Development of a Nanoparticulate Formulation of Docetaxel for the Treatment of
Non-Muscle-Invasive Bladder Cancer

by

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Abstract

Approximately 70-85% of bladder cancer patients present with non-muscle invasive bladder cancer (NMIBC). These patients are usually treated by surgical resection of bladder tumours followed by the intravesical administration of anticancer drugs such as mitomycin C (MMC), doxorubicin or gemcitabine. However, the recurrence rate after 5 years remains high (70%) so that the development of more effective chemotherapeutic strategies is essential. We have previously shown that hyperbranched polyglycerol (HPG-C$_{8/10}$-MePEG-NH$_2$) nanoparticulate carriers of docetaxel (DTX) offered an improved and effective formulation of the drug for intravesical delivery in mice. The present work describes the effect of concentration and exposure times of three HPG-C$_{8/10}$-MePEG-NH$_2$‘s with increasing degrees of amineation on ex vivo porcine bladder tissue morphology and the tissue depth uptake of DTX. The results demonstrated the exfoliation of porcine bladder tissues in a time and concentration-dependent manner. Exfoliation and DTX uptake was significantly enhanced upon treatment with medium or high-density HPG-C$_{8/10}$-MePEG-NH$_2$‘s, as compared to a commercially available DTX/polysorbate 80 formulation. Further studies on the local effect of the chemotherapeutic agents MMC, doxorubicin and gemcitabine, on ex vivo porcine bladder tissue showed that these drugs all caused exfoliation of urothelium and were well taken up by the bladder tissue with no additional effect of HPG-C$_{8/10}$-MePEG-NH$_2$ pre-treatment. The exfoliating effect of these three drugs was shown to enhance the bladder tissue uptake of paclitaxel (PTX) or DTX when the bladder was exposed to combinations of taxanes with either MMC, doxorubicin or gemcitabine. Generally, the exfoliation effect of HPG-C$_{8/10}$-MePEG-NH$_2$‘s, MMC, doxorubicin and gemcitabine is attributed to an interaction of the positively charged amine groups on all these agents with the negatively charged mucosal surface. This binding may modulate tight junction
protein function followed by exfoliation of the protective urothelial layer so that drugs may penetrate the exposed underlying tissue.

In conclusion this thesis supports a novel role of DTX loaded-HPG-C_{8/10}-MePEG-NH_2 nanoparticles as an improved drug delivery vehicle for the potential chemotherapeutic treatment of bladder cancer. Additionally, data suggests promising strategies for intravesical combination drug therapies, to enhance the uptake of taxanes with potential additive therapeutic effects for improved efficacy in the treatment of NMIBC.
Preface

This thesis is comprised of the following two manuscripts of which I am the principal author.

   (The manuscripts are in preparation for submission).

   (The manuscripts are in preparation for submission)

Chapter 2 was based on manuscript 1 and Chapter 3 was based on manuscript 2. I was the primary individual responsible for the design of this study, conduct of the research experiments, analysis and interpretation of the data, and preparation of the manuscript. John Jackson helped with experimental design, conduct of experiments, and preparation of the manuscripts. The drug uptake studies were done in Dr. Helen Burt’s lab in the Faculty of Pharmaceutical Sciences, UBC and exfoliation images were taken at the Bio-Imaging Facility, Department of Botany, UBC. Hyperbranched polyglycerols (HPGs) were a kind gift from the Centre for Drug Research and Development (CDRD). The contribution of the other co-authors was through the provision of intellectual discussion and editorial assistance. There is no ethical approval needed for this research studies.
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Figure 3.8 AUC values for total drug accumulation in bladder tissue following dual drug treatments. Bladders were treated with PTX micelles (0.5mg/ml) or PTX micelles in combination with MMC, DOX, or GEM (1mg/ml). Some tissues were pretreated with HPG-580 (10% w/v) for one hour followed by PTX (0.5mg/ml). The data was compared between control and treatments by one-way ANOVA and significance is shown with p values (*p<0.05, ***p<0.0001).

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<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>CO₂/O₂</td>
<td>Carbon dioxide/Oxygen</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>DTX</td>
<td>Docetaxel</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HPG*</td>
<td>Hyperbranched polyglycerol</td>
</tr>
<tr>
<td>HPG-C8/10-MePEG</td>
<td>Hydrophobically modified HPGs with C8/C10 alkyl chains and Methoxy poly(ethylene glycol)</td>
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<tr>
<td>HPG-C8/10-MePEG-NH₂</td>
<td>HPG-C8/10-MePEG with surface amine groups</td>
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<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>NMIBC</td>
<td>Non-muscle invasive bladder cancer</td>
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<tr>
<td>O/DEG</td>
<td>Octyl/decyl glycidyl ether</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Ta</td>
<td>Papillary tumor confined to the mucosa</td>
</tr>
<tr>
<td>T1</td>
<td>Tumors that invade lamina propria</td>
</tr>
<tr>
<td>Cis/Tis</td>
<td>Carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, nodes, metastasis</td>
</tr>
<tr>
<td>TUR</td>
<td>Transurethral resection</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludins-1</td>
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*In this thesis amine group substitution on HPG’s is represented by the number of moles (x) of amine per mole of HPG’s and is denoted by HPG- (x), where x is 360 (low), 580 (medium) and 780 (high) moles of amine per mole of HPG.
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"The greatest glory in living lies not in never falling, but in rising every time we fall’’

Dr. Nelson R. Mandela

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Dedication

To my Daddy ji and Maa
Chapter 1: Project overview and background

1.1 Project overview

Bladder cancer is the fourth most common form of cancer, following prostate, lung and colon cancer in men, in the western world. Approximately 75-85% of patients will initially be diagnosed with non-muscle invasive bladder cancer (NMIBC) also known as superficial bladder cancer. The gold standard of treatment is the surgical removal of tumors from the bladder, a procedure known as transurethral resection (TUR) followed by an adjuvant intravesical instillation of immunotherapy or anticancer drugs through a catheter. Currently used intravesical chemotherapeutic agents include, thiotepa, doxorubicin, epirubicin, mitomycin C (MMC) and the relatively new drug, gemcitabine. Bacillus Calmette Guerin (BCG) is used in immunotherapy. Although improved diagnosis has led to early detection and improved treatment, approximately 80% of patients show tumor recurrence with 25% progressing to advanced disease (Falke et al., 2011; Statistics, 2013). Recent data also showed that intravesical chemotherapy results in an average short-term decrease in the bladder cancer recurrence rate of 14% (range 3% to 43%) with no significant effect on recurrence at 5 years (Shelley et al., 2010). Intravesical chemotherapy failure is thought to arise from an insensitivity of bladder cancer cells to the drugs or poor drug delivery to the cells (Tammela et al., 1993; Volpe et al., 2010). There are various investigational studies with new intravesical agents (such as taxanes) with the goal of improving efficacy, either alone, or as part of a combination treatment with other drugs (McKiernan et al., 2006; Chade et al., 2009; Barocas et al., 2012; van Lingen et al., 2013).

The main function of the bladder is to hold urine and maintain an effective blood-urine barrier. Accordingly, a robust urothelium provides a tight and impermeable barrier via
specialized superficial cells, termed umbrella cells. In addition to urine retention, this barrier also limits both drug penetration into bladder tissue and the effectiveness of intravesical chemotherapy (Tyagi et al., 2006; Dovedi et al., 2009). It is well established that umbrella cells may be exfoliated upon interaction with certain biological, physical and chemical agents including uropathogenic Escherichia coli or bacterial endotoxins such as lipopolysaccharide (Mysorekar et al., 2002) or chitosan (Grabnar et al., 2003; Kerec et al., 2005; Veranic et al., 2009; Erman et al., 2013), cyclophosphamide (Veranic et al., 2004), protamine sulfate (Lavelle et al., 2002) and sodium saccharin (Romih et al., 1998). Furthermore moderate stress (Dalal et al., 1994), depletion of calcium (Veranic et al., 2000), and irradiation may also contribute to the rapid exfoliation of the superficial urothelium (Jaal et al., 2006). Subsequently, the exposed intermediate cells rapidly differentiate into a new urothelial layer to regenerate the barrier within hours of exfoliation (Veranic et al., 2000; Veranic et al., 2009).

The taxanes, docetaxel (DTX) and paclitaxel (PTX) have similar chemical structures and are antimitotic agents that bind to the beta subunit of tubulin and inhibit cell proliferation. Taxotere® a commercial formulation of DTX is approved by the United States Food and Drug Administration (FDA) and incorporates Tween 80 (polysorbate 80) and ethanol to solubilize the drug. It is currently used to treat breast, gastric, non-small cell lung cancer and metastatic prostate cancer (McKiernan et al., 2006; Balar, 2014).

Previous studies in our laboratory have shown enhanced DTX uptake into bladder tissue exposed to drug loaded nanoparticles composed of amine functionalized, hydrophobically derivatized hyperbranched polyglycerols (HPGs) (Mugabe et al., 2011c; Mugabe et al., 2012). These dendrimer like nanoparticles possess a hydrophobic core composed of C_{8/10} alkyl chains and a hydrophilic shell of methoxy poly(ethylene glycol) (MePEG) in a single molecule and are
abbreviated as HPG-C_{8/10}-MePEG. When amine groups were conjugated to the surface of the nanoparticles, the DTX loaded HPG-C_{8/10}-MePEG-NH_{2} nanoparticles significantly inhibited tumor growth in a mouse model of superficial bladder cancer. Furthermore, *ex vivo* and *in vivo* studies showed evidence of exfoliation of umbrella cells following exposure of bladder urothelium to HPG-C_{8/10}-MePEG-NH_{2} nanoparticles (Mugabe *et al.*, 2012).

The overall goal of this thesis was to develop an increased understanding of the effects of varying concentrations of surface conjugated amine groups on the HPGs and different drugs used in intravesical chemotherapy on urothelial exfoliation and drug uptake into porcine bladder tissue. Our working hypothesis was that the development of a mucoadhesive DTX loaded HPG-C_{8/10}-MePEG-NH_{2} nanoparticle formulation conjugated with an optimized number of amine groups would result in binding of mucoadhesive nanoparticles to the bladder mucosa leading to complete exfoliation of umbrella cells and an increased bladder tissue uptake of DTX.

Chapter 2 describes the effect of concentration and exposure times of three HPGs (HPG-C_{8/10}-MePEG-NH_{2}) with increasing degrees of amination (denoted 350, 580, 780 moles amine per mole of HPG) on *ex vivo* porcine bladder tissue morphology. The study also compared the tissue depth profile of DTX uptake into *ex vivo* porcine tissue.

Chapter 3 describes the effect of intravesical chemotherapeutic agents (such as MMC, doxorubicin and gemcitabine), on *ex vivo* porcine bladder tissue morphology. The study also compared the influence of pretreatment of porcine bladder tissue with HPG-C_{8/10}-MePEG-NH_{2} (HPG-580) on chemotherapeutic drug uptake and whether the combination of each drug with either PTX or DTX influenced the bladder tissue uptake of taxanes.
1.2 Urinary bladder structure and function

The urinary bladder is a hollow, smooth, muscularized organ, located just above and behind the pubic bone. The main function of the bladder is to store and periodically eliminate urine. Urine is produced by the kidneys and travels through two long tubes called ureters to the bladder. The bladder can store up to 400 to 600 mL of urine. Bladder size depends on the stored volume of urine, but when empty it is about the shape of a pear. Storage of urine occurs under low pressure conditions but upon filling with approximately 150-300 mL, the bladder's muscular wall contracts to try and expel the urine. The bladder limits the passage of water and ions between urine and blood until the urine is excreted from the body (Hicks, 1975; Khandelwal et al., 2009) (Figure 1.1).

The bladder wall (Figure 1.2) is composed of several layers including the urothelium, the lamina propria, the muscularis and the fat tissue (Jost et al., 1989). The urothelium forms three layers of continuous cellular sheets and provides an effective barrier to the transport of any chemical agents. The apical surface of the urothelium is composed of hexagonal shaped umbrella cells containing plaques. The plaque arrays are composed of four major proteins: uroplakins Ia, Ib, II, and III (Veranic et al., 2004; Birder et al., 2010). Uroplakins are impermeable proteins that form a transcellular barrier against the passage of water, urea, ammonia and protons (Apodaca, 2004). Immunological studies have shown that uroplakin expression is differentiation related, i.e. these proteins are expressed only in urothelial cells during advanced stages of differentiation (Jost et al., 1989; Jenkins et al., 2007). Tight junctions between cells consist of transmembrane and cytoplasmic proteins such as claudins, occludins and zonula occludins protein-1 (ZO-1) (Khandelwal et al., 2009). ZO-1 forms a continuous, circumferential, purse-string structure at the
periphery of the cell, which mediates adhesion as well as restricts paracellular diffusion of molecules (Acharya et al., 2004; Veranic et al., 2004; Romih et al., 2005; Schulzke et al., 2009).

Beneath the superficial layer, is a mononucleate cell layer known as the intermediate layer. The number of strata of intermediate cells is species dependent and ranges from 5 strata in humans to 1-2 strata in rodents (Khandelwal et al., 2009). The intermediate cells, immediately below the umbrella cells, are partially differentiated and express some uroplakins. These cells have fusiform vesicles and upon loss of, or damage to, umbrella cells, these intermediate cells rapidly differentiate and mature to form the new apical membrane (Khandelwal et al., 2009). The basal cells are a single cell stratum that is in intimate contact with the underlying capillary bed and does not express uroplakins.
Mucins are negatively charged, high molecular weight membrane glycoproteins, found on the apical membranes of umbrella cells (Eroglu et al., 2002). The bound mucin layer forms the barrier between the underlying urothelium and the urine contents of the bladder. Some mucins are secreted in larger quantities in bladder cancer cell lines and in transitional cell carcinomas, while others are overexpressed in the bladders of patients with bladder cancer but are not detected in the normal bladder (Lopez-Beltran et al., 2002; Hollingsworth et al., 2004; Peppas et al., 2004; Lee et al., 2005; Smaldone et al., 2009).

Figure 1.2 Structure of bladder wall. The bladder wall is composed of several layers including the urothelium, the lamina propria (containing blood vessels), the muscularis and the fat tissue. The apical surface of the urothelium is composed of hexagonal shaped umbrella cells (containing impermeable protein uroplakins) and tight junction between them to restrict both paracellular and transcellular diffusion from urine. (Adopted from thesis (Mugabe, 2011))
1.3 Non-muscle invasive bladder cancer (NMIBC)

Bladder cancer is the ninth most common cancer in the world, with 430,000 new cases diagnosed world-wide in 2012 (J Ferlay, 2012). It is estimated that in Canada and the United States there will be approximately 8000 and 75000 people diagnosed, and between 2000 and 16000 deaths from bladder cancer, respectively, in 2013-2014 (Statistics., 2009; Brausi et al., 2011). Approximately 70-85% of patients initially present with bladder carcinomas that are confined to the urothelium and/or lamina propria, which is termed NMIBC (Kassouf et al., 2010; Sexton et al., 2010; Brausi et al., 2011; Mansoor et al., 2011).

1.3.1 Staging and grading

The appropriate classification of bladder cancer is important in order to determine the severity of the disease and suitable treatment options. In 1973, the World Health Organization (WHO) classified bladder tumors as Grade 1 (well-differentiated tumors), Grade 2 (moderately differentiated tumors) and Grade 3 (poorly differentiated tumors). In 2004 a new classification of NMIBC by WHO and the International Society of Urological Pathology was published (Chen et al., 2012b). It incorporated histological descriptions along with specific cytological architecture and graded as follows: urothelial papilloma (benign lesion), papillary urothelial neoplasm of low malignant potential (PUNLMP), and finally low-grade and high-grade urothelial carcinoma. Although there are several discrepancies in the suitability of the old versus new grading system, the new grading seems to have more reproducibility. Despite low inter-observer variability, the 1973 WHO grading system is widely used for clinical trials on NMIBC (Chen et al., 2012b; Gierth et al., 2013).
The American Joint Committee on Cancer TNM (Tumor-Node Metastases) grading system is also followed worldwide to facilitate the uniform description of NMIBC. The group of tumors known as NMIBC contains tumors confined to the urothelium (Ta), invading the lamina propria but not reaching the muscle layer (T1) and carcinoma in situ (CIS/Tis) (Figure 1.3). Approximately 70% of all the newly diagnosed bladder cancers are non-muscle invasive. Of these, 70% present as stage Ta, 20% as T1 and 10% as CIS/Tis. The advanced stage of bladder cancer is known as muscle-invasive bladder cancer (MIBC) and further classified as T2a/T2b (when the tumor just invades the muscle layer), T3a/T3b (it goes past the muscle layer into surrounding tissue) and finally T4a/T4b (spread to neighboring lymph nodes or distant sites) (MacLennan et al., 2007; Cheng et al., 2009).
1.3.2 Risk factors for bladder cancer

Bladder cancer more commonly affects men than women. It is associated with various risk factors including cigarette smoking, occupational exposure to carcinogens, age, chronic infection, and genetic predisposition. However, cigarette smoking is the predominant factor and has been responsible for increasing the risk of bladder cancer by up to four fold (Jemal et al., 2011; Mansoor et al., 2011). The most common clinical presentation of bladder cancer is painless haematuria (blood in urine). Additionally, urinary incontinence, frequent or irritative
urination, abdominal pain or anemia symptoms should alert the clinician to the possibility of bladder cancer (Prasad et al., 2011; Barocas et al., 2012).

1.3.3 Diagnosis
Bladder cancer is diagnosed by a combination of different tests. Initially, most patients with bladder cancer present with haematuria and the first diagnostic step is the imaging of the urinary tract and the bladder. Common imaging procedures used in the diagnosis of bladder cancer include trans-abdominal ultrasound, intravenous urography, computed tomography, and MRI. Further intensive diagnostic tests include urine cytology, tumor markers, cystoscopy, transurethral resection (TUR), bladder biopsies and photodynamic diagnosis (Williams et al., 2010; Brausi et al., 2011; Falke et al., 2011; Neuzillet et al., 2013; Kramer et al., 2014).

1.4 Treatment

1.4.1 Transurethral resection (TUR)
TUR is the gold standard procedure for NMIBC diagnosis, staging and treatment. It consists of the surgical removal of tumors with an electrical probe inserted through the urethra followed by rinsing of cancerous cells from the bladder (Richards et al., 2014a). Despite undergoing TUR, 45% of patients have tumor recurrence within the first few years and 15% of the patients exhibit tumor progression (Johnson et al., 2013). Recurrence may arise from either incomplete removal of tumors or immediate implantation of resected cancer cells into the healthy tissue after TUR. Full resection of the tumors with/or without photodynamic therapy are plausible techniques to reduce perioperative and postoperative comorbidities (Kramer et al., 2014). Additionally, to
reduce the high recurrence rate of tumors, cytotoxic intravesical therapy has been used as an adjuvant therapy after TUR (Gkritsios et al., 2013).

1.4.2 Intravesical therapy

Intravesical therapy is performed by the instillation of anticancer drugs via catheters into the bladder with the goal of eliminating cancer cells. The anatomy of the bladder allows relatively easy access and hence great potential for local drug delivery. Instillation of drugs into the bladder allows direct contact with the urothelium with minimal systemic absorption and side effects. Commonly used intravesical therapies are immunotherapy and chemotherapy agents (Freifeld et al., 2013; van Lingen et al., 2013). Immunotherapeutic agents such as Bacillus Calmette-Guerin (BCG) and chemotherapeutic agents such as MMC are currently used standard of care treatments for NMIBC patients (Falke et al., 2011). A single dose of intravesical chemotherapy after TUR significantly reduces the odds of tumor recurrence by about 39% in patients with low-grade tumor (Lamm et al., 1995; Sylvester et al., 2004). To increase intravesical therapeutic effectiveness, current efforts are mainly focused on defining the ideal treatment schedule, optimizing the efficacy of traditionally utilized therapies, and investigating novel chemotherapeutic agents.

1.4.2.1 Immunotherapy

The intravesical instillation of a strain of Mycobacterium tuberculosis, BCG, has been demonstrated to be partially effective in reducing tumor progression rates. BCG reduces cancer recurrence and progression by about 40%, as compared to TUR alone (Sylvester et al., 2002). Although BCG is the most effective current treatment, approximately 20%-40% of patients fail
to respond to this therapy, increasing the risk of more aggressive muscle invasive bladder cancer from 7% to 30% (Catalona et al., 1987; Sylvester et al., 2002). Furthermore, there is a high incidence of severe toxicities, which include fever, arthritis, granulomatous prostatitis, BCG sepsis, disseminated tuberculosis and tumor recurrence within 5 years of BCG treatment (Neuzillet et al., 2013). For those patients that fail to respond, or with later recurrence, there are other intravesical therapies available including chemotherapy. Some new promising immunotherapeutic agents such as interferons, interleukin-2, interleukin-12, tumor necrosis factor, keyhole limpet hemocyanin, and rubratin, have all shown good activity in BCG-refractory patients in clinical trials (Smaldone et al., 2009; Houghton et al., 2013; Farah et al., 2014).

1.4.2.2 chemotherapy

The immediate instillation of chemotherapeutics after TUR, seems to be an effective method to reduce the incidence of disease recurrence within the first 1–5 years after resection (Sylvester et al., 2004; van Lingen et al., 2013). Patients who do not respond to BCG treatment are treated with either single agent or a combination of intravesical chemotherapeutic agents such as doxorubicin, epirubicin and MMC and gemcitabine. Taxanes (PTX and DTX) are in clinical trials for potential use as intravesical agents (Barocas et al., 2012; van Lingen et al., 2013).
Mitomycin C

MMC is the most frequently used drug for NMIBC (Figure 1.4A). It is an alkylating agent derived from Streptomyces caespitosus with a molecular weight of 328 kDa. MMC binds to DNA and causes a reductive activation reaction resulting in DNA crosslinking and inhibition of DNA synthesis. MMC is typically administered at dosages of 20 to 60 mg/week for 6 to 8 weeks with advised monthly maintenance therapy every year (Williams et al., 2010). Severe chemical cystitis has been reported as a common side effect and is generally manifested by dysuria, frequency, urgency, suprapubic pain, and discomfort (Filson et al., 2014). A meta-analysis of single post-operative instillation of MMC in 1,476 patients (7 trials) showed reduced recurrence rates from 48.4% to 36.7% compared to TUR alone (Sylvester et al., 2004). The low success rates have been attributed to dilution of the drug solution by residual urine or continuous urine production in the bladder during the 2h instillation period, the inability of the drug to penetrate the deep tissue and additionally, in the case of MMC, instability of the drug in acidic urine (Masters, 2001). Moreover, to enhance the effectiveness of MMC, various approaches such as
increasing the dose, reducing the volume of residual urine at the time of treatment, reducing urine production before and during treatment by voluntary dehydration and alkalinization of the urine are practiced (Volpe et al., 2010; Ersoy et al., 2013; Zargar et al., 2014). De Stasi et al. developed a device-assisted approach using electromotive drug administration (EMDA) with MMC to increase tissue penetration in comparison to MMC alone (passive). This study demonstrated that the time to tumor recurrence was prolonged to 35 months with EMDA-MMC, compared with 19.5 months with MMC alone (Turker et al., 2013). Thermochemotherapy (TCT) is another device-assisted therapy that has shown promising results, when used with MMC. This technique includes the use of local microwave hyperthermia to enhance the drug delivery to the bladder (van der Heijden et al., 2004). A comprehensive study performed by van der Heijden et al using a combination therapy of MMC and local microwave hyperthermia was performed on 90 patients with NMIBC (van der Heijden et al., 2004). After 2 years of follow-up there was a lower (24.6%) risk of recurrence, compared to literature data for BCG and/or other intravesical chemotherapy. Various other techniques for optimizing the delivery of MMC, utilizing combination therapies, or sequential anticancer agents, or adding device-assisted approaches are currently undergoing trials (Smaldone et al., 2009; Breyer et al., 2010; Volpe et al., 2010).

1.4.2.2 Gemcitabine

Gemcitabine is a relatively new anticancer nucleoside-based drug that is an analog of deoxycttidine with a chemical formula of C₉H₁₁F₂N₃O₄ (Figure 1.4B). Gemcitabine is a prodrug whose structure mimics cytosine. Once transported into the cell it becomes phosphorylated by nucleoside kinases, thereby forming active metabolites (gemcitabine diphosphate and gemcitabine triphosphate) that results in inhibition of DNA synthesis and apoptosis. Gemcitabine
has shown cytotoxic effects in vitro on bladder cancer cell lines (Kilani et al., 2002). The drug is typically administered in doses of 500 mg, 1000 mg, and 2000 mg in 50 mL saline, instilled for 1 to 2 hours, and administered weekly for 6 weeks (Addeo et al., 2010). A single dose of 2g/100mL gemcitabine as a postoperative instillation was administered in 341 patients with saline as a placebo (Bohle et al., 2009). A two-year follow up suggested that the effect of a single instillation of gemcitabine was not superior to placebo when comparing the rates of tumor recurrence (28% vs 39%, respectively) (Bohle et al., 2009). However, the use of similar single doses of gemcitabine, but with variable dissolution volume, dwell time and frequency of administration showed improved recurrence-free survival rates with no toxicities in a 1 to 2 year follow up (Bartoletti et al., 2005). Several other studies suggested that gemcitabine is a promising drug with a potential role in the management of NMIBC (Dalbagni et al., 2006; Mohanty et al., 2008; Addeo et al., 2010; Gontero et al., 2013; Sternberg et al., 2013).

1.4.2.2.3 Doxorubicin

Doxorubicin (Adriamycin®) is an anthracycline antibiotic with a molecular weight of 580 Da currently used for NMIBC (Figure 1.4C). The mechanism of action of this drug involves binding of the drug to DNA base pairs and inhibition of topoisomerase II thereby preventing protein synthesis (Falke et al., 2011). It is used in doses ranging from 30 to 100 mg given weekly or over three weekly periods. Doxorubicin is known to have more toxic effects than other anthracycline analogs (epirubicin and valrubicin) in the bladder (Ali-el-Dein et al., 1997; Brassell et al., 2006). Lamm et al., demonstrated that in clinical trials, recurrence rates following administration of doxorubicin and epirubicin were lower than those observed for BCG (Lamm et al., 1991; Lamm et al., 1995). Also the use of other anthracyclines including valrubicin and
epirubicin has been widely accepted outside of the United States, showing the most utility in patients with NMIBC and CIS in BCG refractory patients (Patterson et al., 2000; van der Meijden et al., 2001; Witjes et al., 2008).

### 1.5 Taxanes: Paclitaxel and docetaxel

PTX (Figure 1.5A) and DTX (Figure 1.5B) are chemotherapeutic agents belonging to the taxane family with approval for use against breast, lung, ovarian, and bladder cancer (Balar, 2014). PTX is a natural product isolated in 1971 as part of the National Cancer Institute (NCI) cytotoxic agents screening program, from the bark of the Pacific yew tree (*Taxus brevifolia*) (Wani et al., 1971). PTX has a molecular formula of C\textsubscript{47}H\textsubscript{51}NO\textsubscript{14} (Figure 1.5A) with molecular weight of 853 Da. It is a diterpenoid pseudoalkaloid consisting of an eight-member taxane ring with a four-member oxetane ring and a bulky ester side chain at C-13 that is necessary for antineoplastic activity (Wani et al., 1971). DTX is a semi- synthetic chemotherapeutic agent, produced from 10-deacetylbaccatin-III, which is found in the needles of the European yew tree, *Taxus baccata*. The structure of DTX differs from PTX in the 10-position on the baccatin ring and in the 3’-position of the lateral chain with a molecular formula of C\textsubscript{43}H\textsubscript{53}NO\textsubscript{14} and has a molecular weight of 808 Da (Figure 1.5B) (Wani et al., 1971).
Figure 1.5 Chemical structures of paclitaxel (A) and docetaxel (B). Dissimilarities are marked in blue (A) and red (B).

1.5.1 Mechanism of action of taxanes

These drugs are classified as antimicrotubule agents that bind to the β-subunit of tubulin. This binding promotes the polymerization of microtubules and stabilizes the formed structures by inhibiting depolymerization (James M. McKiernan, 2010). Microtubules are ubiquitous in all eukaryotic cells and rely on continuous polymerization and depolymerization. They assemble and mobilize the components of the spindle during cell division as well as maintain the normal functions of cell motility, intracellular transport and the maintenance of cell shape (Kingston et al., 2007). Taxanes stabilize the microtubules, and prevent them from cycling between polymerization and depolymerization states and so become nonfunctional (Kingston et al., 2007). Although binding sites are identical, DTX targets centromere organization, whereas PTX targets the mitotic spindle (James M. McKiernan, 2010). DTX appears to have 1.9-fold higher affinity for the site, and not only affects G2 and M phase stages of division like PTX, but also causes cell damage in S phase making it a more potent drug (Singla et al., 2002; Mackler et al.,
1.5.2 Current formulations of taxanes

PTX and DTX are highly lipophilic drugs with limited water solubility. Taxol® (Bristol-Myers Squibb Co., Princeton, NJ), one of the commercial formulations of PTX, is available as a 6 mg/mL solution in 50% Cremophor-EL (polyoxyethylated castor oil) and 50% dehydrated alcohol. DTX is formulated in 100% polysorbate 80 and comes as a 20mg/mL solution with ethanol as a diluent (Taxotere® Sanofi-Aventis, Bridgewater, NJ). However, both the vehicles have been reported to cause side effects such as hypersensitivity reaction, nephrotoxicity and neurotoxicity (Gelderblom et al., 2001; ten Tije et al., 2003). Additionally Cremophor-EL also results in the sequestration of PTX in micelles, which may lead to low levels of free drug to diffuse through tissue (Knemeyer et al., 1999; Hadaschik et al., 2008; Mugabe et al., 2009).

1.5.3 Intravesical PTX and DTX

The potency and chemical nature of taxanes makes them potentially good candidates for intravesical treatment. It is believed that molecules greater than 300 Da are unlikely to enter the systemic circulation through the bladder urothelium (Crawford, 2002). Accordingly, the high molecular weight of taxanes may restrict systemic uptake. Additionally their lipophilic nature may facilitate their penetration and retention into the bladder urothelium.

Song et.al demonstrated a greater PTX partitioning into dog bladder tissue with minimal systemic absorption compared to MMC and doxorubicin in in vivo studies (Song et al., 1997). An alternative bioconjugate drug delivery system has been synthesized by carboxyl esterification of hyaluronic acid (a glycosaminoglycan abundant in human connective tissues) with paclitaxel.
(Rosato et al., 2006). It has been shown to increase the water solubility of PTX with improved in vitro cytotoxicity against human bladder cancer cells (Rosato et al., 2006; Tringali et al., 2008). In a phase I clinical trial, intravesical instillation of hyaluronic acid-PTX bioconjugate showed minimal toxicity with no systemic absorption in 16 carcinoma in situ, BCG refractory patients (Bassi et al., 2011). The commercially available PTX-albumin nanoparticulate formulation (Abraxane®) was instilled intravesically in a phase I trial and exhibited minimal toxicity and no systemic absorption (McKiernan et al., 2011).

A phase I clinical trial to evaluate DTX (Taxotere®) as an intravesical treatment of NMIBC patients who were resistant to prior BCG or BCG-IFN treatment showed that 44% (8 of 18 patients) patients demonstrated low grade toxicities and 56% (10 of 18 patients) had no evidence of cancer at their post-treatment cystoscopy and biopsy (McKiernan et al., 2006). The long-term clinical outcomes of this phase I trial after 48.3 months of follow-up showed that 22% (4 of 18 patients) had a complete response without maintenance treatment, whereas 17% (3 of 18 patients) had a partial response, defined as a single non-muscle invasive recurrence treated by local resection with median 13.3 months disease-free survival (McKiernan et al., 2006; Laudano et al., 2010). The largest long-term follow up study to evaluate an intravesical DTX regimen, was performed in 54 patients and concluded that the intravesical DTX appeared to be a promising agent with significant efficacy and durability of response in BCG refractory NMIBC patients (Barlow et al., 2013). Adding intravesical DTX maintenance treatments was suggested to potentially increase the duration of tumor recurrence-free survival (Barlow et al., 2013).
1.6 Administration of intravesical agents: urothelial permeability, responses and recovery

For all drugs, the significant barrier properties of the urothelium, the brief contact time of the instilled drug with the bladder wall and urine dilution of the administered dose all serve to limit the efficacy of intravesical chemotherapy. The drug concentration, duration of exposure, urine volume and pH, patient hydration status, urothelial integrity and physicochemical properties of the drug affect the passive diffusion of drug across the urothelium (Farokhzad et al., 2006; Smaldone et al., 2009). Pharmacokinetic and bladder tissue uptake studies in dogs showed higher urothelial tissue level of PTX than doxorubicin or mitomycin C upon intravesical administration (Song et al., 1997). However, it is thought that the formation of micelles of Cremophor-EL (in Taxol®) or polysorbate 80 (in Taxotere®) results in sequestration of PTX or DTX which may reduce drug penetration across the urothelium (Eroglu et al., 2002; Smaldone et al., 2009; Logan et al., 2012).

1.6.1 Urothelial turnover and responses to stress

The human urothelium has the slowest turnover rate of any tissue in the body and requires approximately 200 days for complete regeneration in the healthy state (Lewis et al., 1976; Jost, 1986). However, the umbrella cells rapidly exfoliate within hours in response to microbial invasion, such as uropathogenic Escherichia coli (E.coli) or bacterial endotoxins such as lipopolysaccharide, which is likely an innate defense mechanism to prevent infection (Mysorekar et al., 2002). Furthermore, other chemicals such as chitosan (Grabnar et al., 2003; Kerec et al., 2005; Veranic et al., 2009; Erman et al., 2013), cyclophosphamide (Veranic et al., 2004), protamine sulfate (Lavelle et al., 2002) and sodium saccharin (Romih et al., 1998) as well as
moderate stress (Dalal et al., 1994), depletion of calcium (Veranic et al., 2000), and irradiation may also contribute to the rapid exfoliation of the superficial urothelium (Jaal et al., 2006). In patients with bladder carcinomas, local hyperthermic treatment resulted in rapid exfoliation of urothelial cells, followed by regeneration of urothelium within 3 days, and complete recovery within 10-12 weeks (Jacob et al., 1982).

1.6.2 Urothelial responses to intravesical agents

Intravesically administered BCG bacteria adhere to the bladder mucosa by binding to fibronectin, and become internalized into the urothelium. This results in a complex immune response and a strong local inflammation reaction which is commonly manifested by dysuria and urinary frequency (Neuzillet et al., 2013). BCG is thought to exert antitumor effects by the sloughing of superficial tumour cells (Catalona et al., 1990). Following BCG induction therapy in patients, histological findings in biopsies of non-neoplastic bladder areas showed foci of sloughing in 95% of specimens, severe dysplasia (38%), severe denudation (25%) and extreme inflammatory cell infiltration and edema in specimens from all patients (Koya et al., 2006).

Chitosan and protamine sulfate exposure to the bladder urothelium of rat caused exfoliation followed by urothelial recovery (Lavelle et al., 2002; Kerec et al., 2005; Erman et al., 2013). Protamine sulphate appears to form pores in the apical membrane of the umbrella cells, which is followed by rapid, but selective exfoliation of the umbrella cell layer (Lavelle et al., 2002). The rat urothelium recovery starts with rapid redistribution of UPIIIa to the newly exposed surface of the intermediate cells with subsequently patchy ZO-1 positive tight junctions forming around the border of the cells. By 24 hours, UPs are abundantly expressed at the surface
and the cells are fully surrounded by tight junctions. The injured tissue is fully repaired by 5 days and the newly differentiated cells achieve full size by 10 days (Lavelle et al., 2002).

### 1.7 Mucoadhesive formulation for intravesical drug delivery

Mucoadhesion is generally described as the adhesion between two materials, at least one of which is a mucosal surface (Smart, 2005). Mucoadhesive systems possess numerous advantages compared to conventional dosage forms. Mucoadhesive polymers as a drug carriers can localize and prolong the drug residence time, leading to improved bioavailability and less frequent dosing (Kumar et al., 2014). The exact mechanism of polymer attaching to the mucosal surface is not yet fully understood but there are various theories; for example, physical entanglement (diffusion theory) and/or chemical interactions such as electrostatic, hydrophobic, hydrogen bonding and van der Waals interactions (adsorption and electronic theories) have been used to explain this phenomenon (Edsman et al., 2005; Smart, 2005; Kumar et al., 2014). Intravesical mucoadhesive formulations should have three basic features as follows: the carrier should have rapid attachment or adhesion to the bladder wall after instillation into the bladder, it should not obstruct the flow of urine or any of the normal functions of the bladder and it should be able to stay attached to the affected site even after voiding of urine (Tyagi et al., 2006; GuhaSarkar et al., 2010). Biomolecules, such as chitosan, have been shown to have excellent mucoadhesive properties.

Chitosan is a biocompatible, biodegradable, nontoxic polymer, often used as a permeability enhancer (Kumar et al., 2014). It has been demonstrated that in in vitro and in vivo studies, chitosan enhances drug transportation across the intestinal, nasal, buccal, vaginal and also the urinary bladder wall (Jain et al., 2004; Gao et al., 2008; Nagarwal et al., 2010; de Araujo
Pereira et al., 2012). The main mechanism by which it increases the permeability is a combination of mucoadhesion and its effect on tight junction proteins, as well as F-actin (Schipper et al., 1997; Veranic et al., 2000). Mucoadhesion results from binding between the positively charged amine groups of chitosan and negatively charged sialic acid moieties present on mucin chains on the mucosal surface via electrostatic interactions (Lehr et al., 1992). The *ex vivo* treatment of porcine bladder urothelium with chitosan has been shown to cause significant increases in the penetration of water-soluble solutes such as moxifloxacin and pipemidic acid into the bladder tissue (Kerec et al., 2005). Studies performed by Kerec, showed that such increases in permeability were dependent on urothelial exfoliation (Kerec et al., 2005). The chitosan-induced urothelial exfoliation was shown to be concentration and time dependent in the isolated porcine urinary bladder model. Treatment with low concentrations of chitosan (0.0001% w/v) or for short times (15 min) affected the tight junction structure, but it did not increase the moxifloxacin uptake into porcine bladder tissue (Kerec et al., 2005). The lowest concentrations of chitosan (0.0005% w/v) that resulted in enhanced drug uptake also caused the exfoliation of urothelium (Kos et al., 2006). It was concluded that elimination of the superficial layer was crucial to enhance the permeability of substances into the bladder wall; however, significant damage to the urothelium is likely with high concentrations of chitosan (Kerec et al., 2005). In *vivo* studies done by Veranic et al. investigated the recovery of the mice urothelium after intravesical treatment with chitosan (0.005% w/v) (Veranic et al., 2009). Interestingly, in response to exfoliation caused by chitosan, recovery was rapid with differentiation occurring only 10 min post treatment and within 60 minute a fully recovered urothelium was observed with well-differentiated superficial umbrella cells (Veranic et al). The exfoliation of umbrella cells was suggested to be initiated by necrosis (Veranic et al., 2009).
1.8 Hyperbranched polyglycerols as drug delivery systems

Hyperbranched polymers are structurally related to dendrimers with less perfect branching, so that typically approximately 60% of the monomer might be branched (Kainthan et al., 2007a; Kainthan et al., 2007b). Unlike dendrimers, which are prepared in tedious multistep synthesis and purification steps, hyperbranched polymers are usually prepared in a one-step synthetic procedure which makes them more likely to be commercially possible alternatives to dendrimers (Radowski et al., 2007). Sunder and coworkers described a ring-opening multi-branching polymerization, in which glycidol was slowly added to a solution of a partially deprotonated tri-functional core-initiator for oxyanionic polymerization (Sunder et al., 1999). Because of the slow monomer addition method and fast proton exchange during the polymerization, different chain ends grow simultaneously, resulting in hyperbranched polyglycerols (HPGs) with low molecular weight ($M_n < 20,000$ g/mol) and narrow polydispersities ($M_w/M_n < 1.5$) (Sunder et al., 1999; Sunder et al., 2000).

Brooks and coworkers were the first to report the synthesis of high molecular weight ($M_n$ up to 700,000 g/mol) HPGs with narrow polydispersities ($M_w/M_n = 1.1-1.4$) using the slow monomer addition method with high monomer to initiator ratios, in the presence of dioxane as an emulsifying agent (Kainthan et al., 2007a). These high molecular weight HPGs showed low intrinsic viscosities, with very small hydrodynamic radii (5-10 nm) with more than 10,000 hydroxyl groups/molecule available for derivatization (Kainthan et al., 2007a; Kainthan et al., 2007b; Kainthan et al., 2008a). HPGs have been shown to be highly biocompatible and nontoxic in both in vitro and in vivo studies (Kainthan et al., 2007b).

HPGs were derivatized with hydrophobic groups within the core and varying amounts of MePEG chains on the polymer surface using a single pot synthesis procedure based on an
anionic ring-opening multi-branching polymerization of epoxide (Kainthan et al., 2008b; Mugabe et al., 2009). In the polymerization reaction, first the initiator trimethylol propane (TMP) was activated by potassium methylate and reacted with glycidol and octyl/decyl glycidyl either to create an HPG-C_{8/10}-OH with a hydrophobic core to allow the loading of PTX and DTX. To increase the aqueous solubility, MePEG 350 epoxide was added in the terminal phase of the reaction, resulting in MePEG 350 chains being linked to some of the hydroxyl groups on HPG to form a more hydrophilic shell, relative to HPG-C_{8/10}-OH. Unreacted alkyl chains are removed by extraction and polymer is then purified by a dialysis method (Kainthan et al., 2008a). These polymer systems have no critical micelle concentration (CMC) and are often referred to as “unimolecular micelles” due to the fact that the hydrophobic core and hydrophilic shell are connected by covalent bonds and are present in the single molecule (Kainthan et al., 2008b). Their natural stability to various environmental effects such as dilution, shear force and pH make these nanoparticles excellent candidates as drug delivery systems. Mugabe et al. demonstrated the equivalent in vitro cytotoxicity profile of PTX loaded in either HPGs or Taxol® (Mugabe et al., 2009). In vivo, intravesical instillation of PTX loaded HPGs in a bladder cancer mouse model was shown to effectively reduce orthotopic bladder tumor growth compared to Taxol®. The DTX loaded HPG nanoparticles were more cytotoxic than PTX loaded HPGs against a human urothelial carcinoma cell line (Mugabe et al., 2011a).

1.9 Amine-conjugated HPGs

The amine conjugation on the surface of HPG-C_{8/10}-MePEG resulted in positively charged nanoparticles with improved mucoadhesiveness (HPG-C_{8/10}-MePEG-NH\textsubscript{2}) (Figure 1.6) (Mugabe et al., 2011b; Mugabe et al., 2011c). A two-step procedure was used for surface
derivatization of amine groups on HPG-C_{8/10}-MePEG-OH. In the first step, hydroxyl groups (approximately 10-20\%) on HPG-C_{8/10}-MePEG were deprotonated by potassium hydride and then reacted with N-(2,3-epoxypropyl)-phthalimide (EPP) to create an intermediate product with phthalimide protecting groups (HPG-C_{8/10}-MePEG-EPP). In the second step, the phthalimide functional groups were cleaved to generate primary free amine groups by hydrazinolysis (Mugabe et al., 2011c). The presence of amine groups on HPG-C_{8/10}-MePEG-NH_2, was identified by 1H NMR and HSQC studies (400 MHz) and the amounts of amine groups were calculated from conductometric titration measurements (Mugabe et al., 2011c). In this thesis amine group substitution is represented by the number of moles (x) of amine per mole of HPGs and is denoted by HPG- (x), where x is 360 (low), 580 (medium) and 780 (high) moles of amine per mole of HPG (Kainthan et al., 2008a; Mugabe et al., 2011a; Mugabe et al., 2011b; Mugabe et al., 2011c).
Figure 1.6 Structure of HPG-C₈/₁₀-MePEG-NH₂. In the structure ‘R’ represent the hydrophobic core based on mixture of alkyl (C₈/C₁₀) chains in pink, ()₇, represent the hydrophilic shell based on MePEG 350 in blue and amine groups are shown in red color. (Adopted from thesis (Mugabe, 2011))

Previous studies in our lab have shown that DTX can be loaded in HPGs at high loading levels (5% w/w) and the release profile shows a continuous controlled release (Mugabe et al., 2011c; Mugabe et al., 2012). Furthermore, this formulation had demonstrated significantly increased DTX uptake in isolated porcine bladder tissue using DTX loaded HPG-C₈/₁₀-MePEG-NH₂ nanoparticles, as compared to polysorbate 80 solubilized DTX (Taxotere®) (Mugabe et al., 2011c; Mugabe et al., 2012). A single intravesical instillation of these nanoparticles was more
effective in inhibiting tumor growth in an orthotopic mouse model of bladder cancer compared to the commercial formulation of Taxotere® (Mugabe et al., 2011a; Mugabe et al., 2011b).

Additionally, morphological studies done on both ex vivo porcine and in vivo mouse bladders with DTX loaded HPG-C_{8/10}-MePEG-NH_2 based formulations, showed evidence of exfoliation of umbrella cells, which may be the major mechanism for this increased efficacy (loss of penetration barrier) (Mugabe et al., 2012). Furthermore, upon removal of the formulation, full recovery of urothelium was observed in a mouse model within 24 hours (Mugabe et al., 2012). HPGs have thus been shown to be a potentially very effective drug delivery system for DTX for intravesical treatment of NMIBC.

1.10 Thesis goal, hypothesis and objectives

The overall goal of this thesis was to develop an increased understanding of the effects of varying concentrations of surface conjugated amine groups on the HPGs and different drugs used in intravesical chemotherapy on urothelial exfoliation and drug uptake into porcine bladder tissue. Our working hypothesis was that the development of a mucoadhesive DTX loaded HPG-C_{8/10}-MePEG-NH_2 nanoparticle formulation conjugated with an optimized number of amine groups would result in binding of mucoadhesive nanoparticles to the bladder mucosa leading to complete exfoliation of umbrella cells and an increased bladder tissue uptake of DTX.

The specific objectives were as follows:

1. To compare the effects of different concentrations of HPG-360, HPG-580 and HPG-780 nanoparticles, with or without DTX loading, on ex vivo porcine urothelial exfoliation and drug uptake.
2. To investigate the effect of intravesical chemotherapy agents MMC, doxorubicin and gemcitabine on \textit{ex vivo} porcine bladder tissue morphology and drug uptake.
Chapter 2: Effect of surface amine functionalized, hydrophobically
derivatized hyperbranched polyglycerol nanoparticles loaded with docetaxel
on bladder urothelial exfoliation and tissue uptake

2.1 Introduction
Bladder cancer is the 5th most common form of cancer in Canada, with 7,900 new cases and 2100
deaths per year (Statistics, 2013). The largest group of patients present with non-muscle invasive
bladder cancer (75%-80%) where small tumors are located in the urothelial, lumen–facing tissue
and, for those patients, transurethral resection (TUR) is the gold standard treatment procedure.
Despite undergoing TUR, 70-90% of patients show tumor recurrence within 5 years (Dovedi et
al., 2009; Kassouf et al., 2010; Falke et al., 2011; Gkritsios et al., 2013). In order to decrease the
high incidence of tumor regrowth after TUR, intravesical chemo- or immuno- therapy is used as
an adjuvant therapy (Sexton et al., 2010; Prasad et al., 2011; Richards et al., 2014b). Although
current intravesical therapies such as immunotherapy with Bacillus Calmette Guerin (BCG) or
chemotherapy with drugs such as doxorubicin, epirubicin, gemcitabine or mitomycin C (MMC)
may reduce or delay tumor regrowth, up to 80% of patients still develop recurrent tumors, of
which 20-30% progress into more aggressive tumors (Gierth et al., 2013; Gkritsios et al., 2013).

The anatomy of the bladder wall presents significant challenges to the penetration of
drugs. The main function of the bladder is to hold urine and limit the passage of water and ions
between urine and blood until the urine is excreted from the body (Hicks, 1975; Jost et al., 1989;
Lavelle et al., 2002; Khandelwal et al., 2009). The bladder wall is composed of several layers
including the lumen-facing urothelium, the lamina propria, the muscularis and fat tissue (Hicks,
A robust urothelium forms three layers of continuous cellular sheets. The apical layer of urothelium provides a tight and impermeable barrier through specialized superficial cells, termed umbrella cells, that directly contact urine (Hicks, 1975; Jost et al., 1989; Mysorekar et al., 2002; Birder et al., 2010; Kreft et al., 2010). Between 70% - 90% of the apical plasma membrane of umbrella cells is covered with plaques, composed of transmembrane proteins called uroplakins (Romih et al., 1998; Veranic et al., 2009). Uroplakins are impermeable proteins that form a transcellular transport barrier to water, urea and ammonia (Romih et al., 1998; Veranic et al., 2004; Romih et al., 2005). Furthermore, tight junctions between umbrella cells, which consist of transmembrane and cytoplasmic proteins such as claudins, occludins and zonula occludens protein-1 (ZO-1) form a continuous, circumferential, purse-string structure at the boundary of the cell, which mediates adhesion as well as restricts paracellular diffusion of solutes (Veranic et al., 2004; Schulzke et al., 2009; Veranic et al., 2009). In addition, a bound mucin layer on the urothelium provides a further barrier between the underlying urothelium and urine (Lavelle et al., 2002; Peppas et al., 2004; Farokhzad et al., 2006; Birder et al., 2010). These various features of the lumen-facing bladder surface contribute to a high transepithelial electrical resistance (TEER) of up to 75,000 Ω/cm² resulting in poor tissue penetration of intravesical drugs (Lewis et al., 1976). Furthermore, intravesical drugs are rapidly diluted with urine and only resident in the bladder for approximately 2 hours, resulting in reduced efficacy of intravesical chemotherapy due to both low drug exposure and poor drug penetration into the urothelium (Fraser et al., 2002; Tyagi et al., 2006; Hadaschik et al., 2008; Logan et al., 2012). Under physiological conditions, the human bladder urothelium represents a permanent barrier to the movement of molecules and has the slowest turnover rate of any tissue in the body, taking approximately 200 days (Hicks, 1975; Jost, 1986; Romih et al., 2005;
It is well established that umbrella cells may be exfoliated upon interaction with certain physical triggers and chemical agents such as chitosan (Grabnar et al., 2003; Kerec et al., 2005; Veranic et al., 2009; Erman et al., 2013). Urothelium exfoliation is followed by a rapid recovery process in which urothelium integrity is restored, typically within a few hours post treatment.

Chitosan is a naturally occurring mucoadhesive polymer and has been reported to be a permeability enhancer, increasing drug transport across epithelial tissues, such as intestinal, nasal, buccal and bladder urothelium (Jain et al., 2004; Kerec et al., 2005; Nagarwal et al., 2010; de Araujo Pereira et al., 2012). The mechanism by which chitosan increases epithelial permeability was suggested to be combination of mucoadhesion and its effect on tight junction proteins (ZO-1, ocludins) and F-actin (Veranic et al., 2009). Mucoadhesive drug delivery systems possess numerous advantages such as, the localization of the formulation at a required specific region of the body (improve drug bioavailability at the site of interest), increased intimacy of contact between a formulation and mucous surface (enhance the absorption of the drug) and prolong residence time of the incorporated drugs (leading to less frequent dosing and improved patient compliance) (Kumar et al., 2014). Chitosan is a positively charged polymer due to numerous amine groups in the polymer chain and can bind to mucins via electrostatic interactions with the negatively charged sialic acid moieties on the mucin chains of mucosal surfaces. Chitosan has been shown to increase the permeability of moxifloxacin in isolated porcine bladder tissue in a time and concentration dependent manner (Kerec et al., 2005). It was concluded from the study that elimination of the superficial layer was key to enhancing the permeability of drug into the bladder wall (Kerec et al., 2005). Intravesical treatment with chitosan (0.005%) in mice bladders caused exfoliation of urothelium and was followed by a
rapid urothelial recovery process, beginning 10 min post treatment. Within 60 minutes, the urothelium was fully recovered with well-differentiated superficial umbrella cells observed (Veranic et al., 2009).

Docetaxel (DTX) is a cytotoxic chemotherapeutic agent of interest for the treatment of bladder cancer (Highley et al., 1999; Galsky, 2005; Balar, 2014). DTX promotes the intracellular bundling of microtubules and inhibits microtubule depolymerization resulting in M-phase cell cycle arrest and cell death (James M. McKiernan, 2010). In a Phase I trial of 6-weekly intravesical DTX treatments in 18 patients with recurrent NMIBC, who failed at previous intravesical therapy, McKiernan et al., reported a complete response rate of 56% with no evidence of disease at post-treatment cystoscopy and biopsy, with minimal local toxicity and no systemic absorption (McKiernan et al., 2006). Furthermore, in a median follow-up of 48.3 months of 18 patients, Laudano and co-workers reported, a 39% disease-free survival (without cystectomy or further intravesical therapy) and 89% progression-free survival (Laudano et al., 2010).

Previous studies in our lab have shown a significant increase in DTX uptake into bladder tissue using mucoadhesive DTX-loaded hyperbranched polyglycerol-based nanoparticulate formulations, as compared to DTX solubilized in polysorbate-80 and ethanol (the commercial formulation Taxotere®) (Mugabe et al., 2011c; Mugabe et al., 2012). Furthermore, a single intravesical instillation of these nanoparticles was well-tolerated and effectively inhibited tumor growth in mice with orthotopic bladder cancer xenografts as compared to the commercial formulation of Taxotere® (Mugabe et al., 2011a; Mugabe et al., 2011b). The functionalized hyperbranched polyglycerols (HPGs), synthesized by our group, have shown great potential as a carrier for hydrophobic drugs such as DTX (Kainthan et al., 2008b; Mugabe et al., 2011a). HPGs
were synthesized by the anionic ring opening multi-branching polymerization of glycidol using trimethylolpropane as an initiator (Kainthan et al., 2008a; Kainthan et al., 2008b; Mugabe et al., 2011a; Mugabe et al., 2011c; Mugabe et al., 2012). The HPG core was derivatized with C8/C10 alkyl chains to create a hydrophobic core for DTX binding, whereas the MePEG chains linked to hydroxyl groups on HPGs provided water solubility and biocompatibility. In addition, amine groups were conjugated to the hydroxyl groups on HPG-C8/10-MePEG polymer in a 2-step reaction procedure to increase their mucoadhesiveness, (Mugabe et al., 2011b). DTX (5%) was loaded into these HPG nanoparticles due to hydrophobic interactions between the hydrophobic drug and the hydrophobic core of the HPGs (Mugabe et al., 2011b; Mugabe et al., 2011c; Mugabe et al., 2012). HPGs are small nanoparticles with hydrodynamic radii <10nm and low polydispersity index (<1.5). Amine group conjugation as well as the DTX encapsulation had no effect on HPG particle size (Kainthan et al., 2007b; Kainthan et al., 2008b; Mugabe et al., 2011a). The mole fraction of amine groups derivatized on HPG-C8/10-MePEG polymers was measured by conductometric titration (Mugabe et al., 2011b).

Previous studies demonstrated different extents of exfoliation of ex-vivo porcine bladder tissue following exposure to different solution concentrations of amine functionalized HPGs (Mugabe et al., 2012). The objectives of this study were to investigate the time course and extent of exfoliation on isolated fresh porcine bladders (ex-vivo) exposed to increasing concentrations of HPGs with increasing degrees of amination denoted as low, medium and high amine densities (equivalent to 360, 580 and 780 moles amine per mole HPG, respectively) and to compare the bladder tissue depth profiles of DTX loaded into amine functionalized HPG-C8/10-MePEG-NH2 nanoparticles.
2.2 Materials and methods

2.2.1 Materials

Hyperbranched polyglycerols (HPGs) were a kind gift from the Centre for Drug Research and Development (CDRD). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Ottawa, ON). Tyrode’s salts and glutaraldehyde solutions were purchased from Sigma Aldrich (St.Louis, MO). DTX powder was obtained from Natural Pharmaceuticals Inc. (Beverley, MA) and Taxotere® (DTX in Tween 80) was purchased from Sanofi Aventis Canada Inc. (Laval, QC). Tritium-labeled DTX (³H DTX) in ethanol was purchased from Moravek Biochemicals (Brea, CA) with a specific activity of 50.8 mCi/mmol. Liquid scintillation fluid, CytoScint™-ES, was purchased from MP Biomedicals (Irvine, CA). Chitosan hydrochloride (Protasan Cl 213) was obtained from Pronova Biopolymer (Oslo, Norway). Sodium cacodylate, and 16% formaldehyde solutions were obtained from Canemco Inc. (Lakefield, QC).

2.2.2 Porcine bladder tissue

Porcine bladders were obtained from Britco Inc. (Langley, BC). Freshly excised urinary bladders were removed on-site from 6 to 10 month-old male pigs weighing between 90 and 113 kg and transported on ice in Tyrode’s buffer to our lab within 60-90 min of sacrifice.

2.2.3 Preparation of HPG solutions for exfoliation studies

The HPG-OH (10% w/v) solution was made by dissolving weighed amounts of polymer in Tyrode’s buffer (adjusted to pH 6.4) at 37°C. The chitosan solutions were used as a positive control and made by dissolving a weighed amount of chitosan (0.05% w/v) in Tyrode’s buffer.
(adjusted to pH 6.4) at 37°C. Similarly, HPG-360 (1%, 3.3%, 10% w/v), HPG -580 (1%, 3.3%, 10% w/v) and HPG-780 (1%, 3.3%, 10% w/v) were prepared by dissolving the polymer in Tyrode’s buffer (pH 6.4) at 37°C.

2.2.4 Preparation of DTX-loaded nanoparticles

DTX-loaded HPG formulations were prepared using a solvent evaporation technique as previously reported (Mugabe et al., 2011a). DTX (0.5mg/ml) and HPG (10%) were dissolved in acetonitrile (ACN) and the solvent was then removed under a stream of nitrogen gas. Prior to drying, the polymer/drug solution was spiked with a small amount (50 μCi) of ³H DTX. After solvent evaporation, the resulting polymer/drug matrix was reconstituted in Tyrode’s buffer (pH 6.4) and vortexed for 2 min. The final concentration of drug was 0.5 mg/mL and the concentration of HPG was 10% w/v.

2.2.5 Preparation of DTX/polysorbate 80 micelles

DTX was prepared in polysorbate 80 micelles by diluting the Taxotere® concentrated solution (containing 40 mg of DTX and 1,040 mg of polysorbate 80 per mL, without using the ethanol diluent) with Tyrode’s buffer to yield a final concentration of 0.5 mg/mL DTX. The DTX/polysorbate 80 micellar solution thus contained no ethanol diluent, the latter being a constituent of the Taxotere® commercial formulation. Solutions were doped with a small amount (50 μCi) of ³H DTX prior to dilution.
2.2.6 Tissue sample preparation for drug uptake studies

Freshly excised porcine bladders were cleaned of excess adipose tissue on the exterior wall, opened longitudinally into left and right lateral sides and then cut into pieces measuring approximately 2 × 2 cm in a shallow bath of pH 7.4 Tyrode’s buffer bubbled with carbogen (95% O₂/5% CO₂). All studies were completed within 5 h of bladder collection from the pigs. Each bladder section was mounted onto a Franz diffusion cell apparatus, such that the luminal side of the bladder wall was exposed to the drug solution. These tissue sections were not stretched and measured approximately 2–3 mm thick. Receptor chambers were filled with 10 mL of 37°C Tyrode’s buffer (pH 7.4). The donor chambers contained 0.5 mL of either DTX/polysorbate 80 micelles (0.5 mg/mL) or DTX (0.5 mg/mL) loaded in HPG-360, HPG-580 and HPG-780 (10% w/v) nanoparticles. The diffusion cells were incubated at 37°C for 2 h. After incubation, tissue samples were washed three times with pH 7.4. Tyrode’s buffer to remove any unbound drug. The tissue samples were then trimmed and rapidly frozen with liquid nitrogen on a bed of dry ice.

2.2.7 Cryotome sectioning of tissue

Frozen bladder tissue was mounted with Shandon Cryomatrix™ (Thermo Scientific, Pittsburgh, PA) onto a cryotome object holder. Bladder tissue was sectioned using Shandon MB35 Premier Low Grade Microtome Blades (Thermo Scientific, Pittsburgh, PA) at −20°C on a Shandon Cryotome Electronic (Thermo Electron Corporation, Cheshire, England) with a R404A refrigeration system. Tissues were sectioned into individual 60 μm-thick samples. Tissues between 60 and 240 μm depth from the lumen side (urothelium) were collected individually for analysis. For tissue sections between 240 and 1,260 μm (lamina propria) two pooled 60 μm
sections were collected for analysis. For tissue sections between 1,260 and approximately 2500 μm (muscle layer) three pooled 60 μm sections were collected for analysis. Tissue sections were placed in pre-weighed 1.5 mL eppendorf tubes.

2.2.8 Quantification of drug in tissue
Two hundred microliters of acetonitrile (ACN) was added to the weighed tissue sections for drug extraction. Samples were vortexed until all tissue sections were freely submerged in ACN and left at room temperature for 24 h to ensure complete extraction of drug. The extracted samples including all tissue slices were transferred to scintillation vials and 5 mL of scintillation fluid were added. Counts of ³H DTX were measured by liquid scintillation counting (LS6500 Multi-purpose scintillation counter, Beckman coulter Fullerton, CA) and quantitated using calibration graphs from the original stock solutions.

2.2.9 Analysis of tissue level-depth profiles
The tissue level-depth profile of DTX was analyzed for average drug concentration in the whole bladder tissue as previously described (Tsallas et al., 2011). Briefly, the average tissue levels were calculated as the total amount of drug measured in the tissue layer divided by the total tissue weight for that layer. The area under the tissue level-depth profile (AUC) was calculated using the linear trapezoid rule, as follows:

\[ \text{AUC}_0 = \sum_{i=0}^{n-1} \frac{(t_{i+1} - t_i)}{2} \times (C_{i+1} + C_i) \]
Where,

\[ t = \text{Tissue depth (μm)} \]

\[ C = \text{Concentration (μg/g)} \]

To calculate the total AUC, an estimation by extrapolation of the drug concentration at 0 μm was performed.

### 2.2.10 Ex vivo exfoliation studies

Scanning electron microscopy (SEM) was used to characterize the urothelium morphology. As previously described, freshly excised porcine bladders tissues were mounted onto a Franz diffusion cell apparatus (Mugabe et al., 2009; Mugabe et al., 2011a; Mugabe et al., 2011b; Mugabe et al., 2011c; Mugabe et al., 2012). Receptor chambers were filled with 10 mL of 37°C Tyrode’s buffer (pH 7.4) and the donor chambers (urothelial side) were filled with 0.5 mL of either Tyrode’s buffer, DTX/polysorbate 80 micelles (0.5mg/mL), chitosan (0.005% w/v) or HPG-360, HPG-580, HPG-780 (1%, 3.3% or 10% w/v) and incubated at 37°C for 2 h. The solutions were removed, and the tissues were washed three times with pH 7.4 Tyrode’s buffer. The bladder tissues were fixed with a solution of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer and incubated for 3 h at 4°C. Tissues were transferred in 0.1 M sodium cacodylate buffer (pH 7.4) for 18 h in 4°C and later fixed in osmium tetroxide for 1 h at room temperature. After dehydration in increasing concentrations of acetone (30-100%) and critical point drying, sections were sputter coated to a depth of 8 mm with gold-platinum. Qualitative analysis was performed using SEM for evidence of morphological changes to the urothelial surface cells.
2.2.11 Time course of *ex vivo* exfoliation

As described above, freshly excised porcine bladders were mounted onto Franz diffusion cells. Receptor chambers were filled with 10 mL of 37°C Tyrode’s buffer (pH 7.4) and the donor chambers (urothelium side) were filled with 0.5 mL of either Tyrode’s buffer (pH 6.4), 10% w/v HPG-580 or 10% w/v HPG-780 solution and incubated at 37°C for 5, 10, 15, 30, 60 or 120 min. After each time point, solutions were removed, and the tissues were washed three times with pH 7.4 Tyrode’s buffer. Tissues were prepared for qualitative analysis with SEM for exfoliation studies as described above. Quantitative analysis of exfoliation was carried out using the method described below.

2.2.12 Quantitation of exfoliation

Tissue images were taken using SEM. As shown in Figure 2.1, the whole surface of the tissue was divided into a grid of five vertical and seven horizontally equal squares (square side length 1.2cm). Images were captured from every fourth square of the tissue section. To reduce the bias for each treatment, the sequence of square selection was different for each sample. A total of 10-13 images were captured per tissue sample and the image area was measured using the ImageJ National Institute of Health software (Schneider *et al.*, 2012). Total percent exfoliation was determined as the ratio of exfoliated area to the total area. Final results are represented as a graph comparing treatments (buffer, HPG-360, HPG-580 and HPG-780) with percent exfoliation.
Figure 2.1 Exfoliation was quantified based on SEM images as shown in the schematic above. The whole surface of tissue was divided into a grid and randomly selected images were captured per tissue sample. The image area was measured using the ImageJ National Institute of Health software. Total percent exfoliation was determined as the ratio of exfoliated area to the total area.

2.2.13 Statistical analysis

Data collected from in \textit{ex vivo} bladder tissue uptake studies and exfoliation studies are presented as mean ± standard error of mean. Statistical analysis was performed using GraphPad Prism version 5.0b (GraphPad Software, La Jolla CA). For comparisons between treatment groups, the results were analyzed by using a one-way ANOVA. Differences were considered significant at P<0.05. A Dunnett’s post hoc test was performed when a difference was detected and marked with an asterisk (*).
2.3 Results

2.3.1 Ex vivo exfoliation study

There was no effect of a 2h treatment of bladders with pH 6.4 Tyrode’s buffer solution, 10% w/v HPG-OH (non-aminated), or 0.5mg/mL DTX/polysorbate-80 micelles (negative control for exfoliation) on the morphology of the urothelium as visualized by SEM (Figure 2.2). Similarly, after treatment with HPG-360 at concentrations of 1% and 3.3% w/v, the bladder surface looked intact with a normal appearance (Figure 2.3 A, B). However, after exposure to a 10% w/v HPG-360 solution the surface resulted in a less uniform morphology with minor evidence of the exfoliation of umbrella cells from the bladder urothelium (Figure 2.3C). Treatment with 0.005% w/v chitosan, (positive control and a known inducer of exfoliation), also caused exfoliation in these studies (Figure 2.2D).

The exposure of bladder urothelium to a 1% w/v HPG-580 solution initiated exfoliation with evidence of less intact and missing umbrella cells (Figure 2.4.A). At a 3.3% w/v concentration of HPG-580 there was increased levels of exfoliation as compared to 1% w/v HPG-580 (Figure 2.4B) and at 10% w/v concentrations of HPG-580 there was complete stripping of umbrella cells and an uneven remaining bladder surface (Figure 2.4C). Exfoliation was initiated following exposure of the bladder to a concentration of only 1% w/v HPG-780 (Figure 2.5A). This exfoliation was extensive with 3.3% concentrations (Figure 2.5B) and was complete following exposure of bladder tissue to 10% w/v HPG-780 (Figure 2.5C).

The extent of exfoliation is shown as % exfoliation in Figure 2.6 and compares treatments (pH 6.4 buffer, 10% w/v HPG-360, 10% w/v HPG-580 and 10% w/v HPG-780). The total percent exfoliation value for 10% w/v HPG-360 was 15%, whereas 10% w/v HPG-580 caused 60% exfoliation and the 10% w/v HPG-780 exfoliated 99% of the porcine bladder.
urothelial surface. Exfoliation levels arising from HPG treatments were compared with pH 6.4 Tyrode’s buffer treatment. One-way ANOVA and Dunnett’s post test revealed significant difference in exfoliation, when 10% w/v HPG-360, 10% w/v HPG-580 and 10% w/v HPG-780 were compared with Tyrode’s buffer ($p<0.05$).
Figure 2.2 Scanning electron micrographs of freshly excised porcine bladder exposed to (A) Tyrode’s buffer (pH 7.4), (B) HPG-OH (10% w/v), and (C) DTX/polysorbate 80 micelles (0.5mg/mL), showing distinctive and intact pentagonal or hexagonal shaped superficial cells with tight junctions between umbrella cells. Chitosan (0.05% w/v) (D), as a positive control, showed complete stripping of umbrella cells on bladder tissue.
Figure 2.3 Scanning electron micrographs of freshly excised porcine bladder exposed to (A) 1% w/v HPG-360 and (B) 3.3% w/v HPG-360 showing intact bladder urothelium surface and (C) 10% w/v HPG-360 showing exfoliation initiation with less intact umbrella cells (arrows).
Figure 2.4 Scanning electron micrographs of freshly excised porcine bladder exposed to (A) 1%w/v HPG-580 with evidence of less intact and some missing umbrella cells (arrows), (B) 3.3%w/v HPG-580 showing exfoliation with evidence of complete cell removal from the urothelium (arrows) and (C) 10%w/v HPG-580 showing complete stripping of umbrella cells.
Figure 2.5 Scanning electron micrographs of freshly excised porcine bladder exposed to (A) 1% w/v HPG-780 with evidence of less intact umbrella cells (arrows), (B) 3.3% w/v HPG-780 showing mild exfoliation with evidence of cell removal from the urothelium and (C) 10% w/v HPG-780 showing complete stripping of umbrella cells.
Figure 2.6 Total percent of exfoliation caused by different amine density HPG-360, HPG-580 and HPG-780 on porcine bladder urothelium as quantified by an SEM image analysis method. Porcine bladder tissue was treated with 10% w/v of HPGs for 2h. Data are expressed as mean percent exfoliation ± SEM (n=10). *p<0.05 or 10% w/v HPG-360, ***p<0.001 for, 10% w/v HPG-580 and 10% w/v HPG-780 versus Tyrode’s buffer.
2.3.2 Time course of *ex vivo* exfoliation

The tissue incubated with a solution of 10% w/v HPG-580 w/v for 5 min showed well-preserved tight junctions between superficial cells (Figure 2.7A). Moreover, the urothelial cells showed only minor signs of exfoliation. However, after 10 and 15 min exposure to 10% w/v HPG-580 w/v the tight junctions between umbrella cells were partly exposed and signs of exfoliation with missing umbrella cells were observed (Figure 2.7B&C). Interestingly, at 30 min, 10% w/v HPG-580 exposure resulted in approximately 60% removal of umbrella cells (Figure 2.7D). Furthermore, 60 min and 2 h exposure of the tissue to a 10% w/v HPG-580 treatment results in complete removal of umbrella cell layer (Figure 2.7E&F).

The exposure of bladder tissue to 10% w/v HPG-780 resulted in partial opening of tight-junctions within 5 min of treatment (Figure 2.8A). After 10 and 15 min, approximately 30% of the umbrella cells were exfoliated (Figure 2.8B&C). Further, incubation in 10% w/v HPG-780 for 30 min showed extensive exfoliation of the urothelium (Figure 2.8D). However, at 60 min and 2 h the extent of exfoliation was similar to 30 min treatment but with further exposure of underlying cell layer (Figure 2.8E&F).

The quantitation of exfoliation is showing in Figure 2.9. HPG-580 (10% w/v) caused 80% exfoliation of whole bladder tissue at 60 minutes, whereas 10% w/v concentrations of HPG-780 showed 100% exfoliation with 30 min of exposure to porcine bladder tissue. Two-way ANOVA and Bonferroni posttest showed the elevated drug levels observed for HPG-780 at 30 minutes were significantly higher that the extent of exfoliation observed for HPG-580 at the sametime.
Figure 2.7 Exfoliation time course study of porcine bladder urothelium caused by 10% w/v HPG-580 showing: (A) 5 minute shows distinctive and intact superficial cells with smooth appearance of urothelium, (B, C) After 10 and 15 minute treatment results in initiation of exfoliation seen as a removal of umbrella cell in patches from the urothelium, (D) 30 minute exfoliation of urothelium is seen with many missing umbrella cells and at (E, F) 60, 120 minute there were no remaining umbrella cells and complete exfoliation.
Figure 2.8 Exfoliation time course study of porcine bladder urothelium caused by 10% w/v HPG-780 showing: (A) 5 minutes: gaps between umbrella cells (B, C), 10 and 15 minutes: there is complete removal of umbrella cells (D) 30 minute: exfoliation of urothelium is seen with fully stripped urothelium and (E, F) 60 and 120 minutes full exfoliation.
Figure 2.9 Time course of exfoliation caused by 10%w/v HPG-580 and 10%w/v HPG-780 on porcine bladder urothelium. Porcine bladder tissue was treated with 10% HPGs and treatment was terminated at 5, 10, 15, 30, 60 and 120 minutes to observe complete exfoliation time. Data is represented as ±SD (n=10).
2.3.3 DTX uptake in bladder tissue

The distribution of DTX in bladder tissue treated with 10% w/v HPG formulations using a DTX concentration of 0.5 mg/mL is shown in Figure 2.10. For all incubations, the drug penetration increased and then decreased with tissue depth. A similar average tissue concentration of DTX was observed in tissues treated with either 10% w/v HPG-360 or DTX/polysorbate-80 micelles formulations (approx. 10-30 μg of DTX/g of tissue). However, when tissues were treated with DTX loaded in 10% w/v HPG-580 there were greatly increased tissue levels of DTX of around 40-60 μg/g of tissue, in the sub-urothelial layer around 250-1000 μm (i.e. lamina propria), when compared to the DTX/polysorbate-80 micelles. The tissue levels of DTX in deeper regions remained greater than those for tissues treated with the polysorbate micellar formulation. Incubation of bladder tissues with DTX in HPG-780 at concentrations of 10% w/v resulted in further increases in DTX concentrations in all tissue sections (50-90 μg/g of tissue) as compared to levels in tissues treated with DTX/polysorbate-80 micelles.
Figure 2.10 Tissue level-depth profiles of DTX in bladder tissue following exposure to 0.5 mg/mL DTX/polysorbate-80 micelle commercial formulation (Taxotere®) or 10%w/v HPG formulations. Tissues were incubated for 2 h and sectioned by cryotome. Values are means ± SEM (n = 11).

The area under the curve (AUC) of DTX in pooled samples (60–240 µm: urothelium, 240–1,260 µm: the lamina propria, 1,260–2,160 µm: the muscle layer and 60–2,160 µm (the whole tissue), following incubation with DTX at 0.5 mg/mL in either 10% w/v HPG-360, 10% w/v HPG-580, 10% w/v HPG-780 or DTX/polysorbate-80
micelles formulations for 2h is shown in **Figure 2.11**. The AUCs of DTX in various tissue layers of the bladder wall were calculated using the linear trapezoid rule. One-way ANOVA followed by the Dunnett’s post-test was used to evaluate differences between the AUCs in various layers. There was no statistically significant difference between DTX/polysorbate-80 micelles and 10% w/v HPG-360-DTX uptake in any tissue layer. However, DTX levels were significantly higher for 10% w/v HPG-580-DTX formulations for the lamina propria layer and muscle (p<0.05) compared to DTX/polysorbate-80 micelles. There was a significant difference in drug uptake levels in all tissue layers when comparing bladder tissues exposed to 0.5 mg/mL DTX in 10% w/v HPG-780 formulation versus 0.5 mg/mL DTX/polysorbate-80 micelles (p<0.001).

The average AUCs for the whole tissue (0–2,160 μm) exposure to 0.5 mg/mL DTX/polysorbate 80 micelles, 10% w/v HPG-360-DTX, 10% w/v HPG-580-DTX and 10% w/v HPG-780-DTX, respectively, is shown in Table 2.3.11 & **Figure 2.12**. Statistical significance was done by one-way ANOVA with Dunnett’s post-test with ± SEM (n = 11). The DTX tissue levels were significantly higher for HPGs treatment (10% w/v HPG-580 and 10% w/v HPG-780) formulations compared to treatment with 0.5 mg/mL DTX/polysorbate 80 micelles.
Figure 2.11 The area under the curve (AUC) of DTX in various layers of the bladder wall (60–240 μm: urothelium, 240–1,260 μm: lamina propria, and 1,260–2,160 μm muscle layer) following incubation with DTX at 0.5 mg/mL from 10% w/HPG-360, 10% w/v HPG-580, 10% w/v HPG-780 or DTX/polysorbate-80 micelle formulations for 2h. AUCs of DTX in various tissue layers of the bladder wall were calculated using the linear trapezoid rule.
Table 2.1 The average area under the curve (AUC) between the bladder tissue layer and whole tissue.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Urothelium (µg. µm/g)</th>
<th>Lamina propria (µg. µm/g)</th>
<th>Muscle layer (µg. µm/g)</th>
<th>Whole tissue (µg. µm/g)</th>
</tr>
</thead>
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<tr>
<td>DTX/polysorbate-80 micelles</td>
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<td>23592</td>
<td>13709</td>
<td>46664</td>
</tr>
<tr>
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<td>29525</td>
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<tr>
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<td>47921</td>
<td>26596</td>
<td>87231</td>
</tr>
<tr>
<td>10% w/v HPG-780-DTX</td>
<td>19762</td>
<td>74734</td>
<td>55001</td>
<td>149499</td>
</tr>
</tbody>
</table>

![Figure 2.12 The AUC for the whole tissue (0–2500µm) for 0.5 mg/mL DTX in 10%w/v HPG-780 and 10%w/v HPG-580 formulations versus 0.5 mg/mL DTX/polysorbate-80 micelles. Data are means ± SEM (n = 11). ***p<0.001, * p<0.05.](image_url)
2.4 Discussion

In this study, we investigated the effect of both different surface amine densities on the HPGs and increasing solution concentrations of amine conjugated HPGs on the extent of exfoliation in ex-vivo porcine bladder. Porcine bladder is an excellent model of the human bladder since it is very similar in terms of cell layers and mucosal wall thickness (Dixon et al., 1983; Tsallas et al., 2011). Previous studies have demonstrated that the bladder tissue remains viable more than 5 h following sacrifice and organ retrieval (Tsallas et al., 2011).

Our original hypothesis for enhancing bladder tissue uptake of drugs following intravesical administration was based on developing a mucoadhesive formulation to enhance contact time with the bladder urothelium mucosal layer (Mugabe et al., 2011b). In order to develop a formulation with improved mucoadhesion, we synthesized HPG-C$_8$/10-MePEG nanoparticles, surface modified with amine groups and described earlier (Mugabe et al., 2011b; Mugabe et al., 2012) . The presence of positively charged amine groups on HPG-C$_8$/10-MePEG-NH$_2$ nanoparticles resulted in binding with mucins via interactions with the negatively charged sialic acid moieties to facilitate mucoadhesion, in a manner similar to chitosan. It is also likely that the small size of these HPGs (<10nm) allowed for the movement of the nanoparticles between the urothelial surface bound mucin glycoprotein chains, thus leading to direct interaction of the nanoparticle surface with the urothelium (Mugabe et al., 2011b). Our previous work also showed that the conjugation of amine groups onto the surface of hydrophobically derivatized HPG nanoparticles caused rapid exfoliation of umbrella cells in both mice and porcine bladder tissue upon exposure to these nanoparticles (Mugabe et al., 2011c; Mugabe et al., 2012).
Following removal of the nanoparticles from mice bladders, we showed that the urothelium recovered within 24 hours post treatment (Mugabe et al., 2012).

There are several reports in the literature of biological and chemical agents such as, uropathogenic Escherichia coli, bacterial endotoxins such as lipopolysaccharide (Mysorekar et al., 2002), chitosan (Grabnar et al., 2003; Kerec et al., 2005; Veranic et al., 2009; Erman et al., 2013), cyclophosphamide (Veranic et al., 2004), protamine sulfate (Lavelle et al., 2002) sodium saccharin (Romih et al., 1998) moderate stress (Dalal et al., 1994), depletion of calcium (Veranic et al., 2000), and irradiation, all of which have been shown to cause the rapid exfoliation of the superficial urothelium (Jaal et al., 2006). Lavelle et al. have investigated the effect of protamine sulfate, a polycationic peptide on the rat urothelium exfoliation and recovery processes (Lavelle et al., 2002). Intravesical administration of protamine sulphate caused exfoliation of the umbrella cells, which immediately initiated the urothelium recovery response with rapid differentiation and maturation of underlying intermediate cells and gradual formation of well-defined tight junctions (Lavelle et al., 2002). The urothelium integrity was restored by day 5 post-exposure to protamine sulfate, and full recovery were achieved after 10 days. A similar recovery process was reported with bacterial infection of the urinary bladder (Mulvey et al., 1998). It has been shown that type 1 pilus tips of Escherichia coli are able to interact directly with uroplakins in the umbrella cells of the mouse urothelium resulting in a rapid exfoliation via an apoptosis-like mechanism, which was followed by a rapid recovery process (48 h) for the urothelium. Positively charged polymers, such as chitosan and PAMAM have also shown to modulate tight junctions to enhance the permeability of various agents (Jevprasesphant et al., 2003).
The direct relationship between increasing amine density on the HPG and extent of exfoliation may be clearly seen in these studies. When bladder tissue was exposed to the lowest amine density HPGs (HPG-360) it resulted in only minor evidence of detached umbrella cells and with an otherwise normal looking bladder surface, and only when used at high nanoparticle concentrations, (Figure 2.3). When tissues were exposed to higher density amine terminated HPGs (HPG-580), concentration dependent morphological changes were observed. At lower concentrations, exposure to HPG-580 initiated the loss of tight junctions and weakening of cell-to-cell contact as seen by loosely connected and occasional missing tight junctions along with some missing umbrella cells (Figure 2.4). However, 10% w/v HPG-580 treatment resulted in the complete removal of the umbrella cell layer (Figure 2.4). The bladder tissue exposed to HPG-780, the highest amine density HPGs, resulted in higher levels of exfoliation than observed for HPG-580 (Figure 2.5). Even exposure of the bladder surface to HPG-780 at a concentration of 1% w/v resulted in loosening of umbrella cells and at 3.3 % w/v concentrations there were areas of complete removal of large patches of umbrella cells. At 10% exposure, we observed entire stripping of the umbrella cell layer from the urothelium. These findings are in agreement with our previous preliminary studies presenting a concentration dependent response of urothelium to HPGs exposure (Mugabe et al., 2011b; Mugabe et al., 2011c; Mugabe et al., 2012). In exfoliation time course studies, the high density HPG-780 took 30 min to complete the 100% exfoliation whereas medium density HPG-580 exfoliated the urothelium in 1 hour.

The complete exfoliation of the umbrella cells from the bladder urothelium caused by HPG-580 and HPG-780 removed the normal barrier to drug penetration. Since
the underlying cells are uncovered, it is likely that the DTX-loaded HPG molecules further interact with these cells ensuring a high drug concentration at the tissue surface. Since the DTX is likely bound to the hydrophobic alkyl chains of the HPG nanoparticle core via hydrophobic interactions (Kainthan et al., 2008a; Kainthan et al., 2008b; Mugabe et al., 2012), the drug would then partition from the HPG core to the cell membranes to create a high drug loading in the intermediate cell layers and a concentration dependent driving force for drug diffusion throughout the tissue. Interestingly, the hydrophobic taxane drugs typically have a long residence time in tissues so that, in vivo, the regenerated barrier of the urothelium may subsequently inhibit drug diffusion into the urine, further extending tissue exposure to the drugs (Song et al., 1997; Veranic et al., 2009; Birder et al., 2010; James M. McKiernan, 2010). The studies presented here demonstrated a clear relationship between the degree of exfoliation and an increased tissue accumulation of DTX. Clearly for polysorbate-80 or HPG-360, formulations, which have no effect on urothelial morphology, the levels of drug uptake were low (Figure 2.10). These levels are similar to those previously reported for Taxotere® (Tsallas et al., 2011) and non aminated HPG formulations (Mugabe et al., 2011a; Mugabe et al., 2011b; Mugabe et al., 2011c; Tsallas et al., 2011; Mugabe et al., 2012). However, with intermediate (HPG-580) and high levels of amination (HPG-780) and associated exfoliation observed with these agents, the overall levels of DTX tissue uptake were increased 2-3 fold as compared to polysorbate-80 micelles (Figure 2.10). Since the loss of the urothelial barrier allows improved drug penetration, then an optimal strategy for enhanced DTX accumulation in bladder tissue may rely on a rapid exfoliation mechanism as occurs for HPG-580 and 780 at 10% concentrations (Figure 2.4-2.8).
Since the maximum intravesical residence time of a drug solution is approximately 2 hours, this allows a longer exposure of the underlying tissue to surface bound DTX and a greater time for drug diffusion and accumulation into the bladder tissue.

In conclusion, these studies have clearly mapped out the time course, surface amine density and concentration dependence of the aminated HPG-C_{8/10}-NH\textsubscript{2} exfoliation process. This knowledge may allow for the optimization of an effective mucoadhesive nanoparticulate formulation for intravesical administration of DTX, which would provide a balance between maximum drug uptake, but with minimal tissue toxicity and without adversely affecting subsequent tissue recovery processes. The outcome should be increased drug efficacy whilst minimizing inflammation and patient morbidity.
Chapter 3: To investigate the effect of the intravesical chemotherapeutic agents MMC, doxorubicin and gemcitabine on ex vivo porcine bladder tissue morphology and drug uptake

3.1 Introduction

Globally, bladder cancer is the ninth most prevalent form of cancer usually affecting elderly patients and mostly men (J Ferlay, 2012). Approximately 70% of all newly diagnosed cases of bladder cancer involve non-muscle invasive bladder tumors (NMIBC) and are categorized in terms of increasing severity, as pathologic stages Ta, T1, and carcinoma in situ (CIS). Transurethral resection of bladder tumor(s), or TURBT, is the standard surgical practice performed by urologists for the treatment of NMIBC (Aagaard \textit{et al.}, 2013; Clark \textit{et al.}, 2013; Richards \textit{et al.}, 2014b). To reduce the likelihood of cancer recurrence after TURBT, adjuvant intravesical immunotherapy or chemotherapy is generally administered. The procedure involves the direct instillation of either chemotherapeutic drugs or immunomodulating agents into the bladder via a catheter. Chemotherapeutic agents delivered via the intravesical route include, thiotepa, doxorubicin, epirubicin, mitomycin C (MMC) and gemcitabine (Kassouf \textit{et al.}, 2010; Sexton \textit{et al.}, 2010; Leopardo \textit{et al.}, 2013). However, one study has noted that intravesical chemotherapy only resulted in an average short-term decrease in the bladder cancer recurrence rate of 14% with no significant effect on recurrence at 5 years (Shelley \textit{et al.}, 2010).

The lack of major therapeutic benefit from intravesical chemotherapy is thought to arise from a lack of sensitivity of bladder cancer cells to the drugs or from poor drug
delivery to the bladder tissue (Tammela et al., 1993; Volpe et al., 2010). The high rate of tumor recurrence necessitates lifetime surveillance and leads to high health care related costs. Therefore, there is significant research interest in improving intravesical formulations and evaluating potentially synergistic combination drug treatments. More recent clinical trials evaluated the six weekly sequential instillation of two drugs into patient bladders; gemcitabine (2000mg) for 90 min, removal of drug followed by 90 min instillation of mitomycin C (MMC) (40mg). At 26 months 30% of a total of 47 patients were recurrence free, whereas 10 patients had progressed and required cystectomy (Breyer et al., 2010; Lightfoot et al., 2014). Studies have evaluated the combination effects of gemcitabine with oxaliplatin, cisplatin, paclitaxel or docetaxel (Neri et al., 2007; Chen et al., 2012a; Haggag et al., 2014). New drug delivery strategies such as formulating MMC in chitosan nanoparticles or device assisted drug delivery such as MMC administered together with electromotive drug administration (EMDA) or hyperthermia (HT) are also under investigation to improve existing treatment approaches (Grabnar et al., 2003; Tyagi et al., 2006; Bilensoy et al., 2009; GuhaSarkar et al., 2010; Lu et al., 2011; Erdogar et al., 2012; Milla et al., 2014).

Our group has reported the development of a nanoparticulate drug delivery system for the anticancer drug, docetaxel (DTX), based on the use of functionalized hyperbranched polyglycerols (HPGs) as mucoadhesive drug carriers (Mugabe et al., 2011b; Mugabe et al., 2012). These multi-branched carriers or ‘unimolecular micelles”, consist of a hydrophobic core of alkyl (C₈/C₁₀) chains, providing binding sites for hydrophobic drugs, such as DTX (Kainthan et al., 2008a) and connected by covalent bonds to a hydrophilic shell composed of methoxy poly(ethylene glycol) (MePEG, MW
350) providing water solubility and improved biocompatibility (Kainthan et al., 2008a). Brooks and coworkers were the first to report the one pot synthesis of high molecular weight HPGs with a low polydispersity index ($M_w/M_n = 1.1-1.4$), intrinsic viscosities (less than 10 mL/g) and small hydrodynamic radii (5-10 nm) (Kainthan et al., 2008a).

The conjugation of amine groups to the surface of the HPGs promotes nanoparticle interaction with urothelial mucin chains promoting mucoadhesion (Kainthan et al., 2008a; Kainthan et al., 2008b). Intravesical administration of amine conjugated, taxane loaded HPG formulations showed increased PTX and DTX uptake into bladder tissue as compared to the commercial PTX and DTX formulations (Mugabe et al., 2011a; Mugabe et al., 2012). It was also shown that treatment of bladder tissue with aminated HPGs resulted in the exfoliation of umbrella cells from the urothelial surface so that the barrier properties were temporarily compromised, allowing drug to be transported into the bladder tissue (Mugabe et al., 2012). On the other hand, the intravesical instillation of the polysorbate-80-based commercial formulation of DTX (Taxotere®) showed no exfoliating effect on the bladder urothelium, tissue uptake levels of DTX were low and in vivo studies in an animal model of superficial bladder cancer showed limited efficacy (Mugabe et al., 2011c; Mugabe et al., 2012). In vivo, this strategy of using amine conjugated HPG nanoparticles to both solubilize the hydrophobic drug DTX and to exfoliate and permeabilize the bladder tissue has been shown to be very effective in inhibiting bladder tumor growth in mice (Mugabe et al., 2011a; Mugabe et al., 2011b; Mugabe et al., 2011c; Mugabe et al., 2012). The ability to exfoliate bladder urothelium has also been demonstrated by other amine containing agents such as, chitosan and protamine sulfate (Kerec et al., 2005; Veranic et al., 2009).
The molecular structure of MMC, doxorubicin and gemcitabine are shown in Figure 3.1, and illustrates the presence of amine group on these compounds. Lim et al in a recent case report noted that a patient’s bladder had evidence of exfoliation following an early instillation of a single dose of MMC (Lim et al., 2010). Furthermore, in another study an instillation of epirubicin also showed exfoliation in patients (Tyritzis et al., 2009).

![Chemical structures of mitomycin C (A), gemcitabine (B) and doxorubicin (C).](image)

Figure 3.1 Chemical structures of mitomycin C (A), gemcitabine (B) and doxorubicin (C).

The effects of the water-soluble anticancer drugs doxorubicin, MMC and gemcitabine on the integrity of the bladder urothelium is unknown. The objectives of this work were to investigate the effects of MMC, doxorubicin and gemcitabine on porcine bladder morphology and to determine whether the combination of each of these drugs with either PTX or DTX influenced the porcine bladder tissue uptake of the taxanes. Furthermore, having established the effectiveness of amine conjugated HPGs in exfoliating the bladder urothelium and enhancing drug uptake into bladder tissue, we
investigated the influence of pretreatment of porcine bladder tissue with an HPG formulation with an intermediate amine surface density (HPG-580 with 580 moles amine per mole HPG) on subsequent tissue uptake of MMC, doxorubicin, and gemcitabine.

3.2 Materials and methods

3.2.1 Materials

Hyperbranched polyglycerols (HPGs) were a kind gift from the Centre for Drug Research and Development (CDRD). Acetonitrile, HPLC grade, was purchased from Fisher Scientific (Ottawa, ON). Tyrode’s salts and glutaraldehyde solutions were purchased from Sigma Aldrich (St.Louis, MO). Mitomycin C (MMC) and gemcitabine hydrochloride were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Tritium labeled mitomycin C with a (specific activity of 17.2 mCi/mmol) and tritium labeled gemcitabine (specific activity of 23.4 mCi/mmol) were purchased from Moravek Biochemical CA (Brea, CA). Carbon-14 labelled doxorubicin hydrochloride (specific activity of 57 mCi/mmol) was purchased from GE Healthcare UK. Taxotere® was purchased from Sanofi Aventis Canada Inc. (Laval, QC). Tritium-labeled DTX (³H DTX) in ethanol was purchased from Moravek Biochemicals (Brea, CA) with a specific activity of 50.8 mCi/mmol. Taxol® was purchased from Bristol-Myers Squibb Company (Princeton, NJ, USA). Tritium-labeled PTX (³H PTX) in ethyl acetate solution was purchased from Moravek Biochemicals (Brea, CA) with a specific activity of 25 μCi. Liquid scintillation fluid, CytoScint™-ES, was purchased from MP Biomedicals (Irvine, CA). Chitosan hydrochloride (Protasan Cl 213) was obtained from Pronova Biopolymer
Oslo, Norway). Sodium cacodylate, and 16% formaldehyde solutions were obtained from Canemco Inc. (Lakefield, QC).

3.2.2 Porcine bladder tissue
Porcine bladders were obtained from Britco Inc. (Langley, BC). Freshly excised urinary bladders were removed on-site from 6 to 10-month-old male pigs weighing between 90 and 113 kg and transported on ice in Tyrode’s buffer to our lab within 60-90 min of sacrifice.

3.2.3 Preparation of chemotherapeutic drug solutions for drug uptake studies
MMC and gemcitabine solutions were prepared by dissolution in Tyrode’s buffer (pH 6.4) to yield a final concentration of 1 mg/mL. MMC and gemcitabine solutions were doped with a small amount (20μCi) of ³H labeled drug prior to drug incubation with bladder tissue. For doxorubicin, the drug solution (1 mg/mL) was prepared in Tyrode’s buffer (pH 6.4) and doped with ¹⁴C labeled doxorubicin (50 μCi). HPG-580 (10% w/v) solution for pretreatment was prepared by dissolving in Tyrode’s buffer (pH 6.4).

PTX micelles and DTX/polysorbate-80 micelles were prepared by dissolving in Tyrode’s buffer (pH 6.4) at 0.5 mg/mL concentration. For drug combination experiments, MMC, doxorubicin or gemcitabine dry powder was dissolved in PTX micellar or DTX/polysorbate-80 micellar dispersions at 1 mg/mL concentration. The final drug combination solution contained either of the taxanes at 0.5 mg/mL concentration with one of the water-soluble drugs at 1 mg/mL DTX/polysorbate-80 micelles solutions were
doped with a small amount (20 μCi) of $^3$H DTX. Similarly, PTX micelles solutions were
doped with $^3$H PTX (200 μCi).

### 3.2.4 Bladder tissue sample preparation

Freshly excised porcine bladders were cleaned of excess adipose tissue on the exterior
wall and opened longitudinally into left and right lateral sides and cut into pieces
approximately 2 × 2 cm in size in a shallow bath of 37°C Tyrode’s buffer bubbled with
carbogen (95% O$_2$/5% CO$_2$). All studies were performed within 5 h of sacrifice. Bladder
sections were mounted onto a Franz diffusion cell apparatus, such that the luminal side of
the bladder wall was exposed to the drug solution as previously described (Tsallas et al.,
2011; Mugabe et al., 2012). These tissue sections were not stretched and measured
approximately 2–3 mm thick.

### 3.2.5 Ex vivo bladder tissue exfoliation studies

Receptor chambers of the diffusion cells were filled with 10 ml of Tyrode’s buffer (pH
7.4) and the donor chambers (urothelial side) were filled with 0.5 mL of either Tyrode’s
buffer (pH 6.4), HPG-580 (10% w/v), MMC (1 mg/ mL), doxorubicin (1 mg/ mL) or
gemcitabine solution (1 mg/ mL) and incubated at 37°C for 2h. The solutions were
removed, and the tissues were washed three times with Tyrode’s buffer (pH 7.4). The
bladder tissues were fixed with a solution of 2% glutaraldehyde and 2%
paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and incubated for 3h at
4°C. Tissues were transferred into 0.1 M sodium cacodylate buffer (pH 7.4) for 18h at
4°C and later fixed with osmium tetroxide for 1h at room temperature. After dehydration
in increasing concentrations of acetone (30-100%) and critical point drying, sections were sputter coated with gold-platinum. Qualitative analysis was performed using SEM for evidence of changes to urothelial morphology.

3.2.6 Drug uptake studies

For studies involving pretreatment with HPG-580, receptor chambers were filled with 10 ml of Tyrode’s buffer (37°C, pH 7.4) and the donor chambers were filled with 0.5 mL of HPG-580 (10% w/v in Tyrode’s pH 6.4) solution or 0.5 mL of Tyrode’s pH 6.4 alone. The tissues were incubated with the HPG-580 (10% w/v) at 37°C for 1h and then washed three times with Tyrode’s buffer pH 7.4. Immediately following washing, 0.5 mL of either MMC (1 mg/mL), doxorubicin (1 mg/mL) or gemcitabine (1 mg/mL), were added to the donor chamber and the tissues were incubated at 37°C for 2h. Tissue samples were then removed, trimmed and rapidly frozen on metal plates with liquid nitrogen on a bed of dry ice.

For studies involving combination drug treatments, either PTX micelle or DTX/polysorbate-80 micelle dispersions were used at 0.5 mg/mL with either MMC, doxorubicin or gemcitabine at 1 mg/mL final concentration. The combination drug solutions were added to the donor chamber and incubated at 37°C for 2h. Tissue samples were then removed, trimmed and rapidly frozen on metal plates with liquid nitrogen on a bed of dry ice.
3.2.7 Cryotome sectioning of tissue

Frozen bladder tissue was mounted with Shandon Cryomatrix™ (Thermo Scientific, Pittsburgh, PA) onto a cryotome sample holder. Bladder tissue was sectioned (60 um) with Shandon MB35 Premier Low Grade Microtome Blades (Thermo Scientific, Pittsburgh, PA) at −20°C on a Shandon Cryotome Electronic (Thermo Electron Corporation, Cheshire, England) with a R404A refrigeration system. Tissues between 60 and 240 μm (urothelium) were collected individually for analysis. Two tissue sections (both 60 μm) were collected between depths of 240 and 1,260 μm (lamina propria) and pooled for analysis. Three tissue sections between 1,260 and 2,160 or 3,060 μm (muscle layer) were collected and pooled for analysis. Tissue sections were placed in pre-weighed 1.5 mL eppendorf tubes and frozen at −20°C.

3.2.8 Quantification of drug in bladder tissue

Two hundred microliters of acetonitrile (ACN) was added to the weighed tissue slices for drug extraction. Samples were vortexed until all tissue slices were freely submerged in ACN and left at room temperature for 24 h to ensure complete extraction of drug. The extracted samples, including all tissue slices, were transferred to scintillation vials and 5 mL of scintillation fluid were added. The amount of drug in the sections was then determined using liquid scintillation counting and quantitated using calibration graphs from the original stock solutions.
3.2.9 H&E staining

Slides were fixed in a 60 degree C oven prior to staining, then deparaffinised and rehydrated through xylene, 100% alcohol, 95% alcohol and water. Slides were stained with Gill’s hematoxylin, 1% acidic alcohol, lithium carbonate and eosin solution with a water wash in between each stain. Slides were then dehydrated and cleaned through 95% alcohol, 100% alcohol and xylene. Slides were cover slipped with permount mounting medium. Images were taken using light microscopy.

3.2.10 Statistical analysis

Data collected from bladder tissue drug uptake and exfoliation studies are presented as mean ± standard error of mean. Statistical analysis was performed using GraphPad Prism version 5.0b (GraphPad Software, La Jolla CA). For comparisons between treatment groups, the results were analyzed by using a one-way ANOVA. Differences were considered significant at p<0.05. A Dunnett’s post hoc test was performed when a difference was detected and marked with an asterisk (*).

3.3 Results

3.3.1 Effect of pretreatment with HPG-580 on the tissue uptake of MMC, doxorubicin or gemcitabine

In this study, tissues were pretreated with either buffer (Tyrode’s pH 6.4) or HPG-580 (10%w/v) for 1h followed by 2h incubation with MMC, doxorubicin or gemcitabine drug solutions (radiolabelled). MMC tissue levels reached a maximum of 110 μg/g, followed by a steady decrease in drug concentration (Figure 3.2). Pre-incubation with HPG-580
(10%w/v) had no significant effect on MMC tissue levels (p>0.05). Doxorubicin showed a lower degree of drug penetration into bladder tissue compared to MMC, and pretreatment with HPG-580 (10%w/v) had no effect on tissue concentrations of drug (Figure 3.3). The maximum gemcitabine tissue level was approximately 100 µg/g and similarly, pretreatment with HPG-580 (10%w/v) had no effect on tissue levels of drug (Figure 3.4).

Figure 3.2 Tissue level-depth profiles of Mitomycin C (MMC) in bladder tissue. Tissue was exposed to 1 mg/ml MMC for 2h or pretreated with HPG-580 (10% w/v) for 1h followed by a 2h treatment with MMC (1mg/ml). Values are means ± SEM (n = 15).
Figure 3.3 Tissue level-depth profiles of Doxorubicin (DOX) in bladder tissue. Tissue was exposed to 1 mg/ml DOX for 2h or pretreated with HPG-580 (10% w/v) for 1h followed by a 2h treatment with DOX (1mg/ml). Values are means ± SEM (n = 15).
Figure 3.4 Tissue level-depth profiles of Gemcitabine (GEM) in bladder tissue. Tissue was exposed to 1 mg/ml GEM for 2h or pretreated with HPG-580 (10% w/v) for 1h followed by a 2h treatment with GEM (1mg/ml). Values are means ± SEM (n = 15).

3.3.2 Effect of combination drug treatments on tissue uptake of PTX and DTX

DTX formulated as DTX/polysorbate-80 micelles penetrated bladder tissue at concentrations in the 40-60 µg/g of tissue range at a depth of approximately 500 µm. Gradually drug concentrations dropped to 20 µg/g of tissue or less at depths of 1250 µm. For DTX, the penetration of the drug was enhanced following pretreatment of the bladder with HPG-580 (10% w/v) (Figure 3.5). The simultaneous treatment of tissue with DTX in a solution of MMC gave the highest levels of drug uptake with concentrations above 100 µg/g of tissue dropping to approximately 60µg/g of tissue at a depth of 700µm.
Simultaneous treatment with either doxorubicin or gemcitabine also gave enhanced levels of DTX uptake across the lamina propria layer (to depths of approximately 1200 μm) as seen in Figure 3.5.

PTX penetrated bladder tissues less effectively than DTX so that when presented in the PTX micelles alone, tissue levels remained below 10 μg/g of tissue throughout all layers of the tissue (Figure 3.6). Pretreatment of tissues with HPG-580 (10%w/v) enhanced the uptake of this drug reaching levels of approximately 20 μg/g in the urothelium and between 20 and 10 μg/g of tissue though most of the lamina propria. The combination treatment of bladder tissue with PTX micelles and either MMC, doxorubicin or gemcitabine also resulted in greatly enhanced PTX uptake throughout the whole depth profile of the tissue. For PTX, doxorubicin had the greatest enhancing effect with drug levels similar to those observed following pretreatment with HPG-580 (Figure 3.6). The combination treatment of PTX micelles with either MMC or gemcitabine resulted in similar levels of enhanced PTX uptake in bladder tissue compared to PTX micelles formulation alone.

The total amount of DTX or PTX taken up by the bladder is shown in Figure 3.7 & Figure 3.8 as the total area under the curve (AUC) determined from the data in Figure 3.5 and Figure 3.6 respectively. Both DTX and PTX, combination treatment with gemcitabine, doxorubicin, MMC or pretreatment with HPG-580 (10%w/v) resulted in significantly enhanced drug uptake levels in porcine bladder tissue. The combination treatment with gemcitabine and doxorubicin significantly increased (p<0.05, one-way ANOVA) the DTX uptake compared to DTX/polysorbate-80 micelles. The pretreatment of porcine bladder tissue with HPG-580 (10% w/v) significantly enhanced the DTX
tissue level (p<0.001, one-way ANOVA). However DTX/polysorbate-80 micelles combination treatment with MMC resulted in a greater AUC with significance level of p<0.001, one-way ANOVA.

Similarly, the combination treatment of PTX micelles with MMC (p<0.001), doxorubicin (p<0.001), gemcitabine (p<0.05) and pretreatment with HPG-580 (10% w/v) (p<0.001) followed by PTX micelles treatment overall significantly enhanced tissue levels of PTX compared to PTX micelles alone. Data were analyzed by using one-way ANOVA with Dunnett’s post-test for significant difference.
Figure 3.5 Tissue level-depth profiles of DTX in bladder tissue. Bladder tissue was either exposed for 2h to 0.5mg/ml of DTX/polysorbate-80 micelles as a control or drug combinations of DTX/polysorbate 80 micelles (0.5mg/ml) with MMC, doxorubicin or gemcitabine (1mg/ml). Some tissues were pretreated with HPG-580 (10% w/v) for one hour followed by DTX/polysorbate-80 micelles (0.5mg/ml). Values are means ± SEM (n = 6).
Figure 3.6 Tissue level-depth profiles of PTX in bladder tissue. Bladder tissue was either exposed for 2h to 0.5mg/ml of PTX micelles (a commercial formulation (Taxol)) as a control or drug combinations of PTX micelles (0.5mg/ml) with MMC, doxorubicin or gemcitabine (1mg/ml). Some tissues were also pretreated with HPG-580 (10% w/v) for one hour followed by PTX micelles (0.5mg/ml). Values are means ± SEM (n = 11).
Figure 3.7 AUC values for total drug accumulation in bladder tissue following dual drug treatments. Bladders were treated with (A) DTX alone (DTX/polysorbate-80 micelles 0.5mg/ml) as a control or DTX in combination with MMC, DOX, or GEM (1mg/ml). Some tissues were also pretreated with HPG560 (10% w/v) for one hour followed by DTX/polysorbate-80 micelles (0.5mg/ml). The data was compared between control and treatments by one-way ANOVA and significance is shown with p values (*p<0.05, **p<0.001, ***p<0.0001).
Figure 3.8 AUC values for total drug accumulation in bladder tissue following dual drug treatments. Bladders were treated with PTX micelles (0.5mg/ml) or PTX micelles in combination with MMC, DOX, or GEM (1mg/ml). Some tissues were pretreated with HPG-580 (10% w/v) for one hour followed by PTX (0.5mg/ml). The data was compared between control and treatments by one – way ANOVA and significance is shown with p values (*p<0.05, ***p<0.0001).

3.3.3 Exfoliation studies by SEM

Control treatment of bladder tissue with Tyrode’s buffer alone had no effect on tissue morphology as shown by the intact, tightly packed umbrella cells of the urothelial layer (Figure 3.9A). However, when tissue was incubated with HPG-580 (10% w/v) the umbrella cells were completely exfoliated from the surface (Figure 3.9B). Both MMC
and doxorubicin induced extensive exfoliation of the urothelial surface, characterized by complete removal of umbrella cells from some areas and other areas where cells appeared to be only loosely attached (Figure 3.10 and Figure 3.11). However, gemcitabine induced much less extensive exfoliation with areas of missing cells, along with other areas of an almost intact urothelium but with indications of compromised tight junctions (Figure 3.12).

Figure 3.9 Scanning electron micrograph of porcine bladder urothelium exposed to (A) Tyrode’s buffer for 2h shows distinctive and intact pentagonal or hexagonal shape superficial cells with tight junctions between umbrella cell. (B) HPG-580 (10% w/v) treatment for 2h shows complete exfoliation of umbrella cells from bladder tissue.
Figure 3.10 Scanning electron micrograph of porcine bladder urothelium after exposure to Mitomycin C (1mg/ml) solution for 2h, shows the region of exfoliation of urothelium (red arrow).

Figure 3.11 Scanning electron micrograph of porcine bladder urothelium after exposure to Doxorubicin (1mg/ml) for 2h, shows region of exfoliation of urothelium (red arrow).
Figure 3.12 Scanning electron micrograph of porcine bladder urothelium after exposure to gemcitabine (1mg/ml), shows some areas with (A) Intact urothelium with normal looking urothelium in some places, (B) Patchy removal of umbrella cells (red arrow), (C) loosening of tight junction (red arrow) and (D) region of complete exfoliation.

3.3.4 Exfoliation studies using histopathology H&E staining

Further evaluation of exfoliation was carried out using histopathological staining (H&E) of bladder tissues exposed to drugs or HPG-580 (10%w/v). The results showed the same pattern of exfoliation as observed with SEM (Figure 3.13). The treatment of tissue with MMC or doxorubicin showed exfoliation, whereas gemcitabine treatment of bladder tissue resulted in low levels of exfoliation (Figure 3.13(D,E)).
Figure 3.13 H&E stain images (10X magnification) of porcine bladder urothelium after 2h exposure to (A) tyrode’s buffer shows intact urothelium (arrow) (B) MMC (1mg/ml), shows the intensive exfoliation of urothelium exposing the deep basal layer (arrow), (C) Doxorubicin (1mg/ml) also exfoliating the porcine bladder (arrows), and (D&E) Gemcitabine (1mg/ml) shows (D) some areas with normal urothelium and (E) some region of exfoliation (arrow).
3.4 Discussion

Transurethral resection of bladder tumors is an effective method to eliminate bladder cancer in more than 55% of patients. However, 45% of patients will experience recurrence and there is a 3-15% chance of tumor progression to more invasive or metastatic disease (Williams et al., 2010). Currently it is accepted that the single instillation of drugs such as MMC, doxorubicin or gemcitabine may inhibit short-term recurrence but these drugs have no long-term effect on outcome. Clearly there is a need for improved intravesical chemotherapy and much research has focused on the use of taxane drugs, PTX and DTX, often in combination with drugs like gemcitabine (McKiernan et al., 2006; Neri et al., 2007; McKiernan et al., 2011).

From a pharmaceutical perspective, intravesical chemotherapy offers a rather unique and challenging tissue environment. The bladder is designed to prevent the systemic uptake of molecules from the urine, which would permit the use of high drug concentrations but simultaneously inhibits drug uptake into the local target tissue (Hicks, 1975). Furthermore, the bladder tissue is easily accessible to large volumes of drug solutions but these solutions are immediately diluted with urine and must be eliminated within 2 hours, reducing concentrations and exposure times. Recently, we have adopted a strategy to improve the delivery of taxane drugs to the bladder tissue by encapsulation in custom-synthesized macromolecules called hyperbranched polyglycerols (HPGs) that we believe bind to the bladder wall via mucoadhesive surface conjugated amine groups (Mugabe et al., 2011a). HPG-580 nanoparticles cause exfoliation of the urothelial tissue, allow for enhanced drug uptake in 90 minute instillations and effectively deliver DTX to inhibit bladder tumor development in mice (Mugabe et al., 2011b; Mugabe et al., 2011c;
Mugabe et al., 2012). The disadvantage of using the commercial formulations of Taxotere® (DTX) or Taxol® (PTX) is that although these formulations allow the use of high concentrations of drug, they do not cause exfoliation of the bladder so drug uptake is poor (Tsallas et al., 2011).

Since HPG-580 nanoparticles cause exfoliation, we hypothesized that the pretreatment of bladder tissue with HPG-580 might allow for the subsequent enhanced uptake of water-soluble drugs like MMC, doxorubicin and gemcitabine, which are the first line drugs used in intravesical chemotherapy. However, there was no effect of HPG-580 pretreatment on the uptake of these drugs (Figure 3.2-3.5) and all three drugs penetrated the lamina propria layer of the tissue. Scanning electron microscopy demonstrated that all three drugs elicited an exfoliation effect in the bladder tissue (Figure 3.9-3.12) thus explaining why pretreatment with HPG-580 had no effect on the subsequent permeability of the urothelium to the drugs. These data showed that MMC and doxorubicin caused extensive exfoliation, whereas gemcitabine caused only urothelial cell weakening and partial exfoliation. Since all three drugs contain amine groups that are positively charged at pH 6.4, it is likely that the process of exfoliation is similar to that induced by HPG-580. We have recently reported that the degree of exfoliation caused by aminated HPG’s, like HPG-580, is proportional to both the nanoparticle concentration and the density of amine groups on the nanoparticles (see chapter 2). It is possible that the extensive exfoliation observed with MMC treatment may arise from the presence of two amines on this drug as compared to the single amines on doxorubicin and gemcitabine. Similar exfoliating effects have been reported for other amine-containing compounds such as chitosan (Kerec et al., 2005), protamine (Lavelle et
and cationic peptides (Veranic et al., 2004; Kerec Kos et al., 2009). Lim et al and Tyritzis et al noted in a case report that a patient’s bladder had evidence of exfoliation following an early instillation of a single dose of MMC and epirubicin (Tyritzis et al., 2009; Lim et al., 2010). However, we believe that our findings represent the first comprehensive report of the exfoliating effects of MMC, doxorubicin and gemcitabine.

Interestingly the SEM observations were supported by histopathological studies showing urothelial layers stripped off the underlying tissue following exposure to either MMC or doxorubicin, whereas gemcitabine caused much less disruption (Figure 3.13). Based on our findings that the water-soluble drugs caused exfoliation, we investigated whether co-administration of these drugs with commercial formulations of taxanes might increase taxane uptake similar to taxane loaded HPG-580 nanoparticles. All three co-administered drugs showed large increases in both PTX and DTX uptake through the tissue (Figures 3.5-3.8). Generally these increases, as expressed as the total amount of drug (AUC), were in the range of 50% to 150% and were significant for all drug treatments as compared to drug levels achieved with the commercial formulations alone.

Considering the increased use of taxanes for the systemic treatment of more advanced bladder cancer, it is likely that PTX or DTX will also be used more frequently for intravesical treatment of superficial cancer. The findings of this study are important firstly because these commonly used anticancer drugs (MMC, doxorubicin and gemcitabine) may increase the bladder tissue concentrations of taxane drugs for improved taxane efficacy and secondly because combination treatment with more than one drug may improve overall anticancer effects.
Chapter 4: Summarizing discussion, conclusion and future work

4.1 Summarizing discussion

Previous work in our laboratory concluded that HPG-C8/10-MePEG-NH2 nanoparticles were suitable for the loading of the hydrophobic drugs, PTX and DTX (Mugabe et al., 2011a; Mugabe et al., 2011b; Mugabe et al., 2011c). Mucoadhesive DTX loaded HPG-C8/10-MePEG-NH2 nanoparticles were selected as a lead formulation based on better physical stability and higher drug loading than nanoparticles composed of the same nanoparticle carrier but loaded with PTX (Mugabe et al., 2011b). Further in-vivo studies using DTX loaded HPG-C8/10-MePEG-NH2 nanoparticles showed significantly increased drug uptake in mouse bladder tissues and inhibited tumor growth in an orthotopic model of bladder cancer in mice compared to the commercial formulation of DTX, Taxotere® (Mugabe et al., 2011b). Moreover, morphological studies showed that HPG-C8/10-MePEG-NH2 caused urothelial exfoliation, but no evidence of local inflammation or necrosis in bladder tissues was observed. It was found that upon washout of HPG-C8/10-MePEG-NH2 nanoparticles from exfoliated mouse bladders, there was a rapid recovery process of the exfoliated urothelium, and normal urothelial morphology was restored within 24 hours (Mugabe et al., 2011a; Mugabe et al., 2011b; Mugabe et al., 2011c).

The drug carrier system used in this study, HPG-C8/10-MePEG-NH2, is one large single molecule, often termed a “unimolecular micelle”. It is a less perfectly branched polymer compared to dendrimers (Mugabe et al., 2011a). Kainthan et al., have established the synthesis and characterization of HPGs (Kainthan et al., 2007a; Kainthan et al., 2007b). The group demonstrated that HPGs can be prepared in a single pot
synthetic procedure, in a controlled manner, with predetermined molecular weights and narrow polydispersities (Kainthan et al., 2007a; Kainthan et al., 2007b; Kainthan et al., 2008a; Kainthan et al., 2008b). These HPGs typically possess low intrinsic viscosities (less than 10 mL/g), and smaller nanoparticle size (<10nm) have been reported to be biocompatible and nontoxic both in-vitro and in-vivo (Kainthan et al., 2007a; Kainthan et al., 2007b; Kainthan et al., 2008a; Kainthan et al., 2008b).

In this work, all studies evaluating the effects of HPGs and cytotoxic drugs, both alone and in combination, were carried out using porcine bladder from freshly sacrificed pigs. Porcine bladder is an excellent model of the human bladder since they are structurally and morphologically very similar. Studies have also suggested that porcine bladder possesses comparable urodynamic characteristics to human bladder (Dixon et al., 1983; Teufl et al., 1997; Parsons et al., 2012). Our laboratory has previously established that bladder tissues remain viable for at least 5 h following sacrifice and organ retrieval, in agreement with literature reports that large animal bladders can remain viable when perfused with nutrients in-vitro (Tsallas et al., 2011; Parsons et al., 2012).

The overall goal of this thesis was to develop an increased understanding of the effects of both different densities of surface conjugated amines on HPGs, and drugs used in intravesical chemotherapy, on urothelial exfoliation and drug uptake into porcine bladder tissue. Chapter 2 demonstrated the effect of HPGs with different amine densities on urothelial morphology and DTX uptake. These HPG nanoparticles were designated as low, intermediate and high amine density representing the number of moles of amine per mole of HPG, as follows: HPG-360, HPG-580 and HPG-780, respectively. These HPGs were synthesized and characterized by our collaborators in the Centre for Drug Research
and Development and all synthetic methods and characterization data have been reported in the work of Mugabe et al noted previously. In chapter 2 studies, *ex-vivo* porcine bladder tissues were exposed for 2h treatments and chitosan (0.005%w/v) solution, a known permeability enhancer, was used as a positive control for urothelial exfoliation. The findings in this chapter clearly showed that the extent of exfoliation was both a HPG solution concentration dependent and a surface amine density dependent process, in agreement with previous studies that showed a HPG concentration dependence (Mugabe *et al.*, 2011b; Mugabe *et al.*, 2011c; Mugabe *et al.*, 2012). The time course study was conducted to determine the effect of amine density of HPGs on the time required for exfoliation. HPG-580 (10% w/v) with intermediate amine density showed exfoliation in 1 h, whereas high density amine HPG-780 (10%w/v) completely exfoliated the tissue urothelium within 30 minutes.

These studies demonstrated a clear relationship between the degree of exfoliation and an increased accumulation of DTX. DTX/polysorbate-80 micelles or DTX loaded HPG-360 (10%w/v) formulations, both of which had no effect on urothelial morphology, showed low tissue levels of drug, similar to those previously reported for Taxotere® (Tsallas *et al.*, 2011) and non-aminated HPG formulations (Mugabe *et al.*, 2011a; Mugabe *et al.*, 2011b; Mugabe *et al.*, 2011c; Tsallas *et al.*, 2011; Mugabe *et al.*, 2012). However, DTX tissue levels were significantly higher for highly exfoliating formulations, 10% w/v HPG-580 and 10% w/v HPG-780, compared to treatment with 0.5 mg/mL DTX/polysorbate-80 micelles.

We suggest that the mucoadhesive, aminated HPGs assist in binding to the mucous layer and that the very small size of the nanoparticles facilitates transport
between chains leading to direct contact with the umbrella cells of the urothelium. It appears that the positively charged amine groups on the HPGs modulate tight junctions. Scanning electron micrographs and preliminary immunohistochemistry staining studies showed that aminated HPGs may act on tight junctions resulting in loosening of cell-to-cell contact followed by exfoliation of umbrella cells.

Complete removal of the bladder tissue barrier to drug penetration via exfoliation caused by HPG-580 (10%w/v) and HPG-780 (10%w/v), facilitated the enhanced drug transport from the HPG nanoparticles to the underneath intermediate cell layer. Interestingly, the hydrophobic taxanes drugs have a long residence time in tissues so that, in-vivo, the regenerated barrier of the urothelium may inhibit drug diffusion from the tissue to back into the urine, further extending tissue exposure to the drugs (Kainthan et al., 2008a; Kainthan et al., 2008b; Mugabe et al., 2012).

Chapter 3 reported on use of the intermediate density HPG-580 to enhance the uptake of first line intravesical chemotherapeutic water-soluble drugs like MMC, doxorubicin and gemcitabine. However, in these studies we found that HPG-580 pretreatment had no effect on the uptake of these drugs. SEM confirmed that all three drugs caused exfoliation of the bladder tissue so that any pretreatment with HPG-580 would have little effect on the permeability status of the urothelium. These data showed that the MMC caused extensive exfoliation, doxorubicin’s effects were similar, whereas gemcitabine caused urothelial tight junction loosening of umbrella cells and only partial exfoliation. Structurally all three drugs contain amine groups that are positively charged at pH 6.4. It is likely that the mechanism of exfoliation is similar to that induced by aminated HPGs, namely a charge interaction between the amine group on the drugs with
the bladder mucosal wall resulting in modulation of urothelial tight junction followed by exfoliation.

Since the central component of our HPG-580 formulation-based strategy to increase taxane uptake is based on exfoliation, we investigated whether co-administration of the exfoliating, water soluble drugs with commercial formulations of taxanes could also increase taxane uptake. We observed that that all three drugs significantly enhanced the uptake of both PTX and DTX in *ex-vivo* porcine bladder tissue comparable to the drug levels achieved with the commercial formulations alone.

While exfoliation by HPG-C$_{8/10}$-MePEG-NH$_2$ nanoparticles may allow transiently increased bladder wall permeability to drugs, it is important to be able to establish that rapid recovery of the urothelium to restore normal bladder wall barrier functionality is possible. Based on literature evidence and previous preliminary studies done on mice with HPG-C$_{8/10}$-MePEG-NH$_2$, removal of the positively charged exfoliating agent (bladders of mice were washed with PBS following the 2 h exposure to HPG-C$_{8/10}$-MePEG-NH$_2$ nanoparticles) leads to rapid differentiation of the intermediate urothelial cells into superficial umbrella cells with prominent tight junctions and specialized apical plasma membrane in order to maintain an effective urine-blood barrier (Veranic et al., 2009) This mechanism is illustrated as a schematic representation in Figure 4.1.

In summary, we believe that DTX loaded HPG-C$_{8/10}$-MePEG-NH$_2$ nanoparticles may be a potentially exciting and novel formulation for future development as an intravesical drug delivery system in the treatment of NMIBC. Also the findings from these study are interesting because these commonly used anticancer drugs, MMC, doxorubicin and gemcitabine, may increase the bladder tissue concentrations of taxane
drugs for improved taxane efficacy and also because combination treatment with more than one drug may improve the effectiveness of overall anticancer drug therapy.
Figure 4.1 The mechanism of drug uptake due to the treatment of urothelium with positively charged carrier or drugs causing urothelium exfoliation, drug partition and recovery (Adopted from finding from this thesis and (Mugabe, 2011)).
### 4.2 Conclusion

1. HPG-C_{8/10}-MePEG-NH\textsubscript{2} (at 10%w/v concentration) showed amine density dependent exfoliation (360<580<780).

2. *Ex-vivo* exfoliation time course studies showed that HPG-580 takes 1h to cause major area exfoliation the tissue, whereas HPG-780 exfoliates the tissue urothelium within 30 minute. Morphological observation also showed that HPG acts on tight junction resulting in loosening of cell-to-cell contact followed by exfoliating umbrella cell.

3. Drug uptake studies of DTX loaded HPG-780 nanoparticles results in significantly increased drug penetration into the bladder urothelium via exfoliation induced changes to urothelium barrier function.

4. MMC, doxorubicin and gemcitabine resulted in urothelium exfoliation.

5. The enhanced uptake of PTX or DTX in the presence of MMC, doxorubicin or gemcitabine may be useful in combination intravesical drug strategies, offering improved uptake of taxanes.

### 4.3 Future work

Plans are underway to obtain human bladder samples and evaluation of our lead HPG formulation on human bladder morphology and drug uptake via *ex-vivo* studies will be very interesting. In this study, preliminary drug uptake data from exposure of bladder tissue to combination drug therapy showed an increased tissue penetration of taxanes. It would be important to investigate the doses of drugs, tolerability and toxicity of this approach in *in-vivo* studies.
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