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# Efficacy Of Novel Antifouling/Antimicrobial Coatings For The Prevention Of Urinary Device Associated Infection And Encrustation

Thomas O. Tailly, *The University of Western Ontario*

Supervisor: Dr. Hassan Razvi, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Surgery

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Efficacy Of Novel Antifouling/Antimicrobial Coatings For The Prevention Of  
Urinary Device Associated Infection And Encrustation

by

Thomas O. Tailly

Graduate Program in Surgery

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

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## Abstract

Device associated urinary tract infections are a very important healthcare issue. Despite best efforts in preventing and treating these infections, many patients and physicians are still confronted with this problem. Our research group is involved in testing and evaluating new stent coatings for the prevention of device associated infections and device encrustation. In the current project, we tested two new coating copolymers, both based on a long chain polymer backbone and mussel adhesive protein to this purpose in a rabbit and porcine model. The results demonstrate efficiency of one copolymer in preventing infections in the rabbit model. Interestingly there is an inverse correlation between *E. coli* infections and encrustations. No significant differences were noted in the porcine study, the model itself however seems to have some pitfalls. Finally we identified that the coatings are subject to a certain shelf life. We intend to further investigate the coating and its potential in future research.

## Keywords

Antibiotic resistance, biocompatibility, biofilm, biomaterial, catheter associated urinary tract infection, CAUTI, encrustation, *Escherichia coli*, MAP, mussel adhesive protein, mPEG-DOPA, PEG, DOPA, polyethylene glycol, porcine model, rabbit model, stent coating, ureteral stent, urinary catheter, urinary tract infection, UTI

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# Table of Contents

Abstract.....	ii
Acknowledgments.....	iii
Table of Contents.....	vii
List of Tables.....	xi
List of Figures.....	xii
List of Appendices.....	xiv
Chapter 1.....	1
1 Background.....	1
1.1 Historic Note.....	1
1.2 Urinary Device Associated Urinary Tract Infection.....	2
1.3 Antibiotic Resistance.....	6
1.4 Biofilm.....	8
1.5 Encrustation.....	11
1.5.1 Dependent on Urinary Composition.....	11
1.5.2 Dependent on Indwelling Time.....	13
1.6 Urinary Catheter Biomaterials and Coatings.....	15
1.7 Indications for Stent or Catheter Placement.....	20
1.8 Stent Technology.....	21
1.8.1 Biomaterials.....	22
1.8.2 Coatings.....	25
1.9 mPEG-DOPA.....	29
1.9.1 Polyethylene Glycol (PEG).....	29
1.9.2 Mussel Adhesive Protein (MAP).....	31



1.9.3	mPEG-DOPA.....	32
1.10	mPEG-DOPA Coating For Urinary Devices.....	33
1.10.1	S-095 and the Use of Additional Bactericidal Agents.....	34
1.10.2	Pilot Study.....	38
Chapter 2	.....	40
2	Materials and Methods.....	40
2.1	Rabbit Study.....	41
2.1.1	Transurethral Catheter and <i>E. coli</i> Bacterial Challenge.....	41
2.1.2	Sample Collection.....	43
2.2	Pig Study.....	43
2.2.1	Ureteral Stent Placement and Bacterial Challenge.....	46
2.2.2	Sample Collection.....	47
2.3	Sample Analysis.....	48
2.3.1	Urine Culture.....	48
2.3.2	Device Bacterial Attachment.....	49
2.3.3	Device Encrustation.....	49
2.3.4	Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy.....	50
2.3.5	Tissue Bacterial Attachment and Internalization.....	50
2.3.6	Histopathology.....	50
2.4	Statistical Analysis.....	51
2.5	<i>In vitro</i> Assessment of Catheter Coatings.....	53
Chapter 3	.....	56
3	Results.....	56
3.1	Results Rabbit Study.....	56
3.1.1	Urine Culture.....	56

3.1.2	Device Bacterial Attachment .....	59
3.1.3	Encrustation .....	62
3.1.4	Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy (SEM & EDX).....	66
3.1.5	Tissue Bacterial Counts .....	71
3.2	Results Pig Study .....	73
3.2.1	Urine Culture .....	73
3.2.2	Bacterial Attachment .....	75
3.2.3	Encrustation .....	79
3.2.4	SEM/EDX.....	79
3.2.5	Tissue Bacterial Counts .....	80
3.2.6	Histopathology.....	81
3.3	Post-experimental <i>In Vitro</i> Evaluation of Coating Efficiency .....	84
Chapter 4	.....	87
4	Discussion and Conclusions.....	87
4.1	Rabbit Study.....	88
4.1.1	Urine Sample Analysis .....	88
4.1.2	Device Bacterial Attachment .....	89
4.1.3	Tissue Bacterial Counts .....	89
4.1.4	Encrustations.....	90
4.2	Porcine Study .....	94
4.2.1	Urine Culture, Device and Tissue Bacterial Attachment.....	94
4.2.2	Encrustations.....	95
4.2.3	Histopathology.....	96
4.3	Future Directions .....	97
Bibliography	.....	99

Appendices.....	128
Curriculum Vitae .....	129

## List of Tables

Table 1 .....	57
Table 2. ....	61
Table 3 .....	65
Table 4. ....	72
Table 5. ....	74
Table 6 .....	77
Table 7 .....	80
Table 8. ....	85
Table 9 .....	86

## List of Figures

Figure 1.....	13
Figure 2.....	14
Figure 3.....	22
Figure 4.....	36
Figure 5.....	37
Figure 6.....	42
Figure 7.....	45
Figure 8.....	53
Figure 9.....	55
Figure 10.....	58
Figure 11.....	58
Figure 12.....	60
Figure 13.....	62
Figure 14.....	63
Figure 15.....	64
Figure 16.....	67
Figure 17.....	67
Figure 18.....	68

Figure 19. ....	69
Figure 20. ....	70
Figure 21. ....	71
Figure 22. ....	75
Figure 23. ....	79
Figure 24. ....	83
Figure 25. ....	85
Figure 26. ....	86
Figure 27. ....	92

## List of Appendices

Appendix A.....	128
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# Chapter 1

## 1 Background

This chapter will provide the historical background of catheters and stents and a comprehensive overview of catheter associated urinary tract infection and biofilm formation on urinary devices. A review of previously developed devices, research methods and outcomes in the search for the ideal biocompatibility will demonstrate the extent of this field of research. The novelty of our current research will be highlighted and a summary of the preliminary work done by our research group will aid in the understanding and appreciation of this current project.

### 1.1 Historic Note

Draining the bladder in the treatment of urinary retention has been an essential part of a physician's trade for millennia. Catheterisation of the bladder has been described in ancient Asian, Chinese, Egyptian, Roman, Byzantine and Greek civilizations (Mattelaer and Billiet, 1995). These descriptions report the use of straws, reeds, polished or waxed rolled up leaves or hollow twigs for bladder catheterisation (Mattelaer and Billiet, 1995). Paulus Aegineta, in the 7<sup>th</sup> century AD, was the first to describe bladder drainage with a slender silver catheter, a technique which became very popular in medieval times (Mattelaer and Billiet, 1995). Even in this scientifically naïve era, silver was attributed an antiseptic effect. The popularity of silver catheters remained until the introduction of natural rubber for the manufacture of urinary catheters. Benjamin Franklin suggested the use of a reusable silver flexible catheter for bladder drainage to his brother who suffered from urinary stone disease (Gensel, 2005; Mattelaer and Billiet, 1995). The 19<sup>th</sup> Century was pivotal in the evolution of the urinary catheter. Auguste Nelaton created a tubular soft bladder catheter with a solid straight tip and one side hole, made of vulcanized rubber in 1860, that bears his name (Mattelaer and Billiet, 1995). This design was further developed to hold a retaining mechanism when in the 1930's, Frederick EB Foley



constructed a catheter with an inflatable balloon attached to the catheter tip (Tatem et al., 2013). Ever since this fundamental development, the catheter design has not changed much to the extent that we still use “Foley” catheters to this day.

The use of ureteral stents in surgery has been described as early as the 19<sup>th</sup> century (Shoemaker, 1895). The first urologist to access the ureter endoscopically was Dr. James Brown at Johns Hopkins Hospital in 1893 (Arcadi, 1999). Before becoming popular and globally used to treat obstructed kidneys, Dr. Werner Forssmann used a ureteral catheter to perform the first ever heart catheterization on a human, himself, in 1929. Although initially met with criticism, he went on to win the Nobel Prize for his pioneering endeavor with the ureteral catheter (Morris and Schirmer, 1990). In 1967, Zimskind was the first to describe the cystoscopic placement of indwelling ureteral stents for obstructed kidneys (Zimskind et al., 1967). As ureteral stents were straight, they were very prone to migration and device expulsion, which impaired widespread adoption. As with the urinary catheter, altered designs were suggested to include a retaining mechanism. Gibbons proposed and patented a barbed stent to prevent distal migration (Gibbons et al., 1976). A few years later, Finney developed the “double J” (DJ) or double pigtail stent (Finney, 1978). The DJ stent was an instant success and its use was soon adopted in urology departments worldwide, having a tremendous positive impact on endourologic surgery and patient care. Today, ureteral stents are of fundamental importance to any urologic practice.

## 1.2 Urinary Device Associated Urinary Tract Infection

Bladder catheterization is an everyday practice on almost every hospital ward with 13-25% of hospitalized patients undergoing bladder catheterisation at some point during their stay (Fakih et al., 2012; Glynn et al., 1997; Weinstein et al., 1999). The placement and presence of a urinary catheter may be associated with short and long term complications. Catheter associated urinary tract infection (CAUTI) is one of the most important complications of indwelling urinary catheters. In current guidelines, CAUTI is

defined as significant bacteriuria (defined by convention as  $>10^5$  colony forming units (CFU)/mL) in a patient with symptoms or signs indicating a urinary tract infection with a catheter in situ or within 48 hours after removal. Asymptomatic bacteriuria refers to significant bacteriuria in asymptomatic patients (Hooton et al., 2010). Before 2009, the definition of CAUTI still included asymptomatic bacteriuria. In a large prospective trial, Tambyah et al identified less than 10% of catheterized patients with proven bacteriuria of  $> 10^3$  CFU/ml to be symptomatic (Tambyah and Maki, 2000). The Infectious Diseases Society of America (IDSA) first recommended not screening for bacteriuria in catheterized patients as early as 2005 (Nicolle et al., 2005). The concept of asymptomatic bacteriuria being a different entity that does not need treatment was thus in place for about half a decade before the definition of CAUTI was nuanced in 2009 to exclude asymptomatic bacteriuria. Whereas symptomatic CAUTI deserves to be treated accordingly, bacteriuria in a catheterized patient without any symptoms does not need to be treated with antibiotics. The European Association of Urology (EAU) and the Infectious Diseases Society of America (IDSA) specifically recommend against screening and treatment of asymptomatic bacteriuria as there is insufficient evidence of its benefit (Hooton et al., 2010; Tenke et al., 2008).

CAUTI account for approximately 35% of all hospital acquired infections (Klevens et al., 2007). The other hospital acquired infections are categorized in surgical site infections (20%), pneumonias (11%), bloodstream infections (11%) and “other” hospital acquired infections (22%) (Klevens et al., 2007). Almost 95% of urinary tract infections in the intensive care unit setting are associated with bladder catheters (Richards et al., 2000). Several risk factors influence the incidence of CAUTI. The most important risk factor is prolonged catheterization greater than six days (Maki and Tambyah, 2001). Other risk factors include diabetes, female sex, BMI greater than 30, catheterization outside of the operating room, and the presence of other active sites of infection (Maki and Tambyah, 2001; Stenzelius et al., 2011). The abundant use of urinary catheters and high incidence of CAUTI account for a sizeable economic impact. In the United States alone, the cost associated with CAUTI accumulates to about US\$ 300 million annually (Zimlichman et al., 2013). Several quality improvement and awareness projects have been instituted and are adopted widely, contributing to a significant decrease in the incidence and duration of

catheterization, and ultimately CAUTI rates (Janzen et al., 2013; Parry et al., 2013; Saint et al., 2013). Some of the most important universal recommendations in guidelines for the prevention of CAUTI are 1) avoiding unnecessary catheter use, 2) aseptic catheter placement technique, 3) maintaining a closed drainage system and 4) removal of the catheter as soon as possible (Meddings et al., 2014; Tambyah and Oon, 2012).

Despite best efforts, these preventive measures have not been able to completely eliminate urinary device associated urinary tract infections. Antibiotic prophylaxis and treatment of CAUTI play a definite role in eradication of CAUTI. The revised definition of CAUTI was instituted to prevent overtreatment of patients with antibiotics in instances where it is not needed. Recent research however has investigated the influence of this change in definition. Although it has decreased the number of CAUTIs observed, the clinical practice of antibiotic prescribing for asymptomatic bacteriuria has not changed (Cope et al., 2009; Press and Metlay, 2013).

The antibiotic treatment and prophylaxis of CAUTI poses a challenge and has been the subject of debate and controversy. Multiple different regimens have been proposed and there is a lack of consensus. Prophylactic antibiotics upon removal of a bladder catheter, even after short term catheterization, have been both recommended and discouraged (Hooton et al., 2010; Wolf et al., 2008). The American Urological Association (AUA) guidelines published in 2008 recommended the use of antibiotic prophylaxis after catheter removal if bacteriuria was present, particularly in patients with risk factors such as advanced age, immunodeficiency or corticosteroid use (Wolf et al., 2008). In 2010, the Infectious Disease Society of America (IDSA) discouraged the use of prophylactic antibiotics prior to catheter removal or catheter change (Hooton et al., 2010). Lusardi et al performed a Cochrane meta-analysis on whether or not antibiotic prophylaxis during short-term catheterisation (<15 days) has any benefit (Lusardi et al., 2013). They compared no prophylaxis to antibiotic prophylaxis, prophylaxis with antibiotic A vs. antibiotic B and prophylaxis at catheter removal vs. prophylaxis throughout catheterisation period. Primary outcomes were asymptomatic and symptomatic bacteriuria. Although the meta-analysis was performed after the definition had changed, all the trials included in the meta-analysis were from before 2009. The authors identified

an overall paucity of reliable evidence supporting any conclusions. The evidence available indicated that antibiotic prophylaxis reduces the rate of bacteriuria compared to starting antibiotics when bacteriuria is identified, both in surgical and non-surgical catheterized patients. Taking the 2009 definition of CAUTI into account, no statements can be made according to this meta-analysis on whether or not antibiotic prophylaxis reduces the CAUTI rate in surgical or non-surgical patients.

Marschall et al on the other hand identified in their meta-analysis that antibiotic prophylaxis at time of catheter removal does reduce the incidence of subsequent urinary tract infection (Marschall et al., 2013). They do point out however that adopting this practice would entail an enormous increase in antibiotics usage resulting in the potential for antibiotic resistance over time and a significant economic impact. Therefore, prophylaxis should be considered a recommendation only in patients with risk factors, as previously stated by the AUA guidelines panel.

A meta-analysis comparing suprapubic to transurethral catheter drainage after gynecological surgery concluded that suprapubic catheterization significantly reduces the rate of UTI, but on the other hand was significantly associated with a higher minor complication rate such as hematuria, leakage, blockage or accidental catheter loss. Superiority of one over the other could not be determined (Healy et al., 2013, 2012). When long-term catheterisation is to be expected, one may consider placing a suprapubic catheter taking all possible advantages and disadvantages into account.

Ureteral stents have been demonstrated to be at risk of bacterial colonization and therefore represent a possible source of urinary tract infection. Short-term ureteral stent placement (3 weeks) in a cohort of 209 children following ureteral reimplantation led to urinary tract infection in only 4.8% of patients (Uvin et al., 2011). Asymptomatic bacteriuria was reported in an additional 6.5% of patients. Despite the fact that all patients were receiving antibiotic prophylaxis during the indwelling period of the stent, almost half of the stents were colonized with bacteria. Patients with untreatable ureteral obstruction are often permanently managed with a ureteral stent. In these chronically stented patients, bacterial colonization reaches 100% (Riedl et al., 1999). Indwelling time, female sex, diabetes and chronic kidney disease are factors influencing colonization

of ureteral stents (Kehinde et al., 2002). A negative urine culture has low predictive value for stent bacterial colonization (Kehinde et al., 2004; Rahman et al., 2012). Similar to bladder catheters, routine screening for bacteriuria and treatment of asymptomatic bacteriuria is not recommended in patients with ureteral stents. Antibiotics are only recommended in instance of symptomatic urinary tract infection and appear not to have a role in long-term prophylaxis. A small RCT with 95 patients demonstrated that continuous low-dose antibiotic treatment during the indwelling time of ureteral stents does not influence the incidence or severity of stent-related symptoms or urinary tract infections (Moltzahn et al., 2013).

### 1.3 Antibiotic Resistance

The most commonly prescribed antibiotics for *E. coli* urinary tract infection are ciprofloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole (TMP/SMX) and amoxicillin/clavulanic acid.

Several authors have demonstrated that the resistance patterns for community-acquired and hospital- or catheter acquired urinary tract infections are quite different for the same bacterial species. Bacteria responsible for hospital acquired UTI are more frequently resistant to the most commonly used antibiotics (Lobel et al., 2008). In the comparison of 85 community acquired vs 106 hospital acquired *E. coli* urinary tract infections, Huang et al demonstrated a significantly higher resistance of hospital-acquired *E. coli* to cefazolin, cefuroxim, ceftriaxone, ceftazidim, ampicillin/sulbactam, gentamicin, levofloxacin and trimethoprim/sulfamethoxazole (TMP/SMX) (Huang et al., 2014). Although susceptibility of community-acquired *E. coli* to these antibiotics was significantly higher, the actual susceptibility ranged only from 49% for cefazolin to 87 % for ceftazidim (Huang et al., 2014). Taking *E. coli* as well as other causative organism such as *Klebsiella*, *Enterococcus* species and *Proteus* into account, the overall susceptibility to levofloxacin, amoxicillin-clavulanate and cephalosporines was markedly higher in community acquired UTI compared to hospital acquired UTI (Khawcharoenporn et al., 2012).

Not only is the resistance pattern of hospital-acquired urinary tract infections less favorable than that of community acquired UTI's, the susceptibility to the most commonly used antibiotics for UTI also seems to fade with time. Sanchez et al have identified a significant decrease in susceptibility of *E. coli* to ciprofloxacin (from 97% to 83%) and TMP/SMX (from 82% to 76%) when comparing susceptibility patterns of *E. coli* in 2000 and 2010 in the United States (Sanchez et al., 2012). Interestingly, susceptibility for nitrofurantoin (99.2% to 98.4%) and ceftriaxone (99.8% and 97.7%) were maintained throughout this period (Sanchez et al., 2012). Karlowky and associates demonstrated the same trend in decreasing susceptibility in Canadian hospitals using the CANWARD nationwide surveillance database of the Canadian Antimicrobial Resistance Alliance (CARA). The authors report a significant increase of *E. coli* resistance to ciprofloxacin from 20% in 2007 to 29.2% in 2011 (P=0.0005) (Karlowky et al., 2013).

As Goossens et al pointed out, increased antimicrobial resistance is probably due to a higher use of antibiotics (Goossens et al., 2005). This increase in antibiotic resistance may in part be attributed to inappropriate antibiotics use such as antibiotic treatment of asymptomatic bacteriuria, long-term low-dose prophylaxis in catheterized patients and a short-term antibiotics course where a long-term may be more appropriate.

If this trend continues, bacterial strains responsible for community or hospital acquired urinary tract infections will become so resistant that our current empirical antibiotic therapy will be insufficient and we will have to resort to broad-spectrum IV antibiotics for the treatment of simple, uncomplicated UTI's.

The insufficient effectiveness of preventive measures for CAUTI such as reduced catheterization and continuous monitoring and re-evaluation of catheter need and the increasing antimicrobial resistance of bacterial strains responsible for device associated urinary tract infections has encouraged the search for increasing biocompatibility of urinary devices. To improve biocompatibility of urinary devices, new biomaterials for device manufacturing as well as several coatings have been developed to prevent biofilm formation and urinary tract infection.

## 1.4 Biofilm

The most commonly used definition of the term biofilm is attributed to Costerton who defined it as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” (Costerton et al., 1999). Essential elements of biofilm are bacteria, exopolysaccharides and an attachable surface.

A biofilm is conceptually a survival mechanism for bacteria to remain in a favorable environment. Bacteria within a biofilm have limited exposure to harmful factors (the host’s immune response, antimicrobial agents and waste products) while enhancing the exposure to trophic factors and promoting reproduction.

As previously indicated, foreign bodies implanted in the urinary tract become colonized over time with 90-100% of ureteral stents reaching colonization in chronically stented patients (Reid et al., 1992; Riedl et al., 1999). Trautner and Darouiche indicated that biofilm on the urinary device is the most important factor in inducing catheter associated urinary tract infections (Trautner and Darouiche, 2004). Presence of a biofilm or attached bacteria on the urinary device however does not necessarily translate into a positive urine culture or clinical infection (Reid et al., 1992; Uvin et al., 2011).

A mature biofilm consists of three distinct layers: (1) the innermost layer or conditioning film is deposited onto the surface of the biomaterial and functions as a linking film for bacterial cells and subsequent layers, (2) adhesions of micro-organisms to the linking film form the base film and (3) once matured, the outer layer or surface film of the biofilm can release micro-organisms (Tenke et al., 2012). Ganderton et al measured the thickness of biofilm on urinary catheters and noted a range from 3 to 490  $\mu\text{m}$  composed of multiple cell layers ranging from just a few to up to 400 cells deep (Ganderton et al., 1992).

The innermost layer of the biofilm, the conditioning film, is deposited onto the biomaterial surface as an initial step in biofilm formation. This initial deposition can take place within minutes of insertion (Reid et al., 1995). This conditioning film is composed

of urinary components such as albumin, immunoglobulins, Tamm-Horsfall protein, cytokeratins, polysaccharides, electrolytes and glycoproteins that adhere to the biomaterial surface (Canales et al., 2009; Elwood et al., 2013; Santin et al., 1999). The surface characteristics of the biomaterial are altered due to these depositions, facilitating bacterial adhesions (Reid and Busscher, 1992). The initial bacterial adhesion which is still reversible, is influenced by hydrophobic and electrostatic interactions, ionic forces, osmolality and urinary pH and is still reversible (Gristina, 1987). After attaching to the conditioning film, bacteria produce a matrix of exopolysaccharides and glyocalix, enveloping the micro-colonies and rendering their adhesion irreversible (An and Friedman, 1998). Eradication of the biofilm with antibiotics at this point is extremely difficult and almost impossible due to several defense mechanisms of the biofilm as will become clear in the following paragraphs. Approximately 5-35% of the biofilm consists of bacterial micro-colonies. The remainder of the biofilm consists of matrix and interstitial spaces. These interstitial spaces contain water channels which are filled with fluid, allowing transportation of nutrients and oxygen to the colonies (Tenke et al., 2012).

Micro-organisms present or embedded in biofilm react differently to antibiotic substances compared to planktonic micro-organisms. Ceri et al demonstrated that the concentrations of antibiotics (ampicillin, ciprofloxacin, cefazolin, cefotaxime, TMP/SMX) needed to eradicate *E. coli* embedded in biofilm are 1000-fold higher or more than the concentration needed for eradication of cultured planktonic *E. coli* (Ceri et al., 1999). Similar concentrations were needed to eradicate *Pseudomonas aeruginosa* or *Staphylococcus aureus* from biofilm compared to planktonic *Pseudomonas* or *S. aureus* (Ceri et al., 1999). This relative resistance to antimicrobial treatment of bacteria embedded in biofilm is multifactorial:

**(1)** Bacteria in micro-colonies and biofilm have a means of communicating with each other so that they can respond to the environment in unison, as a multicellular organism. This communication, termed Quorum Sensing, occurs through the bacterial production of signaling molecules called auto-inducers and is population density-dependent (McLean et al., 1997; Salmond et al., 1995; D. J. Stickler et al., 1998). Once a population of bacteria reaches a certain threshold density or ‘quorum’, the cumulative level of signaling



molecules reaches a threshold concentration at which quorum sensing is allowed to regulate the biofilm's response and subsequently induces a population-wide gene expression, thus responding as one behavioral unit or multicellular organism (Fuqua et al., 1994). Quorum sensing is reported to play a role in biofilm formation, swarming, biofilm virulence, biofilm dispersion and antibiotic resistance (Bhardwaj et al., 2013; González Barrios et al., 2006; Li and Nair, 2012; Solano et al., 2014).

(2) The polymicrobial nature of biofilm with the potentially variable susceptibility patterns for each organism may account for the difficulty in treating biofilm induced infection. Frank and associates analysed micro-organisms present in biofilm from urinary catheters in a culture-independent manner (Frank et al., 2009). Using 16S rDNA sequencing and PCR amplification to identify the bacteria embedded in the biofilm, the authors identified *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, and *Propionibacterium acnes* in the specimens and concluded that biofilm is usually polymicrobial (Frank et al., 2009). Holá et al reported that most of urinary catheters collected from in-hospital patients have three to six different micro-organism present on the catheter surface (Holá et al., 2010). Only 12% had a monomicrobial infection. *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Candida albicans* were isolated most frequently (Holá et al., 2010). As the shared gene-pool of a polymicrobial biofilm is advantageous or even necessary for its survival, bacteria seem to actively recruit other bacterial strains and species to their mutual synergistic benefit (Wolcott et al., 2013).

(3) A third factor contributing to antibiotic resistance is the presence of “persister cells” in biofilm. Persister cells contribute up to 1% to a biofilm and appear to be a dormant variant of a regular bacterial cell (Keren et al., 2004). They neither grow nor die and are thus not antibiotic resistant but rather antibiotic tolerant (Lewis, 2010; Wood et al., 2013). When the concentration of the antibiotic decreases, the persister cells can reproduce and repopulate the biofilm (Lewis, 2010). Dormancy is believed to be induced by a variety of toxin/antitoxin systems where the toxin stops the metabolic activity of the cell and the antitoxin can reverse this process (Keren et al., 2004; Lewis, 2012). Recent

research indicates that persister cells can be induced by subinhibitory dosage of antibiotic therapy, suggesting that long-term low-dose antibiotic prophylaxis can actually play a role in multi-drug resistant recurring urinary tract infections (Goneau et al., 2014).

(4) Although antibiotic agents may be able to penetrate the biofilm to reach the embedded bacteria, their antibacterial mechanism of action may be compromised or inactivated due to the micro-environment created within the biofilm. The metabolic activity of the high density of micro-organisms in the biofilm produces a large amount of waste products, altering among others the pH and pO<sub>2</sub> level as well as the pyrimidine concentration within biofilm (del Pozo and Patel, 2007; Stewart and William Costerton, 2001).

The fast deposition of conditioning film and the complex architecture and multiple survival mechanisms of the biofilm entity render biofilm and biofilm associated infections extremely difficult to prevent or treat.

## 1.5 Encrustation

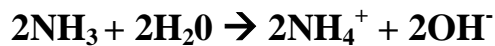
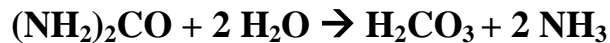
Minor encrustations on a stent or catheter surface are encountered frequently and usually do not cause stent or catheter blockage. With increasing amount of encrustation on the surface or in the lumen of the stent or catheter, this problem becomes more clinically relevant as it can result in stent or catheter blockage or resistance at attempt of removal of the urinary device.

### 1.5.1 Dependent on Urinary Composition

The deposition of encrustations on urinary devices is pH dependent. Hedelin et al demonstrated that a urinary pH of approximately 6.8 is critical to the composition of encrustations (Hedelin et al., 1991). Below this pH, the precipitations mainly consisted of calcium phosphate (~ HydroxyApatite), whereas above this threshold pH, they are composed of magnesium ammonium phosphate (~Struvite) (Hedelin et al., 1991). Choong et al corroborated these results identifying a urinary pH of approximately 7 as a threshold pH above which precipitation occurs. The authors termed this the nucleation

pH ( $\text{pH}_n$ ) (Choong et al., 1999). In a subsequent paper, Choong demonstrated that patients that have encrusted urinary catheters have a pH in the voided urine that is higher than the  $\text{pH}_n$ , thus explaining why they form encrustations more easily (Choong et al., 2001). This nucleation pH is subject to change by modulating urinary constituent concentrations. An increase of  $[\text{Ca}^{2+}]$ ,  $[\text{Mg}^{2+}]$  and [phosphate] decreases the nucleation pH at which these constituents precipitate, whereas the addition of citrate increases the  $\text{pH}_n^{\text{Ca}}$  (Suller et al., 2005).

The presence of urease-producing bacteria may be a prerequisite to the formation of encrustations. The urea-splitting enzyme catalyzes the hydrolysis of urea into carbonic acid which deprotonates and ammonia, which becomes protonated at physiological pH, thus increasing urinary pH (Moblely and Hausinger, 1989).

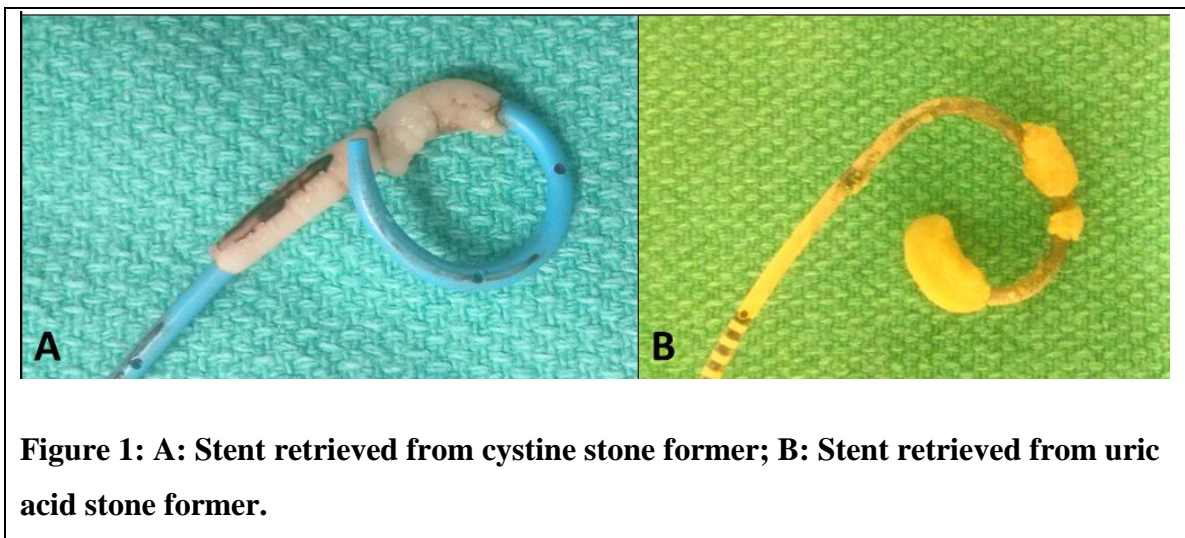


Stickler et al identified *Proteus mirabilis*, *Proteus vulgaris* and *Providencia rettgerii* to be the most potent urease-producing bacteria, able to increase urinary pH well above 8.0 (D. Stickler et al., 1998). Broomfield et al equally identified these bacteria to have higher encrustation rates due to more potent urease activity when compared to for example *E. coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Broomfield et al., 2009). Once precipitations are formed, they can deposit onto the catheter surface and will get embedded in the polysaccharide matrix (Morris et al., 1999).

In contrast to encrustations formed in biofilm containing urease-producing bacteria, the encrustations that form on stents inserted in stone formers are usually composed of calcium oxalate, reflecting the most common stone composition in urolithiasis patients (Bithelis et al., 2004). Calcium oxalate as the major component of stent encrustation was previously reported to occur in the absence of UTI, at pH values  $<5.5$  and in presence of hyperuricosuria, (Grases et al., 2001; Robert et al., 1997).

Canales et al identified components of the conditioning film, alpha-1-anti-trypsin, Ig Kappa, IgH G1 and histone H2B and H3A, to be highly associated with stent encrustation (Canales et al., 2009). Tamm-Horsfall protein and  $\alpha$ 1-microglobulin may have an encrustation protective effect by stabilizing the organic conditioning film and by inhibiting calcium oxalate aggregation (Santin et al., 1999). Santin et al demonstrated that these proteins were only present in the conditioning film of non-encrusted stents and not on encrusted stents (Santin et al., 1999).

Additional risk factors for stent encrustation that depend on the urinary composition include urinary tract infection, history of stone disease, urinary diversions, pregnancy, metabolic or congenital abnormalities and chronic renal failure (Ahallal et al., 2010; Robert et al., 1997; Vanderbrink et al., 2008). **Figure 1 A and B** demonstrate grossly visible encrustations on stents retrieved from stone formers. Biochemical analysis revealed the same composition as the stones the patients formed.



### 1.5.2 Dependent on Indwelling Time

Next to the previously mentioned influencing factors, indwelling time of the urinary device is the most important influence on the development of encrustation. Encrustation has been reported to occur in 9.2-26.8% of stents indwelling <6 weeks, in 47.5-56.9% of stents indwelling 6 to 12 weeks and in approximately 75% of stents indwelling longer

than 12 weeks (el-Faqih et al., 1991; Kawahara et al., 2012). Kawahara additionally reported complete stent obstruction in 8.6% of stents after >12 weeks and 1 % of stents requiring additional treatments to facilitate stent removal (Kawahara et al., 2012). The authors observed stents smaller than 6F to be significantly more prone to encrustations than stents of 7Fr or larger (Kawahara et al., 2012). To prevent severe encrustations, timely stent removal or exchange is an important preventive measure. Most stent manufacturers recommend stent removal or exchange within 4 months of placement. In patients with additional risk factors for encrustation a 6-8 week interval is recommended (Aravantinos et al., 2006). Stent exchange is suggested every 4-6 weeks for pregnant patients as this population is particularly prone to stent encrustation (Denstedt and Razvi, 1992).

All the previously reported influencing factors however cannot always explain or predict the extent of encrustations found on ureteral stents or urinary catheters. Whereas some stents will be encrusted after only a few weeks of indwelling time, in some cases a forgotten stent can show virtually no encrustations even after very long periods of time, which is demonstrated in **Figure 2**.



**Figure 2: Forgotten stent retrieved from a patient with urinary tract stones after two years of indwelling time, showing no appreciable encrustations.**

## 1.6 Urinary Catheter Biomaterials and Coatings

The ideal biomaterial to use for urinary devices should be chemically stable in urine, prevent infection and encrustation, cause no significant discomfort to the patient, be biocompatible, have excellent long-term urinary flow and be affordable (Beiko et al., 2004). The most commonly used catheters are manufactured from latex, rubber, silicone or polyvinylchloride (PVC). Due to widespread availability and lower cost, latex or rubber catheters are preferred for short-term use. Silicone is relatively inert and randomized controlled trials (RCT's) have demonstrated silicone to induce significantly less tissue inflammation and encrustation than latex catheters (Bruce et al., 1974; Nacey et al., 1985; Schumm and Lam, 2008; Talja et al., 1990). Silicone is therefore preferred over latex catheters for long term catheterization.

Several coatings have been studied in an attempt to reduce trauma, urethritis and CAUTI. Coatings of interest to our research are coatings preventing encrustation and device associated urinary tract infection. Coatings with the goal of reducing trauma or catheter associated discomfort other than infection and encrustation will therefore not be discussed here. An important issue in interpreting the results of research on catheter coatings preventing CAUTI, is that many of the papers report on bacteriuria as a primary outcome and not on symptomatic CAUTI. As it is now appreciated that bacteriuria is of low significance in these patients and should not even be screened for in catheterized patients, it may be difficult to compare different approaches in the prevention of CAUTI. It is therefore also not always possible to make statements as to whether or not a certain coating has the ability or the potential to decrease CAUTI.

**Antibiotic-impregnated** silicone catheters have been evaluated in randomized trials in the past. Darouiche et al evaluated the use of **Minocyclin and Rifampin** impregnated catheters in radical prostatectomy patients. In their cohort of 124 patients, they demonstrated a statistically significant benefit of antimicrobial catheters in preventing bacteriuria, both at day 7 (15.2% versus 39.7%) and at day 14 (58.5% versus 83.5%) (Darouiche et al., 1999). It was however later demonstrated that Minocyclin/Rifampicin impregnated catheters have only a low activity against Gram negative bacteria and no

significant effect on enterococci, yeasts, or enterobacteriaceae (Schierholz et al., 2002). These catheters have therefore not been widely adopted and are no longer available. Both **norfloxacin and gentamicin** impregnated catheters have been investigated *in vitro*. Where Gentamicin-coated catheters had demonstrated efficacy against *Proteus vulgaris*, *Staphylococcus aureus* and *Staphylococcus epidermidis* over a short time period (Cho et al., 2003), the Norfloxacin catheters conveyed considerable inhibitory effects against *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus vulgaris* over a 30 day period (Park et al., 2003). *In vivo* trials however have not been pursued. **Nitrofurazone** catheters have been studied more extensively, have been marketed and are more widely accepted. *In vitro* studies have demonstrated the efficacy of nitrofurazone impregnated catheters against common as well as multi-resistant uropathogens (Johnson et al., 1999, 1993). Nitrofurazone-coated catheters have been extensively studied in comparison to uncoated or silver-alloy-coated catheters and have almost unanimously been demonstrated to have a significant influence on reducing catheter associated bacteriuria (Desai et al., 2010; Pickard et al., 2012; Regev-Shoshani et al., 2011; Stensballe et al., 2007). Hachem et al recently researched the activity of an antiseptic coating based on chlorhexidine and gentian violet: Gendine (Hachem et al., 2009). The gendine-coated catheters appeared to prevent both biofilm formation and CAUTI. No human trials have been conducted to date.

The first large randomized controlled trial comparing **silver-oxide** coated silicone-catheters to a silicone-coated latex catheter was published in 1995 and demonstrated that the silver-coated catheters had no benefit with regards to bacteriuria (Riley et al., 1995). Karchmer et al conducted a single-center, hospital-wide crossover study comparing **silver-alloy** coated latex catheters to silicone-coated latex catheters, including 27,878 patients in the trial and using 11032 study catheters (Karchmer et al., 2000). The authors reported a reduced rate of bacteriuria per 1000 patient days, per 100 patients and per 100 catheters placed in the silver-coated catheter cohort. They additionally estimated that the systematic use of silver-coated catheters would be cost-effective. Another large-scale, prospective, crossover study comparing silver-alloy coated silicon-based urinary catheters to non-coated silicon urinary catheters including 3036 patients, could not identify a significant benefit of the silver coated catheter over a non-coated silicone

catheter for the prevention of bacteriuria (Srinivasan et al., 2006). In a more recent multicenter RCT including over 6000 patients, nitrofurantoin-impregnated silicone catheters demonstrated a statistically significant benefit in reducing CAUTI compared to PTFE-coated latex catheters (control), whereas silver-alloy coated latex catheters demonstrated no benefit in reducing CAUTI (Pickard et al., 2012). Although a statistical difference was noted for the nitrofurantoin-impregnated catheters, this difference was too small to be regarded as clinically significant. As no clinically significant benefit was noted for short term catheterization by either catheter, Pickard et al conclude that the routine use of these catheters cannot be recommended (Pickard et al., 2012).

In a systematic review of antimicrobial urinary catheters in the prevention of CAUTI, Johnson et al indicate that both the use of nitrofurantoin- as silver-alloy coated catheters reduces catheter associated bacteriuria in short-term use (Johnson et al., 2006). A 2008 Cochrane meta-analysis of types of catheters used in short-term ( $\leq 14$  days) catheterisation in adults, demonstrated that the use of nitrofurazone- and **silver-alloy** coated catheters but **not silver-oxide** coated catheters significantly reduced the incidence of asymptomatic bacteriuria in short-term ( $>1$  week) use of such catheters (Schumm and Lam, 2008). Silver-alloy catheters demonstrated a benefit for longer than one week, whereas nitrofurazone catheters could not (Schumm and Lam, 2008). Silver-oxide catheters are no longer available. A 2012 Cochrane meta-analysis showed no difference in urinary infection rates in long-term ( $> 30$  days) catheterized patients when comparing different catheter types (Jahn et al., 2012). Although these meta-analyses may indicate that silver-alloy coated and nitrofurazone-impregnated catheters may reduce bacteriuria rates in short-term catheterized patients, no statements can be made regarding the influence on catheter associated urinary tract infections (Hooton et al., 2010). Taking the more recent large multicenter RCT's into account, there is currently insufficient evidence to reliably recommend the systematic use of one catheter type over another for short-term catheterization.

The discrepancy in reported effectiveness of silver-coated urinary catheters may rely on the base compound on which the silver is coated or the compound of the catheter to which the silver-coated catheter is compared to. Crnich et al interestingly reanalyzed the



data from a systematic review on antimicrobial coated catheters (Johnson et al., 2006) and identified a difference in UTI-preventing efficacy of silver-coated catheters when compared to latex- or silicone catheters (Crnich and Drinka, 2007). The benefit of silver-coated catheters over silicone catheters is much smaller than over latex catheters. As previously indicated, an additional factor influencing the differences in effectiveness of silver-coated catheters is the type and manner of silver-coating onto the device.

As the spinal cord injured (SCI) population have a higher need for chronic catheterization and are at higher risk of urinary tract infections due to neurogenic bladder disorders, the results from previously performed RCT's in non-spinal cord populations may not be suited for extrapolation to the SCI population. Bonfill et al published a methodology paper announcing an ongoing RCT in which they intend to compare the effectiveness of silver-alloy-coated, nitrofurantoin-impregnated and PTFE-coated catheters on the reduction of UTI in SCI patients (ESCALE: Efficacy Study of Antimicrobial Catheters to Avoid Urinary Infections in Spinal Cord Injured Patients) (Bonfill et al., 2013). The trial is registered on clinicaltrials.gov and is reported as currently recruiting participants (NCT01803919).

Studies on the feasibility and efficacy of clinical use of **bacterial** coated catheters are ongoing (Darouiche and Hull, 2012; Prasad et al., 2009; Trautner et al., 2007). They have the theoretical benefit of colonizing the urine with a non-virulent strain of *E. coli* and have shown promising results in small pilot trials.

**Hydrogel** coatings have been applied on catheters to render the surface more hydrophilic. The absorption of water by the coating increases the surface lubricity, facilitating catheter insertion and reducing possible trauma (Lawrence and Turner, 2005). Hydrogel-coatings on **indwelling** urinary catheter do not unanimously decrease bacteriuria. Kumon et al demonstrated that hydrogel-coated catheters had a lower bacterial attachment rate than uncoated latex catheters in an *in vitro* study (Kumon et al., 2001). Erickson et al could not identify a significant difference in UTI rate or stricture rate between hydrogel-coated latex catheters versus silicone catheters in a urethroplasty cohort (Erickson et al., 2008). In an *in vitro* study comparing uncoated silicone and latex catheters to hydrogel-coated

and hydrogel/silver coated catheters, the authors demonstrated that uropathogens migrated most easily over hydrogel-coated catheters, insinuating that they may even facilitate catheter associated urinary tract infections (Sabbuba et al., 2002). Additionally, Morris demonstrated that hydrogel-coated latex catheters were blocked with encrustations more rapidly than silicone-coated latex catheters or uncoated silicone catheters (Morris and Stickler, 1998). Yang et al developed a Chitosan/poly(vinyl alcohol) hydrogel coating on polyurethane catheters that demonstrated high lubricity and antiseptic effects on *E. coli* in an *in vitro* setting, identifying its potential for future developments (Yang et al., 2007).

The use of **hydrophilic** coated catheters is of interest in patients performing clean intermittent catheterization (CIC). Such catheters have been associated with less discomfort, fewer traumatic catheterizations and a decreased incidence of symptomatic UTI and urethral strictures in this population (Cardenas et al., 2011; De Ridder et al., 2005; Wyndaele, 2002). A recent meta-analysis by Bermingham was unable to identify a significant difference in the incidence of symptomatic UTI's when comparing different catheter types used for CIC. Li et al on the other hand, in a meta-analysis focusing on the spinal cord injury population, demonstrated that the use of hydrophilic coated catheters for use in CIC significantly reduced UTI and hematuria rates (Bermingham et al., 2013; Li et al., 2013). As the use of clean non-coated catheters for CIC is most cost-effective, and the use of gel-reservoir second most cost-effective in the Bermingham analysis (Bermingham et al., 2013), the routine use of hydrophilic catheters for CIC in non-spinal cord injury patients was not recommended.

New coatings to enhance biocompatibility are still being developed and investigated with the desired outcome of preventing encrustation and urinary tract infections (Siddiq and Darouiche, 2012).

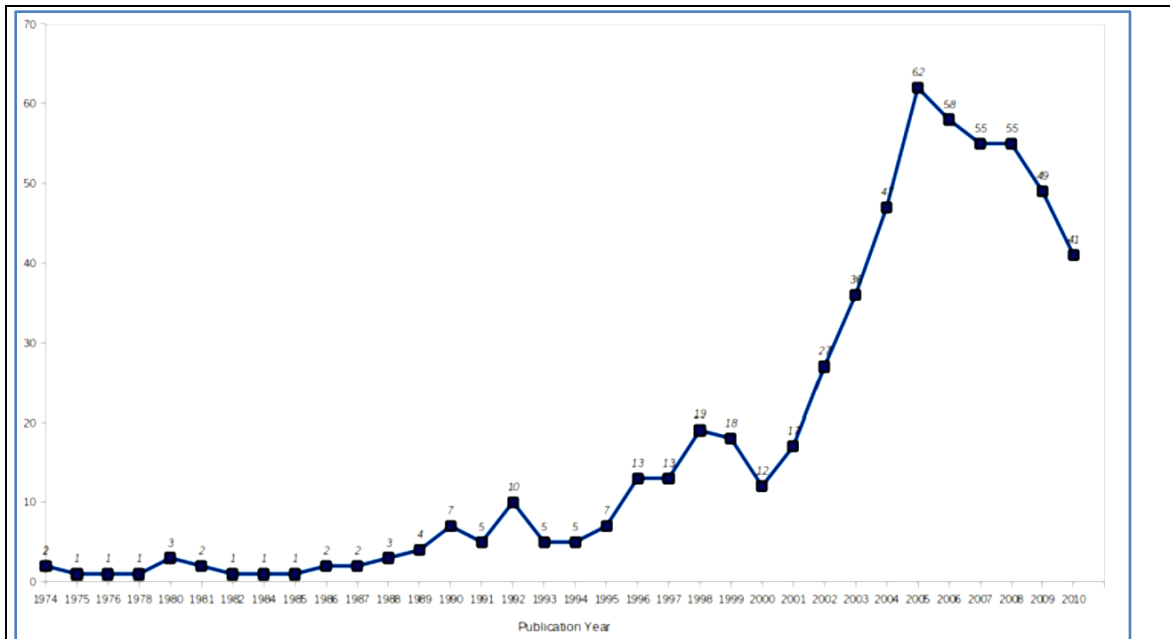
## 1.7 Indications for Stent or Catheter Placement

The indications for inserting an indwelling bladder catheter are numerous and can conveniently be divided into two categories: therapeutic or diagnostic catheterization. This includes catheterization for drainage of urinary retention, drainage after reconstructive surgery, monitoring of urinary output, bladder irrigation of gross hematuria and urethral dilation with urethral catheters. The wide indication for urinary catheterisation accounts for its widespread adoption and abundant use to the extent that the urinary catheter is one of the most commonly placed foreign bodies in humans.

Ureteral stents are most commonly placed to relieve ureteral obstruction. Intrinsic obstruction is typically caused by stones, tumors or strictures, whereas extrinsic obstruction is often due to compression by tumor, overlying vessels, retroperitoneal fibrosis or lymphadenopathy. The need for obstruction relief can be temporary or permanent. Ureteral stents are also frequently placed prior to shockwave lithotripsy treatment or after ureteroscopic treatment of urinary lithiasis after which one may expect some edema or residual fragments of the stone to obstruct the ureter. The most recent guidelines and literature dictate narrower indications for stent placement in these instances (Al-Awadi et al., 1999; Ather et al., 2009; Shen et al., 2011a, 2011b; Song et al., 2012; Tang et al., 2011; Türk et al., 2013). Stents have proven utility in ureteral trauma treatment and reconstructive urological procedures. A particularly important and well-studied post-operative use of ureteral stents is after renal transplantation. A recent meta-analysis in the renal transplant population demonstrated that routine prophylactic stenting significantly reduces the incidence of major urological complications (Wilson et al., 2013).

## 1.8 Stent Technology

The ideal stent is easy to insert, is chemically stable after implantation in a urinary environment, has the ability to relieve intraluminal and extraluminal obstruction, has excellent flow characteristics, does not induce patient symptoms and is resistant to encrustation and infection. The biocompatibility of the biomaterial or coating at the stent surface is of the utmost importance for the prevention of UTI and encrustation. The prevalence of stent related symptoms, encrustation and infection are the main drive for the research and development of new stent biomaterials and coatings. As the worldwide business of DJ stents covers several billion dollars in revenue, industry driven research for new compounds and coatings is very prevalent. The number of new patents filed annually related to ureteral stents is shown in **Figure 3** and demonstrates that development of new designs, biomaterials and coatings for ureteral stents has increased dramatically over the past decade. This once more reflects the vast amount of research dedicated to improving biocompatibility of ureteral stents with the ultimate goal of reducing stent-related symptoms and infections.



**Figure 3:** Graph showing number of patents filed annually related to ureteral stents. Courtesy of Dolcera Patent and Market Research Services.

### 1.8.1 Biomaterials

Initially ureteral stents were manufactured from silicone (Zimskind et al., 1967), which is still the most biocompatible material tested to date (Cormio et al., 1995; Pariente et al., 1998). However, due to the high friction coefficient, flexibility and low tensile strength that are characteristics of silicone, silicone stents are more difficult to insert and may be especially challenging to navigate through a tortuous or obstructed ureter (Mardis et al., 1993). Polyethylene was introduced as the first plastic polymer used in the commonly utilized DJ stents (Mardis et al., 1979). Polyethylene stents however, have a tendency to become brittle after prolonged exposure to the urinary environment and are prone to encrustation, blockage and fragmentation. This led to its discontinuation in stent manufacture and the development of newer polymers. Currently utilized stents are commonly composed of polyurethane, silicone or proprietary copolymers such as Silitek®, C-flex®, Percuflex® or Tecoflex®. The large choice of different stents

available allows the urologist to tailor the choice of stent to the needs and characteristics of the patient in need of a stent. Multiple authors have compared different stent biomaterials and copolymers with the goal of identifying the ideal stent biomaterial. Mitty et al were the first to publish preliminary results on the Percuflex® stent (Mitty et al., 1988). Marx et al observed that C-Flex® and silicone stents induced less inflammation than Silitek® or polyurethane stents in a canine model (Marx et al., 1988). Mardis et al identified C-Flex® and Percuflex® to be the most suitable copolymers for stent manufacturing as they have the best combination of biocompatibility, biodurability, surface friction coefficient, tensile strength, flow capacity and coil retention strength (Mardis et al., 1993). Tunney et al identified Silitek® to be the copolymer most resistant to hydroxyapatite encrustations (Tunney et al., 1996).

Metallic mesh or coil stents such as the **Resonance® stent, the Memokath 051® stent, the Allium® stent or the Uventa® stent**, have been utilized in the treatment of obstructed ureters in which a conventional ureteral stent is unable to relieve the obstruction. This is most commonly due to external compression of the ureteral lumen, often by a malignant process, causing high radial forces that conventional stents are unable to withstand. Conversely, the most important trait of these metal mesh or coil stents is that they can withstand these high radial compressive forces (Blaschko et al., 2007; Pedro et al., 2007).

A common problem among metal mesh stents is reduced patency in long-term follow-up and late complications such as migration, encrustation, erosion and tissue hyperplasia growing into the stent (Agrawal et al., 2009; Chung et al., 2013; Goldsmith et al., 2012; Kadlec et al., 2013; Liatsikos et al., 2010; Moskovitz et al., 2012; Papatsoris and Buchholz, 2010). Coatings to prevent or limit hyperplasia and stent ingrowth in metal mesh stents have been adopted from the endovascular stent realm. Liatsikos et al demonstrated a paclitaxel drug-eluting metal mesh stent to reduce inflammation and hyperplasia of the surrounding tissue compared to a bare metal stent (BMS) in a porcine model (Liatsikos et al., 2007). The use of a zotarolimus-eluting metal stent compared to a BMS has been shown to induce a significantly lower hyperplastic reaction without influencing inflammation rates in a porcine and rabbit model (Kallidonis et al., 2011).

These cytostatic drug coatings have the potential to improve patency and reduce complication rates of metal mesh ureteral stents.

Since the late 1990's, experimental biodegradable ureteral stents have been evaluated with the goal to eliminate the necessity of cystoscopic stent removal and the possibility of forgotten stents (Lumiaho et al., 1999; Schlick and Planz, 1997; Talja et al., 1997). The biodegradable materials assessed to date are composed of high molecular weight polymers such as polylactide (polylactic acid: PLA), polyglycolide (polyglycolic acid: PGA) or variants to these (Lumiaho et al., 1999; Talja et al., 1997). The surface of bioresorbable stents can be chemically modified with for instance hydroxyethylmethacrylate (HEMA), oligo(ethyleneoxide)-monomethacrylate (OEOMA), or acrylic acid (AAC) and has been demonstrated to improve biocompatibility *in vitro* without increasing toxicity of these polymers (Brauers et al., 1998, 1997). The main challenge of biodegradable materials is controlling the rate of degradation. This can be influenced by environmental (urinary pH) and stent-related factors such as basic molecular structure, the degree of the polymerization and the internal arrangement of the material components (Lumiaho et al., 1999). *In vivo* tests with a poly-L,D-lactide polymer in a canine model demonstrated promising results with complete degradation of all stents within 24 weeks without inducing ureteral histologic changes (Lumiaho et al., 2000, 1999). Schlick and Planz demonstrated a pH-dependent controlled degradation of biodegradable copolymers that were stable at a pH of 5.2 and completely dissolved within 20 hours at a pH of 7.9 (Schlick and Planz, 1998). Olweny et al evaluated the use of poly-L-lactide-co-glycolic (PLGA) as a degradable stent in a porcine ureter after endopyelotomy (Olweny et al., 2002). Although the stents degraded, they induced more tissue inflammation than a conventional stent (Olweny et al., 2002).

Uripren<sup>TM</sup> is a biodegradable copolymer composed of L-glycolic acid, polyethylene glycol and barium sulfate showing promising preliminary results in a porcine *in vivo* model. In its current chemical formulation, Uripren<sup>TM</sup> stents reliably achieve degradation after four weeks (Chew et al., 2013). When compared to conventional DJ stents, The Uripren<sup>TM</sup> stents induced a lower degree of ureteral inflammation in a porcine model (Chew et al., 2013, 2010b; Hadaschik et al., 2008).

Most recently MagnesiumYttrium alloy has been investigated *in vitro* for potential bacterial growth inhibition and biodegradability (Lock et al., 2012). The mode and rate of degradation can be influenced through surface modification and alloy design (Lock et al., 2014, 2012).

The only *in vivo* human trial published to date studying the use of biodegradable stents demonstrated adequate drainage while maintaining a high patient tolerance (Lingeman et al., 2003). After 90 days, 96.6% of patients were stent-free. Three patients however required SWL and one patient subsequently required ureteroscopy to clear retained stent fragments. Currently, Uriprene<sup>TM</sup> is being evaluated for feasibility after uncomplicated ureteroscopy in an ongoing trial (NCT02032316).

## 1.8.2 Coatings

Coatings on stent biomaterial are often employed to improve biocompatibility of the device. They may be anti-fouling (preventing deposition of conditioning film constituents), anti-microbial, lubricating or drug-eluting.

**Hydrogel** coatings have been applied to urinary catheters as well as ureteral stents. They are composed of hydrophilic polymers that absorb water, thus reducing friction and rendering the stent easier to insert and theoretically more biocompatible. *In vitro* tests however have not consistently shown a decrease in encrustation rate on hydrogel coated stents. Similar to urethral catheters, hydrogel coated stents have been demonstrated to both reduce and increase encrustation and biofilm formation (Desgrandchamps et al., 1997; Gorman et al., 1998; Tunney et al., 1996). Choong et al even demonstrated in an *in vitro* setting that hydrogel-coated stents (Microvasive Percuflex with Hydroplus) are significantly more prone to encrustation than uncoated silicone stents (Choong et al., 2000).

Urinary device surfaces coated with **Phosphorylchlorine (PC)** were hypothesized to be more biocompatible because PC resembles the major polar head group on the outer



surface of erythrocytes. After performing *in vitro* and *in vivo* preliminary clinical human tests, Stickler et al concluded that PC-coated stents did not stop biofilm formation and encrustation on these devices (Stickler et al., 2002).

**Polyvinylpyrrolidone-iodine (PVP-I)** complex modified polyurethane Tecoflex® stents appear to be highly hydrophilic and reduce encrustation deposits and adherence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* by 80-86% in *in vitro* tests (Khandwekar and Doble, 2011). Tunney and Gorman had previously tested PVP-coated catheters, which have been marketed and are available as the Bard Inlay® stent, *in vitro*, comparing the stent to uncoated polyurethane and uncoated silicone stents (Tunney and Gorman, 2002). The authors demonstrated the PVP-coated stent to be more lubricious than the other two stents in addition to improved resistance to bacterial attachment and encrustation (Tunney and Gorman, 2002).

Diamond-like carbon coatings have shown great benefit in improving biocompatibility of vascular, orthopaedic and other medical implants (Roy and Lee, 2007). Applying a **diamond-like carbon (DLC) coating** onto a polyurethane surface appears to decrease surface friction dramatically, thus improving biocompatibility (Jones et al., 2006). In the only trial published to date, Laube et al demonstrated that DLC-coated polyurethane significantly reduced biofilm formation and microbial adherence in 10 patients, compared to previously used stents in the same patient population. (Laube et al., 2007).

The observation of the absence of *Oxalobacter formigenes* and a lack of calcium oxalate (CaOx) stone formation induced the development and research of silicone stents coated with ***Oxalobacter formigenes* derived oxalate degrading enzymes** (Watterson et al., 2003a). The authors demonstrated a lesser degree of encrustation as compared to non-coated controls in a rabbit model, however the findings were not statistically significant (Watterson et al., 2003a).

Triclosan is an antibacterial and antifungal agent commonly used in deodorants, soap, detergent and many consumer products. *In vitro* evaluation of the efficacy of **Triclosan®-eluting stents (Triumph®)** in artificial urine compared to non-coated Percuflex® stents demonstrated reduced bacterial attachment to the stent and growth

inhibition of *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Proteus mirabilis* (Chew et al., 2006). A subsequent *in vivo* rabbit study showed the Triumph® stent to significantly reduce growth and survival of *Proteus mirabilis* (Cadieux et al., 2006). A preliminary human study in 8 patients with long-term stents demonstrated the Triclosan-eluting stents to reduce but not eliminate device associated urinary tract infection (Cadieux et al., 2009). The stent was further evaluated in a small randomized trial comparing it to the Percuflex® stent for short indwelling periods. Although the Triumph® stent did not significantly reduce infection, biofilm or encrustation, it did reduce other stent-related symptoms such as flank pain and urethral pain during urination (Cadieux et al., 2009; Mendez-Probst et al., 2012).

**Glycosaminoglycans (GAGs)** have been shown to inhibit calcium oxalate crystal growth *in vitro*. **Heparin** appears to be the strongest inhibitor among the GAGs available (Senthil et al., 1996; Yoshimura et al., 1997). Hildebrandt et al performed *in vitro* and *in vivo* (rat model) tests with heparin coated tantalum and stainless steel stents and demonstrated substantially fewer encrustations on the coated devices (Hildebrandt et al., 2001). The first in-human randomized trial with heparin coated stents demonstrated the coated stents to be free from encrustations after 6 weeks of indwelling time, in contrast to uncoated stents that had biofilm and encrustations present on all of the stents (Riedl et al., 2002). Although these numbers are based on a small series, Tenke and Cauda noted that heparin-coated stents may remain indwelling for longer than 6 months and potentially up to 12 months without being affected by encrustations, translating into a possible economic benefit (Cauda et al., 2008; Tenke et al., 2004). In contrast to these positive *in vivo* results, Lange et al demonstrated no significant difference in biofilm formation on commercially available heparin-coated stents compared to a Triumph® stent and uncoated stents when tested *in vitro* in artificial urine incubated with 5 different species of uropathogens (Lange et al., 2009).

**Pentosan polysulphate (PPS)** is a semi-synthetic GAG related to heparin. It has been shown to inhibit CaOx crystallization *in vitro* (Martin et al., 1984). Zupkas et al identified uncoated silicone disks to have significantly more encrustations than PPS coated silicone disks after remaining in a rabbit bladder for 50 days (Zupkas et al., 2000). Although

Jones and Monga appreciated the potential of PPS in a review on the use of the substance in prevention of CaOx stone disease and identified the need for a randomized, double-blind, placebo-controlled trial, the use of PPS in the prevention of CaOx stones has not been revisited since (Jones and Monga, 2003).

Inspired by the antifouling characteristics of placoids in shark skin, Carman et al turned to biomimicry to develop micropatterned surfaces. The most efficient studied thus far which is called **Sharklet AF<sup>TM</sup>**, has demonstrated antifouling characteristics by inhibiting settlement of spores of marine algae (Carman et al., 2006; Schumacher et al., 2007). Reddy et al have applied this technology to surfaces used in urinary environment and demonstrated that micropatterned surfaces inhibit colonization and migration of *E. coli* on silicone in artificial urine (Reddy et al., 2011). This very promising novel technology will undoubtedly be further investigated in the prevention of device associated urinary tract infections.

**Sustained release chlorhexidine varnish** is an antimicrobial method first described and frequently used in the world of dentistry and orthodontics (Balanyk and Sandham, 1985; Beyth et al., 2003). Applying a 1% chlorhexidine sustained release varnish on stent surfaces appeared to significantly reduce bacterial growth and biofilm formation *in vitro* (Shapur et al., 2012). Increasing the dose to a 2% concentration prolonged the inhibitory effect on bacterial growth from 1 to 2 weeks (Segev et al., 2013; Shapur et al., 2012; Zelichenko et al., 2013). A preliminary *in vivo* trial on dogs with the 1% formulation demonstrated less bacterial attachment and reduced biofilm formation when compared to uncoated catheters.

The development of **antibiotic stent coatings** is still in a preliminary phase. *In vivo* experiments on rat models have shown promising results for Rifampin coated stents in combination with systemically administered Tigecycline and for Clarithromycin coated stents in combination with systemic Amikacin (Cirioni et al., 2011; Minardi et al., 2012). No human trials have been performed yet.

In contrast to urethral catheters, **silver** as an antimicrobial agent has not been as widely evaluated on ureteral stent surfaces. Multanen et al have coated biodegradable stents with

silver nitrate to improve biocompatibility (Multanen et al., 2002). They demonstrated that coating a poly-L-lactic acid biodegradable stent with silver nitrate and ofloxacin accelerated stent degradation and decreased encrustation on the surface. There are no other publications mentioning stent surfaces coated with silver.

Our research group has developed a novel copolymer coating for urinary biomaterials based on a long chain polymer backbone conjugated with DOPA, (**Surphys-095**). Preceding copolymer compound coatings leading to the current coating have previously demonstrated *in vitro* resistance to bacterial attachment and biofilm formation (Ko et al., 2008). A cross-linked DOPA-anchored antifouling polymer was identified as the most resistant to *E. coli* adherence (Pechey et al., 2009).

## 1.9 mPEG-DOPA

The strategy of almost all of the ureteral stent coatings mentioned above is binding an anti-fouling polymer onto the device surface to prevent deposition of fouling constituents and bacterial cells on the surface. The efficacy of coated polymers relies on the mechanism of immobilization of the polymer to the surface. Where this can be obtained by a **physisorptive mechanism**, which relies on physical bonds between molecules and surfaces, such as Van der Waals' forces, a **chemisorptive mechanism**, which relies on a chemical bond between surface and polymer, may procure more stability.

### 1.9.1 Polyethylene Glycol (PEG)

Polyethylene glycol (PEG) which is often referred to as polyethylene oxide (PEO) has been extensively studied for its anti-adhesive characteristics, preventing protein deposition and bacterial adhesion on surfaces (Jeon and Andrade, 1991; Jeon et al., 1991; Kingshott et al., 2003; Kjellander and Florin, 1981; Lu et al., 2000). The anti-fouling capability of PEG is thought to rely on multiple factors including PEG density, PEG chain length, molecular weight and immobilization technique. Jeon et al established that high polymer surface density and longer PEO chain length theoretically influence attractive Van der Waals and hydrophobic forces and steric repulsive energy, inducing

optimal coating characteristics for protein resistance (Jeon and Andrade, 1991; Jeon et al., 1991). Creating a copolymer surface coating that has a high density of PEG, conferring these theoretical characteristics to a surface has been shown to be quite a challenge (Kingshott et al., 2003; Lu et al., 2000). PEG, PEG derivatives or PEG copolymers can be attached on surfaces (PEGylation) by a range of methods including adsorption and covalent attachment to a surface anchoring molecule (Holmberg et al., 1997). Holmberg et al compared different PEGylation methods of PEG on solid polyethylene for both PEG density and antifouling properties using a PEG molecule of 3000-4000 Dalton (Da) (Holmberg et al., 1997). They demonstrated that covalent bonding or adsorption of copolymers can procure the same density of PEG onto a surface. However, as the anti-fouling characteristics were different for each of the methods, they concluded that the immobilization method was more important than the PEG density. In subsequent research using PEG of 5 kDa on silica surfaces on the other hand, the same research group conversely showed that both PEG adsorption onto a surface and chemically grafting onto a surface have similar results provided that PEG is grafted in high density indicating that PEG density and PEG molecular weight (chain length) are more important than the grafting method (Malmsten et al., 1998). Kingshott et al then again concluded from their research evaluating different PEGylation strategies (with 5kDa PEG) on bacterial adhesions that the coating's effectiveness is strongly dependent on the PEG immobilization strategy (Kingshott et al., 2003). Not only is it of importance to coat PEG onto a surface in high density, a robust grafting mechanism of the coating onto the surface is preferable so that the surface can retain its anti-fouling characteristics. Park et al grafted polyurethane surfaces with PEG polymers of different chain length and with different end-groups and evaluated their resistance to *E. coli* and *S. epidermidis* attachment in different media (Park et al., 1998). The authors identified all coatings to significantly resist *E. coli* infection in both broth and plasma with results varying between 80% and >90% reduction. It should be mentioned however that a 90 % reduction of 10E7 CFU is still 10E6 CFU and that the efficacy of the coating from a clinical point of view is relative. After reviewing the available literature on PEGylation of surfaces, Dalsin et al pointed out that most PEG coating strategies lack the versatility to allow for wide application of the coating onto different surfaces under different

circumstances (Dalsin et al., 2005). Dalsin and his colleagues were inspired by biomimicry and turned their efforts to developing alternative PEG grafting methods based on mussel adhesive protein (Lee et al., 2002).

### 1.9.2 Mussel Adhesive Protein (MAP)

The Blue Marine Mussel, *Mytilus edulis*, has the unique ability to attach to any wet surface through filamental threads (byssus) attaching to an adhesive pad on the object's surface, both produced by the mussel (Waite and Tanzer, 1981). While the threads consist mainly of collagenous and silk-like protein (Benedict and Waite, 1986; Qin and Waite, 1995), the adhesive pad is composed of polyphenolic mussel adhesive protein (MAP) (Waite and Tanzer, 1981). Waite et al identified that 3,4-dihydroxyphenylalanine (DOPA) containing decapeptides are the main component of these MAP (Waite, 1983). He subsequently postulated that it is this DOPA functional group that is largely responsible for the chemisorption of these polymers to surfaces underwater and for the adhesive covalent cross-linking (Waite, 1990), a statement later corroborated by Yu et al (Yu et al., 1999). The adhesive properties were further emphasized by the work of Yu and Deming, who synthetically formed aqueous solutions of L-Lysine/DOPA copolymers (Yu and Deming, 1998). The authors demonstrated that these copolymers could form moisture-resistant adhesive bonds on surfaces and that with increasing proportion of DOPA in the copolymer, the adhesive potential increased 10-fold (Yu and Deming, 1998). Optimal adhesive bond strength on surfaces such as steel, glass or aluminum, is strongly influenced by the method of oxidization, pH and temperature at which this chemical process is performed (Yu and Deming, 1998). From a biomimetic point of view, the strong adhesive characteristics of 3,4-DOPA and the possibility of synthetically altering DOPA-containing copolymers tailored to the adhesive needs, were soon seen as an asset for industrial applications.

### 1.9.3 mPEG-DOPA

Envisioning the mussel adhesive protein as an anchoring protein to attach molecules to a surface, Dalsin et al realized the anti-fouling potential of 3,4-DOPA (Dalsin et al., 2003; Lee et al., 2002). Through chemical and enzymatical processes, they were able to produce DOPA-modified PEG copolymers capable of forming hydrogels (Lee et al., 2002). The anti-fouling characteristics of PEG and its ability to resist protein adsorption rely mainly but not solely on repulsive steric forces of PEG, hydration or water structuring of the coating, the surface polymer density as grafted on the surface and PEG chain length (Chen et al., 2008; Vermette and Meagher, 2003). By altering the chemical process and PEG structure, hydrogel properties can be tailored to the physical needs (Lee et al., 2002). In subsequent work, they compared hydroxyl-terminated methoxy-PEG (mPEG-OH) surface coatings to DOPA-conjugated mPEG surface coatings on gold (Au) and titanium (Ti) surfaces and identified an mPEG-DOPA 5K coating (single DOPA molecule conjugated to methoxylated PEG of 5kDa molecular weight) to confer strong anti-fouling properties to the coated Au and Ti surfaces whereas the mPEG-OH coating had no anti-fouling effects on the tested surfaces (Dalsin et al., 2003). Where DOPA supplies the strong adhesive to anchor mPEG to the surface, PEG supplies the antifouling properties in this copolymer. By analyzing the differently treated surfaces with X-ray photo-electron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (TOF-SIMS), they demonstrated that PEG was densely attached to the surface of mPEG-DOPA coated surfaces, whereas the mPEG-OH treated surfaces demonstrated only a modest presence of PEG molecules on the surface. This indicates the difficulty of efficiently coating PEG onto a surface and explains why the mPEG-OH coated surfaces had no anti-fouling effects. Continuing their work with mPEG-DOPA, they demonstrated that an mPEG-DOPA coating is rapidly and irreversibly adsorbed onto a surface, providing it with strong anti-fouling abilities (Dalsin et al., 2005). Furthermore, Lee et al demonstrated that DOPA can bind both on organic and inorganic surfaces even in the presence of water and that the interaction between DOPA and a Ti surface is the strongest single molecule binding interaction reported to date (Lee et al., 2006).

## 1.10 mPEG-DOPA Coating For Urinary Devices.

Previous research has demonstrated the mPEG-DOPA copolymer coating to prevent adhesion from fibroblasts (Dalsin et al., 2003; Statz et al., 2005), algae and zoospores (Statz et al., 2006), protein (Dalsin et al., 2005) and bacterial cells (Statz et al., 2008) onto a coated surface. These promising anti-fouling results of coated metal surfaces induced the concept of coating mPEG-DOPA onto urinary devices with the purpose of preventing or reducing biofilm formation, encrustation and device associated urinary tract infection.

Ko et al aimed to ascertain the anti-fouling/anti-bacterial qualities of the coating under urinary circumstances *in vitro* (Ko et al., 2008). The authors submersed TiO<sub>2</sub>-coated silicone disks, half of which were coated with mPEG-DOPA<sub>3</sub>, in sterile pooled human urine and exposed the surfaces to an inoculum of any of 6 different uropathogens: *Pseudomonas aeruginosa* PAO1, *Escherichia coli* GR-12, *Klebsiella pneumoniae* 3 $\alpha$ , *Enterococcus faecalis* 23241, *Proteus mirabilis* 296, and *Staphylococcus epidermidis* 26585. After 24h of exposure, they could identify a uniform film on the surface of the non-coated devices which was not present on the mPEG-DOPA<sub>3</sub>-coated ones. They subsequently compared bacterial attachment data of coated vs. uncoated devices and demonstrated an almost 20-fold reduction of *Pseudomonas* attachment and a more than 200-fold reduction for *E. coli*, *Enterococcus* and *Proteus* on the mPEG-DOPA<sub>3</sub>-coated surfaces. Scanning electron microscopy and Energy Dispersive X-ray Spectroscopy identified a conditioning film on the non-coated devices and the presence of crystalline depositions mainly consisting of Calcium, Phosphate and Magnesium, which were largely absent on the coated devices. With their research, they had demonstrated that the surface of mPEG-DOPA<sub>3</sub>-coated devices, when exposed to a simulated urinary environment, can resist deposition of urinary constituents and adherence of bacterial cells (Ko et al., 2008).

Founded on these results, Pechey et al used an *in vivo* rabbit cystitis/CAUTI model to determine the anti-fouling and anti-bacterial properties of mPEG-DOPA<sub>3</sub> coated devices (Pechey et al., 2009). They aimed to compare the robustness and durability of a linear



(Surphys(S)-002) vs. a cross-linked mPEG-DOPA<sub>3</sub> coating (S-008 and S-009). Rabbits were randomized to have one of 5 possible stent curls placed into the bladder transurethrally: uncoated Sof-Flex<sup>®</sup>, uncoated Percuflex Plus<sup>®</sup> (a hydrogel coated stent), or Sof-Flex<sup>®</sup> coated with S-002, S-008 or S-009, and were all inoculated with 10<sup>7</sup> of *E. coli*. The stent curls were left in place for 7 days and urine samples were taken on Day 1, 3 and 7 via suprapubic aspiration. They identified the group of rabbits with an S-009-coated stent curl in situ to be more efficient in clearing an induced urinary tract infection compared to any of the other groups (P<0.05). Virtually all stent curls had bacterial attachments present on the surface, with S-009-coated devices showing the lowest amount of CFU/cm<sup>2</sup>. This was however not statistically significant. All stent curls without exceptions had encrustations on the surface. The hydrogel coated stent curls had significantly more encrustations than the uncoated stent curls but no significant differences could be demonstrated between the coated and uncoated stent curls. They did however find that stents retrieved from sterile urine had more encrustations than stents from heavily infected (>10<sup>8</sup>) bladders (P= 0.028). Additionally, the SEM pictures depicted that encrustations from infected bladders, arranged more as microspheres, were differently structured than from non-infected bladders which appeared more as large crystalline structures. *In vitro* testing of the coated devices equally identified S-009 as the most efficient coating in resisting bacterial attachment. They thus demonstrated that a cross-linked mPEG-DOPA<sub>3</sub> coating has a more efficient anti-fouling effect than a linear mPEG-DOPA<sub>3</sub> coating (Pechey et al., 2009).

### 1.10.1 S-095 and the Use of Additional Bactericidal Agents

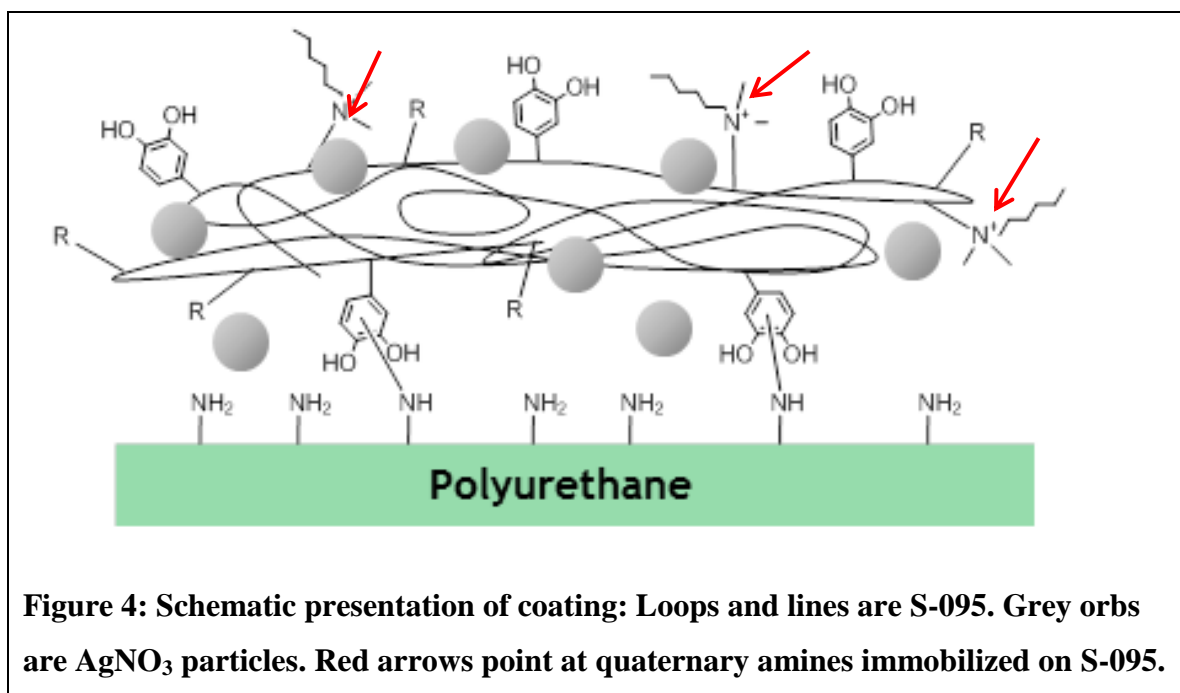
The preliminary work of Pechey and co-workers has helped in the continuous search for and development of newer and more efficient mPEG-DOPA<sub>3</sub> inspired coatings. To increase the efficiency and anti-microbial activity of the coating, a new copolymer was developed (**S-095**) and silver nitrate particles (AgNO<sub>3</sub>) and quaternary amines were added to increase bactericidal effect of the coating. PEG was replaced with a newly developed proprietary long chain polymer backbone. The dual antibacterial effect with use of silver particles (active anti-microbial effect) in the combination with a PEGylated

surface (passive anti-fouling) has been reported by Ho et al (Ho et al., 2004). When the bactericidal effect of silver particles dependent on release from the coating and contact with the bacteria subsides due to depletion of the Ag, the PEGylated surface retains its bacteria-repelling properties and thus the antimicrobial effect of the coating. Li et al had previously demonstrated the dual bactericidal properties of a coating containing both Ag particles and immobilized quaternary amines (Li et al., 2006). The use of the PEG/Quaternary amine combination has been proven to be biocompatible on one hand and have a high bactericidal effect on the other hand (King et al., 2014).

Silver dissociates from the coating and is able to interact with and cross the bacterial cell membrane of both Gram+ and Gram- bacteria. Once inside the cell, silver can interfere with the DNA and enzymatic function of the cell, mediating cell death (Eckhardt et al., 2013). The bactericidal effect of quaternary amines is a kill-by-contact principle, where the quaternary amines disrupt the bacterial outer cell membrane and the cytoplasmic membrane of both Gram+ and Gram- bacteria (Gottenbos et al., 2002; Tiller et al., 2001)

We hypothesize that the triple symbiotic effect of S-095, silver and immobilized quaternary amines will confer a superior anti-bacterial effect to the coating and the coated surface.

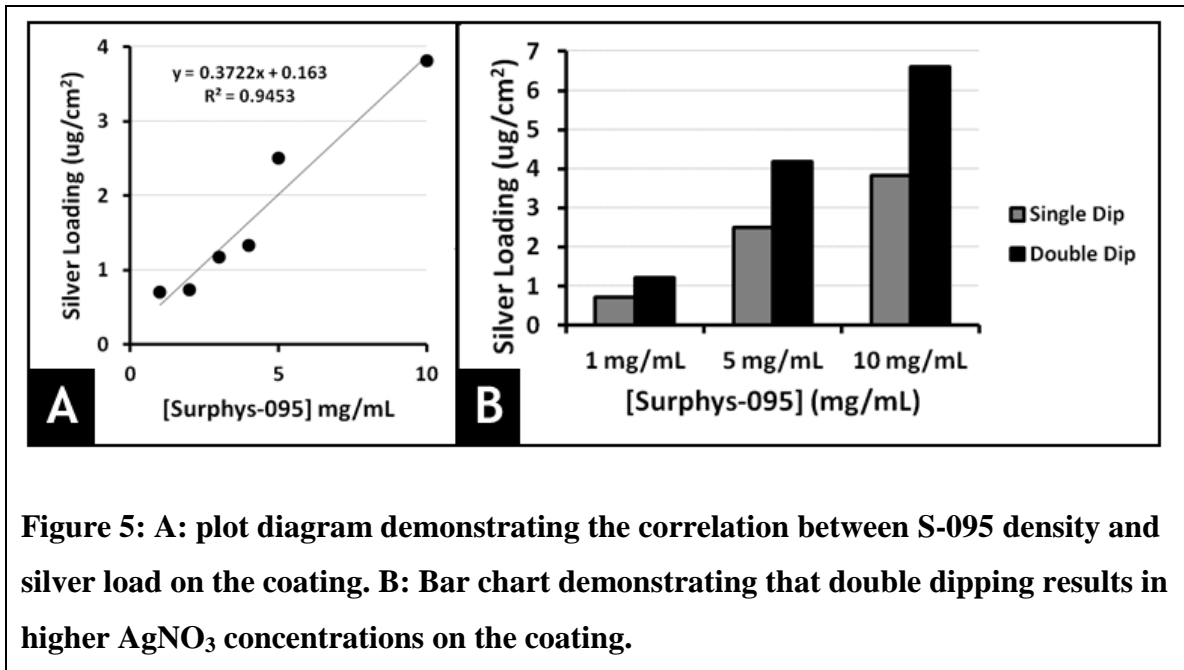
Ammonia treated polyurethane surfaces were coated by chemisorption with S-095 through a dipping method. Quaternary amines were immobilized on the surface and silver nitrate was added to the coating through a subsequent dipping method. The exact details regarding the development and composition of this coating cannot be disclosed due to proprietary restrictions by DSM (DSM Biomedical, Exton, PA, USA).



**Figure 4: Schematic presentation of coating: Loops and lines are S-095. Grey orbs are AgNO<sub>3</sub> particles. Red arrows point at quaternary amines immobilized on S-095.**

The two coatings that were eventually constructed for *in vivo* experiments were developed with two different densities of S-095. For coating A, the dipping solutions had an S-095 concentration of 2mg/ml whereas the dipping solution for the application of coating B had an S-095 concentration of 10 mg/ml. Initially several other dipping solutions at concentrations of 1mg/ml and 5mg/ml were also tested. The devices coated with different densities of S-095 were subsequently air dried and afterwards all dipped in the same dipping solution to add silver with a concentration of 0.25mg/ml AgNO<sub>3</sub> by crosslinking it to the coating.

Post-coating testing for the presence of silver particles demonstrated that the amount of particles present was directly correlated with the surface density of S-095 and that a second dip in the silver solution confers even more silver particles onto the coating (Figure 5A and B).



When implanting a device into a human body, it is of the utmost importance that that device has been thoroughly and efficiently sterilized, which for polyurethane commonly is established with ethylene oxide (EO) gas. Although this is the safest method of sterilizing polyurethane catheters (Shintani, 1995), the effect of EO on experimentally coated polyurethane is unknown. As EO and its metabolites may accumulate on the surface (Lucas et al., 2003), cytotoxicity tests were in order both before and after EO sterilization and to determine residual levels of EO. The coatings were tested before and after sterilization with EO in order to verify that the coated devices retain their anti-microbial properties. Coated devices passed both 10993-5 and 10993-7 tests for allowed cytotoxicity levels for implantable devices and allowed levels of residual EO on the device after sterilization respectively.

*In vitro* analysis demonstrated excellent efficiency of both the 2mg/ml and 10mg/ml S-095 coatings against both Gram-positive and Gram-negative uropathogens. Both coatings significantly decreased the number of both planktonic bacteria and of bacteria attached to the surface. These *in vitro* experiments were repeated after EO sterilization, demonstrating that the coatings retained their activity after the sterilization.

## 1.10.2 Pilot Study

In the core project of this research, the goal is to identify the antimicrobial ability of a coated device to clear or prevent a urinary tract infection from an inoculated individual in an *in vivo* animal model. The control group to which we will be comparing our results should therefore ideally have a urinary tract infection during the experiment, which will be induced at time of device placement. The pilot study was performed to identify the ideal *E. coli* load inoculum that would induce an infection in the control group without inducing urosepsis. If an animal with an uncoated device in situ can clear the infection, we hypothesized that it should be able to clear that infection at least equally as easy or easier with our experimentally coated stent in situ. We used *Escherichia coli* GR12, a bacterial strain initially isolated from a patient with pyelonephritis (Hagberg et al., 1983; Svanborg Edén et al., 1983). This human isolate has been used previously by our group in numerous *in vitro* experiments and a rabbit study involving ureteral stents and has shown to persist and induce an inflammatory response similar to a human urinary tract infection (Pechey et al., 2009).

### 1.10.2.1 Identifying The Ideal Inoculum To Use In The Rabbit Study

To identify the ideal inoculum to use to induce a urinary tract infection in the animals of the rabbit study, we performed a pilot study on 6 rabbits. All of the rabbits were catheterized with an uncoated polyurethane catheter as described in (3.1.1). Half of the rabbits were inoculated with  $10E7$  of *E. coli* GR 12 and the other half was inoculated with  $10E8$  of *E. coli* GR12. Urine samples were collected at day zero, one, three, five and seven under anesthesia as described in (3.1.2). On day 7, the animals were sacrificed after anesthesia and the urinary catheters were collected. Data on urinary bacterial counts and bacterial attachment were quantified as described in 3.3.1 and 3.3.2. The results of this pilot study indicated a bacterial inoculum with  $10E8$  of *E. coli* to be more successful and

consistent at inducing a urinary tract infection and bacterial attachment on the catheters than using a 10E7 inoculum of *E. coli*.

### 1.10.2.2 Identifying The Ideal Inoculum To Use In The Porcine Study

To identify the ideal inoculum to use to induce a urinary tract infection in the animals of the porcine study, we performed a pilot study on 8 pigs. Four of these pigs were stented bilaterally with an uncoated polyurethane stent whereas the other four pigs were not stented. The procedure was performed as described in (3.2.1). Half of the pigs of each group were inoculated with 10E6 of *Escherichia coli* GR 12 and the other half was inoculated with 10E7 of *E. coli* GR12. Urine samples were collected at day zero, one, three and seven under anesthesia as described in (3.2.2). On day 7, the animals were sacrificed after anesthesia. All animals in the 10E7 group had a positive urine culture on day 7 whereas only 2 animals had a positive urine culture at endpoint in the 10E6 group. It should be mentioned that the two animals having a positive urine sample at day 7 in the 10E6 group were stented. The amount of bacterial attachment on the stents was similar in both groups. Bacterial attachment was quantified as described in (3.2.3). The results of this pilot study demonstrated a bacterial inoculum with 10E7 of *E. coli* to be more successful at inducing a urinary tract infection than using a 10E6 of *E. coli*.

## Chapter 2

### 2 Materials and Methods

The study was approved by the University of Western Ontario animal use subcommittee (Appendix 1). As mentioned previously, the novel coatings evaluated in this project were developed based on the results of prior research and noted to contain a new DOPA-anchored long chain polymer backbone with the addition of silver nitrate and quaternary amines. Two different coatings (henceforth referred to as coatings A and B) were tested in this experiment, both containing a different amount of S-095. The test devices were coated with the two experimental coatings by DSM (DSM Biomedical, Exton, PA, USA), sterilized and packaged separately.

Our objective was to evaluate whether or not these new coatings containing silver and quaternary amines would be as effective in an *in vivo* model. As the rabbit model had previously been shown to be suitable for preliminary testing of urinary devices (Cadieux et al., 2006; Morck et al., 1994, 1993; Multanen et al., 2002; Olson et al., 1989), it was selected for our initial *in vivo* work. To test ureteral stents coated with our experimental coating compound, we chose a porcine model for our *in vivo* experiments. The porcine model provides a urinary tract that resembles the macroscopic human urinary tract fairly well and is a very accessible animal for laboratory testing.

All animals were allowed an acclimatization period of at least three days in the animal testing facility after arrival, prior to experimentation. Laboratory feed and drinking water were provided *ad libitum* during their entire stay, including before and during experimentation.

The animal subjects were provided with environmental enrichment in accordance to species-specific standard operating protocol (SOP). The temperature and humidity of the housing space was monitored daily, and animals were provided with a cycle of 12 hours of light followed by 12 hours of darkness.

## 2.1 Rabbit Study

A total of 36 New Zealand White (NZW) male rabbits were randomized 1:1:1 to one of three transurethral catheter groups. The number of animals per group was determined by a power analysis based on the number of recovered bacteria from urine samples in previously performed studies (Chew et al., 2006; Pechey et al., 2009). Given a desired power level of 0.8 and a p-value of 0.05, a sample size of 12 animals in each of the 3 groups will allow detection of a 30% to 40% difference between treatment means.

The study consisted of 3 experimental runs of 12 rabbits per run. Each set of 12 rabbits were randomly assigned to the 3 experimental groups in the following manner: 4 rabbits assigned to the uncoated catheter group, 4 to the coating A group and 4 to the coating B group. This breakdown allowed for uniform representation of each device type on a given test day. The veterinary surgical team was blinded to treatment groups by assigning each rabbit with a generic clinical number. The laboratory technician was then blinded to these labels during post-experimental sample analysis.

### 2.1.1 Transurethral Catheter and *E. coli* Bacterial Challenge

The rabbits in the control group were catheterized with a 5 French (Fr), 15 cm length of uncoated polyurethane catheter. The rabbits in the intervention groups were catheterized with a 5Fr 15 cm long polyurethane catheter coated with coating A or coating B. On day 0, all rabbits were anaesthetized for catheter placement and bacterial challenge. Rabbits were anaesthetized intramuscularly (IM) using ketamine (5 mg/kg body weight) in combination with medetomidine (0.15 mg/kg body weight). Rabbits were intubated during anesthesia and maintained during the procedures via inhalation isoflurane (1.5-5.0%). During anesthesia, the anticholinergic glycopyrrolate (0.01 mg/kg SQ) was given.



During anaesthesia, rabbits were placed in a supine position and the penis was cleaned using two consecutive alcohol preps. A 5Fr catheter from one of the study groups was inserted into the bladder (**Figure 6**).



**Figure 6: 5Fr transurethral catheter used for insertion in rabbit.**

The external section was initially attached to the skin with a stitch to prevent it from migrating further into or out of the bladder. After encountering issues with catheter migration despite this precaution in the first twelve animals, the catheters placed in the subsequent animals were attached to the skin with the aid of a wingtip (catheter attachment aid device resembling butterfly wings that can be placed on the catheter and has small holes in to thread a suture through for more secure skin attachment) that was attached to the catheter. No migrations were noted after this adjustment to the procedure. The bladder was then instilled with  $10^8$  colony forming units (CFU) of the uropathogen *Escherichia coli* GR12 in a 1.5 ml saline solution, followed by 0.5 ml of saline to flush any remaining bacteria from the inside of the catheter into the bladder. The catheter was clamped for 30 minutes to prevent bacterial washout and to facilitate the establishment of infection in the bladder. Before reversing anaesthesia, the animal was given the anti-inflammatory meloxicam (0.3 mg/kg subcutaneous (SC)) and analgesic buprenorphine (0.05 mg/kg SC or IM). After the procedure, atipamezole (stock at 5 mg/mL - volumetric equivalent of medetomidine is given) was given to reverse the anaesthetic effects and speed up animal recovery. This anaesthesia regimen was suggested and performed by the veterinarian of the animal care facility.

### 2.1.2 Sample Collection

Urine and blood samples for culture analysis were collected at day 0, prior to the procedure during the same anesthesia and at day 1, 3, 5 and 7 under a short anesthesia. The same anesthesia regimen as previously described was used for sample collection.

Blood samples were taken by venipuncture in an ear vein after skin disinfection using an alcohol swab. Venipuncture was performed using a 22 gauge needle, collecting approximately up to 1 ml of venous blood according to SOP. Urine sample was taken aseptically by suprapubic, transcutaneous aspiration with a 20 gauge needle after prepping the skin at puncture site with an alcohol swab. This procedure was performed after clamping the catheter for approximately 30 minutes so that the bladder would contain urine for collection. A suprapubic sample was preferred versus collecting the sample through the catheter so as to prevent contamination from skin bacteria or from attached bacteria in the catheter lumen.

Anesthesia was reversed after sample collection except on Day 7. All rabbits were euthanized on Day 7 following anaesthesia using 110 mg/kg intravenous sodium pentobarbital. Following euthanasia, autopsy was performed to collect the implanted device and to collect bladder, ureter and kidney tissue for histopathology analysis. A representative segment of kidney, ureter and bladder were dissected separately for tissue bacterial count analysis. Histopathology samples were submersed in formalin and stored until analysis. All other collected samples and tissue were stored at -80°C following sample collection until time of analysis.

## 2.2 Pig Study

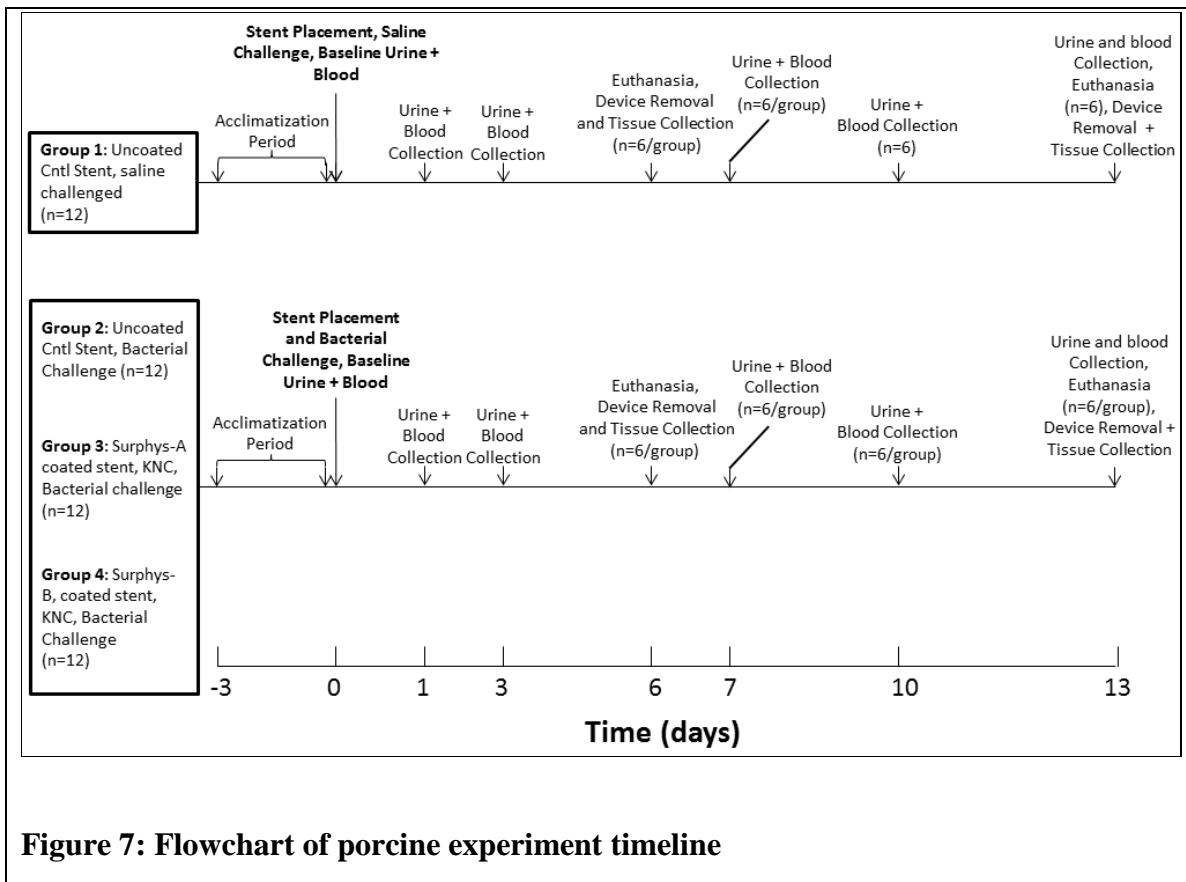
In the porcine study of this project, we used 48 pigs of approximately 3 months old, weighing approximately between 30 and 35 kilograms. For the porcine part of our animal study, we applied a somewhat different approach. Whereas in the rabbit study, we had 3

groups comparing outcomes of control catheters to catheters coated with A or B after an *E. coli* challenge, we had an additional group of pigs stented with uncoated stents and with a saline challenge. This group was added to identify the influence of the devices on tissue inflammation and infection. A total of 48 pigs were randomized to one of four groups in a 1:1:1:1 ratio of uncoated control stent with saline challenge, uncoated control stent with *E. coli* challenge and coating A or coating B with *E. coli* challenge. The control stent was a 7Fr uncoated polyurethane stent (Cook Urological, Bloomington, IN, USA). The coated stents were also 7Fr polyurethane stents treated with either coating A or coating B respectively. Pigs were randomly assigned to treatment groups through an Excel column random order generator.

Of the 24 pigs in the control group, half (N=12) was inoculated with a bacterial challenge of 10E7 of *E. coli* GR 12 in a 5 ml saline solution (the uncoated catheter *E. coli* challenge group, henceforth UnEC) whereas the other half was inoculated with normal saline containing no bacterial load (the uncoated catheter saline challenge group, henceforth UnS). The surgeon was blinded to the contents of the 5ml syringe containing bacteria or saline solution. All animals in both groups stented with coated stents were inoculated with a bacterial challenge of 10E7 of *E. coli* GR 12 in a 5 ml saline solution. These four groups of 12 pigs per group were divided into two different time schedules. Half of the animals in each group were randomized into being stented for a period of 6 days: the short-term stented group. The other half in each group was randomized into being stented for 13 days, thus allowing a longer period of exposure of the stents and tissues to the bacterial or saline challenge: the long-term stented group. At the allocated endpoint of indwelling time of the stents, pigs were euthanized and tissue and urinary devices were collected. A flowchart of the timeline is demonstrated in **Figure 7**.

We based our calculation on the number of pigs to be allocated in each group on previously published literature using a porcine model for stent related research. Hadaschik compared twenty pigs stented with Uriprene™ stents to 16 control stented pigs (Hadaschik et al., 2008). Pigs were sacrificed at 4 different time points, thus comparing 5 Uriprene™ stented pigs to 4 control pigs at any time point. In research of Keterolac-eluting stents, Chew et al. used 20 animals for each of the test groups,

sacrificing 4 animals in each group at 5 different time points (Chew et al., 2010a). Lumiaho et al compared an experimental stent to a control stent in 8 pigs, euthanized at two different time points, thus comparing 4 pigs at any time point (Lumiaho et al., 2011). Both Kallidonis et al. and Liatsikos et al used 10 pigs and inserted an experimental stent in one ureter and stented the contralateral ureter with a control stent. All pigs were euthanized at the same endpoint and acted as their own control (Kallidonis et al., 2011; Liatsikos et al., 2007). Chew et al in a subsequent Uriprene™ study compared 10 pigs stented unilaterally with an experimental stent to 6 pigs stented with a control stent all euthanized at the same time point (Chew et al., 2013).



We decided that having 6 animals in each group at each of two time points was optimal to collect sufficient bacteriological and histological data to allow for statistical analysis to identify significant differences between groups. In addition, we stented all animals bilaterally to increase the number of stents and urinary tract tissue we could analyze while using as few pigs as possible.

The surgeons could not be blinded to stent type during stent placement, though the veterinary technician team was blinded throughout the study. The laboratory technician and pathologist were both blinded during post-study sample analyses.

### 2.2.1 Ureteral Stent Placement and Bacterial Challenge

Pigs were anaesthetized using ketamine intramuscularly (IM) (11-22mg/kg body weight) in combination with one of the following: 1)Acepromazine (1 mg/kg body weight IM), 2)Midazolam (0.5 mg/kg body weight IM) or 3)Xylazine (2.2mg/kg body weight IM). Once anesthetized, the animals were intubated for ventilation. The pigs were maintained during the procedures via inhalation isoflurane (1.5-5.0%).

During anesthesia, pigs were positioned in supine position with the hindlegs strapped back to allow easy access to the genitalia. The genitalia were cleaned with two iodine scrubs followed by an alcohol prep. A rigid 14F pediatric cystoscope (Karl Storz, Mississauga, Canada) with a 30° downward angle lens (Karl Storz, Mississauga, Canada) was used to perform cystoscopy to identify the ureteral orifices of the pig. Sterile normal saline was used as irrigation fluid. With the cystoscope in the bladder, before performing any other steps of the procedure, a baseline urine sample was taken through the cystoscope. Once identified, the ureteral orifice was cannulated with a 0.035 inch hydrophilic guidewire (Terumo Corporation, Tokyo, Japan) under fluoroscopic guidance until the tip of the guidewire was identified in the renal pelvis of the kidney. As the porcine ureter is quite tortuous on occasion, contrast instillation was used through a 5Fr open ended Kumpé catheter (Cook Urological, Bloomington, IN, USA) when deemed necessary to outline the urinary tract. Once the hydrophilic guidewire was confirmed in the renal pelvis of the kidney, a 5Fr Kumpé catheter was advanced over the hydrophilic guidewire into the renal pelvis. At this point the hydrophilic guidewire was removed and exchanged for a 0.035 inch Bentson guidewire (Cook Urological, Bloomington, IN, USA). We subsequently placed the stent allocated to the animal over the guidewire, pushing it into place with the accompanying radiographically visible metal tip pusher device. The stent was placed under fluoroscopic guidance to confirm correct position of

the proximal curl of the stent in the renal pelvis and the distal curl of the stent in the bladder. This procedure was repeated for the contralateral ureter. When both ureteral stents were in situ, cystoscopy was repeated to identify both stent curls in the bladder and to subsequently empty the bladder through the cystoscope shaft. The bladder was then instilled through a working channel of the cystoscope with either 5ml of saline or 10E7 CFU of the uropathogen *E. coli* GR12 in a 5 ml saline solution. One ml of saline was then used to flush the remaining bacteria inside of the working channel into the bladder, ensuring that the entire bacterial load was instilled in the bladder. The animal was then given the anti-inflammatory meloxicam (0.3 mg/kg SC) and analgesic buprenorphine (0.05 mg/kg SC or IM). After the procedure, anesthesia was reversed and all animals were recovered. This anesthesia regimen was suggested and performed by the veterinarian of the animal care facility.

### 2.2.2 Sample Collection

Urine and blood samples for culture and future cytokine analysis were collected at day 0, prior to the procedure during the same anesthesia and at day 1 and 3 under a short anesthesia for all animals. Animals allocated to the six day stent group were additionally anesthetized on day 6 for sample collection. Animals allocated to the 13 day stent groups had additional samples taken in the same manner on day 7, 10 and 13. The same anesthetic regimen as used during stent placement was used for the sample collections.

Blood sample was taken by venipuncture in an ear vein after skin disinfection using an alcohol swab. Venipuncture was performed using a 16-18 gauge needle and collecting approximately 1-2ml venous blood according to SOP. Urine sample was taken aseptically by cystocentesis with a 16-18 gauge needle after sterile prepping the skin at puncture site with an alcohol swab. This procedure was preferred over taking a urine sample via the urethra to prevent perineal contamination of the sample. Upon completion of blood and urine collection, anesthesia was reversed.

On the day of endpoint, pigs were euthanized following anesthesia for sample collection using 150 mg/kg intravenous sodium pentobarbital according to SOP. After euthanasia,

autopsy was performed to retrieve the implanted devices for analysis and to retrieve urinary tract tissue for histopathology evaluation: Kidney, ureter and bladder. A representative segment of kidney, ureter and bladder was excised separately for tissue bacterial count analysis.

Histopathology specimens were submersed in formaldehyde and appropriately stored until analysis. All other collected samples and tissue were stored at -80°C following sample collection until time of analysis.

## 2.3 Sample Analysis

The urinary catheters from the rabbit study and the ureteral stents from the pig study were analysed for bacterial attachment, encrustation and via scanning electron microscopy. The urine samples were analysed for bacteria presence. The urinary pH of every urine sample taken was measured. The blood samples were taken for future cytokine analysis only. The representative segments of bladder, ureter and kidney that were taken post-mortem were processed and analysed for tissue bacterial counts. The bladder, ureter and kidney that were resected whole organ during autopsy were processed and analysed microscopically for histopathology. The only vital sign collected was body temperature at day 0 and at study endpoint.

### 2.3.1 Urine Culture

The presence of *E. coli* in the collected urine samples was quantified by dilution plating on Lysogeny Broth (LB) agar (10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride and 15 g/l granulated agar in Milli-Q H<sub>2</sub>O), a bacteria medium traditionally used for culturing *E. coli*. The agar plates were incubated at 37°C for 24 hours, after which colonies were counted to determine the CFU/ml of *E. coli* for each urine sample. Based on colony appearance and morphology under microscope, several non-*E. coli* colony types were identified which were also counted and recorded. Some, but not all of the other cultured species were later identified with DNA sequencing as *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus suis* and *Lactobacillus* species.

### 2.3.2 Device Bacterial Attachment

For analysis of bacterial attachment, stents or urinary catheters collected post-mortem were cut into three one cm long segments: proximal, middle and distal segment. Each segment was placed in an Eppendorf tube and rinsed 3 times with sterile phosphate-buffered saline (PBS) to remove unattached and loosely attached bacteria from the sample. The segments were then resuspended in 1mL PBS, sonicated for 20min at 50/60 Hz (Branson 1200, Branson Ultrasonic Co., Danbury, CT, USA), and vortexed for 30 seconds to detach the bacteria present on the segments into solution. Bacterial concentration was then determined through dilution plating on LB plates which were incubated for 24 hours at 37°C and the number of colonies recorded. Finally, the bacterial count was divided by the segment surface area to determine CFU/cm<sup>2</sup> on the devices.

### 2.3.3 Device Encrustation

The amount of encrustation on the implanted devices was determined by evaluating a representative 2 cm segment. First, the 2cm segments were air-dried in open Eppendorf tubes for 24 hr. The segments were then weighed on an analytical scale with an accuracy of 0.1 mg (AC100 Analytical Balance, Mettler-Toledo, Mississauga, Canada) and the weights recorded. Next, encrustation from the outer surfaces were scraped off manually using a scalpel, and the devices were then washed vigorously with water via pipette-mixing and vortexing to remove encrustation within the lumen and any remaining material on the outer surface. The segments were air-dried for 24 hours and weighed again. Encrustation was determined by subtracting the final segment weight from the initial weight and was recorded as mg/cm<sup>2</sup>.



### 2.3.4 Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy

Scanning electron microscopy and energy dispersive X-ray spectroscopy analysis (SEM/EDX) of the stents and catheters was performed on representative air-dried segments in the Division of Surface Science at Western University, London, Ontario. The samples were given a thin sputter deposited gold coating in order to alleviate charging during the SEM examination. SEM images and EDX graphs were obtained with a Hitachi S4500 Field emission SEM (Hitachi, Tokyo, Japan) using a 10 kV electron beam voltage with a Quartz XOne EDX system (Quartz Imaging Corp., Vancouver, BC, Canada). EDX can identify elements from Carbon to Uranium for elemental mapping.

### 2.3.5 Tissue Bacterial Attachment and Internalization

Representative tissue segments from bladder, kidney and ureter were analysed for tissue-associated bacterial attachment and internalization. The tissue samples were weighed to 0.1 mg accuracy (AC100 Analytical Balance, Mettler-Toledo, Mississauga, Canada) and transferred to 50 ml conical tubes. Tissue samples were first rinsed with sterile PBS to remove unattached and loosely attached bacteria. The samples were resuspended in varying volumes of PBS, depending on tissue size: rabbit bladder in 5ml PBS, rabbit ureter in 1ml PBS, rabbit kidney in 10ml PBS, and pig bladder, ureter and kidney all in 20ml PBS. The tissue segments were subsequently homogenized at 30,000 rpm with a PT 2100 Homogenizer (Kinematica, Littau, Switzerland) until a homogeneous suspension was established for every sample. The homogenate was then dilution plated, and plates were incubated for 24 hours at 37°C for bacterial quantification. The tissue-associated bacterial counts were measured in CFU/ml and then converted to CFU/mg.

### 2.3.6 Histopathology

Bladder and kidney samples were collected at necropsy and suspended in a 10% neutrally buffered formalin solution. The collected tissues were processed in a paraffin wax tissue

processor (Leica ASP3000, Leica Microsystems Inc., Concord, Canada) and subsequently embedded into paraffin wax blocks (Leica ECG 1150 HC, Leica Microsystems Inc., Concord, Canada) before sectioning. The samples were sectioned into 5µm sections with a microtome (Microm HM335E Microtome, Thermo Fischer Scientific, Waltham, MA USA) and placed on microscopy slides. The slides were stained automatically with hematoxylin and eosin (Leica Autostainer XL, Leica Microsystems Inc., Concord, Canada). The sections were analysed with an Olympus BX 41 microscope (Olympus, Richmond Hill, Canada) by a veterinarian at Western University, London, Ontario, blinded to the stent groups.

Kidney tissue was scored on urothelial inflammation, urothelial fibrosis, ulceration, mucinous metaplasia, squamous metaplasia, suburothelial hemorrhaging and suburothelial inflammation, scoring each as normal = 0, mild = 1, moderate = 2 or severe = 3. Scores were added up to a maximum of 21. Bladder tissue was scored on urothelial inflammation, urothelial fibrosis, ulceration, mucinous metaplasia, squamous metaplasia, suburothelial hemorrhaging, lamina propria fibrosis, lamina propria inflammation, muscle fibrosis and muscle integrity scoring each as normal = 0, mild = 1, moderate = 2 or severe = 3. Scores were added up to a maximum of 30.

To prevent score shifting and assure consistency in his scoring, the blinded pathologist checked his own scores afterwards for score variability and did not identify any scoring variability.

## 2.4 Statistical Analysis

The primary outcomes in this analysis are stent encrustation and urinary tract infection with stent coating considered as an independent variable.

For categorical variables such as the qualitative outcomes of the urine culture (positive or negative), Chi Square and Fisher's exact test were used. Post-hoc between group analysis was likewise performed with a two-sided Fisher's exact.

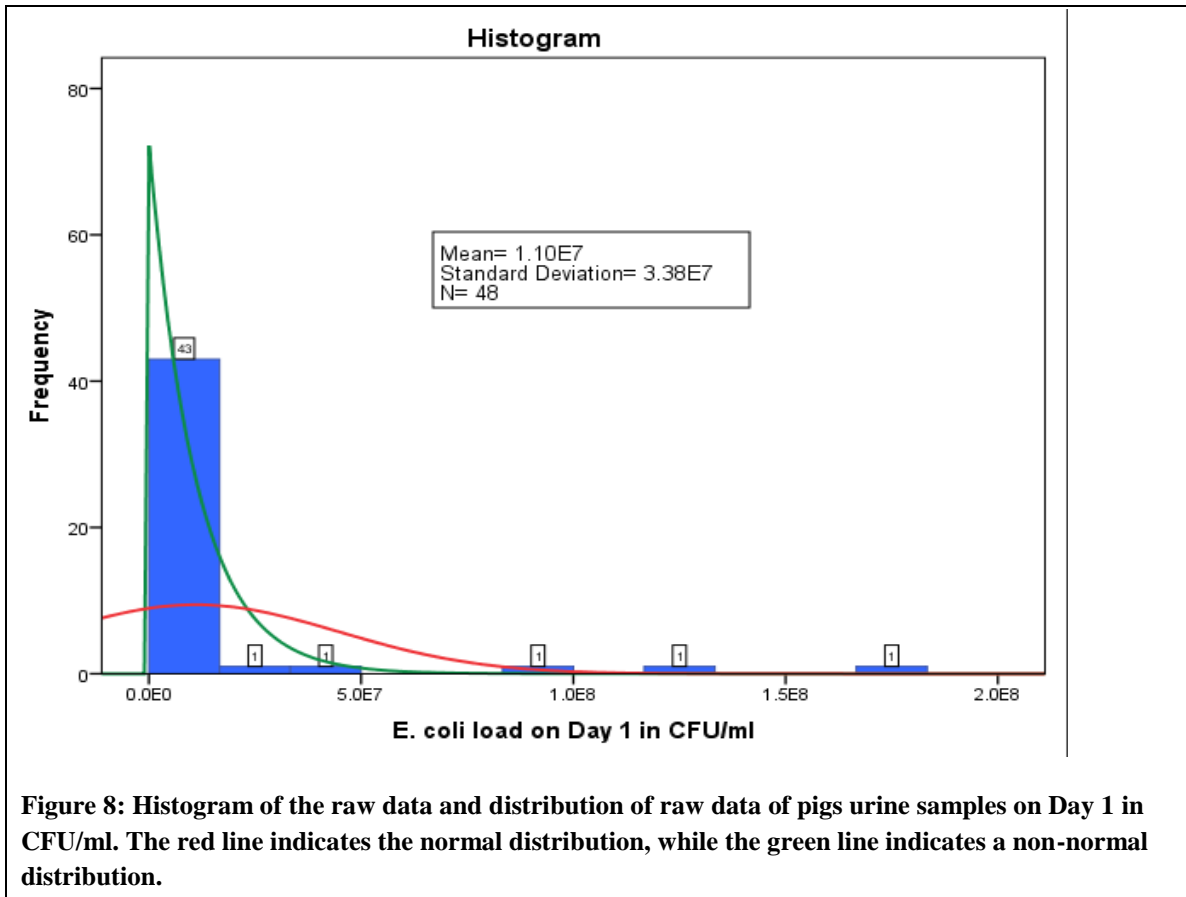
For continuous variables such as the quantitative outcomes of the urine cultures, device bacterial attachment, encrustation and bacterial tissue counts, ANOVA was used with the post-hoc Dunnett's T-test to control for multiple group comparison.

Because of the high CFU counts in our data and the relatively low sample size, we could expect a non-normal distribution of the data (**Figure 8**). The red line in this graph is a normal distribution graph and is quite skewed here, indicating that the data doesn't follow a normal distribution. The data is much better fitted under the green line, which shows the exponential distribution. To have our CFU data follow a normal distribution, we  $\log_{10}$  transformed our data for statistical analysis and transformed them back afterwards (Bland and Altman, 1996; Olsen, 2014). The lowest detectable level of infection is 100 CFU/ml. For some endpoints, some animals did not have a detectable infection, resulting in a 0 value for CFU/ml. As zero values cannot be log transformed, a value of 1 was added to the data so that  $\log(0+1)=0$  and no datapoints are lost for analysis.

Shapiro-Wilk test was performed to explore the normal distribution of data. If despite log-transforming, the data did not follow a normal distribution, non-parametric tests were applied such as Mann-Whitney-U and Kruskal-Wallis where appropriate. Homogeneity of variance of the data was ascertained with Levene's test. Welch's ANOVA was performed on the data where Levene's test was statistically significant, indicating heteroscedasticity. We used Games-Howell for post-hoc analysis after Welch's ANOVA for multiple group comparison instead of Dunnett's T-test, as Games-Howell is more robust for heteroscedastic data.

Statistical analysis was performed using SPSS 22 software (IBM Corp., Armonk, NY, USA). Significance was assessed at  $P < 0.05$ .

Although the data of these in vivo experiments was log transformed for statistical analysis, as is suggested in the literature, statistical analysis was equally performed on the raw data. Interestingly so, this did not influence the statement towards the null-hypothesis in any of our results.



## 2.5 *In vitro* Assessment of Catheter Coatings

We aimed to identify whether or not the coatings retained their efficiency several months after coating the devices by performing *in vitro* analysis on the coated and uncoated stents we had received prior to performing the animal study.

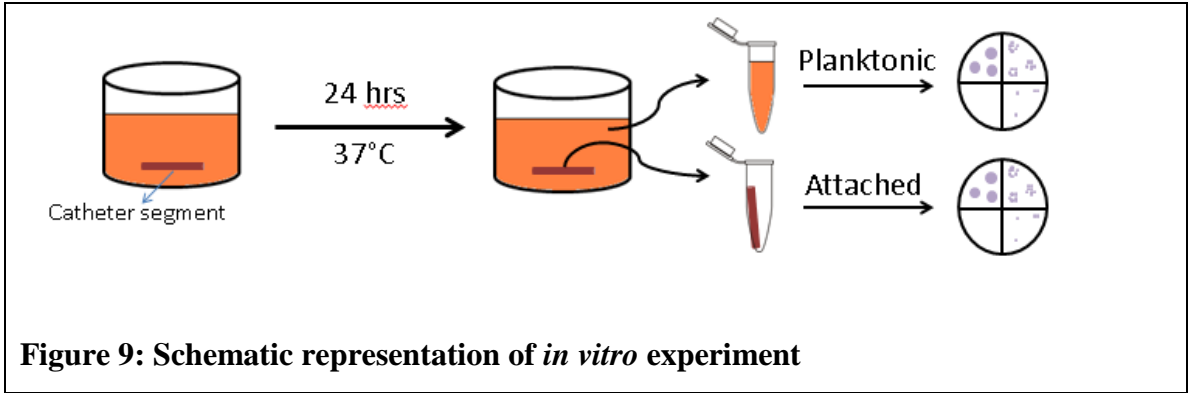
For the *in vitro* experiments, we exposed the stent segments to seven different uropathogenic bacterial strains (*E. coli*, *E. faecalis*, *K. pneumonia*, *P. mirabilis*, *S. saprophyticus*, *S. epidermidis* and *P. aeruginosae*) for the purpose of evaluating the anti-attachment and anti-bacterial capacity. Bacterial cultures were grown for 16 hours in LB at 37°C, diluted 1/100, and incubated for three hours (*E. coli*, *E. faecalis*, *K. pneumonia* and *P. mirabilis*) or five hours (*S. saprophyticus*, *S. epidermidis* and *P. aeruginosae*), depending on the growth rate of the species. Cultures were then diluted to reach a final concentration of  $5 \times 10^5$  CFU/mL. Bacterial strains used for the *in vitro* assessment assay

included one strain from each of 7 clinically relevant uropathogenic bacterial species, including *Escherichia coli* GR-12 (pyelonephritis isolate) (Hagberg et al., 1983), *Staphylococcus saprophyticus* ATCC 15305 (clinical urine isolate) (Erdeljan et al., 2012), *Proteus mirabilis* 296 (catheter isolate) (Watterson et al., 2003b), *Pseudomonas aeruginosa* AK-1 (UTI isolate) (Reid et al., 1994), *Klebsiella pneumoniae* 280 (UTI isolate) (Wignall et al., 2008), *Enterococcus faecalis* 1131 (UTI isolate) (Smeianov et al., 2000), and *Staphylococcus epidermidis* 3399 (clinical skin isolate) (Saldarriaga Fernández et al., 2007). All strains were grown and maintained in LB and on LB agar plates at 37°C in ambient air. To prevent swarming of *P. mirabilis* 296, this strain was grown on non-swarming agar (NSA [10g tryptone, 5g yeast extract, 0.4g sodium chloride and 20g agar per L]).

Uncoated and coated stents were cut into 1 cm segments and placed in 24-well non-tissue culture plates (BD Falcon). We subsequently transferred 1 mL aliquots of the cultures to the wells containing the stent segments. The plates were incubated for 24 hours at 37°C under shaking conditions to mimic urinary flow and to keep the devices uniformly exposed to the bacteria.

Bacterial attachment was quantified as previously described in section 3.3.2. For quantifying the concentrations of planktonic bacteria, cultures from each well were transferred directly to 96-well non-tissue culture plates (BD Falcon) and dilution plated. These experiments were carried out three times in triplicate. The results generated are the means of each triplicate. **Figure 9** schematically demonstrates the *in vitro* experiments performed.

As previously mentioned, the raw data were log transformed before performing statistical analysis. We performed ANOVA with post-hoc Dunnett's T-test on both planktonic and bacterial attachment data.



**Figure 9: Schematic representation of *in vitro* experiment**

## Chapter 3

### 3 Results

After sample collection, all samples were analyzed as described. Complete data was available for urine culture, bacterial attachment, encrustation and tissue bacterial count analysis of both rabbit and pig studies. Histopathology was analyzed for the porcine study. SEM and EDX was performed on stent and catheter segments from both studies.

#### 3.1 Results Rabbit Study

##### 3.1.1 Urine Culture

In contrast to humans, there is no definition of what amount of CFU constitutes bacteriuria or a urinary tract infection in rabbits. Additionally it was not feasible to assess symptoms of a UTI such as frequency, pollakiuria or dysuria. We therefore opted to identify the number of rabbits with a positive culture without a CFU cutoff value. Results on the urine culture analysis are available in **Table 1**. We have some missing data for the *E. coli* CFU counts. We were unable to obtain a urine sample on 3 occasions and we could not reliably count the *E. coli* CFU in 6 samples due to *Proteus mirabilis* swarming in the petri dish. There was no statistical difference in the number of animals having a positive *E. coli* sample for Days 1, 3 and 5. Fisher's exact test for data on day 7 shows a tendency towards significance with a P-value of **0.066**. When comparing the results between groups with a two-sided Fisher's exact test, we see that there are statistically fewer rabbits with a positive urine culture in the coating A group compared to the control group with a P-value of **0.036**. Group B is not significantly different from the control group (**P= 0.193**) (**Figure 10**).

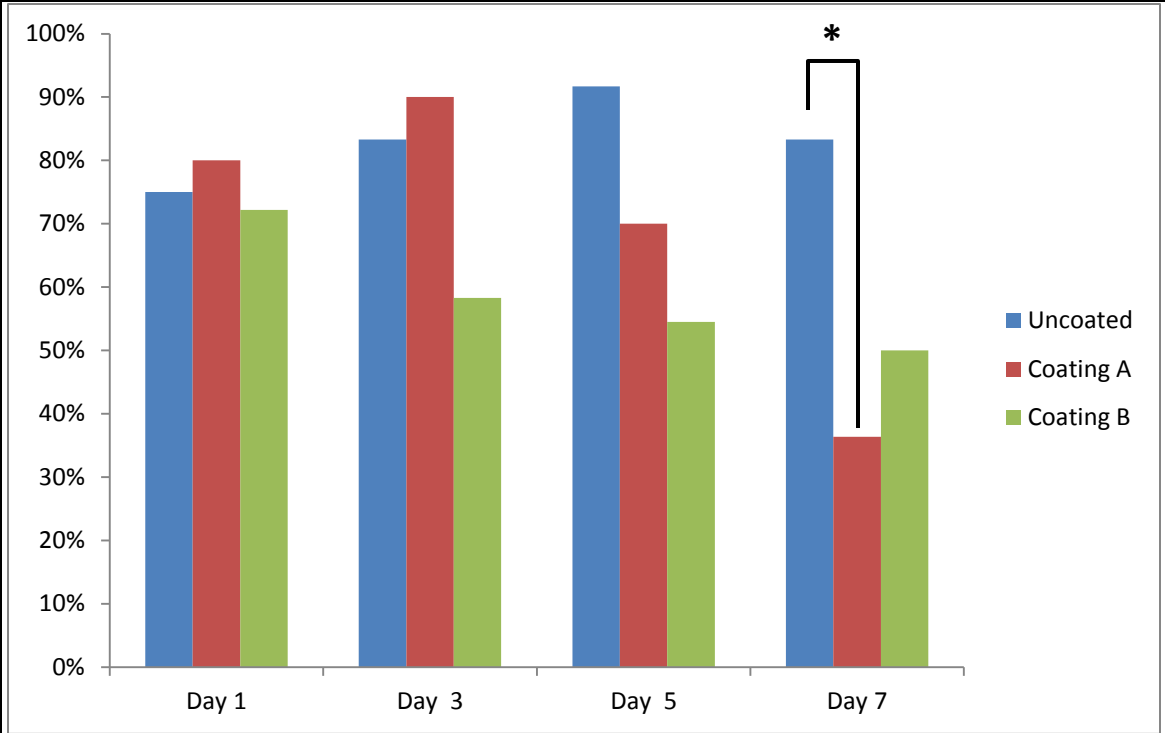
When evaluating the degree of infection between groups by comparing the  $\log(x+1)$  transformed CFU data between groups at any time point, we could not identify any

significant differences between groups at any given time point indicating that the groups don't have a significantly different degree of infection. As the data does not follow a normal distribution, Kruskal-Wallis was performed on this data. There were no other statistical differences between groups at any other time points (**Figure 11**).

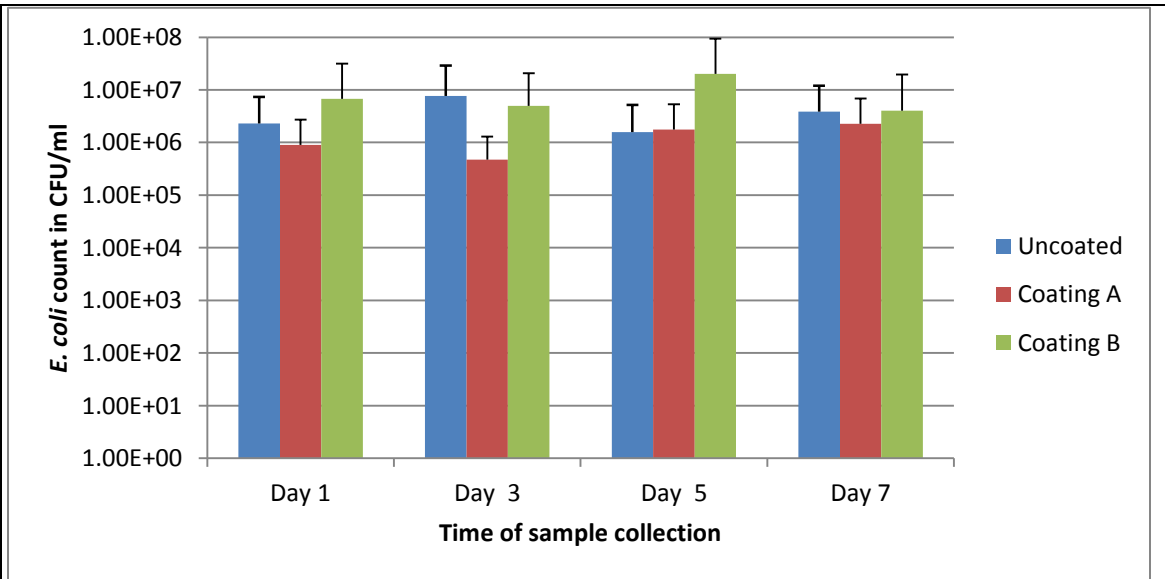
		<b>Uncoated</b>	<b>Coating A</b>	<b>Coating B</b>	<b>P-value</b>
<b>Day 1</b>	#infected/total	9/12	8/10	8/11	1*
	Mean (SD)	2.29E6 (5.06E6)	9.04E5 (2.71E6)	6.74E6 (1.79E)	0.837 <sup>\$</sup>
<b>Day 3</b>	#infected/total	10/12	9/10	7/12	0.216*
	Mean (SD)	7.70E6 (2.13E7)	4.72E5 (1.30E6)	4.98E5 (1.06E7)	0.950 <sup>\$</sup>
<b>Day 5</b>	#infected/total	11/12	7/10	6/11	0.138*
	Mean (SD)	1.59E6 (3.56E6)	1.77E6 (5.35E6)	2.01E7 (5.35E7)	0.707 <sup>\$</sup>
<b>Day 7</b>	#infected/total	10/12	<b>4/11</b>	6/12	<b>0.066*</b>
	Mean (SD)	3.85E6 (8.08E6)	2.28E6 (6.89E6)	4.03E6 (1.16E7)	0.205 <sup>\$</sup>

**Table 1: Rabbits with positive urine sample with the means and standard deviations of the CFU/mL of *E. coli* at the different time points. \*= Fisher's exact test, \$= Kruskal-Wallis**





**Figure 10:** Diagram showing proportion of animals with a positive urine culture at the different time points in %. \* P-value = 0.036



**Figure 11:** Bar graph showing means and standard deviations of CFU/ml for each of the groups at every time point.

When reading out the urine culture plates, we did not only identify *E. coli* on the agar. As mentioned before, some samples even had *Proteus mirabilis* swarming so that we could not reliably read out the *E. coli* count. Other bacteria identified were *Staphylococcus aureus* and *Enterococcus faecalis*. Some colonies observed however could not be identified and were thus grouped according to their appearance.

Fisher's Exact test could not identify a difference in number of rabbits having other bacteria cultured in their urine samples at any of the time points. Interestingly, we did identify an increasing number of other bacterial species present in the urine samples with time. Whereas on day 1 only 2 rabbits had any of the other bacterial species present in the urine, 22 rabbits had one or more of the other species present in the urine sample by endpoint. For analytical purposes, the presence of any of the other identified bacteria was grouped to form the 'non *E. coli*' infection group. No differences between groups were demonstrated with Fisher's Exact test. ANOVA and where applicable Kruskal-Wallis could not demonstrate a difference between groups for the degree of infection with other bacteria. Once again, no differences were unveiled between coating groups with statistical analysis.

### 3.1.2 Device Bacterial Attachment

For catheter bacterial attachment, the number of CFU/ml of *E. coli* were measured and converted to CFU/cm<sup>2</sup>. Proximal, middle and distal segment of the catheter represent different places in the urinary tract. The proximal segment of the catheter was present in the bladder, whereas the middle segment was present in the urethra. The distal segment was outside of the urethra and generally in touch with the skin and often soiled with rabbit's excretions.

In the first group of 12 animals tested, we had an issue with migration of the catheters out of the bladder. In some animals the catheters had disappeared. One animal of the uncoated group and one animal of the Coating A group had a missing catheter at

endpoint. They were retrieved at autopsy in the stomach. We therefore do not have bacterial attachment numbers for these catheters. We did not analyze the distal part of the catheters of the first 4 animals in every group because of the assumption that these were not representative of any infection in the urinary tract of the animal. We collected and analyzed the distal segments of all subsequent animals.

When comparing the amount of catheters that had *E. coli* attached to the surface, there were no significant differences between groups (3X2 contingency table with Fisher's Exact test,  $P=$  ). When performing ANOVA after  $\log(x+1)$  transforming, similarly no statistical differences could be demonstrated between groups (**Figure 12**).

Data reported in **Table 2** are means and standard deviations of *E. coli* CFU/cm<sup>2</sup> and the number of catheters with bacterial attachments.

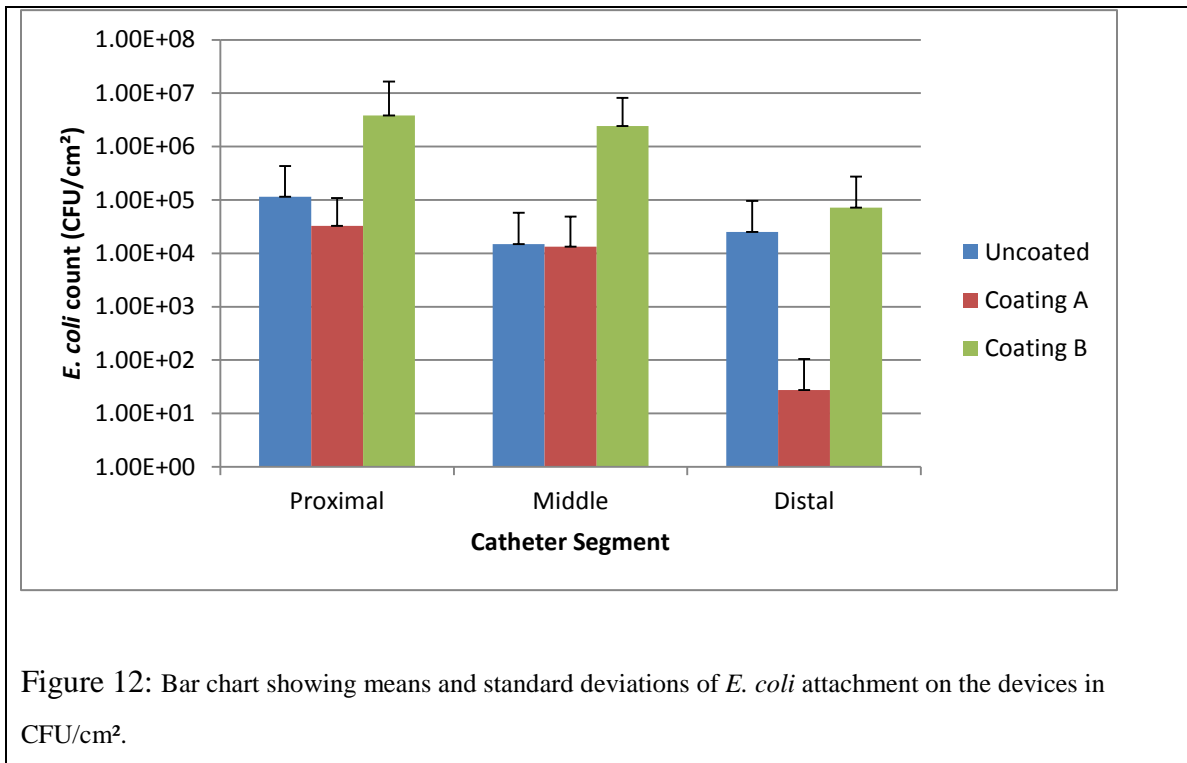
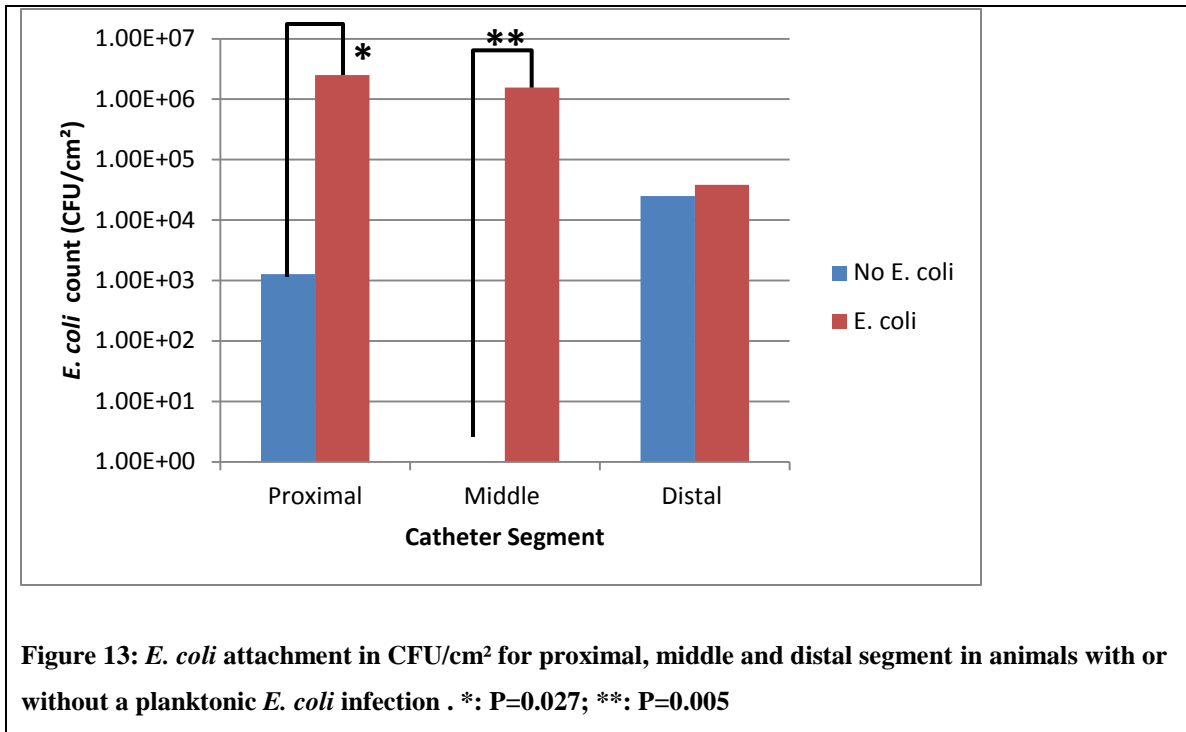


Figure 12: Bar chart showing means and standard deviations of *E. coli* attachment on the devices in CFU/cm<sup>2</sup>.

		Uncoated	Coating A	Coating B	P-Value
<b>Proximal</b>	# infected/total	4/10	6/11	5/12	0.756*
	Mean (SD)	1.15E5 (3.17E5)	3.25E4 (7.54E4)	3.83E6 (1.26E7)	0.950 <sup>†</sup>
<b>Middle</b>	# infected/total	6/10	2/11	3/12	0.138*
	Mean (SD)	1.48E4 (4.26E4)	1.32E4 (3.55E4)	2.43E6 (5.70E6)	0.474 <sup>†</sup>
<b>Distal</b>	# infected/total	2/8	1/8	2/8	0.705*
	Mean (SD)	2.52E4 (7.10E4)	2.75E1 (7.78E1)	7.17E4 (2.03E5)	0.620 <sup>†</sup>

**Table 2: CFU of *E. coli* bacterial attachment on the catheter segments. \*= Fisher's exact test. <sup>†</sup>= ANOVA.**

When comparing the bacterial attachment of the rabbits that had a positive *E. coli* urine sample on day 7 compared to the ones that did not, we see that the infected animals have more bacterial attachment than the uninfected rabbits. Data was  $\log(x+1)$  transformed for the analysis and did not show a normal distribution. Mann-Whitney-U test identified the proximal and middle segments of the catheter to have significantly fewer bacterial attachment in the uninfected animals (P-values 0.027 and 0.005 respectively). None of the uninfected rabbits had any bacterial attachment on the middle segment (**Figure 13**).



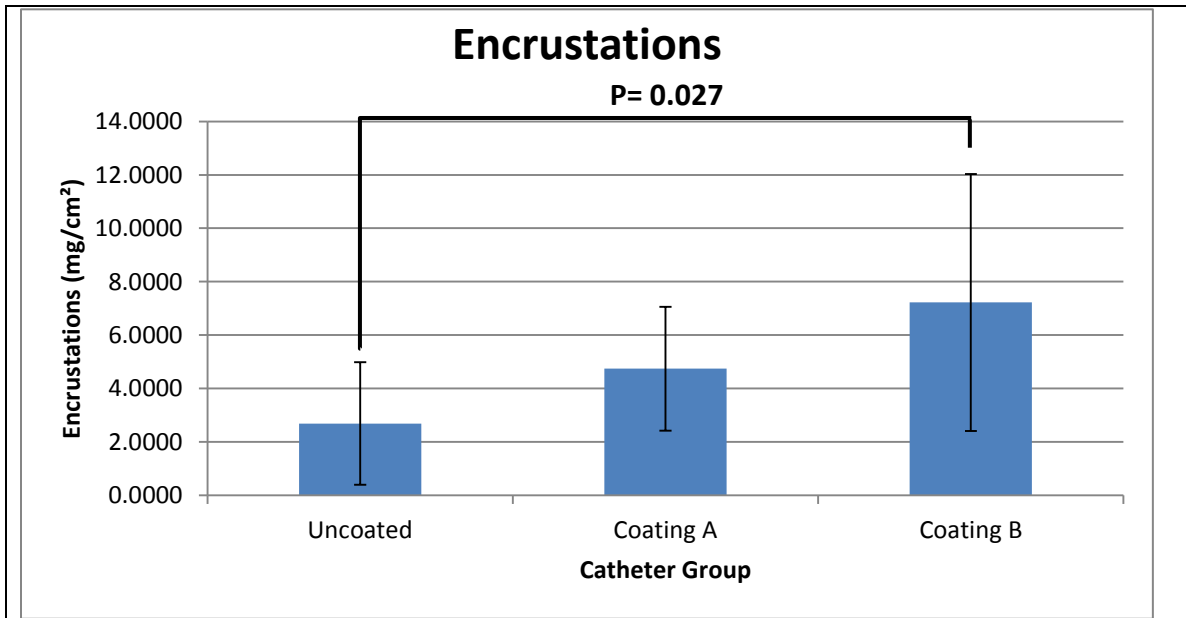
When repeating this analysis for all the different bacterial species cultured from the urine samples, similar results were obtained with uninfected rabbits having significantly less bacterial attachment on the catheter segments.

### 3.1.3 Encrustation

We found encrustations present on every urinary catheter inserted in the rabbits. The encrustations were quantified as described in previous chapter. The uncoated group had on average 2.69 mg of encrustations per cm<sup>2</sup>, ranging from 0.14 to 6.73. The catheters from coating A and coating B had on average 4.74 and 7.22 mg of encrustations per cm<sup>2</sup> respectively, ranging from 2.39 to 11.08 and from 1.48 to 16.9 mg/cm<sup>2</sup> respectively. Statistical analysis comparing the amount of encrustations in the three groups with ANOVA produced a P-value of **0.016** demonstrating a significant difference between groups. As Levene's test was significant indicating heteroscedasticity within this normally distributed data (Shapiro-Wilk was not significant), we performed Welch's ANOVA with Games-Howell post-hoc analysis. There is a statistically significant

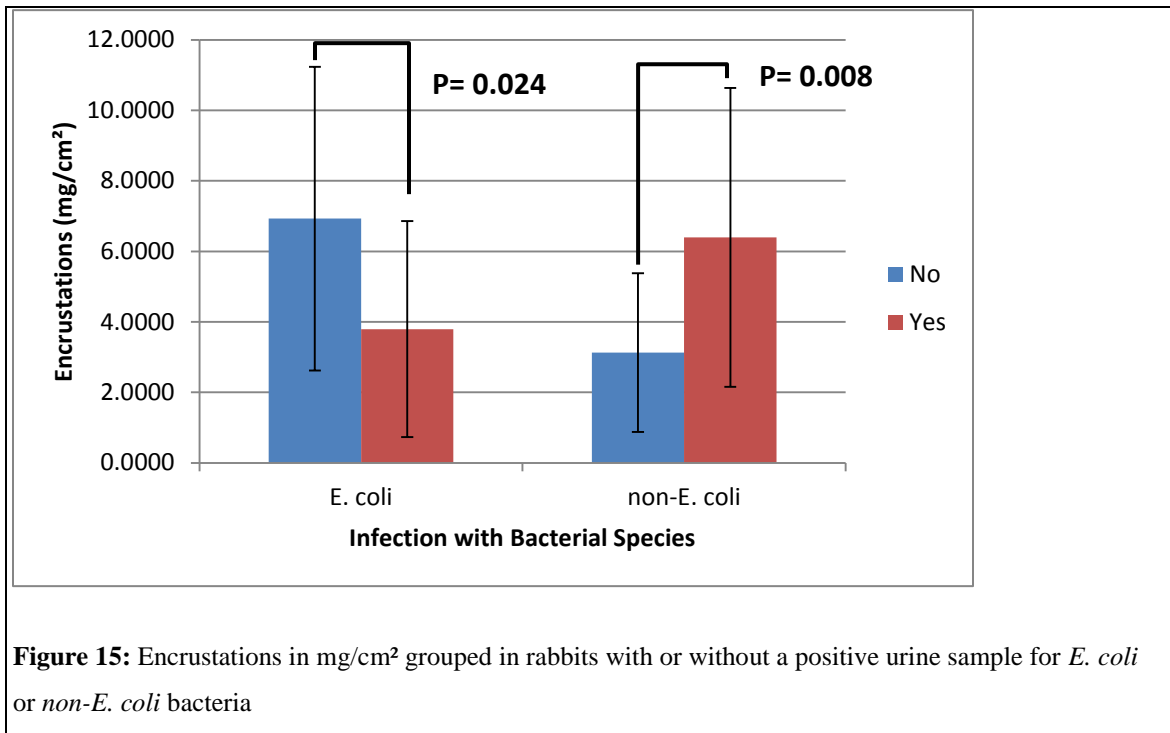
difference between groups with a P-value of 0.022 for all groups comparison. Games-Howell shows that the catheters coated with coating B had significantly more encrustations on the surface than the uncoated catheters (**P=0.027**). (**Figure 14**).

As Pechey (Pechey et al., 2009) et al had identified an inverse correlation of encrustations with infections, we compared the encrustations of infected rabbits to non-infected rabbits with ANOVA. For this purpose, infection was defined as having a positive *E. coli* urine sample on the day of catheter extraction, day 7. The rabbits that had an *E. coli* positive urine culture on day 7 had on average 3.79 mg/cm<sup>2</sup> of encrustations on the catheter whereas rabbits with no *E. coli* in the urine sample on day 7 had an average of 6.93 mg/cm<sup>2</sup> encrustations. The infected group had statistically fewer encrustations than the non-infected group (**P= 0.022**), thus corroborating Pechey’s results (**Figure 15**). The mean and standard deviations of the encrustations in each of the groups, are shown in **Table 3**.



**Figure 14:** Means and standard deviations of Encrustations in mg/cm<sup>2</sup> for the different catheter groups.

As the presence of an *E. coli* infection seems to influence the amount of encrustation inversely, we wanted to verify this for any of the other bacteria identified in some of the urine samples. When considering each of the bacterial species separately, comparison of the amount of encrustations in the infected or uninfected animals showed no statistical difference. However, when grouping these as ‘non-*E. coli*’ infection, we interestingly demonstrated that the rabbits that had a non-*E. coli* infection on day 7 had more encrustations than the non-infected animals (P-value of 0.008 with Welch’s ANOVA), which is the contrary of what we had identified in the *E. coli* infected animals (**Figure 15**).



**Figure 15:** Encrustations in mg/cm<sup>2</sup> grouped in rabbits with or without a positive urine sample for *E. coli* or non-*E. coli* bacteria

	Control	Coating A	Coating B	P-value
Encrustations Mean (SD)	2.69 (2.30)	4.74 (2.32)	<b>7.22 (4.81)</b>	<b>0.022\$</b>
	UTI		No UTI	P-value
<i>E. coli</i>	3.79 (3.06)		6.93 (4.31)	<b>0.022*</b>
Non <i>E. coli</i>	6.40 (4.24)		3.13 (2.25)	<b>0.008\$</b>

**Table 3:** Encrustations on the catheters measured in mg/cm<sup>2</sup>. \$= Welch's ANOVA, \*= ANOVA

Considering that several animals had more than one bacterial species identified on urine culture and considering the opposite effect of *E. coli* vs. *non-E. coli* infection, we aimed to uncover the influence of presence of *E. coli* only, *non-E. coli* only or both *E. coli* and *non-E. coli* in urine on the degree of encrustations on the catheter surface with ANOVA. As expected, the rabbits that had only *E. coli* present in their urine sample on day 5 (18/33 rabbits) and day 7 (12/35 rabbits), had significantly less encrustations than the other rabbits (**P= 0.006 and 0.024** respectively). Conversely, the rabbits that had only *non-E. coli* bacterial species present in their urine sample on day 7 (14/35), had significantly more encrustations than the other rabbits (**P= 0.012**). The opposite effects of both in mind, we analyzed the data for rabbits that had both *E. coli* and any of the other bacterial species present in their urine sample. No significant differences were identified and for the day 7 data, P-value was 0.944.

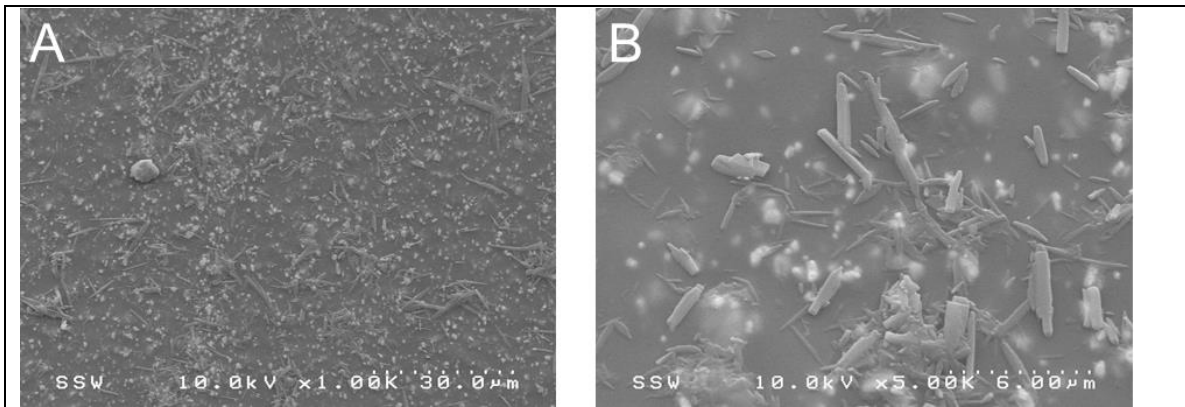
As mentioned previously in this manuscript, the deposition of encrustations on a surface submersed in urine is dependent of multiple factors, among which the urinary pH. We therefore aimed to verify pH as an influence on the amount of encrustations. Unfortunately, the urinary pH was not measured for 5 of the 36 rabbits: 2 in the uncoated and Coating A group and 1 in the coating B group. We could not identify a direct strong



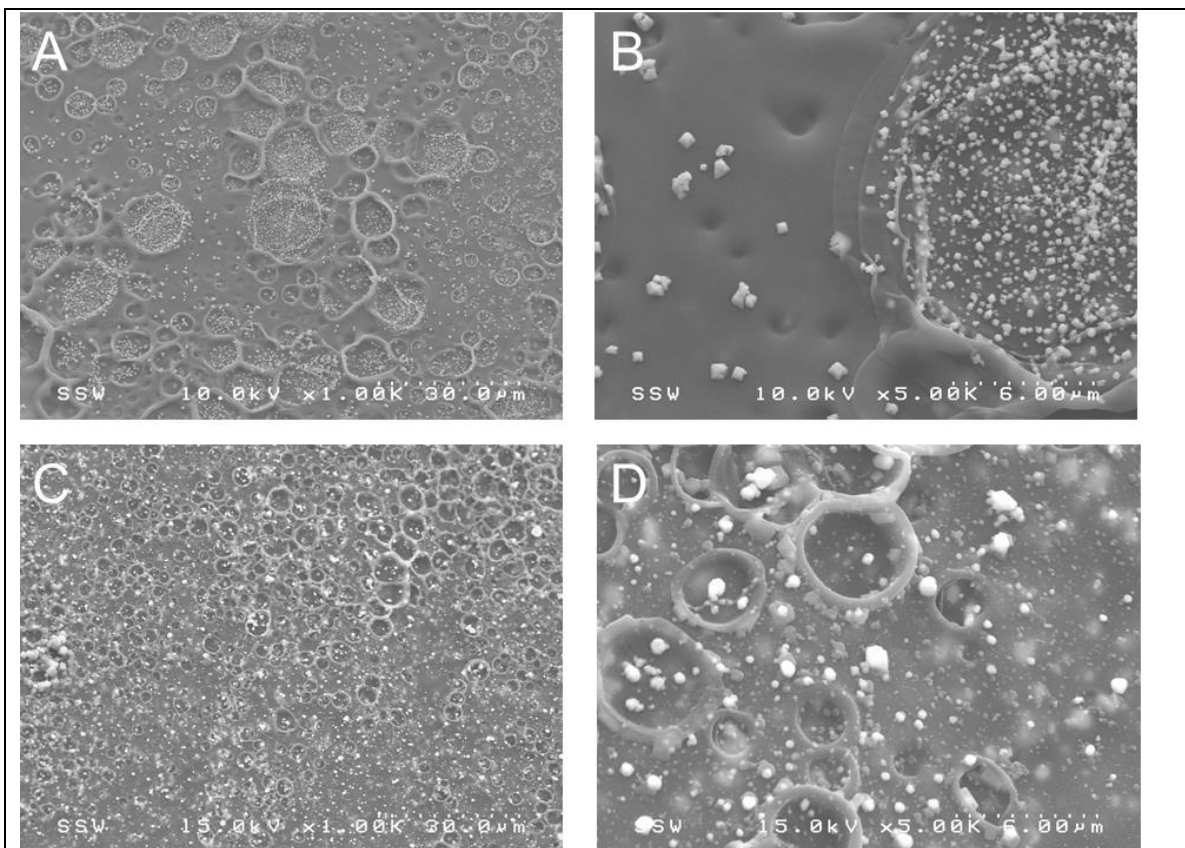
correlation between encrustations and urinary pH at any time point. As *E. coli* seems to have a reducing influence on encrustations, we compared the *E. coli* only rabbits to the other rabbits for pH. Mann-Whitney-U (ANOVA could not be performed due to non-normal distribution) indicates that there is indeed a significant difference between the two groups with the *E. coli* only animals having a significantly lower pH on day 7 compared to the other rabbits in the experiment (mean +/- SD: 7.7 +/-1.2 vs. 8.7 +/-0.4; **P-value=0.031**).

### 3.1.4 Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy (SEM & EDX)

SEM pictures were taken from an uncoated polyurethane control catheter to establish the baseline appearance of an uncoated catheter (**Figure 16**) and subsequently from the coated catheters (**Figure 17**). The main difference between the two coatings is the density of S-095: the density of S-095 in Coating B is 5 times higher than in Coating A. This difference in density is apparent on the SEM **Figures 17 A and C**, showing catheter segments coated with Coating A and B respectively. Both pictures are taken at the same magnification but the crater-like structures are smaller and more numerous in **Figure 17 C** (Coating B).

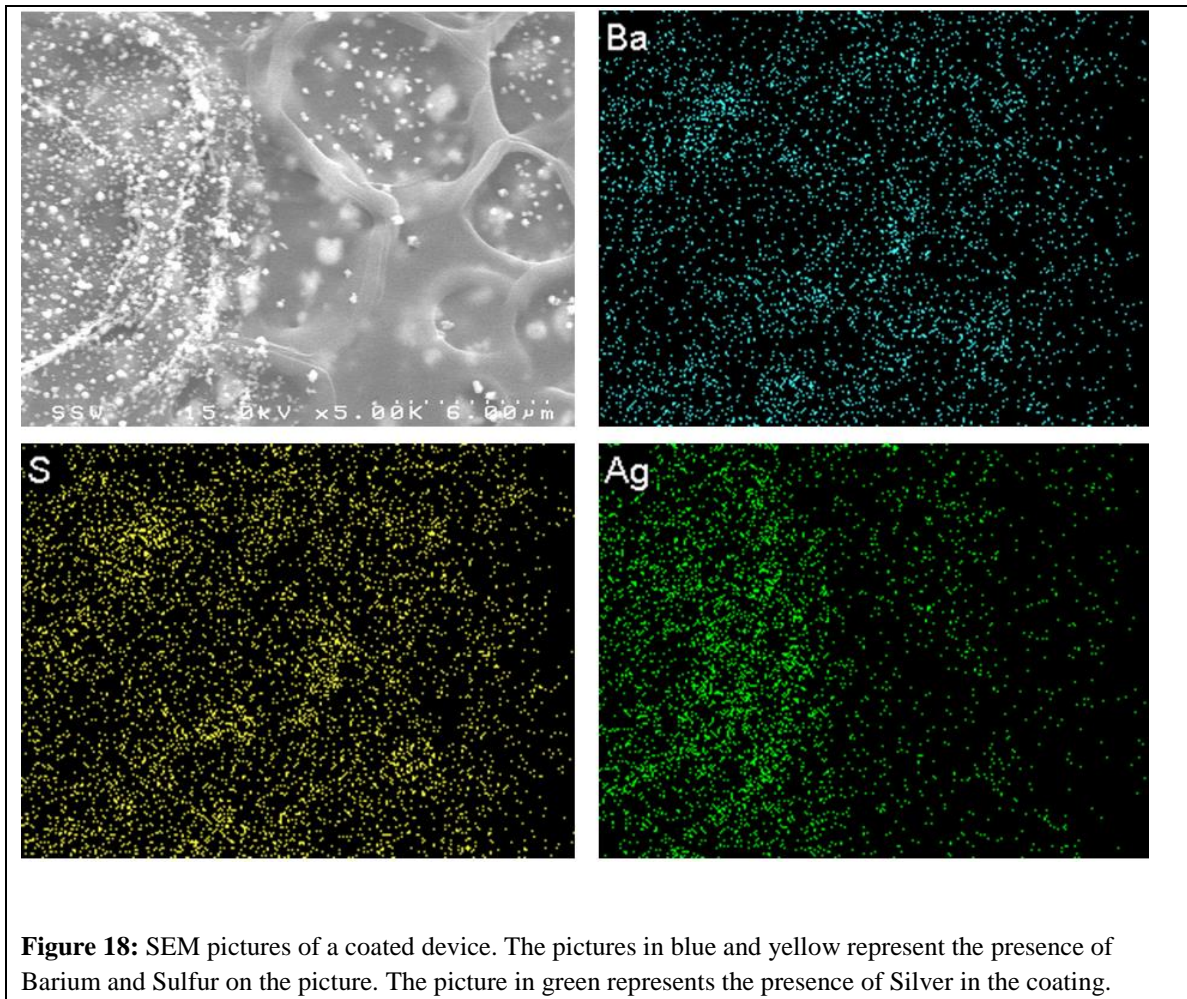


**Figure 16:** SEM pictures at 1000 x (A) and 5000 x (B) magnification of an uncoated polyurethane catheter before implantation.



**Figure 17:** SEM pictures at 1000 x (A/C) and 5000 x (B/D) magnification of catheters coated with Coating A (A/B) and catheters coated with Coating B (C/D). The white spots represent silver particles in the coating.

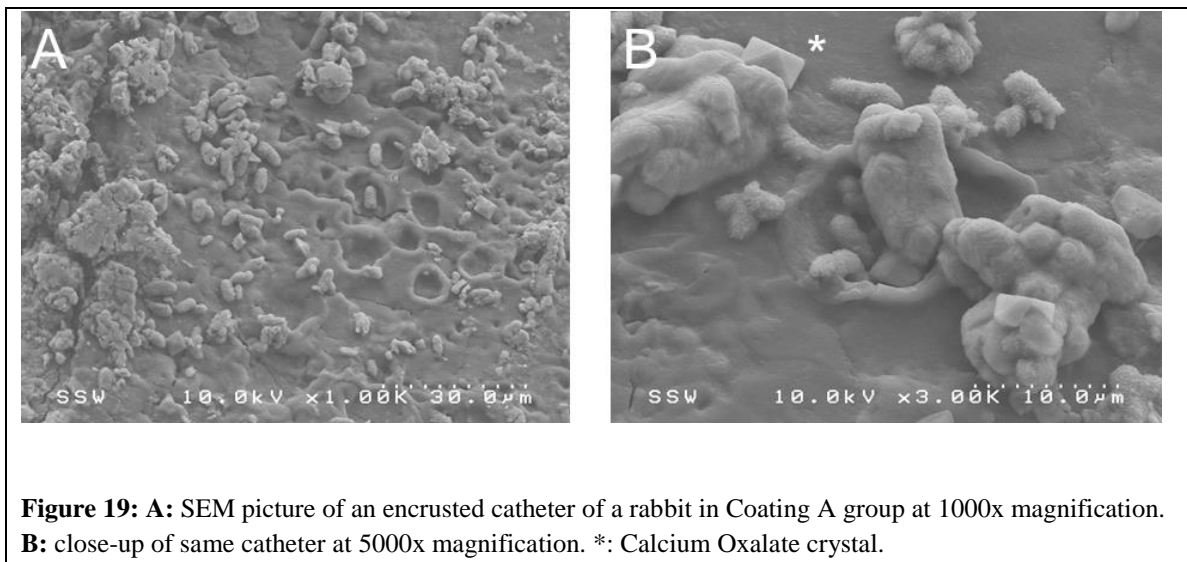
The S-095 coatings contain Barium and Sulfur ions. Due to the capabilities of full spectrum imaging with the elemental mapping, we can select single elements for map creation. The blue and yellow pictures in **Figure 18** depict the mapped content of Barium and Sulfur on a coated stent. These pictures show that the distribution of S-095 is quite homogeneous on the stent surface. The green picture demonstrates the presence of Silver on the coating.

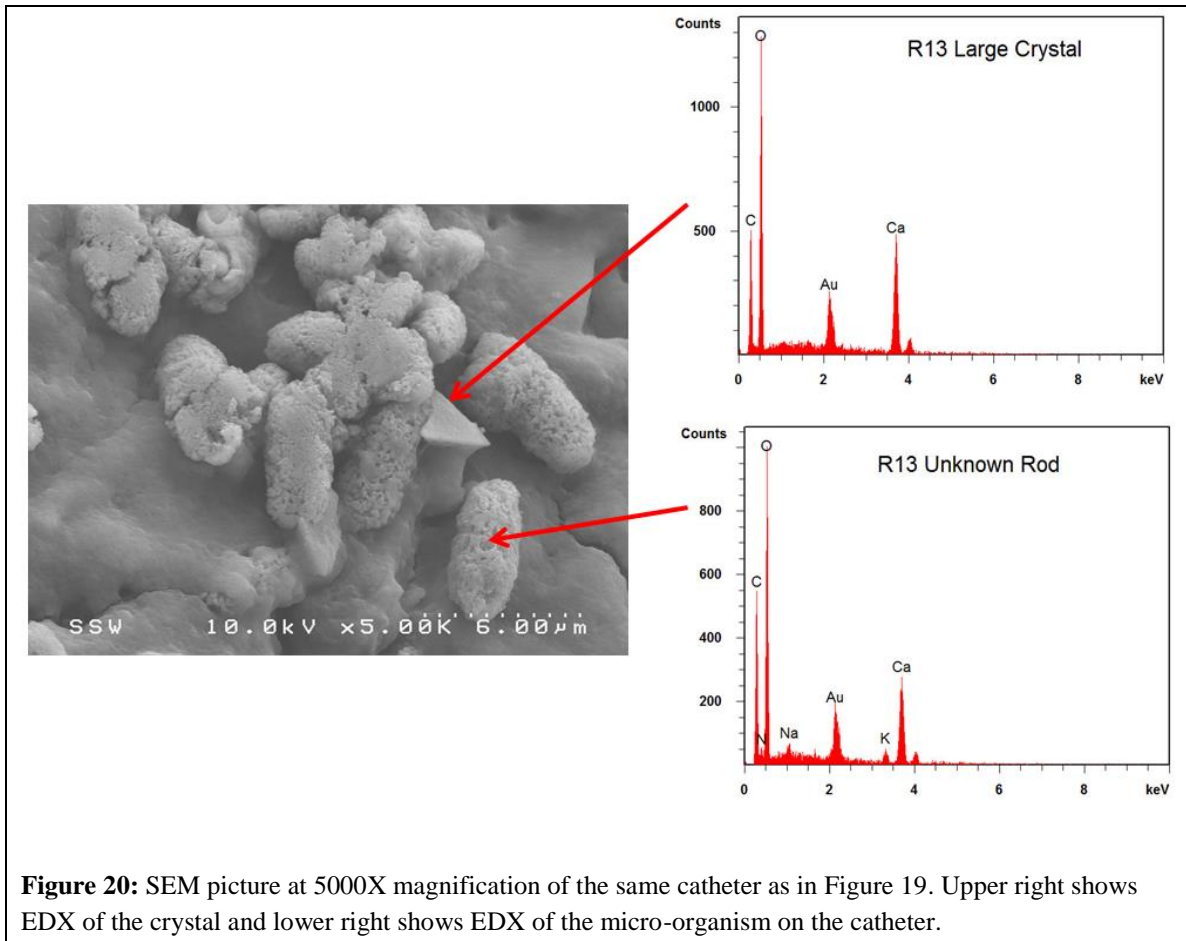


**Figure 18:** SEM pictures of a coated device. The pictures in blue and yellow represent the presence of Barium and Sulfur on the picture. The picture in green represents the presence of Silver in the coating.

We subsequently analyzed the catheters from animals with and without *E. coli* infection for analysis of attached bacteria and encrustations on the catheters.

**Figure 19** shows a SEM of a catheter segment of a rabbit catheterized with a Coating A catheter that did not have an *E. coli* infection on day 7 nor any *E. coli* attached to the surface of the catheter. There were other bacteria present, which unfortunately could not be identified. This catheter had 3.96mg/cm<sup>2</sup> of encrustations on the surface. The asterisk shows a calcium oxalate crystal on the surface. The picture in **Figure 20** is taken from the same catheter segment. Electron dispersive X-Ray Spectroscopy was performed demonstrating a spectrum consistent with calcium oxalate dihydrate (C<sub>2</sub>H<sub>4</sub>CaO<sub>6</sub>).



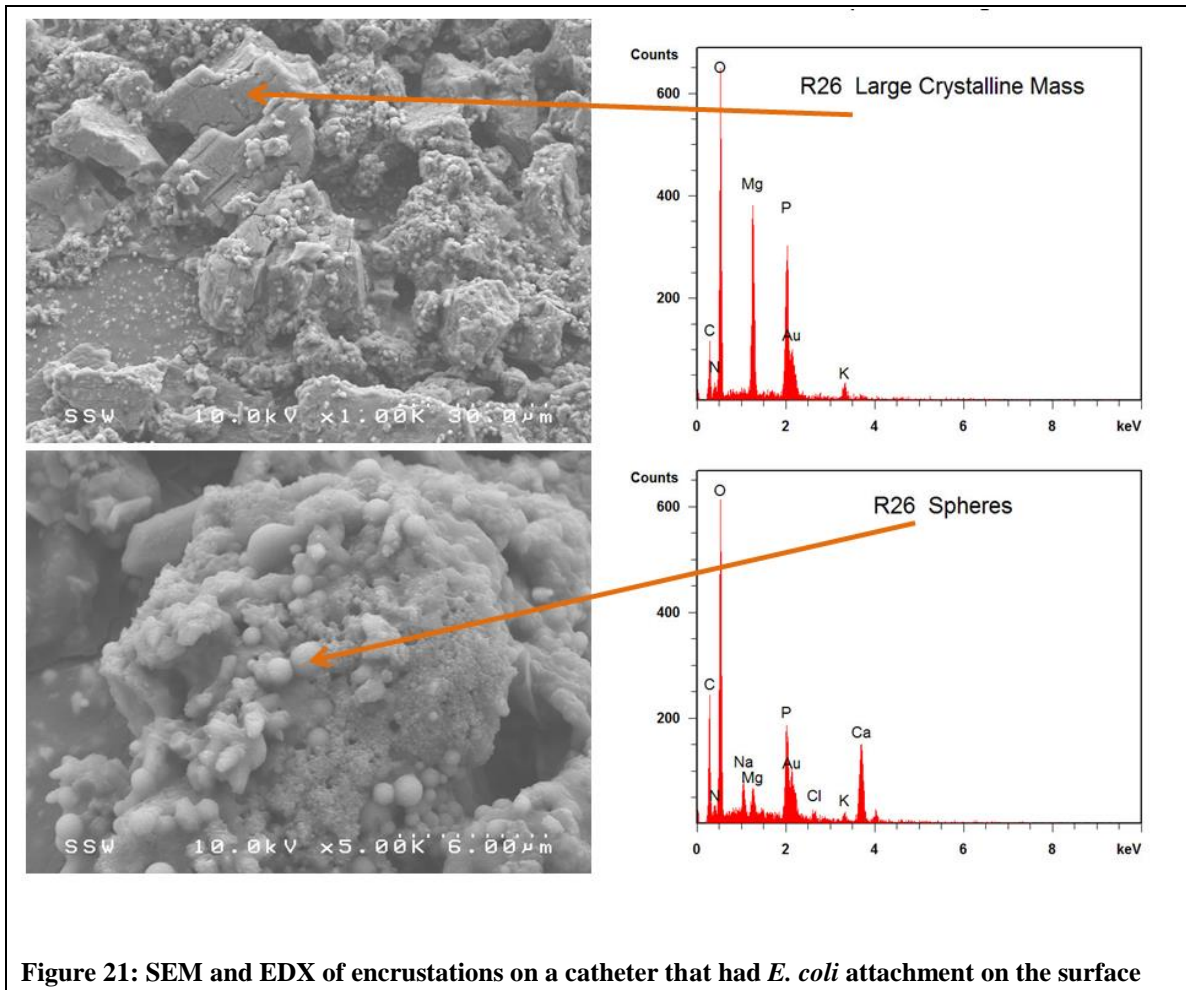


**Figure 20:** SEM picture at 5000X magnification of the same catheter as in Figure 19. Upper right shows EDX of the crystal and lower right shows EDX of the micro-organism on the catheter.

EDX of the encrustations of a rabbit having an *E. coli* infection and *E. coli* attachments on the catheter surface appears to be quite different as is apparent in **Figure 21**. In contrast to the encrustations on the catheters of *non E. coli*-infected rabbits (**Figure 20**), the catheters of *E. coli* infected rabbits do not show any calcium oxalate dihydrate. Two different shapes of encrustations can be identified. The large crystalline mass shows high peaks of Oxygen, Magnesium and Phosphate in its spectrum, which correlates best with struvite ( $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ ). The spectrum of the small spheres on the other hand show high peaks of Oxygen, Calcium and Phosphate. Both EDX spectrum and the SEM appearance are consistent with hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ).

In contrast to analysis of bacterial attachment, we could not identify any *E. coli* on any of the segments that were supposed to have *E. coli* attached to the catheter surface.





**Figure 21: SEM and EDX of encrustations on a catheter that had *E. coli* attachment on the surface**

### 3.1.5 Tissue Bacterial Counts

Comparable to the urine culture and bacterial attachment data, we identified multiple bacterial species, other than the inoculated *E. coli* GR12, inside of or attached to the analyzed tissue. Because of the low bacterial counts for every individual *non-E. coli* species, these were combined as ‘*non-E. coli* bacteria’ number of CFU/mg.

The data on number of rabbits having *E. coli* in bladder, ureter or kidney tissue in each of the groups, are demonstrated in **Table 4**. Statistical analysis with Fisher’s Exact Test

identified that there was a trend towards a difference in infection rate in the bladders between the groups (**P=0.061**). Two-sided Fisher's exact test for between group comparison, showed significantly fewer rabbits in the Coating A group having *E. coli* positive bladders compared to the control group (**P=0.039**). Three of all the ureters analyzed demonstrated *E. coli* on culture. No ureters from the uncoated group had any *E. coli* after culturing, whereas *E. coli* was cultured from one ureter from a coating A rabbit (15.9 CFU/mg) and from ureters from two coating B group rabbits (8100 and 190 CFU/mg respectively). We could not identify any *E. coli* in any of the kidneys. ANOVA on the log(x+1) transformed CFU data in the bladder tissue demonstrated no statistical difference between groups (P= 0.325). When comparing the degree of bacterial counts adherent to or within the bladder tissue of infected vs. uninfected rabbits on day 7 with Welch's ANOVA (as Levene's test indicated heterogeneity of variance), the uninfected animals have significantly lower CFU counts than the infected rabbits (P<0.001 for *E. coli*). When considering any of the other bacterial species present in the tissue, the groups were not statistically different for any of the analyzed tissues.

	Uncoated	Coating A	Coating B	P-value
Bladder	9/12	<b>3/12</b>	6/12	<b>0.061</b>
Ureter	0/12	1/12	2/12	0.758
Kidney	0/12	0/12	0/12	1

Table 4: Number of rabbits that had *E. coli* cultured from the tissue samples.

## 3.2 Results Pig Study

### 3.2.1 Urine Culture

We were not able to collect a urine sample from one pig in the coating A group on day 3. All the other samples were obtained as per protocol.

To identify the influence of the placement and presence of the devices, we compared the UnS group to the UnEC group for presence of UTI and number of CFUs of the different bacteria identified at all time points. Eight of the 12 pigs in the UnS group developed a urinary tract infection by day 1, defined as presence of any *E. coli* in the urine sample. As expected, 11 of the 12 pigs in the UnEC group had developed an infection. In the short-term group, 5 out of 6 of the UnEC pigs had a UTI at endpoint compared to 4 out of 6 in the UnS group at endpoint. In the long-term group, all pigs in the UnEC group had a UTI compared to 4 out of 6 in the UnS group. Two-sided Fisher's exact test could not demonstrate a significant difference between the two groups at any time point.

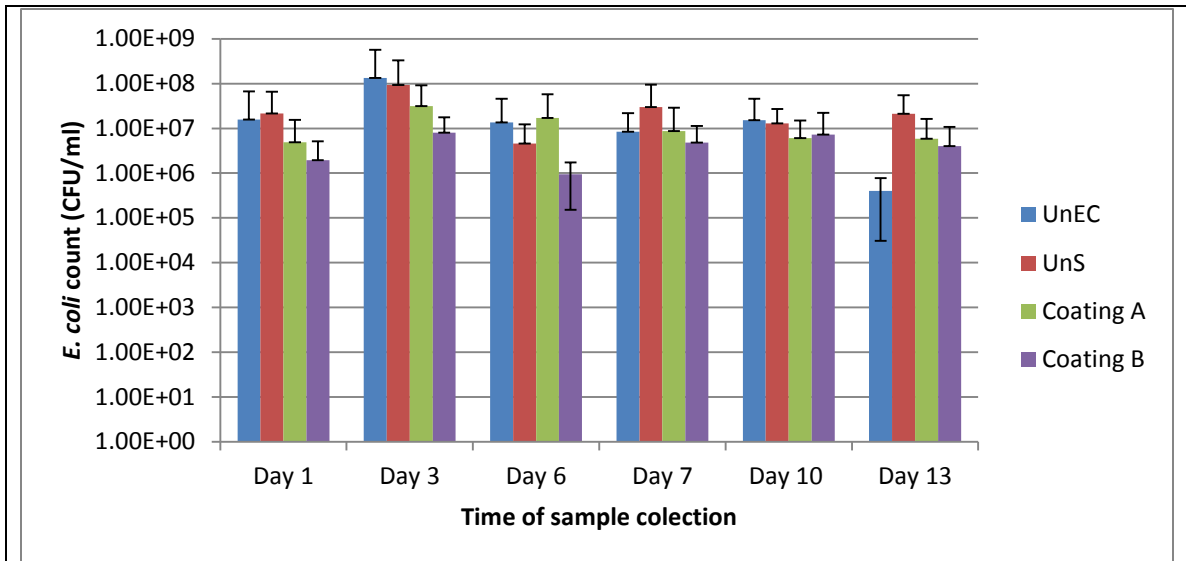
The degree of infection was compared with ANOVA on the  $\log(x+1)$  transformed data. Shapiro-Wilk test showed a significant P-value for the log transformed data, rejecting normal distribution. As such, we performed Kruskal-Wallis as a non-parametric test for continuous variables on the transformed data which resulted in no statistical difference among groups at any time point (**Figure 22**). All data is demonstrated in **Table 5**.

Statistical analysis was repeated for all non-*E. coli* bacteria that were identified. Shapiro-Wilk test demonstrated the data to be non-normally distributed. We therefore applied Kruskal-Wallis tests with post-hoc between group analysis where applicable, which in turn could not identify any significant difference among groups.



		<b>Uncoated Saline</b>	<b>Uncoated EC</b>	<b>Coating A</b>	<b>Coating B</b>	<b>P-value</b>
<b>Day 1</b>	#infected/total	8/12	11/12	12/12	10/12	0.174*
	Mean (SD)	2.15E7 (4.40E7)	1.58E7 (5.06E7)	4.88E6 (1.05E7)	1.96E6 (3.15E6)	0.862 <sup>§</sup>
<b>Day 3</b>	#infected/total	8/12	10/12	10/11	11/12	0.473*
	Mean (SD)	9.31E7 (2.34E8)	1.33E8 (4.31E8)	3.13E7 (6.05E7)	8.07E6 (9.57E6)	0.879 <sup>§</sup>
<b>Day 6</b>	#infected/total	4/6	5/6	6/6	6/6	0.573*
	Mean (SD)	4.61E6 (7.65E6)	1.35E7 (3.26E7)	1.72E7 (4.06E7)	9.43E5 (7.91E5)	0.710 <sup>§</sup>
<b>Day 7</b>	#infected/total	5/6	5/6	6/6	6/6	1.00*
	Mean (SD)	2.98E7 (6.39E)	8.48E6 (1.32E7)	8.64E6 (2.03E7)	4.82E6 (6.44E6)	0.733 <sup>§</sup>
<b>Day 10</b>	#infected/total	4/6	6/6	6/6	6/6	0.217*
	Mean (SD)	1.30E7 (1.41E7)	1.53E7 (3.02E7)	6.08E6 (9.01E6)	7.22E6 (1.52E7)	0.987 <sup>§</sup>
<b>Day 13</b>	#infected/total	4/6	6/6	6/6	6/6	0.217*
	Mean (SD)	2.11E7 (3.36E7)	4.03E5 (3.72E5)	5.88E6 (1.05E7)	3.99E6 (6.73E6)	0.451 <sup>§</sup>

**Table 5:** Number of pigs with *E. coli* infection and severity of infection in CFU/ml. \* = Fisher exact test, § = Kruskal-Wallis on log(x+1).



**Figure 22:** Means and standard deviations of urinary planktonic *E. coli* CFU/ml.

### 3.2.2 Bacterial Attachment

For catheter bacterial attachment, the number of CFU/ml of *E. coli* were calculated and converted to CFU/cm<sup>2</sup>. Proximal, middle and distal segment represent different places in the urinary tract. The proximal segment of the stent was present in the kidney, the middle segment in the ureter and the distal segment in the bladder.

One pig in the uncoated stent saline challenged group did not get a left stent implanted due to technical difficulty of the procedure.

Due to time constraints, the stents collected from one group of 6 pigs that all had surgery on the same date were not immediately processed for analysis after autopsy but kept in storage at -80°C until we were able to process them. After processing, these stent segments showed to have no to very low attachment numbers. To identify whether or not these data influenced the outcomes of statistical analysis, sensitivity analysis was performed with the data from these stents deleted. P-values of sensitivity analysis are reported next to the P-values of analysis of all data in **Table 6 a and b** as ‘P-value b’.

Data was log transformed to fit normal distribution. Shapiro-Wilk test confirmed all log transformed data for this analysis to follow a normal distribution. Levene's test showed the data to be homoscedastic.

No significant differences in bacterial attachment could be identified with statistical analysis. The amount of *E. coli* attached to uncoated or coated catheters was similar between groups at any of the stent segments. P-values obtained after post-hoc analysis with Dunnett's T-test are approaching or equaling 1, emphasizing the similarity between groups. Means and standard deviations of CFU/cm<sup>2</sup> of bacterial attachment on the stent segments are shown in **Table 6 a and b**.

When evaluating the number of stents that had bacterial attachments in the different groups, no statistical difference could be identified with Fisher's exact test. Both in the 6 days and the 13 days indwelling time group, no statistical difference could be demonstrated. The number of stents that had attachments seems however smaller in the coating B group that had stents for 13 days, which is somewhat counterintuitive considering the rest of the data.

For sensitivity analysis, data was ignored from 1 pig in the UnS, 13 day group, 1 pig from the coating A 13 day group and 4 pigs from the coating B 13 day group. This may explain why the number of stent segments displaying attached bacteria seemed so low in the coating B group of the 13 days stented pigs.

After performing sensitivity analysis we can conclude that omitting the results did not substantially change any of the results as the null hypothesis was still retained.

Data from left sided stents were compared to right sided stents and were found to be statistically not significantly different. To increase numbers for statistical analysis, we therefore analyzed all attachment data regardless of stent side. All analyses were repeated including the sensitivity analysis. No significant difference between groups could be identified with Fisher's exact test or ANOVA. Post-hoc analysis could not demonstrate any differences in the between-group analyses.

	<b>Uncoated Saline</b>	<b>Uncoated EC</b>	<b>Coating A</b>	<b>Coating B</b>	<b>P-value</b>	<b>P-value b</b>
<b>Prox Right</b> Mean (SD)	1.20E5 (2.82E5)	8.40E4 (2.25E5)	4.79E4 (7.43E4)	4.54E4 (8.01E4)	0.815*	0.580*
#infected/total	8/12	10/12	10/12	10/12	0.807 <sup>†</sup>	0.507 <sup>†</sup>
6 days	4/6	5/6	5/6	6/6	0.878 <sup>†</sup>	0.878 <sup>†</sup>
13 days	4/6	5/6	5/6	4/6	1.000 <sup>†</sup>	1.000 <sup>†</sup>
<b>Mid right</b> Mean (SD)	1.20E7 (4.13E7)	1.21E5 (4.03E5)	1.37E5 (2.31E5)	7.90E4 (2.35E5)	0.593*	0.883*
#infected/total	7/12	10/12	9/12	6/12	0.359 <sup>†</sup>	0.604 <sup>†</sup>
6 days	3/6	5/6	5/6	4/6	0.766 <sup>†</sup>	0.766 <sup>†</sup>
13 days	4/6	5/6	4/6	2/6	0.463 <sup>†</sup>	0.877 <sup>†</sup>
<b>Distal right</b> Mean (SD)	8.17E4 (1.99E5)	2.46E5 (6.93E5)	4.03E5 (1.26E6)	2.33E5 (7.62E5)	0.673*	0.836*
#infected/total	7/12	9/12	9/12	7/12	0.746 <sup>†</sup>	0.902 <sup>†</sup>
6 days	3/6	4/6	5/6	5/6	0.766 <sup>†</sup>	0.766 <sup>†</sup>
13 days	4/6	5/6	4/6	2/6	0.463 <sup>†</sup>	0.877 <sup>†</sup>

**Table 6A:** Proportions of pigs with bacterial attachment on the right-sided stent and CFU/cm<sup>2</sup> of bacterial attachment on right stent segments. \*=ANOVA, <sup>†</sup>= Fisher's Exact

	<b>Uncoated Saline</b>	<b>Uncoated EC</b>	<b>Coating A</b>	<b>Coating B</b>	<b>P-value</b>	<b>P-value b</b>
<b>Prox left</b> Mean (SD)	3.23E4 (8.00E4)	1.03E5 (2.22E5)	1.70E5 (5.39E5)	2.34E5 (7.07E5)	0.448*	0.580*
#infected/total	5/11	9/12	7/12	5/12	0.366 <sup>†</sup>	0.699 <sup>†</sup>
6 days	2/5	4/6	3/6	4/6	0.843 <sup>†</sup>	0.843 <sup>†</sup>
13 days	3/6	5/6	4/6	1/6	0.180 <sup>†</sup>	0.737 <sup>†</sup>
<b>Mid left</b> Mean (SD)	1.93E3 (5.58E3)	8.71E4 (2.63E5)	5.29E4 (1.28E5)	4.70E4 (8.07E4)	0.541*	0.318*
#infected/total	5/11	7/12	8/12	6/12	0.818 <sup>†</sup>	0.650 <sup>†</sup>
6 days	2/5	4/6	4/6	5/6	0.573 <sup>†</sup>	0.573 <sup>†</sup>
13 days	3/6	3/6	4/6	1/6	0.483 <sup>†</sup>	0.906 <sup>†</sup>
<b>Distal left</b> Mean (SD)	8.55E4 (2.16E5)	4.06E5 (1.04E6)	3.04E4 (4.89E4)	2.888E5 (6.26E5)	0.690*	0.482*
#infected/total	6/11	9/12	9/12	7/12	0.701 <sup>†</sup>	0.600 <sup>†</sup>
6 days	2/5	4/6	5/6	5/6	0.458 <sup>†</sup>	0.458 <sup>†</sup>
13 days	4/5	5/6	4/6	2/6	0.463 <sup>†</sup>	1.000 <sup>†</sup>

**Table 7B:** Proportions of pigs with bacterial attachment on the left-sided stent and CFU/cm<sup>2</sup> of bacterial attachment on left stent segments. \*=ANOVA, <sup>†</sup>= Fisher's Exact

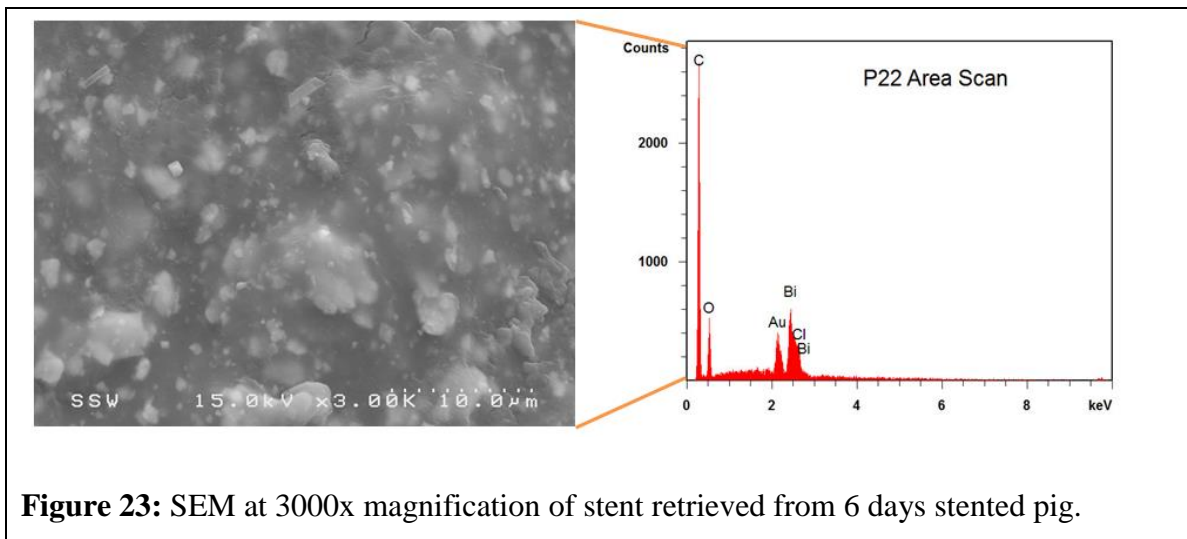
### 3.2.3 Encrustation

In contrast to the rabbits, no stents retrieved from the pigs were found to have any encrustations on the surface or in the lumen, although virtually all the pigs had positive urine cultures at endpoint.

### 3.2.4 SEM/EDX

Scanning electron microscopy with energy dispersive X-Ray Spectroscopy was performed to identify if there were any attachments or encrustations on the stents after indwelling time. As previously mentioned, no encrustations have been found on the stents retrieved from the pigs and the bacterial attachment numbers on the stent surface are fairly low and are not statistically different among groups. With SEM/EDX we aimed to confirm these results and indeed, no encrustations could be detected with SEM.

**Figure 23** is an SEM picture with EDX graph taken from a pig stented for 6 days with an uncoated stent that had an *E. coli* infection and *E. coli* on surface attachments. Although we expected to find bacteria on the surface, we could not find any during SEM analysis. EDX shows no organic material present on the stent. In fact, the stent segment shown in the picture looks almost untouched by the 6 days indwelling in a urinary environment.



**Figure 23:** SEM at 3000x magnification of stent retrieved from 6 days stented pig.

### 3.2.5 Tissue Bacterial Counts

All tissues collected at necropsy were processed and analyzed. As described previously, tissue infection numbers were reported in CFU/mg of tissue. Again, ANOVA showed the groups to have no statistical differences for any of the measured CFU/mg of *E. coli* in tissue samples. Analysis was repeated for other species identified in the tissue and similarly could not demonstrate statistical differences between groups. The means and standard deviations of the CFU/mg of *E. coli* in the tissue samples is available in **Table 7**

	Uncoated Saline	Uncoated EC	Coating A	Coating B	P-value
<b>L Kidney</b> Mean (SD)	1.11E2 (2.33E2)	2.52E2 (6.06E2)	1.09E3 (2.78E3)	6.85E2 (1.77E3)	0.524
<b>R Kidney</b> Mean (SD)	1.82E2 (2.78E2)	5.13E2 (1.57E3)	8.38E2 (1.96E3)	3.35E2 (4.88E2)	0.634
<b>L Ureter</b> Mean (SD)	7.64E2 (2.37E3)	3.39E3 (1.05E4)	3.22E3 (1.00E4)	2.21E3 (5.28E3)	0.281
<b>R Ureter</b> Mean (SD)	1.40E3 (4.23E3)	5.33E2 (1.03E3)	2.15E3 (5.02E3)	6.19E2 (9.08E2)	0.298
<b>Bladder</b> Mean (SD)	1.32E2 (2.53E2)	1.25E3 (4.14E3)	4.65E2 (1.00E3)	3.14E2 (6.14E2)	0.556

**Table 8:** Degree of *E. coli* infection inside of or attached to the tissue in CFU/mg.

When comparing results from the 6 day stented pigs to the 13 days stented pigs, no significant differences were identified. We likewise did not find any differences between

the left sided tissue counts and the right sided tissue counts. As these results were comparable, we decided to group both left and right sided results to increase power of our statistical analysis. Once again however, no statistical differences were found between groups for bacterial tissue counts, demonstrating that the stent coating had no influence on tissue infection numbers.

### 3.2.6 Histopathology

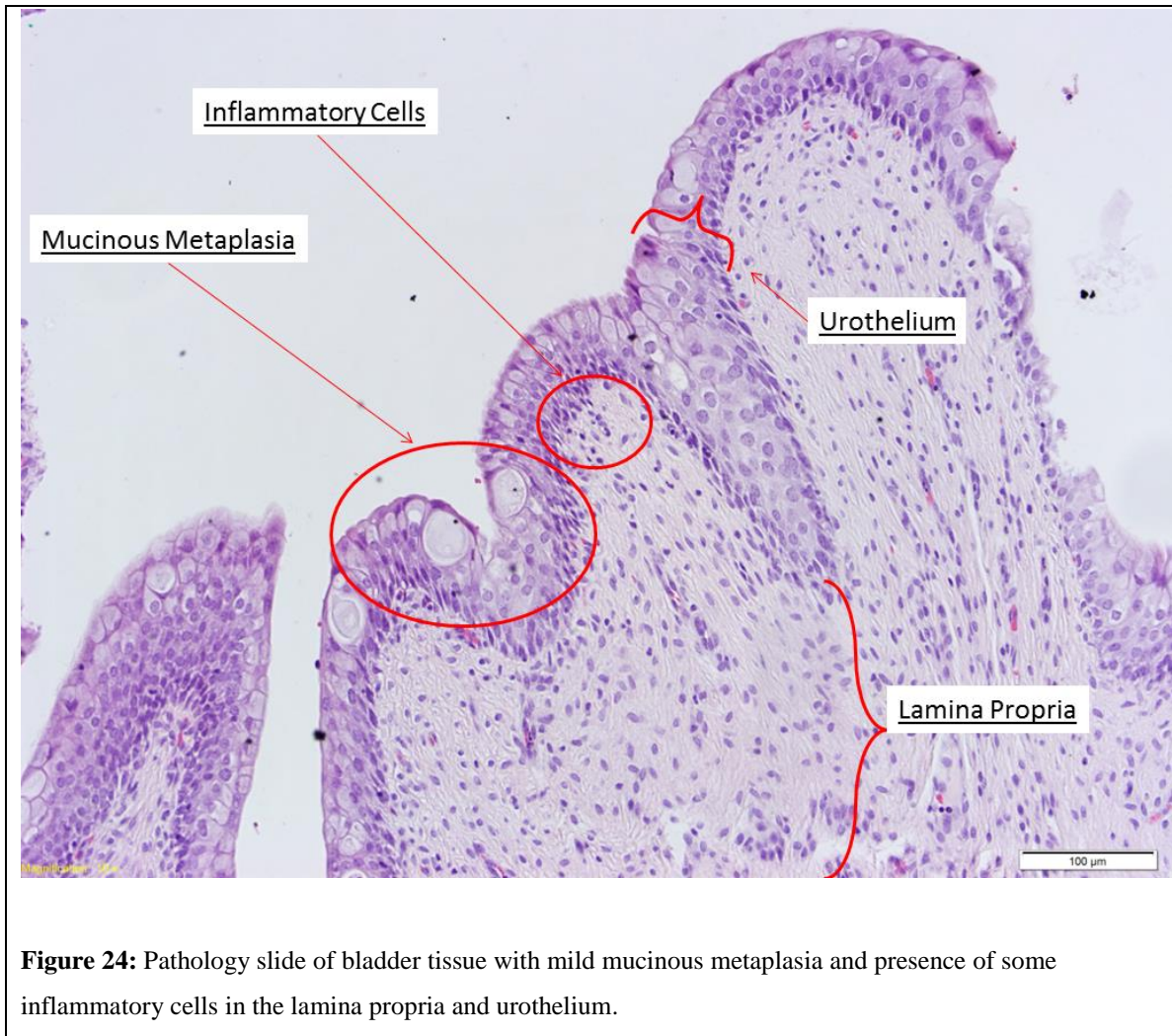
All the tissue from the pigs from the short-term stented group were processed for histopathology analysis. The pathologist was able to accurately read out the slides for all of the samples. As previously indicated he frequently reassessed previously scored slides to prevent score shifting and observed consistency in scoring.

When comparing the scores for all the previously mentioned histopathology changes between coating groups with Fisher's exact test, it appears that there are significantly more pigs from the uncoated saline challenge group that have a higher degree of mucinous metaplasia in the right-sided bladder samples compared to the control group. P-value for the entire comparison was **0.035** and between group analysis identified a significant difference for uncoated saline challenge vs. uncoated *E. coli* challenge with a P-value of **0.03**. The samples from the coating A and B group were not significantly different from the control group. Regression analysis could not point out any confounding factors influencing the mucinous metaplasia after controlling for degree of planktonic infection, amount of attached CFU/cm<sup>2</sup> on the stents, operating time and amount of CFU/mg found internalized in or attached to the bladder tissue. Interestingly, the left-sided bladder tissue samples did not show a significant difference of degree of mucinous metaplasia between groups. There were no other statistical differences between groups compared to the control group for the histopathology scores.

None of the pigs had any fibrosis in the bladder muscle or loss of bladder muscle integrity. Fibrosis was equally absent from the urothelium. Virtually all bladder samples



showed some degree of lamina propria inflammation, mostly mild to moderate. Lamina propria fibrosis (mild) could only be identified in one bladder tissue sample from one pig from the UnS group. A mild degree of sub-urothelial hemorrhage was present in approximately 1/5 of the bladder samples without any significant differences between groups. The bulk of the bladder samples showed no squamous metaplasia. Approximately 15% of pigs had a mild degree and only 4% demonstrated a moderate degree of squamous metaplasia. About a third of pigs showed mild to severe ulceration of the bladder urothelium. A mild degree of urothelial inflammation was present in 83.3% of the right-sided bladder tissue samples with no significance between groups. The left-sided samples on the other hand appeared to be significantly different with Fisher's Exact test for all groups with a P-value of 0.023. Pairwise analysis however could not indicate any group to be significantly different from the control group. **Figure 24** shows a typical example of bladder tissue with mucinous metaplasia and some inflammatory cells close to the bladder epithelium.



**Figure 24:** Pathology slide of bladder tissue with mild mucinous metaplasia and presence of some inflammatory cells in the lamina propria and urothelium.

When comparing the total scores for the bladder tissue, the scores for the right-sided samples are close to being significantly different between groups with a P-value of 0.077. Post-hoc Dunnett's T-Test indicates that the total bladder score for the right-sided samples is significantly higher in the UnS group compared to the UnEC group (Means +/- SD: 5.5 +/- 1.87 vs 3.17 +/- 0.75 respectively, P-value of **0.038**).

For the kidney tissue, we could not identify urothelial fibrosis in any of the pigs. Sub-urothelial and urothelial inflammation was present in virtually all the pigs, ranging from mild to severe (most of the pigs had mild degree) without any statistical differences between groups. Squamous metaplasia was mostly absent in the kidney tissue.

Approximately 38% of the pigs had a mild to moderate degree of sub-urothelial hemorrhage. We found mucinous metaplasia in about 75% of the kidney tissue samples, ranging mainly from mild to moderate with only 2 samples showing severe mucinous metaplasia. Half of the kidney tissue samples of the pigs demonstrated a mild to moderate degree of urothelial ulceration with only severe ulceration in 1 pig. Once again, no statistical differences could be identified with Fisher's Exact test for any of the findings. The total scores for the kidney tissues were comparable.

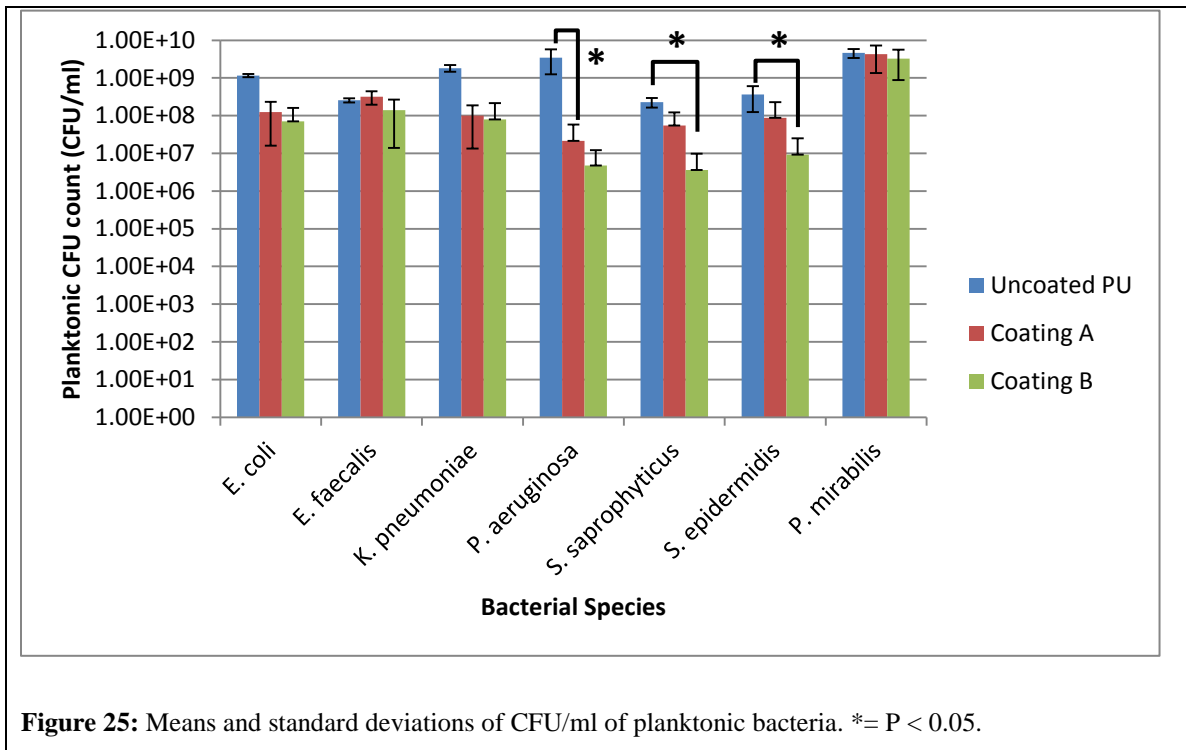
### 3.3 Post-experimental *In Vitro* Evaluation of Coating Efficiency

Analysis with ANOVA of *in vitro* planktonic bacteria demonstrated that there were no statistical differences between groups although there is a trend towards significance for *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus saprophyticus* and *Staphylococcus epidermidis*. Post-hoc analysis with Dunnett's T-test shows that there are indeed significant differences among groups. Stents coated with coating B significantly reduced the CFU/ml of planktonic *S. saprophyticus* and *S. epidermidis*. They showed a trend towards significant influence for *K. pneumonia* and *P. aeruginosa*. The stents coated with coating A demonstrated a significant decrease in CFU for *P. aeruginosa* and a trend towards a significant difference for *K. pneumonia* (**Figure 25**). Interestingly, the catheter coatings don't seem to significantly influence the planktonic *E. coli* infection. All the data on the *in vitro* planktonic infection experiment with means and standard deviations of CFU/ml is available in **Table 8**.

The same analysis was performed for bacterial attachment on the stent segments tested *in vitro*. Significant P-values were calculated for *E. coli*, *P. aeruginosa* and *S. epidermidis*. Post-hoc analysis with Dunnett's t-test identified coating B to induce a significant decrease in bacterial attachment for all of the mentioned species (**Figure 26**). Coating A didn't have a significant influence on bacterial attachment. Data is demonstrated in **Table 9**.

	Uncoated	Coating A	Coating B	P-value	A vs Control	B vs Control
<i>E. coli</i>	1.16E9 (1.07E8)	1.24E8 (1.08E8)	7.11E7 (8.99E7)	0.312	0.519	0.240
<i>E. faecalis</i>	2.56E8 (3.10E7)	3.18E8 (1.23E8)	1.41E8 (1.27E8)	0.214	0.953	0.264
<i>K. pneumoniae</i>	1.82E9 (3.70E8)	9.96E7 (8.61E7)	7.93E7 (1.35E8)	0.092	0.216	0.065
<i>P. aeruginosa</i>	3.48E9 (2.22E9)	2.14E7 (3.70E7)	4.74E6 (7.45E6)	0.055	<b>0.049</b>	0.079
<i>S. saprophyticus</i>	2.29E8 (6.63E7)	5.50E7 (6.79E7)	3.62E6 (6.25E6)	0.064	0.402	<b>0.043</b>
<i>S. epidermidis</i>	3.63E8 (2.40E8)	8.73E7 (1.39E8)	3.62E6 (6.25E6)	0.061	0.281	<b>0.040</b>
<i>Proteus mirabilis</i>	4.62E9 (1.25E9)	4.28E9 (2.93E9)	3.23E9 (2.35E9)	0.714	0.831	0.635

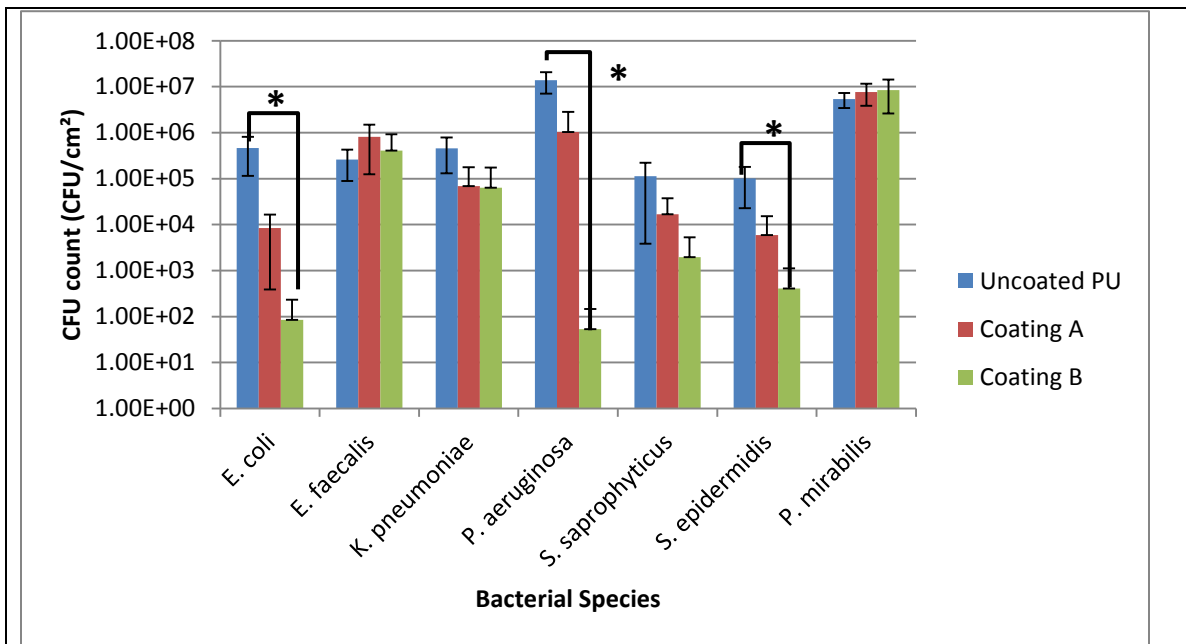
**Table 9:** Means and standard deviations of CFU/ml of planktonic bacteria from *in vitro* experiment.



**Figure 25:** Means and standard deviations of CFU/ml of planktonic bacteria. \*= P < 0.05.

	Uncoated	Coating A	Coating B	P-value	A vs Control	B vs. Control
<i>E. coli</i>	4.61E5 (3.46E5)	8.38E3 (7.99E3)	8.49E1 (1.47E2)	<b>0.028</b>	0.118	<b>0.018</b>
<i>E. faecalis</i>	2.58E5 (1.68E5)	8.10E5 (6.86E5)	4.06E5 (5.15E5)	0.375	0.370	1
<i>K. pneumoniae</i>	4.58E5 (3.27E5)	6.91E4 (1.07E5)	6.37E4 (1.10E5)	0.221	0.409	0.165
<i>P. aeruginosa</i>	1.38E7 (6.69E6)	1.03E6 (1.78E6)	5.30E1 (9.18E1)	<b>0.023</b>	0.080	<b>0.016</b>
<i>S. saprophyticus</i>	1.12E5 (1.08E5)	1.67E4 (2.04E4)	1.94E3 (3.36E3)	0.161	0.419	0.113
<i>S. epidermidis</i>	1.01E5 (7.78E4)	5.92E3 (9.23E3)	1.94E3 (3.36E3)	<b>0.019</b>	0.221	<b>0.012</b>
<i>P. mirabilis</i>	5.34E6 (1.91E6)	7.66E6 (3.83E6)	8.46E6 (5.83E6)	0.848	0.812	0.872

**Table 10:** Means and standard deviations of CFU/cm<sup>2</sup> of bacterial attachment on stents.



**Figure 26:** Means of CFU/cm<sup>2</sup> of bacterial attachment on stents. \*= P < 0.05

## Chapter 4

### 4 Discussion and Conclusions

The projects presented in this thesis have been preceded by years of research and experimenting. Jeff Dalsin and co-workers started off with the notion that DOPA could be of use for coating PEG, a known potent antifouling molecule, onto surfaces in a robust way and in a high density, which had been somewhat of a challenge before (Lee et al., 2002).

Next came the novel idea of applying this coating onto surfaces that reside in a very harsh milieu such as urine (Ko et al., 2008). Ko et al had performed *in vitro* experiments with Titanium coated silicone disks on which they had applied an mPEG-DOPA<sub>3</sub> coating. After submersion in pooled human urine with several uropathogens, the coated disks seemed to strongly resist bacterial attachment (Ko et al., 2008). Once Ko et al had established that the coating was functioning as expected in a urinary environment, the time had come to apply the coating on a urinary device that could be used *in vivo*. Pechey et al applied different formulations of the mPEG-DOPA<sub>3</sub> coating on stent curls and implanted those in rabbit bladders, together with an inoculum of *E. coli* (Pechey et al., 2009). Following their experiments, they identified a cross-linked polymer (Surphys-009) to be clearly more efficient and durable than a linear copolymer in reducing an induced urinary tract infection. Based on this cross-linked structure of mPEG-DOPA<sub>3</sub>, further research and development has led to a new DOPA-anchored copolymer formulation used in this project: Surphys-095. Quaternary amines and silver nitrate were added to the coating to theoretically increase the bactericidal effect of the coating.

After proving the coating's efficiency *in vitro*, we performed *in vivo* experiments with rabbits and pigs.

## 4.1 Rabbit Study

### 4.1.1 Urine Sample Analysis

The rabbit experiment demonstrated the devices coated with coating A to efficiently reduce the chance of getting an infection after *E. coli* inoculation by day 7 (endpoint) of the experiment. Contrary to what we expected, the devices coated with coating B did not have a significant effect on reducing infection. Whereas 83% of the rabbits implanted with uncoated devices had a positive urine sample at endpoint, only 36% of the rabbits in the coating A group and 50% of the rabbits in coating B group had a positive urine sample at endpoint. We assume that a larger sample size might shift the results in favor of the coatings.

The degree of infection was similar between groups at endpoint indicating that if an infection does occur despite the antibacterial effect of the coated device, it is no different than in a rabbit catheterized with an uncoated catheter. This too is somewhat contrary to the expectations. We had expected that the animals getting an infection in the coated catheter groups, would have a lower degree of infection than the rabbits in the uncoated catheter group.

The presence of other bacteria in the urine sample is most probably due to our bladder catheter model in the rabbit. To allow the rabbits a free range of mobility, we did not fit a urine collection bag on the catheter which resulted in an open catheter tip that potentially allows external bacteria to migrate to the inside of the bladder through the catheter. We often noticed rabbit's excretions present on the distal part of the catheter and as such it is very likely that this was the source of the other bacteria in the urine samples. To prevent accumulation of excretions in and on the tip of the catheter as an inducing factor for further infection, about 0.5 to 1 cm was cut off of the external (distal) part of the catheter each time samples were collected. The fact that the number of rabbits with other bacterial species present in the urine sample increased over time (2 on day 1 and 22 on day 7) reflects this problem. The initial rabbit model for catheter associated urinary tract infection as reported by Morck and Olson (Morck et al., 1993; Olson et al., 1989) describes the rabbits residing in a Plexiglas cage and fed mainly with intravenous liquids. On occasion, they would eat. This kind of setup is not possible in our facility due to

general ethical rules in place requiring the animals to have free range of motion and environmental enrichment. The adjusted model, placing stent curls in the bladder, as described by Fung et al (Fung et al., 2003) is a very interesting model that has been used by our research group on several occasions (Cadieux et al., 2006; Pechey et al., 2009). However, as in that model, there is no external portion to the urinary device, it is not fully representative of urethral catheterization, but more of a distal stent curl in the bladder.

#### 4.1.2 Device Bacterial Attachment

Although the bacterial attachment numbers of CFU were not statistically different between coatings, we did demonstrate that the rabbits that did not have an *E. coli* positive urine sample at endpoint had fewer *E. coli* CFU attached to the catheter segments. Similar results were obtained when repeating the analysis for the other bacterial species identified in the urine cultures, with P-values <0.05 indicating fewer CFU on the proximal catheter segments in the uninfected rabbits compared to the infected rabbits. We did indeed expect that if a rabbit does not have certain bacteria in the urine, it would not have that bacteria attached on the catheter surface. However, some rabbits that didn't have *E. coli* in the day 7 urine culture did have some *E. coli* attached on the catheter surface. The most probable explanation for the *E. coli* attachment in uninfected rabbits is that the inoculated bacteria attach on the catheter surface (on conditioning film) quite soon after inoculation, before planktonic bacteria get eradicated by the antibacterial effects of the catheter coating, and that they get covered by encrustations.

#### 4.1.3 Tissue Bacterial Counts

Similar to the number of infected rabbits, there were less rabbits in the coating A group that had bacteria attached to or invaded in the bladder tissue compared to the other groups. Considering that fewer rabbits had *E. coli* infections by day 7 in the coating A group, this was an expected result. However, the number of CFU of adherent or invaded bacteria was not different between groups. Once again we demonstrated that infected animals had higher CFU counts in the tissue than uninfected animals, regardless of which catheter had been placed.



#### 4.1.4 Encrustations

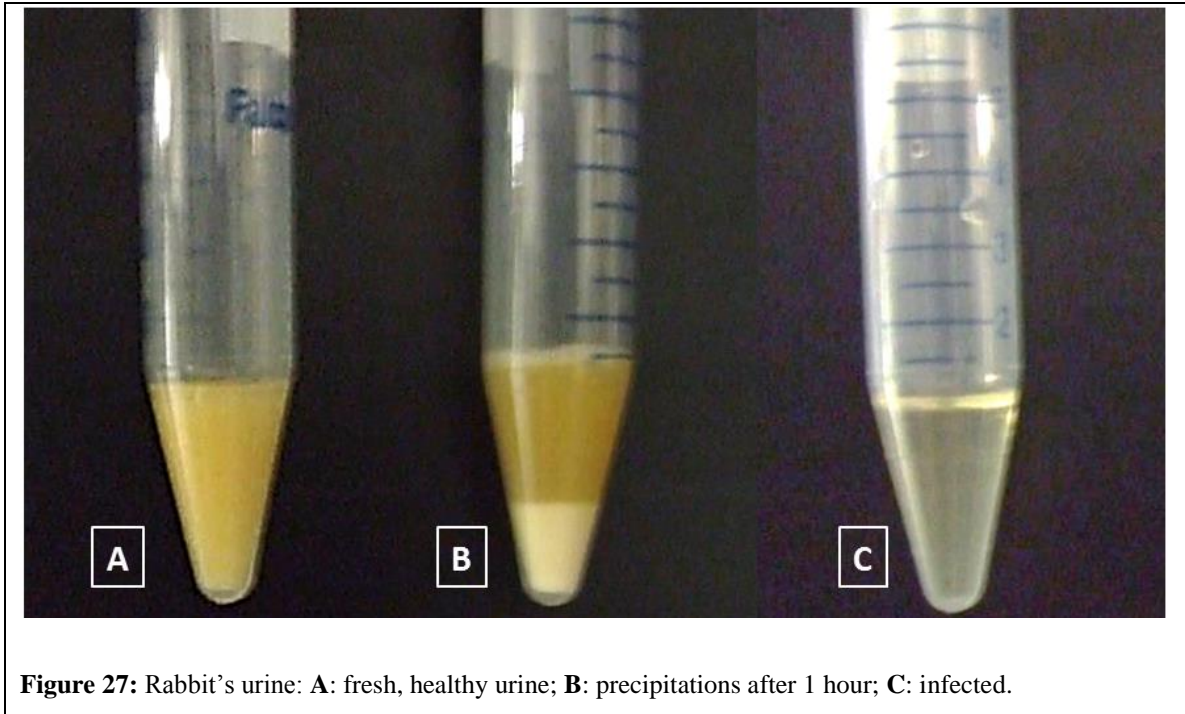
Analysis of the amount of encrustations on the devices has demonstrated that both coating A and B have on average more encrustations on the surface than the uncoated devices, however only the coating B devices had significantly more encrustations on the surface than the control catheters.

Further analysis of our data confirmed the conclusion by Pechey et al that rabbits with an *E. coli* infection have less encrustations than rabbits that didn't have an *E. coli* infection (Pechey et al., 2009). Additionally, in contrast to these findings, we demonstrated that the rabbits that have an infection with *non-E. coli* bacteria have more encrustations than the other rabbits. Whereas an infection with *E. coli* has an inverse correlation with encrustation, the opposite effect appears to be true for infection with *non-E. coli* bacterial species. Furthermore, the composition of the encrustations appears to be different in *E. coli* vs. *non-E. coli* infected rabbits.

As mentioned in section 2.6, encrustations on the device surface are dependent of multiple factors, including the indwelling time, urinary pH and some predisposing and inhibitory protein found in the urine and in the conditioning film (Canales et al., 2009; Choong et al., 2001; Kawahara et al., 2012; Santin et al., 1999; Suller et al., 2005). Indwelling time was identical for all the rabbits and urinary pH did not seem to be strongly correlated to the amount of encrustations on the catheter. The high amount of encrustations in uninfected rabbits is due to the very nature of healthy rabbit's urine. Healthy rabbit's urine has a very cloudy and turbid appearance and is very precipitative in nature as a result of their herbivorous diet (**Figure 27**). Kiwull-Schöne and colleagues have demonstrated that the alkali load of the rabbit's diet directly influences the urinary pH (Kiwull-Schöne et al., 2005). Whereas the urinary pH is above 8 in rabbits on a normal to high alkaline diet, it is strongly reduced to an average of 6.27 in rabbits on a low-alkaline diet (Kiwull-Schöne et al., 2005). When feeding rabbits a meat based diet, apparently the urine rapidly turns clear and acidic (Arunachalam and Woywodt, 2010). The rabbits in our facility were provided with Purina Prolab® High-Fibre Rabbit 5P25 Lab Diet which consists mainly of soy and alfalfa sprouts that both carry a high alkali load. The urinary pH of the rabbits at day 0 was consistently between 8.5 and 9.0. With a

high baseline urinary pH, the magnesium, phosphate and calcium ions already have a high potential for precipitation. Despite the high-alkaline diet, we did however notice changes in the urinary pH during their follow-up to as low as 6.5. Although statistical analysis could not demonstrate a direct correlation between encrustations and urinary pH, interestingly we did see a significantly lower urinary pH in the rabbits that had an *E. coli* infection without any other bacterial species present in their urine culture. The fact that *E. coli* infected animals have fewer encrustations makes more sense now that we know that the *E. coli* infected rabbits have a lower urinary pH. We previously pointed out that a higher urinary pH will predispose the constituents in the urine to precipitate more. Presumably this change in pH is caused by the host's immune response that consists of an influx of plasma, neutrophils, ions, cytokines and multiple other unknown factors, which can react with the constituents in the rabbit's urine and thus decrease the urinary pH. These factors entering the bladder as part of the host's immune response may have inhibitory or precipitative characteristics with regard to deposition of a conditioning film and encrustations onto the device surface. The results from cytokine analysis could potentially aid in answering the question at hand.

Canales et al have identified several proteins influencing deposition of encrustations onto stent surfaces in a human urinary environment (Canales et al., 2009). The authors had already pointed out that injury and defense related proteins are more commonly found in the conditioning film of encrusted stents than in non-encrusted stents retrieved from humans (Canales et al., 2009). They describe immunoglobulin kappa and  $\alpha$ 1-anti-trypsin, proteins as important factors influencing encrustation. Santin and co-workers demonstrated the protective effect of Tamm-Horsfall protein and  $\alpha$ 1-microglobulin (Santin et al., 1999). Sadly enough, the small sample sizes of these papers (5 encrusted stents vs 22 non-encrusted stents in Canales et al and 2 encrusted vs 2 non-encrusted stents in Santin et al) reduce the power of the analysis. It would be very interesting to conduct this research on a vastly larger sample size and to not only analyze the proteome but also the microbiome of both conditioning film and patients' urine sample.



**Figure 27:** Rabbit's urine: **A:** fresh, healthy urine; **B:** precipitations after 1 hour; **C:** infected.

With the aid of SEM and EDX, we were able to identify the encrustations on catheters from *E. coli* and *non-E. coli* infected rabbits. As can be seen on **Figures 19 and 20**, not only the amount but also the composition of the encrustations changes substantially when comparing devices from infected and uninfected animals. Again, we would expect the hydroxyapatite and struvite to deposit more frequently in *non E. coli* infected bladders with a higher pH, which was not the case here. The respective rabbits from which the catheters have been analyzed for SEM, had a urinary pH that never dropped below 8.5. Although no statistical differences were noted, we do believe that the presence of other bacteria and a possible change in pH is the causal factor of the difference in both composition and amount of encrustations on the surface of the catheters.

Overall, coating A demonstrated the best performance in clearing an induced *E. coli* infection and in reducing the infection rate into the bladder tissue. The fact that Coating B has a higher density of both S-095 and silver, built up the expectation that the rabbits in the coating B group would have had even better results than the rabbits in the coating A

group. We did see a trend towards fewer rabbits infected in the coating B group but no statistical analysis could demonstrate a significant difference between the control group and the coating B group. This is quite contrasting with the preliminary *in vitro* results that showed eradication of not only *E. coli* but also other uropathogens by both coatings. It is possible that the rabbit urinary environment is too harsh for the coatings and that the coatings can't cope with the alkaline urine and the quick deposition of crystals onto the catheter surface. Once the coating is covered with encrustations, the kill-by-contact effect of the quaternary amines seems to be nullified

The current literature on stent or catheter related research in a rabbit model uses mainly one of two models: the closed drainage urinary catheter model as described by Morck and Olson (Morck et al., 1994, 1993; Olson et al., 1989) or the transurethral insertion of stent curls into the bladder which are later retrieved at necropsy (Fung et al., 2003). Our own model deviates somewhat from both which makes comparison to the literature somewhat more difficult. The most recent research on catheter and stent coatings in a rabbit model have evaluated Gendine, Triclosan and mPEG-DOPA<sub>3</sub> as potential coatings preventing CAUTI (Cadieux et al., 2006; Hachem et al., 2009; Pechey et al., 2009). Hachem and colleagues adopted the rabbit model from Morck and inoculated the rabbits with *E. coli* by applying 10E9 CFU at the urethral meatus for 4 consecutive days (Hachem et al., 2009). They compared the new coating (8 rabbits) to an uncoated silicone catheter (9 rabbits), a hydrogel (7 rabbits) and a silver-coated catheter (7 rabbits). After 3-5 days, none of the rabbits with a gendine-coated catheter had a positive urine culture, whereas more than half of the rabbits of the other groups did. Despite the promising results, no publications on further research with gendine-coated catheters has emerged. Cadieux and his colleagues have evaluated the efficacy of Triclosan coated stent curls against *P. mirabilis*, using the Fung model (Cadieux et al., 2006). The stent curls showed significant reduction in *P. mirabilis* and were further investigated in human trials. Although they seem to reduce urinary tract symptoms and the incidence of symptomatic CAUTI, reducing the use of antibiotics, they do not significantly reduce the incidence of bacteriuria and stent bacterial attachment (Cadieux et al., 2009; Mendez-Probst et al., 2012). Our own group had previously used the Fung model to investigate mPEG-DOPA<sub>3</sub> copolymer coatings, demonstrating significant efficiency of the cross-linked polymer S-

009. The main issue with our model was the open catheter tip, allowing for external bacteria to migrate into the bladder. Regardless, we did see a beneficial effect in the coating A group, demonstrating efficiency in the face of bacteria flowing in.

## 4.2 Porcine Study

### 4.2.1 Urine Culture, Device and Tissue Bacterial Attachment

Unlike the rabbit project, we included an extra control group to the pig study. 12 pigs had stents inserted but did not get an *E. coli* challenge. This group was added to allow us to identify the effect of the procedure and the presence of the stent on the animal's immune response. In contrast to our expectations, a large amount of pigs developed a urinary tract infection with *E. coli* or any of the other bacterial species. We had expected to see that the saline challenge would be a good control for the *E. coli* challenge.

Urinary tract infections in sows in commercial pig breeding farms are quite common to the extent that they are an important cause of mortality and are thought to result from the housing conditions of these pigs (Bellino et al., 2013; Glock and Bilkei, 2005; Karg and Bilkei, n.d.; Mauch and Bilkei, 2004). The prevalence of urinary tract infections in pigs bred for and housed in research facilities is unknown. 3 out of 48 pigs appeared to have a positive urine sample at day 0, before the intervention. The main reason as to why all the pigs eventually did get a urinary tract infection is most probably due to the procedure. Despite the three day acclimatization period that all the pigs had before proceeding with the procedure, most of the pigs were still dealing with the stress of moving from the farm where they were bred to the University animal facility. Pigs are very prone to stress and then usually get diarrhea. Despite our best efforts to perform a sterile procedure for stent placement, it was almost inevitable to have some fecal soiling of the operation field. This means that by placing the stent in the pig, we probably inoculated the pigs urinary tract with fecal flora and probably in a higher concentration than the  $10E7$  of *E. coli* that we inoculated the pigs with at the end of the procedure.

The fact that we didn't see any statistical differences for any of the groups regarding urinary tract infections, bacterial attachment and tissue attachment and internalization is to be explained by multiple factors. First of all and probably very important in this model, is that the inoculum of fecal bacteria that was inserted with the procedure was probably that concentrated with such a high amount of bacteria that the stent coating was overwhelmed with the plethora of bacteria and the degree of infection and could not deal with the infection because of the sheer abundance of bacteria. Additionally, the post-animal study in vitro experiments should be taken into account. All the devices used for every part of this study were produced at the same time, before the study started. As the rabbit experiment was conducted first, the coated stents had not been stored as long as for the pig study. In contrast to the pre-study in vitro experiments, we could only demonstrate significant in vitro activity of the stents coated with Coating B for *S. saprophyticus* and *S. epidermidis*. Coating A had significant activity against *P. aeruginosa*. Neither of the stents was efficient in clearing the planktonic *E. coli*, which was the core goal of our study. It's safe to say that the coating may have lost some of its activity from the time of production to the time of insertion in the pigs and of in vitro re-assessment. We can only hypothesize why this would have happened. The SEM pictures of the coated stents showed the coating to be homogeneously distributed on the stent surface. They also quite nicely showed the difference in S-095 density and the presence of the silver particles. The DOPA molecule of S-095 was covalently bonded with the amine (NH<sub>2</sub>) groups on the stent surface. We know that the coating is very hydrophilic and 'wants' to interact with water. It is possible that water got through the packaging and that the coating started interacting with it. On the other hand, the coating may have become somewhat brittle and more fragile at the surface. The shear stress of placing the stent into the pigs urinary tract may have been that high that the coating sloughed off during the process.

#### 4.2.2 Encrustations

Although all the pigs had a positive urine sample at endpoint, none of the stents retrieved from the pigs had any encrustations on the surface. This is in stark contrast to our findings in the rabbits, where we found a high amount of encrustations on all of the

devices. In rabbits, an inverse correlation was demonstrated between infection and encrustation. It would however not be correct to extrapolate this statement to the pig model. As mentioned in the background chapter of this manuscript, the presence of encrustations is influenced by multiple factors. It's possible that the lack of encrustations is due to a more acidic urine (at which phosphate and magnesium would not precipitate as easily as at an alkaline pH).

### 4.2.3 Histopathology

Histopathology analysis demonstrated that the right-sided bladder tissue from pigs in the uncoated stent saline group showed a higher degree of mucinous metaplasia than the other animals. We controlled for all the possible other confounders (that we have data for) such as degree of infection of any of the bacterial species cultured, amount of CFU in the bladder tissue, amount of CFU on the stent surface, OR-time and urinary pH. None of these factors had any influence on the mucinous metaplasia. Similarly, the total scores for the right-sided bladder tissue samples was significantly higher for the UnS pigs compared to the UnEC pigs. The strange fact that the uncoated saline challenge pigs had more histopathology changes (they were supposed to be negative controls for the uncoated stent *E. coli* challenge pigs) is probably due to the small sample size of data that we have for the histopathology. For each of the groups, we only have histopathology data for 6 animals in each arm. We assume that the significant differences noted in the statistical analysis are by pure chance alone rather than due to an actual detrimental effect of the polyurethane stent on the bladder tissue. Additionally, we would have expected the results in the left-sided tissue samples to be somewhat similar to the right-sided if the polyurethane catheter would actually have had a negative effect on the tissue. The absence of any significant differences for any of the other histopathology changes measured between the groups strengthens this hypothesis.

The porcine model has been extensively used for stent related research, as mentioned in section 3.2. The bulk of these experiments however deal with biodegradable and symptom-reducing stent designs and biomaterials which is in contrast to our own current study on anti-fouling and anti-bacterial coatings on stents. The theory of biodegradable stents on the other hand is increased biocompatibility, which in turn should translate into

a lower device associated urinary tract infection rate. The Uriprene™ stent has been reported to induce fewer urinary tract infections than a control stent (Hadaschik et al., 2008). All animals in this study were given antibiotic prophylaxis. A newer formulation of the Uriprene™ stent that dissolves in 4 weeks instead of 10 weeks (as the previous formulation did) on the other hand had a higher infection rate than the control stents (Chew et al., 2013). Pigs did not get prophylaxis in this study. In the study by Lumiaho and colleagues on a short helical biodegradable stent, no infections were reported in the pigs (Lumiaho et al., 2011). The short segmental biodegradable stent has the advantage of not having a portion of the stent in the bladder which conceptually could have a lower risk of getting infected. They bypassed the potentially soiled urethra by placing the stents through a small cystotomy. Kallidonis and Liatsikos compared cytostatic-drug-coated metal stents to bare metal stents for long term stenting of malignant ureteral obstruction in a porcine model (Kallidonis et al., 2011; Liatsikos et al., 2007). Infection rates were not mentioned in these studies.

In conclusion, it is currently impossible to adequately compare our results to the literature as our research is currently the only porcine study performed with the goal of testing a ureteral stent coating preventing encrustations and urinary tract infections.

### 4.3 Future Directions

The discrepancy between the rabbit data and the pig data, together with the results of the post-animal study *in vitro* experiments has indicated that the coating is subject to a certain shelf-life. This shelf-life of the device coatings will need to undergo some further evaluation and testing. Durability testing to ensure the coatings are not displaced at the time of insertion as well as testing of the packaging will require further study.

The rabbit model has some limitations due to ethical rules in place. The rabbit model for catheter associated urinary tract infection that was proposed by Morck and others (Morck et al., 1994, 1993; Olson et al., 1989) had the rabbits sitting in a glass or Plexiglas cage without any range of motion and with an intravenous catheter as their main source of



fluid intake and nutrition. This of course allowed the researchers to have a collection bag fitted on the catheter, which prevents the influx of any bacteria from outside to inside. As the animal ethics require the animals to have free range of motion, we could not fit a collection bag on the catheter and thus could not prevent bacteria to migrate into the bladder via the catheter.

The pig model was chosen for our model of stent related infection because the availability of the model and anatomical resemblance to the human urinary tract. Current project shows that although the theory of this model was quite logical and very feasible, the results have raised methodological issues. Alternate models or a different approach of placing the stents (via cystotomy in lieu of transurethrally) need to be considered for future in vivo work.

Taking the theoretical background, preceding and current research into account, especially considering the pitfalls of the current model, we do believe that the currently developed coatings deserve further research and may in the future be applied on stents and catheters for human use.

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## Appendices



**AUP Number:** 2012-034

**PI Name:** Cadieux, Peter

**AUP Title:** Efficacy Of Novel Antifouling/antimicrobial Coatings For The Prevention Of Urinary Device Infection And Encrustation

**Approval Date:** 10/25/2012

**Official Notice of Animal Use Subcommittee (AUS) Approval:** Your new Animal Use Protocol (AUP) entitled "Efficacy Of Novel Antifouling/antimicrobial Coatings For The Prevention Of Urinary Device Infection And Encrustation" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2012-034::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura  
on behalf of the Animal Use Subcommittee  
University Council on Animal Care

**Appendix A:** Animal Use Subcommittee Approval document

## Curriculum Vitae

**Name:** Thomas Tailly

**Post-secondary Education and Degrees:** **The University of Western Ontario  
London, Ontario, Canada  
2013-2014: Master of Science – Surgery**

University of Western Ontario  
London, Ontario, Canada  
2013-2015 Endourology Fellowship

Catholic University of Leuven  
Leuven, Belgium  
2007-2013 Urology Residency

Catholic University of Leuven  
Leuven, Belgium  
2000-2007 MD

**Honours and Awards** Belgian Urology Association Research Grant  
2012-2013

Boston Scientific Endourology Research Grant  
2013-2014

Western Graduate Research Scholarship  
2013-2014

Best Presentation by a Fellow at London Ontario Urology Research  
Day 2014

CUA AUA Canadian Fellows Grant 2014

**Publications** MP8-18 Use of Novel Antimicrobial Coatings on Urinary  
Catheters for Prevention of *E. coli* infection in a rabbit model. *The  
Journal of Urology, Volume 191, Issue 4, Supplement, April 2014,*



*Page e81*, Roderick MacPhee, Justin Koepsel, Ian Welch, Jeff Dalsin, **Thomas Taily**, Peter Cadieux, Jeremy Burton, Hassan Razvi

**Public Lectures** Urology Research Day, April 4, 2014, London, Ontario, Canada