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Advances in intravesical drug delivery systems to treat bladder cancer

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ABSTRACT:

Chemotherapeutic agents administered intravesically to treat bladder cancer have limited efficacy due to periodic dilution and wash-out during urine formation and elimination. This review describes the pathophysiology, prevalence and staging of bladder cancer, and discusses several formulation strategies used to improve drug residence within the bladder. These include the use of amphiphilic copolymers, mucoadhesive formulations, hydrogels, floating systems, and liposomes. Various *in vitro* and *in vivo* models recently employed for intravesical drug delivery studies are discussed. Some of the challenges that have prevented the clinical use of some promising formulations are identified.

Keywords: Bladder Cancer; Intravesical drug delivery; Particulate delivery systems; *In situ* gelling systems; Mucoadhesive; Floating

1 Introduction

Bladder cancer (BC) is the most predominant malignancy affecting the urinary tract, characterised by proliferation of abnormal cells in the urothelial lining of the urinary bladder. It is commonly divided into non-muscle invasive bladder cancer (NMIBC) which makes up 80% of cases at presentation and muscle invasive bladder cancer (MIBC) where the cancer extends into the underlying smooth muscle. The latter makes up 15% of cases at presentation with the remaining 5% presenting with metastases (Cheung et al., 2013). Intravesical therapy is only effective in NMIBC and is never used in MIBC. Haematuria is the commonest symptom in 85% patients but other clinical presentations may include urinary urgency and painful urination (Kaufman et al., 2009). It is ranked 7th and 9th in terms of cancer incidence in the United Kingdom (UK) and worldwide, respectively (Cheung et al., 2013; Torre et al., 2015) as well as being the fifth based on prevalent cancer types among European men (Aziz et al., 2016).

In 2015 in the UK, 6,169 new BC cases were diagnosed in men versus 2,331 new cases in women (Cancer Research, 2017) and it is projected that by 2035 there will be 7,771 BC related deaths per year (Cancer Research UK, 2016). BC is statistically twice as prevalent in the white population than in the black population; however in terms of mortality (Schinkel et al., 2016), with five-year survival rate is considerably higher in the white community (80%) in comparison with the black community (64%) (Torre et al., 2015). The greatest risk factor for BC is smoking and the risk is 2 to 6 fold higher for smokers than non-smokers, and is responsible for almost 31% and 14% of BC deaths in male and female smokers, respectively (Torre et al., 2015). In Africa, 50% of BC patients have a previous history of infection with the water-borne parasite, Schistosoma hematobium, whereas the incidence of such BC cases worldwide is only 3% (Sievert et al., 2009).

Non-muscle invasive bladder cancer (NMIBC), also called superficial BC is the most prevalent form of BC at first diagnosis. It progresses to MIBC in about 15% of patients with a poor prognosis despite transurethral resection of the bladder tumor (TURBT) and Bacillus Calmette-Guérin (BCG) immunotherapy (Williams et al., 2010) and therefore frequent cystoscopic surveillance of the bladder is necessary following initial therapy (Torre et al., 2015). BC has the highest cost of therapy of any cancer over the lifetime of the affected individual due to the associated cost of surveillance and treatment (D. A. Barocas et al., 2012; Sievert et al., 2009; Yeung et al., 2014).Therefore, improved BC therapeutic delivery systems have been investigated over the last two decades (Figure 1) in an attempt to reduce the clinical and economic burden of this disease.

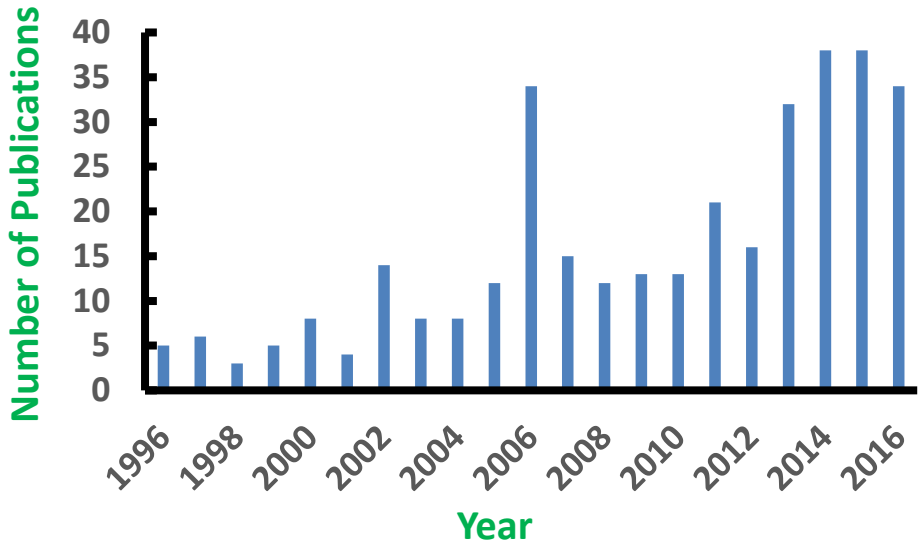

Figure 1: Number of publications reporting bladder cancer therapeutic delivery systems (1996-2016), Source: Web of Knowledge (search terms: bladder cancer, delivery systems).

The degradative hepatic enzymes and gastric acid lowers oral bioavailability of BC therapeutic formulations while systemic therapy is less efficient due to the poorly vascularised urothelium (Elstad and Fowers, 2009; GuhaSarkar and Banerjee, 2010). Increasing systemic drug dose with the aim of improving local drug concentration within the bladder leads to elevated adverse drug reactions and non-selective toxic effects on healthy tissues (GuhaSarkar and Banerjee, 2010). Intravesical Drug Delivery (IDD) (Figure 2) is the instillation of one or multiple therapeutic agents through a catheter, directly into the bladder (Zhang et al., 2014). IDD provides site-specific drug delivery with minimal toxicity (GuhaSarkar and Banerjee, 2010); and this may help to reduce tumor recurrence and progression (van Rhijn et al., 2009) because localised therapy improves therapeutic drug concentrations in the bladder which destroys residual urothelial cancerous cells (Kaufman, 2006; Kaufman et al., 2009; Konety et al., 2007). Due to cellular and physiological limitations posed by the urothelium as well as urine, it is not sufficient to simply administer cytotoxic formulations intravesically; there is a need for careful design of drug delivery systems that would be able to circumvent these barriers.

Some good reviews have been published that considered varieties of nanoparticles and nanotechnology for BC therapy and / or diagnosis (Chen et al., 2015; GuhaSarkar and Banerjee, 2010; Tomlinson et al., 2015) but some of the nanoparticulate systems discussed were developed over a decade ago and recent studies were not discussed fully in some of the articles. Moreover, formulation strategies that require adjunct equipment such as electromotive device assisted therapy

(EMDA) and hyperthermia (HT) has not been widely embraced by urologists because of their complex delivery modalities (Barocas et al., 2012).

This review, therefore, will discuss briefly the pathophysiology, prevalence and staging of BC and will focus on advanced polymeric formulations investigated for BC management that have not been covered previously. Also, *in vitro* and *in vivo* models that are currently used for IDD studies will be discussed. The reviewed advanced drug carriers are categorised into four groups based on non-particulate, particulate, composite systems of hydrogels and particles as well as liposomal drug carriers.

2 Bladder Physiology

2.1 Structure of the bladder

In order to carry out its urine storage and voiding functions, the bladder (Figure 2) can change volume although it remains almost spherical in shape (GuhaSarkar and Banerjee, 2010). The bladder wall is comprised of four layers: the urothelium, lamina propria, detrusor muscle, and serous (adventitia) layers (Konety et al., 2007). The urothelium acts as a permeability barrier and comprises of umbrella cells knitted together by tight junctions and enveloped by uroplakin plaques, mucin, intermediate and basal cell layers. These components prevent diffusion of pathogenic and toxic substances into the systemic circulation; their detailed functions have been discussed in the previous review (GuhaSarkar and Banerjee, 2010). Regulatory information emanating from the bladder lumen is transmitted by the urothelium to the surrounding tissues (myofibroblasts and musculature) via different neurotransmitters including adenosine triphosphate, adenosine and acetylcholine (Khandelwal et al., 2009). The urothelium is covered by a layer of glycosaminoglycans which limits the adherence of negatively charged drug carriers (Lewis, 2000). This also means that, systemic therapeutic agents for BC treatment cannot diffuse efficiently into the bladder (Soler et al., 2008).

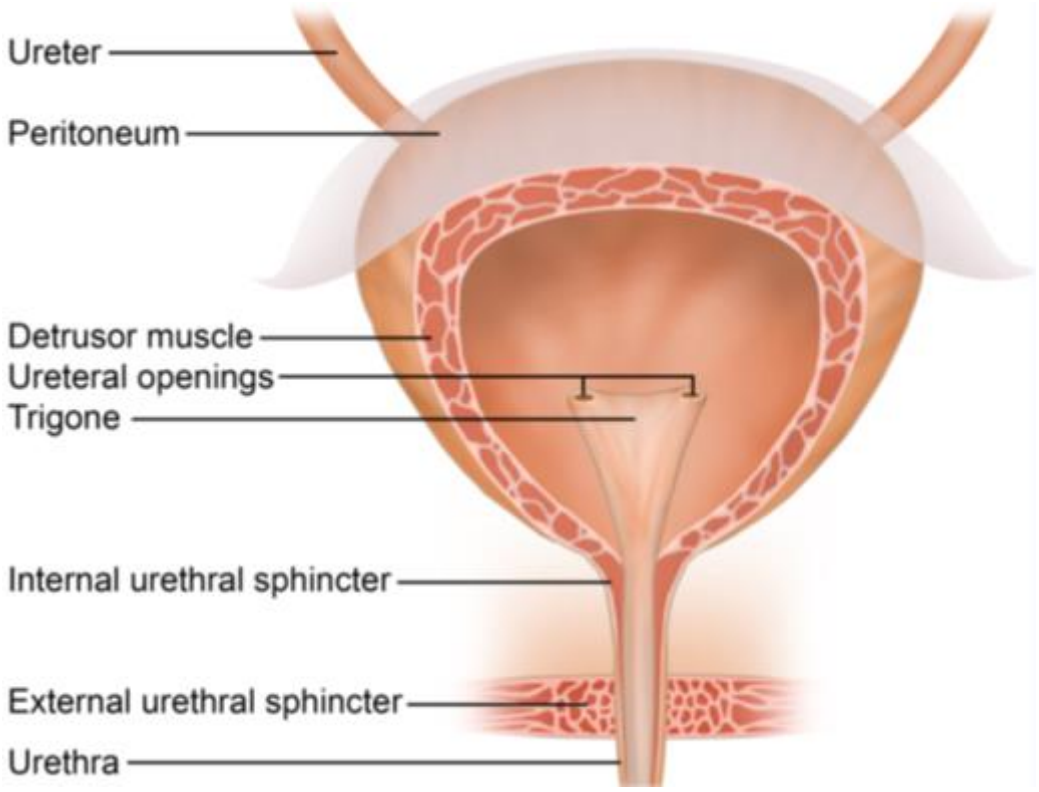

Figure 2: Schematic diagram showing various segments of the bladder. This image was designed and kindly provided by Ms Stephanie Bull.

2.2 Urine Volume and Composition:

The volume of urine in a human bladder is dependent on the sex, race and ethnicity of an individual but in the adult it averages around 350 - 450 mL when full (Gray, 2012) although the first sensation of urination occurs at around 150 to 200 mL of urine (GuhaSarkar and Banerjee, 2010). Urine voiding activity is regulated by the myovesical plexus within the bladder wall which produces a specific sensation when sufficient urine is present in the bladder and sends a voiding signal to the detrusor muscle which relaxes or contracts appropriately in order to regulate the extent and frequency of voiding (Khandelwal et al., 2009).

3 Bladder Cancer

3.1 Genetic / Molecular expressions in Bladder Cancer

Some of the genetic materials that are expressed in BC can be used as molecular targets to design effective therapies. For example, survivin detectable in the urine of BC patients (Smith et al., 2001) has been implicated in BC as it improves the survival rate of cancerous cells by preventing cell death

(Swana et al., 1999). BC patients also are known to express particular mucins (MUC1 and MUC3) associated with bladder malignancies (Cardillo et al., 2000).

The natural immune system of the bladder is achieved through uroplakins, a group of proteins found at the apex of the umbrella cell membrane, namely UPIa, UPIb, UPII, and UPIIIa. They induce bacterial death when infected. They also perform barrier functions along with tight junctions, where they prevent drug diffusion across the urine / bladder tissue interface (Kong et al., 2004; Wu et al., 1994; Yu et al., 1994). Changes in the glycosylation of uroplakins especially UPIII may depict advanced stages of BC, in addition to clinical conditions such as urinary tract infections and interstitial cystitis (Kątnik-Prastowska et al., 2014).

Lewis X glycoantygen, sialyl-Tn (carbohydrate) and its degrading enzyme (sialyltransferase) are expressed on the urothelium and are markers for BC (Kątnik-Prastowska et al., 2014). Metabolomics of urine samples from BC and non-BC subjects' revealed that biomarkers (lipid-metabolic products) such as arachidonite, palmitoyl sphingomyelin, lactate, adenosine and succinate are found in BC patients only (Wittmann et al., 2014). Chemokine ligand 1 (CXCL1), which modulates interaction between stroma and urothelium, in order to accelerate tumor progression and metastasis, is also expressed in urine samples of BC patients (Burnier et al., 2015).

3.2 Stages of bladder cancer

About 90% of BC cases are transitional cell carcinomas while squamous cell carcinomas occurs in 3 - 7% patients and around 2% have adenocarcinomas (Sharma, 2014). Usually, the urothelial layer of the bladder is initially affected by most BC types. The nearby lamina propria and muscular layer becomes involved as the tumor progresses. Afterwards, the lymph nodes or pelvic organs are also affected (Sharma, 2014). The metastatic stage arises when cancerous cells divide and spread to distant organs such as lungs, liver and bone marrow (Sharma, 2014).

The tumor stage defines the extent of disease progression (Bischoff and Clark, 2009); and the tumor node metastasis staging system developed by International Society of Urological Pathology in 1997 for BC classification is still being used by World Health Organization (WHO) (Moch et al., 2016). The American Joint Committee on Cancer TNM system categorises bladder cancer based on growth into the bladder wall (T), spread to neighbouring lymph nodes (N) and metastases (M). Information from these categories is then used to evaluate the overall stage of the disease from stage 0 to IV (Table 1).

Table 1: Bladder cancer stages. Information from taken from (Team, 2016)

Category	Stage	Description, including tumor coverage
Ta, N0, M0	Stage 0a	Non-invasive papillary carcinoma – hollow centre of the bladder
Tis, N0, M0	Stage 0is	Flat, non-invasive carcinoma (carcinoma in situ) – inner bladder lining
T1,N0, M0	Stage I	Invasive – connective tissues beyond the urothelial lining
T2a or T2b, N0, M0	Stage II	Invasive – inner half (T2a); outer half (T2b) of the muscular region
T3a, T3b, T4a, N0, M0	Stage III	Invasive – fatty tissue region visible with microscope (T3a); readily visible (T3b); spread to prostate, uterus and/or vagina (T4a)
T4b, N0, M0, N1-N3, M1	Stage IV	Invasive / metastatic – Pelvic or abdominal wall (T4b); single pelvic lymph node (N1); ≥ 2 lymph nodes (N2); iliac arterial lymph nodes (N3); beyond the bladder to distant sites like bones, liver or lungs

Note: N0 and M0 denotes that lymph nodes and distant sites (metastatic tumors) were not affected, respectively

Eble et al graded BCs based on cell morphology into G1, G2 and G3, with G1 being the most well differentiated, while G3 is the least differentiated BC having the greatest risk of progression (Eble et al., 2004). Recently, WHO proposed a new grading classification for BC based on improved knowledge of its pathology and genetics (Figure 3), where “urothelial dysplasia” and “urothelial proliferation of uncertain malignant potential” were included as well defined forms of non-invasive urothelial lesions (Moch et al., 2016).

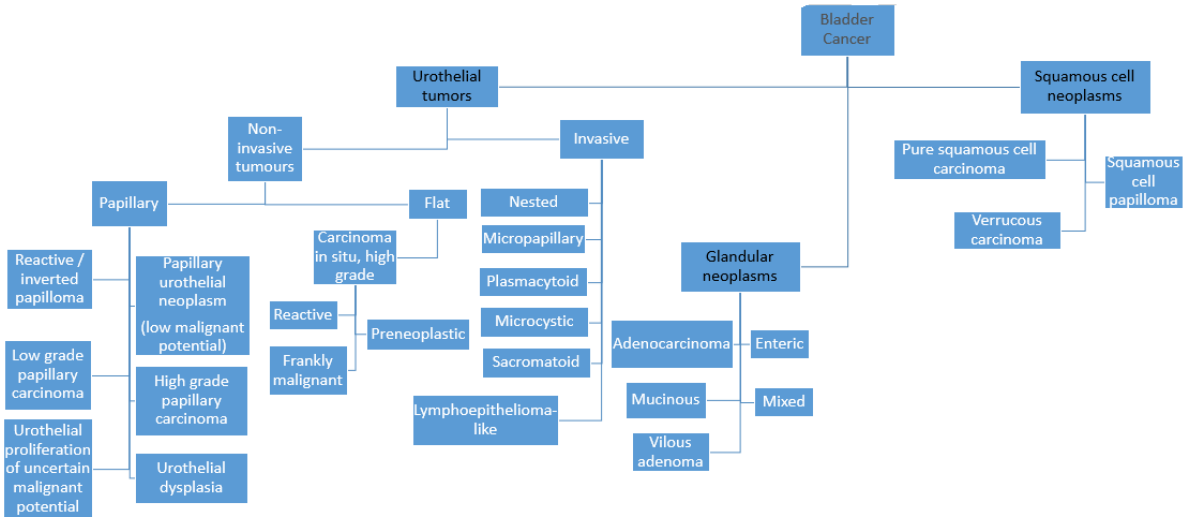

Figure 3: The 2016 WHO Classification of Bladder Cancer (details extracted and presented in a different format) (Moch et al., 2016)

4 Management of bladder cancer

BC treatment is dependent on the stage and severity of disease (Sharma, 2014). The UK National Institute for Health and Care Excellence (NICE) has produced guidelines regarding the diagnosis and treatment of BC. It recommends that both cystoscopy and urine cytology is needed in order to diagnose BC as cystoscopy alone may miss some tumours such as CIS (NICE, 2015). The extent of BC progression is identified using computer tomography (CT) or magnetic resonance imaging (MRI) techniques. NMIBC is initially treated by TURBT (Lerner and Au, 2008; NICE, 2015). Patients with a low risk of disease recurrence post-surgery may be followed up with cystoscopic surveillance alone, whilst those in the intermediate and high risk categories are treated with intravesical chemotherapy (mitomycin C) or immunotherapy (BCG) (Lerner and Au, 2008). The latter triggers an immune response that ensures future recognition of BC cells, and this treatment has been proven to prevent progression in about 85% of BC cases.

5 Intravesical Formulations

The effectiveness of bladder cancer therapeutic dosage forms depends on their ability to overcome the urothelium as well as the drug having suitable physicochemical properties such as molecular weight (≤ 200 Da), water or lipid solubility, aqueous / organic phase partition coefficient (-0.4 to -0.2 or -7.5 to -8.0) and pH 6 to 7 (GuhaSarkar and Banerjee, 2010). Also, the surface charge of drug carriers influences their cellular uptake, for example, positively charged nanoparticles (NPs) are taken up by cells and readily absorbed into tissues in preference to anionic or neutral NPs (He et al.,

2010; B. Kim et al., 2010). The uptake of such drug cargos may be prevented by poor interaction with biorecognitive moieties that facilitate cellular internalisation (Khandelwal et al., 2009); in addition to lack of electrostatic interaction between oppositely charged carrier and bladder mucosa.

5.1 Conventional formulations

The surgical implantation of drug loaded gels into BC patients was expensive and unacceptable (Singh and Lee, 2014). So, local drug delivery using drug instillation via a catheter was adopted which permits easy bladder accessibility and avoids surgery (Shen et al., 2008). The usual volume of drug formulation instilled intravesically is approximately 50 mL (Senyigiti et al., 2015) and micturition is prevented for at least 1-2 h for effective drug transport into the underlying cancerous tissues (Tyagi et al., 2006). Nevertheless, the instilled drug becomes diluted due to residual urine which is often present in the human urinary bladder and/or is washed out prematurely (Grabnar et al., 2006). These limitations mean that frequent catheter insertion, decreased dosing interval and eventual irritation of the urethral lining and possible urinary tract infection are possible complications of intravesical drug delivery (Martin et al., 2014). Also, conventional drug carriers such as microspheres (Bogataj et al., 1999; Le Visage et al., 2004) and gelatin nanoparticles (Lu et al., 2004b) display poor drug loading and uncontrolled drug release profiles.

5.2 Advanced formulations

Hydrophilic drugs are readily soluble in the aqueous urine medium of the bladder but their permeability and cellular uptake is limited due to the lipophilic nature of the urothelial tissues. Thus, chemical enhancers such as dimethyl sulfoxide (DMSO) have been used to improve the cellular uptake of BC chemotherapeutics such as doxorubicin and cisplatin into malignant bladder tissues (Giannantoni et al., 2006). However, their use is now less favourable because of unwanted side-effects such as frequent and painful urination (GuhaSarkar and Banerjee, 2010). Also, hydrophobic drugs such as paclitaxel formulated with DMSO for improved solubility and urothelial permeability are associated with painful sensation post intravesical instillation (Parkin et al., 1997).

Therefore, smart drug carriers were developed to improve the solubility of lipophilic drugs; urothelial permeability of hydrophilic drugs; urothelial adhesion of drug carriers as well as drug uptake / permeation into malignant tissues for prolonged periods of time. They include amphiphilic copolymer based solubilised systems (Tamura et al., 2015), surface modified particulate systems (Cook et al., 2015; Lu et al., 2015; Martin et al., 2014, 2013; Neutsch et al., 2013; Zhang et al., 2014), and composite particulate and hydrogel systems (Lin et al., 2014; Men et al., 2012; Senyigiti et al., 2015; Zhang et al., 2013)(Table 2).

Table 2: Overview of intravesical formulations explored for bladder cancer therapy

Class	Agent	Material	Dosage form	References
Amphiphilic copolymer based solubilised systems	Paclitaxel	Poly (2-methacryloyloxyethyl phosphorylcholine-co-n-butylmethacrylate) diblock copolymer (30mol% MPC unit: 70 mol% hydrophobic BMA unit)	Ionotropic solution	(Tamura et al., 2015)
Surface modified particulate systems	Belinostat	PGON, PLGA	Nanoparticles	(Martin et al., 2013)
	Doxorubicin	ß-cyclodextrin, mesoporous silica	Nanoparticles	(Zhang et al., 2014)
	Survivin SiRNA	Chitosan, PLGA	Nanoparticles	(Martin et al., 2014)
	Stearoyl Gemcitabine	Wheat Germ Agglutinin, PLGA	Microparticles	(Neutsch et al., 2013)
	Doxorubicin	Copolymer of 2-(acetylthio)ethylacrylate and 2-hydroxyethylmethacrylate	Thiolated Microgels	(Cook et al., 2015)
	Docetaxel	Amine functionalized polyacrylamide	Nanogels	(Lu et al., 2015)
	10-hydroxycamptothecin	Poly (L-lysine)-poly (L-phenylalanine-co-L-cysteine)	Nanogels	(Guo et al., 2016)
Composite particulate and hydrogel systems	Adriamycin	Poloxamer 407 (triblock copolymer), sodium hydrogen carbonate, HPMC, Human Serum Albumin	Floating hydrogel-nanoparticle system	(Lin et al., 2014)

	Bacillus Calmette-Guérin	CS, β-glycerophosphate, magnetite	Mucoadhesive nanoparticle - in situ gelling system	(Zhang et al., 2013)
	Deguelin	DOTAP, monomethoxy poly (ethylene glycol)-poly (Ɛ-caprolactone) NP + Pluronic F127 hydrogel	Mucoadhesive nanoparticle - in situ gelling system	(Men et al., 2012)
	Gemcitabine HCl	Chitosan-thioglycolic acid conjugate based NPs + chitosan gel / Poloxamer gel	Thiolated nanoparticle- gel / in situ gelling system	(Senyigiti et al, 2015)
Liposomal systems	Paclitaxel	Soya phosphatidylcholine, gellan gum	Liposomes / ion-triggered gelling system	(GuhaSarkar et al., 2017)

5.2.1 Amphiphilic copolymer based solubilised systems

Solubilised systems are isotropic solutions of a substance in a state of thermodynamic stability generated by dispersing the sparingly soluble substance in an amphiphilic material (Tadros, 2013). Cremophor is conventionally used to improve the solubility of paclitaxel (PTX-CrEL) in the physiological fluid but its use is limited by adverse effects as well as by reduced drug permeation across the mucosal lining due to drug entrapment in polymeric micelles (Knemeyer et al., 1999). However, the amphiphilic copolymeric delivery system, poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate (PMB30W) has been proven to be non-toxic with improved paclitaxel uptake into mammalian cell lines during *in vitro* studies (Goda et al., 2010; Wada et al., 2007). PTX-30W was formulated by solubilising paclitaxel (PTX) in PMB30W in order to improve its aqueous solubility, safety and antitumor activity (Tamura et al., 2015). *In vitro* studies using MBT-2 BC cells to compare cytotoxicity of PMB30W and Cremophor (CrEL) to BC cells showed that PMB30W (1%) displayed no toxic effect to the cells while dose associated toxicity was reported with CrEL after 72 h of instillation. This was confirmed by the lactate dehydrogenase (LDH) activity assay, detecting 0% and 65% LDH release for PMB30W and CrEL, respectively, in the same study (Tamura et al., 2015).

The ability of an orthotopic bladder cancer mouse model (implanted with MBT-2 BC cells) to mimic the human BC environment has been confirmed in several studies (Kikuchi et al., 2007; Matsushima et al., 2011). This orthotopic model of BC was established through transurethral implantation of MBT-2 BC cells into mice. During *ex vivo* studies, mice were treated with various formulations (50 µL of 2mg/mL PBS, PTX-30W and PTX-CrEL) at predetermined time intervals and killed after 22 days to determine the degree of tumor growth or regression (Tamura et al., 2015). It was shown that PTX-MBA improved tumor regression (more than 2 folds) in comparison with PTX-CrEL (Tamura et al., 2015). Also, some of the mice were sacrificed 30 min post intravesical drug instillation and the degree of drug uptake into tumour tissues quantified using liquid chromatography using a tandem mass spectrometer, was shown to be remarkably lower in PTX-CrEL than in the PTX-30W group of mice (4.905 ± 2.412 µg/g vs 7.719 ± 3.274 µg/g).This revealed improved selectivity of the novel paclitaxel carriers for malignant tissues (Tamura et al., 2015). However, studies related to the resistance of the delivery system to urine dilution or wash-out were not evaluated and periodic urine voiding and residual urine in the bladder may have affected product performance. Also, drug release studies were not carried out, thus the pattern of drug release is unknown. This makes it difficult to ascertain that the drug would be released in a controlled manner, which is a critical parameter for improved formulations that would reduce dosing frequency.

5.2.2 Surface modified particulate systems

5.2.2.1 Lectin modified particulate systems

Glycans and lectins are biological entities that are sensitive and responsive to bacterial and malignant invasion by destroying them through endocytosis, where they interact and adhere onto mannose receptors (Bies et al., 2004; Haltner et al., 1997; Yi et al., 2001). It is anticipated that lectins will be useful for malignant conditions associated with the bladder because they attach to the distal portion of *Escherichia coli* pili and destroy the E. coli in bacterial urinary infections (Dhakal et al., 2008). Also, urothelial cellular uptake of plant lectin, like wheat germ agglutinin (WGA, 36 kDa), in pigs and humans, suggested that biologics may be delivered intravesically, as lectins are readily recognised by the glycol-proteins and lipids of the urothelium and facilitate cellular uptake of a bioconjugate (Neutsch et al., 2011). Neutsch and co-workers surface-functionalised fluorescently labelled bovine serum albumin (fBSA) with WGA units and this modification influenced the urothelial cell (SV-HUC-1) adhesive and invasive potential of the fBSA / WGA bioconjugate (Neutsch et al., 2012). Their cytoadhesive features were greatest when the total number of targeting ligands was three and such features did not increase further for bioconjugates with 4-6 WGA units (Neutsch et al., 2012). This glycan-modulated interaction was critical for internalization of the drug carrier (> 175 kDa) within less than 60 minutes, with more than 40% of drug taken up via endocytosis (Neutsch et

al., 2012). The unchanged extent of cell adhesive properties with increased WGA units (≥ 3) may be due to some of their recognitive domains being sterically hindered from binding to glycosylated urothelial cell membranes (Neutsch et al., 2012). WGA metabolism after fBSA release might have taken place via lysosome degradation (Neutsch et al., 2012).

Stearoyl gemcitabine (GEM- C_{18}) loaded PLGA microparticles (MPs) surface modified with WGA or human serum albumin (HSA), WGA-GEM-C_{18}-PLGA MPs or HSA-GEM-C_{18}-PLGA MPs were explored for IDD (Neutsch et al., 2013). *In vitro studies* using cancerous and non-malignant cell monolayers showed that the WGA-GEM-C_{18}-PLGA MPs had better cellular uptake, internalization and cytostatic action than HSA-GEM-C_{18}-PLGA MPs and unmodified MPs within 3 mins of instillation. WGA-GEM-C_{18}-PLGA MPs also had a two-fold greater affinity towards malignant cell lines than healthy cells. The fluorescently labelled drug loaded WGA modified MPs and HSA based MPs required a contact time of 30 mins and 120 mins, respectively, to impart cytotoxic activity on low grade cancerous cell lines using fluorescence microscopy (Neutsch et al., 2013). WGA-GEM-C_{18}-PLGA MPs were more resistant to wash-off by artificial urine, which allowed for superior retention of cytostatic effect (78 ± 12%) compared to free prodrug but the degree of cell inhibitory potential of the latter in the presence of urine was not defined (Neutsch et al., 2013). There was no significant difference in the sustained release effect (after 120 mins) for the three formulations (WGA- , HSA-modified and unmodified PLGA MPs), which could be attributed to the inability of the study design to simulate washout features of the urinary bladder. The WGA-modified MPs also had low drug release of 13.2 ± 1.8% after 5 days with most of the drug retained within the particle matrix. However, studies of bromodeoxyuridine antimetabolic activity (that connotes duration of drug action) suggested that WGA based particles require lower dose than free drug for achieving a particular degree of prolonged action (Neutsch et al., 2013). Also, a cell proliferation assay suggested better antiproliferative properties for drug loaded surface modified particles than free drug (Neutsch et al., 2013).

Overall, the optimisation and translation of this strategy to the clinic may be limited by the relatively high cost of manufacturing associated with the processing in terms of time and materials required for isolation and purification of the bioconjugate of interest. The bioconjugate size may be heterogenous resulting in immunogenic and adverse reactions. However, the authors did not envisage such formulation constraint.

Recently, Apfelthaler et al evaluated a bioconjugate, WGA conjugated to fluorescein cadaverine (F*C*) labelled poly(L*)glutamic acid (PGA)* (WGA/F*C*-PGA), for bladder cancer theranostic application (Apfelthaler et al., 2017). PGA may improve the solubility of the hydrophobic agents while WGA

favours the selective uptake of bioconjugate into malignant bladder tissues by interacting selectively with the glycocalyx components (N-acetyl-D-glucosamine and sialic acid) of BC cells (Wright and Kellogg, 1996). F*C* helps to track transport of the drug carrier into the cancerous cell endosome and/or lysosome. They confirmed earlier findings by Neutsch's group (Neutsch et al., 2013, 2012) that the presence of biorecognitive moieties like WGA was critical for improved cellular uptake and internalization of bioconjugates into BC cells. Flow cytometry was used to evaluate the cell-binding ability of WGA/F*C*-PGA. Size exclusion chromatography was used to purify the synthesized bioconjugate and isolate biorecognitive fractions. Conjugate A (160 kDa) fraction, obtained with 60 to 80 mL eluent, displayed superior cell binding efficiency relative to conjugates B – D due to the presence of WGA conjugated F*C*-PGA system. Five F*C* molecules per PGA were critical for adequate trackability of the bioconjugate as it exhibited greater cell-induced relative fluorescence intensity than bioconjugate with 30 F*C* per PGA. Conjugate A was found to be cytoadhesive at 4°C but become cytoinvasive at 37°C via active transport. On the other hand, *c*onjugates C and D eluted around 90 mL and lacked both cell adhesive and uptake properties (Apfelthaler et al., 2017). There was no hydrophobic agents delivered within the scope of this study, though the authors acknowledged that their future studies will explore optimal drug loading that will not compromise the cell binding and uptake features of the bioconjugate. However, they noted that there is a need for conjugate A (the most promising bioconjugate) to replicate a similar cytoinvasive and cytoadhesive profile when hydrophobic theradiagnostic agents are loaded.

5.2.2.2 Mucoadhesive delivery systems

Over the last decade, researchers have intensified efforts towards investigating materials with ability to adhere readily to mucosal surfaces because they can prolong contact time between dosage form and diseased site, which is desirable for intravesical formulations (Cook et al., 2015; Khutoryanskiy, 2011; Lu et al., 2004a; Senyigiti et al., 2015). Due to their successful applications for other transmucosal routes of administration such as buccal, ocular and vaginal (Bonengel and Bernkop-Schnürch, 2014), mucoadhesive systems have also been explored for intravesical delivery to enhance drug bioavailability and duration of action (Barthelmes et al., 2012; Cook et al., 2015; Men et al., 2012; Zhang et al., 2013, 2014).

5.2.2.2.1 Cationic particulate systems

Poly[D, L-lactide-co-glycolide] (PLGA) nanoparticles have been widely used for biomedical applications because of their biocompatibility, biodegradability, ease of modification with polymers and peptides (Cheng and Saltzman, 2011; Fahmy et al., 2005), ability to protect encapsulated biologics or therapeutics from degradation during transit, as well as the possibility for controlled

release of loaded drugs (Anthony et al., 2005; Cu et al., 2011; Khan et al., 2004). Poly(guanidinium oxanorbornene) (PGON) is a non-toxic synthetic polymer which is comprised of cationic guanidinium groups and acts similarly like a peptide based cell penetration enhancer (Hennig et al., 2008). Martin *et al* explored surface functionalization of PLGA nanoparticles modified with a urothelial cell penetrating polymer such as PGON to improve aqueous solubility, urothelial cellular uptake, internalization, cytotoxic effect, and duration of action of lipophilic drugs such as belinostat, an histone deacetylase inhibitor (Martin et al., 2013). Belinostat promotes hyperacetylation (Martin et al., 2013) with intrinsic IC_{50} in bladder and prostate cancer cells within the range of 1 to 10 µM (Buckley et al., 2007; Qian et al., 2008); and inhibits BC progression to its metastatic and aggressive forms. All PLGA nanoparticles were 140-160 nm but PGON-modified PLGA nanoparticles (NP-Belinostat-PGON) had superior drug loading relative to the unmodified (NP-Belinostat) and biotinylated chitosan conjugated nanoparticles (NP-Belinostat-Chit) by 3.3- and 6.8-folds, respectively (Martin et al., 2013). *Ex vivo* studies of fluorescently labelled NP-Belinostat-PGON using human ureter as well as *in vivo* studies with mouse bladder suggested that the uptake of NP-Belinostat-PGON was ten times greater than that of unmodified nanoparticles (Martin et al., 2013). PGON improved urothelial uptake of nanoparticles by interaction with a negatively charged urothelial surface coated with glycosaminoglycan, or opening tight junctions of the urothelium. This facilitated drug transport across the urothelial membrane into underlying tissues (Martin et al., 2013). The *in vivo* cytotoxic effect of PGON-PLGA nanoparticles was tested using a xenograft murine model generated from the UM-UC-3R human BC cell line. Tumor growth was not significant after 11 days of treatment for NP-Belinostat-PGON, whereas the volume of tumors treated with unmodified PLGA nanoparticles and drug-free PGON-PLGA nanoparticles increased by at least two fold relative to tumors treated with NP-Belinostat-PGON. After 21 days, the volume of tumors treated with NP-Belinostat and NP-Blank-PGON had increased by 77% and 71%, respectively, relative to NP-Belinostat-PGON treated tumors (Martin et al., 2013). The burst release profile of the novel drug carrier was evident by Histone H4 hyperacetylation occurring in RT-4 (non-invasive) and T-24 (highly invasive with metastatic tendency) cell lines within 30 mins following the instillation of NP-Belinostat-PGON, and the protein expression was sustained for 3 days (Martin et al., 2013). Thus, a lower dose of the NP-Belinostat-PGON may be used to achieve the same therapeutic index observed with free belinostat (Martin et al., 2013).Though the degree of tumor regression by the PGON surface decorated PLGA nanoparticles may correlate with their extent of cellular uptake and drug release, the authors have not quantified the amount of belinostat that was taken up for the modified and unmodified nanoparticles. This information would be useful for determining dosage regimen for BC treatment using this drug carrier.

In a later studies, Martin *et al* showed that PLGA nanoparticles functionalised with low molecular weight chitosan (CH) , 2.5 or 20 kDa (CH2.5-PLGA or CH20-PLGA) were able to adhere onto urothelial surface and enhance the uptake of larger therapeutic agents like survivin siRNA (Martin et al., 2014). Survivin siRNA expression within urothelial cancerous cells enhanced destruction of survivin mRNA responsible for preventing death of cancerous cells. *In vitro* release studies showed the encapsulation efficiency of the nanoparticles modified with chitosan was superior compared to unmodified nanoparticles (70 vs 60 %). CH2.5-PLGA nanoparticles demonstrated a superior burst release profile relative to CH20-PLGA. Also, the CH20 decorated carrier displayed 10-fold lower siRNA release than CH2.5-PLGA nanoparticles over 13 days. During e*x vivo* studies, CH20-PLGA and CH2.5-PLGA nanoparticles displayed improved cellular uptake into UM-UC-3 BC cells in comparison with control PLGA nanoparticles, in the magnitude of 5-10 fold and 4-9 fold, respectively, within 120 mins of incubation (Martin et al., 2014). During *in vivo* mice studies, bladder uptake was up to 14-fold and 9-fold greater for CH20-PLGA and CH2.5-PLGA nanoparticles, respectively, compared to the control formulations (Martin et al., 2014). However, CH20-PLGA nanoparticles entrapped greater amounts of siRNA, in addition to forming bulkier surface groups, which prevented their release and bioactivity (Martin et al., 2014).

Thus CH2.5-PLGA NPs may be desirable for therapy where fast onset of action is needed followed by a sustained release profile over a period of time. Moreover, the amount of drug taken up into bladder tissues was sufficient to reduce mRNA expression and promote tumor regression. The varied physicochemical interaction between the surface of carriers and the urothelial membrane is responsible for the different degree of tumor regression, sustained release, and duration of action observed. Their findings indicated that chitosan chain length used for surface modification influenced the carrier's drug loading, release, and cellular uptake behaviour.

5.2.2.2.2 Thiolated particulate systems

Some hydrophilic polymers such as chitosan are intrinsically mucoadhesive due to its cationic amino-groups which promote interaction with mucin (Barthelmes et al., 2011a; Sogias et al., 2008; Van der Lubben et al., 2001). Nevertheless, functional groups such as thiols (Barthelmes et al., 2011b; Bernkop-Schnürch and Greimel, 2005), acrylates (Brannigan and Khutoryanskiy, 2017; Davidovich-Pinhas and Bianco-Peled, 2011), maleimide (Tonglairoum et al., 2016) and catechols (Xu et al., 2012) have been explored to chemically modify polymers in order to improve their mucoadhesion. Irmukhametova *et al* reported the synthesis of thiolated nanoparticles using self-condensation of 3-mercaptopropyltrimethoxysilane (Irmukhametova et al., 2011), which were subsequently used by

Mun et al (Mun et al., 2016) to study retention on porcine bladder surface. Thiol-ene click chemistry involving interactions between pentaerythritol tetraacrylate and tetrakis (3-mercapto-propionate) were employed by Štorha and co-workers to produce thiolated nanoparticles (Štorha et al., 2013) that were shown to be adhesive to porcine bladder mucosa. However, these nanoparticles (Irmukhametova et al., 2011; Štorha et al., 2013) have not been explored for formulation of anticancer agents for intravesical BC therapy.

Zhang and co-workers generated thiol-functionalised cyclodextrin based mesoporous silica nanoparticles (MSNPs-CD-(NH_2)-SH) for potential BC treatment and reported that they possess superior mucoadhesion compared to hydroxyl and amino-functionalised NPs [MSNPs-CD-(OH) and MSNPs-CD-(NH_2)] during mucin-nanoparticles interaction studies (Zhang et al., 2014). *In vitro* MTT cytotoxic testing on UMUC3 BC cells showed that the IC_{50} for doxorubicin loaded thiolated NPs (Dox-MSNPs-CD-(NH_2)-SH) and free doxorubicin were 3.92 ± 1.06 µg mL^{-1} and 0.45 ± 0.05 µg mL^{-1}, respectively (Zhang et al., 2014). The gradual release of doxorubicin from the nanoparticulate formulation into the endosomes /lysosomes of the BC cells may be responsible for the IC_{50} of about 3.92 µg mL^{-1} reported for Dox- MSNPs-CD-(NH_2)-SH. However, IC_{50} values for MSNPs-CD-(OH) and MSNPs-CD-(NH_2) also evaluated in the study, were not provided. Thus there were no means of establishing the cytotoxic superiority of doxorubicin loaded thiolated drug carriers over amino- and hydroxylated nanoparticles. Also, *in vitro* drug release studies revealed that doxorubicin was released faster (63%) from thiolated nanoparticles deposited onto porcine bladder tissues incubated in simulated urine conditions (pH 6.1) relative to PBS (pH 7.4), with drug release of 13% after 48h (Zhang et al., 2014). However, studies that use artificial urine may be more reliable as it is closest to the physiological environment of the bladder.

Cook *et al*, co-polymerised a thiol-bearing monomer, 2-(acetylthio)ethylacrylate (ATEA) with 2-hydroxyethylmethacrylate (HEMA) using ethylene glycol dimethacrylate as a cross-linker (Figure 4) to generate 635-977 nm thiolated microgels (Cook et al., 2015). These microgels were found to exhibit high doxorubicin encapsulation efficiency (75-86%), good colloidal stability, excellent bladder mucoadhesion as well as sustained drug release over 300 min. Doxorubicin was released from the insoluble matrix through Fickian diffusion established from the Higuchi drug release model.

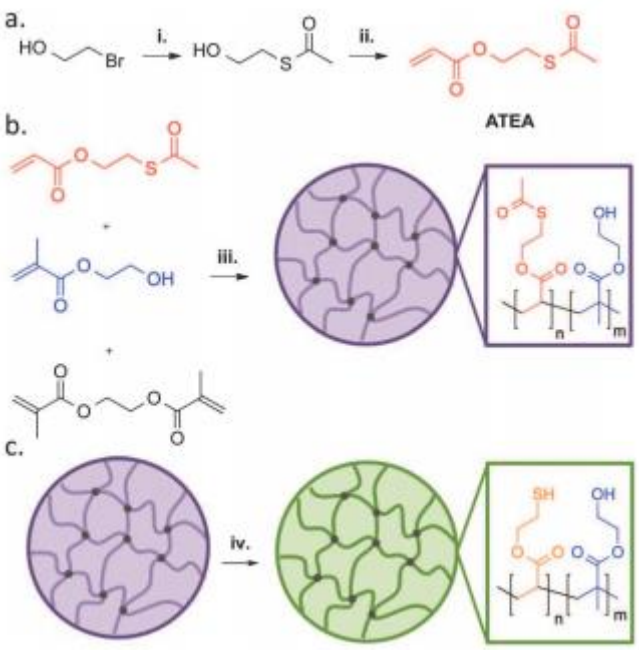

Figure 4: Synthesis of thiolated microgels: (a) Synthetic route to ATEA, a protected thiomonomer. (b) Polymerisation to form ATEA: HEMA copolymer microgels, displaying the pendant functionalities present. (c) Deprotection of ATEA using sodium thiomethoxide to yield thiol-bearing microgels. (i) Potassium thioacetate, acetone, 24 h. (ii) Acryloyl chloride, trimethylamine, DCM, reflux, 24 h. (iii) Ammonium persulfate, ethylene glycol dimethacrylate, water, 70°C, 6 h. (iv) Sodium thiomethoxide, methanol, 30 min. This figure is reproduced from Cook et al., (2015). Reproduced with permission from the Royal Society of Chemistry.

Also, the retention of doxorubicin loaded microgels on the bladder tissues was modulated by variation of the molar proportions of both monomers (ATEA & HEMA) to generate microgels with desirable thiol content. For example, microgels with the greatest and least degree of thiolation were achieved with 80 mol% and 30 mol% ATEA, respectively (Cook et al., 2015). The former resisted wash off by artificial urine during *ex vivo* porcine bladder mucoadhesion studies compared with the latter. This may be associated with a greater amount of thiol groups forming covalent disulphide bridges with the cysteine-rich regions of urothelial mucins as mucosal adherence was independent of surface charge or polarity of carrier. The 30 mol% and 80 mol% ATEA / HEMA based microgels had capacity for loading up to 2.5 mg mL^{-1} and 2.7 mg mL^{-1} doxorubicin, respectively (Cook et al., 2015), which was greater than the therapeutic doses of doxorubicin (1- 2 mg mL^{-1}; 25-100 mL solution) (Amling, 2001; Lamm et al., 2005). However, future *in vivo* studies are desirable to facilitate further development of these drug carriers.

Cationic amine-functionalised polyacrylamide (PAm-NH_2) based nanogels were investigated for intravesical delivery of docetaxel (Lu et al., 2015) due to their safety, mucoadhesive properties and

sustained release potential. They were prepared as a lyophilized solid readily dispersible in water or phosphate buffer saline (PBS). These materials had high drug loading (> 90%) with an initial burst release within 9 h and a sustained release over 9 days when formulations were put in a dialysis membrane and drug release evaluated in artificial urine over predetermined time intervals (Lu et al., 2015). Docetaxel loaded functionalised carrier (DTX-Pam-NH_2) displayed superior inhibition towards UMUC3 cells relative to T24 cells, with a minimum inhibitory concentration (IC_{50}) of 5.6 ng/mL vs 535.6 ng/mL) over 4h and the difference became more pronounced with an exposure time of 72 h, with a calculated IC_{50} of 1.6 ng/mL and 11.6 ng/mL, respectively. Also, the cellular uptake of fluorescently labelled DTX-Pam-NH_2 nanogels into UMUC3 cells at 37°C was more pronounced than into the T24 cells in a concentration dependent manner. This finding was also confirmed in *ex vivo* studies using porcine bladder tissues, where persistent green fluorescence on bladder tissues suggested the adhesion of the fluorescent carrier onto bladder urothelium. The treatment of intact bladder urothelium with the novel formulation also confirmed its safety when analysed using scanning electron microscopy as it induced only mild disruption of the urothelial tissues (Lu et al., 2015). However, the preparation of nanogels was time-consuming as the preparation took about a week. Also, the amine functionalised surface may not have superior mucoadhesive features because its mode of interaction with the bladder mucosa would be via electrostatic attraction rather than covalent bonding which is stronger (Bernkop-Schnürch and Greimel, 2005).

Recently, Mun *et al* developed a new method for evaluating the retention of thiolated and PEGylated silica nanoparticles on porcine bladder epithelium during *ex vivo* studies as drug containing or blank formulations are being washed off using artificial urine (Mun et al., 2016). The parameter "Wash Out_{50}" (WO_{50}) represented the volume of biological fluid (such as artificial urine) required to detach 50% of the adhered particulate carrier from a mucosal tissue. The *in vitro* studies also identified chitosan with superior WO_{50} value and mucoadhesive property relative to thiolated nanoparticles and dextran (89 mL vs 36 mL vs 7 mL). The mucoadhesiveness of thiolated silica NPs was decreased with surface decoration with PEG and porcine bladder mucoadhesion reduced with increase in PEG molecular weight (5000 vs 750 Da) resulting in WO_{50} of 8 mL and 29 mL, respectively (Mun et al., 2016).

Guo et al studied 10-hydroxycamptothecin (HCPT) loaded cationic cross-linked polypeptide (poly(L-lysine)-poly (L-phenylalanine-co-L-cysteine) [PLL-P (LP-co-LC)] nanogels (HCPT/NG) for intravesical delivery. The poly(L-lysine) segment of the peptide is positively charged and interacts favourably with negatively-charged bladder mucosa while PLL-P (LP-co-LC) mimics cell penetrating peptides by promoting drug uptake into BC cells (Shin et al., 2014). The drug carrier had a particle size in a nano-range (≈ 65 nm); was positively charged (+16.3 ± 1.4 mV) as well as displayed good drug loading

capacity and efficiency of 30.6 and 88.2 % (w/w), respectively. Based on Confocal Laser Scanning Microscopic (CLSM) and microplate reading method of analysis, HCPT/NG was taken up into BC cells via endocytosis and drug was efficiently delivered into T24 cell nuclei within 6 h, while the free drug formulation remained into the cytoplasmic region (Guo et al., 2016). During *in vitro* studies, T24 BC cells were treated with HCPT and HCPT/NG for 24 h and the cytotoxic effect of the formulations was investigated using MTT assay. The drug loaded nanogel showed greater cytotoxic effect than the free drug (IC_{50} values of 2.7 mg / L vs. 7.9 mg/L). With the *in vivo* studies using orthotopic BC model, HCPT/NG demonstrated remarkably improved antitumor activity using flow cytometric cell analysis, with cell death occurring predominantly in the nanogel treated regions relative to that treated with free drug. In addition, the drug loaded nanogel exhibited superior tumor necrotic region (46.3 ± 2.2%) relative to the cells exposed to free drug, up to 3.8-fold increase. The *in vivo* biodistribution of the drug carriers was studied: six hours post treatment with nanogel and / or drug, the mice were sacrificed and their bladder and other major organs were excised, homogenised and evaluated by HPLC. The HCPT/NG were preferentially retained in the bladder and rarely in other organs, displaying 3.2-fold greater drug concentration in the bladder than that of the free drug-treated mice. This finding also correlates with the improved safety of the drug carrier due to targeted drug delivery, further confirmed by the insignificant changes in body weight throughout the studies (Guo et al., 2016). This drug carrier appears promising for the treatment of superficial BC. However, *in vitro* drug release and *in vivo* mucoadhesive profile of HCPT or HCPT/NG were evaluated using PBS. It will be more physiologically relevant if artificial urine was used instead of PBS.

5.2.3 Composite system of nanoparticles and hydrogel

Composite systems of nanoparticles and hydrogels were explored to combine the benefits of both formulations including improved drug loading, release, mucoadhesion and urothelial uptake. Hydrogels are three-dimensional hydrophilic or amphiphilic polymer networks, prepared by physical or chemical crosslinking of polymers. These materials exhibit excellent ability to retain water or biological fluids (Kopeček, 2007; Yu and Ding, 2008). They are soft, flexible and biocompatible; this makes them readily fabricated as building blocks for soft tissues (Khetan et al., 2013; Oommen et al., 2013; Rice et al., 2013). The physically cross-linked hydrogels are more readily eliminated after drug release and uptake into the urothelial tissues, than hydrogels prepared using covalently bonded polymers. However, a balance is required between prolonged duration of action and biodegradability so that covalently linked hydrogels do not cause any harm to the body.

In situ gelling systems are liquid formulations with flowing tendency at room temperature and form gels at physiological environment in response to various stimuli such as pH, enzymes or temperature. In recent years, temperature has become the commonly explored stimuli for such formulations (He et al., 2008; Kim and Park, 2002; Narendra and Lee, 20014), where sol-gel transition takes place at 37°C (within the body). It is desirable that dosage forms for mucosal delivery (including the intravesical route) have gelation temperature within the range of 30°C to 36°C (Choi et al., 1998). Over the last decade, biomedical researchers have explored these systems for drug / biomolecule delivery (Gong et al., 2012; Taylor et al., 2010; Wang et al., 2013) and tissue engineering (Niranjan et al., 2013) because they are readily manufactured, exclude use of organic solvents, administered in a minimally invasive mode, and provide sustained release (Supper et al., 2013).Their liquid consistency also allows their easy mixing with therapeutic agents before administration to a patient (Nguyen and Lee, 2010). Thus they have been employed for IDD, serving as drug reservoir, from where drug is released steadily over extended period of time.

5.2.3.1 Floating Composite systems of NPs and hydrogels

Adriamycin, an anthracycline antibiotic, is useful for reducing BC recurrence (Dalbagni, 2007) when administered intravesically after transurethral resection but its application was limited because it causes irritation and scarring of the bladder; additional problem with this drug is its rapid release (Eto et al., 1994). Floating strategies were used for oral formulations to improve residence time of drugs in the gastrointestinal tract (Pawar et al., 2011; Prajapati et al., 2013; Singh and Kim, 2000). Thus composite nanoparticle-hydrogel delivery system, with *in situ* gelling and floating potential within the bladder , was designed to serve as a drug depot to release adriamycin gradually and prevent urinary obstruction associated with the high viscosity of conventional non-mucoadhesive hydrogels (Lin et al., 2014). The safety of adriamycin has also been improved by formulating it as human serum albumin based nanoparticles (103 nm) that are loaded into Poloxamer 407 (P407) and hydroxypropyl methyl cellulose (HPMC) based thermosensitive gel (Lin et al., 2014). The P407 facilitated gelation through micellar packing and entanglements (Alexandridis and Hatton, 1995; Mortensen, 1993), while HPMC enabled attachment of the nanoparticles to the bladder wall and prolonged erosion of the gel which ensured sustained drug release. One of the components of this formulation (sodium hydrogen carbonate) enables the drug carrier to float in urine environment. It produces CO_2 microbubbles in acidic medium which enables the hydrogel system to float, thereby preventing urinary obstruction (Lin et al., 2014). Nanoparticles with adriamycin formulated using P407 and HPMC were reported to undergo sol-gel transition within the shortest time possible, achieving gelation temperature (GT) of 10°C and gelation time (Gt) of 2 mins when evaluated at

37°C, in comparison with other evaluated carriers (nanoparticles-adriamycin or non-floating hydrogel), with GT of 12 to 18°C and Gt of 2-5 mins at 37°C.

The amount of drug released into the urine of rats as well as the amount retained in their bladder following intravesical administration of a suspension of adriamycin nanoparticles and adriamycin loaded nanoparticles dispersed into *in situ* gelling liquid showed that the composite nanoparticles-hydrogel system facilitated a controlled drug release with 81.87% drug released over 10h compared to the nanoparticles (with loaded drug released instantly) (Lin et al., 2014). The *in vitro and in vivo* drug release studies did not correlate well due to the disparity in the volume of evaluation medium as well as hydrogel volume used. This is because the *in vivo* studies had to use hydrogel volume of 0.1 mL that can be accommodated in rat's bladder (volume of ≤ 1 mL), while the *in vitro* studies used 400 mL release medium and hydrogel volume of 12 mL applicable to humans. However, to achieve this excellent retention effect urine needed to be acidified for the formulation to float. This may be less acceptable due to potential irritation of the bladder caused by low pHs (Lin et al., 2014). Thus floating in situ gelling drug carrier with sustained release profile at pH 6-7 (intrinsic pH of the bladder environment) would be worth investigating in the future.

5.2.3.2 Non-floating, mucoadhesive composite systems of polymeric nanoparticles and hydrogels

Some authors investigated delivery systems that form non-floatable gels *in situ* but made of materials that do not potentially obstruct urine elimination as they are flexible and steadily dissolved in urine over time, though mucoadhesive for sufficient length of time to allow for drug uptake into urothelial membranes.

Chitosan (CH) formulated with β-glycerophosphate disodium salt (β-GP) is an example of physically cross-linked temperature-responsive gelling system (Figure 5) that has been evaluated by some researchers (Abdel-Bar et al., 2014; Khodaverdi et al., 2012; S. Kim et al., 2010). They suggested molecular mechanism for the gelation process in terms of increased electrostatic repulsion between chitosan macromolecules in the presence of negatively charged ß-GP which also stabilise the resultant hydrogel system as well as chitosan intermolecular hydrophobic and hydrogen bonds (Lavertu et al., 2008; Qiu et al., 2011). It was suggested by Supper and co-workers (Supper et al., 2013) that these molecular interactions have not explained the influence of temperature on the gelation process, despite the fact that some authors (Patois et al., 2009; Schuetz et al., 2008) have established the role of polyol moiety of gelling material in their thermo-sensitivity.

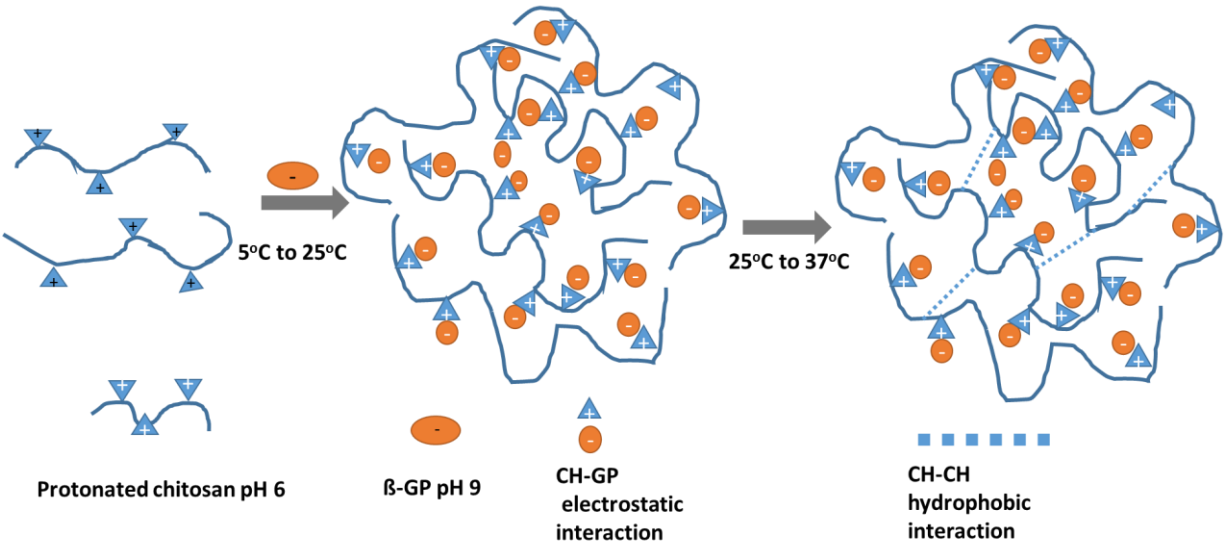

Figure 5: Schematic diagram showing gelation process of chitosan/ß-glycerophosphate mixture at 37°C

Therapeutic agents have been delivered locally and systemically using thermoresponsive chitosan-β-GP in situ gelling system (Abdel-Bar et al., 2014; Aliaghaie et al., 2012; Khodaverdi et al., 2012; S. Kim et al., 2010; Peng et al., 2013). However, drug delivery application of chitosan-ß-GP systems has been limited because of poor drug loading, cellular uptake, rapid drug release and short-lived activity of loaded drugs. (Park et al., 2012; Ruel-Gariépy and Leroux, 2004). Thus they have not been used for IDD.

Magnetic delivery system was studied for the adjuvant treatment of superficial BC. BCG powder and magnetite (Fe_3O_4) nanoparticles were incorporated into chitosan / β-GP solution. The nanoparticulate component targeted the carrier to bladder tissues under the influence of magnetic field. The resultant Fe_3O_4-BCG-chitosan / β-GP system formed gel *in situ* and resisted urine wash-off (Zhang et al., 2013). BCG loaded magnetic gel dosage form stimulated greater Th1 immune reaction with increased expression of interleukin-2 (IL-2, 149.3 ± 8.06 pg/mL) and interferon ɣ (IFN-ɣ, 373.47 ± 40.53 pg/mL) in the urine and superior antitumor activity (tumor volume of 0.53 ± 0.27 mm^3) compared to conventional BCG solution with "1L-2" of 98.84 ± 7.03 pg/mL; IFN-ɣ of 220.28 ± 54.19 pg/mL and tumor volume of 1.82 ± 0.48 mm^3. The duration of BCG residence within the bladder tissues was also extended beyond one month, evident by the CD4+ lymphocyte levels detected in submucosal regions: 3913 ± 467 lymphocytes were recorded for magnetic BCG nanoparticle-hydrogel carrier compared to 2578 ± 269 lymphocytes when BCG solution was used.

Chitosan has an intrinsic mucoadhesive property and may be responsible for improved antitumor immune reaction exhibited by the magnetic carrier in the bladder (Zhang et al., 2013). Also, tight

junctions of the urothelial membrane are loosened up due to their interaction with chitosan, thereby increasing and sustaining local drug concentration (Rosenthal et al., 2012). *In vitro* release studies were not carried out due to the lack of reliable techniques used for detection and quantification of BCG. Moreover, the BCG radiolabelling technique explored by Shen *et al* was not helpful (Shen et al., 2007). So, initial burst release profile was suggested based on persistent cytokine expression in rat urine up to 48 hours during *in vivo* studies. The burst release profile of BCG may result in adverse reactions. So, further studies should be carried out to ascertain if the delivery system is appropriate for BCG or alternative method of BCG quantification may be helpful for dosage determination.

Deguelin has been employed for chemotherapy of lung, breast and colon cancer due to its antiangiogenic potential (Lee et al., 2005; Oh et al., 2007; Peng et al., 2007). However, its clinical use is limited because of its hydrophobicity as well as potential side effects associated with high drug dose (Lee et al., 2005). N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTAP) has cationic hydrophilic head and hydrophobic chain and nanoparticles formulated using this amphiphilic material are able to solubilise lipophilic drugs and enhance urothelial uptake so that the dose required for the therapy is reduced, minimising toxicity effects. This justifies their acceptance by FDA for the gene-based treatment of lung cancer (Díez et al., 2009; Gopalan et al., 2004; Simberg et al., 2004).

Men *et al* reported the improved deguelin's aqueous solubility and residence time in the bladder using the formulations composed of amphiphilic DOTAP and monomethoxyl poly(ethylene glycol)-poly(Ɛ-caprolactone (MPEG-PCL) [DMP] based nanoparticles and thermosensitive in situ gelling Pluronic F127 (F127) (Men et al., 2012). F127 hydrogel improved urothelial uptake and tissue absorption of deguelin while cationic DOTAP/MPEG-PCL nanoparticulate component facilitated sustained drug release (Kabanov et al., 2002). Deguelin loaded nanoparticles had encapsulation efficiency of 98.2% but rate of loading drug into these nanoparticles was low (4.9%). *In vitro* drug release studies were performed using dialysis membrane maintained in a water-bath containing PBS (pH 7.4) and 0.5% w/w Tween 80. This formulation formed gel readily at 25°C *in vitro* and fluorescent gel was observed in mice within 10 minutes of intravesical administration which sustained for 2h (Men et al., 2012). *In vitro* cellular uptake studies using DOTAP modified in situ gelling formulation and unmodified nanoparticles loaded with fluorescent coumarin-6 (with similar hydrophobic nature to deguelin) showed that the former was readily taken up by T24 BC cells than the unmodified nanoparticles. *In vivo* studies using mice confirmed cellular uptake of fluorescent model drug loaded formulation based on fluorescence observed within sections of bladder tissues. The tolerability of deguelin was improved with the intravesical administration of the novel drug

loaded formulation because similar drug amount (2mg kg^{-1}) given intravenously to mice, killed them. Thus, D/DMP-F may be safer, effective without causing urethral blockage due to its gradual elimination from the bladder (Men et al., 2012).

Şenyiğit *et al* have employed chitosan-thioglycolic acid conjugate (CH-TGA) to prepare nanoparticles (CH-TGA NPs) and incorporated them into 2% chitosan gel (CH-TGA NPs/CH) or *in situ* gel forming poloxamer (CH-TGA NPs / Plx gel) for improved intravesical delivery of gemcitabine hydrochloride (Gem-HCl) (Senyigiti et al., 2015). Gem-HCl nanoparticles had greater drug loading than DMP nanoparticles evaluated by Men et al (Men et al., 2012) (9.4% vs 4.9%) but the latter had greater encapsulation efficiency than the former (98.2% vs 19.2%). This finding may be associated with the differences in the physicochemical properties of the formulation. CH-TGA NPs/CH gel was more resistant to dilution by artificial urine (Tyrode solution) than CH-TGA NPs / Plx gel at 37°C based on rheological frequency sweep data (Storage modulus 15Pa vs 6Pa). *In vitro* Gem-HCl release studies also suggested that the rate of drug release following dispersal of nanoparticles into CH-gel and Plx gel decreased by a magnitude of 1.5 and 2.6, respectively as well as release rate of 33.4 ± 5.0% vs 19.6 ± 1.6% in 4 hours (Senyigiti et al., 2015). During bioadhesion test using bovine bladder mucosa, CH-TGA NPs/CH gel also had improved bioadhesive properties (in terms of its force of detachment from the bladder tissues) compared to Plx gel based carrier (1.003 ± 0.048 N˙mm vs 0.378 ± 0.022 N˙mm). The incorporation of the drug carriers with Tyrode solution resulted in a 51% and 80% reduction in bioadhesive properties, respectively (Senyigiti et al., 2015). Greater percentage of the drug permeated the bladder mucosa for the CH-gel based carrier compared to Plx-gel (33.16 ± 5.11% vs 18.78 ± 1.97%) during *ex vivo* studies. Thus CH-TGA NPs/CH gel may be a potential intravesical delivery system for Gem-HCl in order to improve efficacy and drug residence time within the bladder. This investigation was intended to mimic the behaviour of the proposed formulation within the urine containing bladder environment. However, the gelation time reported for Plx gel-Tyrode solution and CH-TGA NPs/Plx gel –Tyrode solution (457 ± 4 seconds vs 483 ± 2 seconds) at 37°C was quite unexpectedly low for formulations with gelation temperature of 51.7±1.0^{o}C and 53.7±1.9^{o}C, respectively.

5.2.4 Liposomal systems

Liposomes were not usually explored for intravesical drug delivery because of their instability in human urine. Recently, Nakamura's group modified cationic liposomal surfaces with cholesteryl-PEG to overcome their urine aggregation and promote uptake into urothelial tissues (Nakamura et al., 2017). N-(carbonyl-methoxypolyethyleneglycol 200)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG (DSPE-PEG) based formulations were the most resistant to aggregation in the presence of human urine relative to 1,2-distearoyl-sn-glycerol, methoxy-PEG 2000 (DSG-PEG)

and cholesterol-PEG (Chol-PEG). This finding was due to the superior flexible conformation of DSPE-PEG in comparison to DSG-PEG and Chol-PEG. Also, the rich density of negative charge on DSPE-PEG shields the cationic liposomes thereby enhancing stability in the urine. Additionally, fluorescently labelled liposomal suspension in human urine was incorporated into MB49 cells and these cells were evaluated using flow cytometry. The surface functionalisation of the cationic liposomes with PEG ensures that the drug carrier is taken up into MB49 murine cells uniformly. Surprisingly, 2 or 5 mol% Chol-PEG functionalized Cat-LPs were taken up into the cells more efficiently compared to unmodified and other PEG modified liposomes. This result was in contrast to their stability behaviour in human urine as more rigid conformation of Chol-PEG based carrier supported their uptake into MB49 cells (Nakamura et al., 2017). Thus lipid based carriers incorporating both DSPE and Chol-PEG may be formulated for improved stability in human urine as well as cellular uptake into cancerous urothelial tissues.

GuhaSarkar *et al* explored paclitaxel loaded composite liposomes in gellan hydrogel (PTX-LP-Gel) for intravesical delivery. Paclitaxel was encapsulated efficiently into the drug carrier (91.2 ± 0.7%) because of the affinity of the hydrophobic drug towards lipid bilayers. Apart from being mucoadhesive, gellan is a temperature- and ion-responsive polysaccharide, so it becomes physically cross-linked in the presence of urine, resulting in prolonged retention of the drug carrier onto the urothelial surface. 0.1% gellan was identified as the optimum concentration for syringeability through the catheter. The liposomal component (124 nm, PDI 0.22, surface charge -16.8 mV) enhances urothelial cell permeation as a result of merging with the lipid content of the cell membranes. Based on cryo-TEM results, the size of the drug loaded liposome was increased with paclitaxel encapsulation to ~ 200 nm. PTX-LP-Gel exhibited a sustained drug release profile over 50 h (17.8 ± 3.0% of loaded drug) due to the smart matrix of the hydrogel; though porous structure facilitates controlled drug and / or liposome diffusion out of the hydrogel. Cellular uptake studies using CLSM confirmed superior NBT-II and T24 cell internalization of loaded rhodamine-6G, relative to the control cell group. Also, cytotoxic testing of the novel drug carrier using NBT-II and T24 cell lines suggested that they retained their cytotoxic effect with IC_{50} values of 55.7 ± 13.0 nm and 1.9 ± 0.5 µm, respectively. LP-Gel was not detected in non-target organs during in vivo retention studies and the amount detected in the rat bladder 7 days post instillation was remarkably greater than that of the commercial product, Taxol (1.71 ± 0.86 µg/g vs. 0.02 ± 0.01 µg/g). The safety of LP-gel was confirmed based on scanning electron and atomic force microscopic images depicting intact urothelium, with residence of the gel formulation for up to 24 h. This work revealed that the limited mucoadhesiveness associated with conventional liposomes can be overcome by incorporation of liposomes within gellan gel. Moreover, toxic effect of cross-linkers (such as glutaraldehyde and 1-

ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) used with mucoadhesive polymers, to improve mucoadhesiveness of liposomes, will be avoided (GuhaSarkar et al., 2017). Overall, this work generated safe, injectable, mucoadhesive, ion-triggered in situ gelling carrier, resistant to pH changes in the bladder with a sustained release profile.

5.3 *In vitro - in vivo* models to study intravesical drug delivery

Various *in vitro* and *in vivo* models have been used to study drug delivery systems intended for intravesical administration. Different types of BC cells have been employed for cell viability studies. Murine sources include MBT-2 (Tamura et al., 2015), MB49 (Nakamura et al., 2017) and NBT-II (GuhaSarkar et al., 2017), while T-24, RT-4, UM-UC-3, 5637 and HT-1376 are human cell lines (GuhaSarkar et al., 2017; Guo et al., 2016; Lu et al., 2015; Martin et al., 2014; Men et al., 2012; Neutsch et al., 2013; Zhang et al., 2014). They differ in terms of their invasive and metastatic tendencies. SV-HUC-1 are healthy bladder cell lines studied to evaluate the biocompatibility of drug carriers (Neutsch et al., 2013). These cells are often maintained in appropriate medium containing 5% carbon dioxide at 37°C for optimal growth (Lu et al., 2015; Martin et al., 2014, 2013; Men et al., 2012; Neutsch et al., 2013; Tamura et al., 2015; Zhang et al., 2014).

During *in vitro* studies, cell viability in the presence of potential drug carriers was evaluated using different methods, such as bromodeoxyuridine and XTT assay (Neutsch et al., 2013), lactate dehydrogenase assay (Tamura et al., 2015), MTT assay (Guo et al., 2016; Lu et al., 2015; Men et al., 2012; Zhang et al., 2014) and sulphorhodamine-B colorimetry (GuhaSarkar et al., 2017). The damage of the intravesical carriers to cell membrane was measured in terms of specific enzymes or antibodies release into the cytosol when carriers were mixed with malignant cells. Some researchers did not carry out *in vitro* cell viability or cytotoxicity testing of their formulations (Cook et al., 2015; Lin et al., 2014; Senyigiti et al.,2015). However, *in vitro* drug release (Cook et al., 2015; Senyigiti et al., 2015) and floating tendency (Lin et al., 2014) studies of their formulation in the presence of artificial urine were evaluated.

There are detailed protocols available for the preparation of artificial urine for *in vitro* cell based studies, which is representative of the components of human urine but they are varied in composition and concentration of their constituents, which imparts on their pH (6.5-7.8), specific gravity (1.008-1.02 g/mL) and osmolality value (430-861 mOsm/kg) (Brooks and Keevil, 1997; Brown et al., 1989; Christmas et al., 2002; Chutipongtanate and Thongboonkerd, 2010; Grases and Llobera, 1998; Mayrovitz and Sims, 2001; Opalko et al., 1997). Examples of constituents include urea, uric acid, creatinine, trisodium citrate, sodium chloride, potassium chloride, ammonium chloride, calcium chloride dehydrate, magnesium sulphate heptahydrate, sodium bicarbonate, disodium oxalate,

sodium sulphate, sodium dihydrogen phosphate and disodium hydrogen phosphate (Brooks and Keevil, 1997; Brown et al., 1989; Christmas et al., 2002; Grases and Llobera, 1998; Mayrovitz and Sims, 2001; Opalko et al., 1997). The artificial urine with pH, specific gravity and osmolality of 6.2, 1.01 g/mL and 446 mOsm/kg, respectively, was appropriate for various biomedical applications (Chutipongtanate and Thongboonkerd, 2010).

Martin *et al* evaluated the uptake of fluorescent carriers suspended in artificial urine into healthy human ureter using *ex vivo* binding assay (Martin et al., 2013). The *in vitro* cytotoxicity of free drug, blank nanoparticles, drug loaded modified and unmodified PLGA nanoparticles, on T-24 cells (metastatic, invasive high grade BC), RT-4 cells (papillary, well differentiated, non-invasive) and UM-UC-3 cells (highly metastatic) were studied using WST-1 reagent and IC_{50} values determined (Martin et al., 2013). These values may help to establish the dose of formulation that would be toxic to urothelial cells.

During *in vivo* studies, mice were induced with human bladder cancer (orthotopic model) by transurethral implantation (Tamura et al., 2015) or subcutaneous injection (Martin et al., 2014, 2013) of BC cells like UM-UC-3 cells as well as by oral intake of water containing 0.05% (w/v) N-butyl-N-(4-hydroxybutyl)-nitrosamine for 20 weeks (Guo et al., 2016). Predetermined concentration of carriers were instilled and the degree of tumor regression determined from the weight of bladder tumor after sacrificing mice (Martin et al., 2014, 2013; Tamura et al., 2015). The actual drug taken up may be evaluated based on LC-MS/MS analysis of extracts from bladder tumor homogenate (Tamura et al., 2015). Other authors quantified *in vivo* retention of drug carriers in terms of observable fluorescence using fluorescence microscopy (Lu et al., 2015; Martin et al., 2014; Neutsch et al., 2013) or acid-fast staining and / or HE staining (Zhang et al., 2013). Men *et al* (Men et al., 2012) also used mice but did not test the antitumor efficacy by induction of bladder cancer. They simply tested the drug uptake into bladder tissues from the delivery system as well as its resistance to urine wash-out. Nevertheless, they evaluated the cytotoxic effects of free deguelin, drug nanoparticles and drug loaded hydrogel-nanoparticle composite formulations on T-24 cells while the anti-angiogenic potential of deguelin was assessed using transgenic zebrafish model (Men et al., 2012).

Similar number (six) of injections were administered to mice and rats based malignant models but dose and dosing interval of the drug carriers differ probably due to pharmacological profile of various chemotherapeutic agents used (Martin et al., 2013; Tamura et al., 2015; Zhang et al., 2013). Therefore, it would be difficult to compare degree of tumor regression across studies. Moreover, some studies were less detailed in terms of the original volume of tumor (Tamura et al., 2015). Thus it is not easy to ascertain the degree of tumor regression by the final volume of tumor observed.

Wistar rats are typically used for *in vivo* studies, so Lin *et al* used these rats to assess the urine wash-off resistance and sustained release profile of Poloxamer-based floating and non-floating carriers (Lin et al., 2014). Zhang *et al* (Zhang et al., 2013) used similar rats to evaluate urothelial cellular uptake of chitosan/ß-GP/ Fe_3O_4-magnetic hydrogel-nanoparticles system. The immunological response of rat bladder to the intravesical instillation of BCG-based carriers was quantified by urinary analysis of cytokines and tissue histochemical analysis of CD4+ T cells (Zhang et al., 2013). GuhaSarkar's group evaluated the *in vivo* retention of rhodamine and paclitaxel loaded liposomes and liposome-gel systems using healthy female and male rats (GuhaSarkar et al., 2017). However, the antitumor effect of the drug loaded formulations was not investigated in rats, in addition to their retention in the bladder.

On the other hand, *in vivo* testing of formulations was not carried out in some studies reported (Cook et al., 2015; Lu et al., 2015; Neutsch et al., 2013; Senyigiti et al., 2015; Zhang et al., 2014). However, *ex vivo* mucoadhesive studies of some formulations were conducted using porcine (Cook et al., 2015; Lu et al., 2015; Zhang et al., 2014) or bovine (Senyigiti et al., 2015) bladder tissues. However, they quantified the mucoadhesiveness of their drug carriers differently using confocal laser-scanning microscope(Lu et al., 2015; Zhang et al., 2014), fluorescence stereomicroscope (Cook et al., 2015) and TA-XT Plus texture analyser (Senyigiti et al., 2015).

5.4 Clinical trials

TCGel®, a polymeric thermoresponsive hydrogel containing Pluronic F-127 (27%), PEG-400 (1.1%), HPMC (0.3%), double distilled water (71.6%) was developed by TheraCoat Ltd (Israel). It exhibited improved safety and residence within the bladder cavity between 6 to 8 hours and gradually eliminated during urine voiding, in comparison to simple mitomycin C solution during preclinical evaluation (Zacchè et al., 2015). Thus it was assessed for intravesical application by mixing TCGel® with 40mg mitomycin C (standard dose prior to surgery) and pharmacological effect compared with same amount of mitomycin C mixed with water, in an ongoing trial for management of low risk recurrent NMIBC (TheraCoat Ltd., 2015).

6 Conclusion

The intravesical route is the most viable means of improving drug delivery for bladder cancer treatment, especially at the early stages, due to the limitations of oral and systemic routes. However, intravesical drug delivery has disadvantages, like drug dilution by urine and drug wash-out during micturition when conventional formulations made of simple solution of the chemotherapeutic agent are used. Moreover, the urothelium limits permeation of potentially useful

therapeutic agents for BC treatment while carrying out its regulatory role. This results in frequent dosing or catheter retention within the urethral tract that leads to bladder irritation and infection.

Drug carriers reviewed are of polymeric nature with potential to treat superficial BC and prevent disease progression to its metastatic and advanced forms. Moreover, the composite nanoparticulate and in situ gelling formulations are able to combine benefits of both delivery systems to generate dosage forms, with improved safety, efficacy and sustained release profile.

There are variations in the design of the *in vitro* and *in vivo* studies as well as evaluation of data generated from such studies. Some studies were not detailed enough to allow for comparison of cytotoxic or mucoadhesive profiles. Also, some authors did not carry out particular *in vitro* and *ex vivo* studies conducted by others, making it difficult to establish dosage regimen for such formulations.

The issue of formulation design (modulation of formulation properties to generate uniform thin gel layer *in situ*, adequate strength and sustained drug release) for higher capacity of human bladder, identified by GuhaSarkar *et al* (GuhaSarkar and Banerjee, 2010) still persist. This has prevented promising formulations from being tested in human bladder during clinical trials. However, their findings have suggested that solubilisation of drugs in amphiphilic systems; surface modification of particulate systems and incorporation of such particulate systems into *in situ* gelling formulations would generate advanced carriers with superior drug loading, safety, *in situ* gelling, mucoadhesive / floating and selective bladder cancer cell penetrating features that would improve the cytotoxic profile of the incorporated therapeutics. Drug carriers that would explore combination of delivery strategies would be desirable in order to prevent BC recurrence and progression.

Promising safety and cytotoxicity data generated from some of the studies offer hope that single drug loaded carriers may be investigated instead of multiple drugs based delivery systems widely explored in ongoing clinical trials for bladder cancer treatment. The harmonisation of the acceptable and realistic protocols for *in vitro* and *in vivo* models for studying intravesical delivery may accelerate the translation of some of the formulations, currently being developed, into the clinic. Therapeutic outcomes of patients would also be improved with promising disease prognosis.

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