

**DEVELOPMENT OF REACTIVE POLYANIONS FOR
ENCAPSULATION OF LIVE YEST CELLS WITHIN
POLYMER HYDROGEL FILMS**

To my parents, Liam and Anne Foley

**DEVELOPMENT OF REACTIVE POLYANIONS FOR
ENCAPSULATION OF LIVE YEST CELLS WITHIN POLYMER
HYDROGEL FILMS**

By

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**Development of Reactive
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Abstract

Conformal coating is a promising method of encapsulating therapeutic cells for immunoprotection during implantation in the human body. Therapeutic cells can include donor-derived primary cells, genetically modified lab lines, or stem-cell derived cells, that can express an enzyme or hormone needed by the recipient. Layer-by-Layer (LbL) assembly, a common method for conformal coating, uses sequential deposition of alternating layers of positively and negatively charged polymers. This permits the preparation of small capsules with thin walls that maximize metabolic exchange while protecting cells from the hosts' immune system. The current work describes the use of auto crosslinking synthetic polyelectrolytes to coat the surface of individual living yeast cells. It is proposed that such crosslinked coatings will provide greater protection than existing, non-crosslinked ones.

The thesis also introduces new reactive polyanions formed by copolymerization of N-acryloxysuccinimide and acrylic acid (AA) using both free radical and photo induced radical polymerization. The resulting reactive polyanions bind under physiological conditions to poly-L-lysine coated calcium alginate hydrogel beads and spontaneously crosslink with the polyamine to form covalent networks of interest for long-term therapeutic cell encapsulation.

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Table of Contents

1. Introduction to Cell Encapsulation.....	1
1.1 Cell Encapsulation.....	1
1.2 Methods of Cell Encapsulation	3
1.3 Microcapsule Preparation.....	5
1.3.1 Extrusion Method.....	5
1.3.2 Co-Extrusion Method.....	6
1.4 Microcapsule Shell Material.....	7
1.5 Conformal Coating	9
1.6 Layer-by-Layer (LbL) deposition of polymers	11
1.7 LbL conformal coating of cells.....	12
1.8 Reactive polyelectrolytes.....	15
1.9 Hydrogel Films	17
1.10 Hydrogel film formation.....	18
1.10.1 Spin Coating	18
1.11 Model Cells.....	20
1.12 Thesis Objectives	20
2. Experimental.....	21
2.1 Materials.....	21
2.2 Characterization	22
2.3 Instruments.....	23
2.4 Procedures.....	24
2.4.1 Functionalization of Poly(acrylic acid) (84:16 mol%).....	24

2.4.2	Thermally initiated Free Radical Polymerization (AA:NAS = 75:25 mol%)	24
2.4.3	Synthesis of P(AA-co-NAS) by Photo-Initiated Copolymerization at Room Temperature	25
2.4.4	Synthesis of Rhodamine-labelled Poly-L-lysine (PII _r)	25
2.4.5	Preparation of PMM ₅₀ and PMM _{f50} by Controlled Hydrolysis of PMM ₀	26
2.4.6	Standard Procedure for the Formation of Alginate-PLL-P(AA-NAS) capsules (work carried out by Carla Brown)	26
2.4.7	Conformal Coating	27
2.4.8	Confocal Microscopy Assessment of coating procedure	28
2.4.9	Thin Film Formation	28
2.4.10	Lamination of Saccaromyces on Glass Slides	29
2.4.11	Investigation of Electrostatic Yeast Immobilisation	30
2.4.12	Identify Budding	30
3.	Results and Discussion	31
3.1	Synthesis of Poly(acrylic acid-co-N-acryloxysuccinimide)	31
3.1.1	Functionalization of Poly(Acrylic Acid)	31
3.1.2	Thermally Initiated Free-Radical Copolymerization	32
3.2	Side reactions of Poly(acrylic acid-co-N-acryloxysuccinimide)	33
3.3	Photo-chemically Initiated Radical Copolymerization	41
3.4	Crosslinking Reactions with Polyamines	43
3.5	Film Formations	46
3.6	Characterization of Polymer and Hydrogel Films	47
3.7	Conformal Encapsulation of Saccaromyces cerevisae	51

3.8	Activation and Lifecycles of Yeast Cells	59
3.9	Lamination of Saccaromyces on glass slides	62
3.10	Budding on surface	65
4.	Conclusions	68
4.1	Synthesis of Reactive Polymers	68
4.2	Coating of Cells	68
5.	Future Work	70
5.1	Further Characterization of Polymer Films	70
5.2	Further Cell Studies with Yeast and Mammalian Cells	70
6.	References.....	72

List of Figures

Figure 1.1 Concept of cell encapsulation, oxygen, nutrients and waste products diffuse across the membrane, antibodies and immune cells are kept out. ⁶	2
Figure 1.2 Devices for cell encapsulation can be broadly divided into macro and micro capsules, as well as conformal coating of cells and cell-surface modifications. ¹	4
Figure 1.3 Schematic of extrusion method ⁹	6
Figure 1.4 LbL assembly of charged polymers onto a solid, cationic substrate. Steps 1 and 3 are the adsorption of polyanion and polycation respectively, while steps 2 and 4 are washing steps.(reproduced with permission) ²⁹	12
Figure 1.5 A scheme showing the sequential deposition of oppositely- charged polycations (red) and polyanions (blue) multilayered films doped with nanomaterials (nanotubes and nanoparticles) onto isolated living cells. ³⁶	14
Figure 1.6 Polyelectrolytes used for crosslinking.....	17
Figure 1.7 A schematic of spin coating ⁴⁰	19
Figure 3.1 Functionalization of poly(acrylic acid) with N-hydroxysuccinimide using DCC coupling.	31
Figure 3.2 Free radical co-polymerization of acrylic acid (AA) and N-acryloxysuccinimide (NAS) using AIBN.	32
Figure 3.3 ¹ H NMR spectrum of 10mol% AA and NAS (25:75) in DMSO-d ₆ at a. time 0; and b. 1 hour.....	35
Figure 3.4 Photo polymerization of AA and NAS to yield co-polymer.	37
Figure 3.5 Two routes to the formation of cyclic anhydride. (A) adjacent ester and acid groups reacting to release NHS, (B1) reaction between two adjacent acid groups to release water and (B2) hydrolysis of the activated ester to release NHS	39
Figure 3.6 Photograph of a photo-initiated precipitation polymerization (60 min, 0 C) of AA with NAS in ACN-d ₃	41

Figure 3.7 1H NMR spectrum of co-polymer, ACN-d3 removed under vacuum dissolved in DMSO-d6.....	42
Figure 3.8 Crosslinking reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage.....	45
Figure 3.9 Crosslinking reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage. PLL is spin-coated onto a glass slide and subsequently spin coated with Poly(AA-co-NAS) and unreacted NAS groups are present for further LbL.....	47
Figure 3.10 Crosslinking reaction between an activated ester and the primary amine of PLL to yield covalent amide linkages. PLLf was spin-coated onto a glass slide followed by spin coating of P(AA-co-NAS). (A) 10 bilayers were built up. (B) NaOH 0.1M was added for x min, and washed away	48
Figure 3.11 Crosslinking reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage. PLLf is spin-coated onto a glass slide followed by spin coating of P(AA-NAS). 2 layers were built up (A), 10 layers (B) and 14 (C).....	49
Figure 3.12 Reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage. Line profiles are taken over each scratch to measure fluorescent intensity. 2 layers were built up (A), 10 layers (B) and 14 (C)	50
Figure 3.13 TEM micrograph of LUDOX® Cl nanoparticles on the surface of a yeast cell	52
Figure 3.14 TEM micrograph of LUDOX® HS nanoparticles on the surface of a yeast cell	53
Figure 3.15 Optical and fluorescence images of PLLr coated yeast cells. A) optical image of PLLr (15-30k) coated cells and its fluorescence image (B), optical image of PLLr (40-60K) (C) and fluorescence image (D). Scale bar is 10 microns.....	55
Figure 3.16 Optical and fluorescence images of PLL-PMM50f coated yeast cells. A) optical image of PLL- PMM50f (15-30k) and its fluorescence image (B), optical image of PLLr- PMM50f (40-60K) (C) and fluorescence image (D). Scale bar is 15 microns.	57

Figure 3.17 . Confocal microscopy images of PLLr / PMM50f coated yeast cells. PLLr (red), PMM50f green and a nuclear stain DAPI (blue). A= 2 layers, B,C = 4 layers and D=8layers.....	59
Figure 3.18 The life cycle of normal yeast cells	60
Figure 3.19 Optical images of yeast cells coated with polyelectrolyte complex – FITC labelled PAH and PSS.....	61
Figure 3.20 Yeast cells deposited on a PLL[(p(AA-co-NAS) – PLL]5 film on glass.	63
Figure 3.21 Yeast cells deposited on PLL-[p(AA-co-NAS) – PLL]5 and coated with p(AA-co-PLL)	63
Figure 3.22 Yeast cells deposited on PLL-[p(AA-co-NAS) – PLL]5 and coated with [p(AA-co-NAS) – PLL]4 – p(AA-co-NAS).....	64
Figure 3.23 Images before addition of NaCl (attempt to break electrostatic interaction) (A) and image B after 2M NaCl was added to the slide. Yeast cells maintained their adhesion to the PLL surface.	65
Figure 3.24 Yeast cells within polymeric multilayer in YPD for 3 days.....	66
Figure 3.25 Yeast cells immobilized within polymeric multilayer films. A) yeast deposited on PLL layer 11 , B) yeast cells in YPD media	67

List of Tables

Table 3-1 Copolymer composition 37

List of Abbreviations

AIBN	Azobis(isobutyronitrile)
AF	Aminofluorescein
APA	Alginate - Poly-L-lysine - Alginate
CaCl₂	Calcium Chloride
DCC	N,N'-dicyclohexyl(carbodiimide)
DCU	Dicyclohexylurea
DMF	N,N'-dimethylformamide
DMPA	2,2-Dimethoxy-2-phenylacetophenone
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
FDA	Fluorescein Diacetate
FITC	Fluorescein isothiocyanate
LbL	Layer-by-Layer
MAA	Methacrylic acid
MOEAA	Methacryloylethylacetoacetate
NAS	N-acryloxysuccinimide
NHS	N-hydroxysuccinimide
PAA	Poly(acrylic acid)
PAH	Poly(allyl amine)
P(AANAS)	Poly(acrylic acid-co-N-acryloxy succinimide)

PEG	Poly(ethylene glycol)
PMM	Poly(methyl vinyl ether- <i>alt</i> -maleic anhydride)
PSS	Poly(styrene sulfonate)
RITC	Rhodamine isothiocyanate
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
VDMA	4,4-dimethyl-2-vinyl-2-oxazoline-5-one

1. Introduction to Cell Encapsulation

1.1 *Cell Encapsulation*

Transplantation of therapeutic allogenic cells has emerged as a promising approach for the treatment of diseases ranging from endocrine disorders such as diabetes¹, to disorders of the central nervous system such as Alzheimer's. While it allows for ongoing delivery of therapeutic agents, including proteins,² the immune response of the patient to free allogenic cells (same species, different individual) represents a key hurdle to the use cell therapy in a clinical setting. Immunosuppressive drugs represent one option to deal with immune rejection, but at the cost of increased risk of infection and cancer. Physical entrapment of therapeutic cells within a semi permeable membrane offers an enticing drug-free approach to immunoprotection of transplanted allogenic cells.

The concept of using encapsulation for the immunoprotection of transplanted cells was introduced in the 1960's by T.M.S. Chang.³

Cell encapsulation involves the entrapment of living cells within a semi-permeable shell or matrix that allows for the in-diffusion of nutrients and oxygen as well as the out-diffusion of waste products and the actual therapeutic protein, while cellular and large molecule components of the immune system are kept out (Figure 1.1.)

Encapsulation of cells can thus allow for extended delivery of the

therapeutic agent expressed by cells, compared to, e.g., the encapsulation of the therapeutic product itself in a reservoir-style fashion. The pioneering work of Chang led the development of the concept of a bio-artificial pancreas,⁴ where transplanted islets of Langerhans produce insulin in feedback with blood sugar levels. A key advance involved the encapsulation of functional islets in alginate hydrogels by Lim and Sun in 1980, and their successful though somewhat short-term control of blood glucose in diabetic mice.⁵

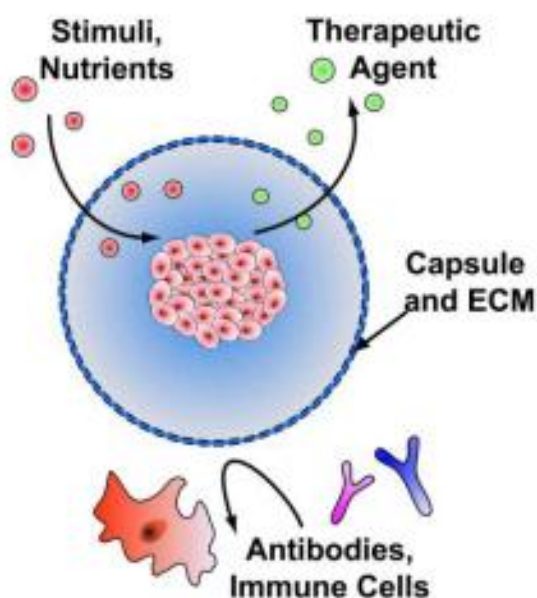


Figure 1.1 Concept of cell encapsulation, oxygen, nutrients and waste products diffuse across the membrane, antibodies and immune cells are kept out.⁶

Since then, many different immunoisolation devices have been developed.

1.2 Methods of Cell Encapsulation

A variety of polymeric and inorganic matrices have been used to create different immunoisolation devices.⁷ Devices for the immunoisolation of cells are shown in Figure 1.2.

Macroencapsulation devices such as vascular tubes and flat disc-shaped devices allow for a large number of cells to be implanted. One advantage of such a system is the minimal risk associated with device retrieval. The main drawback for this technology is the low surface area to volume ratio which limits the diffusion of oxygen and nutrients especially to the cells in the centre of the devices.⁸

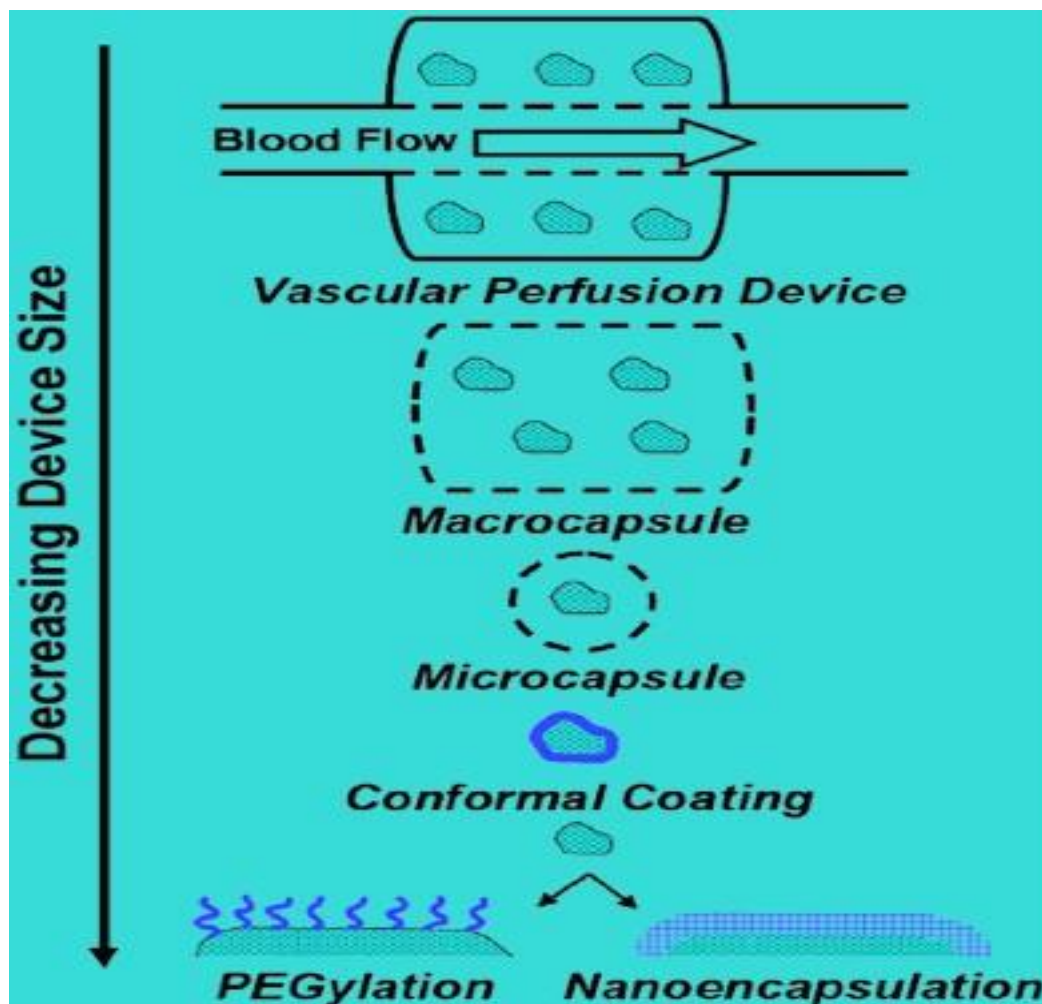


Figure 1.2 Devices for cell encapsulation can be broadly divided into macro and micro capsules, as well as conformal coating of cells and cell-surface modifications.¹

Spherical microcapsules ranging from 50 μm to 1.5mm in diameter have favourable surface to volume ratios, and can be implanted with minor surgery.^{7,8} The size of these microcapsules also permits their implantation in close contact to the patient's blood stream, e.g. through injection into the liver portal vein.

Different parameters need to be considered for the design of an immunoisolation device. Implanted devices need to have sufficient mechanical stability to resist physical and biochemical stresses and prevent rupturing of the capsule. If the capsule breaks, immune rejection of the encapsulated cells will take place, possibly extending to other, still intact capsules nearby. The devices need to be compatible with both the cells contained within the device, and with the host.

1.3 Microcapsule Preparation

Spherical hydrogel microcapsules can be produced in a variety of ways. They almost always consist of synthetic or natural hydrophilic polymer networks. Polymer hydrogels have some resemblance to cells' natural environment, the so called extracellular matrix, which is a complex mixture of hydrophilic highly charged polysaccharides, decorated with peptide and lipid units. Synthetic hydrogels can mimic this highly hydrated environment, and are similarly permeable to low molecular weight nutrients and metabolites. Common methods for microcapsule formation include:

1.3.1 Extrusion Method

Here, the crosslinkable polymer solution containing cells in, e.g., sodium alginate, is extruded through a small needle to drop into a gelation bath, where the hydrogel is typically ionically crosslinked by a multivalent cation, most often calcium chloride or barium chloride, or more recently strontium chloride. The

simplest system uses pressure as the driving force for drop creation as shown in Figure 1.3. This method allows for rapid gelation at room temperature. Air-shearing extrusion is a common variant whereby shearing of the alginate solution is assisted by an annular flow of air. Other variants use electrostatic potential of 1000 – 8000V between the extrusion needle and the gelation bath to facilitate droplet formation, allowing access to droplets of diameters down to 200 micrometer and below.

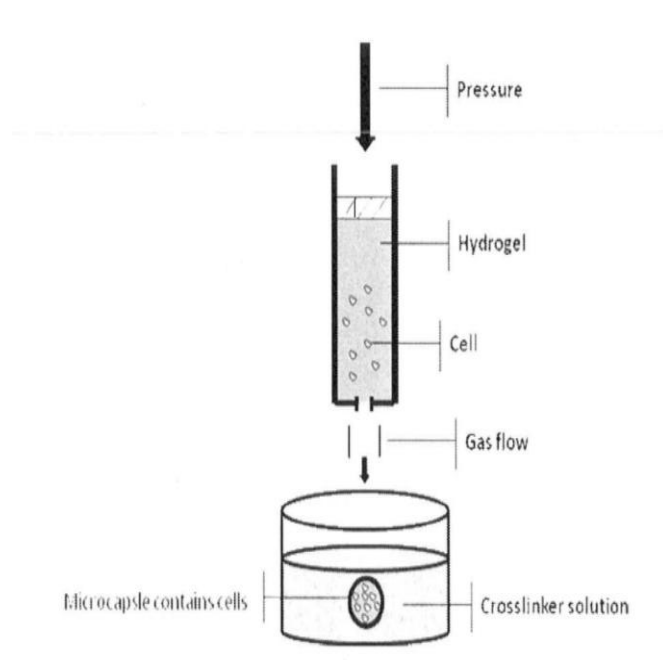


Figure 1.3 Schematic of extrusion method ⁹

1.3.2 Co-Extrusion Method

This technique is based on a double nozzle system, where an aqueous polymer solution with cells is dispensed through the inner nozzle and the shell

forming polymer solution is dispensed through the outer nozzle. The polymer solutions gel upon entering the precipitation bath. The resulting cell-containing cores beads are thus coated with a cell-free hydrogel shell.

1.4 Microcapsule Shell Material

Materials used for long-term microencapsulation must be compatible with both cells and host, have the right permeability, and should be mechanically and chemically stable for periods up to a year.

One of the most widely studied materials is alginate. Alginate, occurring naturally in its sodium form in brown algae in the northern Atlantic, is a polysaccharide, consisting of β -D-mannuronic acid (M) and α -L-gluronic acid (G) residues that can be ionically crosslinked by calcium ions through the G-rich regions of the alginate chain. The classic implementation involves Alginate - Poly-L-Lysine - Alginate (APA), as first described by Lim and Sun.

Formation of APA microcapsules starts with the extrusion of a sodium alginate / cell mixture through a syringe under coaxial air shearing to form droplets that fall into a CaCl_2 solution to crosslink. These capsules are held together by ionic interactions and meet some of the requirements for immunoisolation of cells implanted into mice, though they are have insufficient strength when implanted into larger animals.^{10, 11} This initial calcium alginate beads are hence often coated with poly-L-lysine (PLL), a cationic polyelectrolyte that complexes with alginate to form a strong, permeability-limiting outer shell. To mask this polycation from the host, the beads are again coated with alginate in

attempts to create an overall negatively charged surface. This is thought to prevent undesired protein binding and subsequent immune responses.

Conventional APA capsules' mechanical strength can be improved significantly by covalent crosslinking. Hallé¹², investigated photo-activated crosslinker used to covalently link PLL chains with other PLL chains as well as with alginate chains on the outer shell of the capsules. These capsules showed improved mechanical stability while preserving cell viability and permeability compared to conventional APA microcapsules.

Previously, our group reported on the use of reactive polyanions containing acetoacetate groups that can form covalently crosslinked shells when coated onto poly-L-lysine (PLL)-treated calcium alginate beads.¹³¹⁴ While these capsules showed improved mechanical stability compared to conventional APA capsules, their residual acetoacetate groups caused undesirable protein binding¹⁵.

Gardner et al recently described the use of partially (50%) hydrolyzed poly(methyl vinyl ether-*alt*-maleic anhydride), PMM₅₀, as a water-soluble polyanion that can covalently crosslink with PLL on the surface of PLL-coated calcium alginate capsule.¹⁶ The high reactivity of the anhydride groups offers both a quick crosslinking reaction with the amines of PLL, as well as rapid subsequent hydrolysis of residual anhydride groups to form desirable carboxylate anions. The hydrolysis half-life was measured to be 2.5 minutes, which requires rapid processing to ensure crosslinking before hydrolysis is complete. Our group

developed copolymers of vinyl dimethylazlactone with methacrylic acid that can form permanent amide crosslinks with PLL and PLL-coated calcium alginate beads, and that have hydrolysis half-life of about 30 minutes, though at the cost of using a fairly high molecular weight electrophile. There is hence a need for easily accessible reactive polyanions with a longer hydrolysis half-life, and this represents one aim of this thesis.

1.5 Conformal Coating

Islets of Langerhans have been encapsulated in a variety of gel beads based on synthetic or natural materials, such as poly(vinyl alcohol), alginate and agarose.^{7, 17} Although this approach has been successful in several animal models, it has not led to many clinical trials due to issues with biodegradation of materials, lack of biocompatibility of materials, and in particular, hypoxia of encapsulated islets due to the limited oxygen diffusion into non-vascularised gel beads and capsules.^{7, 17, 18}

The high surface to volume ratio of spherical microcapsules helps with passive transport of oxygen and nutrients to encapsulated cells,¹ but core asphyxia has been noticed for capsules with diameters above 200 micrometers. Most current systems of microencapsulating cells involve different drop generating processes that lead to hydrogel capsules having diameters between 400 and 800 μm , and between 200 and 400 for electrostatically assisted processes.

It has been observed that larger capsule sizes have delayed insulin release in response to glucose level changes.,^{19 20, 21}

Islet encapsulation in beads also suffers from volume issues. When the diameter of microcapsules increases 5-fold over that of the islets (~100 micron), the total volume increases more than 100-fold.²² So, when 500,000 islets (~10 mL) are required for one patient, the total volume of the encapsulated islets is more than 1 L, making it virtually impossible to implant the required amounts of encapsulated islets,²³ even in areas that can handle larger transplant volumes such as subcutaneously or intraperitoneally.

Clinically, however, the portal vein of the liver is a preferred site for islet transplantation,^{24, 25} as it offers an oxygen and nutrient rich environment.¹⁹ The maximum size of hydrogel beads that can be transplanted into the liver portal vein without excessive occlusion is about 200 micrometer, barely larger than the average size of native islets at about 100 to 150 micrometer.²⁶

Engineering the molecular surface of a cell with natural or synthetic compounds can provide a powerful tool for controlling interactions between cells and their environment. Polymers such as polysaccharides along with synthetic polymers have been introduced to the surface of the cell through chemical and physical means.^{27,28} These approaches are called conformal coatings, and in principle give access to cells and clusters of cells coated with immune protecting hydrogel layers ranging from a few nanometer to a few micrometer in thickness.

1.6 Layer-by-Layer (LbL) deposition of polymers

Layer-by-Layer (LbL) polymer assembly allows formation of thin polymer films of different composition and properties..The layer-by-layer technique involves the deposition of alternating layers of oppositely charged polyelectrolytes by electrostatic adsorption from dilute solutions. Introduced in 1991 by Decher, LbL assembly results in alternating surface charges after each deposition step (Figure 1.4).²⁹

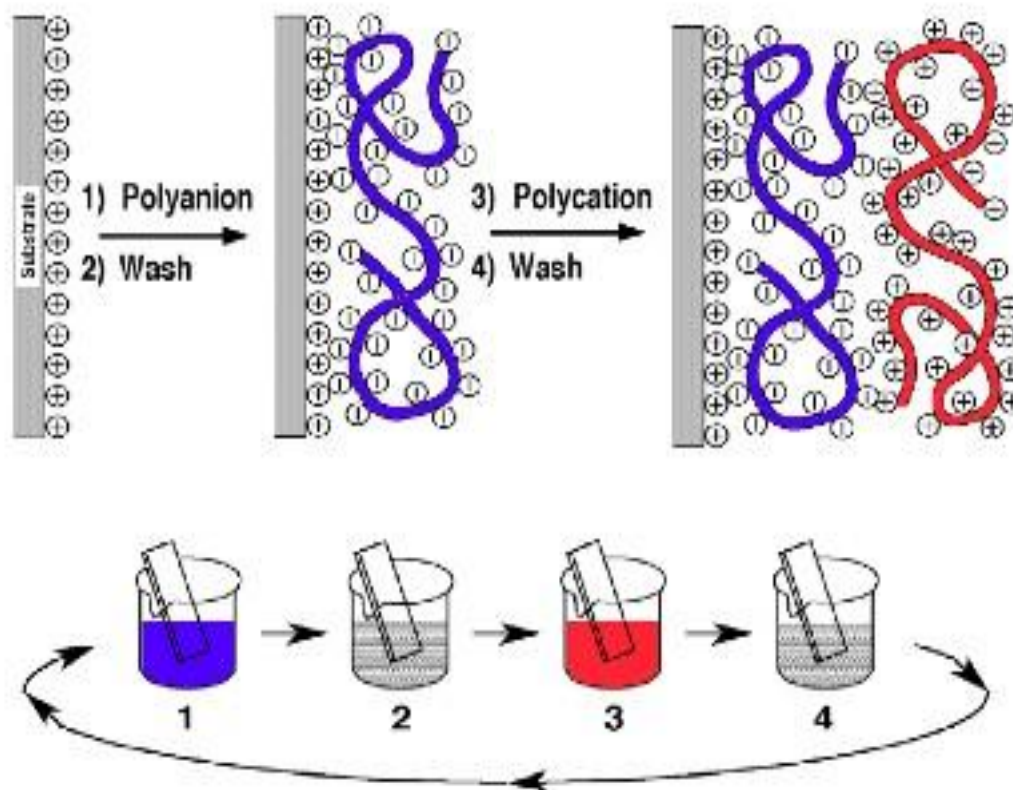


Figure 1.4 LbL assembly of charged polymers onto a solid, cationic substrate. Steps 1 and 3 are the adsorption of polyanion and polycation respectively, while steps 2 and 4 are washing steps. (reproduced with permission)²⁹

One of the main advantages of forming films by LbL deposition of polyelectrolytes is the ease of deposition, and its independence of size and topology.

1.7 LbL conformal coating of cells

This approach rests on the hypothesis that polyelectrolyte coating provides some degree of immunoprotection, while not jeopardising their viability.

One of the first reports of step wise assembly of poly (allylamine) (PAH) and poly(styrene sulfonate) (PSS) multi-layers on cells involved using *Escheria coli* and red blood cells as templates.² This involved the incubation of fixed cells in aqueous solution of the polyelectrolytes. After deposition of the required number of bilayers the cells were dissolved and the shape of the polyelectrolyte multilayer was maintained. Yeast cells were coated with PAH/PSS multi-layers by sequential deposition of PAH and PSS from saline solutions.³⁰ Each coating step was followed by a washing step and subsequent centrifugation. The ‘immunocamouflage’ of red blood cells by LbL coating of polyelectrolytes has also been demonstrated.^{31, 31}

Previously, cell surface modification has been achieved through the introduction of nonbiogenic functional groups by metabolic or genetic engineering.²⁸ Choi et al used polydopamine to covalently bond with thiols or amines on the surface of yeast cells, in what was reported to be the first conformal cell encapsulation using organic materials.³²

It has been reported that the covalent tethering of reactive cell surface moieties, while expanding the options for cell surface re-engineering,^{32, 33} can interfere with cell physiology.³⁴ A need hence exists for non-covalent cell surface modification. Chaikof et al report on using PLL grafted with PEG (poly ethylene glycol) to decrease the cytotoxicity of PLL.²⁸

The surface of cells have been shown to be suitable anionic surfaces for the deposition of a range of materials (Figure 1.5). Cationic nanoparticles have

also been electrostatically deposited onto the surface of cells.³⁵

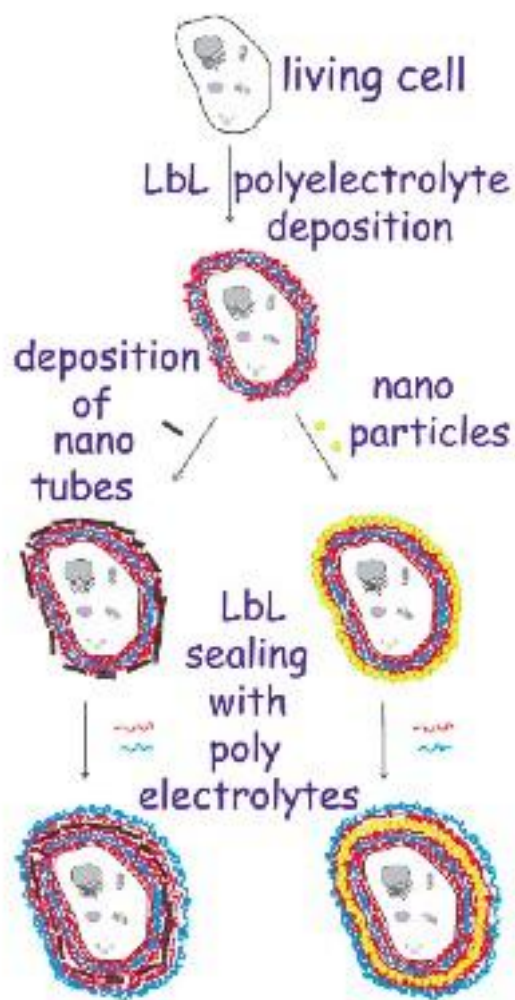


Figure 1.5 A scheme showing the sequential deposition of oppositely- charged polycations (red) and polyanions (blue) multilayered films doped with nanomaterials (nanotubes and nanoparticles) onto isolated living cells.³⁶

1.8 Reactive polyelectrolytes

Polymers containing activated groups such as maleic anhydride and succinimide esters have proven useful in creating protein-polymer conjugates in pharmaceutical research. Ideally, these reactive polymers should react spontaneously and quantitatively with polymeric nucleophiles under physiological conditions, without generating potentially cytotoxic side products. Our group develops water-soluble polyanions containing electrophilic groups, that will electrostatically bind to polyamines, and subsequently form covalently crosslinked networks of interest for long-term immunoprotection of encapsulated cells and thin films. Previous work includes a copolymer of methacrylic acid (MAA) with methacryloyl ethylacetoacetate (MOEAA) that is able to form both shell-crosslinked and core-crosslinked cell-compatible beads that are stable under physiological conditions for up to 4 weeks. Another copolymer contained the electrophilic monomer 4,4-dimethyl-2-vinyl-2-oxazoline-5-one (VDMA) with methacrylic acid.

It has been reported that the hydrolysis of small-molecule succinimidyl esters requires several hours at pH 7,³⁸ while polymers containing analogous activated esters such as N-acryloxysuccinimide (NAS) can rapidly react with amines to yield amides under similar aqueous conditions.³⁷ If the amine component is also attached to a polymer such as PLL, this reaction should lead to stable, amide crosslinked networks. This crosslinking reaction liberates N-hydroxysuccinimide (NHS) which, while not ideal, may be acceptable because of

the low cytotoxicity of NHS and its slow rate of formation. Copolymers of NAS with AA promise to be water-soluble at physiological pH and able to form polyelectrolyte complexes with polyamines. While the crosslinking reaction is expected to be rapid, hydrolysis of residual NAS to carboxylic is expected to be slower than for the anhydride-based copolymers described earlier, promoting covalent crosslinking while still ensuring conversion of electrophilic groups prior to transplantation. The moderate rate of hydrolysis at pH 7 may also allow for post-functionalization with biomolecules before or after coating and crosslinking.

This thesis will hence explore synthesis of copolymers of acrylic acid with NAS, and the ability of these reactive polyanions to form covalently crosslinked networks in combination with PLL as reactive polycation/polynucleophile. In addition, the ability of these networks to immobilize yeast cells will be explored.

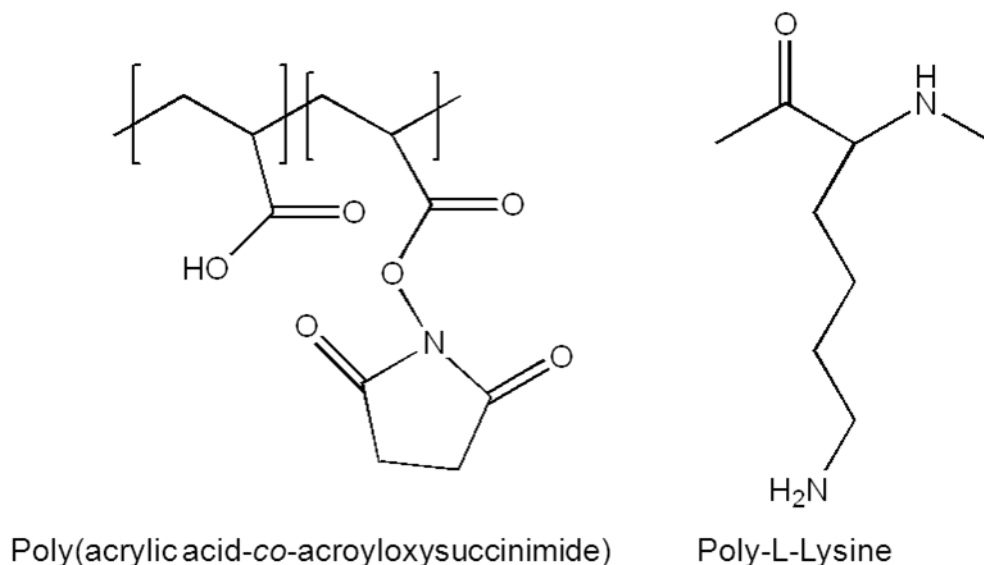


Figure 1.6 Polyelectrolytes used for crosslinking

1.9 Hydrogel Films

In natural habitats microorganisms predominantly exist as biofilms which consist of living cells embedded in an extracellular matrix (ECM) based on charged glycoproteins or carbohydrates. ECM allows microorganisms to (i) adhere to substrate surfaces and (ii) integrate the same type of cells into colonies that are protected from other species.³⁸ This thesis aims to explore formation of artificial biofilms by immobilizing live yeast cells within thin polyelectrolyte networks. Formation of crosslinked polyelectrolyte networks in presence of living cells must be done in a cytocompatible way, using rapid and high yield reactions between polymer-bound functional groups in a click-type fashion.

Click reactions are generally defined as coupling reactions that proceed

rapidly with high yields under mild conditions and also are highly selective.³⁹ The most common methods are 1,3-dipolar cycloadditions and Diels-Alder cycloadditions, but reactions of nucleophiles with activated esters also fall into the class of click chemistry.³⁹

Incorporation of click chemistry with LbL of reactive polyelectrolytes has been shown to form crosslinked polymer films, usually by deposition of mutually reactive polymers from organic solutions. The method used here is based on a reactive LbL process in aqueous solutions, using p(NASl-co-AA) and PLL as the reactive polymer pair.

1.10 Hydrogel film formation

Different methods can be used to deposit polymer films onto a desired substrate.

1.10.1 Spin Coating

Spin coating is a technique used to produce smooth thin films over a large areas. Typically, a drop of a solution of the coating material is placed on a substrate located on a horizontal rotating disc. This results in the centrifugal spreading of the solution followed by rapid evaporation of the solvent, thereby leaving a liquid or solid film on the substrate (Figure 1.7). During application of the solution, the disc is usually stationary or rotates at a low rate. After application the disc is rapidly accelerated to a high spin rate. The adhesive forces at the liquid/substrate interface and the centrifugal forces acting on the rotating

liquid create a strong shearing of the solution, which then causes a radial flow, whereby most of the polymer solution is rapidly ejected from the disc. This process combined with subsequent evaporation of the solvent cause the thickness of the remaining liquid/film to decrease. For a polymer solution, the evaporation process causes the concentration of the polymer to increase at the liquid/vapor interface. This process leads to formation of a concentration gradient within the liquid film, and after complete evaporation of the remaining solvent, a solid polymer film. The process of spin coating can therefore, be divided into four stages: deposition, spin-up, spin-off and evaporation.

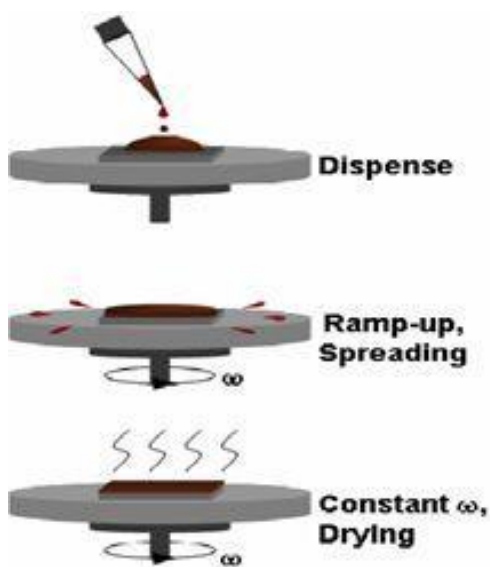


Figure 1.7 A schematic of spin coating⁴⁰

The factors controlling final film thickness include fluid concentration, initial and final rotation speeds, spin time, solution viscosity, rotational acceleration,

substrate temperature, air-flow above substrate and dispense time. Under controlled conditions, spin coating can provide an inexpensive, flexible and reliable method for thin film formation.

1.11 Model Cells

We used *Saccharomyces cerevisiae* (Baker's yeast) as the model cell system in this project. *S. cerevisiae* shares a common life cycle and cell structure with higher eukaryotes, and is hence a popular system for studying eukaryotic biology at cellular and molecular levels.

1.12 Thesis Objectives

The goal of this research is :

- to develop a new water soluble reactive polyanion based on an activated ester electrophile.
- to use reactive polyelectrolytes to conformally coat the surface of living cells. *Saccharomyces cerevisia* is used as model cell.
- to immobilize model cells in flat crosslinked conformal hydrogel films.

2. Experimental

2.1 Materials

All chemicals were obtained from Aldrich (Oakville, Ontario, Canada) unless otherwise noted. The coupling reagents N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were used as received. Poly(acrylic acid) (PAA) (M_w= 100,000, 35 wt% in water) was freeze dried. Monomers (acrylic acid-N-hydroxysuccinimide ester (NAS) (minimum 90% purity) and acrylic acid (AA) (99% purity) were used as received. Sodium alginate was obtained from (Pronova UP MVG) (Novamatrix). Poly-L-lysine (PLL), fluorescein isothiocyanate (FITC) and aminofluorescein (AF), poly(styrenesulfonate sodium salt) (PSS, MW 70,000 Da, 30 wt% in water), poly(allylamine hydrochloride) (PAH, M_w 15,000 Da) and 2,2-azobis(isobutyronitrile) (AIBN, 99.95%) were obtained from Dupont, Mississauga, Ontario.,

N,N'-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) were obtained from Caledon Laboratories, Ontario, Canada, and ethyl acetate from Fischer Scientific, Ontario, Canada.

2.2 Characterization

Optical Microscope. An Olympus BX-51 optical microscope fitted with a Q-imaging Retiga EXi digital camera and ImagePro software was used for conventional optical microscopy as well as fluorescence microscopy of FITC and RITC-labelled polymers and films.

TEM. A JEOL 1230 transmission electron microscope (TEM), equipped with a 4 megapixel AMT digital camera was used. All samples were deposited onto 100 mesh, formvar (polyvinyl formal) coated copper TEM grids. The coated yeast cells were embedded in Spurr epoxy resin and ultramicrotomed to 100nm thickness.

Confocal Microscope. A Nikon Eclipse i90 upright microscope was used, as well as a Nikon C2 confocal with an argon multiline laser for 488 excitation, and a 100x oil immersion lens.

GPC. Gel permeation chromatography was carried out using a Water 515 HPLC pump, Water 717 plus auto sampler, three columns (Waters Ultrahydrogel 120,250,500; 30cm x 7.8mm; 6 μ m particles) and a Water 2414 refractive index detector. The columns were adjusted at 35°C and narrow-dispersed PEG were used as standards (Waters, Mississauga, Ontario).

Nuclear Magnetic Resonance (NMR) spectra were acquired on a Bruker AV600, at 600 MHz. Data were analyzed using Topspin and plotted with Topspin plot.

2.3 Instruments

Mechanical agitator. Lab-line multi-wrist shaker, model 359, 50/60Hz shaker speed setting 10.

Spin Coater. A Headway Research Inc. Model EC101 spin coater was used for thin film formation.

Photo-Reactor fitted with one black-light bulb (350 nm, Ushio F8T5BL, 8 watts).

2.4 Procedures

2.4.1 Functionalization of Poly(acrylic acid) (84:16 mol%)

PAA (1.00g, 17.2mmol), NHS (0.319g, 2.77mmol) and DCC (0.574g, 2.78mmol) were dissolved in DMF (40mL) in a 100mL round bottom flask equipped with a stirring bar. The round bottom flask was placed on a magnetic stirrer and the solution was stirred for 15 hours at room temperature. The side product dicyclohexylurea (DCU) was collected by vacuum filtration. The polymer was precipitated into ethyl acetate (400mL) and dried to a constant weight at room temperature. The polymer was re-dissolved in THF (25mL) and precipitated into diethyl ether (250mL). The purified polymer was dried to a constant weight under vacuum. Yield: 0.754g, 75%.

2.4.2 Thermally initiated Free Radical Polymerization (AA:NAS = 75:25 mol%)

AA (0.561g, 7.78mmol), NAS (0.439g, 2.59mmol) and AIBN (17mg, 0.104 mmol) were dissolved in THF (9mL) in a 25mL glass vial. The solution was bubbled with nitrogen for several minutes before the vial was resealed. The vial was placed in an oven and heated from room temperature to 60°C during 1 hour and maintained at 60°C for 23 hours. The vial was rotated at 4 rpm in the oven to ensure mixing. The polymer was precipitated into anhydrous diethyl ether and dried to a constant weight under vacuum. Yield: 0.22g 22%. Analogous copolymers of 25% AA and 75% NAS, were prepared by analogous copolymerization.

2.4.3 Synthesis of P(AA-co-NAS) by Photo-Initiated Copolymerization at Room Temperature

AA (0.032g, 0.04mmols), NAS (0.072g 0.04 mmols) and DMPA (4mg) were dissolved in acetonitrile (1mL) was irradiated for 60mins in a 4.5mL glass vial. After irradiation the vial was placed under vacuum to remove acetonitrile. Acetonitrile (1mL) was added to remove any remaining monomers. The solvent monomer was decanted off and the residue was dissolved in DMSO-d₆ for NMR analysis.

2.4.4 Synthesis of Rhodamine-labelled Poly-L-lysine (PLL_r)

PLL_r was prepared as described earlier.¹³ For example, PLL (15-30 kDa, 100 mg, 0.48 mmol poly-L lysine hydrobromide) was dissolved in 10 mL of 0.1 M NaHCO₃ buffer (pH 9) in a 20 mL glass vial. Rhodamine B isothiocyanate (RITC) (2.0 mg, 0.005 mmol) dissolved in 0.2 mL of DMF was added to the PLL solution, and the mixture was stirred for 90 min at 20 °C. The resulting solution was adjusted to pH 7 with 1 M HCl and then dialyzed for 5 days in deionised water using cellulose acetate tubing (Spectrum Laboratories, 3.5 kDa MW cut-off) with daily water changes until the dialysate showed no absorbance for DMF. PLL_r(15-30 kDa) was isolated by freeze drying. Yield: 68.5 mg, 68%.

PLL_f (40-60 kDa) was prepared in a similar manner. PLL (40-60 kDa) (100mg, 0.478mmol of lysine-HBr) was dissolved in 0.2M NaCO₃ (10mL) buffer solution at pH 9 in a 20mL glass vial. RITC (1.28mg, 0.0239mmol) in 0.5mL of DMF was added, and reaction and work-up carried out as above, except that

dialysis tubing with a 14 kDa MW cut-off (Membra-Cel, Viskase Corp., Darien, IL) was used.

2.4.5 Preparation of PMM₅₀ and PMMf₅₀ by Controlled Hydrolysis of PMM₀.

As described by Gardner et al¹⁶, PMM₀ (100 mg) was dissolved in 1.0 mL of an ACN-d₃/D₂O (9:1 v/v) mixture in a screw-cap glass vial, forming a 10 w/v % solution. The vial was placed in an oven set to 60 °C for either 14.5 h (20 kDa PMM₀) or 17 h (1080 kDa PMM₀), with reaction times determined by ¹H NMR to result in 50% hydrolysis, forming PMM₅₀ of 20 kDa or 1080 kDa, respectively. After being cooled to room temperature, 0.2 mL of the reaction mixture was diluted to 10 mL with 35 mM HEPES pH 7.8 buffered saline. The resulting slightly turbid solution was immediately agitated on a vortex mixer for 10-30 seconds to give a clear solution and then quickly filtered (0.45 µm) to give a final aqueous coating solution containing 0.2 wt % PMM₅₀. The complete dilution/agitation/filtration sequence was completed within 1 minute to minimize hydrolysis, and the solution was immediately used for coating. Coating solutions containing the fluorescently labeled analog PMMf₅₀ were prepared in the same way except that 2 mol % aminofluorescein (AF) (relative to total anhydride) was added at the beginning of the PMM hydrolysis.

2.4.6 Standard Procedure for the Formation of Alginate-PLL-P(AA-NAS) capsules (work carried out by Carla Brown)

All capsules were prepared by a procedure based on the method reported

by Sun.⁵ Briefly, a 1.0wt% sodium alginate solution in aqueous saline was filtered (0.45 μ m) and extruded through a 27 gauge needle at a liquid flow rate of 0.5mL/minute with a concentric airflow (4 L/min) used to induce droplet formation, into a gelling bath containing a 1.1wt% calcium chloride and 0.45wt% sodium chloride (Figure 1.3). The resulting calcium alginate beads were washed once with fresh gelling bath solution followed by a saline wash. The alginate beads were then coated with PLL (0.1wt%, 15-30kDa or 40-60kDa) for 6 minutes. The resulting AP capsules were washed twice with saline and coated with either 0.25wt% or 0.5wt% P(AA-co-NAS) solution for 6 minutes or with 0.5 wt% PAA (a control) solution for 6 minutes followed by a saline wash. All washing and coating steps involved a ratio of 1 mL beads to 3.3 mL washing or coating solution.

2.4.7 Conformal Coating

S. cerevisiae were suspended in YPD medium overnight at 28°C(5mg/10mL). Subsequently, the cells were centrifuged into a pellet and washed twice with distilled water to remove any remaining YPD. Coating was done using a procedure reported previously.³⁰ Briefly, PMM₅₀ was prepared as described above and used at a concentration of 0.2 wt%. PLL hydrobromide was dissolved in saline, also at a concentration of 0.2 wt%. Polyelectrolyte coatings was performed by adding PLL (15-30k or 40-60k) solutions (250 μ L) to the yeast cells (250 μ L of the stock suspension) for 5 minutes followed by centrifugation

and washing twice with saline, followed by coating with a PMM₅₀ solution (250µL) for 10 minutes, again followed by centrifugation and washing twice with saline . PMM₅₀ solutions were prepared just before the coating step involving PMM₅₀. All of the steps in this coating process were performed in the absence of YPD culture medium. To investigate cell division, coated yeast cells were incubated at 28°C in YPD medium (yeast extract 1%, Peptone 1-2% and Dextran 2%).

2.4.8 Confocal Microscopy Assessment of coating procedure

Dry yeast cells were incubated overnight in YPD medium (5mg/10mL). Then DAPI (2,6-diamidino-phenylindole) was added to a final concentration of 2.5µg/mL. Thirty minutes after staining, the yeast cells were coated as described above. 100µL of the mixture was added to a flat glass slide and covered with a square coverslip. To prevent the sample from dehydrating, the edges of coverslip were sealed with petroleum jelly.

2.4.9 Thin Film Formation

Glass coverslips (VWR micro cover glass 22x40mm No. 1) were cleaned with acetone and ethanol prior to the fabrication of multi-layered films. Solutions of PLL (0.5%, 40-60kDa in deionised water) and P(AA-co-NAS) (3% in DMSO followed by dilution in HEPES buffered deionised water, pH 7.8 to 0.5% solution) were filtered (0.25µm) before use. Films were deposited according to the following layer-by-layer protocol:

- (1) 100uL of PLL solution was deposited on the static glass substrate and spun for 20 seconds at a speed of 2,000 rpm
- (2) 100uL of the reactive polyanion solution was deposited on the previous film and spun for 20 seconds at a speed of 2,000 rpm. This cycle was repeated until the desired number of PLL / P(AA-co-NAS) layers were reached. The films were immediately used in subsequent experiments, without washing or drying.

2.4.10 Lamination of Saccaromyces on Glass Slides

Glass coverslips (VWR micro cover glass 22x40mm No. 1) were cleaned with acetone and ethanol prior to the fabrication of multi-layered films.

Solutions of PLL (0.5%, 40-60kDa in deionised water) and P(AA-co-NAS) (3% in DMSO followed by dilution in HEPES buffered deionised water, pH 7.8 to 0.5% solution) were filtered (0.25um) before use. Films were deposited according to the protocol described above. After deposition of the 11th layer (PLL), the surface was washed with 500uL of distilled water. 100uL of a yeast suspension in distilled water was deposited and allowed to settle for 2 minutes. The yeast solution was spun for 20 seconds at a speed of 2,000 rpm, after which the polymer coating was continued until a total of 20 layer had been deposited, with the yeast cells positioned formally between layers 11 (PLL) and 12 (p(AA-co-NAS)).

2.4.11 Investigation of Electrostatic Yeast Immobilisation

An eleven layer system was created and 100uL of yeast suspension was deposited as described above, to investigate the interaction between the anionic yeast cell and PLL. 100uL each of NaCl in molar concentrations of 0.1M, 0.5M, 1M and 2M were deposited in sequence and spun at 2000 rpm. Optical Microscopy was used to determine if the cells remained.

2.4.12 Identify Budding

To investigate cell division, thin films containing yeast cells were incubated at 28°C in a petri dish containing 3mL of YPD medium (yeast extract 1%, Peptone 1-2% and Dextran 2%).

3. Results and Discussion

3.1 Synthesis of Poly(acrylic acid-co-N-acryloxysuccinimide)

Copolymers can generally be formed by copolymerization of two comonomers, or by post-functionalization of a homopolymer. Both approaches were explored here.

3.1.1 Functionalization of Poly(Acrylic Acid)

A convenient method to synthesize this co-polymer is to functionalize poly(acrylic acid) with N-hydroxy succinimide using DCC coupling (Figure 3.1).

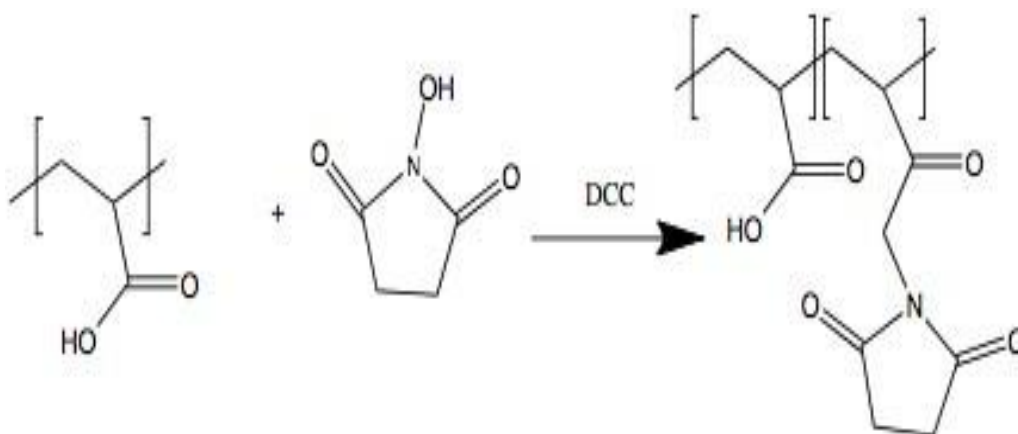


Figure 3.1 Functionalization of poly(acrylic acid) with N-hydroxysuccinimide using DCC coupling.

This approach led to copolymer with a lower than expected degree of functionalization of 5% instead of the expected 16%. In DCC couplings the acid reacts with DCC to produce O-acylisourea, an activated ester. A side reaction is

possible whereby the O-acylisourea can react with a second carboxylic acid to form an acid anhydride. If this second acid is adjacent to the activated ester, formation of the cyclic anhydride would be kinetically favoured over other possible anhydrides.

3.1.2 Thermally Initiated Free-Radical Copolymerization

Random co-polymers of acrylic acid and N-acryloxysuccinimide at a mole ratio of 25:75 were prepared by AIBN initiated free radical polymerization in THF at 60°C for 24 hours and isolated by precipitation in diethyl ether (Figure 3.2).

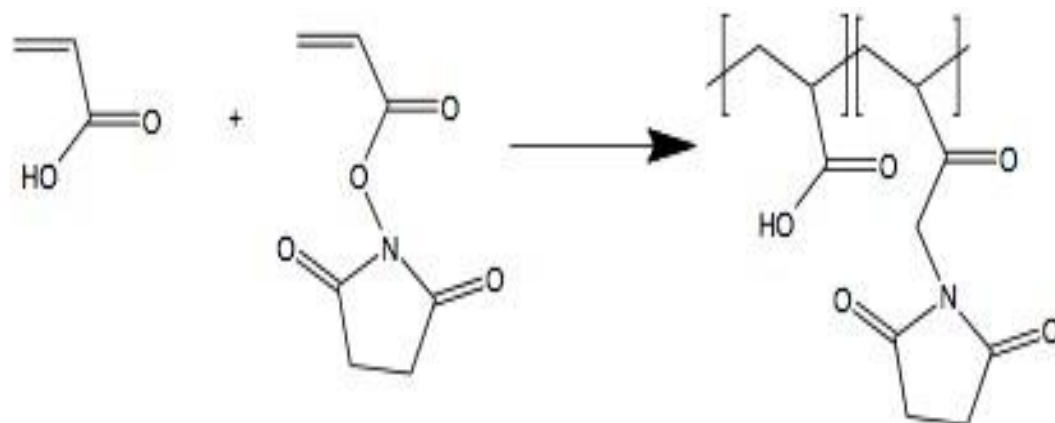


Figure 3.2 Free radical co-polymerization of acrylic acid (AA) and N-acryloxysuccinimide (NAS) using AIBN.

The chemical composition of the polyanion was determined from the ¹H-NMR spectrum measured in DMSO-d₆. Comparing the area under the succinimide methylene signal at 2.8ppm with the carboxylic acid signal at 12.5 ppm showed that the copolymer contained 95% acrylic acid and 5% N-

acryloxysuccinimide, despite the co-monomer feed ratio of 25:75. This low content of succinimidyl esters was seen consistently in the copolymers.

3.2 Side reactions of Poly(acrylic acid-co-N-acryloxysuccinimide)

In order to probe the copolymerization process and determine whether the low NAS content was due to poor incorporation of the NAS monomer or loss of succinimidyl ester during or after copolymerization, a copolymerization was followed by $^1\text{H-NMR}$ at 10% total monomer loading with 25:75 AA:NAS and 2% AIBN in DMSO-d_6 . The NMR tube was placed in an oven at 60°C and NMR spectra were taken at 0, 1, 4, and 6 hours at 60°C . Spectra at 0 and 1 hour are shown in Figures 3.3, a and b, respectively.

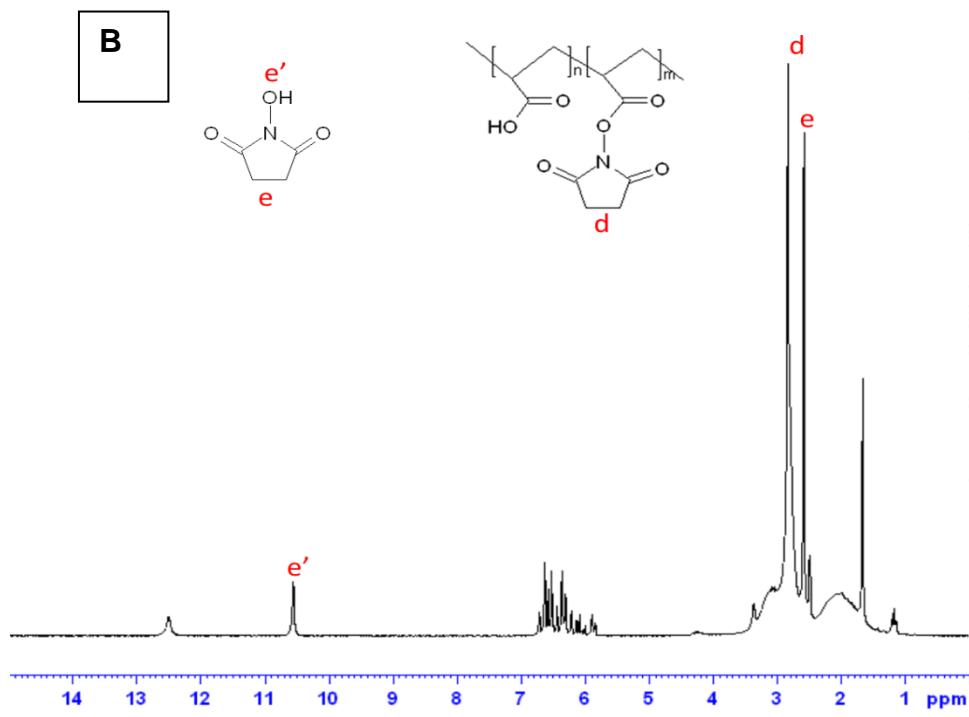
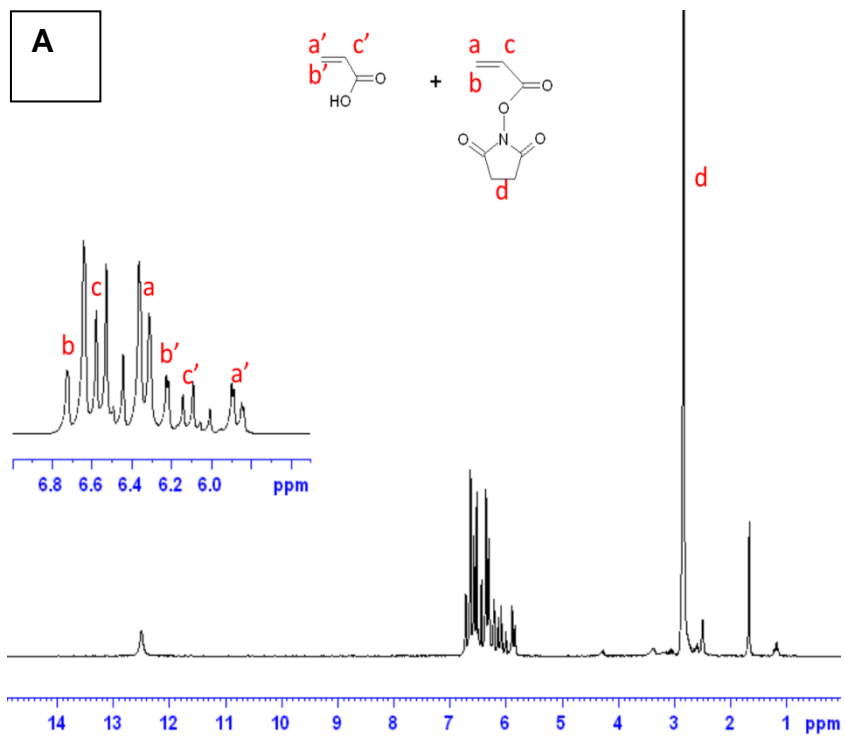


Figure 3.3 ^1H NMR spectrum of 10mol% AA and NAS (25:75) in DMSO- d_6 at a. time 0; and b. 1 hour.

Figures 3.3B show peaks (e and e') for free NHS after 1 hour of heating at 60°C , when conversion to polymer is 75%. The peak for NHS at 10.5 ppm shows that 16% of free NHS has been formed. Further heating leads to release of more NHS as seen in table 1.

This release of NHS could be due to hydrolysis of monomeric or polymeric NAS, or to reaction of monomeric or polymeric NAS with acid groups (trans-esterification).

To test for any reaction between the two monomers alone, a 10% monomer solution (50:50 AA:NAS) in DMSO- d_6 was heated at 60°C in absence of AIBN. NMR spectra recorded after 4 hours and 8 days showed no significant change in the monomer mixture and no NHS was produced, indicating that the free NHS is not formed by a reaction between monomers.

NHS might be produced by hydrolysis, involving water accidentally present in the sample before polymerization, or water produced by cyclization of neighbouring acid groups on the copolymer. The NMR spectra of samples before polymerization (Figure 3.3) show that at time zero no water is present in the reaction (H_2O peak is at 3.35 ppm), while heating at 60°C for 8 days leads to detection of 0.15 equivalents of water compared to total monomers. As there is

no other change in the monomer peaks the most likely source of water is absorption from air during the heating.

PAA has been reported to form 5% cyclic anhydride by heating in the solid state to 130°C.^{41, 42} If so, anhydride/water formation and subsequent hydrolysis of NAS groups releasing NHS should be much slower at 60°C, and especially at room temperature.

To investigate this, photo polymerization (50:50) was carried out at room temperature as depicted in Figure 3.4. The NMR spectrum of this sample showed that polymerization had gone to >95% conversion overall and that 70% of the succinimide was polymer-bound. The amount of free NHS was found to be 30%, less than what had been observed following thermal polymerization of a 50:50 AA:NAS mixture at 60°C. The copolymer reaction solution showed little change in this polymer-bound NAS/NHS ratio after another 17 hours at room temperature. The sample was placed in an oven at 60°C and NMR spectra were recorded over two days (table 2).

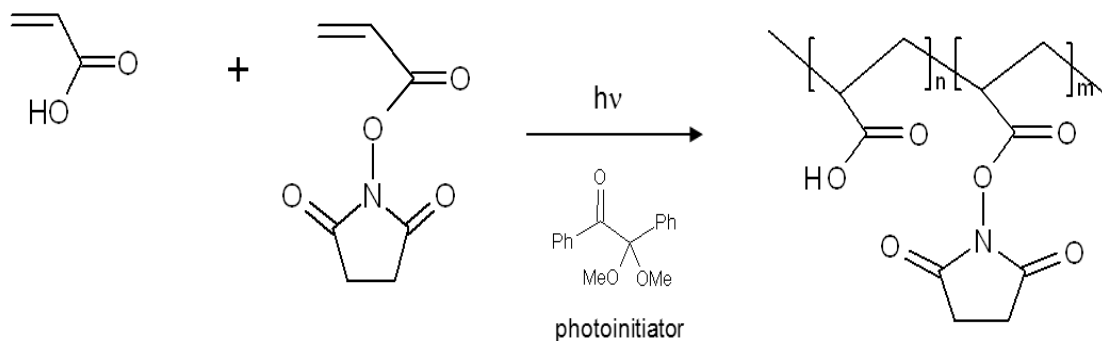


Figure 3.4 Photo polymerization of AA and NAS to yield co-polymer.

It can be observed that the amount of free NHS increases over time, while the amount of polymer bound NAS decreases (table 2).

Table 3-1 Copolymer composition

Time (hours)	Polymerization Temperature	NAS in copolymer [mol%]	Free NHS [mol% of initial NAS]
0	Room Temperature (Photo-initiation)	71	29
17	Room Temperature (Photo-initiation)	70	30
1	60°C	66	34
2	60°C	65	35
3	60°C	63	37
22	60°C	53	47
48	60°C	47	53

It is presumed that the release of NHS is due to a cyclization reaction of either type shown in Figure 3.5:

- adjacent carboxyl and ester groups form a cyclic anhydride releasing NHS (A)
- adjacent carboxyl groups form a cyclic anhydride, releasing water (B1) which in turn hydrolyses polymer bound NAS cleaving NHS (B2)

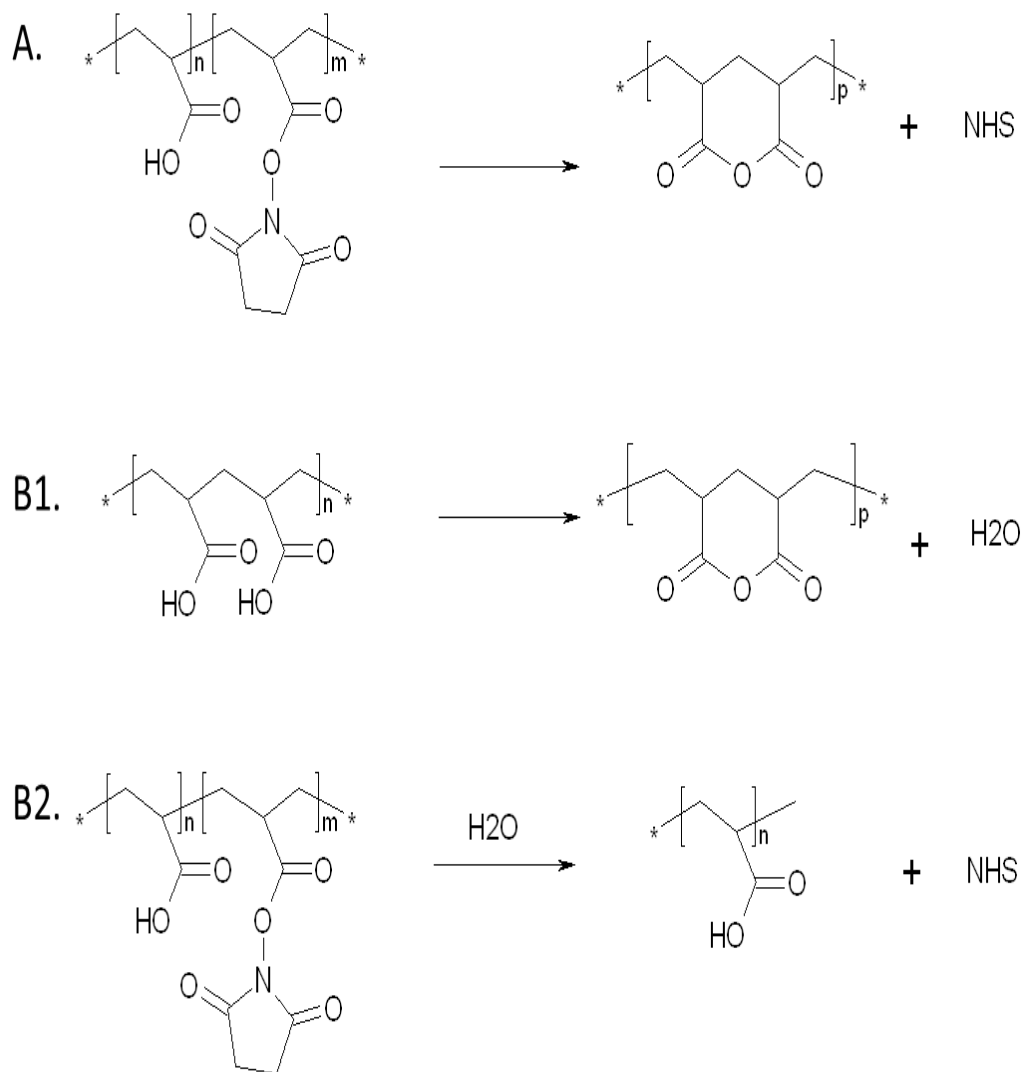


Figure 3.5 Two routes to the formation of cyclic anhydride. (A) adjacent ester and acid groups reacting to release NHS, (B1) reaction between two adjacent acid groups to release water and (B2) hydrolysis of the activated ester to release NHS

Cyclization of adjacent COOH groups of PAA to form anhydrides normally only occurs at much higher temperatures than used in this work and the degree of cyclization is generally small. A low level of cyclization and water release

would not account for the fairly high degree of NHS production. In a control experiment, (data not shown), one equivalent of water was added to the copolymer system depicted in table 1, and heated at 60°C for 19 hours, with no reaction observed. This result provides evidence against mechanism B1/B2.

It has been reported that hydrolysis of esters having neighbouring carboxylic acid groups is much faster than that of isolated esters.⁴³ Hydrolysis of succinate monoester in water at ambient temperatures at pH 2-3 is 230 times faster than hydrolysis of the glutarate ester. This rapid reaction was been shown to be due to the formation of a cyclic anhydride intermediate formed by attack of the adjacent carboxyl group on the activated ester.⁴³ This suggests that cyclization to form a 6-membered anhydride ring is occurring for adjacent acid and polymer bound NAS units as depicted in scheme 1(A).

Interestingly, Jang et al reported on the thermal behavior of PAA and poly(allylamine hydrochloride) (PAH) LbL assemblies in the dry state.¹⁵ They observed that, depending on the number of bi-layers present (5,6 or 8) anhydride formation was observable after heating at 58,60 or 80°C in the solid state for 45 to 70 minutes. As far as we are aware this is the only report of anhydride formation in PAA at temperatures less than 100°C.

3.3 Photo-chemically Initiated Radical Copolymerization

It is known that small amounts of a polar co-solvent can significantly increase the solvency of e.g. THF for polar polymers, so 1–5 wt% of water were added to the anhydrous THF to help keep the forming polymer in solution. It was observed that addition of 4-5wt% water prevented precipitation, but at the cost of increased formation of NHS by 20%.

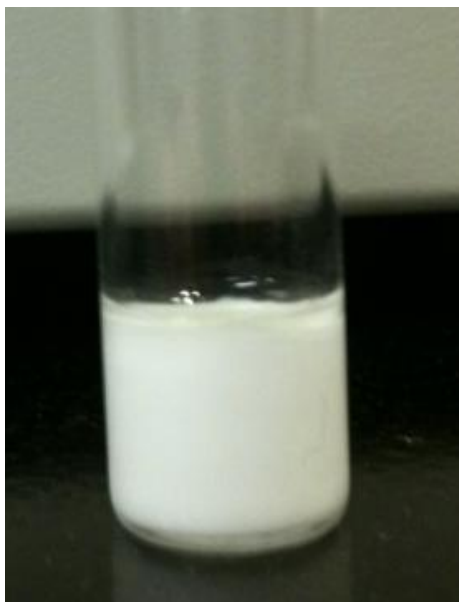


Figure 3.6 Photograph of a photo-initiated precipitation polymerization (60 min, 0 C) of AA with NAS in ACN-d₃.

It has been reported that co-polymers of poly(acrylic acid) can be prepared using precipitation polymerization.⁴⁴ Precipitation polymerizations take place in a medium that is a good solvent for the monomers but a non-solvent for the polymer. To explore this process, a AA:NAS mixture (50:50 mol ratio) in 1mL

acetonitrile- d_3 with photo-initiator was irradiated for 60mins in a 4.5mL glass vial while sitting in ice water. After irradiation the vial was placed under vacuum to remove acetonitrile- d_3 and the residue was dissolved in DMSO- d_6 for NMR analysis (Figure 3.7).

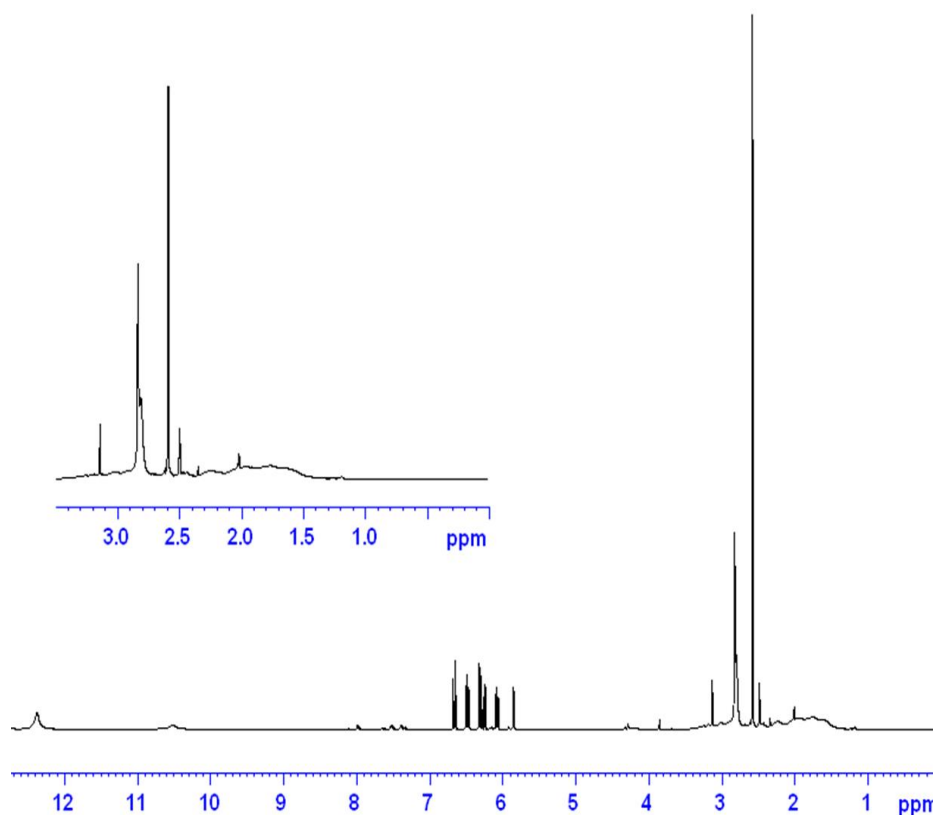


Figure 3.7 ^1H NMR spectrum of co-polymer, ACN- d_3 removed under vacuum dissolved in DMSO- d_6

The NMR spectrum (Figure 3.7) shows 77% conversion of monomers to polymer assuming that no monomer was lost during the brief exposure to

vacuum.. GPC analysis in aqueous solution with PEG standards shows M_n and M_w of 85kDa and 190kDa, respectively, with a polydispersity index of 2.2.

3.4 Crosslinking Reactions with Polyamines

As mentioned earlier, our group has previously used a reactive polyanion which contained acetoacetate groups that can form covalent amide bonds with poly-L-lysine. While the coated capsules had improved mechanical stability compared to conventional APA capsules, it was also shown that the residual acetoacetate groups led to fouling. To combat this issue, PMM₅₀ a water-soluble polyanion was developed. This reactive polyanion can covalently crosslink with PLL absorbed on the surface of AP capsule. Any residual anhydrides hydrolyse to form carboxylate anions. This rapid hydrolysis in physiological conditions requires efficient handling during capsule formation, which can be challenging and may limit the maximum crosslinking density. There is hence a need for analogous reactive polyanions that have somewhat longer hydrolysis half-lives, and still form highly anionic polymers. Azlactone-based copolymers with methacrylic acid have recently shown hydrolysis half-lives of about 30 minutes, but do carry extraneous amide groups formed by crosslinking and hydrolysis of the azlactone groups.⁴⁵

To evaluate the ability of the formed P(AA-co-NAS) copolymers to form crosslinked shells on PLL-coated calcium alginate beads, A-PLL (Alginate-poly-L-lysine) beads were prepared using different molecular weights of PLL(15-30 kDa and 40-60 kDa) and coated with different P(AA-co-NAS) including P(AA) as a control. The

microcapsules were prepared by coating calcium alginate beads first with PLL (0.1%, 15-30kDa or 40-60kDa) and then with P(AA-co-NAS) (5% activated ester group present, work carried out by Carla Brown). To test whether covalently crosslinked shells were formed, the capsules were exposed in sequence to 1M sodium citrate to chelate calcium and dissolve the calcium alginate hydrogel core, and then to 0.1M sodium hydroxide to deprotonate PLL and break its electrostatic interactions with the polyanion. Capsules coated with PAA, the control version, deflate upon extraction of calcium with citrate but still show distinct shells consisting of A-PLL-PAA polyelectrolyte complexes. When challenged with 0.1M NaOH, though, these polyelectrolyte complexes dissolve entirely. The analogous beads coated with P(AA-co-NAS) also form hollow shells following treatment with sodium citrate. At this stage, the shells may be sustained by ionic interactions (Alg-PLL and PLL-P(AA-NAS)) as well as by covalent crosslinking between PLL and P(AA-co-NAS). When exposed to NaOH shell fragments persist, indicating the presence of covalent crosslinking in the shell. These results confirmed the presence of crosslinks in the shells formed by the reaction of PLL(40-60kDa) with P(AA-co-NAS(5%)) (Figure 3.8). Using PLL (15-30kDa), no crosslinking was observed.

The use of higher MW PLL (40-60kDa) results in thin-walled capsules with a higher concentration of PLL on the capsule surface compared to PLL(15-30kDa). It has been shown by our group that such capsules can form stronger shells, possibly because the higher MW PLL on the alginate surface has more dangling PLL segments available for binding to the reactive polyanion.¹⁶

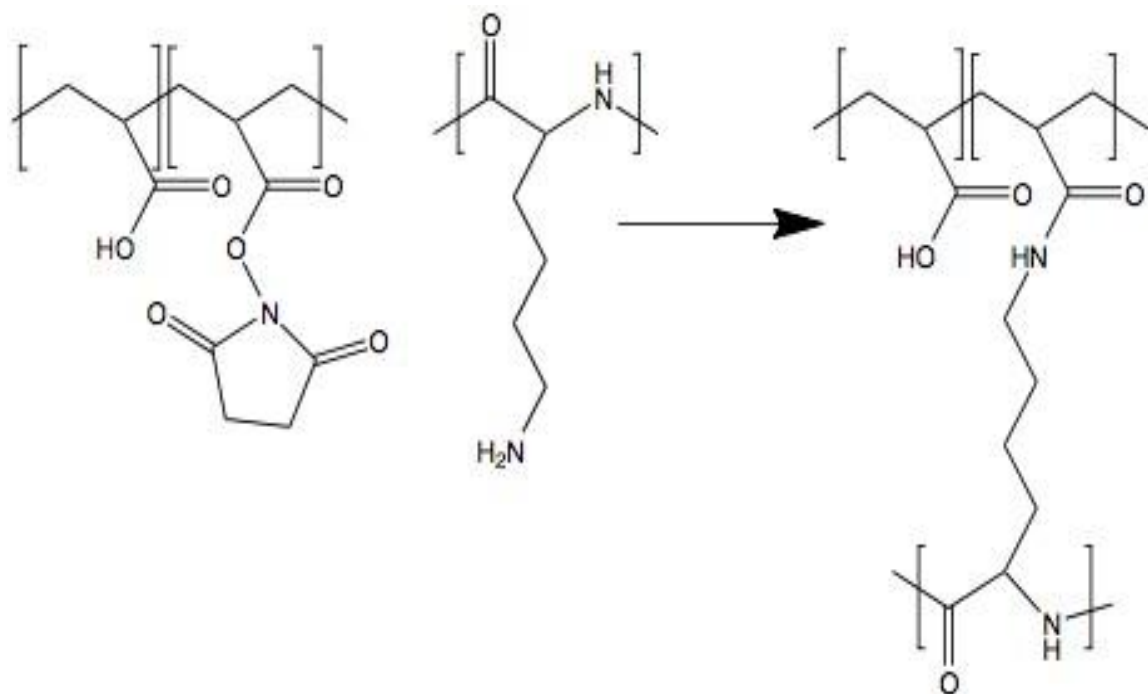


Figure 3.8 Crosslinking reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage.

The thickness and density of the crosslinked shells depend on the ability of the polyelectrolytes to diffuse into the calcium alginate beads. Lower MW PLL has also been shown by our group to diffuse further into the calcium alginate beads and is expected to give rise to thicker crosslinked shells assuming the reactive polyanion can follow.¹³ Gardner et al reported that with low MW PLL (15-30kDa), the concentration of the PLL plays a role in relation to where PLL will be found on the calcium alginate bead. For 0.05% concentration, PLL will be found near the surface, while with 0.25% concentration some PLL will be able to penetrate further into the beads.¹⁶

Future work will include coating calcium alginate-PLL capsules with the p(AA-co-NAS) 50:50 formed by photo-initiated copolymerization at room temperature. The much higher NAS content of these polyanions is expected to lead to much stronger capsules compared to those observed for copolymers containing 5% NAS.

3.5 Film Formations

In the previous section, a reactive polyanion/polycation pair was used to form a spherical shell around calcium alginate beads. In this and the subsequent sections the use of such reactive polymer pairs to form flat films as well as conformal shells, will be explored. Finally, cells will be conformally immobilized within 'flat', crosslinked hydrogel films, combining the two concepts.

One simple and elegant technique to assemble polymer films is by layer-by-layer (LbL) deposition. LbL assembly is a well-known method to prepare versatile thin films with highly tunable properties for diverse applications such as chemical sensors, drug delivery vehicles, and solid electrolyte membranes.³⁹

Covalently bonded polymer thin films were prepared by the reaction between the polymeric activated ester p(AA-co-NAS) (50:50) and PLL during LbL deposition. The reaction schemes for each of the reactive ester polymers and PLL are shown in Figure 3.8. The activated ester groups in the co-polymer are known to be highly reactive toward the primary amine groups present in polyamine such as PLL. The covalent bonds formed between the polymer chains

lead to the irreversible adsorption of one polymer layer as well as crosslinking within the forming polymer film.

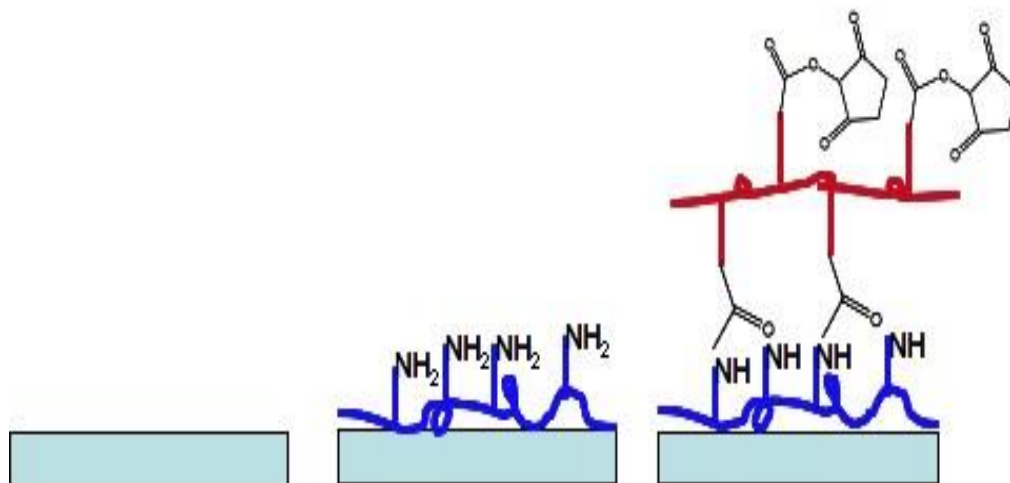


Figure 3.9 Crosslinking reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage. PLL is spin-coated onto a glass slide and subsequently spin coated with Poly(AA-co-NAS) and unreacted NAS groups are present for further LbL.

The hydrolysis of the succinimidyl esters in NAS-containing polymers is also pH dependent however, it is a slower process. This could be due to the aggregation of the relatively hydrophobic succinimide rings, which can reduce hydration of the backbone.

3.6 Characterization of Polymer and Hydrogel Films

Experiments designed to investigate the LbL adsorption of reactive polyelectrolytes on transparent glass substrates were performed. All substrates were cleaned prior to spin-coating by rinsing with ethanol.(10 mL). The results indicate that reactive layer-by-layer assembly can be used to fabricate covalently crosslinked ultrathin films from solutions of PLL and P(AA-co-NAS) (Figure3. 9).

This general approach permits rapid film fabrication and results in films with linear growth profiles and smooth, uniform surfaces, as characterized by ellipsometry.³⁸

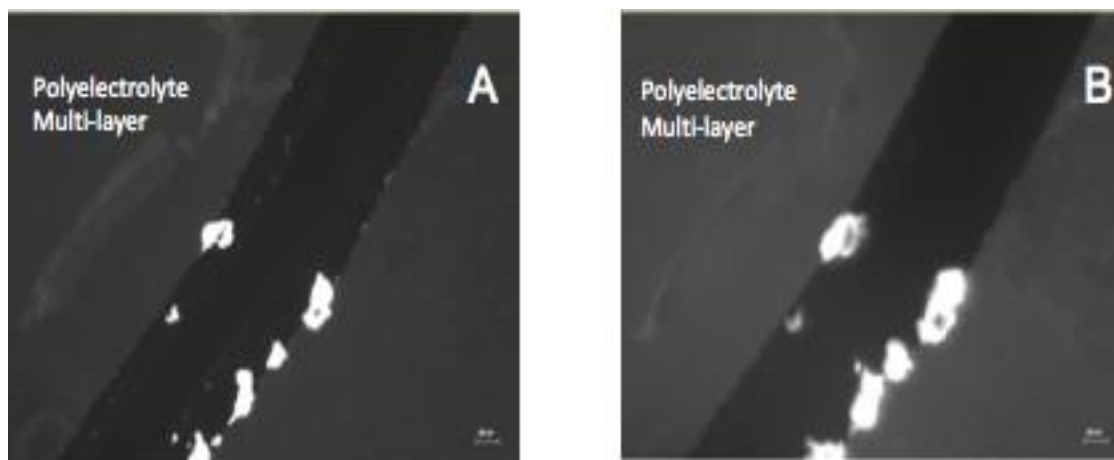


Figure 3.10 Crosslinking reaction between an activated ester and the primary amine of PLL to yield covalent amide linkages. PLLf was spin-coated onto a glass slide followed by spin coating of P(AA-co-NAS). (A) 10 bilayers were built up. (B) NaOH 0.1M was added for x min, and washed away

Thin film growth can often be determined by observing the change in film thickness as a function of bilayer number. Here, four systems were created on the same LbL build-up. After 1, 3, 5 and 7 bilayers were deposited a scratch was made on the surface of the film (Figure 3.11). It can be observed that there is film deposition over each scratch, the exception being the scratch after bilayer 7.

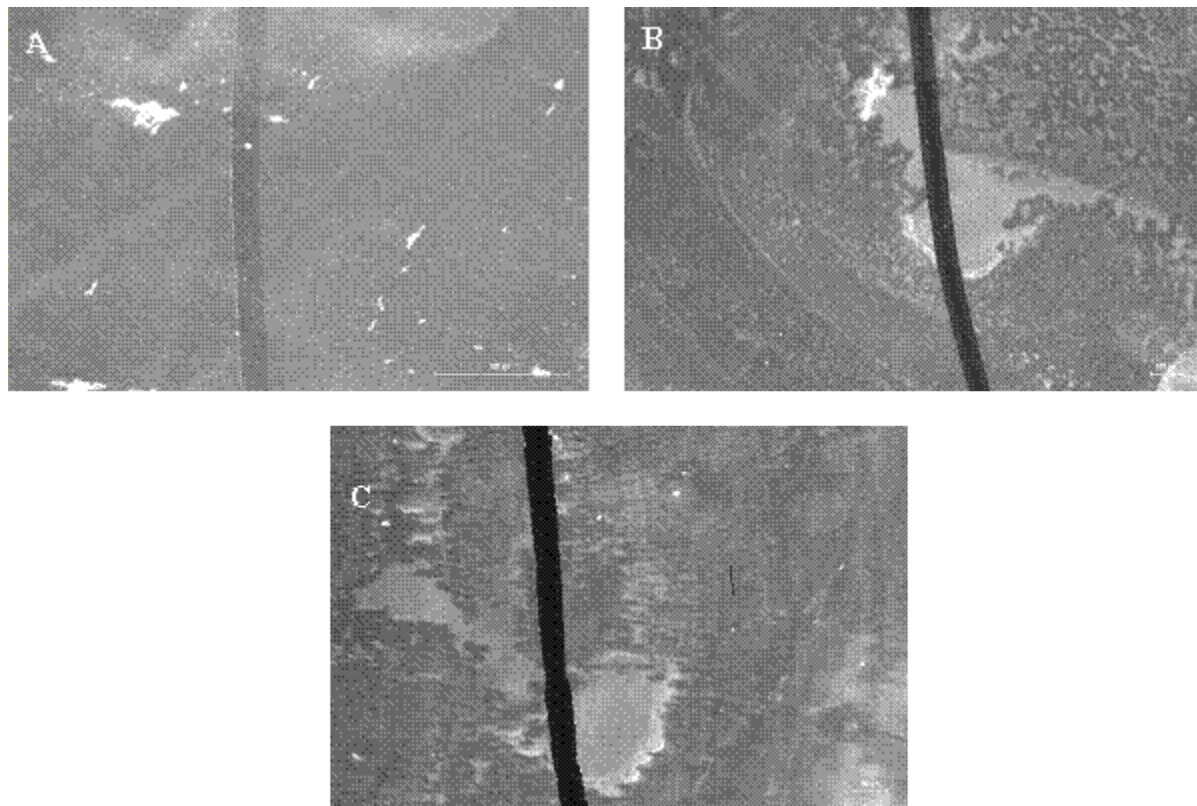


Figure 3.11 Crosslinking reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage. PLLf is spin-coated onto a glass slide followed by spin coating of P(AA-NAS). 2 layers were built up (A), 10 layers (B) and 14 (C)

By measuring the line profiles of the optical microscopy images in (Figure 3.12) we can verify the build up of film formation.

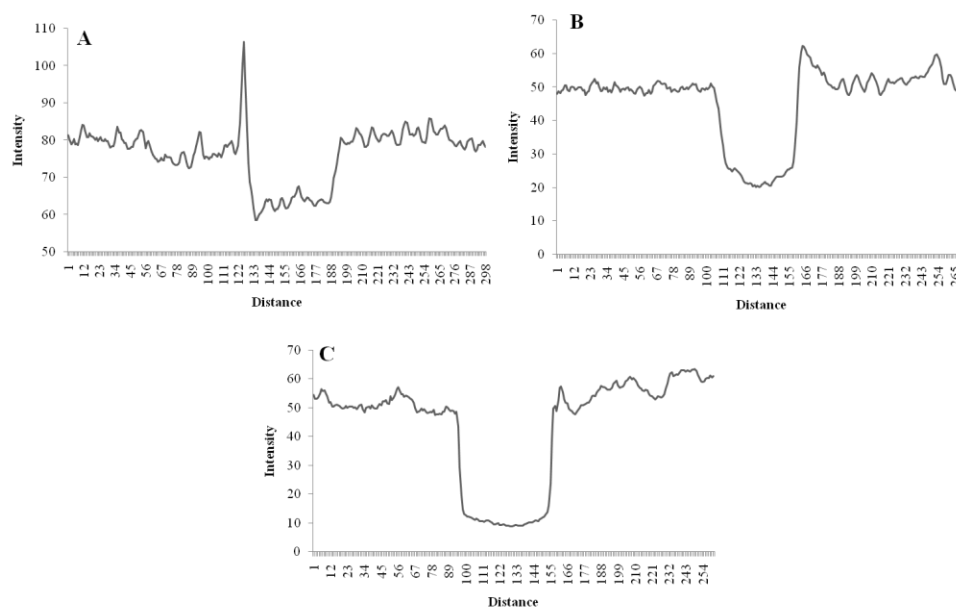


Figure 3.12 Reaction between an activated ester and the primary amine of PLL to yield a covalent amide linkage. Line profiles are taken over each scratch to measure fluorescent intensity. 2 layers were built up (A), 10 layers (B) and 14 (C)

It can be seen that there is a decrease in the intensity of fluorescence over each scratch at 2,5 and 7 bilayers, showing that the intensity of the scratch created after 2 layers is close to the intensity of the polyelectrolyte films that are covering it after subsequent coatings. This gives evidence of polymeric film build-up.

3.7 Conformal Encapsulation of *Saccaromyces cerevisiae*

Encapsulation of living cells in synthetic materials have been reported to show great promise for biomedical applications. Engineering or modifying the surface of living cells with synthetic and/or natural materials can provide a protective coating. For the coating of cells a polycation is deposited onto the negatively charged surface of the cell followed by a polyanion until a desired number of bilayers are obtained. Coated cells are washed after each polyelectrolyte deposition step to remove any excess polyelectrolyte. These washing steps and the centrifuging lowers the overall yield of the number of coated cells due to adsorption onto the sides of the centrifuge tubes. Figures 3.13 and 3.14 provide evidence of surface modification of yeast cells. Using TEM we can observe the change in the surface of the cell. Yeast cells have a net anionic surface charge and so it was proposed to add LUDOX® Cl nanoparticles. These nanoparticles are about 15 nanometer in diameter, positively charged and so ionically interact with the surface of the yeast cell (Figure 3.13).

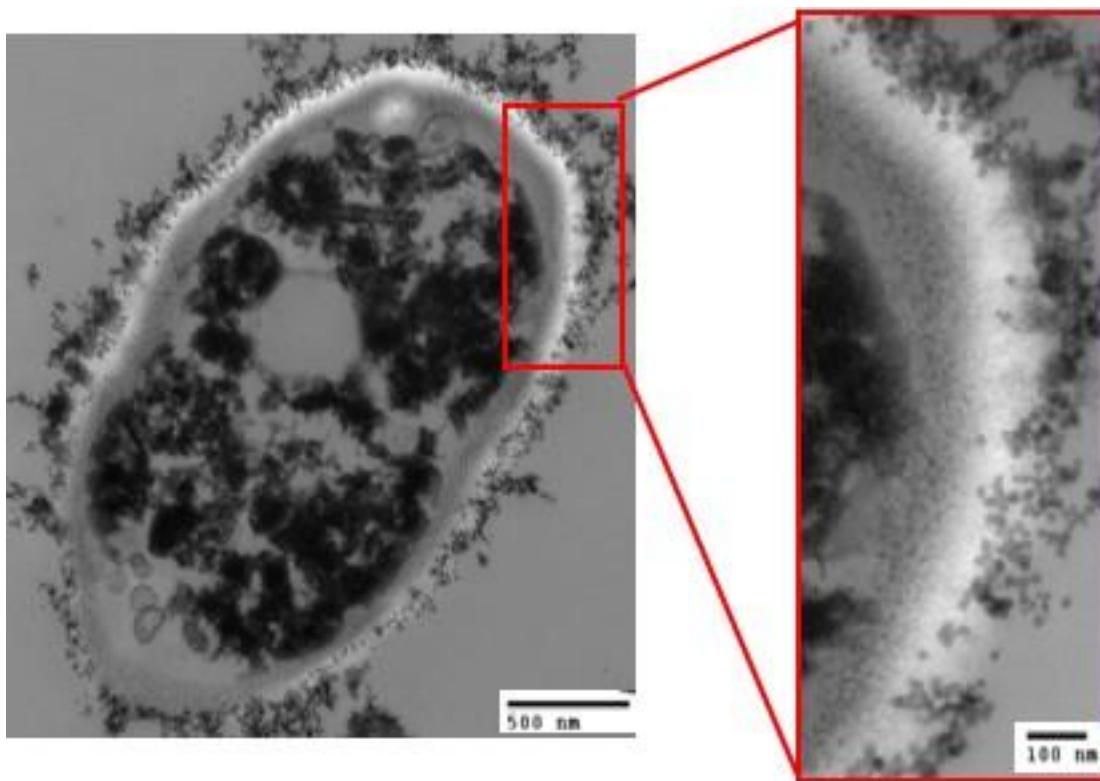


Figure 3.13 TEM micrograph of LUDOX® CI nanoparticles on the surface of a yeast cell

Conversely, when attempting to repeat this with negatively charged nanoparticles, LUDOX® HS, no nanoparticles can be observed on the surface of the cell. Once the yeast cell was coated with a polycation and then with the negatively charged particles, LUDOX® HS were observed on the surface of the cell (Figure 3.14).

Electrostatic deposition of cationic nanoparticles onto the surface of cells has been previously reported³⁵. Gold nanoparticles were deposited on *Bacillus cereus* bacteria. It was reported that the PLL coated gold nanoparticle

are electrostatically attracted to the teichoic acid brushes tethered to the peptidoglycan on the surface of the cell wall.

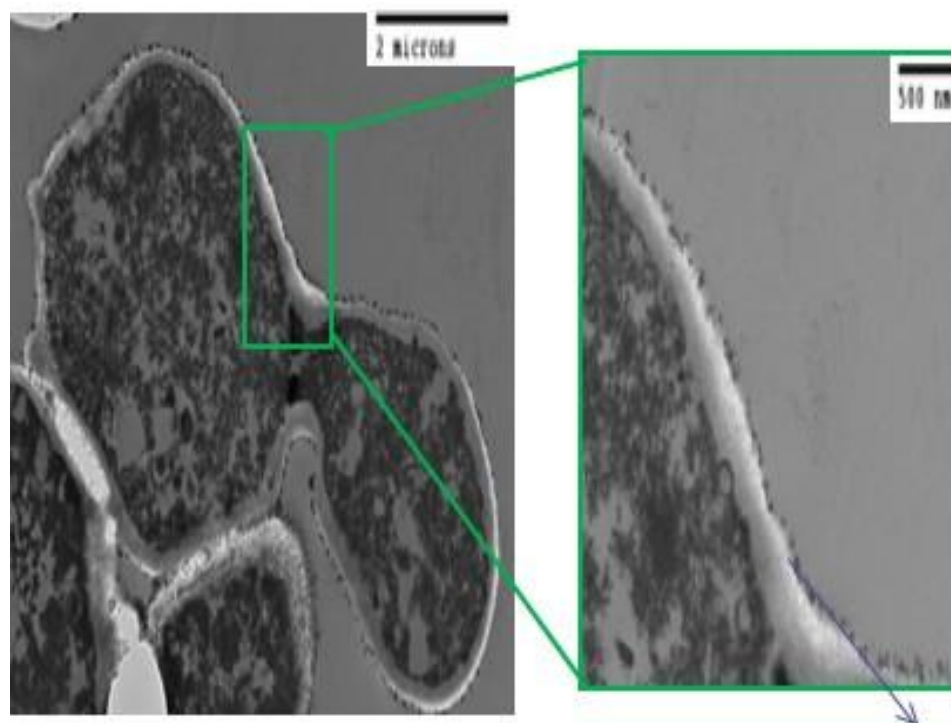


Figure 3.14 TEM micrograph of LUDOX® HS nanoparticles on the surface of a yeast cell

The cell wall of yeast consists of a lipid bi-layer that is surrounded by a layer of glycoproteins and polysaccharides. This layer ensures that the cells are less prone to mechanical stress than mammalian cells which lack the outer polysaccharide layer. It should be important to note that the viability of different cells has the potential to be very different in the same polyelectrolyte solution.

Yeast cells were successfully coated with polyelectrolytes as seen in figures 3.15 and 3.16 where there is evidence the all of the cell was coated with polyelectrolytes.

Previously, cell surface modification was achieved through the introduction of nonbiogenic functional groups by metabolic or genetic engineering.⁴⁶ It has been reported that the covalent tethering of reactive cell surface moieties while expanding the options for cell surface re-engineering modification^{28, 32} can interfere with cell physiology.^{27, 34} Choi et al used polydopamine to covalently bond with thiols or amines on the surface of yeast cell.³² They report that it was the first approach in living cell encapsulation using organic materials. This may be true, however as described above there is a danger that it interferes with cell physiology. A need exists for non-covalent cells surface modification. Chaikof et al report on using PLL grafted with PEG to decrease the cytotoxicity of PLL.²⁷ Polycation toxicity is dependent on charge density and so by grafting PEG chains to the backbone of PEG, cytotoxicity could be reduced. We can postulate that by using our temporary reactive polymers we can reduce the cytotoxicity due to the reaction with amine on PLL to form a covalent amide linkage.

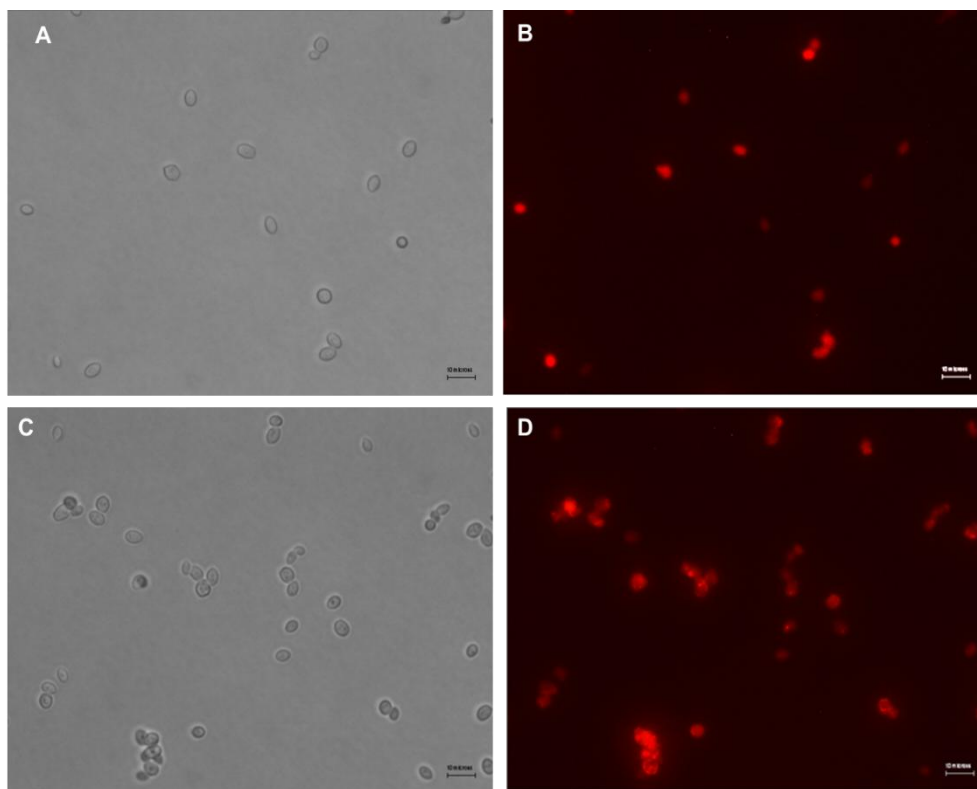


Figure 3.15 Optical and fluorescence images of PLLr coated yeast cells. A) optical image of PLLr (15-30k) coated cells and its fluorescence image (B), optical image of PLLr (40-60K) (C) and fluorescence image (D). Scale bar is 10 microns

Here we use polyelectrolyte multilayers to conformally coat living cells. We have used the non covalent surface modification of yeast cells with PLLr(15-30kDa and 40-60kDa) as seen in figures 3.15a and 3.15c, coating is confirmed in figure 3.15b and 3.15d using fluorescent labeling to view coating efficiency. The new technique of adding the polyelectrolyte solution to the cells rather than the cells to the polyelectrolyte solution has increased coating efficiency.

To investigate the proof of concept for the conformal coating of yeast cells, the previously studied PMM₅₀ was used. Future work will also include using the poly(AA-co-NAS) 50:50 for analogous conformal coatings. Partially (50%) hydrolyzed poly(methyl vinyl ether-alt-maleic anhydride), PMM₅₀, can serve as transiently reactive water-soluble polyanion that permit covalent cross linking with PLL surface layers. The high reactivity of the anhydride groups should ensure both a rapid reaction with the side-chain amines of PLL as well as fast subsequent hydrolysis of residual anhydride units to form carboxylates. We have modified the surface of the cells with PLL. Figures 3.16b and 3.16d show successful coating with PMM50f using different molecular weights of PLL, 15-30kDa (Figure 3.16a) and 40-60kDa (Figure 3.16c).

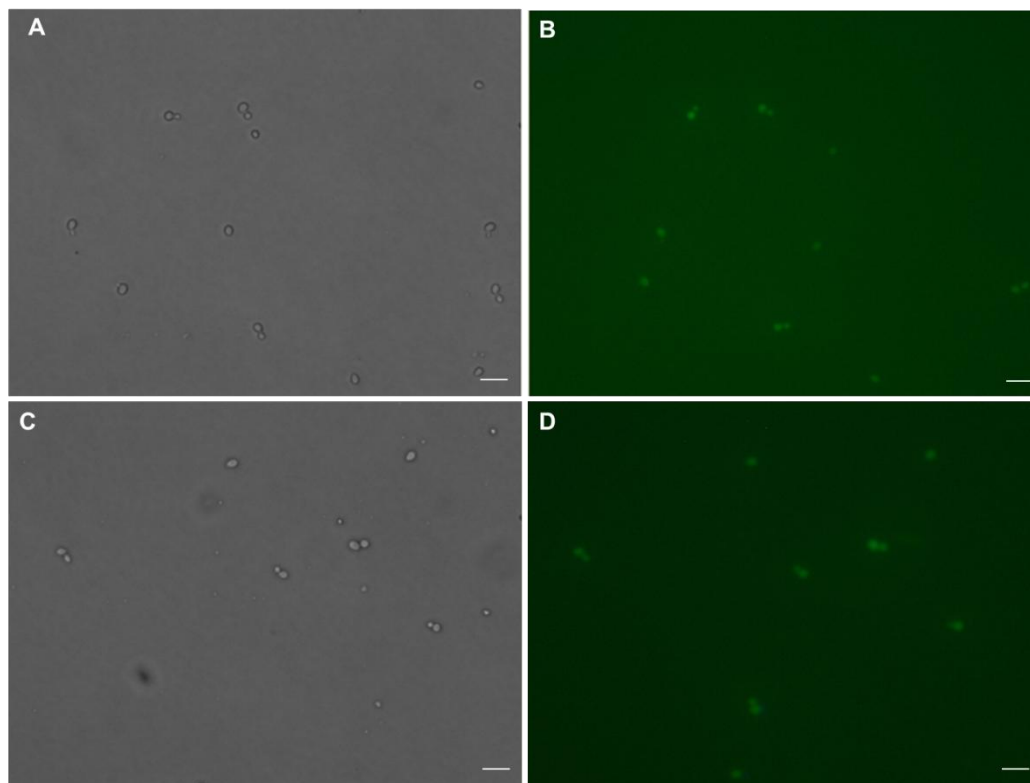


Figure 3.16 Optical and fluorescence images of PLL-PMM50f coated yeast cells. A) optical image of PLL- PMM50f (15-30k) and its fluorescence image (B), optical image of PLLr- PMM50f (40-60K) (C) and fluorescence image (D). Scale bar is 15 microns.

Viability of native yeast and coated yeast was determined using a fluorescent probe, fluorescein diacetate (FDA). FDA is widely used to distinguish between dead and live yeast cells.

FDA examines the activity of intracellular esterases. FDA is a non fluorescent small molecule that passes through the membrane of yeast cells to undergo hydrolysis catalyzed by the intracellular esterases of living cells. This hydrolysis leads to a fluorescent product which shows cellular activity are intact. The product emits fluorescence when excited under a 488nm filter. Cells in green

were considered alive and the others dead. No fluorescence was observed with PLL coated cells and PLL-PMM50 coated cells. Initially this would lead us to believe that the coating killed the cells. This was surprising and so using propidium iodide (PI), a DNA binding red fluorescent dye was used to identify cells that have a compromised cell membrane. It turned out that the initial batch of yeast cells used were dead before coating. This highlights the importance of monitoring cell viability before and after coating.

Using confocal microscopy, the presence of both polyanion and polycation deposited on cell surfaces by LbL processes could be determined (Figure 3.17). The LbL technique also allows us to control the number of layers and the types of layers while controlling the thickness of the shell.

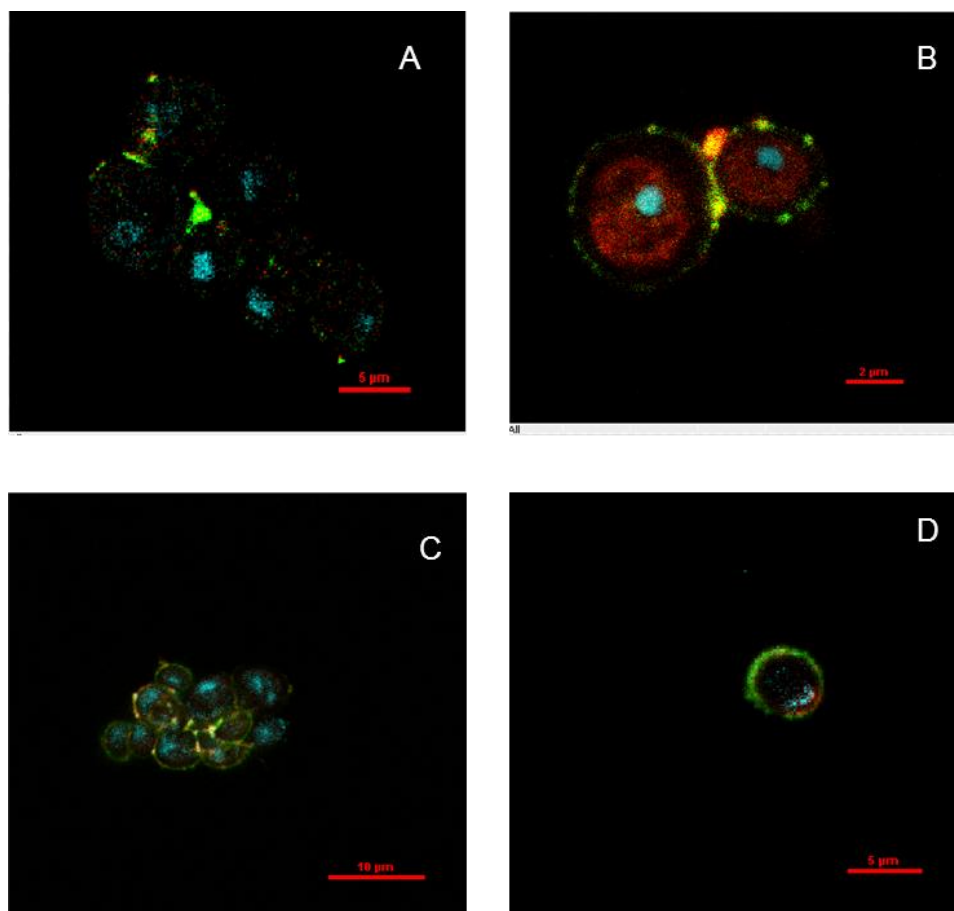


Figure 3.17 . Confocal microscopy images of PLLr / PMM50f coated yeast cells. PLLr (red), PMM50f green and a nuclear stain DAPI (blue). A=- 2 layers, B,C = 4 layers and D=8layers.

3.8 Activation and Lifecycles of Yeast Cells

Wang et al reported that the life cycle of a yeast cell is controlled by specific growth factors and the availability of essential nutrients.⁴⁷ If either of these two signals is lacking, the cell enters into a specialized “dividing resting state”, or stationary phase (G0).

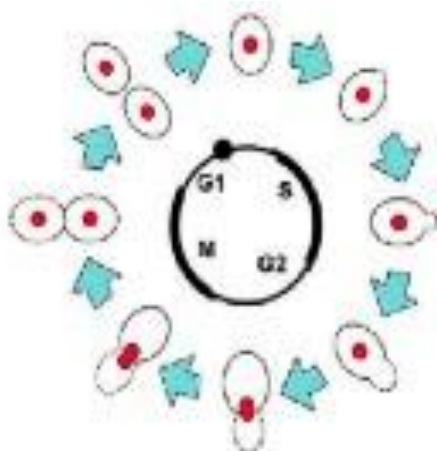


Figure 3.18 The life cycle of normal yeast cells

Yeast has been reported to enter into the G0 state when it is encapsulated by a mineral shell, with proliferation absent even in YPD medium. At 4 °C, bare yeast cells also become inert.. G0 yeast cells can be reactivated (G1 phase). The reactivated yeast can be cultured like normal cells and undergoes DNA synthesis (S Phase), G2 phase and mitosis (M phase). Previously, we mimicked a system by Diaspro et al in the hope of creating a model system using PAH and PSS to form a polyelectrolyte complex around a living yeast cell. Cells were successfully coated using this system (Figure 3.19) while maintaining cell viability (data not shown).

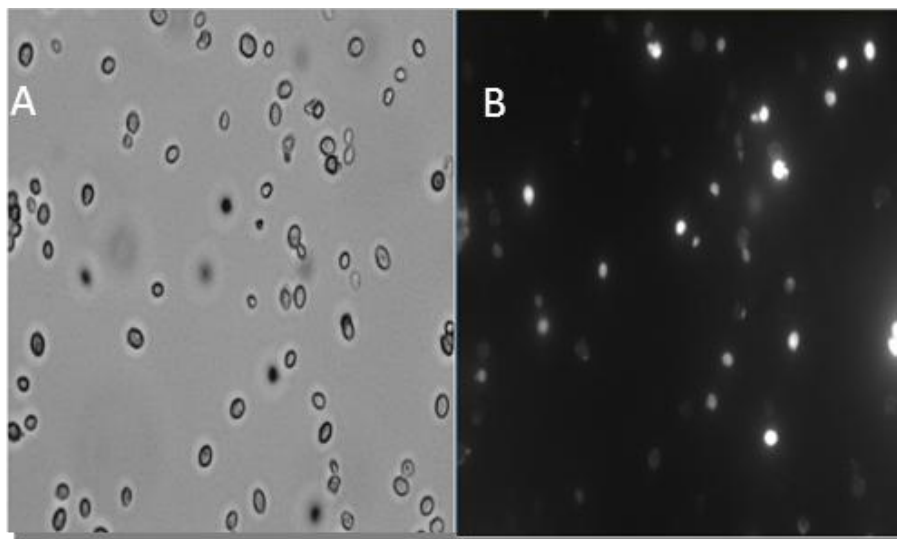


Figure 3.19 Optical images of yeast cells coated with polyelectrolyte complex – FITC labelled PAH and PSS

The next step was to investigate if cells coated with a polyelectrolyte shell are still able to divide, allowing us to determine the viability of the coated cells. PAH/PSS-coated yeast cells were incubated at 28°C in YPD growth medium for the same time periods as those incubated in water. We observed production of carbon dioxide, which is indicative of metabolic activity. Using microscopic techniques we could identify different cell populations. It is observed that as the incubation time in YPD medium increases the number of fluorescently labeled cells decreases. One of the main problems in this system was the fact that there was a lack of fluorescence from some of what were expected to be coated yeast cells, suggesting that not all of the cells were successfully coated.

3.9 Lamination of *Saccaromyces* on glass slides

In this study we focused on the fabrication of thin films that have the potential to become biofilm mimicking substrates. A system was created where yeast cells are immobilised or entrapped in polyelectrolyte multi-layers. First we used LbL to build up 5.5 bilayers of PLL and p(AA-co-NAS) 50:50 to glass coverslips with the polycation, PLL as the initial layer, as well as the outermost layer to promote the adhesion of negatively charged yeast cell that are suspended in distilled water. Yeast cells (100uL) were deposited and allowed to sit on the film for two minutes before being spun off (Figure 3.20) After the deposition of the yeast cells, the LbL process with reactive anionic polyelectrolyte (P(AA-co-NAS) (Figure 3.21) was continued until the desired number of layers was achieved (a total of 10 bilayers) (Figure 3.22).

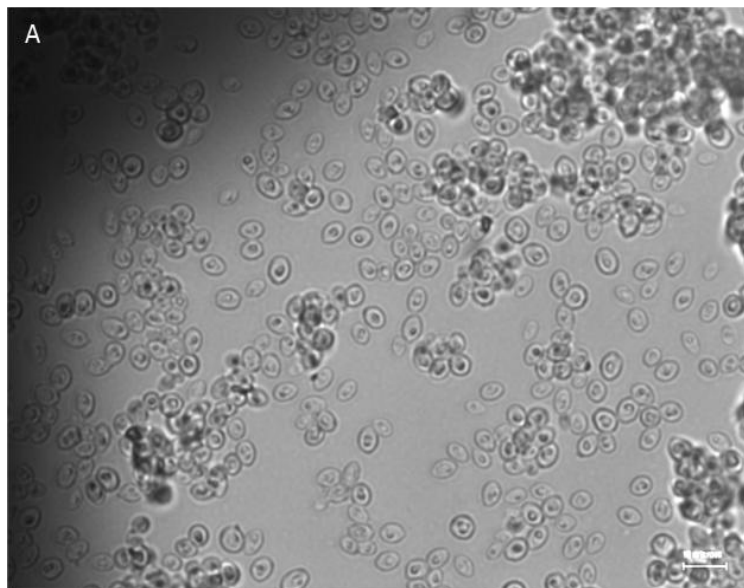


Figure 3.20 Yeast cells deposited on a PLL[(p(AA-co-NAS) – PLL]5 film on glass.

From figures 3.20 and 3.21 it can be observed that cells can be easily identified and there is no loss of cells.

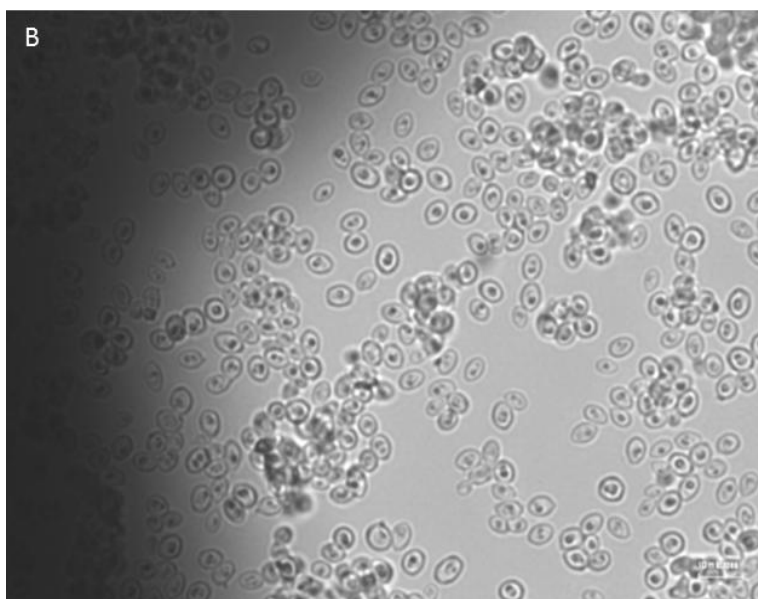


Figure 3.21 Yeast cells deposited on PLL-[p(AA-co-NAS) – PLL]5 and coated with p(AA-co-PLL)

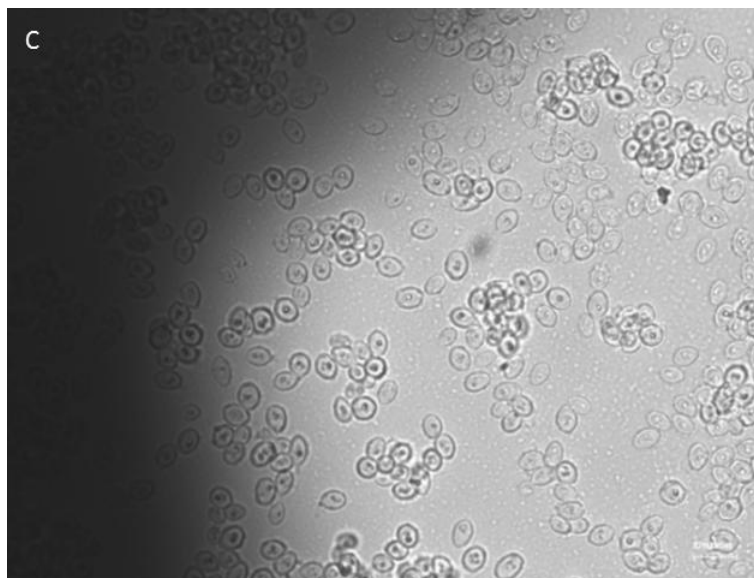


Figure 3.22 Yeast cells deposited on PLL-[p(AA-co-NAS) – PLL]₅ and coated with [p(AA-co-NAS) – PLL]₄ – p(AA-co-NAS)

The glass coverslip substrates were then immersed in polystyrene petri dishes filled with YPD solution. It was observed that similar biofilms created previously seemed to be quite stable as they maintained their integrity over a period of at least 21 days stored at room temperature.

The interaction between the cationic PLL surface and the anionic surface of the yeast cell was investigated. The yeast cells deposited on the 11th layer (PLL) were subjected to different molar concentrations of NaCl (0.1M, 0.5M, 1M and 2M) on the spin coater in an attempt to disrupt the electrostatic interaction (Figure 3.23). There was no disruption to the yeast cells immobilised on the surface.

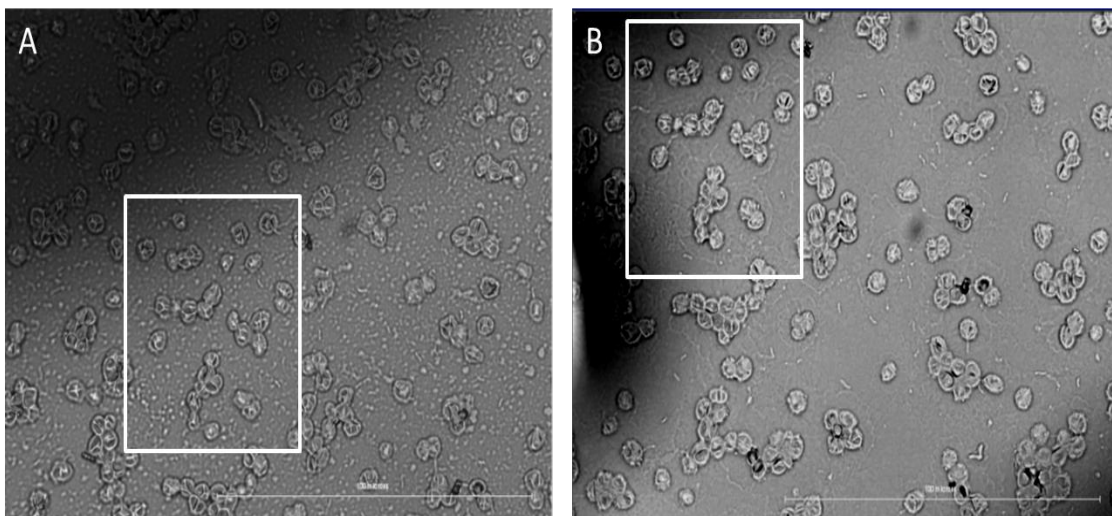


Figure 3.23 Images before addition of NaCl (attempt to break electrostatic interaction) (A) and image B after 2M NaCl was added to the slide. Yeast cells maintained their adhesion to the PLL surface.

3.10 *Budding on surface*

The lamination of yeast cells described earlier was created as a tool to view and observe encapsulated yeast cells. One of the major challenges in this work was tracking cells. It was proposed that if the cells were immobilized with a synthetic polymer film that they could be easily tracked to investigate budding. As mentioned previously yeast cells require YPD growth medium to re-enter into their active G1 phase (figure 3.18). The films with cells embedded within a 10 bilayer film were placed in YPD media, and analyzed by microscopy after 3 days (Figure 3.24).

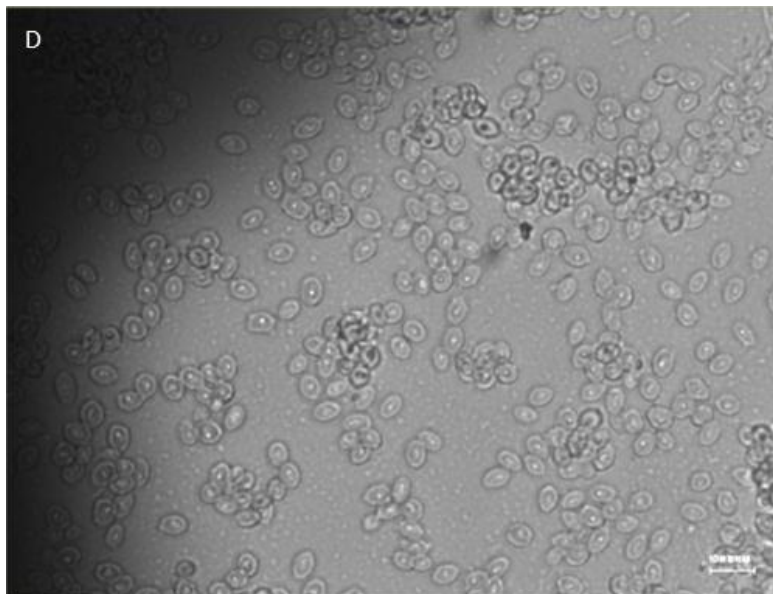


Figure 3.24 Yeast cells within polymeric multilayer in YPD for 3 days

No budding was observed after 3 days, suggesting serious deactivation of the cells by the film. It was noted that after 3 days no free yeast cells were observed within the YPD medium, suggesting that the yeast cells were successfully immobilised within the polymer films. This raises concerns about the viability of the yeast cells as we did not observe budding. This could be due to the toxicity of the polycation, which has been previously reported²⁸ or the tightness of the polymeric film that is encapsulating the cells. Further work will be carried out to investigate cell viability and cell behaviour.

A control was created in depositing yeast cells onto PLL (as layer 11) (Figure 3.25A) and then placed in a petri dish containing YPD and incubated at 28°C (Figure 3.25B). It can also be observed that no budding occurs within 3 days.

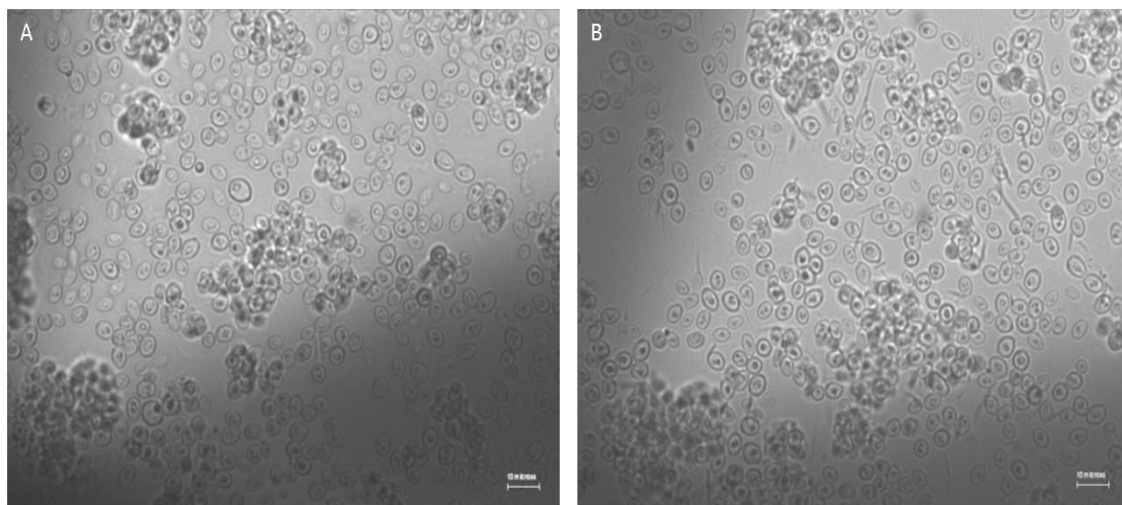


Figure 3.25 Yeast cells immobilized within polymeric multilayer films. A) yeast deposited on PLL layer 11 , B) yeast cells in YPD media

It has been shown that this system shows excellent potential as a tool to immobilize yeast cells and to create a synthetic biofilm. However, conditions may need to be adjusted to ensure survival of the immobilized cells.

4. Conclusions

4.1 *Synthesis of Reactive Polymers*

Using precipitation photo polymerization we have successfully created a water soluble acrylic acid co-polymer with a activated ester group that is reactive towards amines in PLL, capable of creating a covalent amide linkage. There is a need for reactive polyanions that have a longer life-time compared to that of minutes with PMM₅₀, in the region of tens of minutes to a couple of hours. It is hoped this new temporary reactive polyanion will fill this requirement. This new reactive polyanion has been shown to be reactive with polyamines with relatively mild conditions to easily fabricate thin polyelectrolyte multi-layered films. This allows us to propose that post functionalization of both multi-layer coatings and films can be used to introduce e.g. amino PEG, to reduce fouling, or other biomolecules. or specific receptors for targeted delivery of therapeutic proteins.

A possible route to achieve a desired activated ester content is to use the dimer of acrylic acid (carboxyethyl acrylate). This would increase the distance between the acrylate and activated ester, therefore making anhydride formation unfavourable

4.2 *Coating of Cells*

Here we report on the functionalization of cells with both synthetic polymers and nanoparticles. This process allows us to potentially use cells in cell based

therapies. We have shown that a range of materials with different functionalities can be used to coat the surface of a yeast cell.

In this study an approach to layer-by-layer assembly of thin films has been reported. The fabrication of these films takes advantage of the click reaction formation of covalent bonds between a polyamine and a activated ester functionality. Using thin films may allow us to both immobilise and store cells as well as creating a biofilm that could be tuned to mimic the ECM of cells.

5. Future Work

5.1 Further Characterization of Polymer Films

Future work in the area of covalently crosslinked thin films involves research on the accessibility and reactivity of the residual NAS functionality. From these results, it should be possible to tailor the properties of crosslinked thin films post-fabrication.

It will be crucial to determine the following:

- The permeability of the fabricated thin films
- The amount of crosslinking within the film
- The persistence of the residual reactive group

Using thin films will allow for modification with chemical and biological motifs such as peptides, small molecules.

5.2 Further Cell Studies with Yeast and Mammalian Cells

A strategy for cell encapsulation with layer-by-layer shells using covalently crosslinked layers has been shown.

Future work in the area will involve the following:

- Fully investigate cell viability as there are mixed reports in relation to the effect of polycations on the viability of cells.

- Improve shell stability to ensure that cells remain encapsulated and immobilized. This could involve increasing the number of layers.
- Determine the viability of yeast cells entrapped in a polymeric matrix using budding and metabolic activity as indicators.

6. References

- (1) Wilson, J. T.; Chaikof, E. L. Challenges and emerging technologies in the immunoisolation of cells and tissues. *Adv. Drug Deliv. Rev.* **2008**, *60*, 124-145.
- (2) Neu, B.; Voigt, A.; Mitlohner, R.; Leporatti, S.; Gao, C. Y.; Donath, E.; Kieseewetter, H.; Mohwald, H.; Meiselman, H. J.; Baumler, H. Biological cells as templates for hollow microcapsules. *J. Microencapsul.* **2001**, *18*, 385-395.
- (3) Chang, T. M. Semipermeable Microcapsules. *Science (New York, N.Y.)* **1964**, *146*, 524-5.
- (4) Chick, W.L., Like, A.A., lauris, V., Beta cell culture on synthetic capillaries: an artificial endocrine pancreas. *Science* **1975**, 847-849.
- (5) Lim, F.; Sun, A. M. Microencapsulated Islets as Bioartificial Endocrine Pancreas. *Science* **1980**, *210*, 908-910.
- (6) Orive, G., Hernandez, R.M., Gascon, A.R., Calafiore, R., Chang, T.M., Vos, P.D., Hortelano, G., Hunkeler, D., Lacik, I., Shapiro, A.M., Pedraz, J.L. Cell encapsulation: Promise and progress. *Nat. Med.* **2003**, *9(1)*, 104.
- (7) Lanza, R. P.; Hayes, J. L.; Chick, W. L. Encapsulated cell technology. *Nat. Biotechnol.* **1996**, *14*, 1107-1111.
- (8) Chaikof, E. L. Engineering and material considerations in islet cell transplantation. *Annu. Rev. Biomed. Eng.* **1999**, *1*, 103-127.
- (9) Mohajeri, S. Polymers for Reversible Cell Encapsulation, McMaster University, Open Access Dissertations and Theses. Paper 4998., 2011.
- (10) Peirone, M. A.; Delaney, K.; Kwiecin, J.; Fletch, A.; Chang, P. L. Delivery of recombinant gene product to canines with nonautologous microencapsulated cells. *Hum. Gene Ther.* **1998**, *9*, 195-206.
- (11) Shen, F.; Li, A. A.; Gong, Y. K.; Somers, S.; Potter, M. A.; Winnik, F. M.; Chang, P. L. Encapsulation of recombinant cells with a novel magnetized alginate for magnetic resonance imaging. *Hum. Gene Ther.* **2005**, *16*, 971-984.

- (12) Dusseault, J.; Leblond, F. A.; Robitaille, R.; Jourdan, G.; Tessier, J.; Menard, M.; Henley, N.; Halle, J. P. Microencapsulation of living cells in semi-permeable membranes with covalently cross-linked layers. *Biomaterials* **2005**, *26*, 1515-1522.
- (13) Mazumder, M. A. J.; Shen, F.; Burke, N. A. D.; Potter, M. A.; Stoever, H. D. H. Self-cross-linking polyelectrolyte complexes for therapeutic cell encapsulation. *Biomacromolecules* **2008**, *9*, 2292-2300.
- (14) Shen, F.; Mazumder, M. A. J.; Burke, N. A. D.; Stoever, H. D. H.; Potter, M. A. Mechanically Enhanced Microcapsules for Cellular Gene Therapy. *Journal of Biomedical Materials Research Part B-Applied Biomaterials* **2009**, *90B*, 350-361.
- (15) Jang, W.; Jensen, A. T.; Lutkenhaus, J. L. Confinement Effects on Cross-Linking within Electrostatic Layer-by-Layer Assemblies Containing Poly(allylamine hydrochloride) and Poly(acrylic acid). *Macromolecules* **2010**, *43*, 9473-9479.
- (16) Gardner, C. M.; Burke, N. A. D.; Stoever, H. D. H. Cross-Linked Microcapsules Formed From Self-Deactivating Reactive Polyelectrolytes. *Langmuir* **2010**, *26*, 4916-4924.
- (17) de Vos, P.; Hamel, A. F.; Tatarkiewicz, K. Considerations for successful transplantation of encapsulated pancreatic islets. *Diabetologia* **2002**, *45*, 159-173.
- (18) Lanza, R. P.; Kuhlreiber, W. M.; Ecker, D.; Staruk, J. E.; Chick, W. L. Xenotransplantation of Porcine and Bovine Islets without Immunosuppression using Uncoated Alginate Microspheres. *Transplantation* **1995**, *59*, 1377-1384.
- (19) Wilson, J. T.; Chaikof, E. L. Challenges and emerging technologies in the immunoisolation of cells and tissues. *Adv. Drug Deliv. Rev.* **2008**, *60*, 124-145.
- (20) Canaple, L.; Rehor, A.; Hunkeler, D. Improving cell encapsulation through size control. *Journal of Biomaterials Science-Polymer Edition* **2002**, *13*, 783-796.
- (21) Lum, Z. P.; Tai, I. T.; Krestow, M.; Norton, J.; Vacek, I.; Sun, A. M. Prolonged Reversal of Diabetic State in Nod Mice by Xenografts of Microencapsulated Rat Islets. *Diabetes* **1991**, *40*, 1511-1516.

- (22) Iwata, H.; Takagi, T.; Amemiya, H.; Shimizu, H.; Yamashita, K.; Kobayashi, K.; Akutsu, T. Agarose for a Bioartificial Pancreas. *J. Biomed. Mater. Res.* **1992**, *26*, 967-977.
- (23) Teramura, Y.; Kaneda, Y.; Iwata, H. Islet-encapsulation in ultra-thin layer-by-layer membranes of poly(vinyl alcohol) anchored to poly(ethylene glycol)-lipids in the cell membrane. *Biomaterials* **2007**, *28*, 4818-4825.
- (24) Robertson, R. P. Medical progress: Islet transplantation as a treatment for diabetes - A work in progress. *N. Engl. J. Med.* **2004**, *350*, 694-705.
- (25) Shapiro, A. M. J.; Ricordi, C.; Hering, B. Edmonton's islet success has indeed been replicated elsewhere. *Lancet* **2003**, *362*, 1242-1242.
- (26) Leblond, F. A.; Simard, G.; Henley, N.; Rocheleau, B.; Huet, P. M.; Halle, J. P. Studies on smaller (similar to 315 μ M) microcapsules: IV. Feasibility and safety of intrahepatic implantations of small alginate poly-L-lysine microcapsules. *Cell Transplant.* **1999**, *8*, 327-337.
- (27) Wilson, J. T.; Krishnamurthy, V. R.; Cui, W.; Qu, Z.; Chaikof, E. L. **Noncovalent Cell Surface Engineering with Cationic Graft Copolymers.** *Journal of the American Chemical Society* *2009* *131* (51), 18228-18229 .
- (28) Wilson, J. T.; Cui, W.; Kozovskaya, V.; Kharlampieva, E.; Pan, D.; Qu, Z.; Krishnamurthy, V. R.; Mets, J.; Kumar, V.; Wen, J.; Song, Y.; Tsukruk, V. V.; Chaikof, E. L. Cell Surface Engineering with Polyelectrolyte Multilayer Thin Films. *J. Am. Chem. Soc.* **2011**, *133*, 7054-7064.
- (29) Decher, G. Fuzzy nanoassemblies: Toward layered polymeric multicomposites. *Science* **1997**, *277*, 1232-1237.
- (30) Diaspro, A.; Silvano, D.; Krol, S.; Cavalleri, O.; Gliozzi, A. Single living cell encapsulation in nano-organized polyelectrolyte shells. *Langmuir* **2002**, *18*, 5047-5050.
- (31) Mansouri, S.; Merhi, Y.; Winnik, F. M.; Tabrizian, M. Investigation of Layer-by-Layer Assembly of Polyelectrolytes on Fully Functional Human Red Blood Cells in Suspension for Attenuated Immune Response. *Biomacromolecules* **2011**, *12*, 585-592.
- (32) Yang, S. H.; Kang, S. M.; Lee, K.; Chung, T. D.; Lee, H.; Choi, I. S. Mussel-Inspired Encapsulation and Functionalization of Individual Yeast Cells. *J. Am. Chem. Soc.* **2011**, *133*, 2795-2797.

- (33) Uludag, H.; Wong, M.; Man, J. Reactivity of temperature-sensitive, protein-conjugating polymers prepared by a photopolymerization process. *J Appl Polym Sci* **2000**, *75*, 583-592.
- (34) Rabuka, R.; Forstner, M. B.; Groves, J. T.; Bertozzi, C. R. **Noncovalent Cell Surface Engineering: Incorporation of Bioactive Synthetic Glycopolymers into Cellular Membranes.** *Journal of the American Chemical Society* **2008** *130* (18), 5947-5953 .
- (35) Berry, V.; Saraf, R. F. Self-assembly of nanoparticles on live bacterium: An avenue to fabricate electronic devices. *Angewandte Chemie-International Edition* **2005**, *44*, 6668-6673.
- (36) Fakhrullin , R. F.; Zamaleeva , A. I.; Minullina , R. T.; Konnova, S. A.; Paunov, V. Cyborg cells: functionalisation of living cells with polymers and nanomaterials. *Chem Soc Rev* **2012**, *41*, 4189.
- (37) Theato, P. Synthesis of Well-Defined Polymeric Activated Esters. *Journal of Polymer Science Part A-Polymer Chemistry* **2008**, *46*, 6677-6687.
- (38) Buck, M. E.; Breitbach, A. S.; Belgrade, S. K.; Blackwell, H. E.; Lynn, D. M. Chemical Modification of Reactive Multilayered Films Fabricated from Poly(2-alkenyl azlactone)s: Design of Surfaces that Prevent or Promote Mammalian Cell Adhesion and Bacterial Biofilm Growth. *Biomacromolecules* **2009**, *10*, 1564-1574.
- (39) Seo, J.; Schattling, P.; Lang, T.; Jochum, F.; Nilles, K.; Theato, P.; Char, K. Covalently Bonded Layer-by-Layer Assembly of Multifunctional Thin Films Based on Activated Esters. *Langmuir* **2010**, *26*, 1830-1836.
- (40) Peeters, T.; van Remoortere, B.
<http://home.wanadoo.nl/tom.peeters/Subpaginas/spin%20coating.htm>.
- (41) McGaugh, M.C., Kottle, S. *J. Polym. Sci. B.* **1967**, *5*, 817.
- (42) Eisenberg, A. *J. Polym. Sci. B.* **1969**, *7*, 1717.
- (43) Bruice, T. C. *J. Am. Chem. Soc.* **1960**.
- (44) Bunyakan, C.; Hunkeler, D. Precipitation polymerization of acrylic acid in toluene. I: synthesis, characterization and kinetics. *Polymer* **1999**, *40*, 6213-6224.

- (45) Gardner, C. M.; Stoeber, H. D. H. Reactive Polyanions Based on Poly(4,4-dimethyl-2-vinyl-2-oxazoline-5-one-co-methacrylic acid). *Macromolecules* **2011**, *44*, 7115-7123.
- (46) Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. In vivo imaging of membrane-associated glycans in developing zebrafish. *Science* **2008**, *320*, 664-667.
- (47) Wang, B.; Liu, P.; Jiang, W.; Pan, H.; Xu, X.; Tang, R. Yeast cells with an artificial mineral shell: Protection and modification of living cells by biomimetic mineralization. *Angewandte Chemie-International Edition* **2008**, *47*, 3560-3564.