitivity of FITC[148] and the fact that this effect can not be observed when

Polystyrenesulfonate sodium salt is used although environmental properties are almost the same except in terms of free proton concentration. This finding is in accordance with earlier studies that observed a remarkable difference between local pH in close proximity to the polyelectrolyte backbone and the bulk pH that is reflected in different fluorescence intensities of FITC[149] in cases where counter ion condensation occurs.

A basic difference between polystrenesulfonic acid adsorbed at interfaces and in free solution needs to be addressed here in order to understand the different response of FITC to the proton concentration in the bulk and in close proximity to the interface. Once polystrenesulfonic acid is adsorbed on an interface it can not anymore be considered in dilute solution. Thus, according to the Oosawa-Manning theory, the entropic penalty for counter ion condensation becomes less strong. This leads to the fact that protons can be very well confined within close proximity to the interface although polystyrenesulfonic acid has been assembled from dilute solution where no counter ion condensation is expected to occur.

The next fluorophore containing layer shows strong fluorescence intensity, the proton absorbing capacity of that absorbed polyamine layer must thus be greater than the amount of protons that can be provided by the polystyrenesulfonic acid layer for the interaction with the outermost layer.

Layer Thickness and Growth Study

Earlier studies demonstrated a gradual gain in average layer thickness of polyelectrolyte multilayer upon the increasing addition of organic solvents to aqueous polyelectrolyte solutions[135]. Eager to investigate whether earlier results can be extrapolated to an even greater average layer thickness when a pure aliphatic alcohol is used, a layer growth study by atomic force microscopy was conducted. The wall thickness of collapsed hollow polyelectrolyte microcapsules was measured after the assembly of every even layer number and plotted against layer number (Figure 4-9). Average data for each point were acquired from 4-8 AFM pictures each depicting 4 to 10 microcapsules. The average layer thickness is consistent between different batches of microcapsules prepared with the same protocol. The significance of the deposition medium for the average thickness of polyelectrolyte multilayer became especially apparent since various layer thicknesses have been reported to be achievable with PSS and PAH polyelectrolyte of the same molecular weight (most commonly around 70 kDa[105]**).**

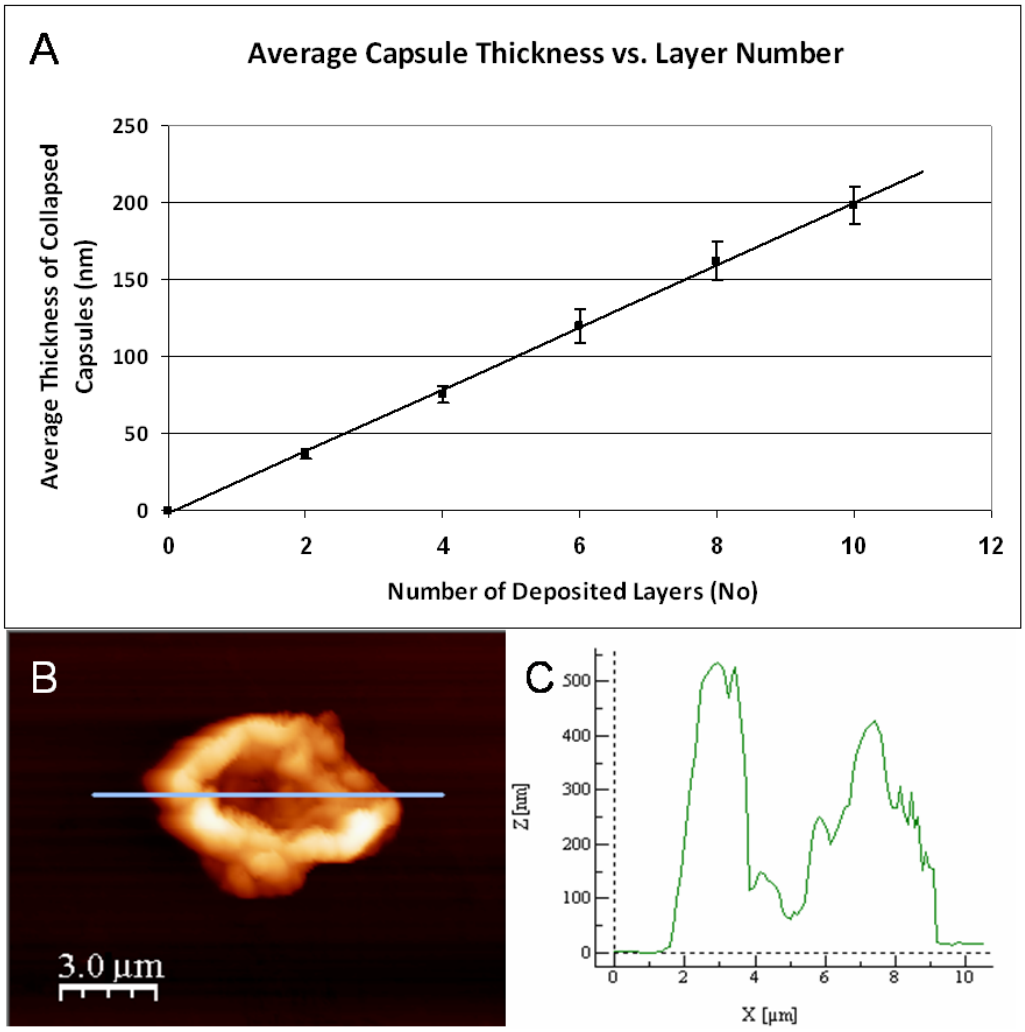

Figure 4-8: Layer thickness growth study by atomic force microscopy of collapsed hollow polyelectrolyte microcapsules comprising of polyallylamine and polystyrenesulfonic acid assembled from 1-butanol.

The averaged thickness of polyelectrolyte microcapsules was plotted against the number of assembled layers (A). Measurements were conducted at lowest folds of microcapsules and are depicted for a capsule comprising of $(PAH/PSS)_2$ (B&C).

More common than adding organic solvents is the usage of salts to tune polyelectrolyte layer thickness in traditional aqueous based approaches[105, 150, 151]. Polyelectrolytes appear to be less stretched due to shielding of the charge/charge interaction at high salt concentrations[113]. The addition of organic solvents to such systems leads to further increase of average layer thickness due to the decrease of

solvent quality, which leads to even more coiled polyelectrolyte conformations[135, 136, 150]. Besides solvent-polymer backbone interaction, Solvent quality may also refer to the ability of the solvent to solvate ions and thus determines ion-polyelectrolyte association[152]. A higher ion-polyelectrolyte association implies a lower charge density and thus to a more coiled polyelectrolyte conformation. In pure organic solvents the charge density of polyacids might approach the same magnitude of charge density; the electrostatic repulsion is however much more far reaching which might annihilate the effect of decreased charge density to some effect.

It is well understood that besides polymer conformation the electrostatic field of the interface is responsible for the average layer thickness of the next adsorbed layer.

The average layer thickness of a polyelectrolyte multilayer could be pushed to 5.5 nm at maximum ethanol concentration of 40% and a salt concentration of 0.5 mol/l measured after deposition of 9 layers of PSS/PAH polyelectrolyte[135]. A clear non-linear growth of polyelectrolyte layers was observed under those conditions but not when low ethanol concentrations were used.

In contrast to those findings, RP-LbL multilayer assembled from pure 1-butanol that comprise of PSS and PAH grow directly proportional to the number of assembled polyelectrolyte layers. The layer thickness was repeatedly found to be 9.75 nm +/- 0.80 nm per layer regardless of the layer number. Notably, the 1-butanol based deposition media is of very low ionic strength that is only determined by the auto-dissociation of the dissolved polymers. Furthermore, the permittivity of 1-butanol is

much lower then that of aqueous based deposition media or of other organic solvents (e.g. ionic liquids or formamide) used for LbL deposition as mentioned before. Thus, screening effects due to ionic strength become minimized while the range of electrostatic interaction becomes greater, which accounts at least partially for the high average layer thickness.

Conclusion

In conclusion, the RP-LbL method that uses polyacid and polyamines dissolved in aliphatic alcohols to assemble polyelectrolyte complexes and multilayer structures has a distinct mechanism compared to established LbL methods such as classical LbL using polyelectrolyte salts or hydrogen bonding based LbL. The mechanism is essentially marked by the fact that no small molecular weight counter ions are released upon polymer complex formation. The polymer-polymer interaction is however of electrostatic nature. This is only possible by an acid base reaction in which an acid moiety protonates an amine moiety to create a salt linkage. This was indeed demonstrated by IR spectroscopic investigation. It was found that in majority amine and acid functionalities exist in their salt form although the spectra resemble properties of free acids and bases to a limited extend. This finding on the same time fulfills the specific aim 1 of this chapter. The multilayer build up was investigated with zeta-potential measurements and fluorescence microscopy based methods (specific aim 2 of this chapter) the multilayer build up is not yet completely

understood although the experimental results suggest that it is primarily due to an acid base reaction in close proximity. Two very distinct mechanisms of polymers approaching an interface can be found in hydrogen bonding LbL and classical LbL using polyelectrolyte salts in high polarity solvents. In hydrogen bonding LbL the polymers approach the interface randomly by diffusion whereas in classical LbL approaches an electric field adds attractive forces. Due to the nature of the polymers that are used in RP-LbL it can be imagined that the nature of polymers approaching the interface is directed towards that of hydrogen bonding LbL when the charge density on polyacid is decreased such as for polacrylic acid in 1-butanol. In the case of polystyrenesulfonic acid an electrostatic attraction is believed to play a major role for the polymer adsorption.

The hypothesis that RP-LbL multilayer build up and polymer interaction is due to an acid base reaction in close proximity to the interface is thus confirmed (specific aim 4). Although the exact nature of polymer adsorption is governed by the charge density inherent to polyacid due to autoprotolysis in the media and might be in between the two extremes of classical polyelectrolyte salt LbL methods and hydrogen bonding LbL.

The specific aims as defined at the beginning of this chapter can thus be considered fulfilled and are stated again for comparison in the following.

1. To investigate the nature of polymer-polymer interactions by IR spectroscopic investigation, in particular to establish whether hydrogen bonds or ionic linkages exist between polyallylamine and polystyrenesulfonic acid when mixed in 1-butanol.
2. To address the mechanism of multilayer build up by measuring of surface properties such as the zeta potential or other fluorescence base methods.
3. To investigate the peculiarities of polymer adsorption at interfaces during the RP-LbL process.
4. To confirm or refute the hypothesis that RP-LbL multilayer build up and polymer interaction is due to an acid base reaction in close proximity to the interface.

Chapter 5: Conclusion & Significance of this Thesis

This chapter of the thesis shall provide a summary of all engineering aspects and scientific hypothesis that were set to be fulfilled and validated at the beginning of the dissertational work. Furthermore the conclusions and particular scientific and engineering advancements that were achieved in this thesis are stated in the light of general significance. A thorough evaluation whether engineering aspects were fulfilled and scientific hypothesis experimentally validated or refuted is provided. The nature of this thesis to address both, engineering aspects as well as basic science is well in line with the policy of the National University of Singapore Graduate School for Integrative Sciences and Engineering (NGS) to synergistically combine engineering and science under one ideational roof. In addition this final conclusion allows to reflect the deliberately chosen structure of this thesis in the light of its value to contribute novel knowledge and designs to the research community. Finally concluding an outlook of current work in progress, that addresses strategies to engineer the RP-LbL process in a way that it will be more suitable for food or pharmaceutical applications.

Background and General Considerations:

The first chapter of this thesis is an introductory chapter that describes a specific sub-part of the physicochemical and applied research field that deals with polyelectrolytes and polyelectrolyte self assembled structures. In particular the first chapter aims to address the limiting parameters in commonly employed protocols to generate polymeric self assembled microcapsules and especially the low encapsulation efficiency for biomolecules. Besides the identification of shortcomings in established protocols, two practical approaches (the RP-LbL and the "inwards build up of polymers" method) that were developed in the NanoBioanalytics laboratory to circumvent those limitations are described. This strategy was chosen deliberately rather then giving a general introduction to polyelectrolyte chemistry and their self-assembly based technologies. The reason for this approach is that a most thoroughly and comprehensive introduction including almost all relevant references to Layer by Layer self-assembly technology can be easily deducted from the latest book by Gero Decher and Joseph B. Schlenoff: *Multilayer Thin Films - Sequential Assembly of Nanocomposite Materials Second Edition.* A general introduction of equal quality would be beyond the scope of a PhD thesis due to the size of the field. To provide the grounds for this thesis with an introduction that anchors the RP-LbL self-assembly technology in the general field of Layer by Layer self-assembly research and to provide specific engineering aspects to be fulfilled and scientific hypothesis to be validated within the scope of this dissertational work seems to be the best introduction possible.

The first chapter reviews almost the entire polymer self-assembly work within the NanoBioanalytics laboratory which I have accomplished and in which I have been involved as collaborator during my dissertational work and provides conclusions and a framework of engineering aspects and scientific hypothesis that shall be fulfilled and investigated in the scope of this thesis.

Specific Engineering Aspects and Scientific Hypothesis of this Thesis

Engineering aspects:

1. To provide a technology that allows reliably the encapsulation of biomacromolecules within nm thin polymeric microcapsules.
2. To achieve control over the concentration of encapsulated materials.
3. To show the applicability of the developed platform technology to life sciences, bio-analytical or diagnostic research.
4. To provide an experimental setup to address specific questions that allows the thorough investigation of the scientific hypothesis.

Scientific hypothesis:

1. That polymeric multilayer formed through the RP-LbL protocol hold together due to electrostatic interactions.
2. That polyacids exhibit a strongly reduced charge density in organic solvents compared to aqueous solutions.

3. That the electrostatic interactions within the polymeric multilayer that were assembled with the RP-LbL protocol are induced by acid base reaction in close proximity to the interface.

The explicit analysis how or whether those specific aims were achieved and hypothesis are refuted or validated will be presented in the following. Further specification of single sub-aims and whether or how they are fulfilled are described detailed within the actual chapters.

Experimental Work and Engineering Aspects

Although the first chapter summarizes the results of scientific experiments, this chapter describes a set of experiments that address the fabrication of polyelectrolyte multilayer capsules using water-soluble template material and their loading with biomacromolecules. In contrast to many other classical theses, the in detail description of the operating principles of equipment is deliberately not stated in this thesis. Detailed instrumental description of standard equipment is available from various sources and its repetition would not add to the understanding of the results or add novel knowledge to the research community. The statement of principles in pH measurements and electrode architecture in chapter 3 constitute an exception since the accurate understanding of the setup and principles is crucial to yield correct scientific data. The second chapter describes the fabrication of self assembled microcapsules from organic solvents using polystyrenesulfonic acid and polyallylamine. The formation of nm thin polymeric capsules and the ability to be loaded with

biomacromolecules at desired concentration was demonstrated. While the first chapter included a report about the utilization of RP-LbL based technologies for the multiplexed observation of biomolecules interactions, the second chapter addresses the question whether microcapsules prepared by the RP-LbL could potentially be used in analogy to conventionally produced microcapsules for biomedical applications. A major concern for the biomedical application has been the possible residue of organic solvents, however it was demonstrated that the residual amount of organic solvents is very low so that cells that could potentially be brought in contact with RP-LbL produced microcapsules would be viable. As finalizing conclusion for RP-LbL derived core shell materials from the engineering point of view, it was demonstrated that RP-LbL produced self assemblies can indeed be applied in life science or biomedical and diagnostic-analytical applications. Chapter 2 thus reports on the successful fulfillment of the aimed engineering aspects 1 to 3. One more aspect that has not yet been demonstrated is to utilize the RP-LbL for specific pharmaceutics or food applications. This would require all materials used to be considered safe for consumption. Reflecting the materials that are used within the RP-LbL process, the solvent might be ethanol and the sacrificial template might chosen to be sucrose so that the challenge to only use materials that are considered safe for consumption lies with the polymers. Two polymers that have not yet be reported to be used fro the RP-LbL process but are generally known to be safe for consumption and are even Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) of the United States of America are polyglutamic acid and ε-poly-L-lysine. Studies to

test their applicability to RP-LbL are currently underway. Unfortunately the pool of polymers that might be used for this application is very limited. Many polymers that are considered GRAS are not soluble in non-aqueous solvents that are considered to be GRAS. Further limitation is exhibited by the fact that not all GRAS considered solvents are suitable for carbohydrate based template materials. The production of uniform, ideally spherical, carbohydrate based template materials with accurate control over cargo concentration seems to be possible by combining the developed technology with spray drying. Spray drying of carbohydrate solutions containing a protein of interest and an emulsifier leads to spherical particles with narrow size distribution[153]. The size distribution might be engineered to be within sub-micron or lower micron range. Options to choose materials in a way that the resulting RP-LbL process derived material are considered safe for consumption is a currently pursued project within the NanoBioanalytics laboratory.

Characterization of Materials and Basic Science in the Light of Engineering

The third chapter addresses the basic need for a method to characterize polyacids in non-aqueous solution. This need is demonstrated by the most versatile applications of solution of polyacids and especially polystyrenesulfonic acid in non aqueous solvents as stated before. Various techniques have been developed based on spectroscopic methods or conductivity measurements to characterize polyacids. Those methods are

however difficult to perform with standard equipment or are labor-intensive. The aim of this thesis is thus to provide an easy method that can be fast and easily employed to characterize polyacids in non-aqueous solutions. Both, potentiometric determination of acid properties for mono protic acids in non-aqueous solvents as well as the characterization of polyacids in water is well investigated. By combining the theories and procedures a simple relation between the pKa value of the acid that describes the monomer unit best and the applicability of potentiometric measurements was established. This is in partial fulfillment to investigate to which extent polystyrenesulfonic acid carries charges in organic solvents due to autoprotolysis. In addition this procedure is a reliable and robust experimental setup to investigate the scientific hypotheses. The creation of such a simple and reliable setup was number 4 of the specific engineering aims as specified before that can be as well considered successfully achieved. In addition the pKa value of PTSA in linear aliphatic alcohols were determined. Despite intensive search no such reference values that were previously published could be found, adding additional significance to this investigation. Furthermore it could be shown that the apparent pKa value of polystyrenesulfonic acid in linear aliphatic alcohols is similar to the acidic strength of polycarboxylic acids in water. The scientific hypotheses that polystyrenesulfonic acid has a significantly lower charge density compared to the same polymer dissolved in water could thus be confirmed.

Novel Mechanism of Multilayer Build Up and Polymer Interaction

The fourth chapter investigates the polymer-polymer interaction by IR spectroscopic, zeta-potential measurements and fluorescence based studies. In addition the adsorption of polymers at interfaces and the polymer multilayer build up is investigated. It could be shown by IR spectroscopic measurements that a acid base reaction occurs to a large extend rather then hydrogen bonding when polymer solution are mixed in the bulk media. Theoretical considerations from the previous chapter indicate that protons condense in close proximity to the interface comprising of a polystyrenesulfonic acid layer. This causes the acid base reaction to happen in close proximity to the interface upon adsorption of the next polyamine layer. The hypothesis that an acid base reaction in close proximity to the interface is responsible for multilayer build up is thus confirmed. Although, electrostatic attraction might provide a contribution for the polymer adsorption due to colloidal polymer molecules in solution being subjected to the same chemistry, which creates two contrary charges at the interface and the colloidal polymer molecule in solution. The uniform polymer adsorption during RP-LbL self-assembly of polystyrenesulfonic acid and polyallylamine from 1-butanol and the empirical rule of thumb to use polyallylamine as first layer during LbL assembly could be explained by Atomic force microscopy studies.

Personal Remarks

My dissertational work has been an extremely fascinating and enriching experience that allowed me to grow professionally and personally. This thesis only reflects a small part of the skills that were acquired during this period and not all scientific publications to which I contributed during my dissertational work are stated here equally detailed. The content of this Thesis was aimed to be selected for its novelty and value for the scientific community. Starting as a chemist specialized in synthetic and polymer chemistry, with some background in microbiology the dissertational work provided me with practical and theoretical knowledge in cell biology (including clean room cell culture training and associated biochemical techniques for characterization), optical, confocal and other fluorescence based microscopy as well as Near Field Scanning Optical (NSOM) Microscopy and Atomic Force Microscopy (AFM). The two AFM based techniques are acquired in special depths since the constant maintenance and utilization of the AFM/NSOM - Multiview 1000 system from NANONICS as well as the comprehensive undergraduate teaching on those systems required in depths practical knowledge. I am also most grateful for the possibility to study the interaction of polymers with living organisms (zebra fish) with much help of my fellow PhD scholar Mr. See Zhenwei Kelvin in the group of Prof. Christoph Winkler and my fellow PhD scholar Mr. Rafi Rashid in the group of Prof. Thorsten Wohland. This work in progress is in the domain of classical biology as well as colloidal chemistry and provides interesting perspectives for further studies. The above mentioned practical skills will facilitate a most promising, fascinating and

exciting postdoctoral project in the field of “nanoparticles and polymer interactions with biological systems” that starts to emerge from our combined efforts and that I believe to be a ideal continuation of earlier research on colloids and polymers. Besides practical skills numerous skills that are associated with the communication of research in written and oral form as well as the protection of innovation and project leadership management skills through the supervision of students were acquired.

This thesis seems to be best concluded by addressing thanks again to all the people that have supported me.

Sebastian Beyer

Singapore, November 2012

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