Dual-functional Polyurea Microcapsules for Chronic Wound Care Dressings: Sustained Drug Delivery and Non-leaching Infection Control

by

Wei He

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Department of Textile Sciences
University of Manitoba
Winnipeg, Manitoba

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ABSTRACT

A new design of dual-functional polyurea microcapsules was proposed for chronic wound dressings to provide both non-leaching infection control and sustained topical drug delivery functionalities. Quaternary ammonium functionalized polyurea microcapsules (MCQs) were synthesized under mild conditions through an interfacial crosslinking reaction between branched polyethylenimine (PEI) and 2,4-toluene diisocyanate (TDI) in a dimethylformamide/cyclohexane emulsion. An *in-situ* modification method was developed to endow non-leaching surface antimicrobial properties to MCQs via bonding antimicrobial surfactants to surface isocyanate residues on the polyurea shells. The resultant robust MCQs with both non-leaching antimicrobial properties and sustained drug releasing properties have potential applications in medical textiles, such as chronic wound dressings, for infection control and drug delivery.

LIST OF ABBREVIATIONS

The most important concepts:

AEA	2-azidoethylamine
DAB	dimethyl-dodecyl-(5-hydroxy-pentyl)-ammonium bromide
MCQ	*in-situ* modified polyurea microcapsule
PU	polyurea
PUC	polyurea model capsule
PUC+Q	post modified PUC
PUCQ	*in-situ* modified PUC
PUMC	polyurea microcapsule
PUMCn	PUMC synthesized without Span™ 85

Chemicals and materials:

CTAC	cetyltimethyl ammonium chloride solution
Cmr	Coumarin-1
EGF	epidermal growth factor
EtOH	ethanol
DMF	dimethylformamide
KBr	potassium bromide
MCT	microcentrifuge tube
MeCN	acetonitrile

MeOH	methanol
PBS	phosphate buffered saline
PEI	polyethylenimine
PEG	poly(ethylene glycol)
PIB	polyisobutylene
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic acid)
QAC	quaternary ammonium compound
SDS	sodium dodecyl sulfate
Span™ 85	sorbitan trioleate
TDI	2,4-toluene diisocyanate
THF	tetrahydrofuran
TSA	trypticase soy agar

Instrumentation:

FTIR	Fourier transform infrared spectroscopy
GPC	gel permeation chromatography
HPLC	high-performance liquid chromatography
^{1}H NMR	proton nuclear magnetic resonance
LS	light scattering
UV-Vis	ultraviolet–visible spectroscopy

Other concepts:

Emulsion science:

O/O	oil-in-oil
W/O	water-in-oil
O/W	oil-in-water
CMC	critical micelle concentration
disp.phase	disperse phase
cont.phase	continuous phase

Microbiology:

cfu	colony forming unit
ESBL	extended-spectrum beta-lactamase
E. coli	Escherichia coli
Gram+	gram-positive
Gram-	gram-negative
MDR	multi-drug-resistant
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*

Others:

–NCO	isocyanate group
–OH	hydroxyl group
–SH	thiol group

Ag+	silver ion
MWCO	molecular weight cut off
Mn	number average molecular weight
PEM	polyelectrolyte multilayer
USP	United States Pharmacopeia

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CHAPTER 1. INTRODUCTION

1.1. Chronic Wounds and Wound Dressings

Chronic wounds are getting increasing concern worldwide, because they not only bring physical and mental sufferings to patients, but also place a financial burden to the healthcare system.[1-4] Wound healing is a complex process that consists of a sequence of overlapping phases including inflammation, reepithelialization, matrix formation, and remodeling.[5] A wound that is unable to heal within the expected time frame is called a chronic wound.[6] The healing process of a chronic wound is disrupted during one or more phases due to malnutrition, infection, excessive exudation, prolonged inflammation, and further trauma. Infection and malnutrition are the major obstacles to successful wound healing.[7]

Wound infection impedes wound healing by excessive protease production: about 94% of the ulcers infected with *S. aureus* heal slowly or recur after discharge, and the presence of *P. aeruginosa* and *S. aureus* significantly reduces skin graft healing.[8, 9] A chronic infection occurs when bio-burden overwhelms host-defense, which leads to prolonged inflammation with the production of exudates that contain higher concentrations of proteases than in acute wounds.[6] The excessive proteases, which destroy proteins, not only kill bacteria on site but also inhibit wound healing by damaging healthy skin tissue, growth factors, and growth factor receptors over the wound area.

Hence, wound bed preparation (disinfection and debridement) is crucial, especially to further expensive treatments like protein therapies, which are prone to fail due to chronic infections.[8, 10, 11] Nonetheless, the misuse or the overuse of topical cytotoxic antibiotics during wound disinfection could become another obstacle to wound healing.[7, 12]

Local nutrition delivery to a disinfected wound can prevent malnutrition induced wound complications.[13] Apart from carbohydrates and fats, which provide cellular energy, vitamin A, vitamin C, vitamin E, selenium thiamine, pantothenic acid, zinc, copper, and manganeses are also essential for wound healing.[7] Compounds that are frequently reported to support wound healing include growth factors, chitosan, collagen, hyaluronan, and beta-glucan.[7, 14]

Growth factors, peptides that regulate cellular proliferation, migration, and survival,[15] are implied as key regulators to wound healing process.[4, 16] They stimulate the growth of epithelial cells, fibroblasts and new blood vessels;[19] guide cell migration toward the wound site; and influence matrix formation and scar remodeling.[14] Topical application of epidermis growth factors (EGF) via ointment, mist, injection or drug release device was found to expedite wound healing.[17 18] Administration method of EGF may affect their bio-efficacy because EGF is prone to degradation at wound sites.[1, 19-22] In a study of second-degree scald burns in rats, EGF formulated in a chitosan gel exhibited higher wound healing efficiency than in solution.[23] In another study, the wound repair process in rats was accelerated when EGF was continuously released from subcutaneous pellets when compared with that in wounds with daily EGF injection.[24] Therefore, dosage

forms that control ingredient release and prevent protein degradation are desirable for the delivery of growth factor with well-preserved bio-efficacy.

Ideally, a wound dressing shall fulfill all wound management requirements,[6] which include wound debridement (to clean the wound site, e.g. TenderWet®, a Ringer solution releasing superabsorbent polymer dressing),[25, 26] moisture regulation (to provide a moist condition), liquid absorption (to remove blood or excess exudates), oxygen exchange; bacteria isolation, non-adherence (to avoid further trauma), cost effectiveness; and infrequent replacement.

Moisture regulation is a fundamental requirement for chronic wound dressings as wounds heals better under moist conditions.[7] A variety of commercialized dressings is available for different wound conditions. For relatively dry wounds (wounds with minimal exudates), semi-occlusive dressings (e.g. Tegaderm®, polyurethane films) were applied to retain liquids (exudates) while permitting water vapor and oxygen exchange.[27]. Alternatively, non-occlusive dressings (e.g. chitin films, which are permeable to liquids) laminated with a top occlusive dressing were applied to retain the moisture.[12] For deteriorated wounds with excessive exudates, super absorbing hydrocolloid dressings (e.g. carboxymethyl-cellulose laminated with an occlusive film) were applied to remove the excessive exudates till saturation.[28] For absolutely dry wounds, moisture donating hydrogel dressings (e.g. calcium sodium alginate gel laminated with an occlusive film) were applied to provide moisture continuously until the gel deteriorates.[29] Recent advances in moist regulation of new dressing materials focused on the introduction of

electro spinning technique. For instance, electro-spun polyurea nano-mats were developed to enhance oxygen exchange for wound dressing, and faster *in vitro* re-epithelialization was observed in nano-mats when compared with ordinary polyurethane films.[30]

In addition to moist regulation, dressings have been endowed with various functionalities by incorporating active ingredients. Various nutrients have been incorporated to wound dressings. Unsaturated fatty acids have been loaded into a flax dressing to reinforce plasma membranes of fibroblasts.[31] EGF has been electro-spun with silk, and the nano silk dressing accelerated the wound closure rate by 3.5 times over the EGF-free dressing in vitro.[32] Vulnamin® (a sodium hyaluronate gel containing four essential amino acids: glycine, l-lysine, l-proline and l-leucine[33]) and sodium ialuronate (Na-Ial) have been loaded into dressings to assist collagen and elastin synthesis.[34] The expedited wound closure and tissue regeneration were observed on the wounds of aged rats.

Protease inhibitors have been loaded into a matrix to neutralize the excessive proteases upon release.[35, 36] The topically delivered protease inhibitors reduced the proteases levels, prevented the proteolytic degradation of growth factors, enabled the accumulation of active growth factors in the wound bed, and thus promoted wound healing. Edwards and coworkers have introduced a controlled release system to wound dressings for oleic acid, a hydrophobic protease inhibitor.[37] The solubility, thus the release dosage, of oleic acid was limited by wound site albumin level, so that the protease

inhibitor was released in response of the amount local exudates. The system performed well on cotton gauze, and was compatible with hydrogel or hydrocolloid dressings.

Antibiotics have also been loaded to wound dressings to serve as a supplement to routine wound pre-disinfection. Most commercial antimicrobial dressings function by releasing encapsulated antibiotics.[38] Metallic silver or silver salts have been incorporated into various substrates including gauze, foam (e.g. Contreet® F), hydrocolloid (e.g. Contreet® H), hydrogel (e.g. Silvasorb®), film or powder (Arglaes®), alginate (Acticoat® absorbent), activated charcoal (Actisorb® Silver 220), hydrofibers (Aquacel® AG), and cream. However, growing concern about silver containing wound dressings rises in terms of drug resistance development,[39, 40] cytotoxicity[41] and potential staining of the wound skin.[42]

Some silver-free antimicrobial dressings have also been commercialized. For example, dressings applied with cadexomer iodine gels or pastes have shown effectiveness in reducing the bacterial count of venous leg ulcers, diabetic foot ulcers, and pressure ulcers.[38] Activon®, a honey containing dressing, has been applied in clinical treatment of early wound infections. The antimicrobial action of honey was attributed to its acidity and osmotic effects.[43] Recent developments in antimicrobial dressings rest mainly in new dosage forms that could be responsive, versatile, or long-lasting. A crosslinked chitosan sponge has been reported to release preloaded norfloxacin in response of moisture. The sponge swelled upon wetting and turned from fibrillar structure to membranous structure, thereby performing swelling controlled drug release.[44]

A dual drug releasing dressing has been developed with electro spinning technique. Two drugs of different hydrophilicity were spun from a dual spinneret apparatus into a single scaffold.[45] A long-term ciprofloxacin releasing wound dressing has been introduced to have sustained antimicrobial action over twelve days.[46]

In summary, commercial wound dressings are designed to optimize the healing conditions for various chronic wounds by providing basic moisture regulation and specialized functionalities such as protease inhibition, nutrition supply and disinfection. More complex dressings are being engineered to combine multiple concepts into one single dressing (for instance, a blood clotting, nano-silver containing, electrospun dressing[35] and a biodegradable, bio-mimicking, thermo responsive peeling, laminated dressing[47]). Most wound dressings achieve their functionalities by releasing active agents. Such strategy nevertheless is not necessarily ideal for all applications, especially in the case of antibiotics. The slow release of antibiotic potentiates the concern of antibiotic resistance. Therefore, a platform with both releasing and non-releasing functionalities is desirable for wound dressings. To explore this possibility, antimicrobial surfaces and drug delivery systems are discussed as follows.

1.2. Antimicrobial Surfaces

Antimicrobial surfaces are categorized into three groups:[48] non-leaching antimicrobial surfaces, biocide-releasing surfaces, and adhesion resistant surfaces. Non-leaching antimicrobial surfaces (e.g. poly-cationic coated surfaces) are inherently antimicrobial: microbes are killed upon direct contact; biocide-releasing surfaces (such as

silver and copper ion releasing surfaces) kill bacteria with biocides released from or generated by the surfaces; adhesion resistant surfaces, or antifouling surfaces, prevent bacteria from initial adhesion and subsequent colonization.

1.2.1. Non-leaching Antimicrobial Surfaces

Since 1980s, cation containing surfaces have been reported to be antimicrobial upon contact by causing physical damage to the bacterial cell membrane.[58] Recently, hydrophobic polycation coatings have been developed to serve as non-leaching antimicrobial surfaces.[49, 50] The hydrophobic cationic arms on the surface were designed to cause cell lysis through penetrating the hydrophobic cell membrane and inducing ion exchange on the exterior anionic cell envelope.[58] Ideally, the cationic moieties repel from each other and thus keep the hydrophobic chains separated and perpendicular to the substrate surface, so that these chains have maximum interaction with bacteria.

To date, a wide variety of antimicrobial peptides and cationic compounds has been grafted onto different matrixes to generate non-leaching antimicrobial surfaces. Antimicrobial peptides are not covered in this thesis in view of their specificity in species and strain. Instead, cationic compounds, which have broader antimicrobial activities, are discussed in the following categories: ammonium compounds,[45, 49, 51-54] guanidine based polymers,[55] and polymeric phosphonium compounds.[56]

Quaternary ammonium compounds (QACs) have been incorporated into polymers as antimicrobial agents. Polyethylenimine (PEI) coated surfaces exhibited antimicrobial properties against *S. aureus*, airborne E. coli, and some viruses thanks to the high density

of protonated amines.[51-54] QAC modified PEI coating showed low mammalian cytotoxicity despite of the medium to high cytotoxicity of QAC monomers.[57] N-dodecyl, N-methyl- linear PEI coated surfaces have been reported to be antimicrobial in various solutions and durable against repeated washing, so that the non-leaching antimicrobial property can be readily rejuvenated by removing the surface accumulated bacteria debris.[54, 58]

The antimicrobial potency of polymeric QACs may be affected by their counter-ions. The QAC dendrimer biocides with bromide anions are more potent than those with chloride anions. However, homopolymers of quaternarized vinylamine or quaternarized methyl methacrylate display no difference in antimicrobial potency among their chloride, bromide or iodide counterparts.[56] Although water soluble polymeric QACs exhibit medium to high antimicrobial potency, their activity has been reported to be suppressed in the presence of biological materials, particularly blood.[59] One alternative to quaternary ammonium salts is a new family of water soluble cationic tertiary poly(diallylammonium) salts, which exhibit strong antimicrobial properties under moderate ionic strength (serum, 0.01 M/0.1 M) or alkaline condition (pH = 10.5).[60]

Polymerized guanidine compounds, which can transform into tertiary ammonium salts, exhibit potent broad spectrum antimicrobial efficacy. Polyvinylguanidine, synthesized through free radical polymerization of N-vinylformamide followed by hydrolysis and guanidinylation, acts both as a hydrolyzing agent against toxic organophosphates and a bactericide against both Gram+ and Gram- bacteria.[55]

Polyhexamethylene biguanide, a commercialized broad spectrum antibiotic against Gram+/Gram- bacteria, fungi, and yeasts, exhibits low mammalian toxicity and environmental impact. It binds with cellulosic materials via acid-base interactions after padding or exhaustion and retains antimicrobial activities for 25-50 wash cycles (e.g. Kerlix® Gauze).[61] Similarly, poly(hexamethylene guanidine hydrochloride) coated antimicrobial surface shows 100% bacterial reduction with good laundry durability.[62]

Polymeric phosphonium compounds also show antimicrobial actions. For example, poly[tributyl(4-vinylbenzyl)phosphonium salt] coated surfaces have been reported to inhibit the growth of *S. aureus*.[56] The chloride salt of polymeric phosphonium possesses the highest antibacterial potency and is followed by its tetraflouride, perchlorate and hexafluorophosphate counterparts. Their antimicrobial potency was believed to be correlated with the solubility of the corresponding polymers.

Allyl derivatives of dimethylhydantoin are durable, regenerable, potent biocides that kill bacteria by chlorine transfer.[63, 64] In one study, monomethylol-5,5-dimethylhydantion was synthesized to be grafted to cellulose fibers. The two-step antimicrobial functionalization of the fabrics was achieved by an acid-condition finishing (grafting the fiber surface with the molecule) and bleach rinsing (to generate an N-halamine group on the hydantoin structure). Then, chlorine could be transferred from the N-halamine group to microorganism based on direct contact. Thanks to the reversible oxidability of the hydantoin N-H, the antimicrobial activity of the fabric can be regenerated through another bleach rinsing process during a normal laundry.[65-67]

1.2.2. Biocide Leaching Surfaces

Different biocides have been incorporated into various surfaces to perform antimicrobial activities upon releasing active agents into the micro-environment. Two common drawbacks of biocide-leaching surface are the function loss upon depletion of active agent and the potential microbial drug resistance development after the continuous exposure to small dosage of antibiotics.

Silver is a strong broad spectrum biocide that have been applied in commercial products.[49] Silver, especially nano silver particles, are widely applied in antimicrobial wound dressings. Most silver containing antimicrobial products, such as AgION® and SilvaGard®, rely on the silver ions (Ag+) diffused from the dressing.[7] It is believed that Ag+ inactivates proteins and enzymes through binding with their thiol groups (–SH).[39, 68] Although metallic silver products may not practically suffer from non-permanency as the production of Ag+ sustains throughout the life span of the product, silver still suffers from potential development of microbial resistance.[39, 40, 68] Additionally, the high dosage of nano silver particles could potentially stain the wound skin[42] Moreover, cytotoxicity of silver could be another concern for silver containing devices or treatments that involve direct contact with human tissues.[41] Finally, the high cost of silver further limits its application.

Other heavy metals apart from silver, such as copper, cadmium, and lead, have long

been used as hygienic materials (e.g. water pipe). Not until recently have copper and its alloys been thoroughly investigated under the topic of antimicrobial surfaces.[49]

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol), a commercial broad spectrum phenolic biocide, has been incorporated in products such as hand wash soaps and toothpastes. The most widespread commercial product of triclosan-based antimicrobial surface is Microban®, in which triclosan is coated onto various surfaces such as door handles in hospital. Apart from the common disadvantages of biocide releasing system, the major concern of triclosan is that it can, under the action of UV light, react with water and produce dioxins which are environmentally hazardous.[49]

An alternative to ordinary molecule/ion-leaching strategy for antimicrobial surfaces is to generate a surface that continuously produces radicals. Radicals are completely nonselective and thus free of resistant development because they have no specific target within a microbe. In addition, radical-producing surfaces are less likely to suffer from non-permanency as radicals are generated by the substrate. In this respect, mammalian cytotoxicity becomes the major concern for radical-producing surfaces.

Photo-sensitizers and photo-catalysts have been incorporated into various surfaces as light-activated antimicrobial agents.[69] Photo-sensitizers immobilized in a polymer matrix can be activated by UV light and thus generate radicals, which are destructive to microbes.[70] In the case of photo-catalysts (e.g. titanium dioxide crystals), hydroxyl radicals are produced from adsorbed water and molecular oxygen on photo-excited catalysts. The resultant hydroxyl radicals are highly reactive and completely non-

selective in oxidizing organic compounds at the surface.[49]

One of the drawbacks of photo-sensitizers and photo-catalysts is that the radicals may cause the degradation of the polymeric matrix and the subsequent loss of mechanical property. Furthermore, because of the high reactivity of the radicals, the healthy tissue may also be damaged through direct contact. Additionally, the requirement of regular exposure to sunlight is also impractical for wound dressings. These factors make the light activated antimicrobial agents less practical for chronic wound dressings.

1.2.3. Adhesion Resistance Surfaces

Another approach to avoid microbial contamination is to prepare an antifouling surface (i.e. a surface that prevents the initial attachment of microbes, proteins, or mammalian cells). Poly(ethylene glycol) (PEG) and modified PEG are usually chosen as coating materials because of their hydrophilicity and lack of binding sites. The resultant surfaces exhibit minimal interactions with the hydrophobic bacterial cell membrane.[49] Antifouling surfaces are not of interest in this study, because bacteria in the microenvironment cannot be killed, and they can still infect the wound site.

In summary, the biocide-leaching surface is the most versatile one among the three categories of antimicrobial surfaces. A variety of biocides can be chosen for infections caused by different bacteria species. However, an antimicrobial surface functionalized biocide-leaching strategy alone will become unprotected when the biocide depletes. Therefore, a versatile platform such as polyelectrolyte multilayers (PEMs),[71] which can be tailored to leverage the key advantages of the three types of antimicrobial surfaces, is

preferred for applications in antimicrobial surfaces.

1.3. Drug Delivery Systems and Microcapsules

1.3.1. Drug Delivery Systems

Drugs can be released from a drug delivery device in three typical ways: diffusion-controlled release, swelling-controlled release, and erosion-controlled release.[72] Driven by concentration gradients, diffusion-controlled drug release exists in all three types of release mechanisms. Drugs loaded in a non-degradable polymer matrix typically exhibit a diffusion-controlled release profile: the drug continuously diffuses from the core to the surface of the matrix and subsequently dissolves in the media. The release rate of the drug is controlled by both the retention effect of polymer matrix and the solubilization effect of the media. Consequently, the diffusion-controlled release rate gradually rises to maximum at first and then slowly drops upon the decreased concentration gradient between the matrix and the media (which is resulted from accumulation of the dissolved drug in the media and depletion of the encapsulated drug in the matrix). In the case of swelling-controlled release, a large increase in release rate occurs when the retention effect drops because of significant swelling of matrix, which could be triggered by environmental changes in pH value, temperature, solvent, or electro-magnetic field. For example, pH responsible hydrogels have been used for triggered or targeted drug delivery in oral drug administration.[73] In the case of erosion-controlled release, the drug leaches from damaged reservoirs, and the overall release rate is controlled by the rate of gradual

dissolution of biodegradable polymer, drug dissolution, and drug diffusion.[74, 75] In the context of this study, a diffusion-controlled drug delivery strategy is more relevant for wound dressings, where a sustained topical supply of drug is needed.

The diffusion-controlled drug delivery devices are further divided into the reservoir type and the matrix type.[72] Drugs in a reservoir type device are surrounded by a thin shell, which is ideally even in thickness. So the drug loading capacity of a reservoir type device is relatively higher than that of a matrix type device as the compartment of reservoir type device allows more room for the drug. In a matrix type device, drugs are dispersed throughout the matrix, and thus the drug loading capacity is limited by the solubility of the drug in the matrix. Though matrix system avoids the risk of pulsate release upon the rupture of a reservoir, the diffusion rate decreases shortly after initial burst, as the drug molecules inside the matrix have a progressively longer diffusion distance and a lower concentration gradient across the matrix. In contrast, the drug release rate of a reservoir device remains constant for a certain period of time before it decreases, because the only structure that limits the diffusion is the polymer shell, which is uniform and unchanged throughout the lifetime of the drug delivery device.[72] Considering the requirement of sustained nutrition delivery for wound dressings, reservoir type devices, such as microcapsules, are most relevant to this study.

1.3.2. Synthesis of Microcapsules

Sustained/controlled drug release has been achieved with microcapsules synthesized from synthetic polymers (e.g. PLA or PLGA), modified natural polymers

(like starches or other polysaccharides) and natural polymers (e.g. albumin or gelatin). Drugs are loaded into microcapsules either before or after the shell formation. Usually, a higher loading ratio can be achieved when drugs are preloaded before the formation of shell. The drug releasing profiles are usually controlled by the shell properties, which include thickness, porosity, hydrophilicity and degradation rate.[14]

Considering the vulnerability of protein drugs to harsh environmental impacts (e.g. heat, strong acid/base, and strong oxidizing and reducing agents), the encapsulation process shall be conducted under mild conditions (room temperature, neutral pH and absence of oxidizer). Thus methods that involve heat-assisted solvent evaporation (e.g. dried-gel droplet method, dried-liquid droplet method and W/O/W solvent evaporation method), or methods that require high temperature or oxidization during reaction (e.g. morphosynthsis method[76]) are unsuitable for protein drugs.[77] In this respect, several methods that are potentially suitable for protein drug encapsulation are reviewed as follows.

Kobaslija and coworkers[78, 79] have developed a safe and economic method of interfacial polymerization to synthesize polyurea microcapsules (PUMCs). This method featured a fast and mild encapsulation of hydrophobic drugs with a high loading ratio. A solvent-in-cyclohexane emulsion (O/O type emulsion) was used in the study, because a classical W/O type emulsion would otherwise render a low loading ratio due to the limited water solubility of the drug.

De Geest and coworkers[80] have applied “chick” reaction to microcapsule synthesis.

An aqueous solution containing both dextran-alkyne and dextran-azide was emulsified in an external aqueous polyethylene glycol solution (modified dextrans and polyethylene glycol do not mix at elevated concentrations). Azides reacted with alkynes and yielded triazole bonds upon the addition of catalyst $CuSO_4$, and biodegradable microcapsules were formed under room temperature. The non-harmful, highly selective, high yielding, and mild reaction conditions are ideal for the encapsulation of protein drugs.

Mohwald and coworkers[81-83] have employed layer-by-layer (LbL) deposition technique in the synthesis of microcapsules. LbL technique is a coating method, in which polyelectrolyte multi-layers (PEMs) are formed on a template by depositing oppositely charged polymers alternatively onto a charged surface. Usually, charged surfaces were selected for strong initial electrostatic adsorption. But weak adsorption could also happen on surfaces that are not inherently charged via van der Waals forces or H-bonding, in this sense polyethylenimine (PEI) and poly (4-styrenesulfonic acid sodium salt) (PSS) have been commonly used as initial layer.[77]

LbL method enables the construction of a uniform shell with tunable properties (thickness, composition, and function). Microcapsules of tunable PEM shells have been applied to investigate the mass transfer during drug release. Chitosan and alginate multi-layers were directly deposited onto indomethacin microcrystals, and the PEM shell effectively reduced the indomethacin release rate.[84] Porous $CaCO_3$ microparticles containing ibuprofen were coated with PEMs to regulate drug release. Protamine sulfate and PSS multi-layers suppressed the initial burst release and reduced the overall release rate in gastric fluids and intestinal fluids in vitro.[85]

LbL technique is also a versatile method: it enables the encapsulation of molecules of different hydrophilicity in a single microcapsule. A hydrophobic drug (pyrene) was initially incorporated into the shell during LbL deposition of chitosan/SDS. After the shell formation, a hydrophilic drug (methylene blue, rhodamine-B, or remazol brilliant blue R) was loaded into the compartment of microcapsules,[86] thus the microcapsules contain both hydrophilic and hydrophobic drugs.

LbL technique is a versatile method that is ideal for protein encapsulation.[76] However, the removal of the template material might require either elevated temperature or addition of another solvent for extraction, which may cause loss of the encapsulated drug. In addition, LbL method is a time-consuming procedure with low loading efficiency, which further limits its applications in industry.[45]

1.3.3. Functionalization of PUMCs

PUMCs (polyurea microcapsules) have been widely applied in the field of agriculture[87-89] and textiles[90-95] thanks to their good mechanical and chemical stability and ease of preparation.[96] PUMCs have also been applied in water treatment,[97, 98] electronic displays,[99] self-healing resins,[100, 101] and artificial red blood cells.[102] The major functions of the shells are to protect the active ingredients from harsh environment and to regulate the mass transfer across the shell. Nevertheless, the surface property also affects the final application of PUMCs in terms of dispersibility[96, 102-104] and interaction with environment.[97, 105, 106]

Recently, non-leaching antimicrobial surface has attracted extensive interest in the battle against nosocomial infections as it offers advantages over conventional antimicrobial coatings in terms of permanent biocide effect and reduction of possible drug resistance.[107] A variety of planar[108] and non-planar[109-111] non-leaching antimicrobial surfaces have been created. For example, Cui and coworkers[111] have developed contact-killing chitosan microcapsules via PEM deposition. The microcapsules with antimicrobial shells offer advantages over conventional biocide loaded microcapsules in the treatment of infections.[112] However, microcapsules with PEM shells are physically unstable:[113] softening of the preformed shell occurs in the presence of electrolytes (e.g. blood, exudates[114]). Therefore, an alternative method for the synthesis of stable robust microcapsules is needed.

In this context, robust PUMCs with chemically modified shells offer good potential in this study because they are versatile drug carriers with durably functionalized surfaces. Current strategies for surface modification of PUMCs include *in-situ* modification and post modification. For *in-situ* modification, functional monomers are incorporated into the shell during polymerization.[102, 115] Functional monomers may interfere in the shell formation and cause adverse effect on shell properties. To avoid changes in the shell matrix, post modification has been applied: Stover and coworkers[88, 96] first covalently bound polyanions onto the preformed polyurea microcapsules and then coated the surfaces with PEM to regulate the mass transfer. Although the described method has solved the softening problem[114] of PEM microcapsules, the long preparation route still impedes its application in large scale.

CHAPTER 2. HYPOTHESES AND OBJECTIVES

To provide a microenvironment that helps to restore normal healing for chronic wounds, efforts should be made in wound disinfection and nutrition supply. In this context, sustained extraneous supply of nutrients or drugs can be achieved by microencapsulation. PUMCs (as reservoir-type drug delivery devices) were selected for nutrition delivery because of their ease of preparation, versatility, good chemical stability, good drug retention and high drug loading capacity. Wound disinfection, on the other hand, can be achieved by non-leaching antimicrobial surfaces. In this respect, the surfaces of PUMCs were proposed to be utilized to carry out the antimicrobial function through chemical modification. We hypothesized that the surface modified PUMCs simultaneously possessed non-leaching antimicrobial properties and sustained drug releasing abilities, so that they could be utilized in chronic wound dressings.

An interfacial polymerization method under room temperature using branched polyethylenimine (PEI) and 2,4-toluene diisocyanate (TDI) was chosen for the PUMC synthesis. PEI, a preformed polyamine, instead of monomers (e.g. ethyldiamine or diehyltriamine) was selected as the building block to minimize the possible adverse impacts on shell property from additional functional agents. We hypothesized that there resides a large amount of isocyanate groups on the surface of freshly prepared PUMCs, and these isocyanate groups can be further utilized for surface modification. Hence, a non-aqueous emulsion system[116, 117] was chosen to preserve the surface isocyanate residues, which may otherwise be hydrolyzed to amines in aqueous emulsions.[90, 96, 118, 119]

QACs have been used as cationic surfactants,[119] and surfactants could also act as functional monomers.[120, 121] Inspired by these studies, we proposed a new surfactant, dimethyl-dodecyl-(5-hydroxy-pentyl)-ammonium bromide (DAB), as the surface modification agent for PUMCs. We hypothesized that DAB could be covalently bonded onto the shells either during or after the PUMC formation, and that DAB would act as a surfactant and a functional monomer with non-leaching surface antimicrobial properties. Two strategies were proposed for surface modification: post modification method, in which DAB was to be introduced after the formation of microcapsules; and *in-situ* modification method, in which DAB was to be introduced before the formation of microcapsules and acts as a surfactant.

The specific technical objectives of this study are listed as follows:

1) to confirm the existence and usability of isocyanate residue on PUMC surfaces;

2) to synthesize drug loaded PUMCs under mild conditions;

3) to develop a modification method for non-leaching antimicrobial PUMCs;

4) to evaluate the antimicrobial and drug release properties of modified PUMCs;

5) to immobilize the microcapsules to a dressing substrate.

The findings of this study would be useful for the design and development of new chronic wound dressings that requires both antimicrobial properties and drug delivery abilities. The concept of *in-situ* modification method proposed for PUMCs could potentially be used in the synthesis of functional microcapsules in similar emulsion systems.

CHAPTER 3. MATERIALS AND METHODS

3.1. Materials

Polyethylenimine branched (PEI, avg. Mw = 25,000 by LS, avg. Mn = 10,000 by GPC), 7-Diethylamino-4-methylcoumarin (coumarin-1, a model drug), 2,4-tolylene diisocyanate (TDI) and Span™ 85 (sorbitan trioleate, surfactant) were purchased from Sigma-Aldrich (Oakville, ON); the PEI solution in anhydrous dimethylformamide (DMF) was dried over molecular sieves (4Å) overnight before use. (Dimethylamino)pentan-1-ol was purchased from Karl Industry (Aurora, OH), and 17-alpha,21-Dihydroxypregn-4-ene-3,11,20-trione (cortisone, a model drug) was purchased from Tokyo Chemical Industry (TCI) America (Portland, OR). All other reagents were purchased from Sigma-Aldrich (Oakville, ON) and were used as received. Multi-drug-resistant extended-spectrum beta-lactamase Escherichia coli (MDR ESBL-E. coli) was obtained from the CANWARD (Canadian Ward Surveillance).

3.2. Experiments

3.2.1. Synthesis of AEA

The labelling agent for isocyanate groups, 2-azidoethylamine (AEA) (**Figure 1a**), was synthesized according to Angelos.[122] Briefly, sodium azide (3.6 g, crystals) was added to an aqueous solution of 2-bromoethylamine hydrobromide (5 g in 30 mL water) and the resultant solution was stirred overnight at 75 °C. After the system was cooled

down to room temperature, NaOH (0.976 g) was added to the reaction mixture. The resultant aqueous solution was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were washed with saturated NaCl solution. The organic phase was dried over anhydrous Na_2SO_4 and was filtrated. CH_2Cl_2 was evaporated with a rotary evaporator to yield a clear, light yellow liquid of 2-azidoethylamine (1.85 g, 88%) that required no further purification. ^{1}H NMR (400 MHz, CD_3SOCD_3): δ 3.21 (t, 2H, J = 6 Hz, CH_2N_3), 2.67 (t, 2H, J = 6 Hz, CH_2NH_2), 1.44 (s, br, 2H, NH_2).

3.2.2. Synthesis of DAB

Dimethyl-dodecyl-(5-hydroxy-pentyl)-ammonium bromide (DAB) was synthesized to serve as an antibacterial cationic surfactant **(Figure 1b)**. To the solution of lauryl bromide (1.48 g, 5.94 mmol) in MeCN (15 mL) was added 5-(dimethylamino) pentan-1-ol (0.72 g, 5.48 mmol), and the resultant solution was refluxed (82 °C) for 12 hours. Evaporation of the solvent and the excess lauryl bromide under vacuum afforded the crude product, which could be further purified on column chromatography eluting with MeOH/CH_2Cl_2 (1:1) to yield DAB, the white solid. ^{1}H NMR (D_2O, 300 MHz) δ 3.62 (t, J = 6.7 Hz, 2 H), 3.37-3.32 (m, 4 H), 3.12 (s, 6 H), 1.75-1.85 (m, 4 H), 1.68-1.59 (m, 2 H), 1.31-1.49 (m, 20 H), 0.91 (t, J = 6.6 Hz, 2 H);

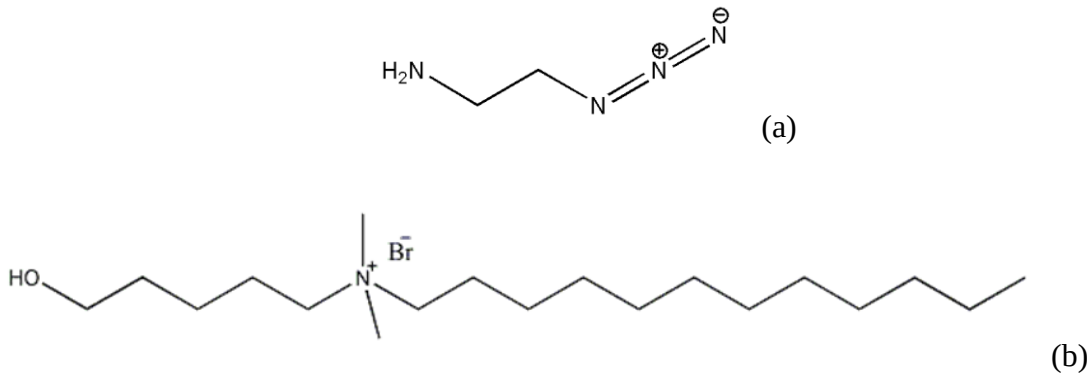


Figure 1. Molecular structure of 2-azidoethylamine (AEA) and dimethyl-dodecyl-(5-hydroxy-pentyl)-ammonium bromide (DAB). (a) AEA, a labelling agent for isocyanate groups); (b) DAB, an antibacterial surfactant as the modifier for microcapsules.

3.2.1. Synthesis of Polyurea Model Capsules (PUCs)

Polyurea model capsules (PUCs) were synthesized to facilitate the study of polyurea capsules surface chemistry and to evaluate modification methods. PEI/DMF solution (10 μL, 50 mg/mL,) was carefully injected into a microcentrifuge tube (MCT, 2 mL, round-bottom) containing anhydrous cyclohexane (1 mL) and DMF phase formed a single droplet at the bottom of the MCT. TDI/cyclohexane solution (10 μL, 25%, v/v) was carefully injected to MCT without disturbing the DMF droplet at the bottom and polymerization occurred instantly at the interface between the DMF phase and cyclohexane phase and formed a capsule. The MCT was gently vibrated and let stand for 5 minutes to allow further diffusion of TDI. The resultant single PUC was ready for further post-grafting reaction if required. Otherwise, system was quenched for 1min with ethanol/hexane solution (20 μL, varied concentrations) after reaction. When DAB was

used to post-modify PUCs after reaction, which will be described later (in section 2.2.5), the post modified PUC was referred to as PUC+Q; when DAB was added into disperse phase before the synthesis, the resultant *in-situ* modified PUC was referred to as PUCQ.

3.2.2. Quantification of Surface Isocyanate Groups on PUCs

Isocyanate groups on PUCs were quantified by applying azide labelling method. AEA was synthesized to serve as a labelling agent: AEA reacted with residue isocyanate groups and were covalently grafted to the surfaces of PUCs. After the freshly prepared PUCs had been partially quenched with an ethanol/hexane solution for 1 minute, they were added into an AEA/anhydrous tetrahydrofuran (THF) solution (typically 20 μL, 10%, v/v) for labelling. After 2 minutes of contact with gentle agitation, PUCs were washed with methanol repeatedly. Finally, PUCs were dried under vacuum and subjected to KBr palletting and IR examination. In the case of control sample, PUCs were quenched with pure ethanol (typically 20 μL) for 20 minutes to ensure maximum quenching effect. The absorbance in the azide band (2108 cm^{-1}) was normalized with that of the Amide I band (1651 cm^{-1})[123] after baseline correction. The normalized data reflected the relative amount of AEA grafted onto PUC.

3.2.3. Post Modified Polyurea Model Capsules (PUC+Qs)

After the synthesis of PUC, the supernatant was carefully removed by pipetting, and DAB/anhydrous THF solution (typically 5 mg DAB/mL anhydrous THF) was added immediately. After MCT was gently vortexed for 5 minutes, soaking solution was

removed and PUCs were washed with methanol repeatedly and were extracted with methanol for an additional 48 hours to remove residue of DAB. The resultant PUCs were referred to as PUC+Qs. In the case of control sample, PUCs were quenched with pure ethanol for 20 minutes before DAB grafting.

3.2.4. Quantification of Surface Quaternary Ammonium Groups on PUCs

DAB density, which was presented as the amount of fluorescein bound to PUC per unit surface area, was measured by fluorescein titration method. PUCs were immersed in fluorescein (1 mL, 1.0%, w/w) solution for 5 minutes under gentle agitation. After removal of fluorescein solution, PUCs were thoroughly washed with Borax buffer (0.1 M, pH = 9.5), and were then extracted with cetyltimethyl ammonium chloride solution (CTAC, 1 mL, 0.1wt%, in Borax buffer: 0.1 M, pH = 9.5) for three times under gentle agitation. The resultant solution of desorbed fluorescein was examined with UV-Vis at 500 nm.

3.2.5. Synthesis of Polyurea Microcapsules (PUMCs)

PUMCs were synthesized following the emulsion interfacial polymerization method[116] with modifications. Briefly, disperse phase (0.6 mL, typically a PEI/DMF solution, 50 mg/mL) was slowly injected into viscous cyclohexane (15 mL, PIB/cyclohexane solution, 7.38 wt%, 158.9 cP, with typically 0.3%, v/v Span™ 85) in a 30-mL beaker. The system was covered with aluminum foil and was agitated with a magnetic flea at 1200 rpm for 10 minutes to obtain an emulsion. TDI was added

immediately after the emulsification and led to instant formation of PUMCs. The system was agitated at 500 rpm for another 2 minutes to allow for further diffusion of TDI into microcapsules. After the reaction, the system was diluted to 50 mL with an ethanol/hexane solution (10%, v/v) and was centrifuged at 1000 rpm (rcf = ca.120 g) for 10 minutes. The PUMCs at the bottom were subjected to repeated washings with ethanol/hexane solution (25% v/v, 50% v/v, 75% v/v) and pure ethanol to remove poly(isobutylene) and TDI residues. When DAB was added into DMF disperse phase to replace the surfactant Span™ 85, the resultant *in-situ* modified PUMCs were referred to as MCQs; when PUMCs were synthesized without Span™ 85, they were referred to as PUMCns, which served as a control sample for MCQ.

3.2.6. Alternative Emulsification Methods for PUMCs

In order to better control the size of droplets, emulsification process was conducted with a high-shear mixer (Sliverson L5M-A, with 1’ tubular emulsor head). After the pre-emulsification through magnetic stirring, the emulsion was subjected to high shear mixer for further emulsification in an ice-water bath. Shearing rate ranged from 3000 rpm to 8000 rpm (typically 8000 rpm) and emulsification time ranged from 120 sec to 3600 sec (typically 500 sec). The resulting emulsion was then agitated with a magnetic stirrer at 500 rpm for reaction.

Emulsification process was also conducted with a vortex mixer (Fisher Scientific Votex Mixer - deluxe, 300 to 3000 rpm) within a micro centrifuge tube (MCT, 2.0 mL, round bottom) to save the chemical and processing time. MCT containing the mixture of

the two phases was reversed and vortexed at 3000 rpm for 2 to 3 minutes.

3.2.7. Microencapsulation of Model Drugs in PUMCs

Model hydrophobic drugs were encapsulated into microcapsule via two different routes: Drugs were encapsulated within the disperse phase before the formation of shell or soaked into the preformed microcapsules. In the former case, concentrated DMF solution of model drug (400 mg/mL coumarin-1/DMF, or 400mg/mL cortisone/DMF) was mixed with PEI/DMF solution and the resultant solution was subject to emulsification. In the latter case, prepared microcapsules were soaked and swollen in concentrated solution of model drug in an organic solvent (e.g. MeOH or EtOH). In this case, the preformed microcapsules were soaked in the concentrated solution of a model drug (200 mg/mL Coumarin-1/EtOH, or 200mg/mL Cortisone/EtOH, or 200mg/mL Rhodamine-B/EtOH) for at least 1 hour at 37 °C. Then the suspension was centrifuged and the supernatant solution was recycled. The pellet of drug loaded capsule was subjected to vacuum drying. Optimized drug loading requires multiple soaking cycles.

3.2.8. Quantification of the Drug Loading of PUMCs

The amount of drug loaded into microcapsules was determined by methanol extraction method. Drug loaded microcapsules (10.0 mg) were extracted with methanol (1.00 mL) for three times in a microcentrifuge tube. For each cycle, the capsule suspension was vigorously shaken with a wrist shaker for 5min and was recovered via centrifugation (1.0 min, 15000 ×G). The resultant supernatant was combined and

subjected to UV-HPLC analysis.

Alternatively, drug loaded capsules (20.0 mg to 40.0 mg) were extracted with 5.00 mL methanol for three times in a 25-mL glass vial. For each cycle, the capsule suspension was vigorously shaken with a wrist shaker for 5min and was recovered via centrifugation (5 min, 1000 rpm).

3.2.9. *In vitro* Drug Release

In vitro drug release of coumarin-1 from microcapsules was conducted under USP Apparatus II on a VanKel® 600 Dissolution Apparatus (Palo Alto, CA, USA).[123] Briefly, microcapsules (ca. 20.0 mg) were accurately weighed and dispersed in PBS (250 mL, 0.1M, pH = 7.4). The paddle speed was set at 75 rpm and the test temperature at 37 ± 0.5 °C. At predetermined time intervals (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 12, 18, 24, 36, 48, 72, 120, and 176 hours), suspension (1.0 mL) was filtered through a 0.2-μm filter and was subjected to HPLC-UV analysis. Fresh PBS (1.0 mL) was then replenished to the dissolution vessels. The HPLC-UV analysis was conducted with a Waters Separation Module equipped with a PDA detector and a C_{18} column (μBoundapak™ C-18 10μm 125A). Chromatographic conditions were as following: mobile phase: 70/30 MeCN/H_2O; flow rate: 1.0 mL/min; retention time: 2.45 min; absorbance: 380nm; detection limit: 0.1 mg/L).

In vitro drug release of coumarin-1 from microcapsules was also conducted by dialysis dissolution tests. Briefly, microcapsules (typically 10 to 20 mg) were accurately

weighed and dispersed in PBS (10 mL, 0.1 M, pH = 7.4), and was transferred into a dialysis tube (7000 MWCO, Snakeskin® Thermo Scientific). The sealed dialysis tube was immersed in PBS (40 mL) in an amber jar. The system was agitated by immersing in the water bath (75 rpm, 37 ± 0.05 °C, Precision, Thermo Scientific). At predetermined time intervals, solution (1.0 mL) in the amber jar was sampled to be subjected to UV-Vis measurement (380nm, Cary 50). Fresh PBS (1.0 mL) was then replenished to the system. Cumulative drug released from the system was calculated from the concentrations of samples and released drug was normalized with the total encapsulated drug in the sample determined by drug loading test.

3.2.10. Antimicrobial Tests

PUMCns and MCQs were extracted three times with methanol and another three times with sterile PBS solution (pH = 7.4, 0.1 M) for an additional 48 hours to thoroughly remove the potential residues of DAB or methanol. Then microcapsules (ca. 400 mg) were suspended in sterile PBS (10.0 mL).

MDR ESBL-E. coli suspension at mid-log growth phase was cultured in MacConkey broth through 3.5 hours incubation of primary culture under 37 °C and was then diluted to desired concentration with sterile PBS.

For surface diffusion test, TSA (trypticase soy agar) plates were inoculated with E. coli suspension with Autoplate® 4000. Microcapsule suspension (20 μL) was placed onto the surface of agar. The agar plates were subjected to incubation (37 °C, 20 hr).

For static contact test, the microcapsule suspension was evenly coated onto the TSA agar surface with a spiral plater (Autoplate® 4000). Multiple layers of microcapsule suspension were coated onto the agar surface at ca. 2 minutes intervals to allow for drying of the previous layer. For each layer, density of microcapsule was 0.22 mg/cm^2). Then microcapsules coated agar plates were inoculated with an E. coli suspension (20 µL, density of bacteria on agar: 3.54 $\log_{10}$ cfu/cm^2) and was subjected to incubation (37 °C, 20 hr).

3.2.11. Microscopic Imaging

On-site microscopic photograph of freshly prepared emulsions or microcapsules were taken under a calibrated optical microscope. One drop of emulsion/suspension was placed on to a glass slide and was covered with a cover slip immediately to prevent evaporation. Size and size distribution of droplets were estimated by manual counting and measuring of photographs with at least 100 counts.

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Synthesis of PUMCs

To synthesize non-leaching antimicrobial PUMCs, five requirements should be met at the same time. First, microcapsules shall be of suitable size. Diameter of microcapsules should be below about 30 μm to provide a significantly large surface area for antimicrobial functionalization. When the drug releasing properties were considered, the diameter shall not be too small (e.g. ca 1 μm). The volume-to-surface ratio (r/3) is one of the major parameters that affect drug release rate. Second, the size distribution of microcapsules shall be reasonably narrow. Small microcapsules are partly responsible for undesired burst release, and nano-sized capsules may stick to the microcapsules and cause poor dispersibility. Hence, nanoparticles resulted from crosslinked micelle should be minimized during emulsification. Third, the shell of microcapsules should be thick and strong enough to sustain the hollow sphere. On one hand, thick shells enable regulation of mass transfer so that drug release rate could be adjusted through either increasing the thickness or the matrix retention effect of the shells. On the other hand, microcapsules bearing stiff shells can retain their hollow spherical structure after drying thus providing easy recovery of microcapsules. Otherwise, thin-shell microcapsules are prone to collapse after drying and can adhere to each other making the re-disperse in solvents extremely difficult. Fourth, the microcapsules shall be capable of sustained drug release. A pseudo-zero-order release profile is desired and an initial burst release shall be minimized. Finally, the shell of resulting microcapsules should exhibit non-leaching antimicrobial

activities, which is the feature of our study. The resultant antimicrobial microcapsules are then coated onto wound dressing surface to provide non-leaching antimicrobial functionality. Ideally, the surface functionalization process shall be mild, fast and, if possible, versatile.

To fulfill the five requirements for microcapsules, a new synthesis process was developed consisting of stock preparation, emulsification, initial reaction, partial quenching, further reaction and recovery. Because of the complicated and sensitive process of synthesis, PUCs were first synthesized and investigated to explore the suitable composition and reaction condition. Then the concept of surface functionalization was also examined with PUCs. After that, emulsification process was separately investigated to make possible synthesis of microcapsules of small size and narrow size distribution. A series of adjustments on emulsion composition, emulsification and reaction conditions gave rise to changes in morphology, drug load/release properties surface hydrophilicity and antimicrobial properties. Finally, *in-situ* modified polyurea microcapsules (MCQs) were synthesized and subjected to further evaluations.

4.1.1. Chemistry of PUMC Formation

The first step in designing the surface modification strategy was to understand the chemistry of PUMC formation.[99, 124-126] Branched PEI was dissolved in DMF as the disperse phase; PIB, an inert hydrophobic thickener, was dissolved in cyclohexane as the continuous phase. Branched PEI molecules at DMF-in-cyclohexane interface were instantly crosslinked by the TDI in continuous phase at room temperature. The

polyaddition reaction resulted in a 3D crosslinked polyurea primary membrane,[102] through which TDI further diffused in and reacted with free PEI inside the capsules. The growth of shell observed moving boundary mechanism[124] which was widely accepted in PUMC synthesis in O/W emulsions, when polymeric isocyanates in the disperse phase were crosslinked by invasive diamines or triamines in the continuous phase.[88, 90, 96, 124, 127] Considering the polyamine nature of PEI and continuous supply of highly reactive TDI at the interface, one can expect a similar shell growth process and a large amount of mono-reacted TDI on the exterior surfaces of primary membranes as illustrated in **Figure 2**.

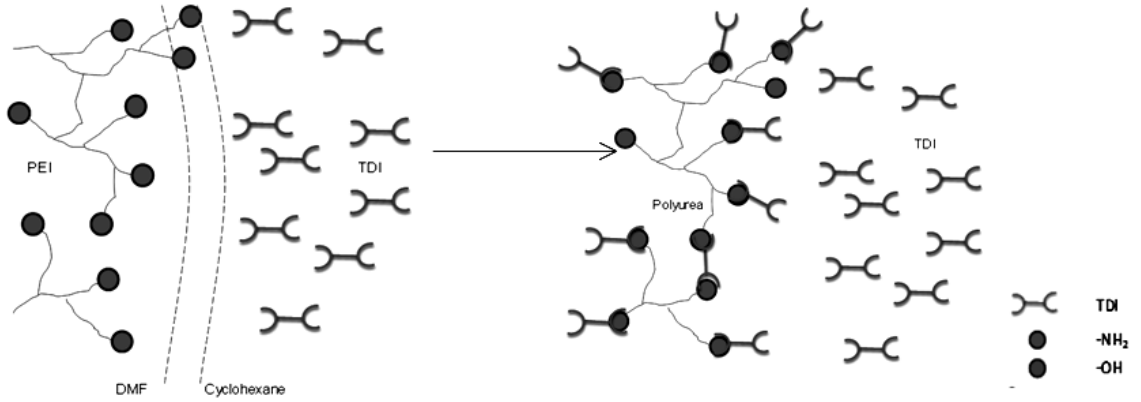


Figure 2. Crosslinking reaction and formation of polyurea shell. Reactive isocyanate residue is expected to be available at the exterior surface for further surface modifications.

To confirm the existence of residue isocyanate groups on the shell, PUCs were synthesized, as shown in **Figure 3a (**PUCs, PUC+Qs, PUCQs, and their cross-sections are also shown in **Figure 3,** they will be discussed later**).** PUCs were synthesized without surfactant, thickener or emulsification process to eliminate possible loss of active isocyanate group during washing process. Freshly prepared PUCs were partially

quenched by ethanol and labelled by AEA, which had strong IR absorbance. AEA reacted instantly and stoichiometrically with isocyanate groups, thereby labelling unquenched isocyanates. Finally, AEA labelled capsules were subject to IR examination. Results are shown in **Figure 4** and **Table 1.**

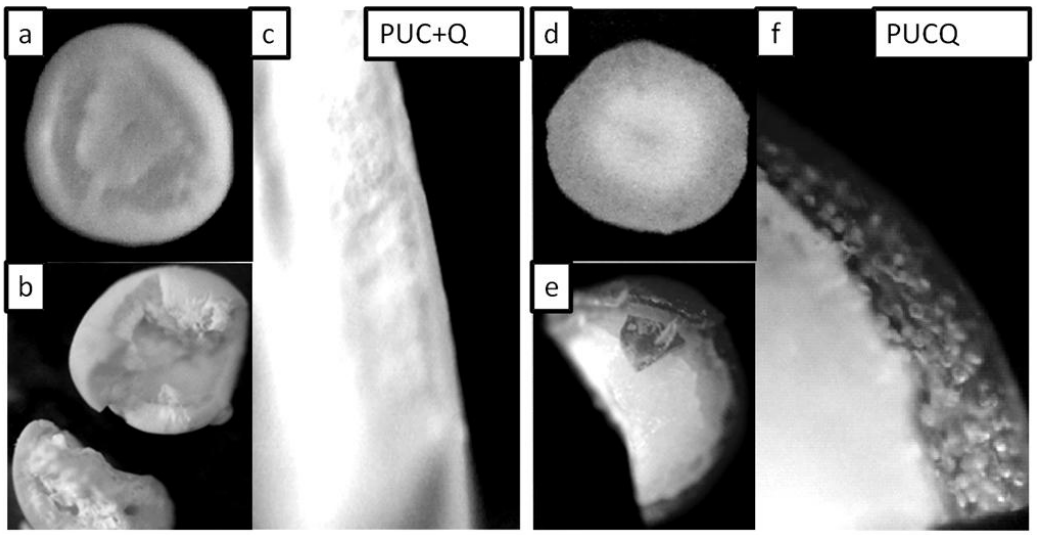


Figure 3. PUCs and cross-section of fluorescein stained PUCs. (a), (b) and (c): post modified PUC (PUC+Q); (d), (e) and (f): *in-situ* modified PUC (PUCQ); (a) and (d): freshly prepared PUC; (b) and (e): cracked fluorescein stained PUC after CTAC extraction; (c) and (f): magnified of (b) and (e), cross-sections

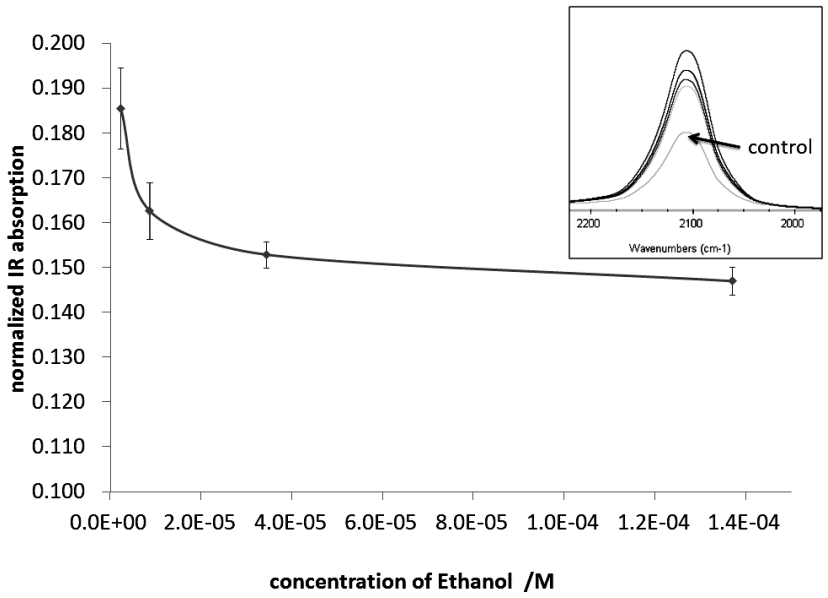


Figure 4. Semi-quantification of free isocyanate groups. Normalized IR absorbance for 2-azidoethylamine labelled PUCs at 2108 cm^{-1}. Inset: the original spectra at azide band

Table 1. Semi-quantification of free isocyanate groups

Sample	conc. EtOH/ M	-OH/-NCO molar ratio	normalized IR absorbance (accessible free –NCO)
A	2.1×10^{-6}	0.02	0.186 ± 0.009 (100%)
B	8.6×10^{-6}	0.07	0.163 ± 0.006 (73%)
C	3.4×10^{-5}	0.29	0.153 ± 0.003 (62%)
D	1.4×10^{-4}	1.18	0.147 ± 0.003 (55%)
Control	1.4×10^{-3}	11.8	0.100 ± 0.009 (0%)

Normalized IR absorbance is presented by average ± 1 standard deviation (n = 3). An estimation of accessible free isocyanate group in the shell is presented in the bracket.

The IR absorbance at 2108 cm^{-1} was normalized with the amide-I band which was predominant in polyurea capsule,[128] so that the resultant relative absorbance represented

the free isocyanate group in the shell after partial quenching. For control samples, PUCs were thoroughly quenched by excess amount of ethanol for 20 minutes, so the relative IR absorbance of which was attributed to the adsorbed AEA that had not been completely extracted from the shell by repeated washing process. The introduction of small amount of ethanol (less than 3.4×10^{-5} M in the environment) resulted in a fast initial drop of IR absorbance (from A to C) followed by a plateau (from C to D), which suggested that the surface isocyanate groups were highly sensitive to nucleophiles such as hydroxyl groups, thus made possible the surface modification of PUC through reaction with surface isocyanate residues. Difference between sample D and control sample (**Table 1**) was attributed to the active free isocyanate groups beneath the surface of shell. Ethanol molecules reached deeper part of shell in control sample than in sample D because of the 10-time higher concentration of ethanol, longer diffusion time (20 min vs. 2 min) and the swelling effect of the shells, thus more active isocyanate groups were quenched in control sample than in sample D. Assuming that all free isocyanate groups in sample A were labelled by AEA, and all free isocyanate groups in control sample were quenched by ethanol, thus accessible free isocyanate groups on the surface were estimated (**Table 1**). At least 28% of free isocyanate groups in the polyurea shell resided on the outer surface of PUCs (calculated by subtracting A with B), while 55% of isocyanate groups resided in the depths of the shells (calculated by subtracting D with control).

Providing that the surfaces of PUCs were covered with a layer of free isocyanate groups which could be quenched by ethanol, DAB, a quaternary ammonium salt ended with a hydroxyl group, could be anchored onto the shell in the same manner. PUCs were

post grafted with DAB, and the densities of which were determined with fluorescein staining method (**Figure 5)**.[129]

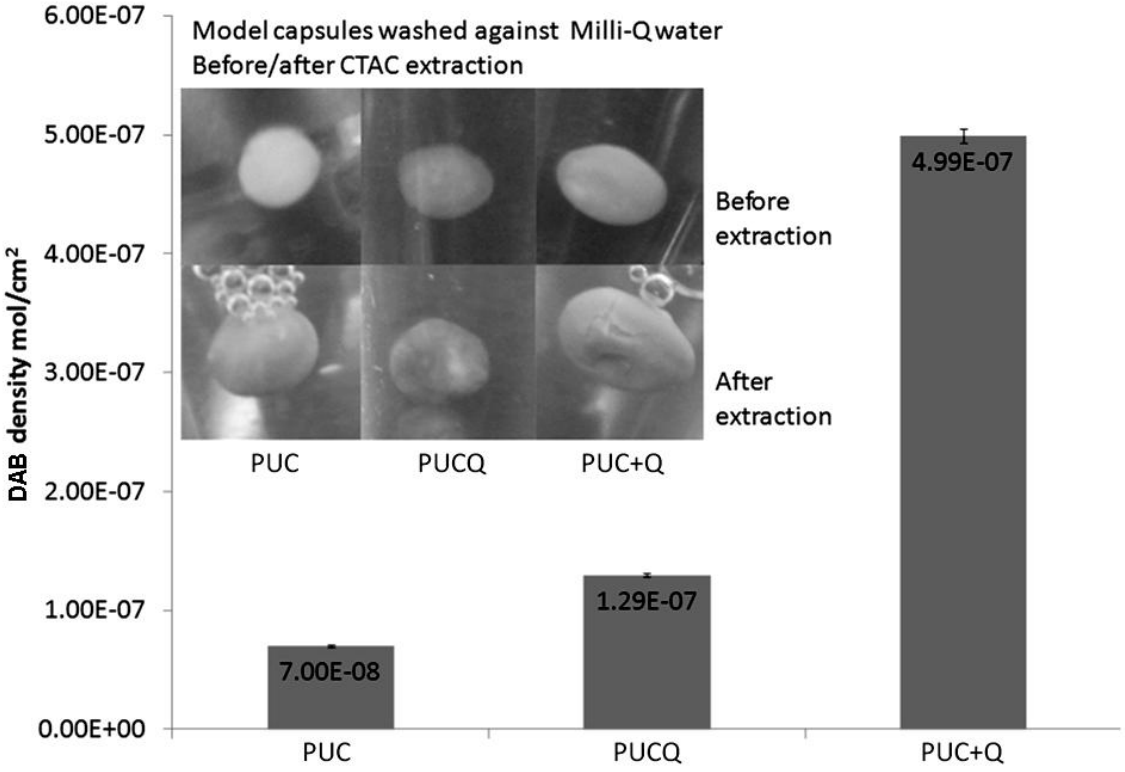


Figure 5. Quantification of DAB density of PUCs. Error bar was generated from 3 batch-to-batch measurements. PUC: control model capsule; PUCQ: *in-situ* modified model capsule; PUC+Q: post-modified model capsule;

PUC+Q bore significantly higher surface DAB density compared to PUC (control sample). The estimated surface group density was 4.29×10^{-7} mol/cm^2 (calculated by subtracting PUC+Q with PUC). The quantitative result also accorded with the staining result: PUC+Q bore deeper color than that of the control capsule before being extracted with CTAC, while color difference between PUC+Q and PUC disappeared after the

extraction (**Figure 5** insets). Hence, isocyanate residues on PUC surface could be preserved and utilized for post modification with a molecule end-capped with a hydroxyl group, such as DAB, in a non-aqueous system at room temperature. Because the surface chemistry of PUMCs was the same as that of PUCs, DAB post modification method that utilize isocyanate residue on polyurea shells shall also work on PUMCs.

4.1.2. Morphology Control of PUMCs

PUMCs to be modified with DAB were synthesized by the emulsion interfacial polymerization method[116] with some modifications. The PUMC synthesis differed from PUC synthesis in the introduction of emulsification process: emulsions were generated by magnetic stirring, high-shear mixing, or vortex mixing; Additionally, Span™ 85 (a surfactant) was added to stabilize the emulsion. TDI was introduced right after emulsification process and was allowed least 5 minutes for thorough reaction to form PUMCs that were robust enough for further applications and tests.

In selection of diluents for the disperse phase, DMF was chosen in substitute of methanol. Although the higher solubility of methanol over DMF in cyclohexane was believed to contribute to thicker shells,[116] we found that the drawbacks of methanol can override the advantage of higher solubility. Methanol is highly reactive to TDI, thus it produces by-products during the synthesis of PUMCs. The IR spectrum of the reaction product of methanol and TDI (**Figure 6** top) is identical to that of N-[5-(methoxycarbonylamino)-2-methylphenyl]carbonate (**Figure 6** bottom), which confirms that large amount of TDI was consumed by methanol during PUMC synthesis. Moreover,

methanol impeded PEI conversion and thus limited the shell growth, especially after formation of primary membrane when the shell growth was limited by the TDI diffusion rate rather than its reaction rate with PEI.[95, 124] The PUMCs that have a methanol core tended to burst (swell fast and explode) when they were immersed in water, ethanol, or methanol. We speculate that such phenomena may be caused by osmotic pressure induced by the intact PEI inside the PUMCs. In this respect, DMF is a better candidate for disperse phase diluents because PEI conversion will not be influenced as DMF do not react with TDI.

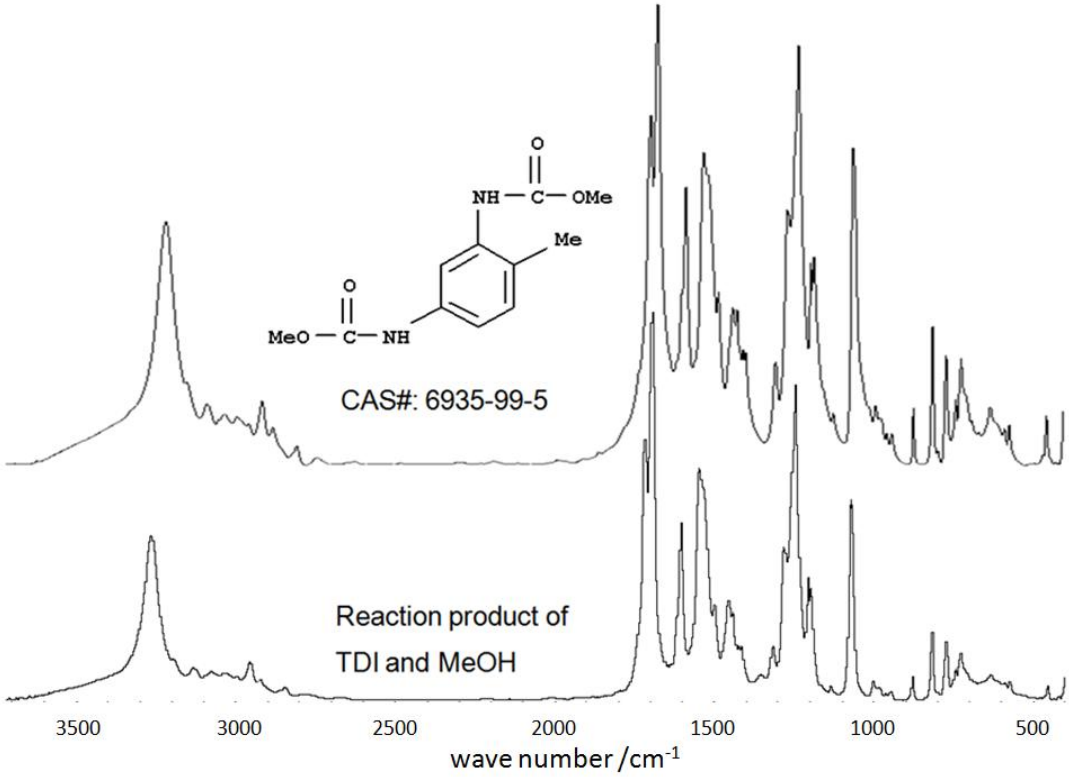


Figure 6. FTIR spectra of the by-product of TDI and methanol. Top spectra of N-[5-(methoxycarbonylamino)-2-methylphenyl]carbamate was obtained from Wiley Subscription Services, Inc. (US); Bottom spectra was obtained from the crystalline byproduct collected during PUMC synthesis when methanol served as disperse phase;

However, DMF-core PUMCs also suffered from morphological problems: coexistence of nanoparticles caused severe aggregation of PUMCs (**Figure 7a**), which finally resulted in poor dispersibility and thus low capsule recovery. Reduction of nanoparticles could be achieved via either suppressing micelle formation by reducing Span™ 85 dosage (**Figure 7b**, dosage of Span™ 85 was reduced from 0.3% (v/v) to 0.1% (v/v)) or inducing the coalescence of nano-droplets by long-term (20min) emulsification in the presence of a non-polar drug (**Figure 7c**, cortisone was loaded as a hydrophobic model drug, drug load = 18wt%, efficiency = 45%, without optimization).

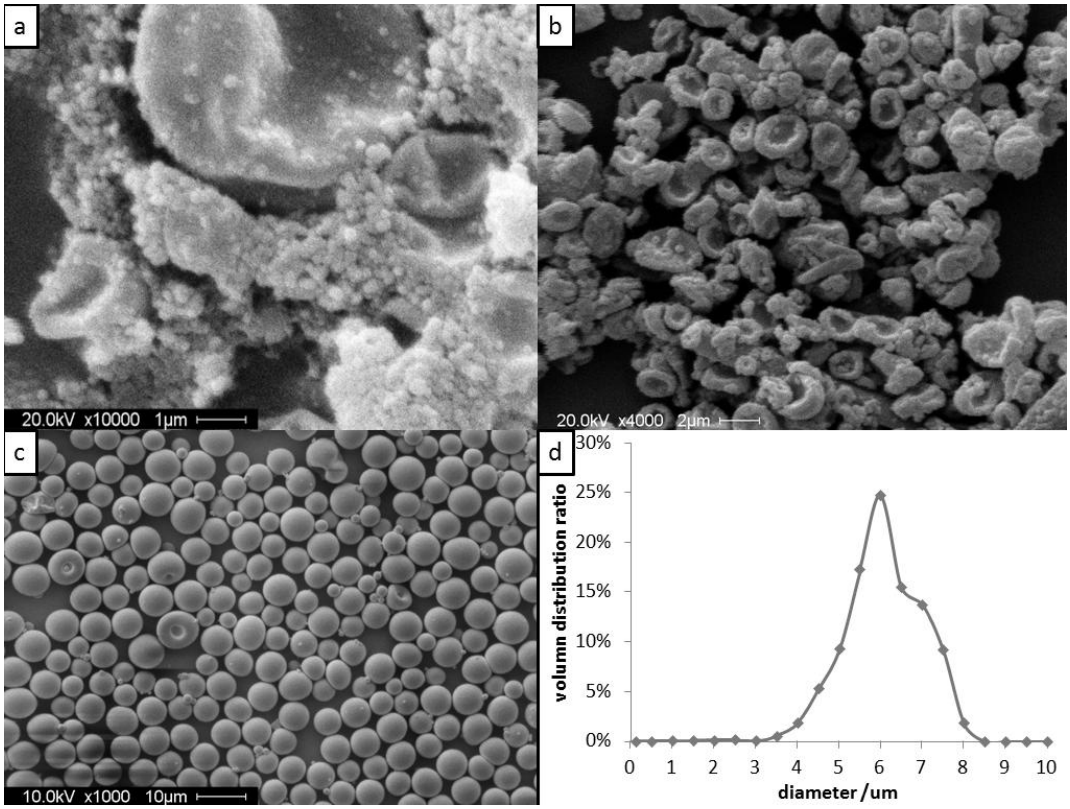


Figure 7. SEM pictures of PUMC synthesized in DMF. (a) 0.3v/v% Span™ 85, empty capsules; (b) 0.1% (v/v) Span™ 85, empty capsules; (c) 0.3 % (v/v) Span™ 85, cortisone loaded; (d) diameter distribution for cortisone loaded capsule by volume (200 counts)

4.1.3. Size Distribution Control of PUMCs - Emulsion Preparation

The size distribution of PUMCs synthesized by emulsion interfacial polymerization was determined by the correspondent droplets size distribution in the emulsion. Therefore the careful preparation of emulsion was the key to successful PUMC size control, even though the optimization of size distribution was not the major focus of this study.

During emulsification process, large droplets were deformed, elongated and finally broken into smaller droplets because of the shearing force between two phases. At the same time, droplets may collide, coalesce and thus growing into larger droplets. The two opposite tendencies reached a dynamic equilibrium after a reasonably long period of emulsification resulting in a stabilized droplet size distribution. In this sense, parameters that would influence shearing force, collision and interface stability were controlled.

Parameters concerning the composition of emulsion (properties and dosages of disperse phase, continuous phase and surfactants) and emulsification process (the intensity and time for shearing and cooling) were of our concern. In this study, the phi value was kept under 4.4 % (v/v); the viscosity ratio of two phases was adjusted close to 1 (the viscosity of continuous phase was kept 158.9 cP, the concentration of PEI was typically 50 mg/mL); the minimum emulsification time was 500 sec at 8000 rpm agitation (with a high-shear mixer); and the concentrations of Span™ 85 varied from 0.1% (v/v) to 0.3% (v/v).

The Phi value is the ratio of disperse phase volume to total volume. It indicates the

spatial density of droplets in an emulsion and plays an important role in droplet dynamic stability. The collision probability of droplets in an emulsion rises when phi value increases, and consequently the average droplet diameters increases.[130]

The viscosity of continuous phase can be controlled by the concentration of PIB. The viscosity of PIB/cyclohexane solution under room temperature was 158.9 cP at 5% (v/v), and 5.1 cP at 1% (v/v).[116] It was reported that the viscosity ratio of two phases affects the droplet size distribution: the viscosity ratio (disp.phase /cont.phase) for narrowly distributed droplets was controlled between 0.1~100, when phi value ranged between 1%~33% and emulsification time exceeded 500 sec.[131] In our case, the viscosity ratio of the two phases could be adjusted by concentrations of PEI, PIB, and drug content, among which the PEI concentration dominated the change in viscosity ratio. The viscosity of the disperse phase increased at high PEI concentration and subsequently the average droplet size.

Span™ 85, a surfactant, was introduced to stabilize the droplets by reducing interfacial tension. However, a high dosage of Span™ 85 reduced the viscosity of continuous phase, thus compromised emulsification efficiency. Moreover, excessive micelle formation occurring at high concentration of Span™ 85 (0.3%, v/v, ca. 3 times of CMC) led to the formation of nanoparticles. Finally, the hydroxyl groups in Span™ 85 may react with free surface isocyanate groups and reduce the post modification efficacy. In this context, the concentration of Span™ 85 was set near its CMC (around 0.1%v/v).

High temperature was avoided during emulsification to ensure low droplet size and

size distribution. Increased temperature reduced the spontaneous curvature of Span™ 85 and thus unstablized the interfaces. In addition, high temperature lowered the viscosity of continuous phase, thus reduced the actual shearing force. Larger droplets (droplets over 20 μm) were observed in systems emulsified at room temperature when compared with that under ice water cooling bath. Furthermore, high-shear mixer generated heat and increased the regional and overall temperature of the emulsion, which compromised the emulsification effect. In this respect, intense emulsification (long-term, high shear) was avoided and temperature of emulsion was monitored and strictly controlled by efficient heat dissipation. The thermo equilibrium was achieved through moderate emulsification condition (one example is described in Section 3.1.4).

4.1.4. Size Distribution Control of PUMCs - Influence of Drugs

PUMC ranging from 1 μm to 10 μm was synthesized with emulsion prepared with a high-shear mixer. The microphotographs of PUMCs with size distribution charts are shown in **Figure 8**, and their size distribution data are shown in **Table 2**. Generally, cortisone loaded PUMCs possessed larger size and size distributions than those of empty PUMCs. Even though an increased dosage of surfactant was applied in cortisone loaded PUMCs (0.1% v/v for empty PUMCs and 0.3% v/v for cortisone loaded PUMCs), the average diameter of empty PUMCs by number (ca. 1.8 μm) were smaller than that of drug loaded PUMCs (ca. 4.0 μm). The encapsulated cortisone increased the droplet viscosity and thus more energy was required for deformation, elongation and breakage of large droplets.

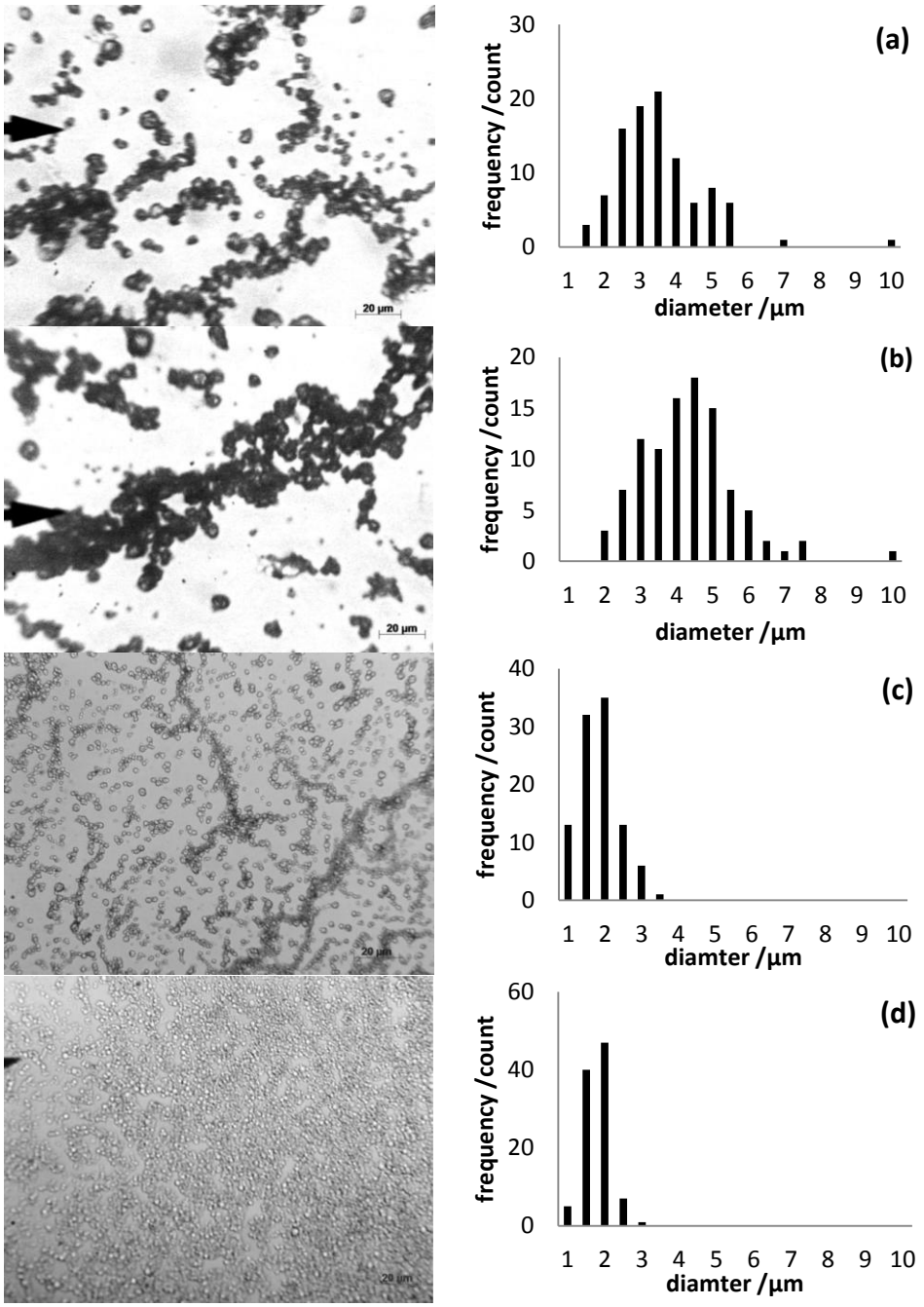


Figure 8. Size distribution of cortisone loaded PUMCs and empty PUMCs: (a)MC68a, drug loaded bare PUMCs; (b) MC68b, drug loaded DAB grafted PUMCs; (c) MC69a, bare empty PUMCs; (d) MC69b DAB grafted empty PUMCs.

Table 2. Diameter distribution of empty and cortisone loaded PUMCs

	MC68a	MC68b	MC69a	MC69b
d_{avg} /μm	3.5	4.3	1.9	1.8
SD /μm	1.2	1.3	0.5	0.3
RSD	35%	31%	29%	19%
Count	100	100	100	100

d_{avg}: average diameter; SD: standard deviation; RSD: relative standard deviation; MC68a, drug loaded bare PUMCs; MC68b, drug loaded DAB grafted PUMCs; MC69a, bare empty PUMCs; MC69b DAB grafted empty PUMCs.

Narrowly size distributed PUMCs were synthesized by stabilizing droplets with surface crystallizable moieties and by controlling the coalescence of small droplets. During the synthesis of cortisone containing PUMCs, extended emulsification (over 20 min) was conducted under ice-water bath (0 °C), and the resultant emulsion was shown in **Figure 9**. SEM examination of the resulting PUMCs further confirmed that nano-particles no longer co-exist with cortisone loaded PUMCs and the surfaces of which become smooth (**Figure 7c**). The elimination of nanoparticles was attributed to excessive coalescence of nano droplets, which could be explained by "salt out" effect:[132] cortisone molecules in the vicinity of the DMF-cyclohexane interface retracted the DMF molecules and thus reduced the effective hydrophilic head volume of surfactants. As a result, the spontaneous curvature of the Span™ 85 was reduced and the interface was unstablized. Subsequently, the reduced interface membrane stability caused the excessive coalescence of nano droplets. On the other hand, micro-scale droplets were believed to be stabilized by cortisone nanocrystals produced by long-term emulsification.[133] The cyclohexane insoluble cortisone was partially crystallized at the DMF/cyclohexane interfaces when

cortisone concentration in DMF phase was high. The resultant nano-crystals provided steric repulsion between two colliding micro-scale droplets. In case of nano-droplets, there was insufficient amount of cortisone nano-crystals to cover the surfaces of nanodroplets because of their extremely high surface/volume ratios (surface/volume ratio = 3/radius, smaller the droplets, larger the surface/volume ratio). Consequently, nano droplets tend to coalesce into big droplets due to insufficient nano-cortisone barriers, whereas the resultant large droplets possess surface/volume ratios that are small enough to allow the formation of stabilizing crystals that inhibit further coalescence. Furthermore, the high concentration of cortisone inside the disperse phase also suppressed the Ostwald ripening effect, thus the tendency of size diversion of droplets was further inhibited.

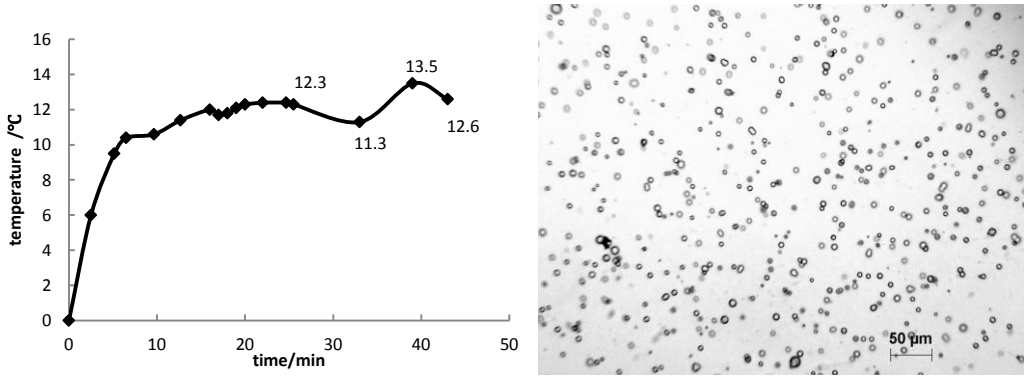


Figure 9. Emulsification of cortisone loaded emulsion. Shearing rate: 4000 rpm; cooling conditions: 0 °C, ice-water bath. A microscopic picture of freshly prepared emulsion at 40 min (magnification = 1200×) is shown on the right.

4.1.5. Water Dispersibility of PUMCs

Though the morphology problems of PUMCs were solved, PUMCs still suffered from poor water redispersibility (PUMCs tended to flocculate and float on the top of water suspensions). The poor water redispersibility indicates that the shells of the PUMCs are hydrophobic. The most probable reason is that Span™ 85 (a sorbitan surfactant bearing a hydroxyl group) was anchored onto the polyurea shell during the reaction. One similar case[99] was reported when Span 80 (another sorbitan surfactant) was grafted onto PUMC. Hence, most non-ionic surfactants may consume the isocyanate residues and render hydrophobic PUMC surfaces during synthesis. Moreover, moisture or trace amount of nucleophilic agents also consumed isocyanate groups on PUMC surfaces, which made the post-modification method even more difficult. In this context, an alternative method for PUMC surface modification was desired.

4.1.6. Method of *In-situ* Modification

To resolve the dilemma that the surfactants required for emulsification also quenched isocyanate residue, DAB was introduced as a multipurpose surfactant (**Figure 1**). As a cationic surfactant, DAB reduce interfacial tension at the interface of droplets; as an antimicrobial agent, DAB exhibits optimal bactericidal properties when carbon chain length reaches twelve.[121] Additionally, the hydroxyl group solubilizes DAB in DMF phase and enables anchoring via free isocyanate group on the polyurea shell during or after the polyaddition reaction. Furthermore, DAB competes with PEI during reaction with TDI, thus efficiently reduce the crosslinking density of the primary membrane and

facilitates diffusion of TDI. Consequently, thicker shells and more rigid microcapsules were synthesized with DAB *in-situ* modification method (DAB was encapsulated into the disperse phase before emulsification (**Figure 10**).

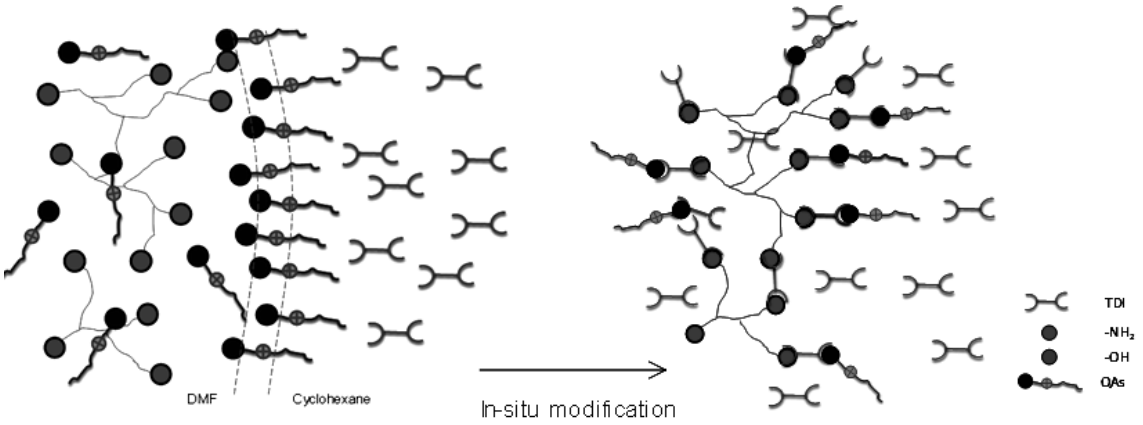


Figure 10. Mechanism of DAB encapsulated PUMC synthesis. DAB was added before the introduction of TDI and served as surfactants.

4.1.7. Shell Thickness of PUMCs

PUMCs of thick shells are ideal for our purposes: mechanically, the thick shells of the PUMCs can better retain the hollow spherical structures (thus the reservoirs), especially when drying process is required. In terms of drug retention effect, the mass transfer of drugs is better regulated by the thick shells, because the porosity and hydrophilicity of the shell can be further adjusted if necessary.

The PEI concentration was one of the major factors for relative shell thickness (the ratio of absolute shell thickness to the diameter of a capsule). Assuming a constant shell density over batches and the complete conversion of PEI, a calculation model was

developed to estimate the relative shell thickness. According to the calculation result (**Figure 11),** increased PEI concentration can result in increased relative shell thickness.

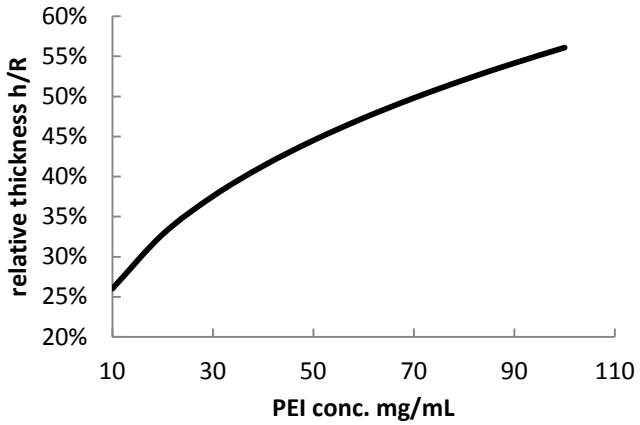


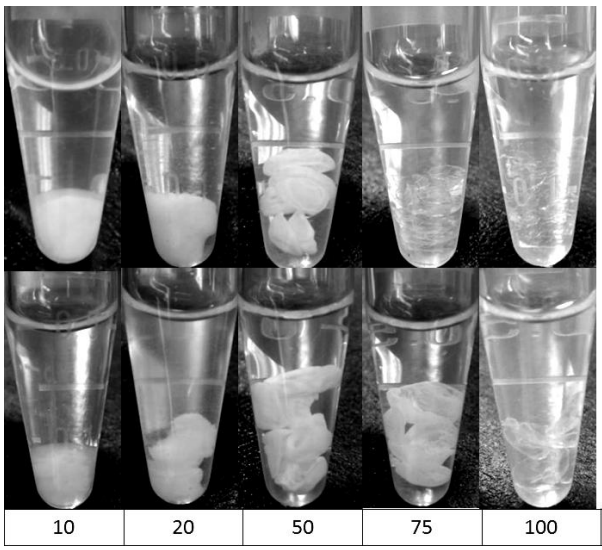
Figure 11. Calculation of influence of PEI on relative shell thickness based on model. Relative shell thickness = (shell thickness (h) / radius (R)) = 1 - (1 - k'x) ^ - 3; k' = k / d = 0.001764; k = 1.9402 (mass polyurea/mass PEI); d = 1100 mg/cm^3 (density of polyurea).

However, the assumption of complete conversion of PEI is true only when TDI penetrates through the shell and reacts with PEI residues inside the PUMCs. Therefore, the TDI dosage required for full PEI conversion shall not only meet the stochiometry ratio to amine group in PEI, but it shall also reach a concentration that is high enough to allow efficient TDI diffusion into the preformed shell. A series of PUCs were synthesized to determine the optimum TDI level for different PEI concentrations (**Figure 12**).

TDI conc. →

PEI conc. ↓	2%	5%	10%	20%	50%	100%
10g/L	X	X	-	-	-	-
20g/L	X	X	-	-	-	-
50g/L	10	10	10	10	10	10
75g/L	4	6	-	-	-	-
100g/L	1	2	-	-	-	-
200g/L	0.5	0.5	0.5	0.5	0.5	0.5

(a)



(b)

Figure 12. Reactivity tests on PUCs: effect of PEI and TDI concentrations. Model capsules were synthesized with various PEI/DMF (mg/mL), TDI/Cyclohexane solutions (%, v/v). (a) Shell thickness scores (0 to 10) were manually marked according to observations: high scores refer to thick shells; "-" refers to no data; "X" refers to failure to form capsules; (b) pictures accords with the samples of first two columns in the table.

Row six in **Figure 12(a)** shows that high PEI concentrations alone do not guarantee thicker shells: when PEI concentrations was 200 mg/mL, shell thickness did not increase at elevated TDI concentration. No change was observed even when pure TDI was used for crosslinking (not shown in the graph). A large amount of intact PEI was encapsulated inside PUCs (PEI inside PUCs could still react with TDI when the PUCs were destroyed

by manually squeezing the PUC with a spatula after 10min of reaction). The highly crosslinked primary membrane was believed responsible for the inhibition of TDI diffusion. When PEI concentrations reached 75 mg/mL, increased TDI level provided larger concentration gradient, allowing deeper penetration of TDI and thicker shells. When PEI concentration was 50 mg/mL, thick shell was obtained even at lower TDI levels compared with 70 mg/mL PEI. When PEI concentration was below 20 mg/mL, the crosslinking reaction speed was so slow that only weak primary shell was formed. In conclusion, PUCs synthesized with 50 mg/mL PEI and 2~5% (v/v) TDI possessed optimal PEI conversion.

In the case of PUCQ, when DAB was loaded into the disperse phase before crosslinking reaction, DAB concentrations also affected the shell thickness (as shown in **Figure 13**). In the first 5 minutes of reaction, DAB slowed down the growth of shell: thinner shells were produced at higher DAB concentrations. However, after 4 hours of reaction, the shells thickness increased slowly and differences in samples of varied DAB concentrations disappeared. When PEI concentration was 75 mg/mL, DAB (40 mg/mL) slightly facilitated penetration of TDI and resulted in slightly thicker shell compared to that of control sample (0 mg/mL DAB). DAB reacted with invading TDI and was subsequently anchored onto the shell (**Figure 10**). After the formation of a primary membrane, the reaction frontline moved inward gradually. The DAB in the disperse phase competed with PEI in reacting with TDI. This reduced the crosslinking density of the shell, and finally facilitated deeper and faster TDI diffusion.

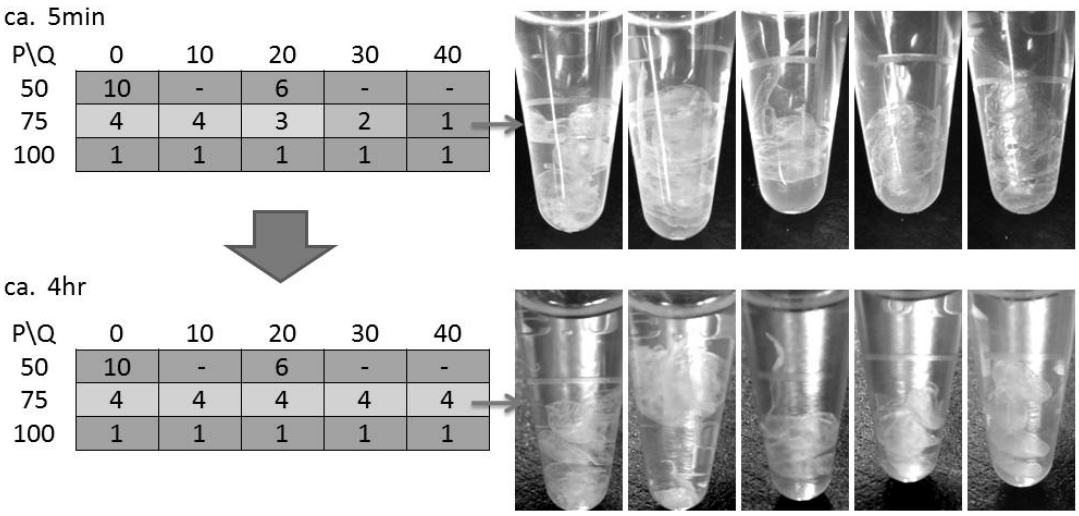


Figure 13. Reactivity tests on PUCs: effect of PEI and DAB concentrations. PUCs were synthesized with various PEI (P) and DAB (Q) concentrations (mg/mL), with TDI/cyclohexane (2%, v/v) solutions. The shell thickness scores in the table were manually marked according to observations (high scores refer to thick shells, "-" refer to no data). Pictures in the upper and bottom row were taken at 5 minutes and 4 hours after reaction, respectively.

4.1.8. Morphology Problems Caused by TDI Dimers

TDI may form dimers during room temperature storage (**Figure 14**). TDI dimers are poorly soluble in TDI, hexane or cyclohexane, and their crystals were regularly found in TDI that was purchased over half a year. TDI dimers were often observed during synthesis of the PUMCs, especially when TDI was in excessive amount (e.g. 5% v/v, during a 20-min reaction). Although TDI dimers can be removed from PUMCs through excessive methanol or ethanol extraction after synthesis, co-existence of TDI dimers became a problem for drug loaded PUMCs as extraction was no longer suitable.

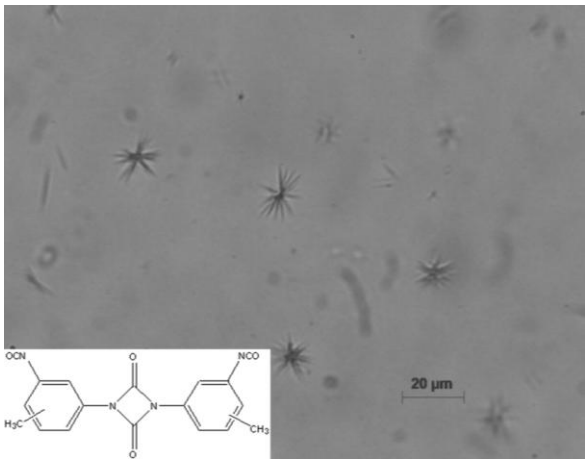


Figure 14. TDI dimers in hexane. Needle shaped TDI dimer crystals were observed under a microscope, the structure of which is shown in the inset.

To avoid the formation of TDI dimers, reducing TDI dosage could be one of the strategies (e.g. synthesis of MC42 series, TDI conc. = 0.12% v/v). However, low TDI concentrations did not meet the requirement for thick shell PUMCs. Alternatively, increasing the solubility of TDI dimers in cyclohexane by reducing Span™ 85 and PIB level in the system also reduced the TDI crystal formation (e.g. synthesis of MC33, no surfactant was used; PIB concentration = 5.0 % v/v). Nonetheless, such strategy may affect the emulsification and complicate the synthesis process. A last resort was critical storage management: TDI should always be tightly sealed and frozen to minimize exposure to heat or air. A good practice was to dispense small amounts of fresh TDI into separate containers and use freshly thawed TDI every time (thawing temperature was below 40 °C). In practice, any TDI stock solution that was left at room temperature for more than 24hr after its first opening was no longer considered fresh.

4.1.9. Surface Chemistry of PUCQs

Surface chemistry of PUCQs was examined with the fluorescein titration method (**Figure 5**). PUCQ showed deep red color during fluorescein staining, which indicated higher amount of quaternary ammonium salt in the shell (**Figure 5** inset). However, little or no color change was observed for PUCQs during CTAC extractions. The titration results generated from desorbed fluorescein also showed lower apparent DAB density (5.90×10^{-8} mol/cm^2, calculated by subtracting PUCQ with PUC) than that for PUMC (4.29×10^{-7} mol/cm^2, **Figure 5**). In order to elucidate these results, cross-sections of PUCs were studied under a microscope (**Figure 3**). The fluorescein presented not only on the surface of the capsule, but they also penetrated into the shell. So the adsorption and desorption of fluorescein reflected the bulky properties of the shell (such as porosity, hydrophilicity and polarity), rather than those properties of the very exterior surfaces. Consequently, quantitative data of PUCQs were incomparable with that of PUC+Qs as PUCs were no longer a proper control sample for PUCQs.

Nevertheless, fluorescein staining still provided qualitative information: deeper color of PUCQ shell indicated higher density of fluorescein in the shell. The presence of DAB inside the shell of PUCQ inhibited the diffusion of CTAC into the shell by electronic repulsion resulting in selective extraction of surface adsorbed fluorescein in the staining test. Hence, absolute surface quantification of PUCQ fluorescein desorption was an approximate estimation of surface DAB (7.0×10^{-8} mol /cm^2), which was still much higher than the lower threshold for QAC densities for antimicrobial surface (0.8×10^{-8}

mol /cm^2).[134] To summarize, volume DAB density in the shell of PUCQs was higher than that of PUC+Qs depth of color. Surface DAB density of PUCs or PUMCs was not accurately measured with fluorescein titration method because of their porous outer surface.

4.1.10. Synthesis of the *In-situ* Modified Microcapsules (MCQs)

Since PUCQs offered better physiochemical properties (thicker shells and higher DAB densities) than that of PUC+Qs, *in-situ* modification method was more suitable for our purpose: synthesis of microcapsules for drug release and infection control. A series of tests were carried out to evaluate the drug release properties and antimicrobial property of MCQs.

DAB *in-situ* modified polyurea microcapsules (MCQs) and control microcapsules (PUMCns) were synthesized (**Table 3**) through the emulsion interfacial polymerization method[116] with some modifications. In case of MCQs, DAB was loaded into disperse phase to serve as a multifunctional surfactant. The resultant MCQs had thicker shells compared with that of PUMCns when compare **Figure 15a** with **Figure 15c**. MCQ retained their spherical shape after drying (**Figure 15b**). Such property allowed easy re-dispersion of microcapsules, which was critical for handling and further examinations or applications such as drug release. Moreover, PUMCns was observed to swell in water and had a wider size distribution than MCQs.

Table 3. Composition of disperse phase for synthesis of MCQs

Sample	phi value*	disp.phase ingredients (g/mL)			vol. of TDI (mL)
		PEI	DAB	Coumarin-1	
MCQ-Cmr	3.8%	0.050	0.100	0.133	0.330
MCQ	3.8%	0.050	0.100	0.000	0.330
PUMCn	3.8%	0.050	0.000	0.000	0.330

*phi value= volume ratio between disperse phase and emulsion; solvent for disperse phase is anhydrous DMF. PUMCn is the control sample in antimicrobial test for MCQ.

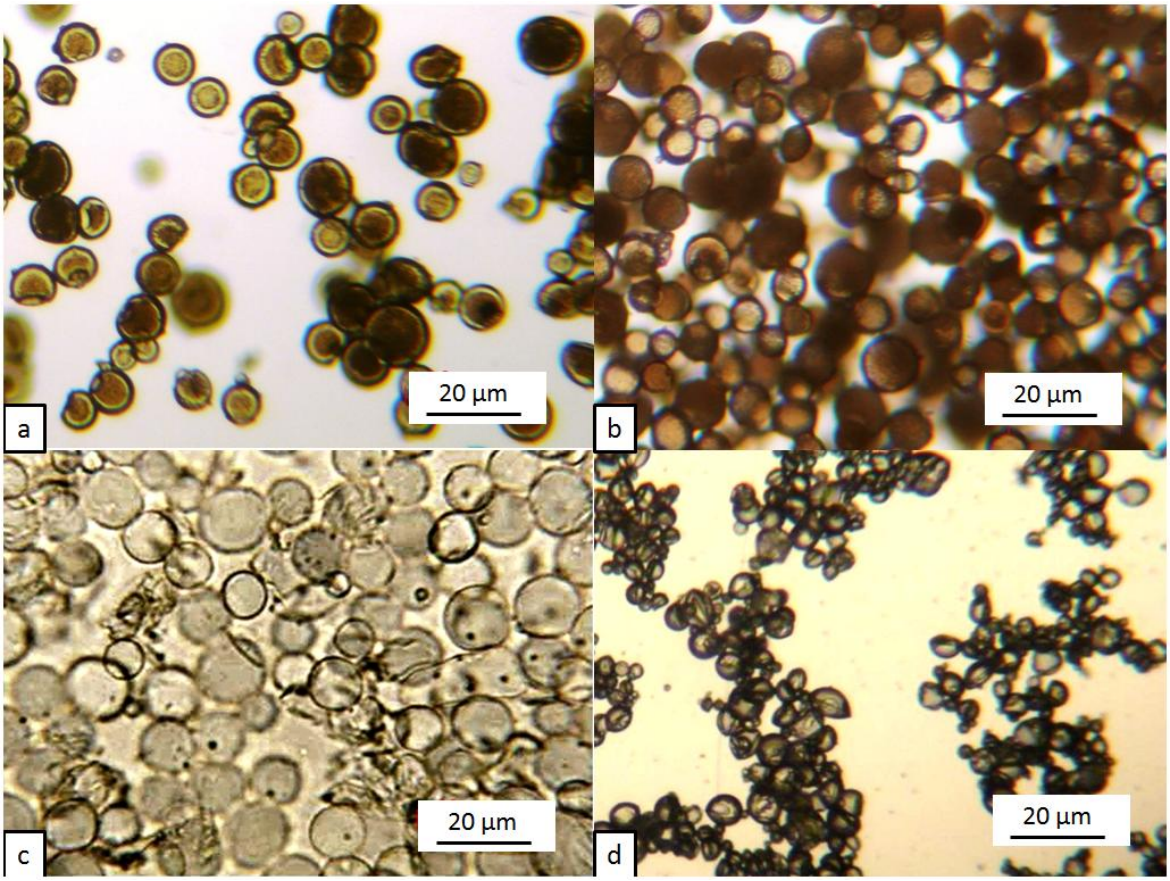


Figure 15. Morphology of MCQs and PUMCns. (a) MCQs dispersed in water; (b) dry MCQs; (c) PUMCns dispersed in water; (d) dry PUMCns.

4.2. PUMCs as Drug Carriers

4.2.1. Drug Loading Properties of PUMCs

Drugs can be encapsulated inside the disperse phase by premixing a concentrated drug solution with disperse phase before reaction. The influence of a high concentration of drug content on both particle morphology and shell properties was discussed in section 4.1.4. Several properties on shell structure are assessed as follows: drug loading capacity, drug loading efficiency, and drug release profile.

Ideally, drug loading properties in an emulsion depend on the partition coefficient of a certain drug in a specific emulsion system and are independent from the droplet size. However, we found that emulsion based microcapsules that have various sizes do have different loading properties: larger microcapsules, in general, possessed higher loading capacities (comparing row 1, 2, 3 to the rest of **Table 4**). We attributed such phenomena to drug leaching, which could take place during either emulsification or washing. The co-effect of lower phi values (during emulsification) and higher surface-volume ratios (during both emulsification and washing) probably resulted in low loading capacities and efficiencies of smaller microcapsules. A lower phi value was usually required for the production of smaller droplets in our system, thus more drugs could be dissolved into the continuous phase even though partition coefficient was unchanged. Apart from the lower phi value, a higher surface–volume ratio of the resultant smaller droplets further aggravated the drug leaching, especially during washing process, when the encapsulated drugs are prone to be extracted by washing solution. Efforts had been made to

compensate the drug loss by increasing the drug concentration (comparing row 2 to 3). We found that simply increasing the drug concentration (by ca. 9 times) had very limited improvement on loading capacity (an 11% increase). Rather, it resulted in a reduction of efficiency by 71%. However, the loading capacity could be improved via adjusting the partition coefficient by substituting the disperse phase diluent DMF for MeOH (comparing row 1 to 2). The improvement in loading capacity probably resulted from the lower solubility of DMF in cyclohexane than that of MeOH, thus less coumarin-1 was leached into the continuous phase during emulsification. At lower phi values (comparing row 5 to 4), the effect of increased drug concentration and reduced surfactant level was even overridden by reduced phi values, and the loading efficiency was unacceptably low. Efforts had also been made to increase the capsule shell thickness by increasing PEI concentration (comparing row 6 - 8). No significant improvement was found and the loading efficiencies are still unacceptably low. In summary, phi values have a stronger effect on loading capacity compared to other factors such as surfactant dosage and drug concentration, and the loading properties of small PUMCs (1 – 10 μm) were too low to be acceptable for further tests.

Table 4. Coumarin-1 loading properties of PUMCs

	loading capacity (w/w)	loading efficiency (w/w)	d_{avg} (μm)	phi. value/ diluent	PEI conc. (g/mL)	drug conc. (g/mL)	surfactant dosage (v/v)	shearing rate (rpm)
1	17.0%	10.3%	60.0	16.7% / DMF	0.15	0.42	2.0%	1200
2	5.9%	6.9%	18.0	16.7% / MeOH	0.15	0.42	2.0%	1200
3	5.3%	23.9%	50.0	16.7% / MeOH	0.15	0.05	2.0%	500
4	1.8%	0.05%	5.0	0.3% / DMF	0.15	0.20	0.3%	5000
5	0.6%	0.1%	2.0	0.2% / DMF	0.15	0.42	0.1%	5000
6	0.9%	0.4%	2.0	1.0% / DMF	0.05	0.42	0.1%	4000
7	1.2%	0.2%	2.0	1.0% / DMF	0.20	0.42	0.1%	5000
8	0.5%	0.2%	2.0	1.0% / DMF	0.40	0.42	0.1%	7500

Loading capacity = mass (encapsulated drug) / mass (drug loaded microcapsules); loading efficiency = mass (encapsulated drug) / mass (total drug added); d_{avg} refers to average MCQ diameter by number; phi value = volume ratio of disperse phase in the emulsion; drug/PEI concentration refers to the concentration of courmarin-1 or PEI in the disperse phase before emulsification; surfactant dosage refers to the volume concentration of Span™ 85 in the continuous phase.

To achieve higher loading properties of PUMCs for drug release tests (section 4.2.2), cortisone was chosen in substitute for coumarin-1 (**Table 5**), and significant improvements in both loading capacities and loading efficiencies were achieved. These improvements could be attributed to the increased molecular mass (cortisone, 360.46 Da; coumarin-1, 231.29 Da) and slightly increased hydrophilicity (cortisone has two hydroxyl groups whereas coumarin-1 gives none) of the model drug. Hence, drug properties strongly affect loading properties of PUMCs. Similarly, MCQs have improved coumarin-1 loading properties (drug load = 3.33wt%; efficiency = 31%) compared to PUMCs (**Table 4)**, which could be attributed to both the presence of DAB in the disperse phase and the improved morphology, shell thickness, and dispersibility of microcapsules.

Table 5. Cortisone loading properties of PUMCs

	loading capacity (w/w)	loading efficiency (w/w)	d_{avg} (μm)	phi. value/ diluent	PEI conc. (g/mL)	drug conc. (g/mL)	surfactant dosage (v/v)	shearing rate (rpm)
1	26.0%	57.0%	5.0	2.2% / DMF	0.05	0.30	0.1%	8000
2	20.7%	48.9%	5.0	4.2% / DMF	0.05	0.30	0.3%	8000
3	15.2%	43.6%	5.0	4.2% / DMF	0.05	0.30	0.3%	8000

Drug loading efficiencies of PUMCs were also affected by microcapsule recovery rates: smaller PUMCs were more difficult to be collected through centrifugation, especially in cases of micron-scale PUMCs (comparing row 4 - 8 to the row 1 - 3 in **Table 4**). Moreover, poor redispersibility of PUMCs required intense washing conditions, which in turn aggravated the drug leaching, especially when capsule shells were thin and fragile.

In summary, drug loading properties are sensitive to emulsification and recovery conditions. PUMCs suffered from severe drug leaching problems during emulsification and washing processes, especially in cases of micron-scale PUMCs due to their low phi value and high surface-volume ratio. The drug loading efficiency could be improved by controlling microcapsule morphology and shell thickness, and further optimizations of emulsion system are required to improve the drug loading properties.

4.2.2. Drug Releasing Properties of PUMCs

Because the coumarin-1 loading properties of PUMCs were unsuitable for releasing test, cortisone was loaded into PUMCs (refer to **Table 5**, row 1) as model drug for *in vitro* release tests. Cortisone loaded PUMCs sustained the cortisone release during a 7-day *in vitro* release test (**Figure 16**). The release profiles of cortisone loaded of PUMCs were similar to each other when microcapsules were extracted with PBS solution (0.1 M) of different pH value (pH = 7.4, 6.5, 5.5).

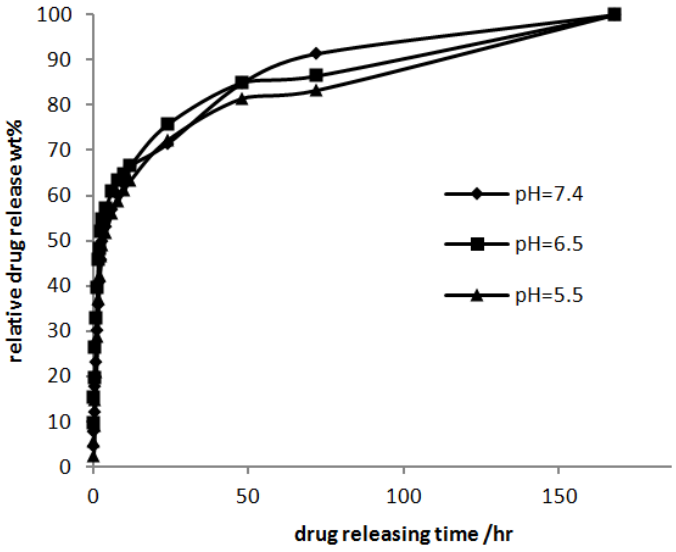


Figure 16. *In vitro* drug release of cortisone loaded PUMCs.

To investigate the influence of surface DAB on drug release, freshly prepared PUMCs were post-modified with DAB in a THF solution and were subjected to *in vitro* release test (MC68a, **Table 5** row 2; MC68b, **Table 5** row 3). Release profiles of both MC68b (DAB post-modified PUMCs) and MC68a (the control sample, which was

treated in pure THF without DAB) were shown in **Figure 17**. MC68b reduced the initial release rate by ca. 4 folds in comparison to that of MC68a (**Figure 17,** inset). The suppression of initial burst release was attributed to the hydrophobic layer of DAB moiety on the surface, which inhibited the diffusion of releasing media, water, into the shell and reduced the swelling effect of capsule shell. Thus the drug releasing rate during first 5 hours could be controlled by adjustment of the DAB density on the surface.

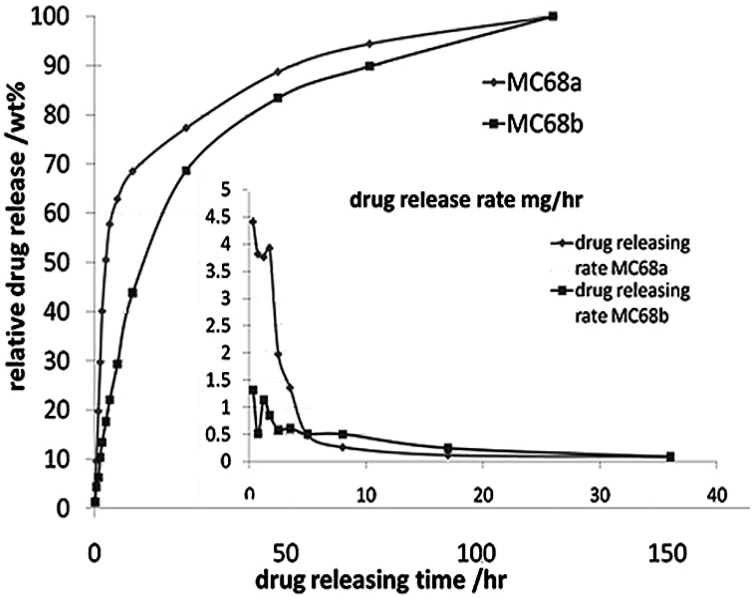


Figure 17. Influence of surface DAB on cortisone releasing profile. MC68a bare microcapsules; MC68b DAB post-modified PUMCs.

With improved coumarin-1 loading properties of MCQs (cap% = 3.33wt%, eff% = 31%), there is no need to use cortisone as a target drug for MCQs. Thus, coumarin-1 encapsulated MCQs (MCQ-Cmr, **Table 3**) were synthesized and subjected to paddle dissolution test (USP #2).[123, 135] A sustained release profile is shown in **Figure 18**. During

a 7-day continuous release, the release curve stabilized at 2.14 mg/L at the 5th day, with 51.8% total drug released. During the first 24-hour of dissolution, 35% drug was released following a pseudo-zero-order profile (**Figure 18** inset) after an 8.5% initial burst within 15mins. The result accorded with typical release curves for reservoir-type drug delivery systems, in which the thick shells of capsules could efficiently retain the drug, so that constant concentration gradient could be maintained across the shell.[72]

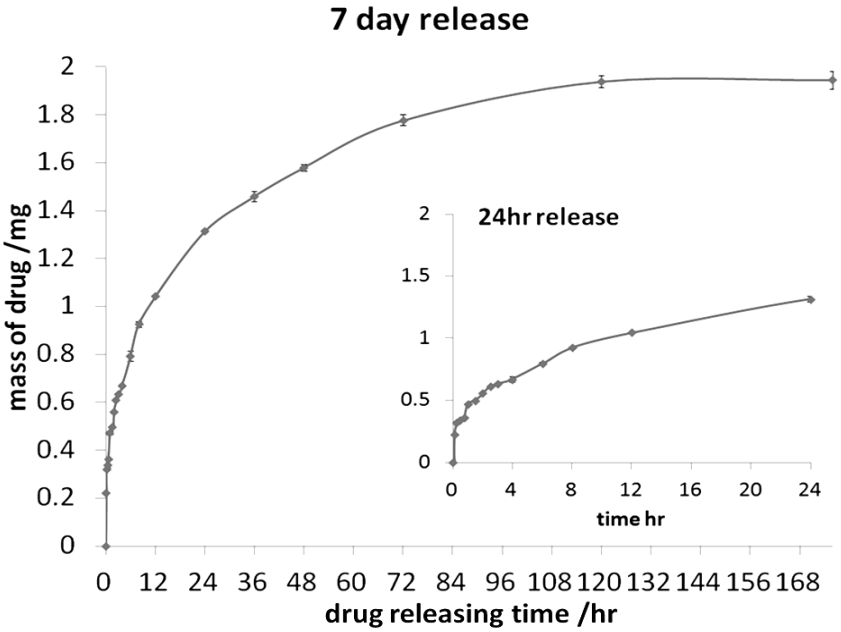


Figure 18. *In vitro* cumulative drug release profile of MCQ-Cmr; insert: first 24hr release curve; (drug load = 3.33wt%; efficiency = 31%).

4.3. MCQs as Antimicrobial Agents

MCQs and the control sample, PUMCns, were synthesized to evaluate the non-leaching antimicrobial properties. All samples were extracted repeatedly with methanol and sterile PBS solution for additional 48hr after regular washing process to eliminate the potential influence of chemical residues (e.g. TDI, PEI, free DAB). The last batch of PBS extraction solution was used as the blank solution for antimicrobial tests. A strain of gram negative bacteria, (MDR - ESBL - E. coli) was chosen to serve as a model bacterium for antimicrobial evaluations.

The surface diffusion test was applied to evaluate the non-leaching antimicrobial properties of MCQs. A MCQs suspension (20 μL) was dropped onto the E. coli inoculated agar (capsule density: 0.88 mg/cm^2, bacteria load: 4.54 $\log_{10}$ cfu/cm^2). As shown in **Figure 19**, a clear spot of inhibition was observed in the MCQ coated area. In the control sample coated area, colonies could still be observed while the shape of colony was altered. Blank solution coated area showed little or no effect on the bacteria, which excluded the possibility that antimicrobial activity of MCQ was a result of leached antimicrobial agent.

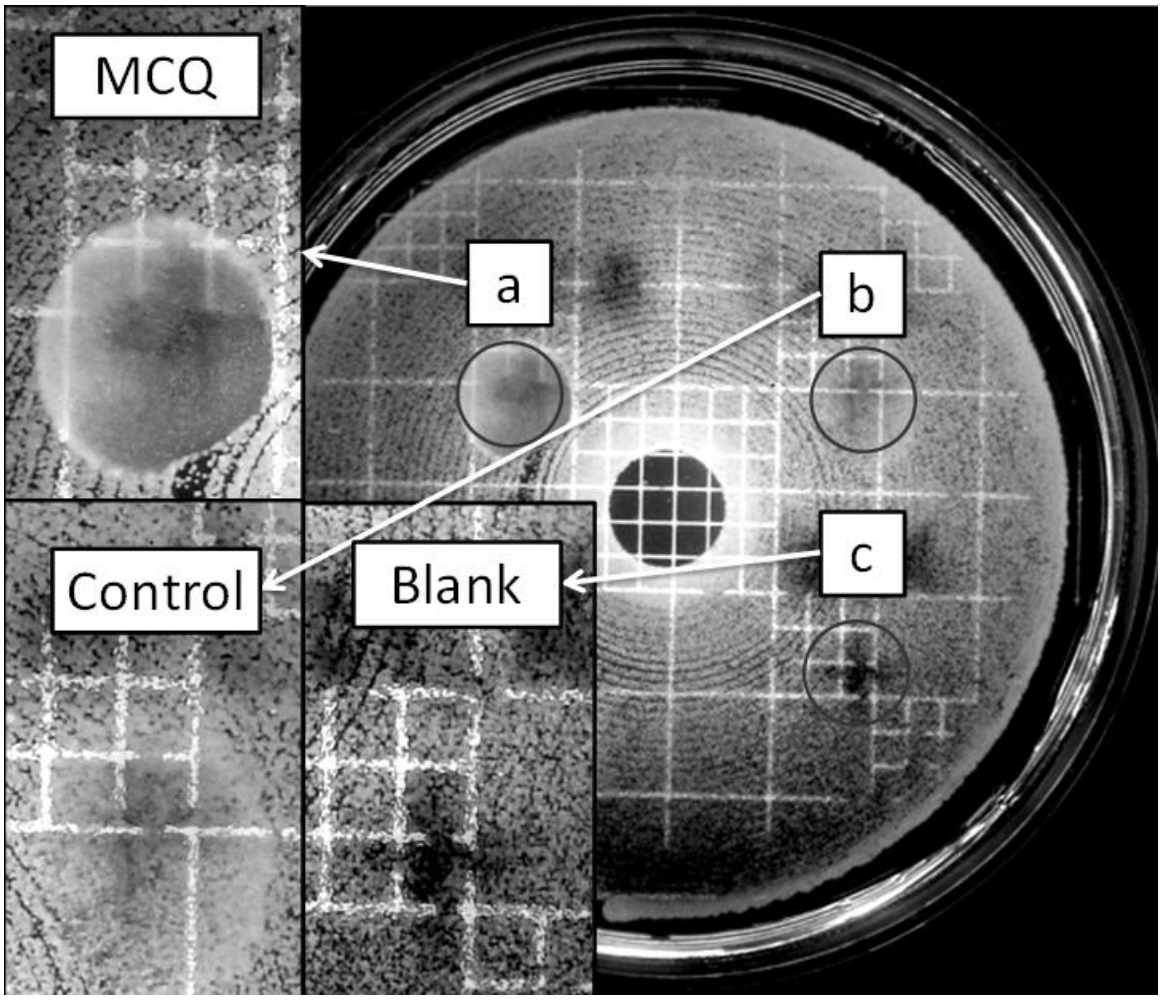


Figure 19. Diffusion antimicrobial test: a 10-uL droplet of microcapsule suspension was dropped onto inoculated agar; pictures were taken after a 20hr incubation under 37 °C (a): MCQ, DAB *in-situ* modified capsule; (b): PUMCn, control sample,;(c): Blank, last batch of washing solution of MCQ. Bacteria load: 4.54 $\log_{10}$ cfu/cm^2; microcapsule density: 0.88 mg/cm^2.

To further investigate the influence of microcapsule coating density on the antimicrobial properties with different levels of bacteria loading, a static contact antimicrobial test was utilized. Bacteria suspension was evenly spread on a microcapsule coated agar plate with a spiral plater. The capsule density was controlled by the numbers

of layers of coating (0.22, 0.44, 0.66, 0.88 mg/cm^2). Bacteria densities were controlled by the concentrations of bacteria suspensions (3.54 $\log_{10}$ cfu/cm^2). Results are shown in **Figure 20**. Inhibition effect was observed when MCQ density reached 0.66 mg/cm^2. The size and number of colony were reduced, which indicated a lower population of bacteria colonized on the surface of agar. The growth of bacteria that attached to an MCQ surface was inhibited while those attached to the interspaces between capsules can still multiply. PUMCn exhibited little or no inhibition effect except that they caused the bacteria to spread over the capsules with increasing density of capsules. The antimicrobial action was resulted from the cell lysis caused by the alkyl chains and the quaternary ammonium salt of DAB which penetrated the cell membrane and induced ion exchange.[49, 50]

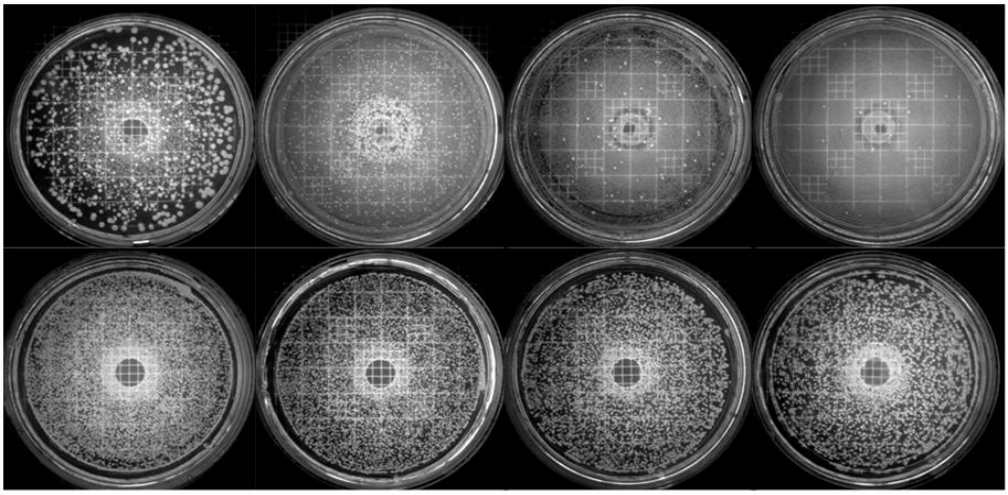


Figure 20. Static contact antibacterial test. Bacteria load: 3.54 $\log_{10}$ cfu /cm^2; for each layer, 0.22mg /cm^2 (3.2mg) capsules are coated over TSA in 100 mm petri dish with spiral autoplater. MCQ sample is the DAB modified microcapsule and control sample PUMCn is the polyurea microcapsule without DAB modification.

4.4. Preliminary Study of PUMC Immobilization

In an attempt to immobilize freshly prepared PUMCs onto cotton fabrics, a hexane suspension of freshly prepared PUMC (3 mL, 1.63 mg/mL) was sprayed onto pre-dried cotton fabrics. Fabrics were washed gently with water and were subjected to SEM examination after vacuum drying. The microcapsules were expected to be anchored onto cotton fibers via the reaction between surface isocyanate group and hydroxyl group on cotton fiber. As shown in **Figure 21** A and C, a few of the PUMCs was attached onto the surface of cotton fibers. Because larger particles required more binding force to be immobilized, PUMCs over 5 μm could hardly be anchored to the cotton fabrics. That is why only microcapsules of ca. 2 μm were found on the surface of cotton fabric. In addition, lots of TDI dimer crystals were also observed in **Figure 21** C (MC32). They could be partially removed by one cycle of gentle hexane washing of PUMC loaded fabric (compare D to C). Alternatively, formation of TDI dimer crystals could be minimized by reducing the PIB concentration (PIB in MC33 was 4 times lower in comparison to that MC32). In summary, PUMCs of ca. 2 μm were immobilized onto cotton fiber via spraying method, while PUMCs over 5 μm were too large for immobilization without a crosslinker.

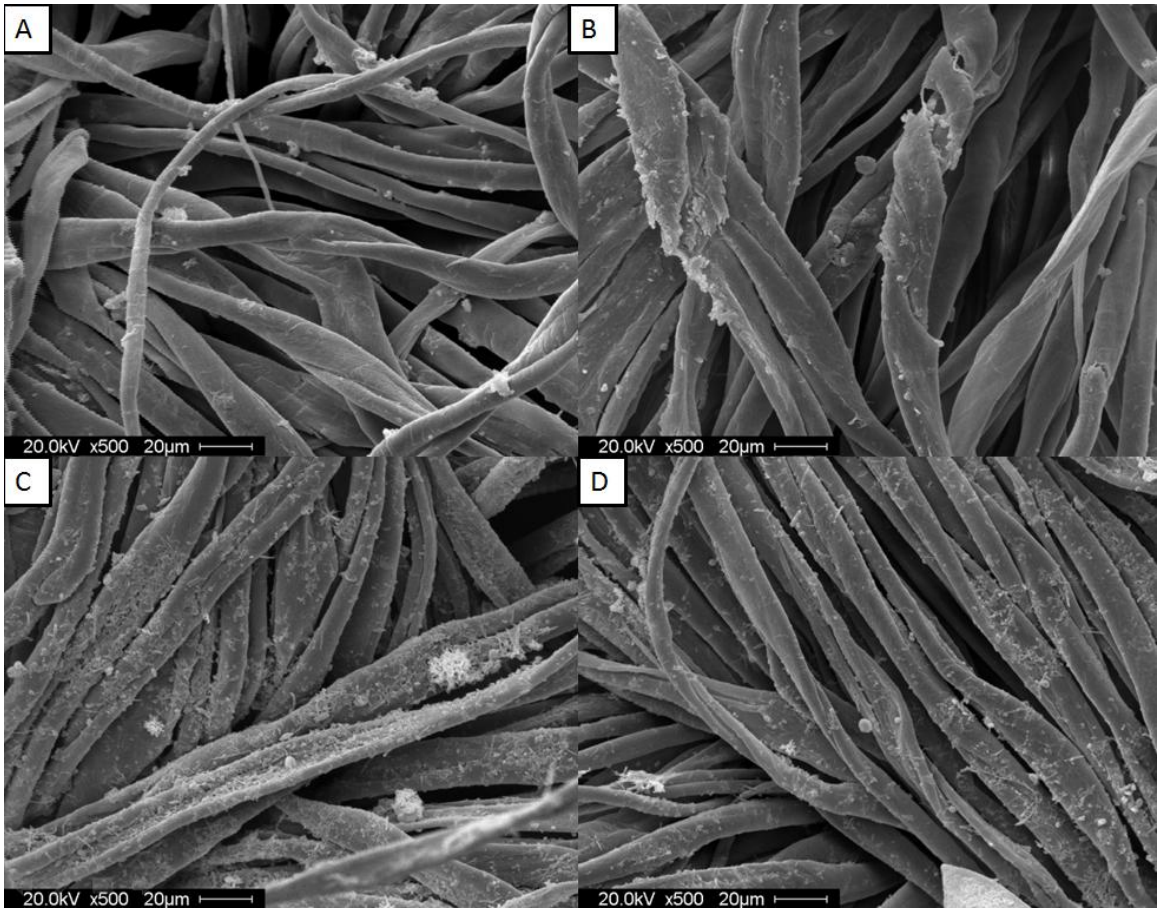


Figure 21. Immobilization of PUMCs on cotton fibers: (A):MC33 before hexane wash; (B):MC33 after hexane wash; (C):MC32 before hexane wash; (D):MC32 after hexane wash; MC32/MC33: freshly prepared microcapsule, synthesis conditions: phi = 2.0%v/v, PEI conc. = 0.30g/mL, Span™ 85 conc. = 2.5%v/v, shearing rate 2500 rpm, $d_{avg.}$= 2.5µm. PIB concentration in MC33 synthesis was 1/5 of that in MC32 synthesis

CHAPTER 5. CONCLUSIONS AND FUTURE STUDIES

Nutrition supply and infection control are the two major tasks for chronic wound treatment. Microcapsules, as reservoir-type drug carriers, are suitable for sustained drug release, thus they can be applied in chronic wound dressing to achieve long term nutrition supply. The large surface area of microcapsules, on the other hand, can be utilized as antimicrobial surfaces for wound infection control.

PUMCs and PUCs were successfully synthesized via interfacial polymerization in a DMF/cyclohexane non-aqueous emulsion under room conditions. The exterior surfaces of PUMCs were modified with DAB, a quaternary ammonium salt, to produce non-leaching antimicrobial surfaces. Two methods of modification were explored: post-modification method and *in-situ* modification. Both methods are based on the surface isocyanate residues on the freshly prepared microcapsules. The surface isocyanate groups are capable of anchoring hydroxyl-group ended small molecules. The existence of isocyanate residues and the subsequent DAB grafting on PUC surfaces was confirmed and semi-quantified through IR labelling method and fluorescein staining method respectively. After the model study, two modification methods were tested and compared on microcapsules. *In-situ* modification method has advantages over post-modification method because of their ease of preparation, absence of various morphological problems, good redispersibility, and thick capsule shells. As a result, *in-situ* modification method was chosen as the modification strategy for the synthesis of antimicrobial polyurea microcapsules.

The drug release properties of *in-situ* modified microcapsules (MCQs) were evaluated by drug dissolution test (USP#2, paddle test). MCQs are capable of releasing model drug in a sustained manner over a 7-day period. The release rate from 15min to 24hr follows an psudo-zero order kinetics with an initial burst of 8.5%, and 35.0%, 51.8% of model drug was released at the end of the first day and the fifth day. The non-leaching antimicrobial properties of MCQs were confirmed on model wound dressing surface with diffusion test. Then the efficacy of the MCQ coated surface was evaluated by surface contact antimicrobial test. Obvious inhibition effect was observed when MCQ density reached 0.66 mg/cm^2 under an *E.coli* load of 3.54 $\log_{10}$ cfu/cm^2.

The concept of *in-situ* modified dual-functional microcapsules in this study could be applied in the synthesis of other kind of microcapsules: functional surfactants can be loaded into the disperse phase, either to minimize the formation of large amount of micelles in the continuous phase, or to endow new functions to shells by participating in shell formation. Further research directions include: optimization of the synthesis conditions for PUMC in terms of formulation, encapsulation, and emulsification process; adjustment in shell property for better mass regulation, improved antimicrobial property, higher encapsulation efficiency, and narrower size distribution; design and optimization of new functional surfactant for better stabilization and enhanced functions (easier recovery or redispersion in various media or durable immobilization of the synthesized capsules on various substrates); design and synthesis of bio-degradable polymers or crosslinkers as building blocks for the synthesis of degradable, non-toxic PUMCs; and development of multifunctional surfactants as a versatile platform for chemical grafting.

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