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Masters of Engineering Thesis

Artificial Cell Live Yeast Microcapsule Formulation for Use in Renal Failure Uremia

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ABSTRACT

Renal failure uremia occurs when the kidneys fail to function properly. Despite being the main treatment, dialysis and other therapeutic approaches are not only associated with numerous long-term adverse complications often leading to morbidity and mortality events, but are also not affordable. Orally administrating Alginate-Poly-$_L$-Lysine-Alginate microcapsules entrapping live yeast cells to treat renal failure uremia has not yet been investigated. In this thesis, the growth and microencapsulation of yeast were optimized. The efficacy of these microcapsules in removing unwanted electrolytes was tested *in vitro* in simulated gastro-intestinal media, *in vitro* in a column bioreactor and *in vivo* in an uremic rat model. Results showed that these novel microcapsules can not only maintain morphological stability and membrane integrity under gastro-intestinal environments and mechanical stresses, but also, preserve the viability of yeast. These microcapsules were successful in reducing urea concentrations while not harming the human GI tract's microbial flora.

RESUME

L'insuffisance rénale se produit lorsque les reins ne fonctionnent plus correctement. Cette maladie peut être aiguë ou chronique. Alors que la condition aiguë peut être inversée lorsqu'elle est traitée convenablement, la condition chronique conduit souvent au stage final de l'insuffisance rénale. En dépit d'être le principal traitement, la dialyse est associée à de nombreuses néfastes et longs termes complications conduisant souvent à des événements de morbidité et de mortalité. L'administration par voie orale d'Alginate-Poly-$_L$-Lysine-Alginate microcapsules contenant de la levure pour traiter les défaillances rénales n'a jamais été investiguée. Dans cette thèse, la croissance et la microencapsulation de la levure ont été optimisées. L'efficacité de ces microcapsules à éliminer des électrolytes indésirables a été tester *in vitro* dans des solutions gastro-intestinales et *in vivo* en utilisant un modèle de rats urémiques. Les résultats ont montré que lorsqu'elles sont soumises à des conditions gastro-intestinales combinées avec des forces mécaniques, ces nouvelles microcapsules peuvent non seulement maintenir une stabilité morphologique et l'intégrité de leurs membranes, mais aussi, préserver la viabilité de la levure. Egalement, ces microcapsules ont pu réduire les concentrations d'urée sans nuire à la flore microbienne du system gastro-intestinal humain.

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PREFACE

In accordance with the McGill Thesis Preparation guidelines, I have decided to write the experiment section of this thesis as a compilation of original papers appropriate for publication. These papers are presented in chapters 3, 4, 5 and 6. Each chapter is divided into sections consisting of an abstract, introduction, materials and methods, results, discussion and a conclusion. Also included in this thesis are a common abstract, a general introduction, a literature review, a summary of the results obtained, a conclusion and references.

TABLE OF CONTENT

Chapter I

GENERAL INTRODUCTION

Renal failure uremia is the condition in which the kidneys fail to function properly. Physiologically speaking, renal failure is manifested by a decrease in the glomerular filtration rate (GFR). Under normal physiological conditions, Gonwa *et al.*[1] reported GFRs of 114 ± 17 mL/min and 102 ± 15 mL/min per 1.73 m^2 for men and women, respectively, aged between 21 to 30 years. With age, the filtration capacities of the kidneys decrease significantly and may reach GFR's values of 84 ± 13 ml/min and 79 ± 15 ml/min per 1.73 m^2, respectively, for men and women aged between 51 to 60 years[2].

Broadly speaking, renal failures can be divided into two categories: acute renal failure (ARF) and chronic renal failure (CRF). ARF is a rapid and progressive loss of renal function, which is characterized by decreased urine production, body water and fluid and electrolyte derangements. CRF, however, is manifested by a much slower progression characterized by few initial symptoms. Various diseases and factors can lead to CRF including IgA nephritis, glomerulonephritis, chronic pyelonephritis and urinary retention. Unlike ARF, CRF, which results in end-stage renal failure/disease (ESRF or ESRD), is not a reversible process and once a patient loses 90% of his/her kidney functions, renal replacement therapy (RRT) is highly needed. Thus, ESRD is the ultimate consequence, which, if not treated, leads to a certain death.

In North America, ARF is quite common, affecting approximately 5% of hospitalized patients and up to 30% of patients in intensive care units (ICUs)[3]. In 2005, 8.3 million Americans were reported to have a mature chronic kidney disease with a significant number developing ESRD[4,5]. ARF affects up to 200,000 people in the United States annually, or approximately 5% of all long-term hospitalized patients[6-8]. ARF and ESRD have high mortality characterized by an overall survival rate of approximately 50% or lower depending on the severity of the renal malfunction[9].

Faced with the seriousness of kidney failures, numerous RRTs, such as kidney transplantation (KT) and dialysis, have been developed since the 1950s. KT, despite

being the only treatment assuming complete renal functioning, is limited in its reach. This is mainly explained by the shortage of donors and the persistent increase in the age of ESRD patients[10]. In fact, in the United States between 1994 and 2003, more than 60% of total organ waitlist, including intestine, pancreas, lung, heart and liver, is represented by kidney failure patients[11].

Dialysis is another current solution to kidney failures. It removes accumulated wastes by connecting a patient to an external filtration system at regular time intervals. Dialysis only replaces the filtration properties of the diseased kidneys. There are two major types of dialysis: hemodialysis (HD) and peritoneal dialysis (PD). The former utilizes hemodynamic pressures to achieve the filtration whereas the latter, a gentler method, uses the peritoneum as a semi-permeable membrane. Although dialysis is widely used in renal failures, it is not devoid of complications, which range from cardiomyopathy-related morbidities and moralities[12-14] to infection[15] and inflammation[16] mediated by the use of synthetic, bio-incompatible Cuprophan or cellulosic membranes[16, 14]. Furthermore, the need for an access site in both HD and PD increases the likehood of infection[18-21]. Another major problem is the contamination of dialysate solutions by endotoxins and bacteria[22].

Ideally, a RRT should provide the patient with native metabolic renal functioning, which includes synthesis of cytokines, antigen presentation, and oxidative deamination. It must present a low power and other resources (water for example) expenditure profile. This is particularly important for the treatment's instauration in the developing world. The RRT must also be portable and user friendly so that patients would acquire a freer movemental capabilities and an easier operability[23]. Unfortunately, most of the actual treatments do not fulfill these requirements despite the medical advantages they confer and, for this reason, new therapeutic modalities have been investigated and developed.

Artificial cell technology, for instance, is one novel approach to potentially treat renal failures. Cells with synthetic biomaterials are currently recognized as a "new category of therapeutic products" by the U.S. Pharmacopeia and National Formulation[24]. It is at McGill University that T. Chang first reported artificial cells in 1957[25-30]. An artificial cell refers to man made structures to supplement or replace deficient cell functions.

"Encapsulation was proposed as a means to protect the enclosed materials from the external environment, thereby helping to prevent rejection by the immune system"[28-30]. In other words, there is no direct contact between the active materials inside the cells and the external environment, such as the gastrointestinal (GI) tract, or entities such as tryptic enzymes, leukocytes and antibodies[31]. The ingenious design of the polymeric membrane allows smaller molecules, such as hormones and peptides, to diffuse inside the artificial cell and, thus, equilibrate rapidly across its membrane. This is explained by the membrane's ultra-thinness and the large surface-to-volume ratio of the artificial cells[31].

Depending on the nature of the application and the type of material used in synthesizing the membrane, it is possible to vary its permeability, composition and configuration, which allows, in consequence, for extensive variations in the properties and functions of artificial cells. The cells size can range from macro-dimensions of up to 2 mm diameter, through micron- and nanodimensions down to molecular dimensions[28-32].

All of these properties made of artificial cells excellent candidates for efficient toxins and drugs removal. In fact, the most common and earliest application is the use of microscopic polymeric artificial cells that enclosed activated charcoal and oxidized starch (oxystarch)[33-35] for the removal of metabolic toxins[31,36]. Currently, this is in routine clinical use after it has been proven successful in initial clinical trials for poisoning, kidney failure, and liver failure[31,36]. Note that this approach is of particular practical usefulness in situations where HD units are not easily accessible or available. Other possible applications of artificial cells concern the encapsulation of enzymes or blood cells substitutes. For instance, Setala *et al.*[37,38], in the 1970s, tried to degrade nitrogenous wastes using enzymes isolated from soil bacteria.

Later, Prakash *et al.*[39] microencapsulated genetically engineered nonpathogenic *E. coli* DH5 cells containing *Klebsiella aerogenes* urease gene for the purpose of lowering systemic urea in renal failure subjects[40,41]. As microorganisms cannot be injected directly in the body, the only solution to safely administer these microcapsules is through oral delivery. This way, these structures can act as microscopic dialyzer–bioreactors as they travel through the intestine[31]. The oral route does not require neither cannulation nor feeding apparatus. The authors found that these bioreactors can lower up

to 99.99% of plasma urea within 30 min using only 100 mg of bacterial cells[39]. Unfortunately, some problems, including capsules strength, long-term biocompatibility, microorganism's cells entrapment in membrane or surface exposure and the genetic engineering of the cells limit the usefulness of this novel technique[31].

Given the capacity of *Saccharomyces cerevisiae*, commonly known as yeast, to efficiently degrade urea[42], its high environmental adaptive acquaintance, the easiness of its large scale production as well as the benefits of microencapsulation, using encapsulated live yeast cells for urea removal may prove a rewarding attempt. The potential of administrating such artificial cells to treat human renal failures has not yet been investigated. Hence, this project is focused on investigating the feasibility of designing artificial cells containing live *Saccharomyces cerevisiae* as an alternative approach to renal failures. Studying the uremic metabolites and electrolytes removal capacities of these artificial cells both *in vitro*, in simulated gastric and intestinal media, and *in vivo*, in experimental renal failure animal models, is also part of this project.

1.1. RESEARCH OBJECTIVES

As mentioned earlier, the current study investigates the feasibility of using microencapsulated live *Saccharomyces cerevisiae* for use in renal failure uremia. This research study is divided into 3 phases, each characterized by several objectives:

Phase I: Preparation of Yeast Microcapsules and Process Optimization

1. To optimize the encapsulation of live yeast cells in order to design a novel Alginate-Poly-$_L$-Lysine-Alginate (APA) microcapsule for the immobilization of *Saccharomyces cerevisiae* for use in renal failure uremia.

Phase II: *In vitro* Studies

1. To evaluate the stability and the mechanical strength of yeast microcapsules with respect to mechanical agitation, peristaltic shear stress and osmotic pressure.
2. To test yeast microcapsules in simulated gastric and intestinal fluid environments with respect to urea removal and select the optimal yeast microcapsules loading.
3. To design and test a column bioreactor containing yeast APA microcapsules for use in renal failure uremia.

Phase III: *In vivo* Studies

1. To assess the pre-clinical efficacy of microencapsulated yeast cells in experimental uremic rat models.

Chapter II

COMPREHENSIVE LITERATURE REVIEW

2.1. THE KIDNEYS

A normal healthy human adult possess a pair of bean-shaped kidneys located in the posterior part of the abdomen. "There is one on each side of the spine; the right kidney sits just below the liver, the left below the diaphragm and adjacent to the spleen" (figure 2.1)[43]. Each kidney weights approximately 150 g[45] and is "about 10 cm long, 5.5 cm in width and about 3 cm thick"[43]. Anatomically speaking, each kidney consists of a renal cortex (the outer portion) and a renal medulla, which comprises between ten to twenty cone-shaped renal pyramids (figure 2.2).

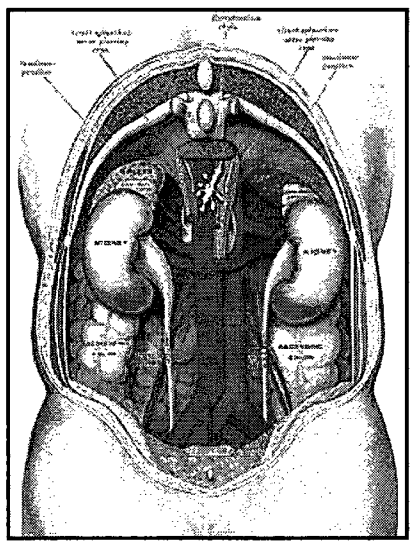

Figure 2.1: Human Kidneys Viewed from Behind with Spine Removed [43]

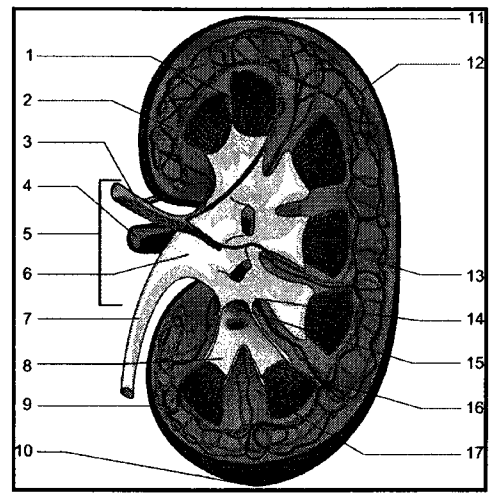

Figure 2.2: Parts of a Kidney. 1. Renal pyramid. 2. Efferent artery. 3. Renal artery. 4. Renal vein. 5. Renal hilum. 6. Renal pelvis. 7. Ureter. 8. Minor calyx. 9. Renal capsule. 10. Inferior renal capsule. 11. Superior renal capsule. 12. Afferent vein. 13. Nephron. 14. Minor calyx. 15. Major calyx. 16. Renal papilla. 17. Renal column [43]

The renal artery (figure 2.2), in part branching form the aorta, supplies the kidneys with blood. Upon renal entrance, this artery divides into smaller interlobar arteries, which divide further into smaller branches known as the cortical radial or interlobular arteries[43]. The afferent arterioles, which branch from these cortical radial arteries, are the smallest blood carrying units supplying the glomerular capillaries. The peritubular capillaries, which result from the glomerular capillaries' division, provide an extensive blood supply to the renal cortex.

The human kidney is composed of approximately one million nephrons (figure 2.3) located between the cortex and medulla and considered to be the basic functional unit of the kidney[46]. Each nephron is composed of a glomerulus and a tubule. The former consists of glomerular capillaries and the Bowman capsule whereas the latter consists of a proximal tubule, ascending and descending portions of the Henle loop, a distal tubule, and a collecting duct (figure 2.3). Nephrons regulate water and electrolytes balance by first filtering the blood, then reabsorbing necessary fluids and molecules and secreting unneeded ones. The nephritic reabsorption and secretion functionalities are achieved by co-transport and counter-transport mechanisms[43].

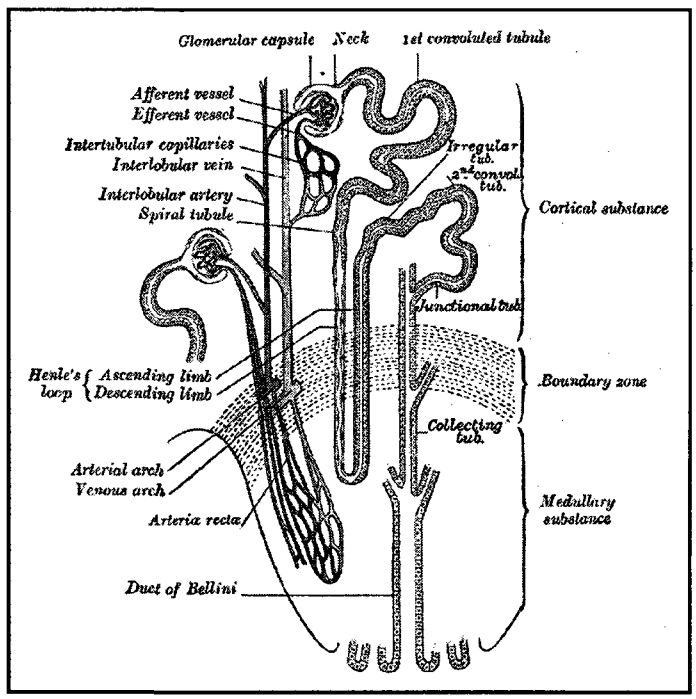

Figure 2.3: Nephron of the Kidney [44]

The kidneys are major bodily organs involved in whole-body homeostasis. Their primary role is to maintain the homeostatic balance of bodily fluids. This is achieved *via* the filtration, absorption, secretion of specific metabolites (such as urea, nitrogenous wastes and uric acid) and minerals (such potassium and chloride) as well as their excretion, along with water, as urine. Furthermore, this organ plays a crucial role in blood pressure regulation, glucose metabolism, acid-base balance and erythropoeisis[1],[43].

Renal diseases and disorders can be either congenital or acquired. Congenital hydronephrosis, congenital obstruction of urinary tract, duplicated ureter, Horseshoe kidney, Polycystic Kidney disease, renal dysplasia, unilateral small kidney, Multicystic Dysplastic and Big Kidney are examples of congenital kidney diseases. Among the most common acquired kidney diseases are Diabetic nephropathy, Glomerulonephritis, Nephritis, kidney stones kidney tumors, Pyelonephritis, and of course ARF and CRF[43]. One kidney is normally sufficient to provide daily renal functions and humans can live with a single kidney. Only when the amount of functioning kidney tissue is greatly diminished that RRTs become a necessity.

2.2. KIDNEY FAILURE

The improper function of the kidneys is an indication of kidney failure. As mentioned earlier, kidney failures are divided in acute and chronic. Biochemically speaking, both conditions are detected by elevated serum creatinine and urea levels but are however characterized by different creatinine and urea trends. Physiological indications of kidney failures can be: abnormal bodily fluid levels, deranged acid balance, or abnormal concentrations of electrolytes such as potassium, calcium, phosphate. Long-term persisting kidney problems have drastic "repercussion on other diseases, such as cardiovascular disease"[48]. Uremia is currently "used to loosely describe the illness accompanying kidney failure"[46].

[1] The process by which red blood cells (erythrocytes) are produced [47]

2.2.1. Acute Renal Failure (ARF)

2.2.1.1. Generalities

ARF, which is also known as acute kidney failure or acute kidney injury[49,50], is a rapid loss of renal function originating from kidneys' damage. This condition leads to nitrogenous (such as urea and creatinine) and non-nitrogenous waste products retention, which are all excreted by this organ under physiological normal functioning.

This adverse accumulation might be paralleled with other complications including metabolic acidosis[2], hyperkalaemia[3], and body fluid imbalance. ARF can be characterized either by Oliguria, a condition manifested by decreased urine production and "quantified as less than 400 mL/day in adults[51], less than 0.5 mL/kg/hr in children or less than 1 mL/kg/hr in infants", or by anuria, a condition defined by urine production's cessation[50].

2.2.1.2. Causes

ARF is usually subdivided into three categories based on *pre-renal, renal* and *post-renal* causes. The *pre-renal* causes are related to the blood supply and include hypovolemia[4], which usually results from dehydration or excessive diuretics use, hepatorenal syndrome, "in which renal perfusion is compromised in liver failure and vascular problems, such as atheroembolic disease and renal vein thrombosis"[50].

The *renal* causes are the most numerous and are related to kidneys' damage. Damage can be induced by sepsis[5], medication abuse, rhabdomyolysis[6] , in which myoglobin's blood release affects the kidney, hemolysis[7], in which hemoglobin damages the tubules, and acute glomerulonephritis, caused by "a variety of causes, such as anti-glomerular basement membrane disease/Goodpasture's syndrome, Wegener's granulomatosis or acute lupus nephritis with systemic lupus erythematosus"[50].

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[2] Acidification of the blood
[3] Elevated potassium levels
[4] Decreased blood volume
[5] Systemic inflammation due to infection
[6] Breakdown of muscle tissue
[7] Breakdown of red blood cells

Finally, the *post-renal* causes, which are characterized by urinary tract obstruction, are due to "medication interfering with normal bladder emptying, benign prostatic hypertrophy or prostate cancer, kidney stones, abdominal malignancy (e.g. ovarian or colorectal cancer) or obstructed urinary catheter"[50].

2.2.1.3. Diagnostic

According to Groeneveld *et al.*[52], the ARF age-related yearly occurrence is only 17 per million population (pmp) in adults less than 50 years old and around 950 pmp for individuals between 71 to 89 years old[53]. Either high creatinine or blood urea nitrogen levels are usually used in ARF's diagnostic. Such elevated levels may be backed-up by previous measurements of renal failures and this offers a comparative basis. When the underlying causes of ARF are not apparent and, thus, unknown, blood tests and urine examination are required. Moreover, to provide a definite diagnosis and a possible prognosis, kidney biopsy, in which live tissues are extracted for examination, should be performed.

Consensus criteria for the diagnosis of ARF have been established and agreed on by the scientific community. These include a risk criterion, characterized by a 1.5 serum creatinine increase or a urine production lower than 0.5 ml/kg body weight for 6 hrs; an injury criterion, characterized by a two folds increase in serum creatinine or a urine production lower than 0.5 ml/kg body weight for 12hrs; a failure criterion, reached when creatinine concentrations top 355 µmol/L (more than 3 times the normal) or when the urine output is 0.3 mL/kg for 24 hrs; and a loss criterion, which occurs in persistent ARF cases or when there is a complete loss of kidney function for more than 4 weeks period[51,54].

2.2.1.4. Treatment

When immediately diagnosticated and properly treated, ARF can be reversible, as opposed to CRF. The key physiological aspects that should be resuscitated are blood pressure and cardiac output. This can be done by closely monitoring fluid balance and

urine output *via* the insertion of a urinary catheter[8]. Typically, the administration of intravenous fluids improves the renal functionalities in the absence of fluid overloads.

The two most serious biochemical manifestations of ARF are metabolic acidosis and hyperkalemia, which require sodium bicarbonate administration and anti-hyperkalemic measures. When the patient's condition does not improve with fluid resuscitation, therapy-resistant hyperkalemia, metabolic acidosis and fluid overload, RRT is needed. It is worth mentioning that a good proportion of ARF patients will never re-gain full renal function, and, thus, require lifelong RRTs.

2.2.2. Chronic Renal Failure (CRF)

2.2.2.1. Generalities

The CRF, also identified as chronic renal disease or chronic kidney disease, is a progressive and somewhat slow loss of renal function over a period of months or years. It consists of five stages, in which the GFR, determined indirectly by the serum creatinine's level, deteriorates drastically until it reaches abnormally low values[55].

The first stage of CRF is characterized by mildly diminished renal functions with a normal or increased GFR (>90 mL/min/1.73 m^2). Stages 2 to 4 are reached when GFR is reduced to 89-60 mL/min/1.73 m^2 (mild reduction), 59-30 mL/min/1.73 m^2 (moderate reduction), 29-15 mL/min/1.73 m^2 (severe reduction), respectively. The mild GFR's reduction of the second stage is accompanied by kidney damage[56]. Finally, stage 5 is characterized by a GFR lower than 15 mL/min/1.73 m^2 and requires permanent RRTs due to the severity of the illness. This particular stage is generally referred to as end-stage renal disease, or simply ESRD.

As mentioned in the introduction, no specific symptoms alert for the onset of CRF, which can only be detected by an "increase in serum creatinine or protein in the urine"[57]. With continuous kidney malfunction, potassium accumulates, blood pressure increases due to fluid volume overload[9], urea accumulates leading, in consequence, to

[8] It is "useful for monitoring urine output as well as relieving possible bladder outlet obstruction, such as with an enlarged prostate" [52]

[9] "symptoms may range from mild edema to life-threatening pulmonary edema" [59]

azotemia and eventually uremia. Furthermore, anemia, which occurs due to the decrease in erythropoietin synthesis, hyperphosphatemia and metabolic acidosis might also be seen in CRF[58]. Moreover, CRF-affected individuals are reported to have higher incidence of cardiovascular diseases (CVDs).

Individuals with a GFR lower than 60 mL/min/1.73 m^2 for a period equivalent to three months are automatically classified as CRF-affected subjects, irrespective of the presence or absence of kidney damage. The rationale behind this classification "is that reduction in kidney function to this level or lower represents loss of half or more of the adult level of normal kidney function"[59]. Similarly, individuals with kidney damage are grouped under chronic kidney disease, irrespective of the level of GFR. The rationale for including these individuals is that such patients present increased risk of kidney function losses and CVDs' development[60]. Regardless of whether stage 5 is reached or not, CVDs are qualified as number one killer in patients with chronic kidney diseases[61-63].

2.2.2.1. Causes

Diabetic nephropathy, hypertension, and glomerulonephritis are considered to be the most common causes of CRF, and represent together approximately 75% of all adult cases. Historically, there has been a tendency to classify kidney disease with respect to human anatomy. For instance, "large vessel disease, such as bilateral renal artery stenosis, and small vessel disease, such as ischemic nephropathy, hemolytic-uremic syndrome and vasculitis"[57], are all grouped under vascular kidney diseases. The glomerular diseases comprise focal segmental glomerulosclerosis and IgA nephritis, both considered as Primary Glomerular disease, but also, Secondary Glomerular disease, such as diabetic nephropathy and lupus nephritis. Diseases concerned with the tubule and the intestine are referred to as Tubulointerstitial diseases and include rug and toxin-induced chronic tubulointerstitial nephritis, reflux nephropathy and polycystic kidney disease. Finally, obstructive diseases can either be due to kindey stones or prostate's diseases[57].

2.2.2.2. Diagnostic

Due to the reversible aspect of ARF, it is very important to differentiate it from CRF. Commonly, abdominal ultrasound, in which the size of the kidneys is measured, is used as a diagnostic means for distinguishing between ARF and CRF. Generally, kidneys of CRF's patients are 9 cm smaller than normal ones. A gradual rise in serum creatinine over several months or years is an indication of a chronic condition, and can, thus, be used as another diagnostic tool. This progressive increase is opposed to the ARF's serum creatinine sudden rise that takes place over several days to weeks.

2.2.2.3. Treatment

The principal goal of therapy is to "slow down or halt the otherwise relentless progression" of CRF to its last stage[57]. This is achieved *via* blood pressure control and treatment of the original disease by administrating angiotensin II receptor antagonists (ARBs) or angiotensin converting enzyme inhibitors (ACEIs) to slow the progression to stage 5[64,65]. Erythropoietin and vitamin D3 hormonal replacements are, in many cases, necessary. The use of phosphate binders to control this molecule's elevated serum level is also required. However, when stage 5 is reached, RRT, which severely impart the quality of life (QOL)[66,67], is highly needed.

In some cases, ARF can occur on top of CRF. This condition is called acute-on-chronic renal failure (AoCRF). As in ARF, the acute part of AoCRF may be reversible if it is promptly and appropriately treated by re-adjusting the patient's renal function to baseline thresholds.

2.3. UREMIA AND DIALYSIS

The need to quantify uremia is of crucial importance in renal failure because it helps nephrologists decide when to administer RRTs without being too late. To date, there is still no global consensus for satisfactory urea levels in kidney failures. Katirzoglon *et al.*[68] reported blood urea nitrogen (BUN) levels between 90–100 mg/dL. Others, such as Cameron *et al.*[69] and Indraparasit *et al.*[70] considered urea clearance levels of 12–20 mL/min to be indicative of renal failure onset. However, all these

"studies suffer from limited sample size and inappropriate measurements for catabolism state"[71]. For this reason, relying on GFR measurements seems to be the safest predictive parameter for kidney failure onset.

Historically, dialysis dosage has been quantified based on creatinine clearance. However, dialysis dosage can also be estimated using three separate parameters: the urea depuration, k in mL, the dialysis treatment duration, t in minutes, and the patient's urea distribution volume, v in mL. kt/v is the mathematical formula expressing the prescribed or delivered dialysis dose. Another way of quantifying dialysis dose uses the Solute Removal Index (SRI) "which shows urea reduction per dialysis session"[71]. According to Chitalia *et al.*[72], a score greater than 20% is considered adequate and sufficient as a dialysis dose. In the absence of accepted standards for renal failure, urea and creatinine's clearances and kt/v values are compared to benchmarks for ESRD patients set by the National Kidney Foundation[72-76].

2.4. CURRENT TREATMENT MODALITIES

Current renal replacement therapies consist of kidney transplantation (KT), dialysis modalities or hemofiltration (HF). Dialysis constitutes an enormous domain by itself and can be broadly divided into hemodialysis (HD) and peritoneal dialysis (PD). Each one of these branches can then be further divided into subcategories. All of these modalities will be discussed in great details in the subsequent sections below.

2.4.1. Kidney Transplantation (KT)

2.4.1.1. Generalities

Each kidney is thought to have about 4 to 5 times the minimal required renal function. Hence, the rational behind KT is that the donation of one kidney should still leave a donor with more than sufficient renal function. Nonetheless, donating a kidney reduces the GFR by approximately 25%[77], but this should not jeopardize daily renal functions due to the elevated work capacity of each kidney[78]. The average lifetime for a donor kidney is 10-15 years conferring the patient a high flexibility and maneuverability.

KT is the closest alternative to a definite cure and the need of a transplant is always indicated by ESRD, regardless of its primary underlying cause(s). To date, the most common cause behind KT is still diabetes, which accounts for approximately 25% of all North American transplantation[79]. Due to the chronic shortage of available organs for transplantation, KT is recognized as an impractical solution in most cases of ESRD. The number of KT performed each year is less than 10,000 while the number of patients awaiting transplantation is six times greater[80].

KT is in the majority of the cases accompanied with a life-lasting immunosuppressive therapy, which is supposed to help prevent organ rejection. The metabolic advantages of the transplantation therapy are somehow lessened due to the drawbacks of the administered immunosuppressants, which can lead to infections and/or changes in appearance (e.g. fuller face, weight gain, facial hair growth) by weakening the immune system[81]. Kidney transplant's patients must follow a restricted diet in which salt and calories must be limited. This is due to the given medications that increase both appetite and sodium retention[81].

Transplantation and donation are not for everyone. Cardiac and pulmonary insufficiencies, hepatic disease, concurrent tobacco use, morbid obesity and recent cancer occurrence are contraindicators putting a patient at a higher risk for surgical complications[79]. Post-transplantation problems include transplant rejection[10], which occurs in 10% to 25% of the cases during the first two month[82], delayed kidney functioning, infections and sepsis resulting from the immunosuppressant drugs, lymphoproliferative disorder, electrolytes imbalances[11], hirsutism, hair loss, obesity, acne, Type II Diabetes Mellitus and hypercholesterolemia[79].

Requirements for KT, such as donor and recipient age, their health histories, "vary from program to program and country to country. Many programs place limits on age[12] and require that one must be in good health"[79].

[10] Rejection does not necessarily mean loss of the organ, but may require additional treatment.
[11] such calcium and phosphate which can lead to bone problems, GI inflammation, stomach ulceration
[12] e.g. the person must be less than 69 years old when put on the waiting list

2.4.1.2. Sources of Kidneys and Other Related Issues

The kidney's source can either be from deceased-donors[13] (DD) or from living-donors (LD). DD can be divided in two groups: brain-dead (BD) donors ("heart-beating") or donation after cardiac death (DCD) donors, which "are patients who do not meet the brain-dead criteria, but have no chance of recovery whatsoever"[79]. BD kidneys, because they have not been exposed to warm ischemia[14], are generally of a superior quality when compared to kidneys from DCD. LD renal transplants are also further divided into genetically related (a.k.a. living-related) or non-related (a.k.a. living-unrelated) transplants, depending on biological relationship between the donor and recipient[79]. Due to the shortage in DD transplants, LD offers a bigger advantage by avoiding the long waiting times.

Currently, there are three factors used in matching kidneys with potential recipients. Firstly and most importantly, there must be blood type compatibility between donor and recipient. Secondly, human leukocyte antigens (HLAs) can differ between the donor and recipient and still allow a compatible transplant[15]. Thirdly, cross-matching antigens result in negative/positive cross-match upon mixing a sample from the recipient's blood with a sample of the donor's blood[81]. Ideally, in order to decrease the risk of transplant rejection, the donor and recipient should share the least amount of antigens and as many HLAs as possible[79].

Traditionally, most donated kidneys come from DD; however, LD transplantation is rising recently in the US[2]. In 2006, about 47% of all donated kidneys came from LD[82]. Moreover, recent statistics show that unrelated LD represent more 30% of the all live donations and, thus, are almost as common as related LD[2]. This is explained by the FDA approval, in 2004, of the Cedars-Sinai High Dose IVIG protocol, which, based on the efficiency of immunosuppressive therapy, eliminates the need for donor-recipient blood type (ABOcompatible) compatibility or tissue match[79]. In a study conducted by Gloor et *al.*, 41 patients (20 with a positive cross-match and 21 with ABO incompatible grafts) have undergone transplantation[83,84]. 43% of the positive cross-match recipients

[13] Formerly known as cadaveric
[14] Time between the stopping and the kidney being cooled
[15] 17% of all American DD kidney transplants have no HLAs mismatch

developed acute, humoral and cellular, rejection while "no hyperacute rejection has been seen in the ABO incompatible grafts. The 1-yr graft survival was 79% for positive cross-match transplants, 94% for ABO incompatible grafts, and 96% for conventional"[84].

3-5 days vs. 7-15 days are normally required to reach normal functioning levels with living and cadaveric donations respectively. 85 to 90 % of DD transplants are still functioning one year after the surgery. Living-related donation are, however, known to work better than either living-unrelated or DD transplants, simply because they are usually a closer match (figure 2.4).

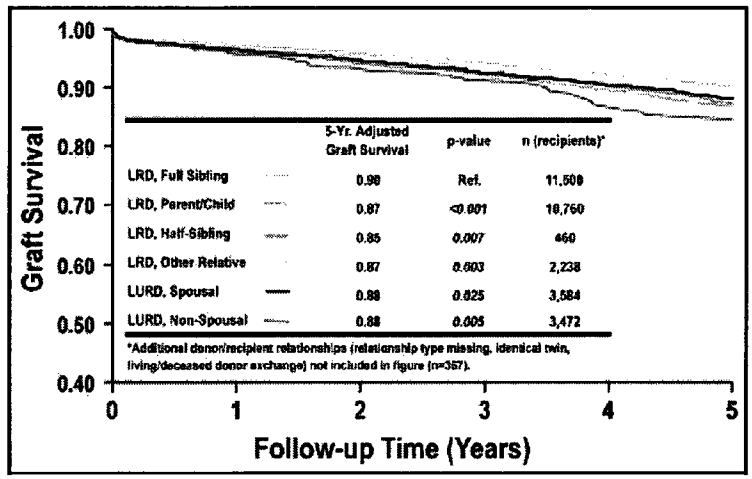

Figure 2.4: Five-year graft survival according to donor relationship to the recipient[2]

2.4.1.3. Procedure and Further Safety Complications

The removal of the barely functioning existing kidney during the transplant operation was reported to increase the rates of surgical morbidities[79]. For this particular reason, it is advisable to keep the native kidney in position and place the donated one in a location different from the original kidney. This then forces the use of a different blood supply route, which consists of connecting the renal artery to the external iliac artery in the recipient and connecting the renal vein of the new kidney to the external iliac vein in the recipient. In rare occasions, the kidney is transplanted together with the pancreas. This is generally done for Type I Diabetes Mellitus patients, "in whom the diabetes is due to destruction of the Beta cells of the pancreas and in whom the diabetes has caused renal

failure (diabetic nephropathy)"[79]. Such procedures are commonly referred to as "SKP transplant", which stands for "simultaneous kidney-pancreas transplant" and "PAK transplant", which stands for "pancreas after kidney transplant"[79].

Laparoscopic surgery is used in live KT. Compared to open procedures, this technique has been credited for the shorter hospital stays (2-4 days vs. 3-7 days), less incisional discomfort, and an earlier return to work (12-21 days vs 30-60 days)[84-90]. Currently, laparoscopic nephrectomy accounts for over 50% of the donor nephrectomy procedures in the US[91].

The underlying premise of living kidney donation is that the removal of one kidney does not impair survival or long-term kidney function. Reports of relatively homogeneous northern European populations after nephrectomy suggested that this procedure is safe [91-94]. Nevertheless, Ellison *et al.* [93] found that ESRD's rate in donors was comparable to that in the general U.S. population (0.04% vs. 0.03% respectively). In another report by Ramcharan and Matas[94], around 1% (5 out of 464) of the donors developed ESRD and three others had abnormal renal function. Ergo, there might be a little long-term medical risk to a healthy donor after unilateral nephrectomy. The significance of this risk is however not fully assessed yet.

2.4.2. Dialysis

2.4.2.1. Generalities

Dialysis is majorly employed to provide an artificial RRT for those individuals with kidney failures[64]. Dialysis uses a countercurrent flow system between blood and dialysate solution. It works on the principles of the diffusion of solutes and fluid across a semipermeable membrane. Small molecules such as urea and creatinine, usually excreted by the kidney, can pass across this membrane down their high to low concentration gradients. Removal of fluid from blood is achieved by applying hydrostatic pressure across the membrane[65]. Currently, HD and PD are the two types of dialysis available. Several acute or chronic factors influence the decision to initiate HD or PD in renal failure's patients.

2.4.2.2. Hemodialysis (HD)

2.4.2.2.1. Generalities

HD, which is also called haemodialysis, (figure 2.5) uses the same operational modality as the one described in 2.4.2.1. Briefly, it involves the diffusion of solutes across a semipermeable membrane and utilizes countercurrent flow between the dialysate and the blood, which flows in the extracorporeal circuit. This flow scheme maintains the concentration gradient across the membrane at a maximum and increases the efficiency of the dialysis. Ultrafiltration is the basis of the fluid removal and is controlled "by altering the hydrostatic pressure of the dialysate compartment, causing free water and some dissolved solutes to move across the membrane"[96].

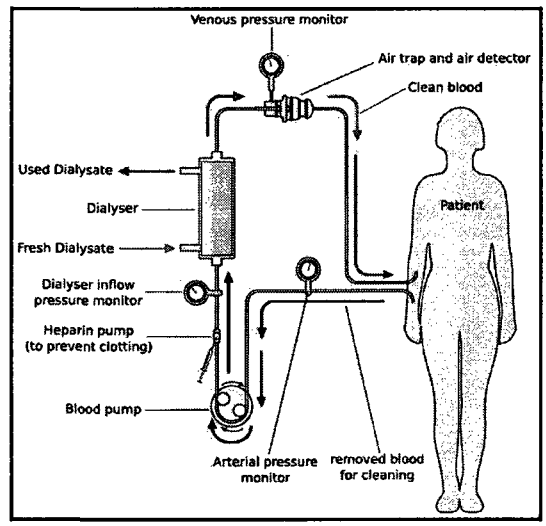

Figure 2.5: Schematic of a HD Circuit[96]

HD can be either an outpatient or inpatient therapy. In general, it is proportional to body mass: "the larger the body size, the more dialysis a patient will need"[96]. In North America and the UK, routine HD, which is conducted in dialysis outpatient facilities, is done three times a week with each treatment lasting from 3-5 hrs depending on the severity of the renal failure and, hence, the final concentrations that are targeted. "Twice-a-week sessions are limited to patients who have a substantial residual kidney function. Four sessions per week are often prescribed for larger patients, as well as patients who have trouble with fluid overload"[96]. Usually, due to the lengthy treatment hours, patients

can occupy themselves with other activities, such as reading or watching TV[81]. Recently, there has been a growing interest in daily home HD (DHHD)[96], which consists of 1.5-4 hrs sessions given 5-7 times per week, and in nocturnal home HD (NHHD), which is ideally administered 3-6 nights per week for 8-10hrs per night.

A sterilized solution of mineral ions is used as the dialysis solution. In general, potassium, phosphate urea and other waste products, which are expected to diffuse out of the blood, are absent in the dialysis solution. Sodium and chloride concentrations in the dialysis solution are, however, kept similar to those of normal plasma to avoid any loss. Acidity is, most of the time, corrected by the addition of high bicarbonate concentrations in the dialysis solution. A small amount of glucose is also commonly used.

A proper restricted diet is required for patients under HD to reduce the wastes' generation in their blood and, consequently, limits, as much as possible, the dialysis's sessions. Potassium's amounts and liquid intake should be carefully monitored to decrease the risk of hypertension, heart trouble, and cramps during dialysis. Furthermore, salty food should be limited to the maximum because it makes the body hold more water. Food containing large amounts of phosphorus, such as milk, cheese and nuts, should also be reduced because it render bones weak and brittle and can cause arthritis by affecting calcium bone resorption[81].

Most of the dialyzers in use today are cylindrical hollow fibers, characterized by semipermeable wall membranes. It is through these hollow fibers, which form very thin capillary-like tubes, that blood is pumped while the dialysate flows through the space surrounding the fibers. High-level disinfection procedures are required after each session to allow the re-use of the dialyzer[97]. The newest dialysis machines available on today's market are highly computerized to "continuously monitor an array of safety-critical parameters, including blood and dialysate flow rates, dialysis solution conductivity, temperature, pH and analysis of the dialysate for evidence of blood leakage or presence of air"[96]. Alarming systems are used to notify any reading that is out-of-bound. Fresenius, Gambro, Baxter, B. Braun and Bellco are the principal dialysis machines' manufacturers.

2.4.2.2.2. Vascular Access

Vascular access is usually required to gain into the patient's blood stream. There are three primary methods for this purpose: an intravenous catheter, an arteriovenous (AV) fistula or a synthetic graft. Factors such as the expected time course of a patient's renal failure and the condition of his/her vasculature affect the type of vascular access (figure 2.6).

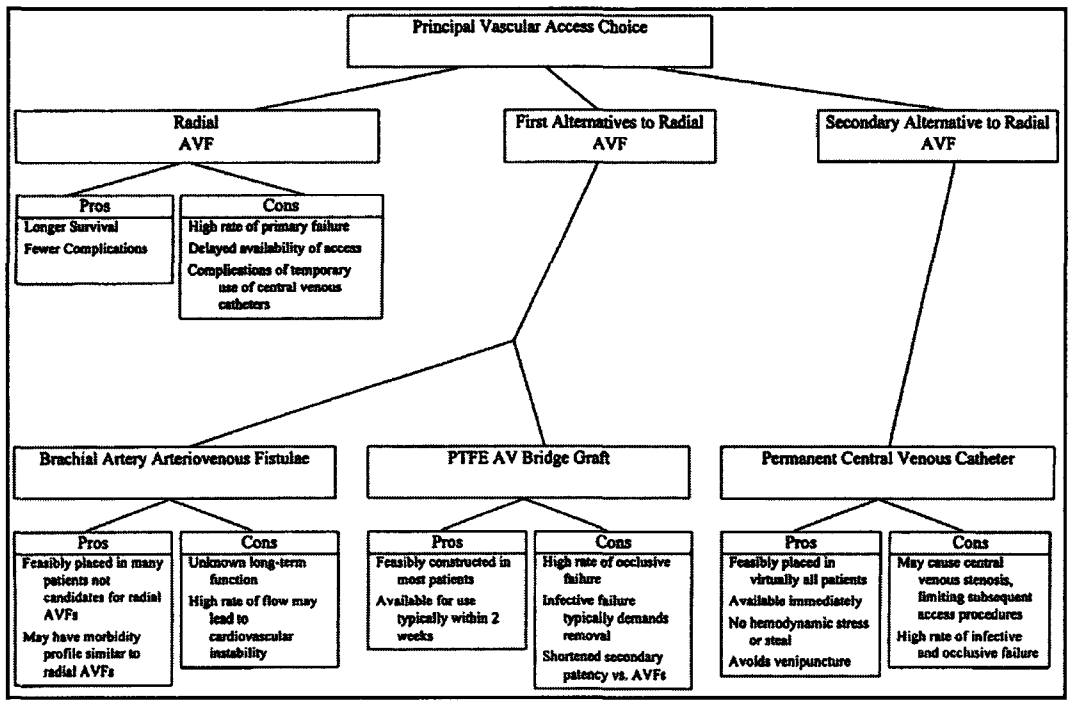

Figure 2.6: Vascular access selection: what are the tradeoffs?[101]

Catheter access consists of inserting a plastic catheter with two lumens into the vena cava. It is typically used in immediate dialysis for rapid access, in patients with reversible ARF and ESRD's patients "who are either waiting for alternative access to mature or who are unable to have alternative access"[96]. Because this type of access does not require needles for the attachment to the dialysis machine, it is often popular with patients. Two varieties of catheters, tunnelled and non-tunnelled, exist for vascular access. For a 10 days short-term access or for a single dialysis session, non-tunnelled catheters, which emerge from the skin at the site of entry into the vein, are used. In the

case of a long treatment period, tunnelled catheters are inserted under the skin and link the venous insertion point to a distant exit site. Usually, the insertion point is the internal jugular vein in the neck and the exit site is located on the chest wall.

AV grafts are made surgically by joining an artery and an artificial vein together *via* anastomosis. Generally, the graft can either be of synthetic nature, such as poly(tetrafluoroethene) (PTFE), or it can be chemically treated, sterilized xenograft. AV fistulas are the preferred access method. They are very similar to AV graft expect that no artificial material is used. The hand, the forearm or the elbow of the nondominant arm are the usual location to insert an AV fistula, which on average takes 4-6 weeks to mature. For vascular access two needles are used to draw and return blood to the patient. Only when the patient's native vasculature does not permit a fistula, a graft is then used. Note that on average because of the synthetic nature of AV grafts, these catheters are more prone to infection and, thus, can deteriorate faster compared to fistulas[96].

2.4.2.2.3. Side Effects and Possible Complications

The continuous or rapid removal of too much fluid leads to the dialysis hangover or dialysis washout[98]. This term refers to a collection of symptoms including hypotension, fatigue, chest pains, leg-cramps, nausea, vomiting and headaches, which complicate 20-30% of HD dialysis sessions[81,99,100]. The severity of these symptoms is proportional to the amount and speed of fluid removal. By limiting fluid intake or increasing the dialysis dose, achieved by dialyzing more often or for longer periods, it is possible to avoid and/or lessen the severity of these symptoms.

Endocarditis[16] or osteomyelitis[17] may be seen in patients undergoing HD. Due to need of anticoagulant therapy, some patients might develop heparin allergies, which can reduce the platelets count. Another possible complication is First Use Syndrome. Even though it is rare, this syndrome is a severe anaphylactic reaction manifested *via* "sneezing, wheezing, shortness of breath, back pain, chest pain, or sudden death"[18,[96]].

[16] An infection affecting the heart valves
[17] An infection affecting the bones
[18] In recent years, the incidence of First Use Syndrome has fallen off, due to an increased use of gamma irradiation, steam sterilization, or electron-beam radiation instead of chemical sterilants, and the development of new semipermeable membranes of higher biocompatibility"

Other HD complications concern the rise in the hospitalization of vascular access related morbidities. Common problems include infection, blockage from clotting, and poor blood flow[81,101]. Aside from infection, venous stenosis is another serious problem with catheter access due to the non-self or foreign nature of the catheter. This often leads to inflammatory reactions which result in venous lumen narrowing, and, if not treated might results in clotting or thrombosis. Amyloidosis, neuropathy and various forms of CVDs are the most commmon long-term complications of HD. These can be improved by also increasing the frequency and length of the HD's treatment[102,103].

2.4.2.3. Peritoneal Dialysis (PD)

2.4.2.3.1. Generalities

PD (figure 2.7), which is a special version of dialysis, relies on the use of the peritoneal membrane, a physiological membrane surrounding the intestine, as a natural semipermeable membrane. A PD catheter, surgically placed in the patient's abdomen, is used to instill dialysis fluid. The exit site depends on the surgeon's or patient's preferences, which are highly influenced by the patient's anatomy and/or hygiene issues. The generated excess fluid can be removed by altering the glucose concentration *via* osmotic manipulations. PD, unlike HHD, does not require help from a trained partner[81] and can be done almost anywhere; all that the patient needs is "a way to elevate the bag of dialysis fluid and a method of warming the fluid"[104]. However, due to psychological annoyances, most of the patients undergoing PD do it in private places, such as home and workplace.

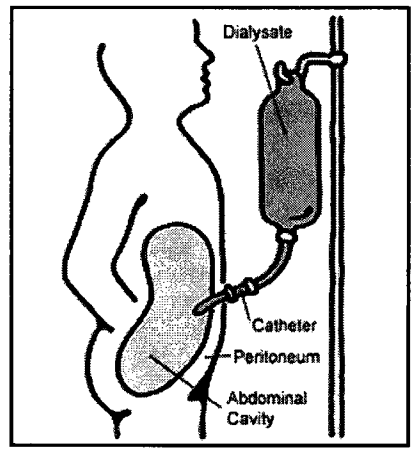

Figure 2.7: Schematic of PD[104]

Conventional PD modalities, which require the use of dialyzers, are based on intermittent techniques and are, thus, referred as intermittent PD (IPD). With increasing dialysate flow, the dialytic downtime, represented by the inflow and outflow phases,

becomes highly important and results in an increasingly negative influence on the overall dialytic efficiency. From this observation stemmed the concept of continuous flow PD (CFPD). It was "developed as a technique in which a certain amount of fluid was constantly kept in the abdomen and the inflow and outflow were maintained constant, without interruption, thanks to two dwelling catheters"[19,[105]].

Currently, there are two types of CFPD. Continuous ambulatory PD (CAPD) is the most popular because it does not necessitates any machine and can be done in any clean, well-lit place. Usually, it takes around 30 to 40 min to drain the used dialysis solution and replace it with a fresh one. Most people change the dialysis solution at least four times a day[20] and, thus, sleep with it at night. The biggest advantage of CAPD is the continuous cleaning of the patient's blood[78,106,107].

The other type is cyclic PD (CCPD), which is usually performed at night. CCPD uses a cycler, which is similar to the one used in IPD, to nocturnally fill and empty the abdomen 3-5 times. This modality is, hence, characterized by a 24 hrs dwell time single exchange[81,104]. A combination of CAPD and CCPD is, also, used if the patient weighs more than 175 pounds or if his/her peritoneum filters wastes slowly. In such cases, a nocturnal cycler is used joint with one exchange during the day. In other cases, it is, however, possible to do four exchanges during the day and use a minicycler to perform one or more exchanges during the night[81].

The technical simplicity and the lack of extracorporeal circuit and bleeding risk are among the advantages conferred by PD. This RRT permits solute and liquid removal with excellent cardiovascular tolerance due to its gradual and continuous operability. It is, therefore, less hemodynamically instable and this "reduces both kidney aggression by ischemia and risk of hydroelectrolytic imbalance"[110]. It can also be used to maintain volemic control in patients with congestive heart failure[111,112], control hyperthermia and treat necrohemorrhagic pancreatitis. PD is an excellent candidate to treat kidney failures in children and patients with difficult vascular access[113,114].

The dialysis solution comprises known amounts of glucose dissolved in water. The needed osmotic gradient is determined by the strength or dilution extent of the

[19] Catheters usually take 10 days to 3 weeks for the insertion site to heal [48]
[20] Which is equivalent to a dwell time of at least 6 hr

glucose solution. Typical strengths of glucose are 0.5%, 1.5%, 2.5% and 4.25%. More concentrated solutions lead to greater removal of fluid and wastes into the abdominal cavity, increasing, in consequence, both "early and long-dwell exchange efficiency"[104]. Moreover, heating the dialysis solution to body temperature avoids causing cramping.

As in most dialysis modalities, salt and liquids must be limited while protein intake must be increased. Unlike the other modalities, patients may need to eat high-potassium foods and cut back on the number of calories in the dialysis fluid that may cause weight gain.

2.4.2.3.2. Possible Complications and Limitations

PD does have some limitations, such as the need for an intact peritoneal cavity. Recent abdominal surgery and, to some extent, the presence of a dynamic ileum and peritoneal fibrosis or adherences are other counter-indications[113,114]. It is less effective, compared to HD, in treating emergency situations such as acute pulmonary edema, poisoning, or drug intoxication. Concern exists that PD can not properly control uremia due to a lower solute clearance compared to daily HD. There are also controversies concerning its use in patients with severe hypercatabolism[72,115]. Technical problems related to fluid handling, inadequate peritoneal access and poor quality of sorbents hamper, to some extent, the use of PD[108].

The most common problem with PD is infection, which is limited to the skin or soft tissue around the catheter. "Infections that reach the peritoneum (peritonitis) are more serious, and will likely require antibiotics and/or supportive care. If the peritonitis is severe, removal of the catheter and a change of RRT modality to HD may be necessary"[81,104]. Redness, tenderness and drainage are all indicators of infection, which must be quickly treated to avoid any subsequent complications.

Long-term PD was reported to cause changes in the peritoneal membrane, decreasing, in consequence, its permeability till it can no longer act as a dialytic membrane. Other complications are related to fluid leaks, which are manifested by hernias. The latter occurs due to the abdominal fluid load and often requires immediate repair before the recommencement of PD. Fibrin accumulation in the effluent is another

problem. This can lead to draining and/or filling issues "if too much fibrin collects in or around the catheter inside the peritoneum"[104]. Heparin injection in the dialysate bags is used to break up fibrin accumulation.

2.5. ISSUES AND LIMITATIONS OF CURRENT RRTS

In the U.S., the incidence of ESRD and its related problems will increase sharply in the near future and this rise will unfortunately not be counterbalanced by an increase in the capacity of current methods of RRTs[120,121]. This is partially explained by the continuous aging of the ESRD population, which engenders in consequence more health related problems. The elderly portion of the population, those older than 65 years, represents the fastest growing sector of the general population in the Western world. This portion has grown from 4% in 1900 to its current level of approximately 12.7% in the U.S. Demographic studies predict that this proportion will reach 20% in 2030. In Europe, the population having more than 60 years of age is expected to be around 224 million individuals in 2025[122]. It can, therefore, be expected that the incidence of renal failure in the elderly population will certainly increase[53].

To date, numerous fundamental management issues concerning RRTs remain unresolved both in ARF and CRF. The indications and timing of initiation of therapy, modality selection, and the optimal dosing strategy for each modality are examples of such issues[123]. The role of the kidneys "in reclamation of metabolic substrates, synthesis of glutathione, gluconeogenesis, ammoniagenesis, catabolism of peptide hormones and growth factors, as well as production and regulation of multiple cytokines [...] are not addressed by current treatment modalities"[124-129]. These same modalities are at the same time uncomfortable and inconvenient for a significant number of patients due to multimedication requirements and QOL's derangements.

All these realizations have boosted the need for novel, non-invasive, practical RRTs characterized by rapid, if not instantaneous, operability and outstanding efficiency. Furthermore, there is also a considerable drive to reduce morbidity, mortality and the overall economic impact associated with kidney failure. Due to medicine's monofactorial approach in treating this disease, such ambitions lie beyond conventional medical reach.

"Into this breach steps the nascent and expanding field of cell therapy"[130] and tissue engineering, which "offers the promise of harnessing the native abilities of the cell"[131] and allows implementing new potential treatment modalities surpassing the qualitative and quantitative limitations of the current RRTs[121].

2.6. POTENTIAL NOVEL TREATMENTS FOR RENAL FAILURE AND UREMIA

Innovative approaches to overcome the limitations of current RRTs include therapies centered on tissue engineering, cell technology and mechanical renal replacement devices.

2.6.1. Tissue Engineering of Renal Failures

The proximal tubular cells play a crucial role "in supporting the metabolic, endocrine, and immune functions of the kidney, all of which, in addition to the reabsorption function, are compromised in renal failure"[132]. Isolation and culture of these cells with the objective of integrating them into a more optimal RRT device for the ARF or CRF patients has been reported by Humes *et al.* back in 1992[132]. This device is called the bioartificial renal tubule assist device (RAD)[121].

Critical to the success of this cell therapeutic strategy is the isolation and *in vitro* growth of proximal tubular cells (figure 2.8). Tubule cells cultures are expanded from adult kidneys[23,132], which are then grown along the inner surface of the hollow fibers in a standard HF cartridge[130]. Biocoating the membrane with an extracellular matrix promote comprehensive cell attachment. The source of the tubule cells dictates the matrix selection. "For porcine RADs, either proNectin-L (a laminin-derivative) or murine laminin have been used and for the human RADs, either murine laminin or bovine collagen type IV are used"[133]. Combining the high-flux HF membrane with the extracellular matrix forms an immune-barrier as well as scaffold for the cells of the bioartificial tubule[133].

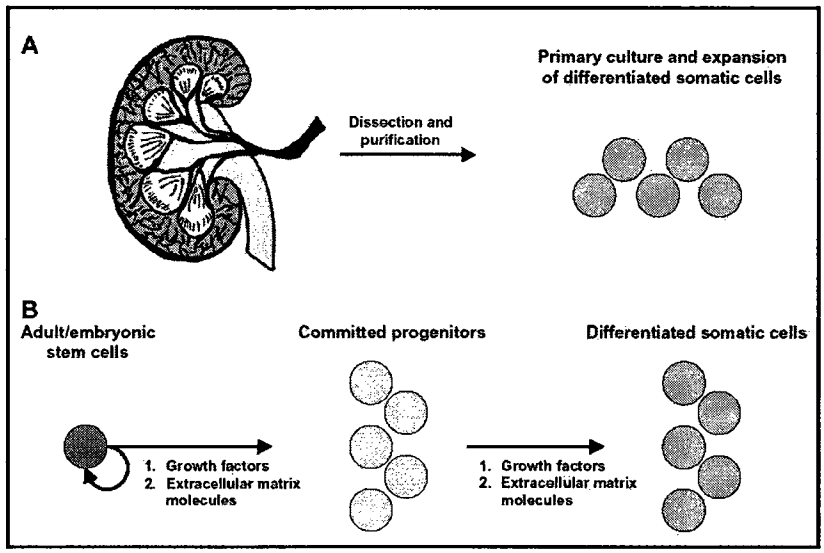

Figure 2.8: (A) A methodology for the purification and expansion of primary cells from adult kidney exists. Primary cells are retrieved from postmortem kidneys through a regimen that includes enzymatic digestion, sieving, and centrifugation. Cells are expanded in vitro before seeding into a bioartificial renal tubule assist device (RAD). Progenitor and stem cells have been purified from other tissue sources, including muscle and skin. **(B)** Alternative methodologies for generation of fully functional differentiated somatic cells are currently being investigated by researchers in the stem cell field. The working hypothesis is that adult or embryonic stem cells can differentiate into somatic cells if placed into the appropriate chemical and physical environment. A major challenge of this effort is to reproduce the complex temporal, spatial, and signaling events that occur during development[80]

The setup of the bioartificial renal tubule is comprised of a passive conventional hemofilter followed in series by the tubule RAD unit (figure 2.9)[23,130]. Using peristaltic pumping and ultrafiltration[21], blood is pumped out of a large animal and is delivered into the RAD's tubule lumens. "Direct contact between the ultrafiltrate and the tubule cells lining the membrane allows them to carry out their metabolic regulated transport functions. [...] Processed ultrafiltrate exiting the RAD is collected and discarded as "urine" while the filtered blood exiting the hemofilter enters the RAD through the extracapillary space port and disperses among the fibers of the device. The direct bathing of the tubule cells within the RAD by ultrafiltrate and transmembrane contact with the filtered blood delivers the metabolic substrates, low-molecular weight growth factors, and

[21] generated as the blood passes through the hemofilter

oxygen required to maintain their viability while preserving immunoisolation of the heterologous tissue"[130]. A third pump, which is used to maintain hydraulic pressures within the unit, delivers the processed blood exiting the RAD back into the animal (figure 2.9). To diminish clotting risks within the device itself, Heparin is continuously injected into the blood before entering the RAD. The device is oriented horizontally and is continuously controlled at physiological temperature (37°C) to ensure optimal functional cells functionality[130].

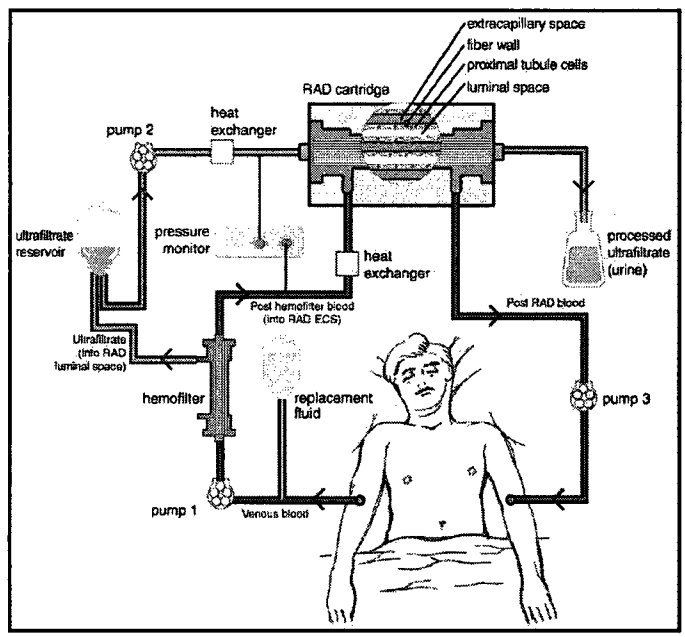

Figure 2.9: Schematic of the Bioartificial Kidney[130]

Stem cell therapy is another new and exciting therapeutic approach to acute and chronic diseases[134-136]. "The potential success of the treatment lies in the growing appreciation that most disease processes are not caused by the lack of a single protein but result from alterations in the complex interactions of a variety of cell products"[121]. Briefly speaking, stem cells are characterized by a high capacity for self-renewal and the ability to differentiate under defined conditions into specialized cells that develop into the structure and functional components of a physiologic organ system. Three different classes of stem cells have been identified based on their levels of competence. Totipotent stem cells are those which can give rise to all three embryonic germ layers as well as

extraembryonic tissues. Pluripotent cells can contribute to all three germ layers of the embryo and multipotent cells possess the potential to differentiate into multiple cell types excluding derivatives of all three germ layers[130].

Numerous researches studied the feasibility of using stem cells for kidney failure treatment purposes. In experimental glomerulonephritis, bone-marrow-derived endothelial and mesangial cells were found to contribute to renal repair[137-139]. In 2005, Feng Z *et al.* showed that transplanting bone marrow from young mice into older animals reversed insidious glomerular hypertrophy and sclerosis in the animal[140]. These findings suggest that bone marrow contain "a subset of cells that can be released "into the circulation and replace endothelial, mesangial and tubular cells"[120]. Braam *et al.* used this advantageous fact to propose a "refurbishing strategy for extensive kidney repair" mainly based on using mesenchymal or bone-marrow-derived stem cells[120].

Another group investigated the feasibility of using nuclear transplantation with stem cells in order to culture kidneys[141]. Therapeutic nuclear cloning generates autologous stem cells, which when used to treat kidney disease can prevent immunological reactions in the nucleus donor. The transplantation of autologous stem-cells is based on the expansion of pluripotent cells from a diseased organ. Making use of the theory, Lanza *et al.*[142] generated bovine uterus blastocysts by the nuclear transplantation from fibroblasts into bovine oocytes. After 12 weeks of *in vitro* development, resulting kidney-like structures were reported to contain "glomeruli and tubules and produced urine-like fluid" erythropoietin and 1,25-dihydroxyvitamin-D3. Furthermore, these structures presented proteins specific to different renal cell types, such as aquaporin-1, which is found in proximal tubules, and Tamm–Horsfall protein, which is a characteristic of the ascending loop[143].

Distributed renal functionality, which is still in its infancy, is another strategy which could redirect RRT. This approach consists of injecting cells that degrade a variety of substances into the peritoneum[144]. Transfection of the gut with a phosphate transporter for extrusion of phosphate, and manipulation of the liver to express the erythropoietin gene are other possible manifestations of this promising strategy.

2.6.2. Artificial Cells Based Renal Treatment

The concept of artificial cell, mediated through microencapsulation, offers a potential window of opportunity in treating renal failures. First developed in 1957 by T.M.S. Chang[27], an artificial cell refers to man made structures to supplement or replace deficient cell functions. Cells with synthetic biomaterials are currently recognized as a "new category of therapeutic products" by the U.S. Pharmacopeia and National Formulation[24].

Microencapsulation consists of enclosing bioactive material, such as enzymes, eukaryotic cells or other microorganisms, in an ultra-thin semi-permeable membrane, forming, in consequence, microscopic capsules (figure 2.10). Serving as a delivery means, these microcapsules have several advantageous properties. For instance, enclosed microorganisms can not attach to and colonize the GI tact or be absorbed in the intestine and disperse throughout the body. This way the effect of the enclosed biomaterial can be rendered transient until the whole micro-capsules are excreted in the stool.

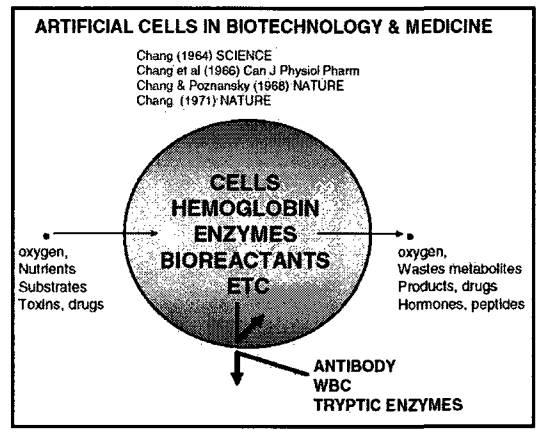

Figure 2.10: The basic principle of artificial cells[36]

Additionally, the entrapped biomaterial is protected from the host immune cells, which usually reject foreign and non-self entities, and from the harsh gastro-intestinal environment[145,146]. All these advantages greatly preserve the integrity and viability of the microcapsules content. Studies have suggested that immobilized microorganisms are more stable and more productive than free ones[147-149]. Furthermore, microencapsulation increase the retention time of the biomaterial in the GI tract conferring it, in consequence, more time to exert it effect[150,151].

2.7. ARTIFICAL CELLS: A NEW HOPE FOR RENAL FAILURE

The ingenious design of the polymeric membrane allows smaller molecules, such as hormones and peptides, to diffuse inside the artificial cell and, thus, equilibrate rapidly across its membrane. Hence, the membrane is what governs the exclusion and selective bi-directional diffusion of molecules. This is governed by the membrane molecular-weight-cut-off (MWCO), which is defined as the largest molecular size capable of passing through the membrane. Therefore, it is possible to vary the MWCO by using different coatings. Furthermore, the membrane's ultra-thinness and large surface-to-volume ratio allow more diffusion compared to a conventional dialyzer membrane. Chang *et al.*[31] reported that about 10mL of artificial cells with a diameter of 20μm have a total surface area equivalent to that of a hemodialysis machine[31].

Three routes of delivering microcapsules have been reported: implantation, hemopurfusion and oral delivery. The first two have witnessed a decrease in their usage due to short term functionality in the implantation route and the need of vascular access, which reduces the patient's movements, in the hemopurfusion route. Only the oral route appears to be convenient for self-administration, and hence, will most likely end up in wider acceptance[152].

2.7.1. Materials Used in Microencapsulation

Depending on the nature of the application and the type of material used in synthesizing the membrane, it is possible to vary its permeability, composition and configuration. This allows, in consequence, for extensive variations in the properties and functions of artificial cells. However, in all cases, the material used must not only be biocompatible and permeable, but also, must provide the microcapsule with enough mechanical strength in order to conserve its integrity.

Initially, synthetic materials, such as acrylic/methacrylic acid and dimethylaminoethyl methacrylate/dimethylaminoethyl acrylate, were used as membranes constituents. However, these materials were toxic[153], and researchers needed naturally occurring coatings. One such material, which is today considered a benchmark in cell encapsulation, is alginate, or alginic acid. Alginate is a polymer naturally extracted form

brown algae. Its biocompatibility and biodegradability are suitable for encapsulation purposes.

Alginate has been extensively used in numerous applications including the transplantation of microencapsulated xenogenic and mammalian cells[154-159] as well as the encapsulation of microbial cells for drug delivery purposes[35,39]. Considered a mild process, alginate encapsulation consists of ionotropically gelating or cross linking this anionic polymer with divalent cations, such as calcium.

Despite all alginate's advantages, its integrity and functionality may be compromised by positively charged proteins attached to the carboxylic acid sites of the outer alginate matrix[160]. This may result in membrane perfusion leading, in consequence, to potential leakage[161]. For this particular reason, coating the cross linked alginic membrane with additional layers was perceived as the only technique to preserve the microcapsule integrity and stability and benefit from the microencapsulation.

In the 1980s, Lim and Sun[24] first combined poly-$_L$-lysine (PLL) with alginate. Briefly, the alginate beads coating the material of interest were formed through a droplet technique and then immersed in a PLL solution to form the second layer. This has contributed to a more stable structure and prevented cells from agglomerating. It was not until the addition of a third alginate layer, creating the alginate- poly-$_L$-lysine-alginate (APA) membrane, that further potential of this formulation was realized. The APA capsules have high biocompatibility and biodegrability stemming from alginate[162], as well as immunoprotective properties due to the use of PLL[153]. The third alginic layer prevents the attraction of immune cells by neutralizing the cationic PLL[163].

Another possibility of additional coating is the use of chitosan, a naturally occurring cationic polysaccharide, which form strong ionic complexes with alginate due to these materials' structural similarities[164]. Other materials that have been studied for coating purposes include cellulose, polyethylene glycol, polymethylene-co-guanidine, pectin and aggarose.

2.7.2. Oral Delivery of Artificial Cells

Several advantages render the oral route superior over parental ones; such advantages range from the absence of cannulation, apparatus and trained personnel, the wide and simple application without concern of any existing disease and age to its non-invasive character and its easy self-administration[165].

On top of all these factors, the rational behind orally administrating microencapsulated agents, such as biomolecules and microorganisms, to remove undesired and/or excessive uremic metabolites from the GI tract, lies in the fact that exogenous and endogenous nitrogenous compounds are metabolized in the GI tract. Such removal is made possible by the extensive capillary network surrounding the GI tract that ensures a continuous exchange of uremic metabolites between the systemic circulation and the gastrointestinal lumen[166,167]. Furthermore, villi and microvilli, contributing to the large surface area of the small intestine, allow an efficient absorption of solutes from the GI tract. Therefore, during microcapsules' passage in the GI tract, a concentration gradient will be established between the inside of microcapsules and its surrounding environment, forcing the uremic metabolites of interest to diffuse into the spherical entities (figure 2.11).

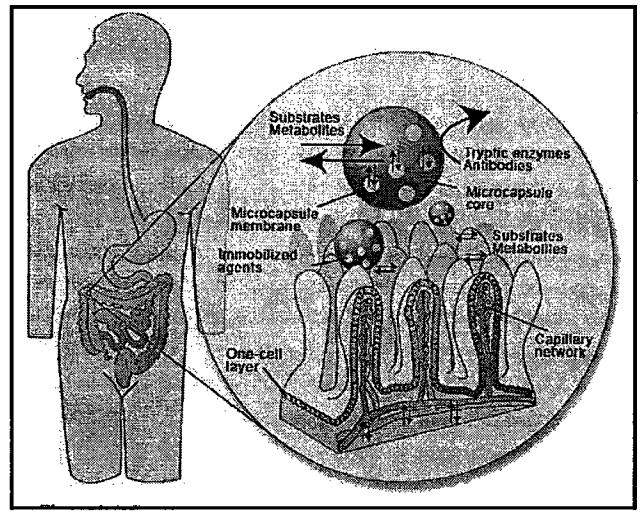

Figure 2.11: Theorized mode of action of orally administered microcapsules containing bioagents[168]

2.7.3. Biomolecules vs. Microorganisms

Therapies based upon the oral delivery of biomaterials are seen to hold great potential for mediating GI disorders. When first implemented, there were two schools of thoughts concerning the content of the microcapsules. Some researchers preferred entrapping whole organisms, such as eukaryotic cells and bacteria while others used purified therapeutics, such as enzymes and proteins. For instance, urease combined with zirconium phosphate were encapsulated for the break down of urea and the removal of ammonium ions[169]. However promising this combination may be, the zirconium phosphate had limited affinity for the ammonium ions[170]. Ristau *et al.*[171] and Elcin *et al.*[172] have each tried to immobilize urease but without convincing results. This impracticality is mainly related to the high enzymatic specificity, which engenders not only high isolation costs, but also, additional purification steps required to remove any intermediates.

For all these reasons, encapsulating whole microorganisms was considered more practical due to the fact that no extensive purification is required to produce the desired enzymes. The first attempt to make use of microorganisms was in the 1970s, when Setala *et al.* encapsulated lyophilized soil bacteria in gelatin oral capsules to make use of their antiazotemic enzymes as potential degraders of uremic metabolites[37,38]. Despite the poor quantification of their results, the group provided solid evidence that such encapsulated microorganisms proved effective in lowering nitrogenous compounds.

Further attempts to use microorganisms in renal failures were achieved by Malchesky *et al.*[173,174] who used *in vitro* cultured batches of *Pseudonomas alcaligenes*, *Diphteroid bacillus* and *Alpha streptococci* to degrade nitrogenous metabolites. The authors reported successful urea, uric acid and creatinine consumptions over a period of two consecutive months.

Finally, Prakash *et al.*[39] showed the effect of microencapsulated genetically engineered nonpathogenic *E. coli* DH5 cells containing *Klebsiella aerogenes* urease gene on the lowering systemic urea in renal failure subjects[40,41]. The novel APA oral microcapsules served as microscopic dialyzer–bioreactors as they travel through the intestine[31]. The authors found that these bioreactors can lower up to 99.99% of plasma

urea within 30min using only 100mg of bacterial cells[39]. Unfortunately, some problems including capsules strength, long-term biocompatibility, microorganism's cells entrapment in membrane and surface exposure limit the usefulness of this novel technique[31].

2.8. PROPOSED YEAST CELLS MICROCAPSULES DESIGN AND IT'S POTENTIAL

For thousands of years, the yeast species *Saccharomyces cerevisiae* has been used in baking and fermenting alcoholic beverages. Presently, it is extremely important as a model organism in modern cell biology research, and "is the most thoroughly researched eukaryotic microorganism"[175].

Given the capacity of *Saccharomyces cerevisiae* to efficiently degrade urea[42], its high environmental adaptive acquaintance, the easiness of its large scale production, the cost effectiveness of its manipulation compared to other microorganisms as well as the benefits of microencapsulation, using encapsulated live yeast cells for urea removal may prove a rewarding attempt. The potential of orally administrating such artificial cells to treat human renal failures uremia has not yet been investigated. Ergo, for the first time, this project shows a proof-of-concept combination of yeast and artificial cells for the purpose of removing nitrogenous wastes.

The goal of this research is to design microcapsules containing live yeast cells for chronic renal failures or ESRD endeavors. During their passage in the GI tract, these novel microcapsules will function as bioreactors capable of lowering nitrogenous wastes, mainly urea, by metabolizing or deactivating them, and entrapping them for complete fecal removal. If proven successful after oral administration, this treatment can serve, for instance, as an adjunct to conventional dialysis, improving in consequence the quality of life of concerned individuals.

A mild and gentle microencapsulation technique must be used in order to preserve the integrity, viability and functionality of the yeast cells. Since alginate has been extensively studied and widely used for encapsulation purposes, it will be the first and most suitable chemical for the immobilization of the yeast cells. Being administrated orally, these yeast cells must be structurally solid and must withstand physiological

stresses. For this particular reason, further coating is needed to protect the capsules and its content from the external environment. The use of PLL and an additional alginic coating improve the mechanical properties of the microcapsules. Hence, in this study, live yeast cells will be entrapped in newly designed APA microcapsules.

2.9. RESEARCH JUSTIFICATION

Currently, there is an obvious and persistent need for new and accessible therapies to treat renal failures. Despite their advantages in alleviating the uremic syndrome in renal failures, current RRTs are neither practical, due to the QOL restrictions, nor desirable, due to the complications associated with their use. Hence, a biocompatible, safe, convenient, economic and efficient treatment modality is needed. It is for this objective that this research project investigates the feasibility of designing artificial cells containing live *Saccharomyces cerevisiae* for removal of uremic metabolites both *in vitro* in simulated gastric and intestinal media and *in vivo* in experimental renal failure animal model.

Preface for Chapters 3, 4, 5 and 6

All the experimentation carried out during this research project is summarized with the corresponding results in chapters 3, 4, 5 and 6. Chapters 3 investigates the feasibility of formulating APA microcapsules containing live yeast cells for oral administration in renal failure. Mechanical properties in various environments and under different stresses as well as viability studies are reported as essential characteristics of these novel capsules. Chapter 4 describes the *in vitro* studies conducted to investigate the efficacy of the APA live yeast cells microcapsules in lowering urea concentrations. The best microcapsules' dosage is chosen. Furthermore, a series of experiments, in which a newly designed column reactor packed with APA yeast microcapsules was tested for its urea lowering capacities, are reported in chapter 5. In chapter 6 are presented the results of the *in vivo* study conducted using rats as the uremic model. In this animal trial, additional metabolites and electrolytes, including potassium, calcium, creatinine, phosphate and uric acid, have been studied in order to cover a large spectrum of renal failure' indicators.

Research articles presented in Chapters 3, 4, 5 and 6:

1. **Razek Coussa,** Christopher Martoni, Jasmine Bhathena, Aleksandra Malgorzata Urbanska, Arun Kulamarva and Satya Prakash. Microencapsulation of Live *Saccharomyces cerevisiae* Cells in Alginate-Poly-$_L$-Lysine-Alginate for Use in Renal Failure Uremia: *in vitro* Analysis.
2. **Razek Coussa**, Christopher Martoni, Jasmine Bhathena, Aleksandra Malgorzata Urbanska and Satya Prakash. *In vitro* Analysis of APA Microcapsules for Oral Delivery of Live *Saccharomyces cerevisiae.*
3. **Razek Coussa**, Christopher Martoni, Jasmine Bhathena and Satya Prakash. Alginate-Poly-$_L$-Lysine-Alginate Live *Saccharomyces cerevisiae* Microcapsules Column Bioreactor for Use in Renal Failure Uremia.

4. **Razek Coussa**, Christopher Martoni, Jasmine Bhathena, Aleksandra Malgorzata Urbanska and Satya Prakash. Microencapsulated Live *Saccharomyces cerevisiae* Cells Administered Orally to Maintain Plasma Urea Level in Uremic Rats.

N.B.: All the manuscripts presented in this thesis are under preparation.

Contributions of Authors

All the original research articles are included as individual chapters in this thesis, I am the first author. I was responsible for designing studies, conducting experiments, analyzing data and preparing manuscripts. Dr. S. Praksah, reported as the last author in all articles, is my supervisor, the research advisor and the corresponding author. Christopher Martoni, Jasmine Bhathena, Aleksandra Malgorzata Urbanska and Arun Kulamarva have provided suggestions and assistance in performing experiments as well as proofread the articles.

Chapter III

Original Paper 1

Microencapsulation of Live *Saccharomyces cerevisiae* Cells in Alginate-Poly-L-Lysine-Alginate for Use in Renal Failure Uremia: *in vitro* Analysis

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Preface: The present study investigates the mechanical stability of the yeast microcapsule membrane. This study characterizes key physical properties in regards to swelling behavior, mechanical stability, long-term storage and resistance to the gut environment. Also, the viability of the cells within the APA microcapsules was investigated.

3.1. Abstract

Oral administration of microcapsules containing live yeast cells presents a novel alternative therapy for urea removal. Safety concerns mandate complete entrapment of the microorganism, preventing any possible leaking into the host's GI system. This article evaluates the *in vitro* behavior and feasibility of orally administrating alginate-poly-$_L$-lysine-alginate (APA) microcapsules containing live yeast cells, using several mechanical shear and osmotic pressure tests. Results showed that alginate concentration ranging from 1% to 1.75% did not have any effect on either the microcapsule's diameter or the yeast's viability inside the microcapsule ($p>0.05$). However, with increasing alginate concentration, more spherical capsules were obtained, reaching a maximum of 99% at 1.75% alginate. Incubating the yeast cells for 48 hrs yielded a higher cell mass (34 ± 2 mg/ml vs. 46.5 ± 1.4 mg/mL) and a higher free cells count ($5.7X10^7 \pm 1.4X10^6$ CFU/mL vs. $8.4X10^7 \pm 1.8X10^6$ CFU/mL) than incubating them for 24 hrs. Microencapsulated yeast cells were one and a half times more viable than free yeast cells at 37°C ($p<0.05$). No difference was noticed between microenapsulated yeast viability sampled along a 1 year period ($p>0.05$). When the yeast microcapsules were subjected to saline concentrations varying between 1x and 10x, the percentage of broken capsules increased with increasing concentrations (25 ± 2% for 1x and 62 ± 6% for 10x). After 120 min in SGF pH < 2 under mechanical stress generated by a peristaltic pump, the number of broken yeast APA microcapsules and empty APA microcapsules were 93 ± 3% and 97 ± 5%, respectively. Under the same conditions in SIF pH ≥ 6.5, 38 ± 7% of the yeast APA microcapsules and 65 ± 5% of the empty control APA microcapsules were broken after 360 min, respectively. In the same experiment, simulated GI conditions did not affect the viability of yeast cells ($p>0.05$); the CFU/mL in SGF and SIF averaged $3.38X10^8 \pm 1.09X10^7$ and $3.55X10^8 \pm 1.6X10^7$ CFU/ml, respectively. After 120 min of orbital shaking at 50 rpm,100 rpm or 200 rpm in SGF, the percent breakage varied between 1 ± 8% and 57 ± 8% for yeast APA microcapsules and between 3 ± 8% and 87 ± 5% for empty APA microcapsules ($p<0.05$). This microcapsule formulation was effective in protecting yeast cells and, thus, holds promise in fulfilling the requirements for oral delivery of yeast cells. Results of this study suggest the adequacy of APA microcapsules

and provide a basis for future research on the APA microcapsule therapeutic based potential.

Key Words

Microencapsulation, alginate, yeast, morphology, mechanical stresses, osmotic pressure, viability.

3.2. Introduction

Microencapsulation, which was developed by Chang[40] in the 1960s, is a procedure by which biologically active materials, such as microorganisms, are enclosed within microscopic semi-permeable membrane to achieve a specific treatment or replace a deficient biological function[25,26,27,32]. Given the capacity of *Saccharomyces cerevisiae*, commonly known as yeast, to efficiently degrade urea[42], its high environmental adaptive acquaintance, its effortless large scale production as well as the benefits of microencapsulation, then using encapsulated live yeast cells for urea removal may prove rewarding. To be considered an effective treatment, the oral administration of the capsules should result in neither deleterious metabolic activities nor systemic infections. This implies that the microencapsulated organisms must be trapped inside the artificial cells at all times during their transit along the GI tract and must be excreted in the stool with the intact capsules after the appropriate digestion time.

Previous work show that the capsules' mechanical integrity depends on their membrane thickness, which is correlated with permeability, toughness, mechanical strength, drug release capacity and biocompatibility[176-182]. For *in vitro* and *in vivo* applications to be feasible, there must be a definite qualitative and quantitative understanding of the mechanical stability of the microcapsules when subjected to mechanical and environmental stresses. Microcapsules should be strong enough to withstand environmental constraints during processing, oral administration and both short- and long-term *in vivo* utilization.

The mechanical properties of microcapsules are an intriguing object to study for physical reasons including the small size, spherical shape, hydrogel nature and the fragility of the membrane. Though no standard testing is currently available, a number of

assessment methods have been explored[183-188] including electron microscopic methods, gravimetric measurements and compression tests. Most of these techniques are limited by resolution problems, and it is time consuming to test enough capsules to avoid sampling errors. An easier method for characterizing the mechanical stability of microcapsules is the use of an osmotic pressure test, as described by Van Raamsdonk and Chang. It consists of exposing the microcapsules to a sequence of hypotonic/hypertonic environment and, then estimating the number of broken artificial cells[189].

Alginate has been extensively used in microencapsulation of live microorganisms due to its superior biocompatibility, mild process conditions and its FDA approval. Typically, Calcium ions, are used to gel the alginate coating and additional polymers are used to envelop the alginate-calcium beads, thus, creating a stable and stronger semi-permeable membrane. For instance, alginate-poly-$_L$-lysine-alginate (APA)[190] has been extensively employed for the microencapsulation of microorganisms microencapsulation purposes[24,39, 176, 180-182, 191-194].

To date, the potential of administrating live *Saccharomyces cerevisiae* APA microcapsules for the removal of urea in renal failure uremia has not been investigated yet. Towards this goal, we have, in this article, optimized the encapsulation procedure and its respective parameters to accommodate yeast cells. The present work investigates the suitability and the feasibility of using these microencapsulated yeast cells.

3.3. Materials and Methods

3.3.1. Chemicals

Sodium alginate (low viscosity) and poly-$_L$-lysine (*MW* 27,400) were both purchased from Sigma-Aldrich. Simulated gastric fluid (SGF) is composed of 1.0 g/L arabinogalactan, 2.0 g/L pectin, 1.0 g/L xylan, 3.0 g/L starch, 0.4 g/L glucose, 3.0 g/L yeast extract, 1.0 g/L peptone, 4.0 g/L mucin, 0.5 g/L mucin and the pH was adjusted using 0.2 N HCl. Simulated intestinal fluid (SIF) consists of SGF and the appropriate amount of pancreatic juice, 12 g of $NaHCO_3$, 6 g of oxgall and 0.9 g of pancreatin all suspended in 1L of sterile water.

3.3.2. Microorganism and culture conditions

Saccharomyces cerevisiae, strain reference number 9896, was purchased from ATCC[195]. Yeast mold broth (YMB) growth medium was used for primary cell cultivation. YMB is composed of 10 g/L of glucose, 3 g/L of malt extract, 5 g/L of peptone and 3 g/L yeast extract. The whole mixture was incubated aerobically at 37°C. The log phase culture was then centrifuged at 3500 rpm for 15 min at 4°C.

3.3.3. Preparation of APA microcapsules containing live yeast cells

Log phase cultures of *Saccharomyces cerevisiae* were harvested by centrifugation at 3500 rpm for 15 min at 4°C. Pellets were then mixed with 2% alginate solution in order to form a final mixture consisting of 8% (v/w) pellets and 1.75% alginate. Alginate microcapsules were prepared by generating droplets of sodium alginate solution (15 mg/mL) using an IER-20 encapsulator machine manufactured by Inotech. Corp. Extruded through a 300 μm nozzle by a syringe driven pump, these droplets were gelled for 30 min in a well-stirred calcium chloride solution (11 mg/mL). The same droplets were then coated with a 0.1% (w/v) solution of PLL for 10 min to form AP beads and washed again with physiological saline (PS) for 5 min. Finally, the final alginate coating is applied by immersing the beads in a 0.1% (w/v) sodium alginate solution for 10 min followed by a final 5 min PS wash. The resulting microcapsules, whose average diameter was 638 ± 17 μm, were then collected and stored in a 10/90 YMB/PS solution at 4°C. All solutions used in the preparation of these APA microcapsules were either filtered using 0.22 μm filter or autoclaved to ensure sterility. Moreover, the entire procedure was carried out in a biological hood.

3.3.4. Evaluation of alginate concentration, yeast microcapsule morphology and cell viability

To investigate the effect of sodium alginate initial concentration on the microcapsules' diameter and shape, pictures of 20 μL of APA microcapsules were taken using an inverted phase contrast microscope (Nikon Eclipse TE20004) equipped with a digital camera (Nikon DXM 1200F). Different microcapsules were prepared using sodium alginate at four different concentrations of 1 g/100 mL PS (1%), 1.25%, 1.5% and 1.75%.

To estimate the viability of the encapsulated yeast cells, 0.1 mL of microcapsules were collected in a 1.5 mL graduated Eppendorf tube and mechanically ruptured using a sterile tissue pestle. Several 10-fold serial dilutions were performed using autoclaved PS and 0.1 mL aliquots of suspended yeast were plated on selective YM agar plates. The plates were then incubated aerobically for 48 hrs at 37°C. The colonies were enumerated against free yeast cells and yeast loaded microcapsules serving as positive and negative controls, respectively. The same amount of yeast cells present in microcapsules was used in free cells' viability studies. All experiments were done in triplicate. We have also followed the viability of yeast cells in APA microcapsules for a 12 month period. This can provide vital information about the efficacy of the microorganisms over extended storage conditions.

3.3.5. Microcapsule mechanical shaking shear test

20 μL of yeast microcapsules were suspended in deionized H_2O solution and were magnetically stirred at 300 rpm in a Lab Line Environ Shaker for 3 hrs. The temperature was fixed at 37°C in order to mimic physiological conditions. The APA microcapsules were placed in small Petri dishes during the testing period. Using an optical microscope (LOMO, PC) equipped with a digital camera (Canon Power shot G2), images were taken every hour in triplicate. The number of intact cells was counted and the percentage breakdown was determined by subtracting the initial number of intact capsules from the final one.

3.3.6. Microcapsule saline osmotic pressure test

Osmotic pressure was applied using a modification of an earlier procedure[189]. 0.1 mL of APA yeast microcapsules were equilibrated for 30 min in different hypertonic PS solutions having concentrations of 1, 2, 5 and 10 times the normal physilogical 0.85 wt% PS solution. Afterwards, the cells were transferred into a hypotonic deionized water solution, which created a high osmotic pressure inside the microcapsules. Using an optical microscope (LOMO, PC) equipped with a digital camera (Canon Power shot G2), images were taken after 1 hr suspension in the hypotonic solutions. The number of intact cells was counted and the percentage breakdown was determined by subtracting the

initial number of intact capsules from the final one. This experiment was performed in triplicate.

3.3.7. Microcapsule simulated gastric and intestinal fluid mechanical stability test using a peristaltic pump

To date, no one has subjected APA microcapsules containing live yeast cells to shear stresses and peristaltic loads similar to those generated by human gastric and intestinal systems. For that, 3 g of yeast and empty APA microcapsules were immersed in 20 mL of SGF pH < 2 in a 100 mL beaker. A Watson-Marlow peristaltic pump (model 101U/R) was used to empty the content of the input beaker into the output one. The pump was calibrated to transfer the mixture in 2 hrs, a time equivalent to retention period the bolus spends in the stomach during digestion. After the first 2hrs, the content of the output beaker was sieved and suspended in 20 mL of SIF pH $\geq$ 6.5 to mimic digestion in the small intestine. Again, the same setup was used for a 6 hr period.

To estimate the percentage breakdown over time, images of 20 µL samples of the tested solution were recorded in triplicate using an optical microscope (LOMO, PC) equipped with a digital camera (Canon Power shot G2). The microcapsules percentage breakdown and the cell viability were estimated as described earlier. Note that in the first 2 hrs of the tests, samples were taken at 0 min from the input beaker and at 5 min, 1 hr and 2 hrs from the output beaker. The following 6 hrs were sampled at 0 min, 5 min, 2 hrs, 4 hrs and 6 hrs.

3.3.8. Microcapsule simulated gastric and intestinal fluid mechanical shaking shear test

In another test to study the yeast microcapsule mechanical resistance and viability during the human GI transit, 3 g of empty APA mircocapsules, yeast microcapsules and the corresponding amount of free yeast were suspended, each, in 20 mL of SGF pH < 2 solutions and were magnetically stirred at 50, 100 and 200 rpm for 2 hrs (period equal to the stomach retention time) using a Lab Line Environ Shaker. Once again, the percentage breakdown and the cell viability were estimated as described earlier at the following time intervals: 0 min, 5min, 1 hr and 2 hrs.

3.3.9. Statistical Analysis

The statistical Analysis System (SAS Enterprise Guide 4.1 (4.1.0.471) by SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Data was expressed as means ± SEM. Group and time effects were statistically investigated by ANOVA mixed models. Data were considered significant at $p<0.05$.

3.4. Results

3.4.1. Optimization of microencapsulation procedure: effect of alginate concentration

In this study, it was necessary to investigate the effects of alginate concentration on the diameter, the cell viability and the microcapsules' surface morphology, which is extremely important in ensuring proper microorganism repartition and mass transfer. All the results are shown in Table 3.1. For alginate concentration of 1%, 1.25%, 1.5% and 1.75%, the average microcapsule's diameter was 645 ± 48 μm, 625 ± 64 μm, 593 ± 46 μm and 638 ± 17 μm, respectively. For the same range of concentrations, the percentage of spherical microcapsules in a 20 μL sample were 9.8%, 45.12%, 73% and 99%, respectively. Similarly, the cell Colony Forming Unit (CFU) of freshly microencapsulated yeast cells per mL was $4.0X10^8$, $3.9X10^8$, $4.2X10^8$ and $4.2X10^8$ for alginate concentration of 1%, 1.25%, 1.5% and 1.75%, respectively.

It can be inferred that the concentration of alginate has no significant effect on either the size of the microcapsule or the cell viability inside it ($p>0.05$). In this experiment, the viability and morphology were estimated 1 week after producing the microcapsules. However, our results suggest that alginate's concentration noticeably affects the morphological quality of the microcapsules; lower alginate concentrations generated microcapsules characterized by tear-like shapes (figure 3.1a-d). Furthermore, the percentage of spherical capsules reported increased with an increase in alginate concentration.

3.4.2. Growth optimization & viability of live yeast cells inside APA microcapsules

Another important criterion that had to be considered in the study of live yeast cells is their actual growth. Incubating the yeast cells for a period of 24 hrs allows harvesting this microorganism in its late log phase. This differs from the 48 hrs proposed by Richards[185]. In order to investigate this matter, we decided to inoculate two batches of yeast culture and incubate each one for 24 hrs and 48 hrs. Both the cell mass and the Colony Forming Unit (CFU) per mL of growth medium and were then assessed as indicators of cell growth.

Another point to investigate was the fate of differently incubated yeast cells inside the microcapsules under storage conditions. This is of particular importance because it indicates the extent of growth after each respective incubation period. All microcapsules for this test have been stored for 1 week in a 10/90 YMB/PS solution at 4°C.

Figures 3.2a and b clearly indicate that incubating the yeast cells for 48 hrs rather than for 24 hrs yields a higher cell mass (34 ± 2 mg/mL vs. 46.5 ± 1.4 mg/mL, figure 3.2a) and a higher free cells' CFU/mL ($5.7X10^7$ ± $1.4X10^6$ CFU/mL vs. $8.4X10^7$ ± $1.8X10^6$ CFU/mL, figure 3.2b). Moreover, microencapsulated cells that were incubated for 48 hrs were found to be more numerous than those incubated for 24 hrs ($2.5X10^8$ ± $1.8X10^7$ CFU/mL vs. $4.9X10^8$ ± $1.9X10^7$ CFU/mL, figure 3.2b).

The results of the one year viability study under storage conditions indicate that the CFU/mL at t=0 month and at t=12 month were $3.9X10^8$ ± $3.6X10^7$ and $3.95X10^8$ ± $2.1X10^7$, respectively (figure 3.2c). Our statistical analysis reveals no apparent significance among readings taken within the 3 days period and those taken during the 1 year period for the microencapsulated yeast cells ($p>0.05$). Similarly, no statistical significance was reported after comparing the 3 days readings of microencapsulated yeasts with those of the 1 year study ($p>0.05$).

3.4.3. Mechanical stability of APA microcapsule membrane

The mechanical properties of the microcapsules' membrane are of crucial importance due to their integrity preservation and *in vivo* performance. Previous reports indicated that stronger membranes are more likely to confer microcapsules more durability, and mechanical strength[198-200]. In this study, the membrane structural strength

was evaluated using four different tests relying on either osmotic pressure or shear stress or both.

3.4.3.1. Microcapsule mechanical shaking shear test in PS

The strength of the yeast APA microcapsules was investigated by exposing the microcapsules to shear stresses generated by orbital shaking at 300 rpm for 3 hrs. At the beginning of the test, all microcapsules were intact and spherical in shape (figure 3.3a). However, with increasing exposure to shear stress, more microcapsules were reported to break in half (figure 3.3b). Specifically, after 1 hr, 2 hrs and 3 hrs, 7 ± 3%, 29 ± 4% and 63 ± 9% of the microcapsules were broken respectively. Thus, the percentage of broken capsules increased significantly with an increase in the shaking time (figure 3.3c) ($p<0.05$).

3.4.3.2. Microcapsule saline osmotic pressure test

Another way to investigate the mechanical strength of the microcapsules' membranes is to subject them to osmotic pressure, which is a function of the ionic concentration in a given solution. Hence, the higher the salt concentration, the greater the osmotic pressure, expected to drive water out of the microcapsules, will be. Figure 3.4 confirms the dependence of microcapsules' breakage on the osmolarity of the bathing solution. For saline concentrations of 1x, 2x, 5x and 10x, the percentage of broken microcapsules were 25 ± 2%, 56 ± 4%, 57 ± 9% and 62 ± 6%, respectively. Although such dependence exists, it does not, however, follow a linear trend due to the closeness of the values for 2x and 5x; in fact, there was no significant difference between the percentage breakage at 2x and 5x ($p>0.05$). The values obtained for the 1x and 10x experiments are not only statistically different from each other but also from those at 2x and 5x ($p<0.05$).

3.4.3.3. Microcapsule simulated gastric and intestinal fluid mechanical stability test using a peristaltic pump

In order to assess the capsular resistance to the GI environment and, thus, evaluate the suitability of the APA microcapsules for oral delivery, it is essential to understand their behavior under physiological conditions. In this study, two mechanical stability tests

were carried out in physiological conditions which mainly consisted of immersing the microcapsules in gastro-intestinal fluids and exposing them to mechanical shear stresses and/or an osmotic pressures.

After 5 min, 60 min and 120 min in SGF pH < 2, approximately 8 ± 3%, 84 ± 8% and 93 ± 3% of all yeast APA microcapsules and 19 ± 8%, 87 ± 2% and 97 ± 5% of all empty ones were broken, respectively (figure 3.5g). For both types of microcapsules, there appears to be a correlation between immersion time and extent of breakage: the larger the retention time, the greater the percentage of broken microcapsules will be ($p<0.05$).

Similar trends were observed when the microcapsules were immersed in the SIF pH ≥ 6.5 (figure 3.5h). Specifically, % breakage after 5 min, 120 min, 240 min and 360 min was 8 ± 3%, 19 ± 10%, 34 ± 5% and 38 ± 7% for yeast APA microcapsules and 12 ± 9%, 25 ± 9%, 45 ± 6% and 65 ± 5% for empty control APA microcapsules, respectively. However, empty APA microcapsules were more prone to breakage than yeast ones after 6 hrs immersion in SIF. In fact, the percentage of broken empty microcapsules was almost double that of the yeast microcapsules. Statistical analysis revealed that values for breakage percentages in SGF did not significantly differ for yeast and empty microcapsules ($p>0.05$). However, when comparing SIF alone and both SGF and SIF together, it appears that values for yeast APA microcapsules are significantly lower from those of empty ones ($p<0.05$).

CFU/mL of microencapsulated yeast cells after 0 min, 5 min, 60 min and 120 min in SGF pH < 2 were $3.5X10^8 \pm 1.6X10^7$, $3.46X10^8 \pm 1.8X10^7$, $3.76X10^8 \pm 1.5X10^7$ and $2.8X10^8 \pm 1.5X10^7$, respectively. Similarly, the CFU/mL after 0 min, 5 min, 120 min, 240 min and 360 min in SIF pH ≥ 6.5 were $3.45X10^8 \pm 1.5X10^7$, $3.55X10^8 \pm 1.8X10^7$, $3.61X10^8 \pm 1.5X10^7$, $3.48X10^8 \pm 1.6X10^7$, $3.6X10^8 \pm 1.5X10^7$ and $3.58X10^8 \pm 1.7X10^7$. The viability of the yeast cells inside the microcapsules did not vary dramatically in both SGF and SIF with respect to time ($p>0.05$). Moreover, the viability in the SGF, for all sampling times, was found to be not significantly different from the one in SIF ($p>0.05$) (figures 3.5i and j). Microcapsules that were not broken under both stomach and small intestine conditions were found to maintain their spherical morphology; however, the broken ones had, in most cases, swelled and some had ghost-like shapes (figures 3.5a-f).

3.4.3.4. Microcapsule simulated gastric fluid mechanical shaking shear test

During their GI transit, microcapsules will be first subjected to the gastric environment before reaching the small intestine, where they are hypothesized to act as bioreactors. For this reason, investigating the effect of the stomach conditions on these structures is more than necessary. In the previous experiment, a peristaltic pump was used to incorporate shear forces. In the experiment, we decided to expose the microcapsules to orbital shear stresses at different rates while being immersed in SGF for 2 hrs.

After 5 min, 60 min and 120 min of orbital shaking at 50 rpm, 1 ± 8%, 25 ± 4% and 41 ± 4% of the yeast APA microcapsules and 3 ± 8%, 46 ± 2% and 65 ± 3% of the empty APA microcapsules were broken, respectively (figure 3.6a). Similarly, after 5 min, 60 min and 120 min of orbital shaking at 100 rpm, 11 ± 4%, 39 ± 4% and 51 ± 5% of the yeast APA microcapsules and 14 ± 5%, 49 ± 6% and 65 ± 6% of the empty APA microcapsules were broken, respectively (figure 3.6b). Also, 11 ± 1%, 42 ± 2% and 57 ± 8% of the yeast APA microcapsules and 20 ± 7%, 54 ± 6% and 87 ± 5% of the empty APA microcapsules were broken, respectively, after 5 min, 60 min and 120 min of orbital shaking at 200 rpm (figure 3.6c).

Hence, with increasing speed, the microcapsules, whether empty or containing yeast, were found to break to greater extents. Again, in this experiment, yeast APA microcapsules were found to be more resistant to harsh conditions than empty ones, especially after 2 hrs shaking at 200 rpm. In particular, no differences were noticed between values at 100 and 200 rpm for yeast APA microcapsules ($p>0.05$). When comparing yeast APA microcapsules to empty ones, there were significant differences in breakage rates at the three different speeds ($p<0.05$).

After 0 min, 5 min, 60 min and 120 min of orbital shaking at 50 rpm, the CFU/mL of encapsulated yeast cells was $5.91X10^8 \pm 1.3X10^7$, $5.7X10^8 \pm 1.4X10^7$, $5.2X10^8 \pm 1.4X10^7$ and $5.01X10^8 \pm 2.7X10^7$ and that of free yeast cells was $4.07X10^8 \pm 1.1X10^7$, $4.6X10^8 \pm 2.8X10^7$, $3.65X10^8 \pm 1.3X10^7$ and $3.67X10^8 \pm 2.4X10^7$, respectively (figure 3.6d). Similarly, the CFU/mL of encapsulated yeast cells was $5.50X10^8 \pm 4.2X10^7$, $5.20X10^8 \pm 2.8X10^7$, $4.9X10^8 \pm 1.8X10^7$ and $5.30X10^8 \pm 1.4X10^7$ and that of

free yeast cells was $4.08X10^8 \pm 7.2X10^7$, $4.65X10^8 \pm 2.1X10^7$, $3.63X10^8 \pm 1.6X10^7$ and $3.5X10^8 \pm 2.8X10^7$, respectively, after 0 min, 5 min, 60 min and 120 min of orbital shaking in SGF at 100 rpm (figure 3.6e). Values of CFU/mL after 0 min, 5 min, 60 min and 120 min of orbital shaking in SGF at 200 rpm were $5.80X10^8 \pm 2.8X10^7$, $5.8X10^8 \pm 1.7X10^7$, $5.0X10^8 \pm 2.3X10^7$ and $5.1X10^8 \pm 1.8X10^7$ for microencapsulated yeast cells and $4.7X10^8 \pm 1.2X10^7$, $4.8X10^8 \pm 1.7X10^6$, $4.3X10^8 \pm 1.8X10^7$ and $3.9X10^8 \pm 1.3X10^7$ for free yeast cells, respectively (figure 3.6f).

The viability of the microencapsulated yeast compared to free yeast was significantly larger at the three chosen speeds ($p<0.05$). However, within each category, the viability did not neither vary with time nor with shaking speed ($p>0.05$). The shape of the capsules at the end of the experiment was similar to those observed in the previous experiment (figures 3.5a, b, d and e).

3.5. Discussion

An essential criterion for the success of the proposed treatment is the production of proper APA microcapsules capable of not only withstanding physiological stresses, but also, allow adequate mass exchange between the inside and the outside of the microcapsule. Thus, the membrane characteristics of the microcapsule are essential and need to be optimized to accommodate live yeast cells. Parameters such as membrane permeability, thickness, composition are key elements in this optimization. Luckily, some previous work has been done in our laboratory concerning the effect of the additional coatings (i.e. alginate and PLL) as well as the permeability of the APA membrane.

Chen *et al.*[197], who studied the effect of PLL, reported that the diameter of APA microcapsules tend to increase with decreasing concentration of PLL solution. Moreover, higher the PLL concentrations yield thicker membranes, which, at a first glance, might seem advantageous from a stability point of view. However, this increased thickness might jeopardize the molecular weight cut off (MWCO) and prevent, in consequence, the urea molecules from entering the capsule. For this reason, this group suggested using PLL concentrations as low as 0.1% (w/v)[197].

The rational behind using one week old microcapsules as opposed to fresh ones lies in the fact that the morphology of the microcapsules over long-term culture is one of

the indicators of their stability[201]. Ergo, it would not be credible to use freshly produced microcapsules because these would not have been exposed to storage conditions.

In investigating the effect of alginate concentrations, it was found to significantly alter the quality, shape and number of spherical entities (Table 3.1). Spherical morphology is needed not only because it does spread yeast cells evenly in the confined space, but also, because it confers better stability and mass transfer. Low concentration of alginate (1-1.5%) generated microcapsules possessing a characteristic tail or having tear-like shapes. This is particularly undesirable and not practical because it eases the rupture and leakage of the microcapsules. Furthermore, proper mass transfer nutrient exchange may not be efficient due to a plausible improper spreading of the microorganism.

The greater number of spherical microcapsules observed when using higher alginate concentrations might be explained by the gelation's physics of the microencapsulation process. The higher the alginate is, the heavier the microcapsule will be. This increase in weight is actually beneficial because it overcomes surface tensions generated by the nozzle's tip. In this line of reasoning, higher alginate concentrations render the falling microcapsule more compact and robust, thus, allowing it to preserve it spherical entity upon gelation in the calcium chloride solution. For this particular reason, higher alginate concentrations are thought to be correlated with a greater number of spherical microcapsules.

Note that for any given material a thicker capsule wall is stronger and more desirable from a mechanical strength perspective, but not very practical from a diffusive point of view. The diffusion properties of the microcapsules for cell viability and secretion must be also taken into consideration when optimizing the coatings concentrations. One reason why we decided not to exceed 1.75% of alginate is the possibility of altering the alginic membranes' MWCO estimated to be between 60-70 kDa[202]. Too much or not enough alginate might reduce or increase, respectively, this threshold, which can, thus, reach the MWCO of urea (60 Da) or urease (483 kDa)[202]. Another reason is related to the difficulty, and sometimes the impossibility, of encapsulating solution with high alginate concentrations. Overall, 1.75% (w/v) alginate and 0.1% PLL are the optimum concentrations for producing APA microcapsules

entrapping yeast cells because such dimensions respect the MWCO of urea and urease. Under these parameters the average thickness of an APA microcapsule is 4.5± 0.6 μm[197]

Optimizing the yeast growth is of crucial importance because it allows the generation of productive (easier scale-up) and adaptive yeast cells. According to ATCC's recommendations, this strain of the yeast best grows at 30°C. Incubating the yeast cells at this temperature is problematic because their subsequent exposure to physiological temperatures (37°C) might not only affect its viability, especially after several generation of continuous environmental acquaintance, but also, disrupt the yeast ability in breaking down urea, which is undesirable for the sake of this application.

Late lag phase yeast cells are desirable for two specific reasons. Firstly, little or no growth inside the microcapsules under storage conditions is highly targeted. The possibility of the microcapsule bursting or changing properties under extensive cell growth strengthens this first reason. Secondly, late lag phase was reported to have the highest number of maximally grown cells[42].

Incubating yeast cells for 48 hrs showed a 36.7% increase in cell mass/mL compared to 24 hrs incubation. Similarly, under the same conditions, the CFU/mL of free and encapsulated yeast cells increased by 47% and almost 100%, respectively (figures 3.2a-b). We hypothesize that the greater cell viability inside the microcapsule may be correlated with a possible cell-to-cell communication. However, we have, yet, no means of verifying this hypothesis. The long-term viability study we conducted (figure 3.2d) proved that there is no time effect on this variable; in other words, the yeast cells maintain their viability for up to one year under storage conditions. This outcome, which further proves that our cells are indeed in their late lag phase, is of particular importance for large scale operations.

The effect of time was significant in the mechanical shear stress experiment (section 3.4.3.1, figure 3.3). Our results were consistent with those observed by Chen *et al.*[203], who subjected their APA microcapsules to 3 hrs of magnetically stirring at 600 rpm. It is worth noting that 300 rpm is not physiologically possible because neither stomach propulsion nor intestine peristaltic movements can reach such high shear values. We conducted this shear experiment in order to define the maximum shear stresses that our APA microcapsules can withstand.

In the saline osmotic pressure test (section 3.4.3.2), the 30 min equilibration time in hypertonic solution was used to force the microcapsules shrinking while the hypotonic immersion that follows created the reverse effect. Surrounding water molecules are expected to enter the microcapsules, thus, forcing them to swell. Physical diffusive laws imply that less equilibration time[22] is needed as the salt concentration increases. In this fashion, APA microcapsules shrink and swell to a greater extent and at a faster rate when immersed in more concentrated solutions; this tendency appears to have important effects on the stability and mechanical strength of the microcapsule.

A linear trend is expected for the dependence of the mechanical stability on the saline concentration. Although, a correlation can be drawn between 1x and 10x, there was no difference in the sensitivity to 2x and 5x ($p>0.05$). This suggests that yeast APA microcapsules are not affected by specific concentrations but by ranges of concentrations varying from low (1x) to high (10x). Chen *et al.*[203] found that more than 95% of the APA capsules containing *Lactobacillus Plantarum* 80, produced using the same parameters as ours, ruptured within 10 min of exposure to the saline osmotic test. Our yeast APA capsules show about 35% increase in the number of intact capsules when compared to Chen *et al.*'s results, suggesting a possible halting effect from the yeast microorganisms themselves.

Chen *et al.*[203] investigated the effect of incubating APA microcapsules in various physiological GI fluids without exposing them to any stress. For this reason, we decided to upgrade the experimental protocol by adding mechanical shear stresses and osmotic pressures. Furthermore, because most yeast microcapsules are designed to work in the small intestine, we decided not to invest much in testing the structural stability in the colon. Time effects were important during the first 2 hrs in the SGF and 6 hrs in the SIF. Although no noticeable differences were detected between empty and yeast capsules in the SGF ($p>0.05$), there were significant differences between yeast and empty capsules breakage in the SIF as well as in the combined SGF/SIF as well ($p<0.05$). Overall, this suggests that yeast microcapsules are stronger than empty ones.

[22] Between the inside and outside of the microcapsule.

Originally, APA microcapsules were spherical and uniform in shape (figures 3.5a and d). Although most of the yeast capsules ruptured under the mechanical forces by the peristaltic pump, the 62 ± 7% remaining maintained their spherical shape in both the SGF and SIF. Thus, as they passed through the pump, the integrity of the microcapsules declined after 8 hrs of continuous pumping. We believe that using the peristaltic pump underestimated the microcapsules strength, and, thus, exaggerated their breakage rate. This is explained by the very high surface area exposure of the conveying tubing to the volume of the capsule; in reality, microcapsules will be mixed within the bolus, which shields them, and this prevents the high exposure to high shear stresses. Therefore, the breakage of yeast APA microcapsules should be lower than the ones we found after exposure to SGF and SIF.

In the SGF mechanical shaking shear experiment, we found that exposure time affects the structural strength of both empty and yeast APA microcapsules (figures 3.6a-c). Even though shear stresses through orbital shaking affected each type of capsules differently, there was no apparent difference between microcapsules' breakage values at 100 and 200 rpm for each group ($p>0.05$). This suggests a possible plateau kind of behavior in that APA microcapsules, whether empty or full, exhibited similar breakage behavior for speeds equal or above 100 rpm. In other words, there was no noticeable sensitivity within each group for a change in shaking speed above 100 rpm. In a study done by Van Raamsdonk and Chang[189], the presence of cells was reported to weaken the APA microcapsule. Their results contradict ours because, in our experiments, yeast APA microcapsules were less prone to rupture than empty ones when exposed to the same mechanical and environmental conditions.

Our results also suggest that the viability of microencapsulated yeast cell are affected by neither the mechanical forces generated by either a peristaltic pump or orbital shaking nor by the environment in which they were present, whether in SGF or in SIF. Thus, the acidity of the stomach and basicity of the intestine do not impair the yeast viability. It appears that the stomach acidity affects the capsular strength to a greater extent than the intestine basicity.

According to Raamsdonk and Chang, the osmotic pressure test, although being one of the few possible assessments of mechanical stability, can not adequately mimic the

forces that act to weaken microcapsules *in vivo*. Although the main cause of microcapsules breakage under physiological conditions was reported to be the osmotic swelling of the alginate core against the more rigid PLL-alginate membrane[204], "orally administered microcapsules will never be exposed to such environments"[189].

The precise mechanisms weakening the microcapsules *in vivo* are for the most part unknown. However, our data reveal critical indications on the possible breakage mechanism. In most cases, the spherical microcapsules have tendency to break into two halves. For this breakage mechanism to occur, a crack must first be created in the membrane's wall. This is most plausibly due to the effect of osmotic pressure. Then, the crack must propagate along the surface of the microcapsule in order to tear it into tow entities. Shear stresses are most likely responsible for this propagation. Thus, the fracture mechanism appears to be mediated by a combination of osmotic pressure and shear stresses. Further research is highly needed in order to investigate this finding.

3.6. Conclusion

Sufficient mechanical stability, appropriate permeability, proper immunoprotection and biocompatibility are major considerations in developing microcapsules for cell therapy. However, the exact requirements for artificial cells are dictated by the bioactive species of interest and the desired function, and are, therefore, not identical for all applications. The present study evaluates the feasibility of orally administrating APA microcapsules containing live yeast cells for renal failure purposes. In this paper, the structure and mechanical properties of the yeast microcapsules have been characterized. Results showed that the novel yeast microcapsules possessed strong membrane and morphological stability as well as potent resistance to mechanical stress, calcium sequestration and GI acidic and basic impediments. Results also showed that the viability of microencapsulated yeast cells is not only higher than that of free yeast, but also, is not affected by the changing gastrointestinal environment. Furthermore, microencapsulated yeast cells maintained their viability under storage conditions for one year. This microcapsule formulation was effective in protecting yeast cells and is, thus, promising in fulfilling the requirements for oral delivery of yeast cells. Further *in vitro*

studies may advance the understanding of microcapsule performance in conditions pertinent to the human GI tract.

Table 3.1: Effect of alginate concentration on APA microcapsules' shape, diameter and yeast cell viability

Alginate Concentration	Average Diameter [μm]	% Spherical Microcapsules	Cell Viability after 48hr incubation CFU/mL
1%	645 ± 48	9.8	4.0×10^8
1.25%	625 ± 64	45.12	3.9×10^8
1.50%	593 ± 46	73	4.2×10^8
1.75%	638 ± 17	99	4.2×10^8

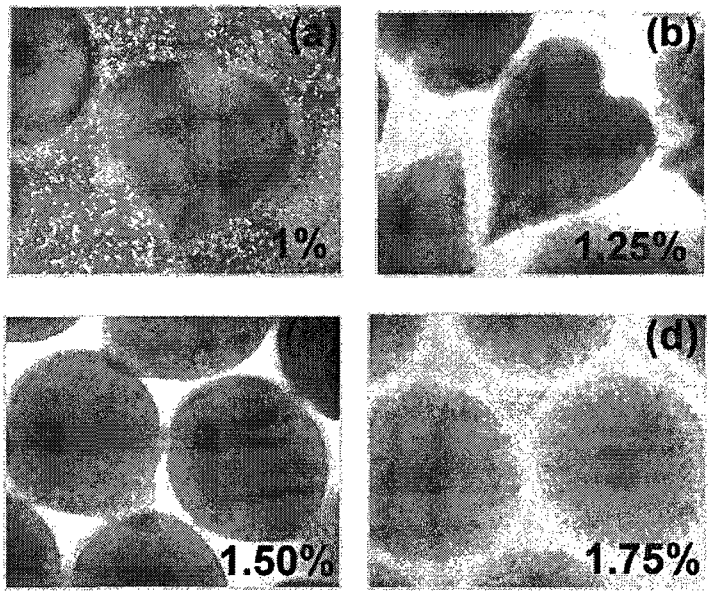
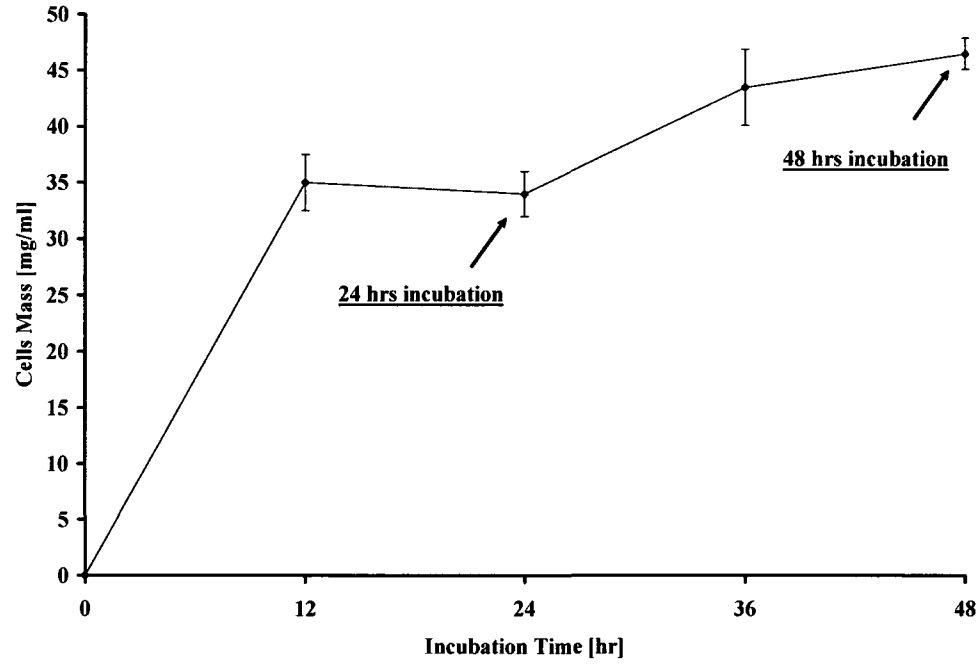
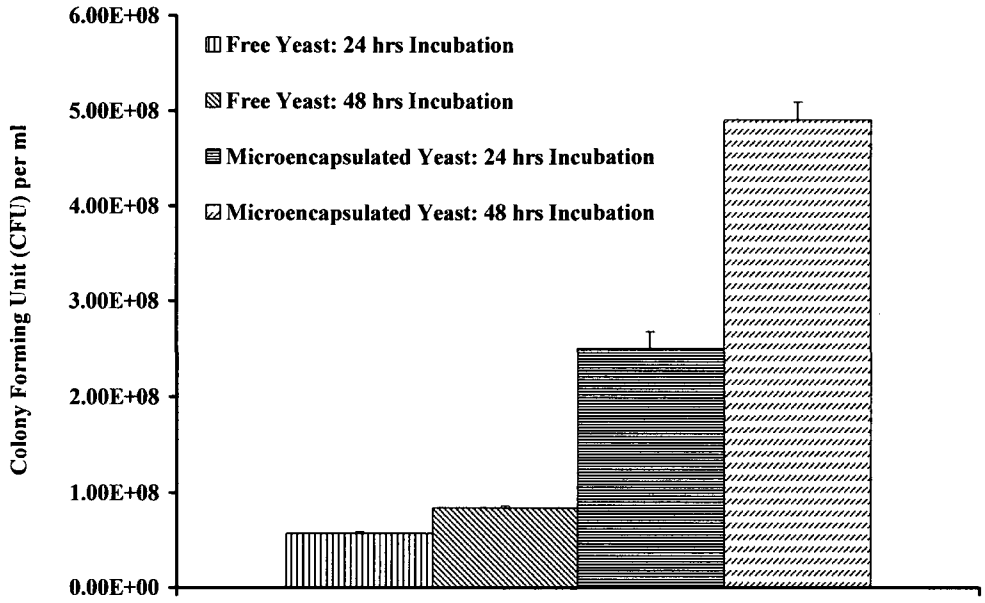


Figure 3.1: Effect of alginate concentration on yeast APA microcapsules' shape and diameter. Photomicroscopic images of yeast APA microcapsules having different alginate concentrations: (a) 1.0% (645 ± 48 μm), (b) 1.25% (625 ± 64 μm), (c) 1.5% (593 ± 46 μm), and (d) 1.75% (638 ± 17 μm).

(a)


Cells Mass [mg/ml]
50
45
40
35
30
25
20
15
10
5
0
0
12
24
36
48
Incubation Time [hr]
24 hrs incubation
48 hrs incubation

(b)


Colony Forming Unit (CFU) per ml
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
Free Yeast: 24 hrs Incubation
Free Yeast: 48 hrs Incubation
Microencapsulated Yeast: 24 hrs Incubation
Microencapsulated Yeast: 48 hrs Incubation

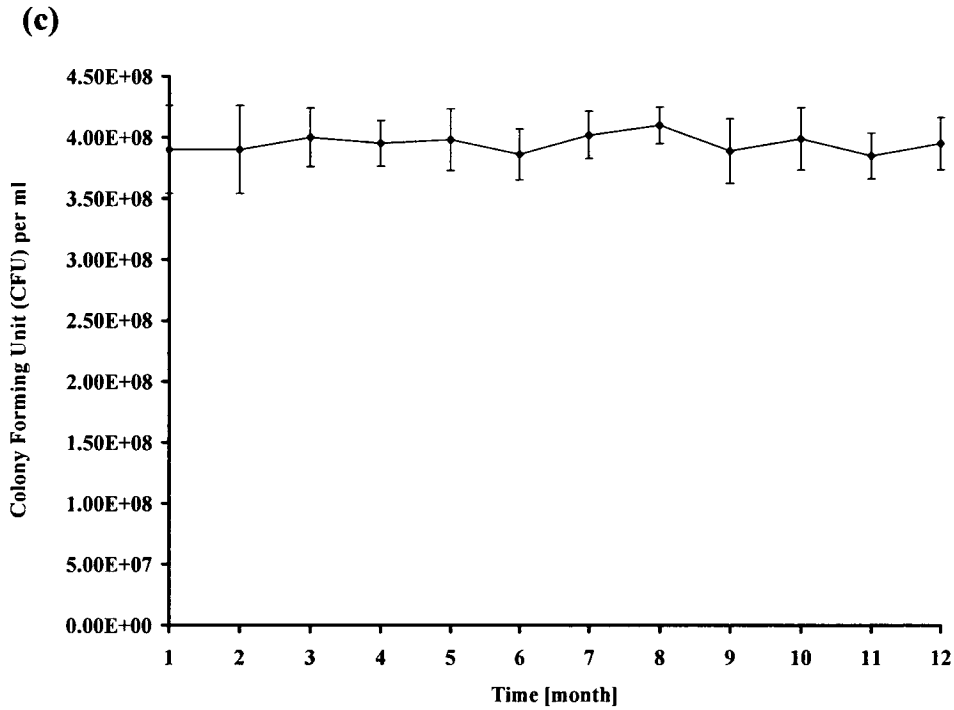
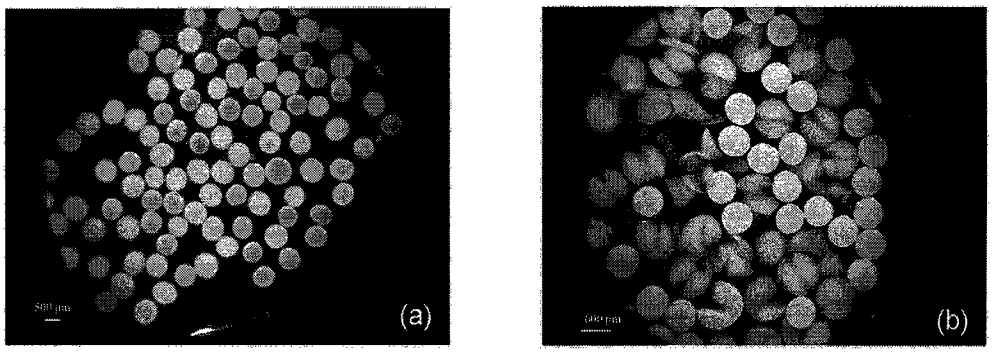


Figure 3.2: Viability and growth studies of free and encapsulated live yeast cells. (a) Yeast cell mass as a function of incubation time, (b) Effect of incubation time on yeast cell Colony Forming Unit (CFU), (c) 1 year viability study of APA microencapsulated live yeast cells after incubation for 48hrs.



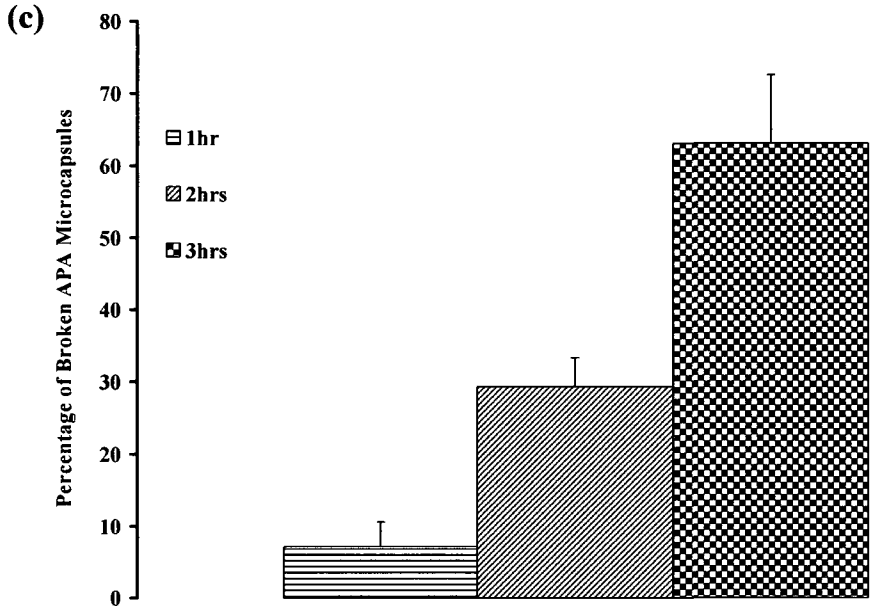


Figure 3.3: Mechanical shaking shear test in physiological saline. (a) Photomicroscopic image of yeast APA microcapsules without orbital shaking, (b) Photomicroscopic image of yeast APA microcapsules after 3 hrs of orbital shaking at 300 rpm, (c) Percentage of broken yeast APA microcapsules as function of shaking time.

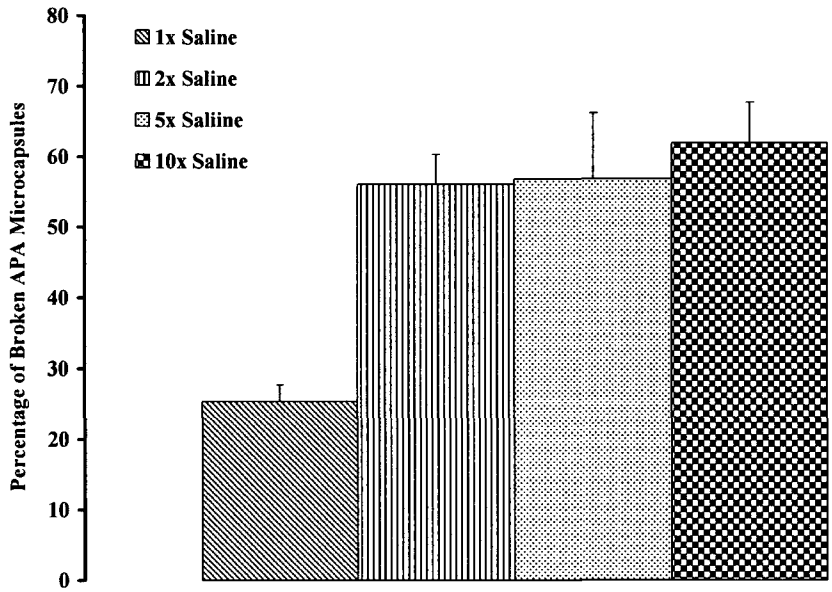
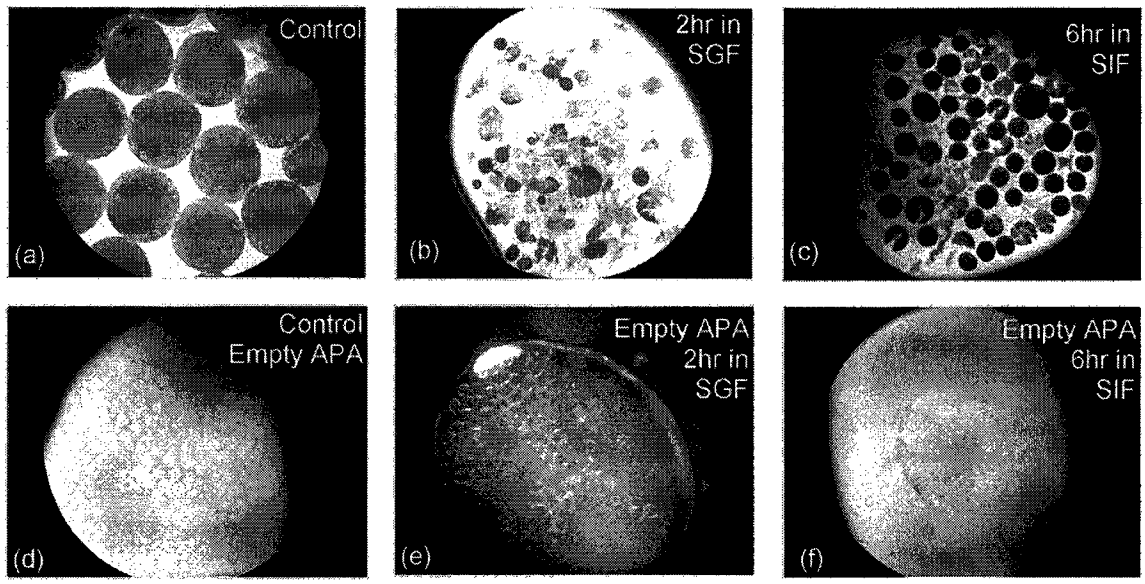
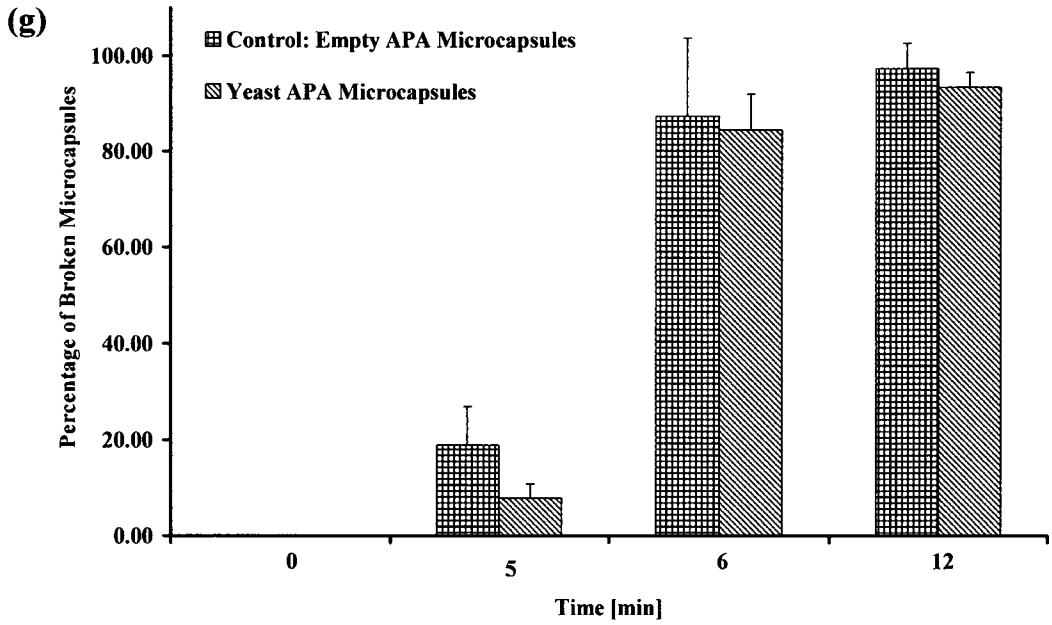
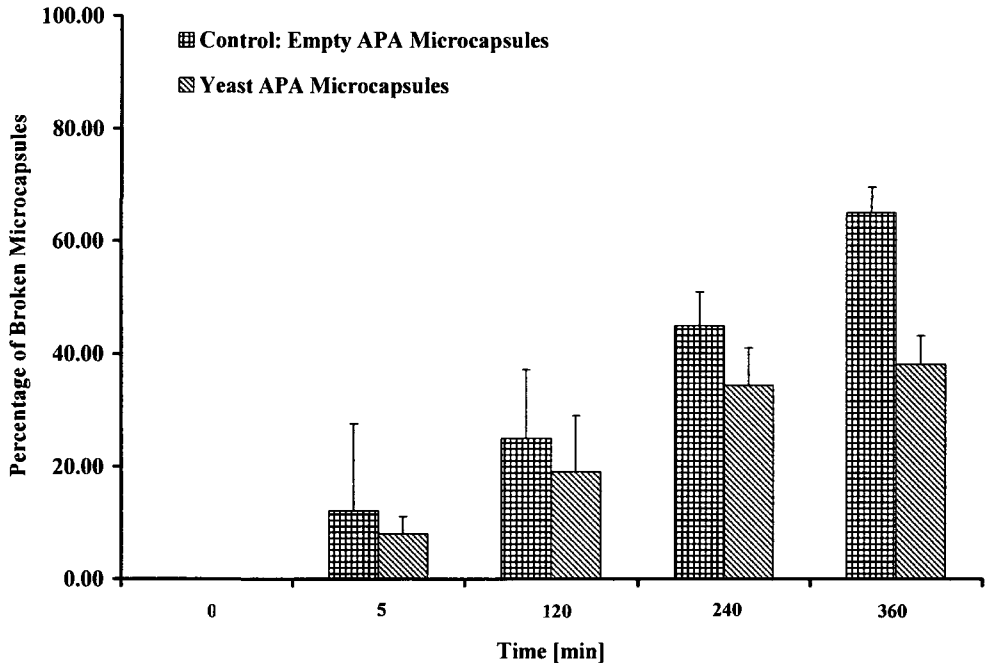


Figure 3.4: Physiological saline osmotic pressure test: percentage of broken APA microcapsules as function of NaCl concentration

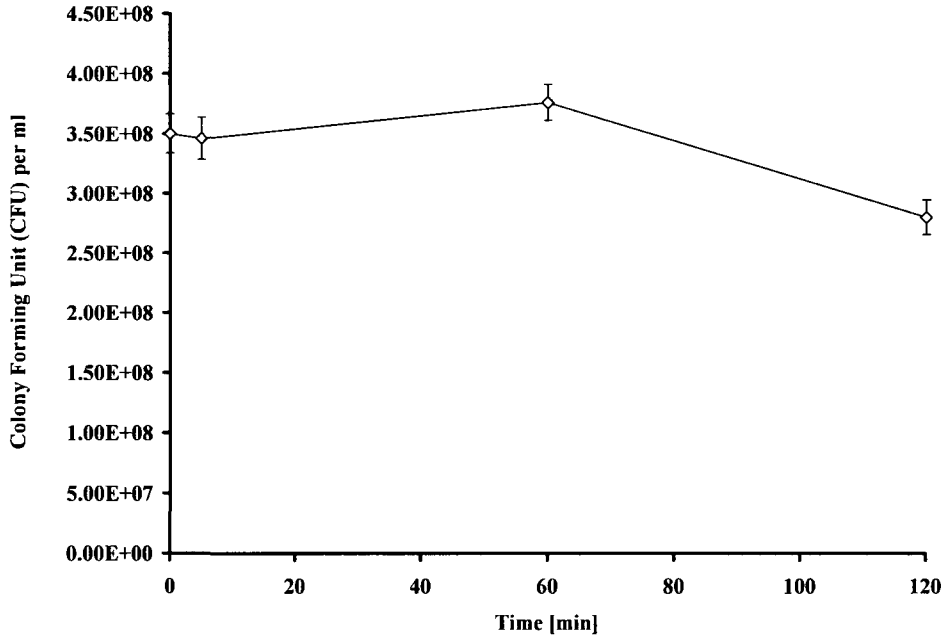




(h)


100.00
80.00
60.00
40.00
20.00
0.00
Percentage of Broken Microcapsules
Control: Empty APA Microcapsules
Yeast APA Microcapsules
0
5
120
240
360
Time [min]

(i)


4.50E+08
4.00E+08
3.50E+08
3.00E+08
2.50E+08
2.00E+08
1.50E+08
1.00E+08
5.00E+07
0.00E+00
Colony Forming Unit (CFU) per ml
0
20
40
60
80
100
120
Time [min]

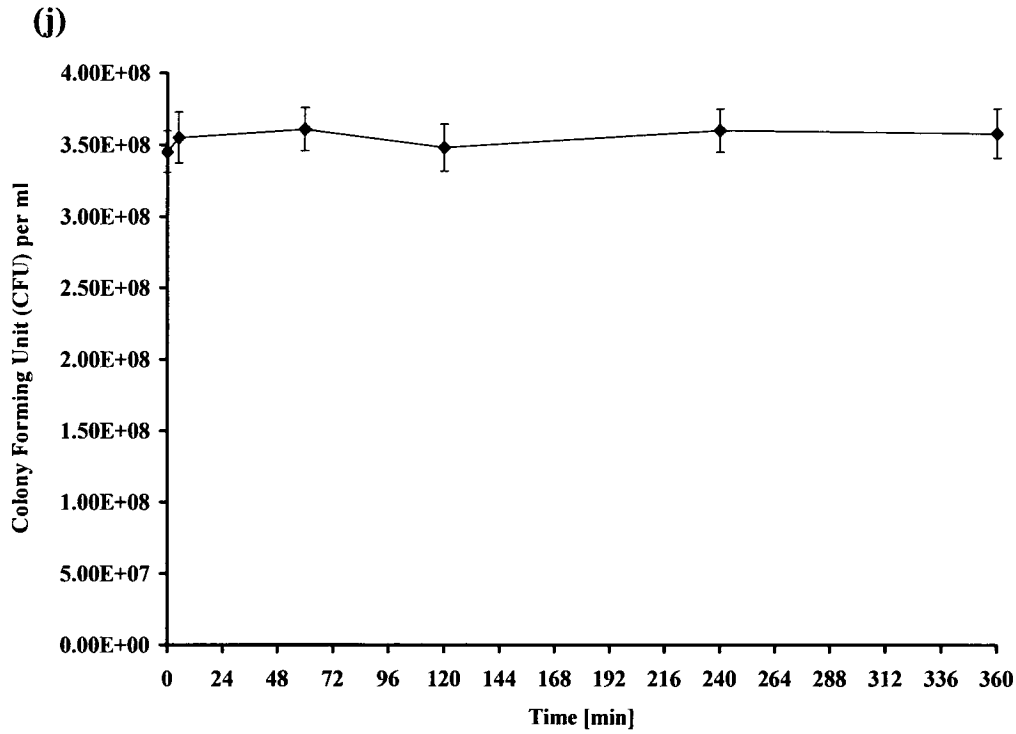
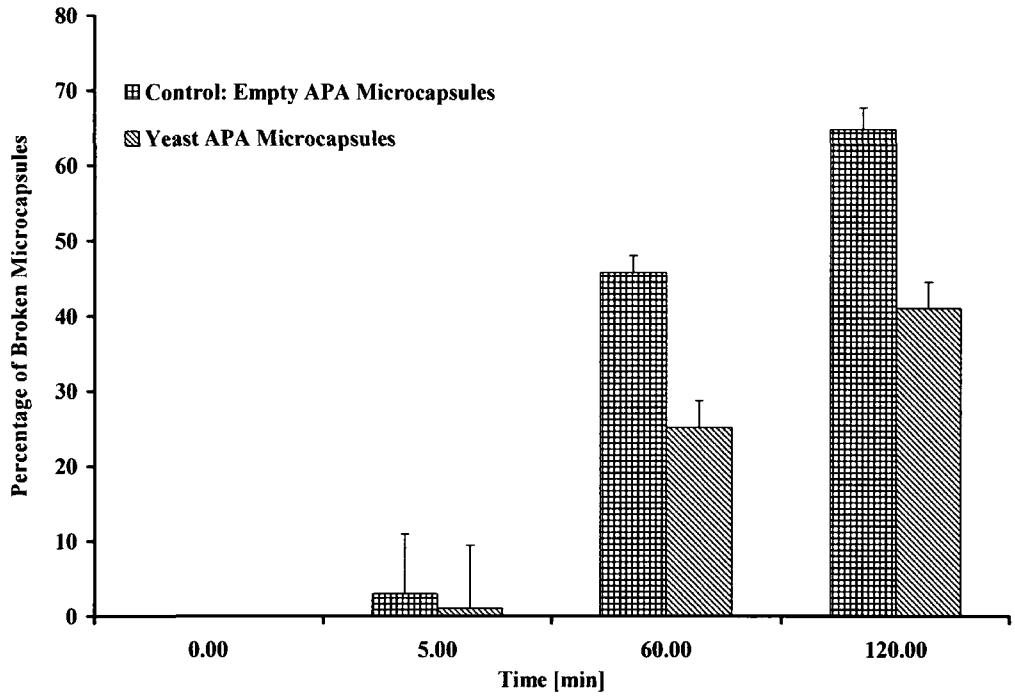
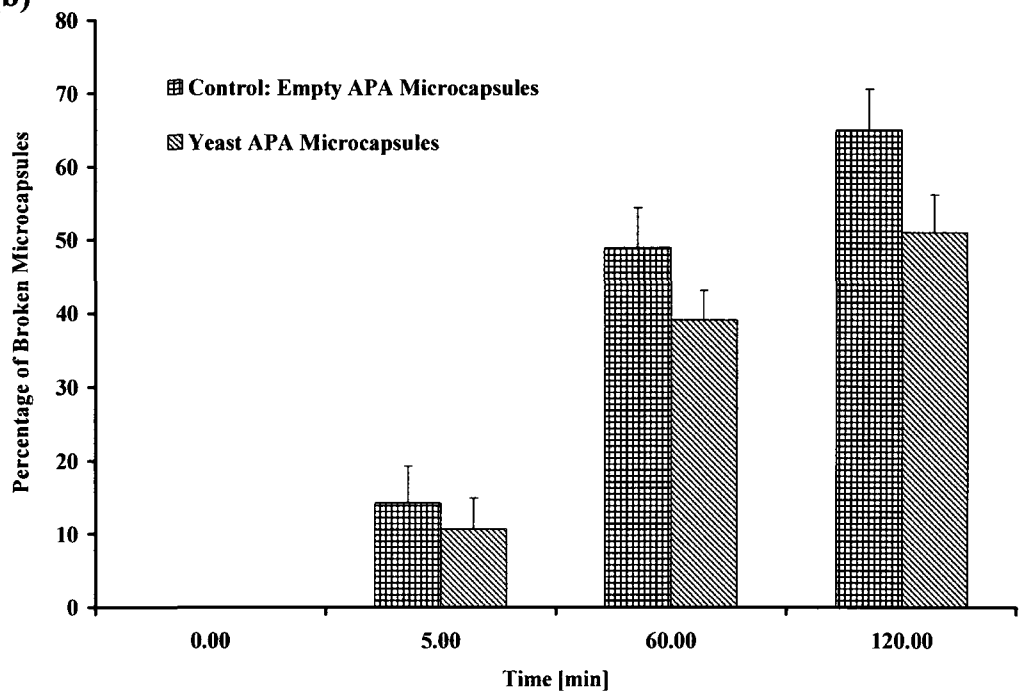


Figure 3.5: Resistance of APA microcapsules to SGF (stomach conditions: pH < 2) and SIF (small intestine conditions: pH ≥ 6.5) after exposure to mechanical shear stress and osmotic pressure. Photomicroscopic study: (a) Yeast APA microcapsules at t=0 min, (b) Yeast APA microcapsules after 2 hrs in SGF, (c) Yeast APA microcapsules after 6 hrs in SIF, (d) Empty APA microcapsules at t=0 min, (e) Empty APA microcapsules after 2 hrs in SGF, (f) Empty APA microcapsules after 6 hrs in SIF, (g) Percentage of broken yeast and empty APA microcapsules as function of time after 2 hrs in SGF, (h) Percentage of broken yeast and empty APA microcapsules as function of time in SIF, (i) Viability of microencapsulated yeast cells as function of time in SGF, (j) Viability of microencapsulated yeast cells as function of time in SIF.

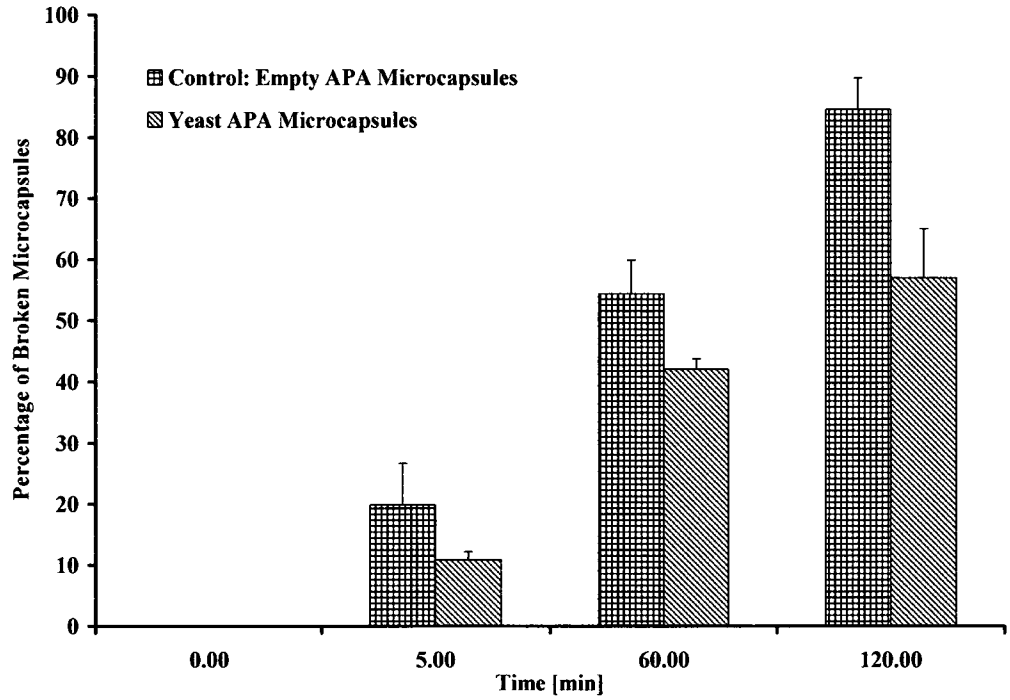
(a)


Control: Empty APA Microcapsules
Yeast APA Microcapsules
Percentage of Broken Microcapsules
0
10
20
30
40
50
60
70
80
0.00
5.00
60.00
120.00
Time [min]

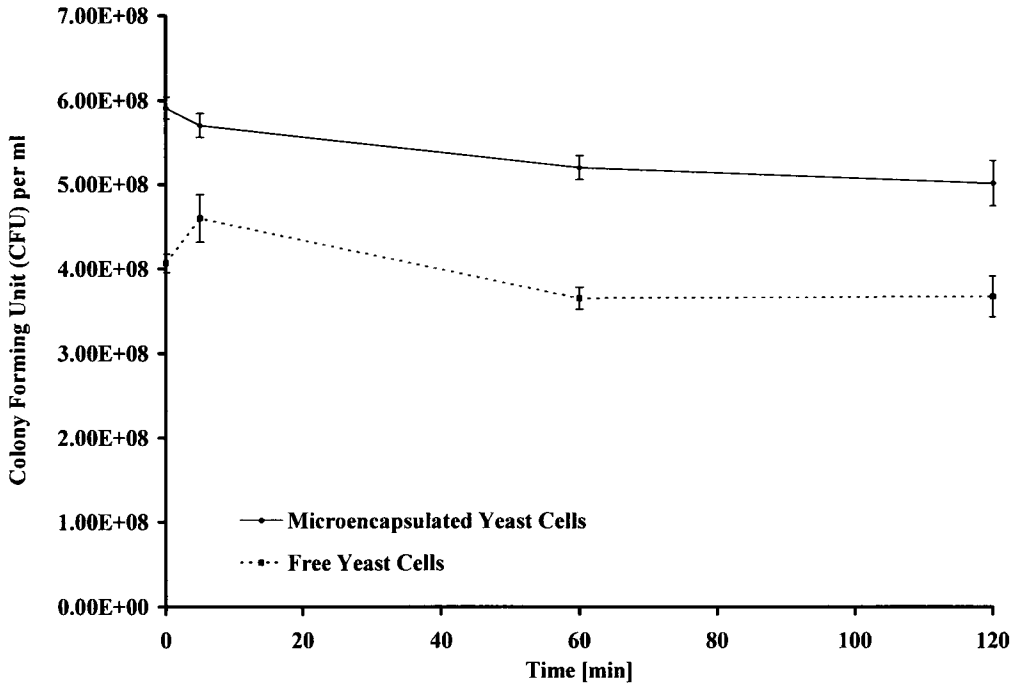
(b)


Control: Empty APA Microcapsules
Yeast APA Microcapsules
Percentage of Broken Microcapsules
0
10
20
30
40
50
60
70
80
0.00
5.00
60.00
120.00
Time [min]

(c)


Control: Empty APA Microcapsules
Yeast APA Microcapsules
Percentage of Broken Microcapsules
100
90
80
70
60
50
40
30
20
10
0
0.00
5.00
60.00
120.00
Time [min]

(d)


Colony Forming Unit (CFU) per ml
7.00E+08
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
Microencapsulated Yeast Cells
Free Yeast Cells
0
20
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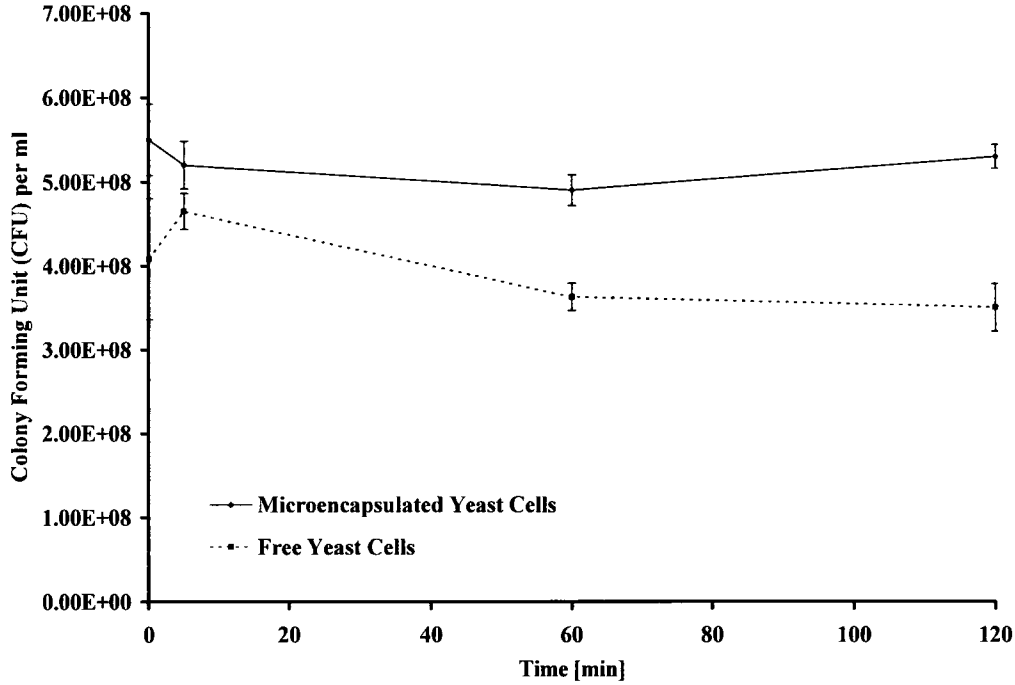
Time [min]

(e)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Colony Forming Unit (CFU) per ml
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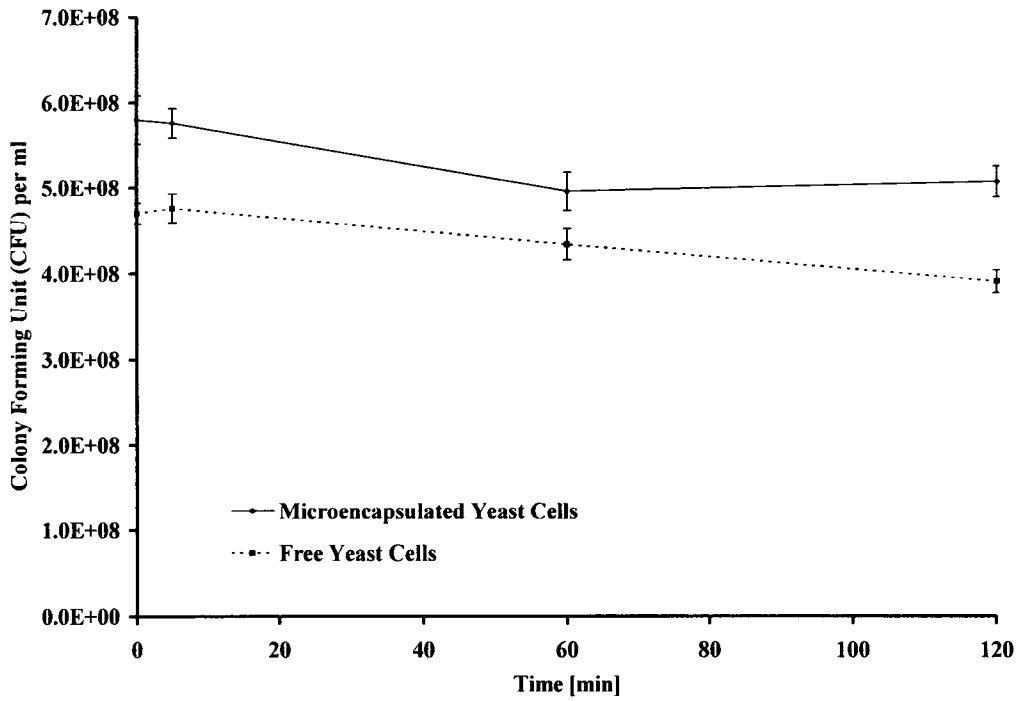
Time [min]
Microencapsulated Yeast Cells
Free Yeast Cells

(f)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Colony Forming Unit (CFU) per ml
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100
120
Time [min]
Microencapsulated Yeast Cells
Free Yeast Cells

Figure 3.6: Resistance of APA microcapsules in SGF (stomach conditions: pH < 2) exposed to mechanical shear stress and osmotic pressure generated by orbital shaking. (a) Percentage of broken yeast and empty APA microcapsules as function of time in SGF orbital shaking at 50 rpm, (b) Percentage of broken yeast and empty APA microcapsules as function of time in SGF in orbital shaking at 100 rpm, (c) Percentage of broken yeast and empty APA microcapsules as function of time in SGF in orbital shaking at 200 rpm, (d) Viability of microencapsulated yeast cells and free yeast cells as function of time in SGF in orbital shaking at 50 rpm, (e) Viability of microencapsulated yeast cells and free yeast cells as function of time in SGF in orbital shaking at 100 rpm, (f) Viability of microencapsulated yeast cells and free yeast cells as function of time in SGF in orbital shaking at 200 rpm.

Chapter IV

Original Paper 2

In vitro Analysis of APA Microcapsules for Oral Delivery of Live *Saccharomyces cerevisiae*

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Preface: As a consecutive study to that in Chapter 3, the present study investigates the capabilities of the free and microencapsulated yeast used to hydrolyze urea. It also characterizes the dependence of urea hydrolysis on yeast loading and the effect of GI conditions on urea removal. This work demonstrates the *in vitro* potential of using yeast APA microcapsules as an oral alternative treatment in renal failures.

4.1. Abstract

This article investigates the *in vitro* capacity of microencapsulated yeast cells to hydrolyze urea under several environments and evaluates the feasibility of designing a yeast column bioreactor. Results showed that the yeast loading of 1mg/100mL is capable of decreasing the urea concentration down to 10.93 ± 0.06% when immersed in SIF pH ≥ 6.5 and to 12.91 ± 1.04% when immersed in Minimal SD Base pH ≥ 6.5. Moreover, when first immersed in SGF pH < 2, the acidity of the stomach was found to lower the yeast cells' urea hydrolysis to 0.91 ± 0.02% after 6hrs in SIF pH ≥ 6.5. Results of this study proved that microencapsulated yeast cells are capable of hydrolyzing urea in *in vitro* physiological conditions. These results provide a basis for future research on the APA microcapsule therapeutic based potential.

Key Words

Microencapsulation, yeast, urea hydrolysis, loading, *in vitro* environmental effects, column bioreactor.

4.2. Introduction

Microencapsulation, which was developed by Chang[39] in the 1960s, is a procedure by which biologically active materials are enclosed within microscopic semi-permeable membrane, forming in consequence artificial cells[25,26,37,32]. This procedure does actually provide a means of routing and controlling potential cells and microorganisms with desired metabolic activities into the human body. In fact, this technology has proven successful in facilitating a wide range of biomedical and pharmaceutical processes including drug delivery, artificial organs and cellular therapy[203].

Previous research has demonstrated the feasibility and the potential of administrating microencapsulated microorganisms as an alternative oral therapy. Examples include microencapsulating genetically engineered *Escherichia coli* DH5 cells over-expressing the *Klebsiella aerogenes* urease gene for urea removal in renal failure[39], *Oxalobacter formigenes* producing oxalate-degrading enzymes for removal of

accumulated oxalate in urolithiasis[205,206] and genetically engineered *Lactobacillus Plantarum* 80 (pCBH1) for bile acid deconjugation lowering[176].

The occurrence of renal failures, characterized mainly by extensive accumulations of toxic levels of urea, is increasing consistently both in magnitude and widespread. Microencapsulating microorganisms capable of reducing urea is of particular interest since it implements a possible novel renal replacement therapy. *Saccharomyces cerevisiae*, commonly known as yeast, can be an excellent bioagents since it efficiently degrade urea[42,196]. Furthermore, it is highly adaptive to changing environments and is easily scaled up for large scale production purposes.

The strain of yeast used in this paper produces[170] urea amidolyase that is currently thought of as a second pathway in urea metabolism. This pathway is characterized by three activities: biotin carboxylase, urea carboxylase, and allophanate hydrolase (figure 4.1). According to Jitrapakdee *et al.*[207] a biotin cofactor, which is covalently attached to a carboxyl carrier domain of the protein, is carboxylated in an ATP-based and bicarbonate-dependent step. Urea reacts with the carboxyl group from the carboxy-biotin entity translocated to the second active site, forming in consequence allophanate[42,196]. The latter is then hydrolyzed by the third active site. The reduced resonance stabilization involving the carbonyl carbon makes the hydrolysis of the latter compound far more easier than that of urea[208-210].

To date, little is known about the potential of orally administrating live *Saccharomyces cerevisiae* APA microcapsules for the removal of urea in renal failure cases. Towards this goal, we have recently optimized the encapsulation procedure and its respective parameters to accommodate yeast cells. The present work investigates the suitability and the feasibility of using microencapsulated yeast cells in kidney failure as bioreactors for urea removal. For that, the dependence of urea hydrolysis on yeast loading and the effect of GI conditions on urea removal is investigated. Furthermore, the feasibility of designing and using a yeast column bioreactor as an external means of reducing urea are also studied.

4.3. Materials and Methods

4.3.1. Chemicals

Sodium alginate (low viscosity) and poly-$_L$-lysine (*MW* 27,400) were both purchased from Sigma-Aldrich. Simulated gastric fluid (SGF) is composed of 1.0 g/L arabinogalactan, 2.0 g/L pectin, 1.0 g/L xylan, 3.0 g/L starch, 0.4 g/L glucose, 3.0 g/L yeast extract, 1.0 g/L peptone, 4.0 g/L mucin, 0.5 g/L mucin and the pH was adjusted using 0.2 N HCl. Simulated intestinal fluid (SIF) consists of SGF and the appropriate amount of pancreatic juice, 12 g of $NaHCO_3$, 6 g of oxgall and 0.9 g of pancreatin all suspended in 1L of autoclave water. Minimal SD Base was purchased from Clontech and consists of 26.7g minimal SD Base with appropriate DO supp./1L. Urea in the form of white crystal beads was also purchased from Sigma-Aldrich.

4.3.2. Microorganism and culture conditions

Saccharomyces cerevisiae, strain reference number 9896, was purchased from ATCC[195]. This organism is capable of producing urease urea amidolyase, which in turn metabolizes urea[196]. Yeast mold broth (YMB) growth medium was used for primary cell cultivation. YMB is composed of 10 g/L of glucose, 3 g/L of malt extract, 5 g/L of peptone and 3 g/L yeast extract. The whole mixture was incubated aerobically at 37°C for 48 hrs.

4.3.3. Preparation of APA microcapsules containing live yeast cells

Log phase cultures of *Saccharomyces cerevisiae* were harvested by centrifugation at 3500 rpm for 15 min at 4°C. Pellets were then mixed with 2% alginate solution in order to form a final mixture consisting of 8% (v/w) pellets and 1.75% alginate. Alginate microcapsules were prepared by generating droplets of sodium alginate solution (15 mg/mL) using an IER-20 encapsulator machine manufactured by Inotech. Corp. Extruded through a 300 μm nozzle by a syringe driven pump, these droplets were gelled for 30 min in a well-stirred calcium chloride solution (11 mg/mL). The same droplets were then coated with a 0.1% (w/v) solution of PLL for 10 min to form AP beads and washed again with physiological saline (PS) for 5 min. Finally, the final alginate coating is applied by immersing the beads in a 0.1% (w/v) sodium alginate solution for 10 min followed by a

final 5 min PS wash. The resulting microcapsules, whose average diameter was 638 ± 17 μm, were then collected and stored in a 10/90 YMB/PS solution at 4°C. All solutions used in the preparation of these APA microcapsules were either filtered using 0.22 μm filter or autoclaved to ensure sterility. Moreover, the entire procedure was carried out in a biological hood.

4.3.4. Viability of live encapsulated yeast cells

To estimate the viability of the encapsulated yeast, 0.1 mL of freshly made yeast microcapsules, stored in a 10/90 YMB/PS solution at 4°C, was collected in a 1.5 mL graduated Eppendorf tube and mechanically ruptured using a sterile tissue pestle. Several 10-fold serial dilutions were performed using autoclaved PS and 0.1 mL aliquots of suspended yeast were plated on selective YM agar plates. The plates were then incubated aerobically for 48 hrs at 37°C. All experiments were done in triplicate.

4.3.5. Detection of urea hydrolysis by the specific strain of *Saccharomyces cerevisiae* used

Urea agar base, which was purchased form BD Biosciences, had been used for detecting urea. The same procedure used in 4.3.4. was used also employed for this experiment. The only exception is that the 0.1 mL aliquots of suspended yeast were plated on selective urea agar plates. The plates were then incubated aerobically at 37°C and were observed daily for 5 days. The reactions were considered positive after the appearance of a deep pink color. This experiment was done in triplicate.

4.3.6. *In vitro* 72 hrs soaking in SIF pH ≥ 6.5: investigation of the effect of yeast loading

To investigate the effect of the small intestine environmental conditions on the yeast hydrolysis capacities as well as the effect of the yeast loading on the urea hydrolysis, 6 different loadings were immersed in 50 mL SIF (pH ≥ 6.5) solution to which was added pure urea beads in the concentration of 54 mmol/L. The 6 loading groups consisted of 2 controls containing either empty APA microcapsules or no microcapsules and 4 yeast groups containing either 0.25 g or 0.5 g or 0.75 g or 1 g of yeast microcapsules per 100 mL of SIF solution. Microcapsules' mass was assessed by first weighing an Erlenmeyer, in which they will be soaked, and then adding the

correspond amount of yeast microcapsules. An AG204 DeltaRange balance was used for this purpose.

The experiment lasted for 72 hrs during which samples were taken at 0 min, 10 min, 20 min, 30 min, 1 hr, 2 hrs, 4 hrs, 6 hrs, 12 hrs, 18 hrs continuing in 6hrs increment until the 72nd hour. Thus, in total there were 19 samples per group. Each sample was taken in triplicate to ensure a significant analysis. All groups were fixed to the metallic plate of a Lab Line Environ Shaker rotating at 50 rpm. The temperature was fixed at 37°C. Using sterile pipette tips, 1 mL of the solution, excluding microcapsules, was collected at the respective sampling times, filtered using 0.22 μm filter and stored in graduated 1.5 mL Eppendorf tube at -80°C for subsequent analysis. The samples were analyzed using the Hitachi Clinical Chemistry Analyzer (Roch Diagnostics) which was loaded with a BUN urea kit (purchased from Roch Diagnostics).

4.3.7. *In vitro* 72 hrs soaking in Minimal SD Base: investigation of possible presence of other urea sources preferred by yeast

The same experiment as the one described in section 4.3.6 was carried out in Minimal SD Based instead of SIF (pH $\geq$ 6.5) medium in order to verify that the microencapsulated yeast cells are not actually using alternative sources of nitrogenous compounds. 3 groups were used for this experiment: 2 control groups containing either empty APA microcapsules or no microcapsules and 1 loading group consisting of 1 g of yeast capsules per 100 mL of minimal medium. Samples were taken in triplicate every 6 hrs. The pH of the medium was fixed at 6.5 and 54 mmol/L of urea was added. The same setup for the orbital shaker was used in this experiment. The sampling and the analysis procedures were identical to those of section 4.3.6.

4.3.8. *In vitro* 2 hrs soaking in SGF pH < 2 followed by 6 hrs soaking SIF pH $\geq$ 6.5: investigation of the effect of stomach acidity on urea hydrolysis by yeast microcapsules

To investigate the effect of the stomach conditions, mainly its acidity, on the microencapsulated yeast hydrolyzing capacities, 2 control groups containing either empty APA microcapsules or no microcapsules and 1 yeast loading group 1 g/100 mL were first immersed in 50 mL of SGF pH < 2 for 2 hrs prior to transferring them into 50 mL of SGF

solution pH ≥ 6.5. Although the experiment lasted for 8 hrs, samples were only taken during the 6 hrs soaking in SIF. The sampling procedure is described in section 4.3.6. Around 54 mmol/L of urea was added to the 50 mL SIF solution. Similarly, the Lab Line Environ Shaker rotating was used at 50 rpm and the temperature was fixed at 37°C. The analysis was again carried out using the Hitachi light scattering Multistat III Clinical Chemistry Analyzer (Roche Diagnostics) loaded with a BUN urea kit.

4.3.9. Statistical Analysis

The statistical Analysis System (SAS Enterprise Guide 4.1 (4.1.0.471) by SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Data was expressed as means ± SEM. Group and time effects were statistically investigated by ANOVA mixed models. Data were considered significant at $p<0.05$.

4.4. Results

4.4.1. Detection of urea hydrolysis by the specific strain of *Saccharomyces cerevisiae* used

When one conducts a research project based mainly on a microorganism's ability to carry out a certain task, one must verify that the microorganism in question can actually fulfill this task. The use of urea agar to detect urea hydrolysis by yeast is based on the fact that urease activity is indicated by the liberation of ammonia when a microorganism, in this case yeast, is cultivated with urea as a substrate. The production of ammonia causes the pH to rise, forcing, in consequence, the indicator phenol red to change color from yellow to pink. After exactly 48 hrs of continuous aerobic incubation at 37°C, several noticeable pink areas were observed on the surface of the agar. This proves that the yeast used the urea as the carbon source in order to grow.

4.4.2. In vitro 72 hrs soaking in SIF pH ≥ 6.5: investigation of the effect of yeast loading

After verifying that the yeast can actually hydrolyze urea, it is necessary to expose this organism to real physiological conditions and measure its urea hydrolysis efficiency. According to some unpublished work in our laboratory, it was hypothesized that a yeast loading of 1 mg per 100 mL shows the highest urea hydrolysis rate. In order to verify this

hypothesis and investigate, further, the effect of yeast loading, we decided to use 4 different yeast loadings in our experiment.

Figure 4.2 illustrates the results of this experiment. While the two control groups consisting of either no microcapsules or empty ones seemed to have relatively constant urea concentrations through out the 72 hrs, the other 4 yeast loading groups demonstrated a noticeable urea hydrolysis effect. In fact, the readings for both control groups, which were not significantly different from each other ($p>0.05$), were significantly higher than those of the other groups ($p<0.05$). The urea concentrations in each yeast loading group exhibited both a time and loading effects ($p<0.05$).

All groups had the same starting urea concentration of 54.22 mmol/L. As mentioned before, after 72 hrs, the urea concentration in the no capsules and empty APA control groups did not vary and averaged 54.32 ± 0.56 mmol/L and 54.32 ± 0.88 mmol/L, respectively. However, the urea concentrations in the other 4 loading groups followed a continuous decrease along the test period reaching a minimum of 49.74 ± 0.13 mmol/L, 48.72 ± 0.88 mmol/L, 48.96 ± 0.39 mmol/L and 48.29 ± 0.06 mmol/L, for the 0.25 g/100ml, 0.5 g/100ml, 0.75 g/100ml and 1 g/100ml yeast loadings, respectively.

4.4.3. In vitro 72 hrs soaking in Minimal SD Base: investigation of possible presence of other urea sources preferred by yeast

In order to enforce our hypothesis, it was necessary to make sure that the microencapsulated yeast cells are actually using urea as their carbon source and not some other nitrogenous compounds present in the SIF. Figure 4.3 illustrates the behavior of the 3 different groups in regards to urea hydrolysis when immersed in minimal SD Base (pH $\geq$ 6.5) for 72 hrs. The urea concentrations for each control group did not vary significantly with respect to time ($p>0.05$). Also, there was no significant differences in the urea concentrations at the same time interval between the two control group ($p>0.05$). Along the 72 hrs, the urea concentration averaged 53.14 ± 0.72 mmol/L and 53.91 ± 0.85 mmol/L for the no microcapsules and empty APA microcapsules groups, respectively. The urea hydrolysis by the yeast loading group (1 g/100mL) did vary with respect to time starting at 54.22 ± 0.61 mmol/L at t=0 hr and reaching 47.22 ± 1.04 mmol/L at t=72 hrs. The values for the latter group were significantly different from those of the control

groups ($p<0.05$). Therefore, the results of this experiment confirm that the yeast cells trapped inside the APA microcapsules are actually hydrolyzing urea and not some other nitrogenous compound.

4.4.4. In vitro 2 hrs soaking in SGF pH < 2 followed by 6 hrs soaking SIF pH ≥ 6.5: investigation of effect of stomach acidity on urea hydrolysis by yeast microcapsules

Considering the effect of the stomach acidity on yeast's urea hydrolysis efficiency is extremely important in that it determines the extent of damage, if any exists, that may challenges the yeast's performance. This study will give insights into possible mechanisms by which the yeast performance is affected. As it was noticed in the previous experiments, the no microcapsules and empty microcapsules control groups did not display significant variations in the urea concentrations along the 6 hrs sampling period (figure 4.4). The urea concentrations for the no microcapsules and empty microcapsules groups averaged 54.19 ± 0.09 mmol/L and 54.27 ± 0.09 mmol/L, respectively. This suggests that there is neither a group effect nor a time effect for the control readings ($p>0.05$). On the contrary, the 1 g/100mL yeast loading group had its starting urea concentration (t=0 hr) at 54.22 ± 0.05 mmol/L and reached 54.07 ± 0.12 mmol/L, 53.91 ± 0.11 mmol/L, 53.75 ± 0.18 mmol/L, 53.74 ± 0.08 mmol/L and 53.73 ± 0.02 mmol/L at the t=30 min, 1 hr, 2 hrs, 4 hrs and 6 hrs, respectively. Note that the change in the urea concentration differed significantly between sampling times for the latter loading ($p<0.05$).

4.5. Discussion

According to Paliwal *et al.*[211], no color change was found when yeast cells were plated on urea agar plates. Our findings contradict this finding. Furthermore, the group found that the fasted organisms capable of hydrolyzing urea are *Cryptococcus neoformans* with minimum and maximum times of 12 hrs and 72 hrs, respectively. Therefore, reporting that our yeast strain took 48 hrs to hydrolyze urea is not surprising knowing that this particular strain is not genetically engineered.

The whole design and feasibility of this novel renal failure uremia treatment relies on the fact that the newly designed APA microcapsules entrapping live yeast cells will

remove excess urea concentrations. This will only be possible when a concentration gradient will be established between the inside and the outside of the artificial cells. With the exception of all electrolytes diffusion occurring in the renal system, most of remaining diffusion, which is mediated by an extensive network of blood streams, occurs in the intestine region. This then implies that the yeast APA microcapsules, once in the small intestine, will be able to hydrolyze urea once a concentration gradient has been set. For this particular reason, we tested the effect of SIF conditions on the yeast's urea hydrolysis efficiency. The rational behind testing the capsules for 72 hrs is that digestion may take up to 72 hrs in some cases in the following sequence: 4 hrs in the small intestine, then 18 hrs in ascending colon followed by 24 hrs in the tranverse colon and finally another 24 hrs in the descending colon[203]. Moreover, to minimize experimental bias and ensure homogeneous concentrations upon sampling, the solutions had to be continuously and gently mixed.

Investigating the yeast loading effect is crucial since it will provide insights into the optimum dosage of yeast microcapsules needed. Since yeast is a foreign material, it must be administered in the least possible amounts while still keeping a certain level of acceptable efficiency. In our experiment, only the loading groups (0.25 g/100mL, 0.5 g/100mL, 0.75 g/100mL and 1 g/100mL) displayed a noticeable urea hydrolysis effect ($p<0.05$) when compared to the 2 control groups. Based on that, it is possible to infer that the highest yeast loading group (1 g/100mL) displayed the fasted hydrolysis per unit time (figure 4.2). When the data is expressed as percent decrease in urea concentration, the 1 g/100mL yeast loading group would have achieved approximately an 11 ± 0.59% decrease versus 10 ± 0.81%, 9 ± 0.65% and 8 ± 0.41% for the 0.75 g/100mL, 0.5 g/100mL and 0.25 g/100mL loading groups, respectively. This can also be observed when approximating the curves in figure 4.2 to straight lines; by doing so, one can notice that the line for the highest yeast loading has, also, the steepest slope.

Due to the presence of nitrogenous compounds in the SIF medium, the possibility that yeast cells use alternative sources of carbon can not be overruled. Minimal SD Base medium provides the minimum nutrients needed for the yeast to grow. When urea is added, the yeast cells, with no other possible source of carbon, will have to break down urea in order to sustain themselves. Following the results of the first experiment, we

noted that the 1 g/100mL of yeast loading was the fastest in hydrolyzing urea. For this it is though to be the optimal dosage for the purpose of this treatment. That is why the other loadings were not tested. Even when urea is the only available substrate, similar results in regards to urea hydrolysis were observed. Both control groups performance, which were not significantly different from each other ($p>0.05$, figure 4.3), varied significantly from that of the yeast loading ($p<0.05$). All that to confirm that yeast cells are actually breaking down urea for their survival.

Comparing the performance of the 1 g/100mL loading in both experiments, no significant differences in urea concentrations were noticed ($p>0.05$). Specifically, this yeast loading hydrolyzed approximately 11% and 13% of the initial urea concentration in the SIF and Minimal SD Base experiments, respectively. This would then prove that in presence of other nitrogenous compounds, yeast cells do use urea as their primary source of carbon. If this was not the case, yeast cells would have taken more time to break down urea, since other easier carbon sources are available. This will be translated by a time lag phase in figure 4.2 with respect to figure 4.3.

When yeast cells were first soaked in SGF pH < 2 followed by 6 hrs soaking SIF pH ≥ 6.5, these microorganisms hydrolyzed urea, but not to the same extent as before. Without the acidity of the stomach, the 1 g/100mL yeast loading group decreased the urea concentration by 1.95 ± 0.58% and 1.78 ± 0.95% after 6hrs in SIF or Minimal SD base, respectively. However, when first exposed to the stomach's environment, the microencapsulated yeast cells hydrolyzed 0.91 ± 0.02% of the initial urea after 6hrs immersion in SIF. This result tends to be significantly different from the first two ($p<0.05$), suggesting that stomach acidity have a considerable effect on the yeast's urea hydrolysis. The stomach's acidity does that by damaging some of the microcapsule's membrane, which at the end, starts leaking. Without any membrane to protect them, free yeast cells will not be able to survive long enough in such harsh environments. Therefore, the stomach's acidity limits the yeast's urea hydrolysis performance by decreasing the number of yeast cells capable of undertaking this task. Even a small decrease in yeast cells appears to have a larger effect on the urea hydrolysis efficiency.

4.6. Conclusion

Results showed that the yeast cells, whether in SIF or in Minimal SD Base, use solely urea as their carbon source for their survival. Results also showed that a loading of 1 g/100mL of yeast microcapsules generates the fastest urea hydrolysis rate per unit time. The acidity of the stomach was reported to jeopardize the yeast urea hydrolysis efficiency by damaging some of the membranes of certain weak APA microcapsules. Finally, this microcapsule formulation shows promising outcomes in hydrolyzing urea.

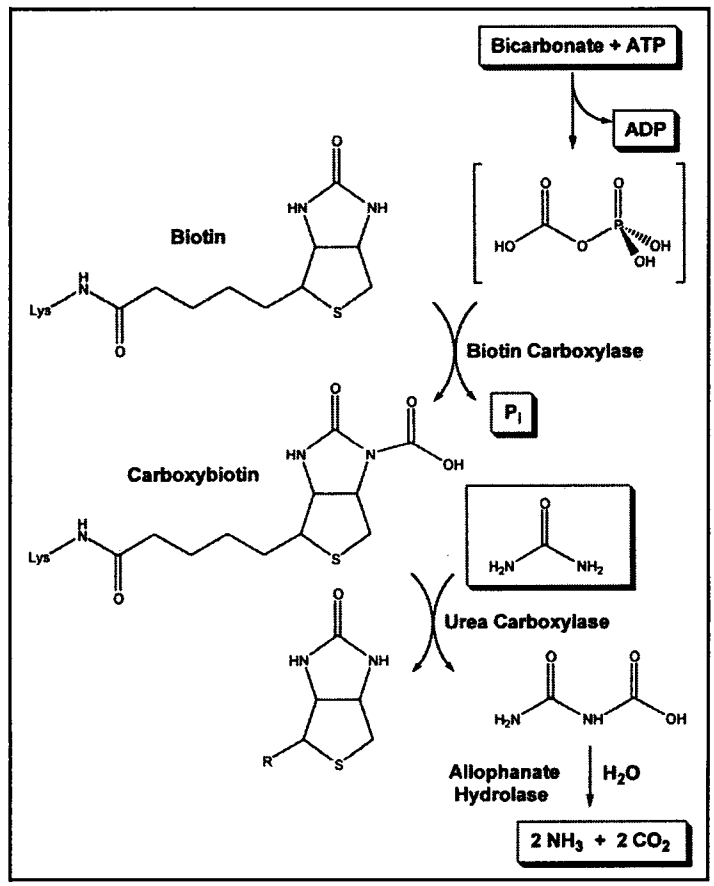

Figure 4.1: Urea hydrolysis pathway: the three activities of urea amidolyase, biotin carboxylase, urea carboxylase, and allophanate hydrolase[196]

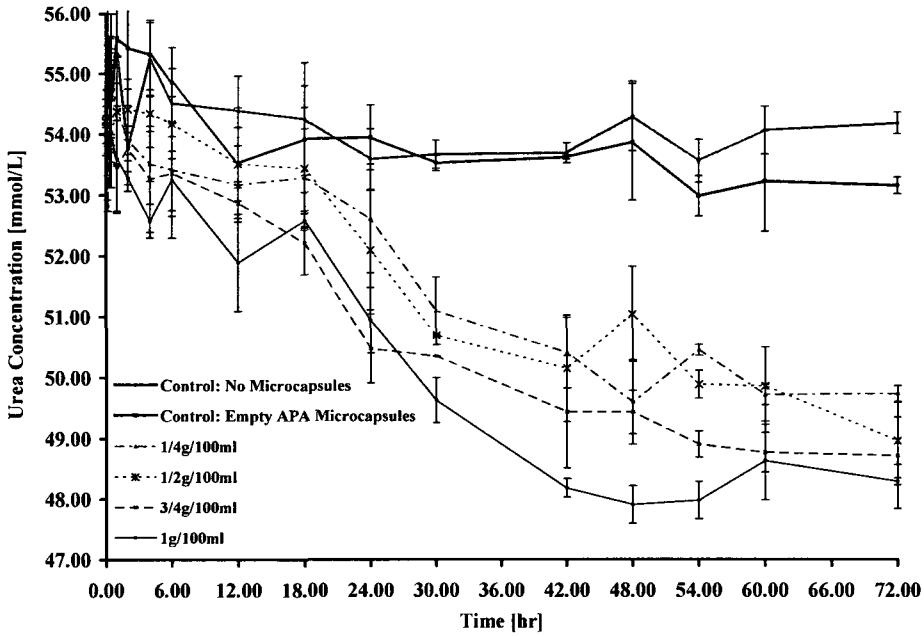

Figure 4.2: In vitro exposure to SIF pH ≥ 6.5: Investigation of the effect of yeast loading. Urea concentration as function of time and yeast microcapsules' loading.

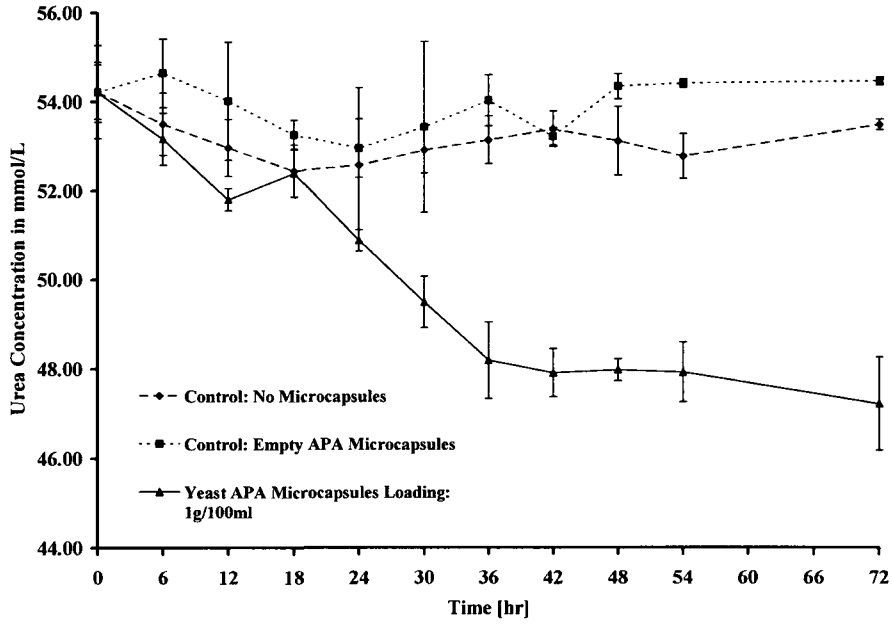

Figure 4.3: In vitro exposure to Minimal SD Base pH ≥ 6.5: Investigation of possible presence of other urea sources preferred by yeast. Urea concentration as function of time and yeast microcapsules' loading.

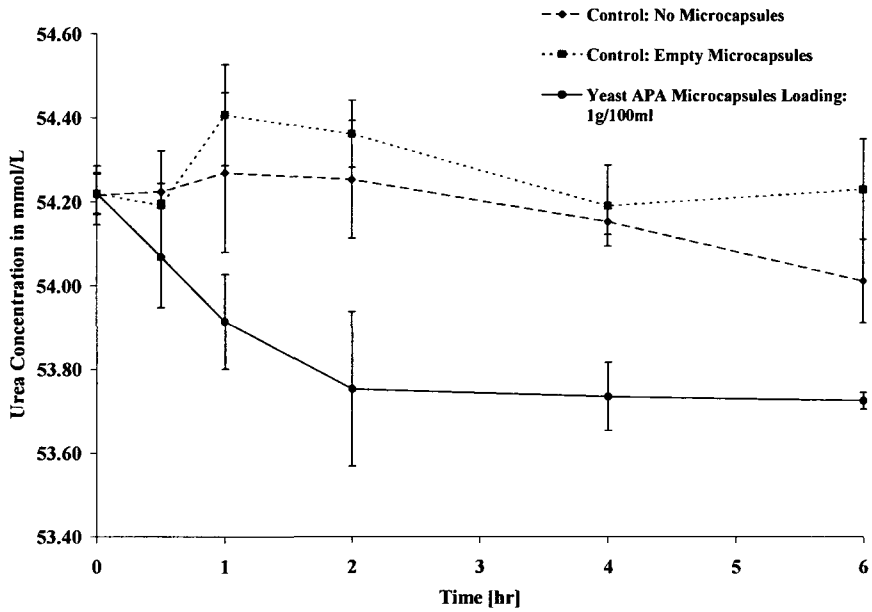

Figure 4.4: In vitro exposure to SGF pH < 2 followed by 6hrs in SIF pH ≥ 6.5: Investigation of the effect of stomach acidity on urea hydrolysis by yeast microcapsules. Urea concentration as function of time and yeast microcapsules' loading.

Chapter V

Original Paper 3

Alginate-Poly-$_L$-Lysine-Alginate Live *Saccharomyces cerevisiae* Microcapsules Column Bioreactor for Use in Renal Failure Uremia

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Preface: As a consecutive study to those in Chapters 3 and 4, the present study investigates the feasibility of designing and using a column bioreactor packed with APA live yeast microcapsules for use in renal failure uremia. It also characterizes the dependence of urea hydrolysis on the column yeast loading.

5.1. Abstract

A new bioartificial bioreactor filled with APA microcapsules entrapping live yeast cells for use in renal failure uremia has been designed. In this present study, the feasibility of using this bioreactor is investigated with respect to the yeast cells' urea hydrolysis capacities. Rat plasma was used as the perfusion fluid and each experiment lased for 4 hrs. Plasma samples were collected and analyzed for urea, creatinine, calcium and phosphate concentrations. Also, the effect of yeast column loading was studied using 3 different packing. As the column loading increased from 25% to 50% and then to 100%, the urea hydrolysis, after 4 hrs of continuous perfusion, reached 18.2%, 18.5% and 28.2%, respectively ($p<0.05$). Our analysis of the other compounds present in the rat plasma revealed no significant change in their concentrations with respect to either time or loading ($p>0.05$). In particular, for the 25%, 50% and 100% yeast column loading, creatinine concentrations averaged 26.26 ± 1.55 μmol/L, 23.92 ± 1.33 μmol/L and 26.71 ± 1.79 μmol/L, respectively. The concentrations of calcium for the same three groups averaged 4.88 ± 0.21 mmol/L, 5.74 ± 0.31 mmol/L and 5.59 ± 0.17 mmol/L, respectively. Finally, phosphate concentrations averaged 1.97 ± 0.11 mmol/L, 2.00 ± 0.10 mmol/L and 2.07 ± 0.08 mmol/L, respectively, for the 25%, 50% and 100% yeast column loading. Results of this study proved that microencapsulated yeast cells are capable of hydrolyzing urea in an *in vitro* plasma perfusion setup. These results provide a basis for future research on the APA yeast microcapsule therapeutic based potential.

Key Words

Microencapsulation, yeast, column bioreactor, urea hydrolysis, loading effect, plasma perfusion.

5.2. Introduction

Around 8.3 million Americans were reported, in 2005, to have a mature chronic kidney disease with a significant number developing end-stage renal disease (ESRD)[4,5]. Approximately 200,000 people are affected by ARF in the United States annually. This represent around 5% of all long-term hospitalized patients[6-8]. ARF and ESRD have high

mortality characterized by an overall survival rate of approximately 50% or lower depending on the severity of the renal malfunction[9].

Faced with the seriousness of kidney failure uremia, numerous renal replacement therapies (RRTs), such as kidney transplantation and dialysis, have been developed since the 1950s. To date, numerous fundamental management issues concerning RRTs remain unresolved both in ARF and CRF. The indications and timing of initiation of therapy, modality selection, and the optimal dosing strategy for each modality are examples of such issues[123]. The role of the kidneys "in reclamation of metabolic substrates, synthesis of glutathione, gluconeogenesis, ammoniagenesis, catabolism of peptide hormones and growth factors, as well as production and regulation of multiple cytokines [...] are not addressed by current treatment modalities"[124-129]. These same modalities are at the same time uncomfortable and inconvenient for a significant number of patients due to multimedication requirements and QOL's derangements.

All these realizations have boosted the need for novel, non-invasive, practical RRTs characterized by rapid, if not instantaneous, operability and outstanding efficiency. Furthermore, there is also a considerable drive to reduce morbidity, mortality and the overall economic impact associated with kidney failure. *Ex vivo* perfusion treatment of renal failure uremia using novel bioreactors has proven successful. Using microencapsulated live microorganisms is of particular importance because the membranes of these artificial cell can "have the required permeability characteristics to allow the rapid equilibration of external molecules to be acted on by the enclosed bioreactants"[212]. Furthermore, such therapeutic alternative can hydrolyze toxic wastes and other unwanted metabolites without deranging the physiological hemostasis or resulting in neither deleterious metabolic activities nor systemic infections.

In this study, the efficacy of using a novel bioreactor packed with APA microcapsules entrapping live yeast cells is investigated. Towards this goal, we have recently optimized the encapsulation procedure and its respective parameters to accommodate yeast cells. In this study, we report the results of our *in vivo* plasma perfusion studies in to evaluate the practicability of developing a novel extracorporeal approach for urea removal. Moreover, this study can serve as a feasibility study for other works using similar microorganisms for different endeavors.

5.3. Materials and Methods

5.3.1. Chemicals

Sodium alginate (low viscosity) and poly-$_L$-lysine (*MW* 27,400) were both purchased from Sigma-Aldrich. Simulated gastric fluid (SGF) is composed of 1.0 g/L arabinogalactan, 2.0 g/L pectin, 1.0 g/L xylan, 3.0 g/L starch, 0.4 g/L glucose, 3.0 g/L yeast extract, 1.0 g/L peptone, 4.0 g/L mucin, 0.5 g/L mucin and the pH was adjusted using 0.2 N HCl. Simulated intestinal fluid (SIF) consists of SGF and the appropriate amount of pancreatic juice, 12 g of $NaHCO_3$, 6 g of oxgall and 0.9 g of pancreatin all suspended in 1L of autoclave water. Minimal SD Base was purchased from Clontech and consists of 26.7g minimal SD Base with appropriate DO supp./1L. Urea in the form of white crystal beads was also purchased from Sigma-Aldrich.

5.3.2. Microorganism and culture conditions

Saccharomyces cerevisiae, strain reference number 9896, was purchased from ATCC[195]. This organism is capable of producing urease urea amidolyase, which in turn metabolizes urea[196]. Yeast mold broth (YMB) growth medium was used for primary cell cultivation. YMB is composed of 10 g/L of glucose, 3 g/L of malt extract, 5 g/L of peptone and 3 g/L yeast extract. The whole mixture was incubated aerobically at 37°C for 48 hrs.

5.3.3. Preparation of APA microcapsules containing live yeast cells

Log phase cultures of *Saccharomyces cerevisiae* were harvested by centrifugation at 3500 rpm for 15 min at 4°C. Pellets were then mixed with 2% alginate solution in order to form a final mixture consisting of 8% (v/w) pellets and 1.75% alginate. Alginate microcapsules were prepared by generating droplets of sodium alginate solution (15 mg/mL) using an IER-20 encapsulator machine manufactured by Inotech. Corp. Extruded through a 300 μm nozzle by a syringe driven pump, these droplets were gelled for 30 min in a well-stirred calcium chloride solution (11 mg/mL). The same droplets were then coated with a 0.1% (w/v) solution of PLL for 10 min to form AP beads and washed again with physiological saline (PS) for 5 min. Finally, the final alginate coating is applied by immersing the beads in a 0.1% (w/v) sodium alginate solution for 10 min followed by a

final 5 min PS wash. The resulting microcapsules, whose average diameter was 638 ± 17 μm, were then collected and stored in a 10/90 YMB/PS solution at 4°C. All solutions used in the preparation of these APA microcapsules were either filtered using 0.22 μm filter or autoclaved to ensure sterility. Moreover, the entire procedure was carried out in a biological hood.

5.3.4. Viability of live encapsulated yeast cells

To estimate the viability of the encapsulated yeast, 0.1 mL of freshly made yeast microcapsules, stored in a 10/90 YMB/PS solution at 4°C, was collected in a 1.5 mL graduated Eppendorf tube and mechanically ruptured using a sterile tissue pestle. Several 10-fold serial dilutions were performed using autoclaved PS and 0.1 mL aliquots of suspended yeast were plated on selective YM agar plates. The plates were then incubated aerobically for 48 hrs at 37°C. All experiments were done in triplicate.

5.3.5. Column Bioreactor Design

The column bioreactor's design criterion was the average surface area of conventional dialyzer membrane, which is estimated to be $1.8m^{2}$,[213]. Knowing the average diameter of our APA microcapsules (638 ± 17 μm), it is possible to calculate the bioreactor's volume. Having the volume and fixing either the diameter or the height, one can then solve for the missing variable. According to these specifications, the column was 12 cm high and 22 cm wide from the inside (figure 5.1). A robust material capable of withstanding harsh conditions while maintaining its integrity and stability is highly desired. For that Poly(methyl methacrylate) (PMMA), more commonly known as Plexiglass, was chosen as the ideal material of construction of the column.

To the 12 cm cylindrical portion were added 2 fittings measuring each 3cm high and 22 cm wide. The purpose of these parts is to allow a fully developed flow inside and outside the column. This will be extremely beneficial to ensure an even widespread of the circulating fluid around the microcapsules. Furthermore, 2 identical meshes equipped with rubber O-ring were used in order to prevent any microcapsule from being drifted with the flow. Note that because these meshes are movable, it is possible to vary the

yeast loading inside the column and, hence, investigate the effect of this variation on urea hydrolysis.

5.3.6. Column Bioreactor Experimental Procedure

200 mL of heparinized sterile rat plasma (purchased from Equitech-Bio Inc.) was re-circulated using two identical Watson-Marlow peristaltic pumps (model 101U/R). Pure urea beads in the concentration of 54 mmol/L were added to the initial 200 mL of rat plasma. The temperature of re-criculating plasma was fixed at 37°C using heating magnetic stirrers. 3 different loading ratios of 25%, 50% and 100% were used in this experiment. Each ratio corresponds to the portion of column's volume occupied by microcapsules. Each experiment lasted for 4 hrs. 1 mL samples were taken using sterile pipette tips at 0 min, 5 min, 30 min, 1 hr continuing in 30 min increments until the 4^{th} hour. The samples, which were stored at -80°C, were analyzed using the Hitachi Clinical Chemistry Analyzer (Roch Diagnostics) loaded with a BUN urea, creatinine, calcium and phosphate kits. In order to keep a sterile environment, the column and all of its accessories were soaked in a 90% alcohol solution for 48 hrs before each usage.

5.3.7. Statistical Analysis

The statistical Analysis System (SAS Enterprise Guide 4.1 (4.1.0.471) by SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Data was expressed as means ± SEM. Group and time effects were statistically investigated by ANOVA mixed models. Data were considered significant at $p<0.05$.

5.4. Results

Designing a yeast column bioreactor as an external mechanical device to lower urea concentrations serves more than one purpose. It does provide insights into yeast urea saturation, extent of yeast APA microcapsules' packing and flow rheology around the microcapsules. Figures 5.2a-d present the results of this set of experiments.

As expected, the higher the column yeast packing is, the greater the urea removal will be. This is in particular illustrated in figure 5.2a. Using 25% of column volume, the urea concentration changed from 54.22 ± 0.26 mmol/L at t=0 hr to 44.42 ± 0.27 mmol/L,

42.11 ± 0.20 mmol/L, 41.49 ± 0.18 mmol/L and 44.17 ± 0.37 mmol/L at t=1 hr, 2 hrs, 3 hrs and 4 hrs, respectively. Similarly, for the 50% packing, the urea concentration was 54.22 ± 0.88 mmol/L, 43.57 ± 0.50 mmol/L, 44.33 ± 1.28 mmol/L, 44.20 ± 1.11 mmol/L and 44.08 ± 1.42 mmol/L at t=0 hr, 1 hr, 2 hrs, 3 hrs and 4 hrs, respectively. The 100% yeast packing, which generated the highest urea hydrolysis, decreased the urea concentration from 54.22 ± 0.30 mmol/L at t=0 hr, to 39.50 ± 0.20 mmol/L, 39.02 ± 0.02 mmol/L, 39.04± 0.01 mmol/L and 38.93± 0.29 mmol/L at t=1 hr, 2 hrs, 3 hrs and 4 hrs, respectively. The statistical analysis suggested that group and time effects are important ($p<0.05$); therefore, increasing the yeast loading yields a higher urea hydrolysis.

Our analysis of the other compounds present in the rat plasma revealed no significant change in their concentrations with respect to either time or loading ($p>0.05$). In particular, for the 25%, 50% and 100% yeast column loading, creatinine concentrations averaged 26.26 ± 1.55 μmol/L, 23.92 ± 1.33 μmol/L and 26.71 ± 1.79 μmol/L, respectively (figure 5.2b). The concentrations of calcium for the same three groups averaged 4.88 ± 0.21 mmol/L, 5.74 ± 0.31 mmol/L and 5.59 ± 0.17 mmol/L, respectively (figure 5.2c). Finally, phosphate concentrations averaged 1.97 ± 0.11 mmol/L, 2.00 ± 0.10 mmol/L and 2.07 ± 0.08 mmol/L, respectively, for the 25%, 50% and 100% yeast column loading (figure 5.2a).

The yeast loading had also a noticeable effect on the plasma flowrate through the column. In particular, the flowrates for the 25%, 50% and 100% yeast column loading were 20 mL/min, 10 mL/min and 5 mL/min, respectively. Hence, there is a linear relationship between the flowrate and the column yeast packing.

Investigating the viability of the yeast cells before and after the experiment is of particular importance since it provides information about the effect of plasma elution on the yeast viability. The viability of microencapsulated yeast cells at t=0 hr was $3.78X10^8 \pm 1.7X10^7$ CFU/ml, $3.65X10^8 \pm 1.4X10^7$ CFU/mL and $3.72X10^8 \pm 1.5X10^7$ CFU/mL for the 25%, 50% and 100% loading groups, respectively. After 4 hrs of continuous plasma elution, the viability for the same three groups was $3.69X10^8 \pm 1.2X10^7$ CFU/ml, $3.58X10^8 \pm 1.6X10^7$ CFU/mL and $3.68X10^8 \pm 1.5X10^7$ CFU/mL, respectively. Clearly, varying the yeast loading does not seem to affect the yeast viability ($p>0.05$).

5.5. Discussion

Designing a column bioreactor is an attempt to implement an alternative to external dialysis machines. The rational behind testing the column for 4 hrs lies in the fact that most dialysis sessions last for an average of 4 hrs[96]. Our results suggest that urea hydrolysis by yeast microcapsules depends on the yeast loading. The higher the loading, the greater the urea breakdown will be. As the column loading increased from 25% to 50% and then to 100%, the urea hydrolysis reached 18.2 ± 0.21%, 18.5 ± 0.15% and 28.2 ± 0.05%, respectively ($p<0.05$). However, based on figure 5.2a, the urea concentration seems stagnant after almost 1 hr of re-circulation. In fact, there were no significant differences among urea concentrations after 1 hr within each column loading group ($p>0.05$). Microencapsulated yeast cells seem to either saturate after 1 hr or simply stop hydrolyzing urea. The last hypothesis is not possible because it suggests that yeast cells, by doing so, fail to survive after 4 hrs, which contradicts our viability study's results. Saturation might be explained by an inefficient re-circulation possibly jeopardizing the urea concentration gradient. The exact cause of this saturation can not be explained at this stage and further research is needed in this regard.

Another interesting point to note is the difference in flowrates due to the difference in yeast loading. This may seem problematic at first glance, but is logic after all. As the packing increase, less space is available, inside the column, for plasma particles to flow. Thus, there is an increase in the retention time as the yeast loading increases. A urea particle will, thus, spend more time in the column and has, in consequence, more chance of being hydrolyzed than when the same particle travels several times more through the emptier column – which is the case with lower yeast loading. For this reason, the 100% loading had the highest hydrolysis extent compared to the others.

In a previous study, we have found that the optimal oral yeast microcapsule dosage is 1 g/100mL. When comparing the urea hydrolysis by the 100% yeast loading group in the column experiment to that dosage, a clear difference can be noticed (28.2 ± 0.29% for the column vs. 10.93 ± 0.06% for the SIF experiment and 12.91 ± 1.04% for the Minimal SD Base experiment – *please refer to original paper 2, chapter 4*).

However, this does not suggest that the latter loading is inefficient simply because each one of these optimal loadings has its own application. The 1 g/100mL is the yeast APA microcapsules loading that should be used when these microcapsules are administered orally. Obviously, due to medical concerns, it would not be feasible to orally administer a yeast loading equivalent to that of the column. Similarly, loading the column with a ratio of 1 g of microcapsules for each 100 mL will not yield any noticeable urea hydrolysis since such loading implies using very few microcapsules.

5.6. Conclusion

We have investigated the urea hydrolysis capacity of microencapsulated live yeast cells when packed in a column Plexiglas bioreactor for *in vitro* plasma perfusion treatment of renal failure uremia. Results showed that fully loading the column with yeast microcapsules generates the fastest urea hydrolysis rate per unit time. Even though our design is not as complex and elaborated as those of dialysis machines, it provides, nonetheless, comprehensive results in regards to urea hydrolysis. The yeast microcapsule formulation in a column bioreactor shows promising outcomes in hydrolyzing urea.

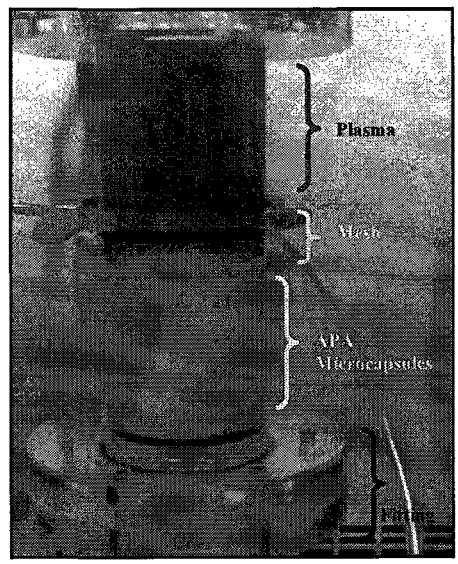
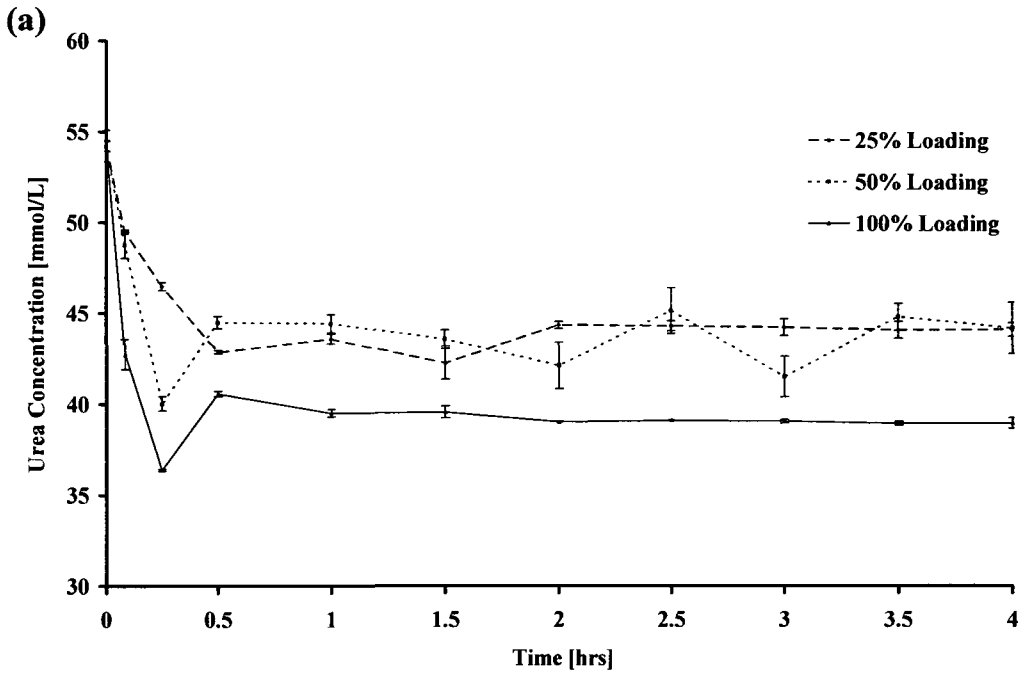
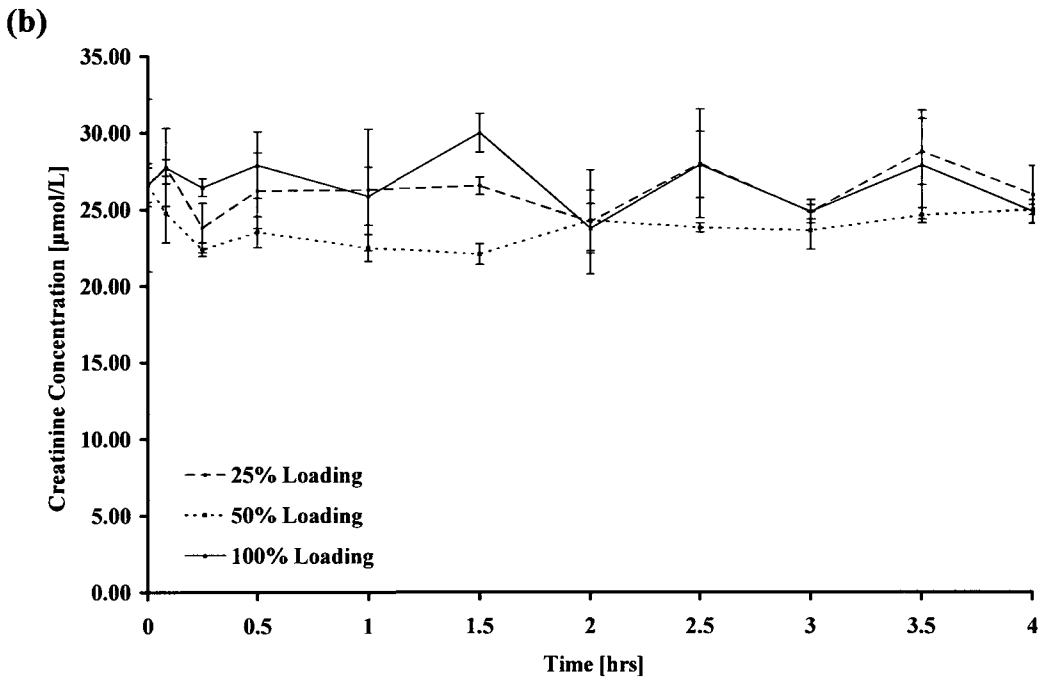


Figure 5.1: Image of the column bioreactor packed with yeast APA microcapsules (60% loading).


(a)
Urea Concentration [mmol/L]
60
55
50
45
40
35
30
0
0.5
1
1.5
2
2.5
3
3.5
4
Time [hrs]
25% Loading
50% Loading
100% Loading


(b)
Creatinine Concentration [µmol/L]
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0
0.5
1
1.5
2
2.5
3
3.5
4
Time [hrs]
25% Loading
50% Loading
100% Loading

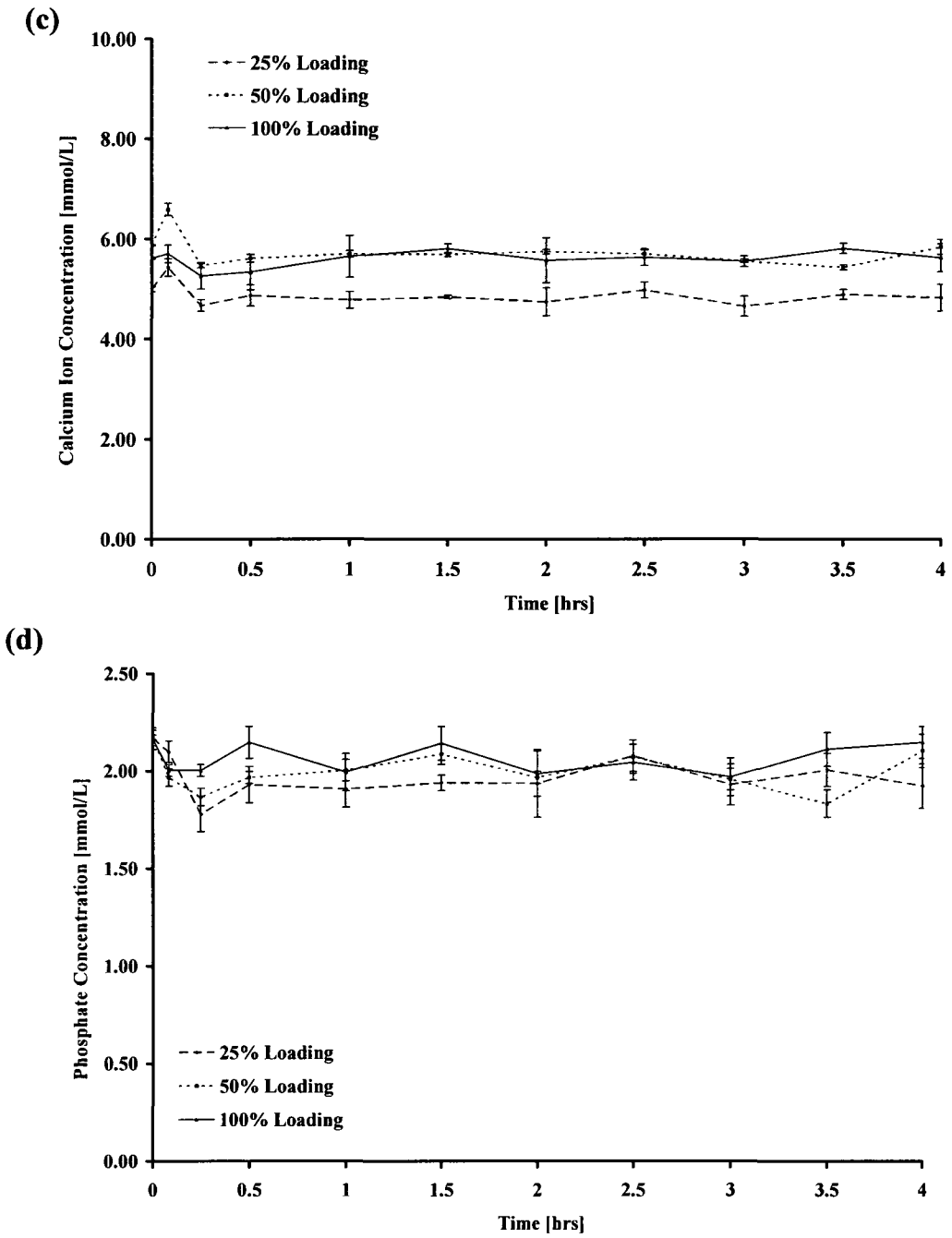


Figure 5.2: Yeast column experiment using sterile rat plasma. (a) Urea concentration as function of time and yeast microcapsules' loading, (b) Creatinine concentration as function of time and yeast microcapsules' loading, (c) Calcium concentration as function of time and yeast microcapsules' loading, (d) Phosphate concentration as function of time and yeast microcapsules' loading.

Chapter VI

Original Paper 4

Microencapsulated Live *Saccharomyces cerevisiae* Cells Administered Orally to Maintain Plasma Urea Level in Uremic Rats

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Preface: As a consecutive study to those in Chapters 3, 4 and 5 the present study investigates the feasibility of *in vivo* administrating APA microcapsules enclosing live yeast cells as an oral therapeutic alternative to renal failure in uremic rats.

6.1. Abstract

Entrapping live yeast cells in orally administered APA microcapsules presents a novel alternative treatment for renal failure. When given orally, the yeast cells remain at all times inside the microcapsules, which are then excreted in the stool. During their passage in the intestine, small molecules like urea diffuse into the microcapsules and are hydrolyzed, in consequence, by the yeast cells. Orally administrating these microcapsules to uremic rats was found to decrease urea levels from 7.29 ± 0.89 mmol/L to 6.12 ± 0.90 mmol/L over a treatment period of eight weeks. After stopping the treatment, the urea concentration increased back to an average uremic value of 7.64 ± 0.77 mmol/L. The analysis of creatinine concentrations averaged 39.19 ± 4.33 μmol/L, 50.83 ± 5.55 μmol/L and 50.28 ± 7.10 μmol/L for the normal and uremic control as well as the uremic treatment group, respectively. While values for both uremic groups did not differ among each other ($p>0.05$), they were, however, significantly higher than those of the normal control group ($p<0.05$). Uric acid concentrations averaged 80.08 ± 26.49 μmol/L, 99.92 ± 26.55 μmol/L and 86.49 ± 28.42 μmol/L for the normal and uremic control as well as the uremic treatment group, respectively. Calcium concentrations averaged 2.65 ± 0.25 mmol/L, 2.66 ± 0.15 mmol/L and 2.63 ± 0.22 mmol/L for the normal, uremic-control and uremic treatment groups, respectively. There were neither time nor group effects in the data for calcium concentrations ($p>0.05$). Finally, phosphate concentrations, which averaged 2.84 ± 0.31 mmol/L, 2.78 ± 0.28 mmol/L and 2.75 ± 0.33 mmol/L for the normal, uremic-control and uremic treatment groups, respectively, did not display any time or group effects ($p>0.05$). Furthermore, the microbial populations of five tested types of bacteria were not substantially altered by the presence of the yeast APA microcapsules ($p>0.05$). This suggests, in consequence, that there were no perceivable adverse effects of oral administration of these capsules on the microbial flora of the human GI tract.

Key Words

Microencapsulation, yeast, oral administration, urea hydrolysis, renal failure rat model.

6.2. Introduction

In North America, renal failure is quite common, affecting approximately 5% of hospitalized patients and up to 30% of patients in intensive care units (ICUs)[3]. In 2005, 8.3 million Americans were reported to have a mature chronic kidney disease with a significant number developing end-stage renal disease (ESRD)[4,6]. ARF affects up to 200,000 people in the United States annually, or approximately 5% of all long-term hospitalized patients[6-8]. ARF and ESRD have high mortality characterized by an overall survival rate of approximately 50% or lower depending on the severity of the renal malfunction[9].

In most cases of renal failures, there is usually an increased retention of waste metabolites (mainly urea and creatinine) in the blood and body tissues due to a substantial decrease in their urine's excretion[215-218]. In humans, blood urea nitrogen (BUN) and creatinine levels increase from 18 mg/dL to 100-300 mg/dL and from 1 mg/dL to 10-25 mg/dL, respectively, in case of major renal failures[219,220]. Treating renal failures has been a persistent challenge for almost two decades. Dialysis is generally used to correct for these excess metabolites[218,222]. However, all current renal replacement therapies (RRTs), including kidney transplantation, dialysis and hemofiltration, display inherent disadvantages and are characterized by serious long term complications that undermine the efficacy of these renal failure treatment modalities. Hence, new therapeutic modalities have been investigated and developed in order to bridge the gap between efficacy and operability.

Among such novel potential treatments is the microencapsulation of live microorganisms capable of depleting toxic wastes and other unwanted metabolites. Previous research has demonstrated the feasibility and the potential of administrating microencapsulated microorganisms as an alternative oral therapy. Examples include microencapsulating genetically engineered *Escherichia coli* DH5 cells over-expressing the *Klebsiella aerogenes* urease gene for urea removal in renal failure[39], *Oxalobacter formigenes* producing oxalate-degrading enzymes for removal of accumulated oxalate in urolithiasis[205,206] and genetically engineered *Lactobacillus Plantarum* 80 (pCBH1) for bile acid deconjugation[176] lowering.

In the realm of renal failures, there have been three major attempts to design novel potential alternative approaches to remove nitrogenous wastes. The first uses adsorbents, such as oxystarch, to directly bind urea[218-225]. The second method consists of hydrolyzing urea *via* co-immobilized enzyme urease and removing it *via* adsorbents[218, 221-225]. However, using such adsorbents requires very large doses, which is medically not advisable[219,220,223,224,226,227]. The third method consists of microencapsulating multienzyme systems to convert urea and ammonia into essential amino acids[222,223,228-230]. The problem with the last method is its poor conversion rate[229, 230]. Thus, there is a persistent need for new RRTs, which not only overcome the issues and limitations of the current ones, but also, efficiently remove nitrogenous wastes.

This article investigates the feasibility of orally administrating APA microcapsules entrapping live yeast cells in renal failure uremia. Towards this goal, we have recently optimized the encapsulation procedure and its respective parameters to accommodate yeast cells. In this study, we report the results of our *in vivo* studies in experimental uremic rats.

6.3. Materials and Methods

6.3.1. Chemicals

Sodium alginate (low viscosity) and PLL (*MW* 27,400) were both purchased from Sigma-Aldrich.

6.3.2. Microorganism and culture conditions

Saccharomyces cerevisiae, strain reference number 9896, was purchased from ATCC[195]. This organism is capable of producing urease urea amidolyase, which in turn metabolizes urea[196]. Yeast mold broth (YMB) growth medium was used for primary cell cultivation. YMB is composed of 10 g/L of glucose, 3 g/L of malt extract, 5 g/L of peptone and 3 g/L yeast extract. The whole mixture was incubated aerobically at 37°C for 48hrs.

6.3.3. Preparation of APA microcapsules containing live yeast cells

Log phase cultures of *Saccharomyces cerevisiae* were harvested by centrifugation at 3500 rpm for 15 min at 4°C. Pellets were then mixed with 2% alginate solution in order to form a final mixture consisting of 8% (v/w) pellets and 1.75% alginate. Alginate microcapsules were prepared by generating droplets of sodium alginate solution (15 mg/mL) using an IER-20 encapsulator machine manufactured by Inotech. Corp. Extruded through a 300 μm nozzle by a syringe driven pump, these droplets were gelled for 30 min in a well-stirred calcium chloride solution (11 mg/mL). The same droplets were then coated with a 0.1% (w/v) solution of PLL for 10 min to form AP beads and washed again with physiological saline (PS) for 5 min. Finally, the final alginate coating is applied by immersing the beads in a 0.1% (w/v) sodium alginate solution for 10 min followed by a final 5 min PS wash. The resulting microcapsules, having an average diameter of 638 ± 17 μm, were then collected and stored in a 10/90 YMB/PS solution at 4°C. All solutions used in the preparation of these APA microcapsules were either filtered using 0.22 μm filter or autoclaved to ensure sterility. Moreover, the entire procedure was carried out in a biological hood.

6.3.4. Preparation of a uremic rat model

Renal failure Wistar rats were purchased from H. Sprague Dawley Laboratory. The surgical procedure for making the rat model involve two steps: first to perform right nephrectomy and second to ligate the left artery, vein and ureter. This procedure was performed by trained staff at H. Sprague Dawley Laboratory.

To prepare this rat model, anaesthetized animals are placed in ventral recumbency with its tail towards the surgeon. The hair in the right dorsal lumber area is clipped and the skin swabbed thoroughly with surgical scrub. A 2-3 cm incision is made into the skin caudal to the rib cage on the right side of the animal. Another 2-3 cm incision is then made into the underlying muscle wall. The kidney is pooled through the muscle wall, and the renal artery, vein and ureter are then ligated before completely removing the kidney by incising the vessels and ureter between the kidney. The remaining tissue is returned to the peritoneal cavity and the muscle is sutured. The skin incision is closed with 2-3 com wound clips. Similarly, the left side of the rat is prepared as if to perform a left

nephrectomy. After an incision (2-3 cm) is made in the muscle wall, the left renal artery, vein, and ureter are located. Using blunt forceps, the left renal vessels and ureter are isolated and separated from the peritoneal connective tissue. The renal vessels and ureter are ligated using sterile silk suture. The muscle wall is sutured. The skin incision is closed with 2-3 metal wound clips.

6.3.5. Treatment phases and animal monitoring

In this study, 36 (12 control normal and 24 uremic rats) male Wistar rats were purchased from Sprague Dawley Laboratory. All rats' aged between 60 and 80 days and weighted between 300 and 340 g. Both normal and uremic rats were tested upon arrival for determination of baseline urea levels (18-30 mg/dL or 3-5 mmol/L approximately for normal rats). At reception, rats were be placed two per cage and fed rodent chow for 1 week in order to acclimatize them to the facility (sterile room with controlled temperature (22-24°C) and alternating light and dark cycles). Food and water were provided ad libitum. The animals were then be randomized in 3 groups: 1) normal control-treatment (TP) consisting of 12 normal rats fed with APA microcapsules containing live yeast cells, 2) uremic control-no-TP consisting of 12 uremic rats fed with empty APA microcapsules and 3) uremic-TP consisting of 12 uremic rats fed with yeast APA microcapsules.

During the experiment, the 3 groups of animals were monitored over time to evaluate changes in their urea, creatinine, phosphate, calcium and uric acid levels. As mentioned before there was a 1 week pre-TP period to acclimatize the animals. The treatment period lasted for 8 weeks during which the rats were gavaged daily and bleeded twice a week. There was a post-TP period of 2 weeks during which the animals were only bleeded respecting the same frequency.

6.3.6. Gavaging

3 mL syringes (purchased from Fisher Scientific) were used to gavage the animals. Each syringe contained 2.5 mL of APA microcapsules mixed with 0.5 mL of PS. The filling procedure was conducted in a sterile biological hood using a beacker filled with the desired mixture. Before gavaging the animals, syringes were incubated for 2hrs at 37°C in order to activated the microorganisms. The microcapsules were then orally

forced fed to the experimental rats using curved 16G stainless steel gavage needle. For this, hand restraint was applied to the animal and no anesthesia was used.

6.3.7. Blood collection

200 µL of saphenous blood was collected in SST microtainers (purchased from BD Biosciences) without sedating the animals. The samples were centrifuged and analyzed using a Hitachi light scattering Multistat III Clinical Chemistry Analyzer (Roche Diagnostics), which was loaded with the appropriate kits. Animal health indicators and clinical sign verifications (body weight, body condition, respiration rhythm, agitation, etc...) were all checked and assessed twice a week for all the trial period (12 weeks).

6.3.8. Effect of oral administration of yeast APA microcapsules on the gut microbial flora

To investigate the effect of oral administration of yeast APA microcapsules on the gut microflora, parts of rats' seccum and large intestine were obtained after the sacrifice. Form each group, 5 rats were chosen randomly for this purpose. The organs were crushed mechanically until a fluid mixture was obtained. Following that, 1 mL of this slurry was collected in a 1.5 mL graduated Eppendorf tube. Several 10-fold serial dilutions were performed using autoclaved PS and 0.1 mL aliquots of final dilution were plated on 5 different agar plates. The plates were then incubated aerobically according to their corresponding conditions. Bacterial enumeration for specific fecal marker microorganisms, total aerobes, total anaerobes, *Escherichia coli, Staphylococcus sp.* and *Lactobacillus sp.* was performed in triplicate using an agar-plate-count assay. The platting media and incubation conditions used are all listed in Table 6.1.

6.3.9. Statistical Analysis

The statistical Analysis System (SAS Enterprise Guide 4.1 (4.1.0.471) by SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Data was expressed as means ± SEM. Group and time effects were statistically investigated by ANOVA mixed models. Data were considered significant at $p<0.05$.

6.4. Results

One of the important parameters indicating the efficiency of the treatment is the body weight, which was sampled twice a week along the trial period. In figure 6.1a are graphed the average body weights of each rat group. The average body weight of the normal control group increased from 347.83 ± 11.86 g at t=1 week to 464.03 ± 11.86 g at t=11 weeks, respectively. Similarly, body weights for the uremic control and uremic treatment groups increased from 341.64 ± 11.86 g to 461.73 ± 29.01 g, and from 345.50 ± 9.28 g to 465.00 ± 34.56 g, respectively, between week 1 and 11 of the trial. The statistical analysis suggested that there are significant time and group effects in rats' body weight ($p<0.05$).

The urea concentration, which is one of the essential indicators of the success of the propose treatment, followed a different trend (figure 6.1b). Specifically, values for the latter group during the pre-TP and post-TP phases were not statistically different from those for the uremic control group ($p>0.05$). However, urea concentrations for the uremic treatment group in between weeks 2 and 9 were significantly different from those for the other two controls ($p<0.05$). All along the 11 weeks of the trail, urea concentrations averaged 5.37 ± 0.67 mmol/L and 7.39 ± 0.99 mmol/L for the normal and uremic control groups, respectively. The average urea concentration for the uremic treatment group decreased from 7.29 ± 0.89 mmol/L to 6.97 ± 0.48 mmol/L once the yeast microcapsules were orally administered. During the treatment, the urea concentration for this same group averaged 6.01 ± 0.47 mmol/L before increasing again to 7.64 ± 0.77 mmol/L once the gavage had stopped.

The analysis of creatinine concentrations averaged 39.19 ± 4.33 μmol/L, 50.83 ± 5.55 μmol/L and 50.28 ± 7.10 μmol/L for the normal and uremic control as well as the uremic treatment group, respectively (figure 6.1c). While values for both uremic groups did not differ among each other ($p>0.05$), they were, however, significantly higher than those of the normal control group ($p<0.05$).

Uric acid concentrations averaged 80.08 ± 26.49 μmol/L, 99.92 ± 26.55 μmol/L and 86.49 ± 28.42 μmol/L for the normal and uremic control as well as the uremic treatment group, respectively (figure 6.1d). Calcium concentrations averaged 2.65 ± 0.25

mmol/L, 2.66 ± 0.15 mmol/L and 2.63 ± 0.22 mmol/L for the normal, uremic-control and uremic treatment groups, respectively (figure 6.1e). There were neither time nor group effects in the data for calcium concentrations ($p>0.05$). Finally, phosphate concentrations, which averaged 2.84 ± 0.31 mmol/L, 2.78 ± 0.28 mmol/L and 2.75 ± 0.33 mmol/L (figure 6.1f) for the normal, uremic-control and uremic treatment groups, respectively, did not display any time or group effects ($p>0.05$).

An important pre-requisite when orally administrating APA microcapsules containing live yeast cells is that these entities should not disturb the natural colonic gut flora. This importance is accentuated when prolonged and repeated oral intake of large quantities of microcapsules is required. Table 6.2 shows no marked differences in the population of the tested microbes for the uremic treatment group and those for both the normal and uremic control groups ($p>0.05$).

6.5. Discussion

The rational behind using an experimental uremic rat animal model stems from the striking similarities between rats and humans' GI tracts. In particular, this experimental uremic rat model has shown earlier high levels of plasma urea and other unwanted metabolites compared to normal rats, with a similar experimental endpoint. With 12 animals in each group, differences in mean uremic metabolites could be determined with a power larger than 80% and an alpha error of 5%. In other words, using 36 animals allows us to detect small differences, if any exist, and, thus, infer properly about the feasibility of the treatment.

The major objective of this animal study is to determine the differences in animals with respect to the lowering of renal failure uremic toxic metabolites due to oral administration of microcapsule formulation. This article reports, for the first time, the use of polymeric membrane artificial cells[28,29,30,35] containing live yeast cells in renal failures. Our studies show that live yeast cells can be microencapsulated and retained inside the artificial cells during their passage down the GI tract. During passage through the intestine, urea molecules diffuse through out the permeable membrane of the artificial cells. This is rendered possible due to the molecular-weight-cut-off (MWCO) of these

artificial cells. In particular, the MWCO of the alginic membrane is estimated to be between 60-70 kDa[202], while that of urea and urease are 60 Da and 483 kDa, respectively. Theoretically speaking, urease should always stay entrapped inside the cell. Once inside the cell, urea molecules get hydrolyzed due to yeast cells, which use urea as their carbon source. The same reasoning applies for the other electrolytes.

In humans, normal urea concentration ranges approximately between 3-5 ± 0.5 mmol/L (or 18-30 mg/dL) of plasma. Anything above this range is considered uremic. The normal and uremic control rats are expected to display urea concentrations falling respectively inside and outside the normal urea range (figure 6.1b). Noting that the urea concentrations of the uremic treatment group decreased at the start of the treatment and increased to uremic values after stopping the treatment suggests that the yeast APA microcapsules do actually serve their purpose and work as hypothesized. Looking closely at the data, one can notice that urea in uremic treated rats decreased by approximately 18% from the pre-TP phase level. During the treatment eight weeks period, the urea concentrations for the uremic-TP group stagnated around 6.01 ± 0.47 mmol/L, which is around 1.1 times greater than the average urea concentration of the normal control group. Upon terminating the oral administration of the yeast APA microcapsules, the plasma urea level returned rapidly to the uremic values (figure 6.1b). This means that the artificial cells were all excreted with the stool and were not, thus, retained in the intestine.

The concentrations of creatinine and uric acid (figures 6.1c and d) did not vary significantly within and among each uremic group but were significantly different from those of the control group. Specifically, the creatinine concentrations of uremic treated and not treated groups were 1.29 and 1.28 times greater than those of the normal group, respectively. Similarly, those of uric acid for the same uremic groups were 1.23 and 1.07 times greater than those of the normal group, respectively. These trends suggests that the orally administered yeast APA microcapsules did not have any effect on lowering neither creatinine nor uric acid, which are both correlated with decreased renal function[231, 232]. Similarly, the artificial cells did not influence calcium (p>0.05) and phosphate (p>0.05) plasma electrolytes because there was no variation in their concentrations neither with respect to time nor among groups (figures 6.1e and f).

Body weight, which is an indication of the overall health condition, increased through out the trial period (figure 6.1a). This suggests that the animals are under a constant bodily growth. Furthermore, each rats' group displayed body weight values that are statistically different from the other two groups with respect to time. However, renal failure individuals have usually their body weight decreasing with time. If the TP is proven successful, we would have expected the body weight of the uremic control rats to decrease and that of the normal and uremic-TP groups to increase. Specifically, the body weight of the normal and uremic controls increased by 33.41 ± 2.1% and 35.15 ± 1.9%, respectively, at the end of the 11^{th} week. Similarly, the uremic-TP had their body weight increased by 34.59 ± 0.9%. During the TP period, the increase in the body weight of the uremic-TP group is significantly larger than that of the uremic control group. This fact is not surprising and suggests that the treatment is somehow effective in alleviating some of the uremia effects. However, the only possible explanation to the rise in the body weight of the uremic control rats is that the decrease in this parameter due to uremia is outweighed by its increase due to growth.

It is known that a well-balanced gut microbiota plays a crucial role on the *ensemble* of the human health[233]; upsetting the intestinal flora may lead to many unwanted problems[234]. The results from the *in vivo* viability study (Table 6.2) suggest that the materials used to produce the microcapsules combined with the yeast itself did not evoke any appreciable adverse effects on the human intestinal flora.

6.6. Conclusion

Normally, the kidneys are responsible for removing wastes metabolites and adjusting water and electrolytes. In case of renal failures, these tasks can not be fulfilled by the damaged kidneys and patients need alternative treatment to survive. Dialysis and kidney transplantation are considered effective but expensive. Other alternatives such as the use of absorbents are challenged with the need of large doses of absorbents[218-225,228]. This work presents a novel yeast-based approach to treat renal failure uremia. Orally administrating these microcapsules to uremic rats was found to decrease urea levels by 18 ± 0.31% over a period of eight weeks. After stopping the treatment, the urea

concentration increased back to the uremic values. However, this formulation had no perceivable effects on the concentrations of creatinine, uric acid, phosphate, and calcium suggests that yeast cells are only sensitive to urea. The microbial populations of the five tested types of bacteria were not substantially altered by the presence of the yeast APA microcapsules. This proves, in consequence, that there were no perceivable adverse effects of oral administration of these capsules on the microbial flora of the human GI tract.

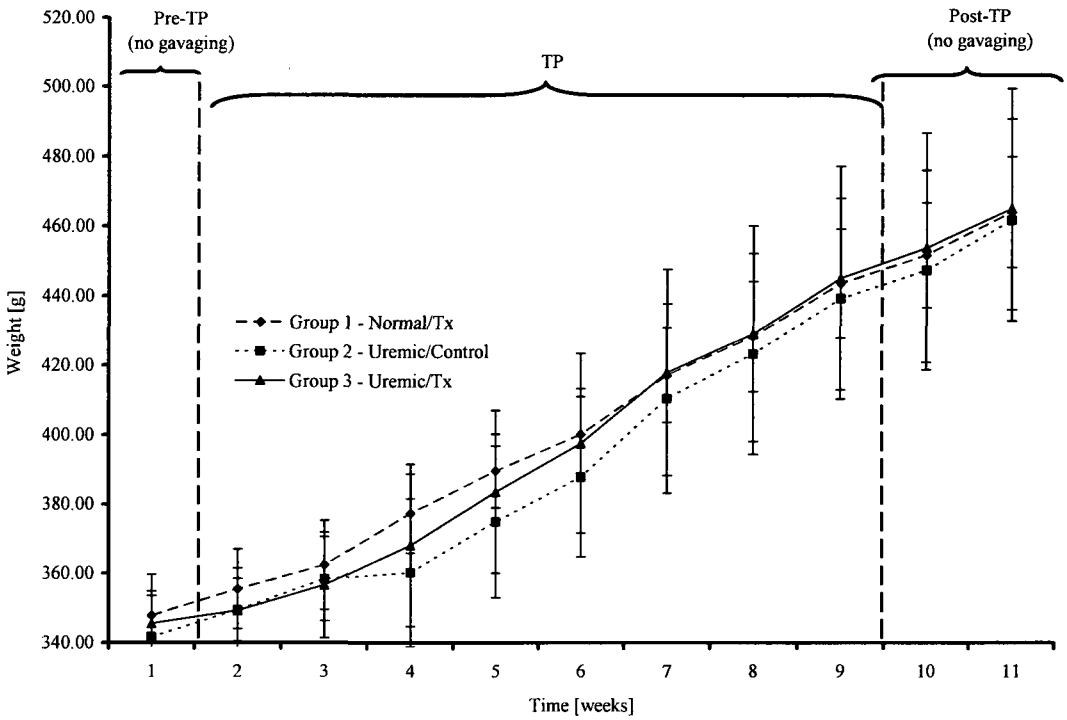
6.7. Acknowledgments

We would like to forward special thanks to Ms. Ayla Coussa, Ms. Genevieve Berube and Ms. Sonia Kajla for helping us conduct this animal trial.

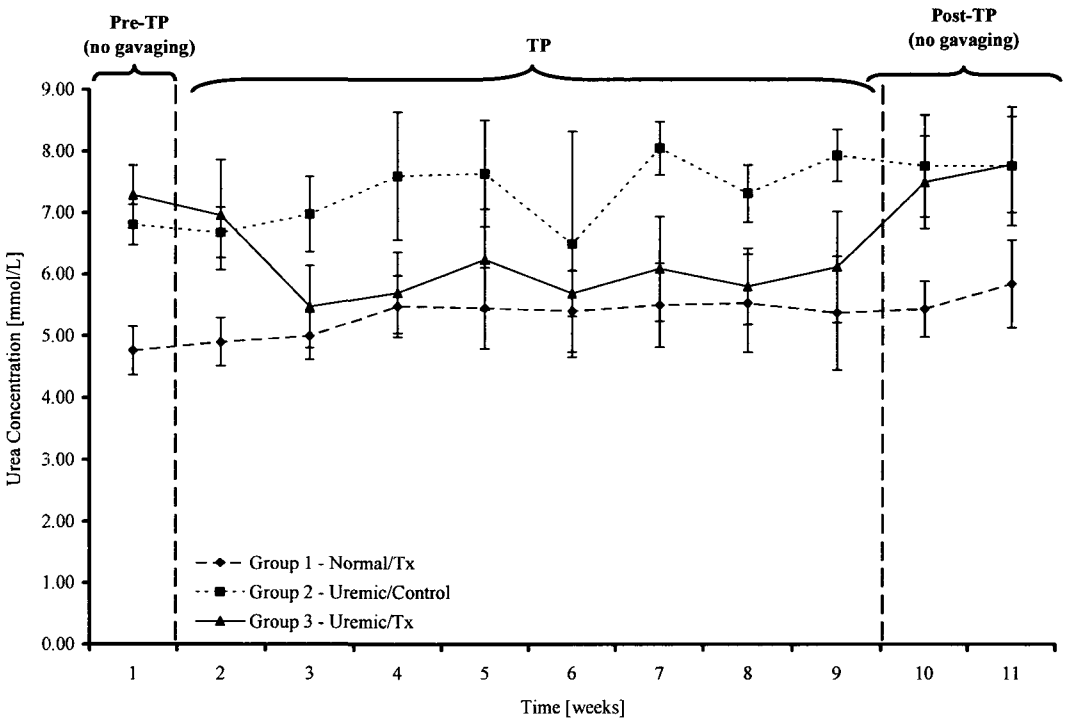
Table 6.1: Media and incubation conditions used for enumeration of representative microbes in the human colon

Microbial Group	Medium	Incubation Conditions and Time	Colonies Formed
Total aerobes	Brain heart infusion agar	Aerobic, 37C, 24h	White
Total anaerobes	Brain heart infusion agar	Anaerobic, 37C, 72h	White
Escherichia coli	Mc Conkey agar	Aerobic, 43C,24h	Red-purple
Staphylococcus sp.	Mannitol Salt agar	Aerobic, 37C, 48h	White with yellow/purple zone
Lactobacillus sp.	Rogosa agar	Anaerobic, 37C, 72h	White

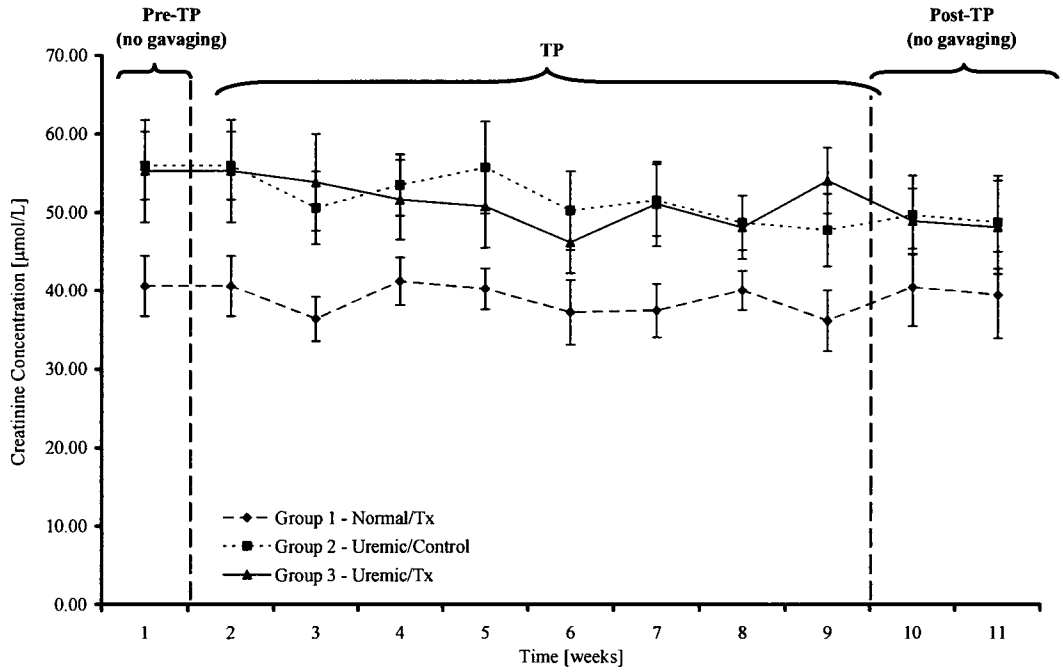
(a)


Pre-TP
(no gavaging)
TP
Post-TP
(no gavaging)
520.00
500.00
480.00
460.00
440.00
420.00
400.00
380.00
360.00
340.00
Weight [g]
Group 1 - Normal/Tx
Group 2 - Uremic/Control
Group 3 - Uremic/Tx
1
2
3
4
5
6
7
8
9
10
11
Time [weeks]

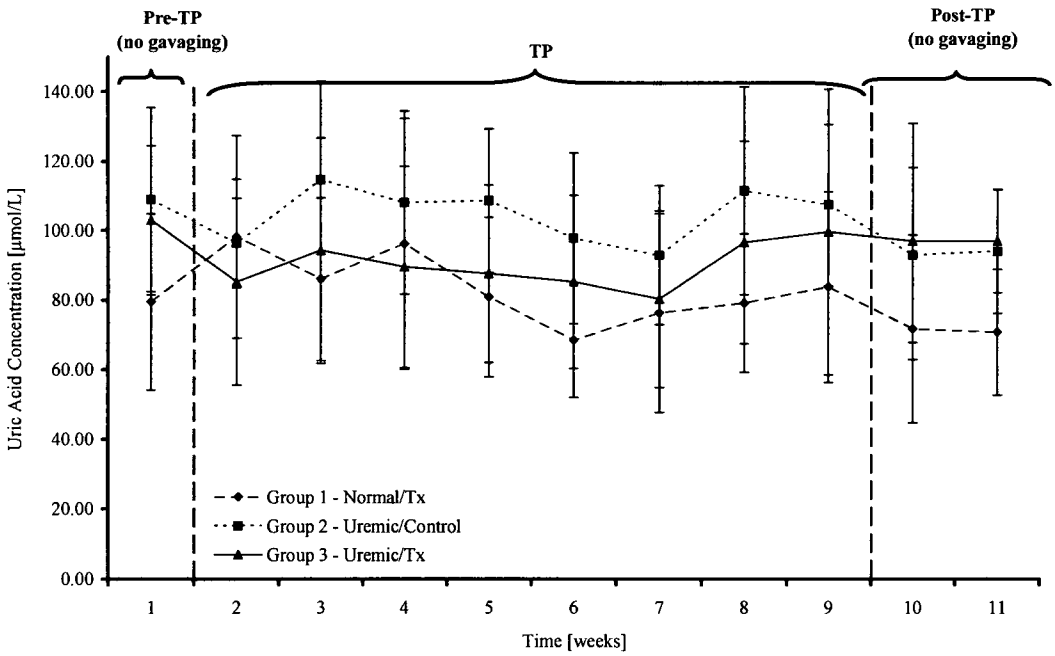
(b)


Pre-TP
(no gavaging)
TP
Post-TP
(no gavaging)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Urea Concentration [mmol/L]
Group 1 - Normal/Tx
Group 2 - Uremic/Control
Group 3 - Uremic/Tx
1
2
3
4
5
6
7
8
9
10
11
Time [weeks]

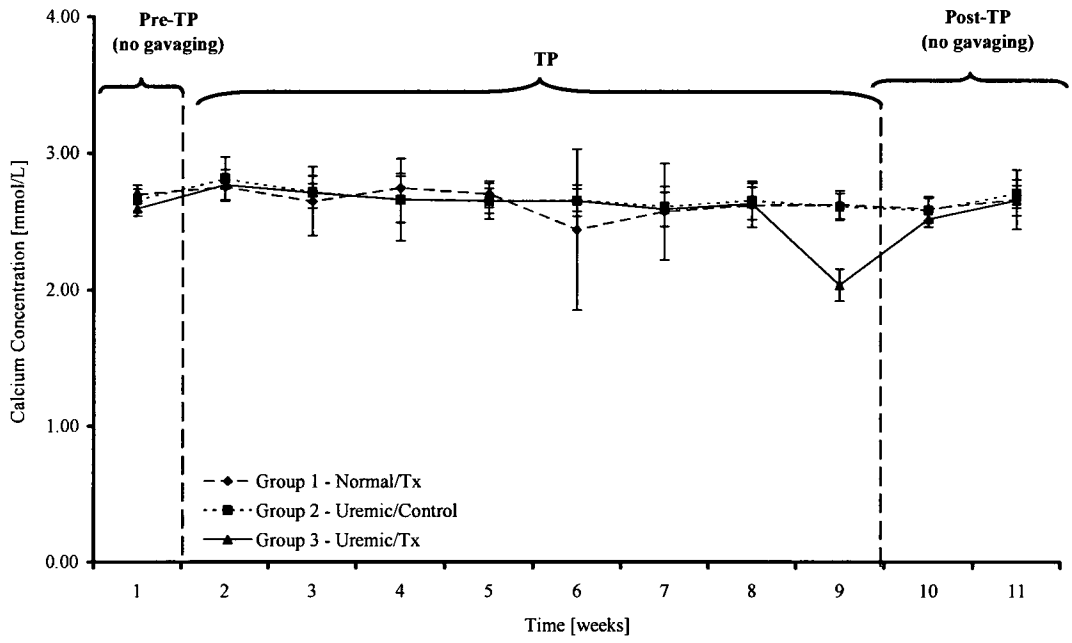
(c)


Pre-TP
(no gavaging)
TP
Post-TP
(no gavaging)
Creatinine Concentration [µmol/L]
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
1
2
3
4
5
6
7
8
9
10
11
Time [weeks]
Group 1 - Normal/Tx
Group 2 - Uremic/Control
Group 3 - Uremic/Tx

(d)


Pre-TP
(no gavaging)
TP
Post-TP
(no gavaging)
Uric Acid Concentration [µmol/L]
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
1
2
3
4
5
6
7
8
9
10
11
Time [weeks]
Group 1 - Normal/Tx
Group 2 - Uremic/Control
Group 3 - Uremic/Tx

(e)


Pre-TP
(no gavaging)
TP
Post-TP
(no gavaging)
Calcium Concentration [mmol/L]
4.00
3.00
2.00
1.00
0.00
Group 1 - Normal/Tx
Group 2 - Uremic/Control
Group 3 - Uremic/Tx
1
2
3
4
5
6
7
8
9
10
11
Time [weeks]

(f)

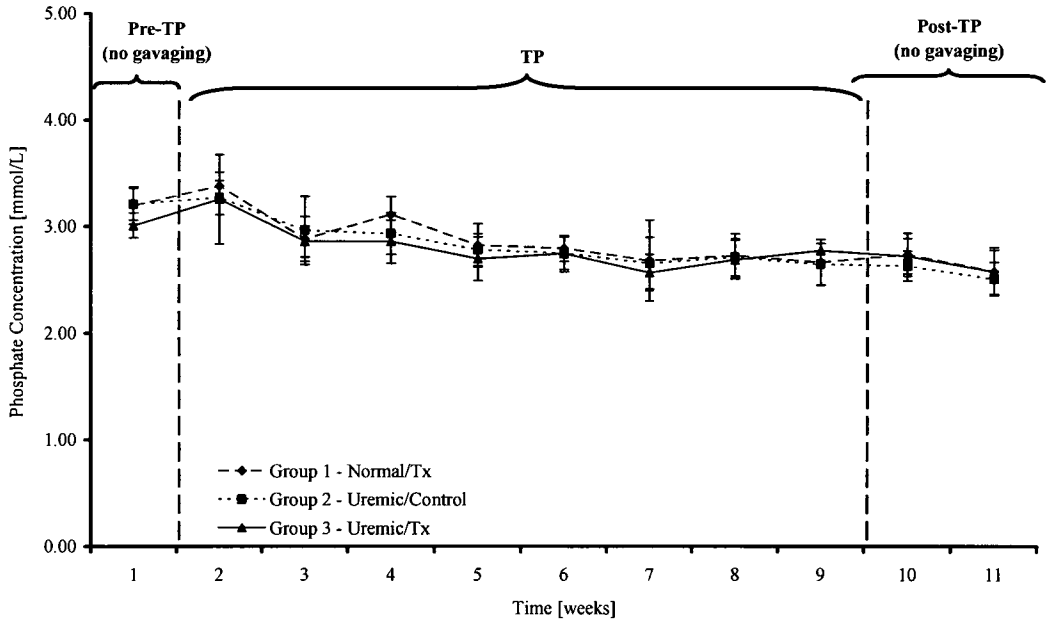

Pre-TP
(no gavaging)
TP
Post-TP
(no gavaging)
Phosphate Concentration [mmol/L]
5.00
4.00
3.00
2.00
1.00
0.00
Group 1 - Normal/Tx
Group 2 - Uremic/Control
Group 3 - Uremic/Tx
1
2
3
4
5
6
7
8
9
10
11
Time [weeks]

Figure 6.1: (a) Rats' body weight before, during and after oral administration of APA microencapsulated yeast cells, (b) Plasma urea concentration before, during and after oral administration of APA microencapsulated yeast cells, (c) Plasma creatinine concentration before, during and after oral administration of APA microencapsulated yeast cells, (d) Plasma uric acid concentration before, during and after oral administration of APA microencapsulated yeast cells, (e) Plasma calcium concentration before, during and after oral administration of APA microencapsulated yeast cells, (f) Plasma phosphate concentration before, during and after oral administration of APA microencapsulated yeast cells.

Table 6.2: Effect of APA microcapsules on selected microbes in the human colon

Microbes	Incubation Time [hr]	Log CFU/ml medium		
		Normal-TP	Uremic-Empty APA Microcapsules	Uremic-TP
Total aerobes	0	8.22 ± 0.12	8.22 ± 0.11	8.22 ± 0.09
	6	8.27 ± 0.01	8.17 ± 0.16	8.26 ± 0.08
	12	8.24 ± 0.14	8.34 ± 0.04	8.31 ± 0.07
	24	8.29 ± 0.1	8.32 ± 0.02	8.28 ± 0.03
Total anaerobes	0	8.37 ± 0.03	8.37 ± 0.01	8.37 ± 0.01
	6	8.35 ± 0.09	8.39 ± 0.02	8.31 ± 0.1
	12	8.38 ± 0.11	8.41 ± 0.07	8.38 ± 0.12
	24	8.37 ± 0.14	8.4 ± 0.11	8.36 ± 0.16
Escherichia coli	0	8.29 ± 0.13	8.29 ± 0.08	8.29 ± 0.11
	6	8.24 ± 0.16	8.33 ± 0.05	8.29 ± 0.12
	12	8.27 ± 0.11	8.24 ± 0.17	8.32 ± 0.1
	24	8.31 ± 0.07	8.28 ± 0.12	8.27 ± 0.11
Staphylococcus sp.	0	6.65 ± 0.14	6.65 ± 0.12	6.65 ± 0.1
	6	6.74 ± 0.07	6.57 ± 0.16	6.72 ± 0.03
	12	6.59 ± 0.15	6.61 ± 0.06	6.69 ± 0.01
	24	6.64 ± 0.13	6.62 ± 0.12	6.58 ± 0.08
	48	6.71 ± 0.07	6.74± 0.12	6.6 ± 0.17
Lactobacillus sp.	0	5.32 ± 0.01	5.32 ± 0.06	5.32 ± 0.03
	6	5.4 ± 0.05	5.41 ± 0.01	5.36 ± 0.08
	12	5.45 ± 0.03	5.39 ± 0.05	5.33 ± 0.11
	24	5.39 ± 0.09	5.37 ± 0.12	5.37 ± 0.11
	48	5.36 ± 0.1	5.35 ± 0.08	5.41 ± 0.01
	72	5.38 ± 0.17	5.42 ± 0.12	5.45 ± 0.15

Chapter VII

SUMMARY of OBSERVATIONS

The mechanical strength and membrane's integrity of the APA yeast microcapsules novel artificial cells were assessed in SGF and SIF while being challenged by osmotic pressures and shear stresses. These microcapsules were also tested for their effect on uremic metabolites including urea, creatinine, phosphate, uric acid and calcium both *in vitro* and *in vivo*. Viability studies in storage conditions and after exposure to stress and changing environmental conditions were performed. A yeast column bioreactor was also designed and *in vivo* experiments were performed. The research work can be summarized as the following:

1. Preparation of microcapsules containing live yeast cells

The microcapsule groups used in this study were either APA microcapsules containing yeast or empty loading. Varying the alginate concentration between 1% and 1.75% was reported to significantly affect ($p<0.05$) the number and shape of spherical microcapsules (Table 3.1). In particular, using 1.75% of alginate generated 99% spherical capsules with an average diameter of 638 ± 17 μm.

2. Growth and viability of microencapsulated yeast cells

Compared to incubating yeast cells for 24 hrs, incubating them for 48 hrs was found to yield a higher cell mass (34 ± 2 mg/mL vs. 46.5 ± 1.4 mg/mL, figure 3.2a) and a higher free cells' count ($5.7X10^7 \pm 1.4X10^6$ CFU/mL vs. $8.4X10^7 \pm 1.8X10^6$ CFU/mL, figure 3.2b). Moreover, cells incubated for 48 hrs were found to be more numerous than those incubated for 24 hrs after being entrapped in the APA microcapsules for a 1 week period ($2.5X10^8 \pm 1.8X10^7$ CFU/mL vs. $4.9X10^8 \pm 1.9X10^7$ CFU/mL, figure 3.2b).

The viability of microencapsulated yeast cells was not significantly affected by neither shear stresses nor changing environmental conditions, especially the stomach's acidity ($p>0.05$) (figures 3.5i-j and 3.6d-f). Over extended storage conditions (4°C and 10/90 YMB/PS), the entrapped yeast cells were found to maintain their viability after 1 year around an average of $3.95X10^8 \pm 7.0X10^6$ CFU/mL (figure 3.2c).

3. Mechanical strength of the yeast APA microcapsules

The osmotic pressure tests revealed that APA microcapsules shrink and swell to a greater extent and at a faster rate when immersed in more concentrated saline solutions; this tendency appears to have important effects on the stability and mechanical strength of the microcapsule. A linear trend characterized the dependence of the mechanical stability on the saline concentration. Although, a correlation can be drawn between breakage rates at 1 and 10 times the physiological saline concentration, there was, however, no difference in the sensitivity at 2 and 5 times this concentration ($p>0.05$). This suggests that yeast APA microcapsules are not affected by specific concentrations but by ranges of concentrations varying from low (1x) to high (10x).

Whether in SGF pH < 2 (figure 3.5g) or SIF pH ≥ 6.5 (figure 3.5h), both empty and yeast APA microcapsules displayed a perceivable correlation between shaking time and extent of breakage: the larger the retention time is, the greater the percentage of broken microcapsules will be. However, empty APA microcapsules were more prone to breakage than yeast microcapsules after 6hrs in SIF ($p<0.05$). In fact, the percentage of broken empty microcapsules was almost double that of the yeast microcapsules.

Statistical analysis revealed that values for breakage percentages in SGF did not significantly differ when yeast and empty APA microcapsules were challenged by a peristaltic pump ($p>0.05$). However, when comparing SIF alone and both SGF and SIF together, it appeared that values for yeast APA microcapsules were significantly different from those of empty ones ($p<0.05$). Microcapsules that were not broken under both stomach and small intestine conditions were found to maintain their spherical morphology; however, the broken ones had, most likely, swelled and some had ghost-like shapes (figures 3.5a-f). The fracture mechanism appears to be mediated by a combination of osmotic pressure and shear stresses.

Results for the mechanical shaking shear stress in SGF pH < 2 show that exposure time affects the structural strength of both empty and yeast APA microcapsules (figures 3.6a-c). Even though these shear stresses affected each type of capsules differently ($p<0.05$), there were no apparent differences between microcapsules' breakage values at 100 and 200 rpm for each group ($p>0.05$). This suggests a plateau kind of behavior in that

APA microcapsules, whether empty or full, exhibited similar breakage behavior for speeds equal or above 100 rpm. Again, in this experiment, yeast APA microcapsules were found to be more resistant to harsh conditions than empty ones, especially after long hours of exposure to mechanical stresses. The presence of yeast inside the capsule appears to decrease the osmotic driving force responsible for the capsules' rupture.

4. *In vitro* and *in vivo* effect of yeast APA microcapsules on urea concentrations

Monitoring the extent of urea hydrolysis by microencapsulated yeast cells for 72 hrs suggested that using 1 g of capsules for each 100 mL of SIF yielded the largest and fastest hydrolysis per unit time (11 ± 0.59%) (figure 4.2). Soaking yeast APA microcapsules (1 g/100mL) in Minimal SD Base, which provides the minimum nutrients needed for the yeast's growth, gave the same urea hydrolysis profile as soaking these capsules in SIF pH ≥ 6.5 (p>0.05, figure 4.3).

When yeast cells were first soaked in SGF pH < 2 followed by 6hrs soaking SIF pH ≥ 6.5, these microorganisms hydrolyzed urea, but not to the same extent as before (figure 4.4). Without the acidity of the stomach, the 1 g/100mL yeast loading group decreased the urea concentration by 1.95 ± 0.58% and 1.78 ± 0.95% after 6 hrs in SIF or Minimal SD base, respectively (figures 4.2 and 4.3). However, when first exposed to the stomach's acidic environment, the microencapsulated yeast cells hydrolyzed 0.91 ± 0.02% of the initial urea after 6hrs immersion in SIF (figure 4.4). This result tends to be significantly different from the first two (p<0.05), suggesting that stomach acidity have a considerable effect on the yeast's urea hydrolysis.

Results of our uremic rat experimental animal model confirmed the yeast's urea hydrolysis capacity. In particular, urea concentrations in uremic treated rats decreased by approximately 18 ± 0.31% from the pre-TP phase level. During the treatment eight weeks period, the urea concentrations for the uremic-TP group stagnated around 6.01 ± 0.47 mmol/L, which is around 1.1 greater than the average urea concentration of the normal control group (p<0.05). Upon terminating the oral administration of the yeast APA microcapsules, the plasma urea level returned rapidly to the uremic values (figure 6.1b). This means that the artificial cells were all excreted with the stool and were not, thus, retained in the intestine.

5. *In vivo* Effect of yeast APA microcapsules on other molecules

The concentrations of creatinine and uric acid (figures 6.1c and d) did not vary significantly within and among each uremic group ($p>0.05$) but were significantly different from those of the control group ($p<0.05$). Specifically, the creatinine concentrations of uremic treated and not treated groups were 1.29 and 1.28 times, respectively, greater than those of the normal group. Similarly, those of uric acid for the same uremic groups were 1.23 and 1.07 times, respectively, greater than those of the normal group. These trends suggests that the orally administered yeast APA microcapsules did not have any effect on lowering neither creatinine nor uric acid, which are both correlated with decreased renal function. Similarly, the artificial cells did not influence calcium ($p>0.05$) and phosphate ($p>0.05$) plasma electrolytes because there was no variation in their concentrations neither with respect to time nor among groups (figures 6.1e and f).

6. Yeast column bioreactor

Designing a column bioreactor is an attempt to implement an alternative to external dialysis machines. Our results suggest that urea hydrolysis by yeast microcapsules highly depends on the yeast loading: the higher the loading is, the greater the urea breakdown. As the column loading increased from 25% to 50% and then to 100%, the urea hydrolysis reached 18.2 ± 0.21%, 18.5 ± 0.15% and 28.2 ± 0.05%, respectively ($p<0.05$). However, based on figure 5.2a, the urea concentration seems stagnant after almost 1 hr of plasma re-circulation. In fact, there were no significant differences among urea concentrations after 1 hr within each column loading group ($p>0.05$). In our experiment, microencapsulated yeast cells seem to saturate after 1 hr. The exact cause of this saturation can not be explained at this stage and further research is needed in this regard.

7. Effect of oral administration of yeast APA microcapsules on the gut microflora

The microbial populations of total aerobes, total anaerobes, *Escherichia coli, Staphylococcus sp.* and *Lactobacillus sp.* were not substantially altered by the presence of the yeast APA microcapsules ($p>0.05$) (Table 6.2). This suggests, in consequence, that

there were no perceivable adverse effects of oral administration of these capsules on the microbial flora of the human GI tract.

Chapter VIII

CONCLUSION

Although current RRTs such as dialysis and kidney transplantation have proven successful in reducing the magnitude of renal failures, they nonetheless do not answer the needs of the ESRD and chronic renal failure population. Orally administered APA microcapsules containing live yeast cells are hypothesized to act like mini-bioreactors capable of hydrolyzing unwanted metabolites, such as urea, as they pass through the GI tract.

To investigate this matter, a research project with the following objectives was carried out: i) to optimize the microencapsulation process of yeast cells, ii) to assess the mechanical stability of the novel microcapsules in simulated gastric and intestinal fluids and challenged by shear stresses and osmotic pressures, iii) to investigate the efficacy of the yeast APA microcapsules in removing urea and other electrolytes both *in vitro* and *in vivo* using an experimental uremic renal failure rat animal model and iv) to design a yeast column bioreactor and study its performance as an external device for removing urea. After reviewing the results, the following conclusions can be drawn:

1. The novel APA yeast microcapsules possessed strong membrane and morphological stability as well as potent resistance to mechanical stress, calcium sequestration and GI acidic and basic impediments. The viability of microencapsulated yeast cells is not only higher than that of free yeast, but also, not affected by the changing gastrointestinal environments. Microencapsulated yeast cells maintained their viability over extended storage conditions. This microcapsule formulation was not only not toxic to the yeast cells but also effective in protecting these cells. APA yeast microcapsules are excellent candidate for oral delivery use in renal failure uremia.

2. For their survival, yeast cells use urea as their primary carbon source. A loading of 1 g/100mL of yeast microcapsules generated the fastest urea hydrolysis rate per

unit time, reaching 11 ± 0.59% after 72 hrs. The acidity of the stomach was reported to jeopardize the yeast urea hydrolysis efficiency by damaging some of the membranes of certain weak APA microcapsules and affecting the viability of the leaking yeast cells.

3. The yeast column bioreactor experiments suggest that fully loading this device with yeast microcapsules yielded the highest hydrolysis rate (28.2 ± 0.05% after 4 hrs). Even though our design is not as complex and elaborated as those of dialysis machines, it provides, nonetheless, comprehensive results in regards to urea hydrolysis.

4. Orally administrating APA yeast microcapsules to uremic rats decreased urea levels by 18 ± 0.31%. After stopping the treatment, the urea concentration increased back to the uremic levels. The fact that this formulation had no perceivable effects on the concentrations of creatinine, uric acid, phosphate, and calcium suggests that yeast cells are only sensitive to urea.

5. The microbial populations of total aerobes, total anaerobes, *Escherichia coli, Staphylococcus sp.* and *Lactobacillus sp* were not substantially altered by the presence of the yeast APA microcapsules. This proves that orally administered yeast APA microcapsules do not cause adverse effects on the microbial flora of the human GI tract.

Chapter IX

RECOMMENDATIONS AND FUTURE APPLICATIONS

1. Researchers have extensively investigated the use of orally administered microcapsules entrapping therapeutic bioagents for the removal of unwanted uremic metabolites such as creatinine and urea. Such removal is made possible due to the exchange of these unwanted metabolites between capillaries in the systemic circulation and the lumen of the small intestine[167,220]. To date nobody has ever investigated the microencapsulation of yeast cells for use in renal failure uremia. The current study has shown the feasibility of lowering urea *in vitro* and *in vivo* by using APA microcapsules containing live *Saccharomyces cerevisiae*. This novel formulation can withstand harsh physiological gastro-intestinal conditions while keeping the desired microorganism intact. This approach of lowering urea may serve as an oral adjuvant for current RRTs. However, more research should be conducted in order to understand the precise mechanism by which a microcapsule ruptures. Having more insights into this subject will allow us to construct more suitable membranes capable of resisting to a greater extent with the physiological conditions.

2. Although the yeast APA microcapsules were designed for oral delivery, it is still nonetheless possible to design a column bioreactor packed with yeast APA microcapsules. These APA yeast microcapsules can be used in the hemopurfusion of renal failure patients. Our study showed promising results but more research should be carried out in order to optimize the rheology of the circulating fluid in order to maximize capsules elution, urea diffusion and urea-yeast contact.

3. Compared to the APA microcapsules containing genetically engineered *Escherichia coli* DH5 cells over-expressing the *Klebsiella aerogenes* urease gene, which removed almost all plasma urea in 30 min[39], our yeast microcapsules seem inefficient. However, the use of genetically engineered microorganisms is

subject to regulatory obstacles concerned with severe safety issues. While our yeast microcapsules overcome these obstacles, the extent of urea hydrolysis is still low. More research should be invested in inducing yeast cells to hydrolyze urea. The proposed approach of removing urea opens the door to numerous possibilities and potential applications in renal failure uremia.

REFERENCES

1. Gonwa TA, Atkins C, Zhang YA, Parker TF, Hunt JM, Lu CY. White MG: Glomerular filtration rates in persons evaluated as living-related donors—Are our standards too high? *Transplantation* 55: 983–985, 1993.
2. Connie L. Davis and Francis L. Delmonico. Living-Donor Kidney Transplantation: A Review of the Current Practices for the Live Donor. *Am Soc Nephrol* 16: 2098–2110, 2005.
3. Brady HR, Clarkson MR, Lieberthal W. Acute renal failure. In: Brenner BM, editor. Brenner & Rector's the kidney. *Philadelphia*7 WB Saunders; 2004. p. 1215– 92.
4. Coresh J, Astor BC, Greene T, et al. Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. *Am J Kidney Dis* 2003;41:1– 12.
5. Obrador GT, Pereira BJ, Kausz AT. Chronic kidney disease in the United States: an underrecognized problem. *Semin Nephrol* 2002;22:441–8.
6. Thadhani R, Pascual M, and Bonventre JV. Medical progress: acute renal failure. *N Engl J Med* 334:1448–1460. 1996.
7. Lieberthal W and Nigam SK. Acute renal failure. II. Experimental models acute renal failure: imperfect but indispensable. *Am J Physiol Renal Physiol* 278:F1–F12, 2000.
8. Nigam SK and Lieberthal W. Acute renal failure. III. The role of growth factors in the process of renal regeneration and repair. *Am J Physiol Renal Physiol* 279:F3–F11, 2000.
9. Schrier RW, Wang W. Acute renal failure and sepsis. *N Engl J Med* 2004; 351:159–69.
10. Izhar H. Kan & Graeme R.D. Catto. *Complications of Long-term Dialysis.* Edwina Brown & Patrick Parfrey (eds.), pp. 53-71 (Oxford University Press, Inc., New York, 1999).
11. 2004 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 1994-2003. Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation, Rockville, MD; United Network for Organ Sharing, Richmond, VA; Arbor Research Collaborative for Health, Ann Arbor, MI.
12. Varma R, Garrick R, McClung J, Frishman WH. Chronic renal dysfunction as an independent risk factor for the development of cardiovascular disease. *Cardiol Rev.* 2005 Mar-Apr;13(2):98-107.
13. Disney AP. Demography and survival of patients receiving treatment for chronic renal failure in Australia and New Zealand: report on dialysis and renal transplantation treatment from the Australia and New Zealand Dialysis and Transplant Registry. *Am J Kidney Dis* 1995; 25: 165-175.
14. Foley RN, Parfrey PS, Sarnak MJ.Epidemiology of cardiovascular disease in chronic renal disease. *Am Soc Nephrol.* 1998 Dec; 9(12 Suppl):S16-23.

15. Andrzej Borzecki, Anna Pikuła, Adam Stadnik, Justyna Janowska-Nowosad, Dorota Dyczko, Halina Borzecka. Occurrence of infectious diseases in dialysed patients. *Ann Univ Mariae Curie Sklodowska [Med].* 2004; 59 (1):351-5 16146011.
16. Morena M, Delbosc S, Dupuy AM, Canaud B, Cristol JP. Overproduction of reactive oxygen species in end-stage renal disease patients: a potential component of hemodialysis-associated inflammation. *Hemodial Int.* 2005 Jan; 9(1):37-46.
17. Colì L, Tumietto F, De Pascalis A, La Manna G, Zanchelli F, Isola E, Perna C, Raimondi C, De Sanctis LB, Marseglia CD, Costigliola P, Stefoni S. Effects of dialysis membrane nature on intradialytic phagocytizing activity. *Int J Artif Organs.* 1999 Feb; 2 2(2):74-80.
18. Lowrie EG, Lew NL. Death risk in hemodialysis patients: the predictive value of commonly measured variables and an evaluation of death rate differences between facilities. *Am J Kidney Dis.* 1990 May; 15(5):458-82.
19. Lewis SL, Van Epps DE. Neutrophil and monocyte alterations in chronic dialysis patients. *Am J Kidney Dis.* 1987 May; 9(5):381-95.
20. Kaslow RA, Zellner SR.Infection in patients on maintenance haemodialysis. *Lancet.* 1972 Jul 15; 2(7768):117-9.
21. R Gabriel. Morbidity and mortality of long-term haemodialysis: a review. *J R Soc Med.* 1984 July; 77(7): 595–601.
22. Baurmeister U, Travers M, Vienken J, Harding G, Million C, Klein E, Pass T, Wright R. Dialysate contamination and back filtration may limit the use of high-flux dialysis membranes. *ASAIO Trans.* 1989 Jul-Sep; 35(3):519-22.
23. Fissell WH, Fleischman AJ, Humes HD, Roy S. Development of continuous implantable renal replacement: past and future. *Transl Res.* 2007 Dec; 150(6):327-36.
24. Lim, F. & Sun, A. M. Microencapsulated islets as bioartificial endocrine pancreas. Science 210, 908–909 (1980).
25. Chang TMS. Semipermeable microcapsules. Science 1964; 524–525.
26. Chang TMS, MacIntosh FC, Mason SG. Semipermeable aqueous microcapsules: I. Preparation and properties. Can J Physiol Pharmacol 1966; 44:115–28.
27. Chang TMS. Semipermeable aqueous microcapsules [artificial cells]: with emphasis on experiments in an extracorporeal shunt system. Trans Am Soc Artif Intern Organs 1966; 12:13–9.
28. Chang, T. M. S. Semipermeable microcapsules. Science 146, 524–525 (1964).
29. Chang, T. M. S. Semipermeable Aqueous Microcapsules Ph.D. Thesis. McGill Univ. Montreal (1965).
30. Chang, T. M. S., MacIntosh, F. C. & Mason, S. G. Semipermeable aqueous microcapsules: I. Preparation and properties. Can. J. Physiol. Pharmacol. 44, 115–128 (1966).
31. Chang TM. Therapeutic applications of polymeric artificial cells. Nat Rev Drug Discov. 2005 Mar; 4(3):221-35.
32. Chang, T. M. S. *Artificial Cells* (Thomas, Springfield, 1972).
33. Friedman EA, Fastook J, Beyer MM, Rattazzi T, Josephson AS. Potassium and nitrogen binding in the human gut by ingested oxidized starch (OS). *Trans Am Soc Artif Intern Organs.* 1974; 20A:161-7.

34. Giordano C, Esposito R, Pluvio M. Oxycellulose and ammonia-treated oxystarch as insoluble polyaldehydes in uremia. *Kidney Int Suppl.* 1975 Feb; (3):380-2.
35. Meriwether LS, Kramer HM. In vitro reactivity of oxystarch and oxycellulose. *Kidney Int Suppl.* 1976 Dec; (7):S259-65.
36. Chang TM. Artificial cells for cell and organ replacements. *Artif Organs.* 2004 Mar; 28(3):265-70.
37. Setälä K.Treating uremia with soil bacterial enzymes: further developments. *Clin Nephrol.* 1979 Mar; 11(3):156-66
38. Setälä K. Bacterial enzymes in uremia management. *Kidney Int Suppl.* 1978 Jun; (8):S194-202.
39. Prakash S, Chang TM. Microencapsulated genetically engineered E. coli DH5 cells for plasma urea and ammonia removal based on: 1. Column bioreactor and 2. Oral administration in uremic rats. *Artif Cells Blood Substit Immobil Biotechnol.* 1996 May; 24(3):201-18.
40. Chang TMS. *Live* E.coli cells to treatment uremia. *Nat Med* 1997; 3:1997.
41. Chow KM, Liu ZC, Prakash S, Chang TMS. Free and microencapsulated Lactobacillus and effects of metabolic induction on urea removal. *Artif Cell Blood Sub* 2003.
42. Sumrada RA, Cooper TG. Urea carboxylase and allophanate hydrolase are components of a multifunctional protein in yeast. *J Biol Chem.* 1982 Aug 10; 257(15):9119–9127.
43. http://en.wikipedia.org/wiki/Kidney.
44. http://en.wikipedia.org/wiki/Nephron
45. Martini F. *Fundamentals of Anatomy and Physiology 5th edition.* Prentice Hall International Inc. 2001.
46. http://en.wikipedia.org/wiki/Renal_failure
47. http://en.wikipedia.org/wiki/Erythropoeisis
48. Meyer TW and Hostetter, TH. Uremia. *N Engl J Med* 357 (13): 1316. PMID 17898101, 2007.
49. Himmelfarb J. *Continuous renal replacement therapy in the treatment of acute renal failure: critical assessment is required.* Clin J Am Soc Nephrol. 2007 Mar; 2(2):385-9.
50. http://en.wikipedia.org/wiki/Acute_renal_failure
51. Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky P. *Acute Dialysis Quality Initiative workgroup. Acute renal failure - definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group.* Crit Care. 2004 Aug; 8(4):R204-12.
52. Mehta RL, McDonald B, Gabbai F, Pahl M, Farkas A, Pascual M Fowler W. Collaborative Study Group: Continuous versus intermitten dialysis for acute renal failure in the ICU: results from a randomized multicenter trial. *Am SocNephrol* 7:1457, 1996.
53. Van de Noortgate N, Verbeke F, Dhondt A, Colardijn F, Van Biesen W, Vanholder R, Lameire N. The dialytic management of acute renal failure in the elderly. *Semin Dial.* 2002 Mar-Apr; 15(2):127-32.

54. Lameire N, Van Biesen W, Vanholder R. Acute renal failure. *Lancet* 2005; 365:417-30.
55. http://www.kidney.org/professionals/KDOQI/guidelines_ckd/p4_class_g1.htm NKF.
56. http://www.kidney.org/professionals/KDOQI/guidelines_ckd/Gif_File/kck_t12.gif
57. http://en.wikipedia.org/wiki/Chronic_renal_failure
58. Adrouge HJ, Madias NE. Changes in plasma potassium concentration during acute acid-base disturbances. *Am J Med* 1981; 71: 456-67.
59. http://www.kidney.org/professionals/KDOQI/guidelines_ckd/p6_comp.htm
60. http://www.kidney.org/professionals/KDOQI/guidelines_ckd/p7_risk.htm. Part 7 STRATIFICATION OF RISK FOR PROGRESSION OF KIDNEY DISEASE AND DEVELOPMENT OF CARDIOVASCULAR DISEASE.
61. Perazella MA, Khan S. *Increased mortality in chronic kidney disease: a call to action.* Am J Med Sci. 2006 Mar; 331(3):150-3.
62. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW. *American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention.* Circulation. 2003 Oct 28; 108(17):2154-69.
63. Tonelli M, Wiebe N, Culleton B, House A, Rabbat C, Fok M, McAlister F, Garg AX. *Chronic Kidney Disease and Mortality Risk: A Systematic Review.* Am Soc Nephrol. 2006 May 31.
64. Ruggenenti P, Perna A, Gherardi G, Gaspari F, Benini R, Remuzzi G. Renal function and requirement for dialysis in chronic nephropathy patients on long-term ramipril: REIN follow-up trial. Gruppo Italiano di Studi Epidemiologici in Nefrologia (GISEN). Ramipril Efficacy in Nephropathy. *Lancet.* 1998 Oct 17; 352(9136):1252-6.
65. Ruggenenti P, Perna A, Gherardi G, Garini G, Zoccali C, Salvadori M, Scolari F, Schena FP, Remuzzi G. Renoprotective properties of ACE-inhibition in non-diabetic nephropathies with non-nephrotic proteinuria. *Lancet.* 1999 Jul 31; 354(9176):359-64.
66. Heidenheim AP, Kooistra MP, Lindsay RM. Quality of life. *Contrib Nephrol.* 2004; 145:99-105.
67. de Francisco AL, Pinera C. Challenges and future of renal replacement therapy. *Hemodial Int.* 2006 Jan; 10 Suppl 1:S19-23.
68. Katirtzoglou A, Kontesis P. Continuous equilibration peritoneal dialysis (CEPD) in hypercatabolic renal failure. *Perit. Dial. Bull.* 1983; 3:178–180.
69. Cameron JS, Ogg C, Trounce JR. Peritoneal dialysis in hypercatabolic acute renal failure. *Lancet* 1967; 1:1188–1191.
70. Indrapasit S, Charoenpan P, et al. Continuous peritoneal dialysis in acute renal failure from severe falciparum malaria. *Clin. Nephrol.* 1988; 29:137–143.

71. Daniela Ponce Gabriel, Ginivaldo Victor R. Nascimento, Jacqueline Teixeira Caramori, Luís Cuadrado Martim, Pasqual Barretti, André Luís Balbi. Peritoneal Dialysis in Acute Renal Failure. *Renal Failure,* 28:451–456, 2006.
72. Chitalia V, Almeida AF, Rai H, Bapat M, Vipul KC, Khannal R. Is peritoneal dialysis adequate for hypercatabolic acute renal failure in developing countries? *Kidney Int.* 2002; 61:747–757.
73. Schiff HMD, Lang MS. Daily hemodialysis and the outcome of acute renal failure. *N. Engl. J. Med.* 2002; 348:305–310.
74. Ronco C, Bellomo R, Honel P. Effects of different doses in continuous veno-venous haemofiltration on outcomes of acute renal failure: a prospective randomized trial. *Lancet* 2002; 356:26–30.
75. Evanson JA, Kisler TA, Wingard R, et al. Measurement of the delivery in acute renal failure. *Kidney Int.* 1999; 55:1501–1508.
76. Eknoyan G, Levin N, Esbach JW. Continuous quality improvement DOQI becomes K/DOQI and is updated. *Am. J. Kidney Dis.* 2001; 37:179–194.
77. Gossman J, Wilhelm A, Kachel HG, et al. Long term consequences of live kidney donation follow-up in 93% of living kidney donors in a single transplant center. *Am J Transplant* 2005; 5:2417–2424.
78. Paramesh AS, Killackey MT, Zhang R, Alper B, Slakey DP, Florman SS. Living donor kidney transplantation: medical, legal, and ethical considerations. *South Med J.* 2007 Dec; 100(12):1208-13.
79. http://en.wikipedia.org/wiki/Kidney_transplantation
80. Tiranathanagul K, Eiam-Ong S, Humes HD. The future of renal support: high-flux dialysis to bioartificial kidneys. *Crit Care Clin.* 2005 Apr; 21(2):379-94.
81. Choosing a Treatment That's Right for You. U.S. Department of Health and Human Services. NATIONAL INSTITUTES OF HEALTH National Kidney and Urologic Diseases Information Clearinghouse.
82. McDonald SP, Russ GR (2002). Survival of recipients of cadaveric kidney transplants compared with those receiving dialysis treatment in Australia and New Zealand, 1991-2001. *Nephrol. Dial. Transplant.* 17 (12): 2212–9.
83. Gloor JM, Lager DJ, Moore SB, Pineda AA, Fidler ME, Larson TS, Grande JP, Schwab TR, Griffin MD, Prieto M, Nyberg SL, Velosa JA, Textor SC, Platt JL, Stegall MD: ABO-incompatible kidney transplantation using both A2and non-A2 living donors. *Transplantation* 75: 971–977, 2003.
84. Gloor JM, DeGoey SR, Griffin MD, Larson TS, Lager DJ, Fidler ME, Moore SB, Stegall MD. Living donor kidney transplantation with and without splenectomy in positive crossmatch patients. *Am J Transplant* 4: 258, 2004.
85. Schweitzer EJ, Wilson JS, Jacobs S, Machan CH, Philosophe B, Farney A, Colonna J, Jarrell B, Bartlett ST. Increased rates of donation with laparoscopic donor nephrectomy. *Ann Surg* 232: 392–400, 2000.
86. Ratner LE, Montgomery RA, Kavoussi LR. Laparoscopic live donor nephrectomy. A review of the first 5 years. *Urol Clin North Am* 28: 709–719, 2001.
87. Greenstein MA, Harkaway R, Badosa F, Ginsberg P, Yang SL. Minimal incision living donor nephrectomy compared to the hand-assisted laparoscopic living donor nephrectomy. *World J Urol* 20: 356–359, 2003.

88. Jacobs SC, Cho E, Foster C, Liao P, Bartlett ST. Laparoscopic donor nephrectomy: The University of Maryland 6-year experience. *J Urol* 171: 47–51, 2004.
89. Simon SD, Castle EP, Ferrigni RG, Lamm DL, Swanson SK, Novicki DE, Andrews PE. Complications of laparoscopic nephrectomy: The Mayo Clinic experience. *J Urol* 171: 1447–1450, 2004.
90. El-Galley R, Hood N, Young CJ, Deierhoi MH, Urgan DA. Donor nephrectomy: A comparison of techniques and results of open, hand assisted and full laparoscopic nephrectomy. *J Urol* 171: 40–43, 2004.
91. Najarian JS, Chavers BM, McHugh LE, Matas AJ. 20 years or more of follow-up of living kidney donors. *Lancet* 340: 807–810, 1992.
92. Narkun-Burgess DM, Nolan CR, Norman JE, Page WF, Miller PL, Meyer TW. Forty-five year follow-up after uninephrectomy. *Kidney Int* 43: 1110–1115, 1993.
93. Fehrman-Ekholm I, Elinder CG, Stenbeck M, Tyden G, Groth CG: Kidney donors live longer. *Transplantation* 976–978, 1997.
94. Ramcharan T, Matas AJ: Long-term (20–37 years) follow-up of living kidney donors. *Am J Transplant* 2: 959 964, 2002.
95. http://www.dialysistips.com
96. http://en.wikipedia.org/wiki/Hemodialysis
97. http://www.kidney.org/professionals/kdoqi/guideline_upHD_PD_VA/hd_rec5.ht m.
98. Maggiore Q, Pizzarelli F, Dattolo P,MaggioreU,Cerrai T. Cardiovascular stability during haemodialysis, haemofiltration and haemodiafiltration. *Nephrology Dialysis Transplantation* 2000; 15 Suppl 1: 73.
99. Santoro A, Panzetta G, Tessitore N, AttiM, Mancini E, Esteban J. A prospective randomised European multicentre study of mediumL ong runmortality andmorbidity comparing acetate-free biofiltration and bicarbonate dialysis. *Journal of Nephrology* 1999; 12(6):375–82.
100. Rabindranath KS, Strippoli GF, Daly C, Roderick PJ, Wallace S, MacLeod AM. Haemodiafiltration, haemofiltration and haemodialysis for end-stage kidney disease. *Cochrane Database Syst Rev.* 2006 Oct 18; (4):CD006258.
101. Feldman HI, Kobrin S, Wasserstein A. Hemodialysis vascular access morbidity. *J Am Soc Nephrol.* 1996 Apr; 7(4):523-35.
102. http://www.homedialysis.org/pros/abstracts/20051014/
103. Weinreich T, De los Ríos T, Gauly A, Passlick-Deetjen J. Effects of an increase in time vs. frequency on cardiovascular parameters in chronic hemodialysis patients. *Clin. Nephrol.* 66 (6): 433–9, 2006.
104. http://en.wikipedia.org/wiki/Peritoneal_dialysis
105. Greene T. What did we learn from the HEMO Study? Implications of secondary analyses. *Contrib Nephrol.* 2005; 149:69-82.
106. Rocco-M. More Frequent Hemodialysis: Back to the Future? *Advances in CKD.* July, 2007.
107. Bonomini V, Mioli V, Albertazzi A, Scolari P. Daily-dialysis programme: indications and results. *Nephrol Dial Transplant.* 1998 Nov; 13(11):2774-7.
108. Ronco C. Continuous flow peritoneal dialysis: is there a need for it? *Contrib Nephrol* 2006; 150:310-20.

109. Vale L, Cody J, Wallace S, Daly C, Campbell M, Grant A, Khan I, Donaldson C, Macleod A. Continuous ambulatory peritoneal dialysis (CAPD) versus hospital or home haemodialysis for end-stage renal disease in adults. *Cochrane Database Syst Rev.* 2004 Oct 18; (4):CD003963.
110. Alarabi AA, Petersson T. Continuous peritoneal dialysis in children with acute renal failure. *Adv. Perit. Dial.* 1994; 10:289–293.
111. Methrotra R, Kharma R. Peritoneal UF for congestive heart failure: rationale, evidence and future. *Cardiology 2001*; 96(Suppl. 3–4):177–182.
112. Parvathaneni LS, Gugleilni K, Silver MA. Difficult cases in heart failure: role of continuous ambulatory peritoneal dialysis in refractory heart failure. *Congest. Heart Fail.* 1999; 5(Suppl. 6):283–285.
113. Tenckhoff H, Schechter H. A bacteriology safe peritoneal access device. *Trans. Am. Soc. Artif. Intern. Organs* 1968; 14:181–187.
114. Diaz-Buxo JA. Automated peritoneal dialysis: a therapy in evolution. *Contrib. Nephrol.* 1999; 129:1–14.
115. Mehta RL, Letteri JM, for the National Kidney Foundation Council on Dialysis. Current status of renal replacement therapy for acute renal failure. Am. *J. Nephrol.* 1999; 19:377–382.
116. http://en.wikipedia.org/wiki/Home_hemodialysis
117. Palevsky PM. *Dialysis modality and dosing strategy in acute renal failure.* Semin Dial. 2006 Mar-Apr; 19(2):165-70.
118. Bellomo R, Parkin G, Love J, Boyce N. *A prospective comparative study of continuous arteriovenous hemodiafiltration and continuous venovenous hemodiafiltration in critically ill patients.* Am J Kidney Dis 21:400–404, 1993.
119. Ozdemir FN, Akcay A, Haberal M. *Dialysis modalities in patients with acute renal failure.* Nephrol Dial Transplant. 2001;16 Suppl 6:18-20.
120. Braam B, Verhaar MC, Blankestijn P, Boer WH, Joles JA. Technology insight: Innovative options for end-stage renal disease--from kidney refurbishment to artificial kidney. *Nat Clin Pract Nephrol.* 2007 Oct; 3(10):564-72.
121. Tiranathanagul K, Eiam-Ong S, Humes HD. *The future of renal support: high-flux dialysis to bioartificial kidneys.* Crit Care Clin. 2005 Apr; 21(2):379-94.
122. Smith T. Medicine in Europe. European health challenges. *BMJ* 303:139 1397, 1991.
123. Han MK, Hyzy R. Advances in critical care management of hepatic failure and insufficiency. *Crit Care Med.* 2006 Sep; 34(9 Suppl):S225-31.
124. Kida K, Nakajo S, Kamiya F, Toyama Y, Nishio T, and Nakagawa H (1978) Renal net glucose release in vivo and its contribution to blood glucose in rats. *J Clin Investig* 62:721–726.
125. Tannen RL and Sastrasinh S (1984) Reponse of ammonia metabolism to acute acidosis. *Kidney Int* 25:1–10.
126. Deneke SM and Fanburg BL (1989) Regulation of cellular glutathione. *Am J Physiol* 257:L163– L173.
127. Maak T (1992) Renal handling of proteins and polypeptides in *Handbook of Physiology* (Windhager EE ed) pp 2039–2118, Oxford University Press, New York.

128. Frank J, Engler-Blum G, Rodemann HP, and Muller GA (1993) Human renal tubular cells as a cytokine source: PDGF-B, GM-CSF and IL-6 mRNA expression in vitro. *Exp Nephrol* 1:26–35.
129. Stadnyk AW (1994) Cytokine production by epithelial cells. *FASEB J* 8:1041–1047.
130. Brodie JC, Humes HD. Stem cell approaches for the treatment of renal failure. *Pharmacol Rev.* 2005 Sep; 57(3):299-313.
131. Humes HD, Buffington DA, Lou L, Abrishami S, Wang M, Xia J, and Fissell WH (2003) Cell therapy with a tissue-engineered kidney reduces the multiple-organ consequences of septic shock. *Crit Care Med* 31:2421–2428.
132. Humes HD and Cieslinski DA (1992) Interaction between growth factors and retinoic acid in the induction of kidney tubulogenesis in tissue culture. *Exp Cell Res* 201:8–15.
133. Humes HD, Fissell WH, Weitzel WF, Buffington DA, Westover AJ, MacKay SM, and Gutierrez JM (2002) Metabolic replacement of kidney function in uremic animals with a bioartificial kidney containing human cells. *Am J Kidney Dis* 39:1078–1087.
134. Gage FH. Cell therapy. *Nature* 1998; 392:18– 24.
135. Dove A. Cell-based therapies go live. *Nat Biotechnol* 2002; 20:339–43.
136. Petit-Zeman S. Regenerative medicine. *Nat Biotechnol* 2001; 19:201– 6.
137. Rookmaaker MB *et al.* (2002) Bone-marrow-derived cells contribute to endothelial repair after thrombotic microangiopathy. *Blood* 99: 1095.
138. Cornacchia F *et al.* (2001) Glomerulosclerosis is transmitted by bone marrow-derived mesangial cell progenitors. *J Clin Invest* 108: 1649–1656.
139. Imasawa T *et al.* (2001) The potential of bone marrow-derived cells to differentiate to glomerular mesangial cells. *J Am Soc Nephrol* 12: 1401–1409.
140. Feng Z *et al.* (2005) Glomerular aging in females is a multi-stage reversible process mediated by phenotypic changes in progenitors. *Am J Pathol* 167: 355–363.
141. Atala A and Koh CJ (2004) Tissue engineering applications of therapeutic cloning. *Annu Rev Biomed Eng* 6: 27–40.
142. Lanza RP *et al.* (2002) Generation of histocompatible tissues using nuclear transplantation. *Nat Biotechnol* 20: 689–696.
143. Koh CJ and Atala A (2004) Tissue engineering, stem cells, and cloning: opportunities for regenerative medicine. *J Am Soc Nephrol* 15: 1113–1125.
144. Henderson LW (2000) Future developments in the treatment of end-stage renal disease: a North American perspective. *Am J Kidney Dis* 35 (suppl s): S106–S116.
145. Soon-Shiong P, Lu ZN, Grewal I, Lanza R, Clark W. Prevention of CTL and NK cell-mediated cytotoxicity by microencapsulation. *Horm Metab Res Suppl.* 25, 215-219 (1990).
146. Chang PL. Microencapsulation--an alternative approach to gene therapy. *Transfus Sci.*17, 35-43 (1996).
147. R. Walker, S. Rossall, M.J.C. Asher. Comparison of application methods to prolong the survival of potential biocontrol bacteria on stored sugar-beet seed. *Journal of Applied Microbiology*, 97, 293-305 (2004).

148. Ellerby LM, Nishida CR, Nishida F, Yamanaka SA, Dunn B, Valentine JS, Zink JI.Encapsulation of proteins in transparent porous silicate glasses prepared by the sol-gel method. *Science.*, 28, 1113-1115 (1999).
149. Scannell AG, Hill C, Ross RP, Marx S, Hartmeier W, Arendt EK. Continuous production of lacticin 3147 and nisin using cells immobilized in calcium alginate. *J Appl Microbiol.*, 89, 573-579 (2000).
150. Moës AJ. Gastroretentive dosage forms. *Crit Rev Ther Drug Carrier Syst,* 10, 143-95 (1993).
151. Talukder R, Fassihi R. Gastroretentive delivery systems: a mini review. *Drug Dev Ind Pharm.*, 30, 1019-1028 (2004).
152. Josias H Hamman, Gill M Enslin, Awie F Kotzé. Oral delivery of peptide drugs: barriers and developments. *BioDrugs.* 2005; 19 (3):165-77.
153. H. Uludag, P. De Vos, P. Tresco. Technology of mammalian cell encapsulation. *Adv. Drug Del. Rev.* (2000) 42: 29-64.
154. Soo-Jin Park and Seung-Hak Kim. Preparation and characterization of biodegradable poly(l-lactide)/poly(ethylene glycol) microcapsules containing erythromycin by emulsion solvent evaporation technique. *J. Colloid Interface Sci.* 271, 336-341 (2004).
155. Chandy T.; Mooradian D.L.; Rao G.H.R. Evaluation of Modified Alginate-Chitosan-Polyethylene Glycol Microcapsules for Cell Encapsulation. *Artificial Organs*, 23, 894-903 (1999).
156. Dawson RM, Broughton RL, Stevenson WT, Sefton MV.Microencapsulation of CHO cells in a hydroxyethyl methacrylate-methyl methacrylate copolymer. *Biomaterials.* 1987 Sep; 8(5):360-6.
157. S M Chia, A C A Wan, C H Quek, H Q Mao, X Xu, Lu Shen, M L Ng, K W Leong, H Yu. Multi-layered microcapsules for cell encapsulation. *Biomaterials.* 2002 Feb; 23 (3):849-56.
158. Sawhney AS, Hubbell JA. Poly(ethylene oxide)-graft-poly(L-lysine) copolymers to enhance the biocompatibility of poly(L-lysine)-alginate microcapsule membranes. *Biomaterials.* 1992; 13(12):863-70.
159. G. Orive, R. M. Hernández, A. R. Gascón, M. Igartua and J. L. Pedraz. Survival of different cell lines in alginate-agarose microcapsules. *Eur. J. Pharm. Sci.*, 18, 23-30 (2003).
160. Gombotz W.R., Wee S. Protein release from alginate matrices. *Advanced Drug Delivery Reviews*, 31, 267-285 (1998).
161. Douglas B. Seifert and Janice A. Phillips. Porous Alginate-Poly(ethylene glycol) Entrapment System for the Cultivation of Mammalian Cells. Biotechnol. Prog., 13 (5), 569 -576, 1997.
162. Gorka Orive *et al.* History, challenges and perspectives of cell microencapsulation. *Trends Biotechnol.*, 22, 87-92 (2004).
163. Strand BL, Ryan TL, In't Veld P, Kulseng B, Rokstad AM, Skjak-Brek G, Espevik TPoly-L-Lysine induces fibrosis on alginate microcapsules via the induction of cytokines. *Cell Transplant.*10, 263-275 (2001).
164. J. Li, J. Pan, L. Zhang, X. Guo and Y. Yu, Culture of primary rat hepatocytes within porous chitosan scaffolds. *J Biomed Mater Res. A*, 67, 938-943 (2003).

165. Sparks RE, Mason NS, Meier PM, Litt MH, Lindan O. Removal of uremic waste metabolites from the intestinal tract by encapsulated carbon and oxidized starch. *Trans Am Soc Artif Intern Organs.* 1971; 17: 229-38.
166. Frank Hinman and A. E. Belt. An Experimental Study of Uretero-. Duodenostomy. *J. Amer. Med. Ass.*, 79, 1917 (1922).
167. Pendleton, W. R., West, F. E. Passage of urea between the blood and the lumen of the small intestine. *Amer. J. Physiol.* 101, 391 (1932).
168. Trisna Lim, Microcapsules immobilizing activated charcoal and metabolically induced *Lactobacillus acidophilus* cells as potential oral renal failure therapy formulation. Masters Thesis, McGill University, 2006.
169. Kjellstrand, C., Borges, H., Pru, C., Gardner, D. & Fink, D. On the clinical use of microencapsulated zirconium phosphate-urease for the treatment of chronic uremia. *Trans. Am. Soc. Artif. Intern. Org.* 27, 24–30 (1981).
170. Gardner, D. L., Kjellstrand, C. M., Hassler, C. R., Fink, D. J. and Emmerling, D. C., An Orally Administered Microcapsule System for Treating Chronic Renal failure Patients, *Appl. Biochem. Biotech.*, 10, 27040 (1984).
171. Ristau O, Pommerening K, Jung C, Rein H, Scheler W. [Activity of urease in microcapsules]. *Biomed Biochim Acta.* 1985; 44(7-8):1105-11.
172. Elcin Y.M. Encapsulation of urease enzyme in xanthan-alginate spheres. Source: Biomaterials, 16, 1157-1161(1995).
173. Malchesky PS, Nosé Y. Biological reactors as artificial organs. Concept and preliminary in vitro study. *Cleve Clin Q.* 1975 Fall; 42(3):267-71.
174. Malchesky PS, Nosé Y. Biological reactors as renal substitutes. *Artif Organs.* 1979 Feb; 3(1):8-10.
175. http://en.wikipedia.org/wiki/Yeast.
176. R Gugerli, E Cantana, C Heinzen, U von Stockar, I W Marison. Quantitative study of the production and properties of alginate/poly-L-lysine microcapsules. *J Microencapsul.* ; 19 (5):571-90.
177. Rehor A, Canaple L, Zhang Z, Hunkeler D.The compressive deformation of multicomponent microcapsules: influence of size, membrane thickness, and compression speed. *J Biomater Sci Polym Ed.* 2001;12(2):157-70.
178. Thu B, Bruheim P, Espevik T, Smidsrød O, Soon-Shiong P, Skjåk-Braek G. Alginate polycation microcapsules. II. Some functional properties. *Biomaterials.* 1996 Jun;17(11):1069-79.
179. Gåserød O, Sannes A, Skjåk-Braek G.Microcapsules of alginate-chitosan. II. A study of capsule stability and permeability. *Biomaterials.* 1999 Apr;20(8):773-83.
180. Ma X, Vacek I, Sun A. Generation of alginate-poly-l-lysine-alginate (APA) biomicrocapsules: the relationship between the membrane strength and the reaction conditions. *Artif Cells Blood Substit Immobil Biotechnol.* 1994;22(1):43-69.
181. Thu B, Bruheim P, Espevik T, Smidsrød O, Soon-Shiong P, Skjåk-Braek G. Alginate polycation microcapsules. I. Interaction between alginate and polycation. *Biomaterials.* 1996 May;17(10):1031-40.
182. A. Bartkowiak, D. Hunkeler. Alginate-oligochitosan microcapsules: II. Control of mechanical resistance and permeability of the membrane. *Chemistry of Materials* (2000), 12 (1), 206-212

183. A. Prokop, D. Hunkeler, A. C. Powers, R. R. Whitesell and T. G. Wang. Water Soluble Polymers for Immunoisolation II: Evaluation of Multicomponent Microencapsulation Systems. *Advances in Polymer Science*, 136, 53-73 (1998).
184. Peyratout CS, Dähne L. Tailor-made polyelectrolyte microcapsules: from multilayers to smart containers. *Angew Chem Int Ed Engl.* 2004 Jul 19;43(29):3762-83.
185. Oscar W. Richards. The Growth of the Yeast Saccharomyces cerevisiae: I. The Growth Curve, its Mathematical Analysis, and the Effect of Temperature on the Yeast Growth. *Annals of Botany*, 42, 271-283 (1928).
186. Maruyama O, Yamane T, Tsunemoto N, Nishida M, Tsutsui T, Jikuya T.A preliminary study of microcapsule suspension for hemolysis evaluation of artificial organs. *Artif Organs.* 1999 Mar;23(3):274-9.
187. Fery, A., Dubreuil, F., & Mohwald, H. Mechanics of artificial microcapsules. *New Journal of Physics,* 6 (2004).
188. Gleb B. Sukhorukov, Andreas Fery, Milan Brumen and Helmuth Möhwald Physical chemistry of encapsulation and release. *Phys. Chem. Chem. Phys.*, 2004, 6, 4078 – 40.
189. Van Raamsdonk JM, Chang PL. Osmotic pressure test: a simple, quantitative method to assess the mechanical stability of alginate microcapsules. *J Biomed Mater Res.* 2001 Feb;54(2):264-71.
190. C. P. Champagne, C. Gaudy, D. Poncelet and R. J. Lactococcus lactis release from calcium alginate beads. *Applied and Environmental Microbiology,* 58, 1429-1434 (1992).
191. de Groot M, Schuurs TA, van Schilfgaarde R.Causes of limited survival of microencapsulated pancreatic islet grafts. *J Surg Res.* 2004 Sep;121(1):141-50.
192. Umehara Y, Hakamada K, Seino K, Aoki K, Toyoki Y, Sasaki M. Improved survival and ammonia metabolism by intraperitoneal transplantation of microencapsulated hepatocytes in totally hepatectomized rats. *Surgery.* 2001 Sep;130(3):513-20.
193. Juste S, Lessard M, Henley N, Ménard M, Hallé JP. Effect of poly-L-lysine coating on macrophage activation by alginate-based microcapsules: assessment using a new in vitro method. *J Biomed Mater Res A.* 2005 Mar 15;72(4):389-98.
194. Strand BL, Mørch YA, Espevik T, Skjåk-Braek G.Visualization of alginate-poly-L-lysine-alginate microcapsules by confocal laser scanning microscopy. *Biotechnol Bioeng.* 2003 May 20;82(4):386-94.
195. http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=9896&Template=fungiYeast
196. http://jb.asm.org/cgi/content/full/186/9/2520.
197. Chen H, Ouyang W, Lawuyi B, Lim T, Prakash S. A new method for microcapsule characterization: use of fluorogenic genipin to characterize polymeric microcapsule membranes. *Appl Biochem Biotechnol.* 2006 Sep;134(3):207-22.
198. Rokstad AM, Holtan S, Strand B, Steinkjer B, Ryan L, Kulseng B, Skjåk-Braek G, Espevik T. Microencapsulation of cells producing therapeutic proteins: optimizing cell growth and secretion. *Cell Transplant.* 2002;11(4):313-24.

199. van Raamsdonk JM, Cornelius RM, Brash JL, Chang PL. Deterioration of polyamino acid-coated alginate microcapsules in vivo. *J Biomater Sci Polym Ed.* 2002;13(8):863-84.
200. Hsu BR, Ho YS, Fu SH, Huang YY, Chiou SC, Huang HS. Membrane compactness affects the integrity and immunoprotection of alginate-poly-L-lysine-alginate microcapsules. *Transplant Proc.* 1995 Dec;27(6):3227-31.
201. Wen-tao Q, Ying Z, Juan M, Xin G, Yu-bing X, Wei W, Xiaojun M. Optimization of the cell seeding density and modeling of cell growth and metabolism using the modified Gompertz model for microencapsulated animal cell culture. Biotechnol Bioeng. 2006 Apr 5;93(5):887-95.
202. Chang TMS, Prakash S: Artificial cells and genetically engineered microencapsulated for *E. coli* cells, for urea and ammonia removal. *Methods Molec Biol* 1996;23:343-357.
203. Hongmei Chen, A new alginate-chitosan based microcapsule formulation, its characterization, suitability for cell encapsulation and potential gastrointestinal applications Ph.D Thesis, McGill University, 2006.
204. Hu, B.J. Alginate Polycation Microcapsules: A Study of Some Molecular and Functional Properties Relevant to Their Use as a Bioartificial Pancreas. PhD Thesis. Norwegian University of Science and Technology, Trondheim, Norway. 1996
205. Hoppe B, von Unruh G, Laube N, Hesse A, Sidhu H. Oxalate degrading bacteria: new treatment option for patients with primary and secondary hyperoxaluria? *Urol Res.* 2005 Nov; 33(5):372-5.
206. Mitch Lawrence Jones, Hongmei Chen, Wei Ouyang, Terrence Metz, and Satya Prakash (2004). Microencapsuled Genetically Engineered *Lactobacillus Plantarum* 80 (pCBH1) for bile acid deconjugation. *J. Biomedicine and Biotechnology*, 2004(1):61-69.
207. Jitrapakdee, S., and J. C. Wallace. 2003. The biotin enzyme family: conserved structural motifs and domain rearrangements. *Curr. Protein Pept. Sci.* 4:217-229.
208. Roon, R. J., and B. Levenberg. 1968. An adenosine triphosphate-dependent avidin-sensitive enzymatic cleavage of urea in yeast and green algae. *J. Biol. Chem.* 243:5213-5215.
209. Roon, R. J., and B. Levenberg. 1970. CO_2 fixation and the involvement of allophanate in the biotin-enzyme-catalyzed cleavage of urea. *J. Biol. Chem.* 245:4593-4595.
210. Roon, R. J., and B. Levenberg. 1972. Urea amidolyase. 1. Properties of the enzyme from *Candida utilis*. *J. Biol. Chem.* 247:4107-4113.
211. Paliwal DK, Randhawa HS.Rapid method for detection of urea hydrolysis by yeasts. *Appl Environ Microbiol.* 1977 Feb;33(2):219-20.
212. Chang TM. Artificial cells as bioreactive biomaterials. *J Biomater Appl.*, 3(1):116-25 (1988).
213. K. Jainkiatfu. Effects of vitamin E-coated membrane dialyzer on reduction of inflammation. *Hemodialysis International,* 9, 88-88 (2005).
214. Chang TM. Coated charcoal haemoperfusion. *Life Support Syst.* 2(2):99-106 (1984).
215. Charles, S., David, M. & Stacia, M. Control of body fluid, electrolyte, and acid–base balance. in *Human Physiology: Foundations and Frontiers.* (ed.

Deborah, A.) 526–527 (Times-Mirror College Publications, St. Louis, Missouri, 1990).

216. Asaba, H. *et al.* Plasma middle molecules in asymptomatic and sick uremic patients. in *Middle Molecules in Uremia and Other Diseases.* (ed. Klinkmann, H. *et al.*) 137–141 (International Society for Artificial Organs, Cleveland, Ohio, 1980).
217. Cohen, B.D. Uremic toxins. in *Uremia, an International Conference on Pathogenesis, Diagnosis and Therapy.* (eds. Kulte, R., Geoffrey, B. & Benjamin, B.C.) 1–11 (Thieme, Stuttgart, 1972).
218. Kolff, W.J. Artificial kidney and artificial heart: Future considerations. *Int. J. Artif. Org.* 13, 404–406 (1990).
219. Esposito, R., Carmelo, G. & G. Polyhalides. in *Sorbents and their Clinical Applications*, (ed. Caramelo, G.) 133–153 (Academic Press, London, 1980).
220. Kjellstrand, C., Borges, H., Pru, C., Gardner, D. & Fink, D. On the clinical use of microencapsulated zirconium phosphate-urease for the treatment of chronic uremia. *Trans. Am. Soc. Artif. Intern. Org.* 27, 24–30 (1981).
221. Drukker, W., Parsons, P.M. & Mahar, J.F. *Replacement of Renal Functions by Dialysis.* (Martinus Nijhoff, Boston, 1983).
222. Friedman, E.A. Future treatment of renal failure. in *Strategy in Renal Failure*, (ed. Friedman, E.A.) 521–528 (Wiley, New York, 1978).
223. Sparks, R.E., Saleman, R.M., Meier, P.M., Litt, M.H. & Lindon, O. Binders to remove metabolite from the GI tract. *Tram. Am. Soc. Artif. Intern. Org.* 18, 458–464 (1972).
224. Sparks, R.E. Review of gastrointestinal perfusion in treatment of uremia. *Clin. Nephrol.* 11, 81–85 (1979).
225. Walker, J.M., Jacobson, R.L., Stephen, W.J. & Rose, D. The role of adsorbents in the wearable artificial kidney. in *Artificial Organs.* (ed. Gilchrist, T.) 137–149 (Macmillan Press, London, 1977).
226. Friedman, E.A., Fastcook, J., Beyer, M.M., Rattazzi, T. & Josephson, A.S. Combined oxystarch-charcoal trial in uremia, sorbent induced reduction in serum cholesterol. *Kidney Int.* 13, S273–S276 (1976).
227. Agishi, T., Yamashita, N. & Ota, K. Clinical results of direct charcoal hemoperfusion for endogenous and exogenous intoxication. in *Hemoperfusion, Part I, Kidney and Liver Support and Detoxification* (eds. Sidemen, S. & Chang, T.M.S.) 255–263 (Hemisphere, Washington, DC, 1980).
228. Gu, K.F. & Chang, T.M.S. Conversion of alpha-ketoglutarate into L-glutamic acid with urea as ammonia source using multienzyme system and NAD immobilized by microencapsulation with artificial cell in bioreactor. *Biotechnol. Bioeng.* 32, 363–368 (1988).
229. Gu, K.F. & Chang, T.M.S. Conversion of urea or ammonia into essential amino acids, L-leucine, L-valine and L-isoleucine using artificial cells containing an immobilized multienzyme system and dextran-NAD: Yeast alcohol dehydrogenase for enzyme recycling. J. Biotech. Appl Biochem. 12, 227–236 (1991).
230. Cattaneo, M. & Chang, T.M.S. The potential of microencapsulated urease-zeolite oral sorbent for the removal of urea in uremia. *J. Am. Soc. Artif. Intern. Org.* 37, 80–87 (1991).

231. Iseki K, Oshiro S, Tozawa M, Iseki C, Ikemiya Y, Takishita S: Signi. cance of hyperuricemia on the early detection of renal failure in a cohort of screened subjects. *Hypertens Res* 2001; 24: 691–697.
232. Wu X, Wakamiya M, Vaishnav S, Geske R, Montgomery C Jr, Jones P, Bradley A, Caskey CT: Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. *Proc Natl Acad Sci USA* 1994; 91: 742–746.
233. Hirayama K, Rafter J. The role of lactic acid bacteria in colon cancer prevention: mechanistic considerations. *Antonie Van Leeuwenhoek*, 76, 391-394 (1999).
234. Simon GL, Gorbach SL. Intestinal flora in health and disease. *Gastroenterology*, 86, 174-193 (1984).