Chitosan and carboxymethylcellulose functionalized graphene oxide as nanocarriers for chemotherapy of bladder cancer

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**CHITOSAN AND CARBOXYMETHYLCELLULOSE
FUNCTIONALIZED GRAPHENE OXIDE AS NANOCARRIERS
FOR CHEMOTHERAPY OF BLADDER CANCER**

LOW YING ZHEN

**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

2020

**CHITOSAN AND CARBOXYMETHYLCELLULOSE
FUNCTIONALIZED GRAPHENE OXIDE AS NANOCARRIERS
FOR CHEMOTHERAPY OF BLADDER CANCER**

LOW YING ZHEN

INTERDISCIPLINARY GRADUATE SCHOOL

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2020

Statement of Originality

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Authorship Attribution Statement

This thesis contains material from one paper published in ACS Journal of Applied Bio Materials where I am the first author.

Sections from Chapter 2.8, Chapter 3 to Chapter 6 are included in the paper titled “Investigating the Behavior of Mucoadhesive Polysaccharide-functionalized Graphene Oxide in Bladder Environment.”

The contributions of the co-authors are as follows:

- A/Prof Li Lin provided the initial project direction.
- A/Prof Tan Lay Poh provided project guidance and edited the manuscript drafts.
- I prepared the manuscript drafts which was revised by A/Prof Tan Lay Poh
- I designed the study and analyzed all data with guidance from A/Prof Li Lin and A/Prof Tan Lay Poh. All laboratory works are performed at the School of Materials Science and Engineering and School of Mechanical and Aerospace Engineering.
- All microscopy excluding AFM, and sample preparation, are conducted by me in Materials Lab I, School of Mechanical and Aerospace Engineering.
- AFM was conducted with assistance from Lai Hui Ying in Organic Materials Service Laboratory, School of Materials Science and Engineering.

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Abstract

As the seventh most common malignancy worldwide, bladder cancer is the costliest cancer to treat due to high rate of recurrence which necessitates life-long monitoring. There is hence, a paramount need for effective treatment options to be established. Accompanying intravesical BCG instillation as standard treatment of bladder cancer, is intravesical treatment by chemotherapy drugs. Intravesical treatment of bladder cancer involved instillation of therapeutic agent into the bladder cavity via a catheter, introduced as a strategy to enhance localized treatment concentration and efficacy, as well as reducing systemic absorption. This method has been proven to have high success rate for treatment of the superficial Non-Muscle Invasive Bladder Cancer (NMIBC). The inherent reflexes of the organ, however, poses a long-standing hurdle to full utilization of treatment. The constant formation of urine in the bladder cavity constitutes a race against time as drugs are diluted gradually while maximum residence time is entirely bounded by the urge to urinate. The non-permeability of the bladder wall which is a characteristic to its function to contain waste substances, acts as an inflexible barrier to treatment effectiveness.

In the hunt for a solution, drug delivery systems have been widely proposed and proven to improve effective transport of drugs in the intravesical environment. The primary approach is focused on strategizing increased drug residence and permeability in order to achieve optimal therapeutic effects without compromising safety. Taking advantage of the robust versatility of graphene oxide, a novel research direction for bladder cancer targeted nano-sized drug delivery system employing functionalized graphene oxide is proposed. The potential of functionalized graphene oxide as drug delivery systems has been widely reported in recent decades, with spotlight on the high drug loading capacity of a variety of drugs via π - π interactions. At the same time, the same loading mechanism allow synergistic interaction with other molecules, promising an idealized context where co-loading of two or more therapeutic agents in a single delivery is possible. The selection of the polysaccharides, chitosan and carboxymethylcellulose as functionalizing polymers on the other hand, introduces dispersion stability, biocompatibility and mucoadhesive property to

the otherwise ill-performing graphene oxide. These characteristics, in addition to the drug loading and release capabilities as well as exposure safety, are key factors to consider in the development of a new system and are therefore explored in this work.

The effect of polysaccharide functionalization degree on the mentioned characteristics are investigated carefully and one of each chitosan and carboxymethylcellulose functionalized graphene oxide is selected. Further study on dispersion stability in physiological conditions, drug loading and release capability, in vitro cytocompatibility, behaviour in simulated bladder conditions as well as interaction responses with *ex-vivo* bladders follow. The retention ability in *ex-vivo* porcine bladder demonstrated muco-adherence for up to 20 urine voiding, which is beyond the standard intravesical therapy with one voiding retention. Particle stability in physiological conditions surpassed that of graphene oxide and was reasonably sufficient for mucoadhesion to occur before aggregative events, especially in high protein and salt conditions of simulated disease urine. Meanwhile, drug loading and release profile of model drug demonstrated great potential for high loading capacity and sustained releases, not to mention the nanocarriers are able to load most common chemotherapeutic drugs utilized for bladder cancer as reviewed. Drug loading capacities for the functionalized graphene oxide stood at the higher range among other reported bladder drug delivery systems. Without a doubt at this juncture, both nanocarriers show promises as effective intravesical drug delivery systems for bladder cancer treatment, with chitosan functionalized graphene oxide prevailing over carboxymethylcellulose functionalized graphene oxide due to the greater stability in simulated disease environment and minimal alteration to the bladder mucosa surface.

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Finally, I would like to dedicate this thesis to my grandmother, who had envisioned this granddaughter as a medical doctor, but oh well, this is as close as it gets!

Table of Contents

Abstract.....	i
Acknowledgements	v
Table of Contents	ix
Table Captions	xiii
Figure Captions.....	xv
Abbreviations	xxi
Chapter 1* Introduction	1
1.1 Background	2
1.2 Hypothesis/Problem Statement	4
1.3 Objectives and Scope	5
1.4 Dissertation Overview.....	6
1.5 Findings and Outcomes.....	7
References	8
Chapter 2* Literature Review	11
2.1 Bladder Cancer and the Chemotherapy Treatment	12
2.2 Human Bladder and its Resistance to Therapeutic Drugs.....	16
2.3 Current Strategies for Enhancing Treatment Efficiency	17
2.3.1 Enhancing bladder permeability to drugs	20
2.3.2 Prolonging drugs residence time within bladder	22
2.3.3 Alternative approaches	24
2.3.4 Challenges and outlook for intravesical drug delivery	26
2.4 Graphene Oxide and Functionalized Graphene Oxide: The Properties	28
2.5 Graphene Oxide in Drug Delivery	32
2.6 Chitosan and Chitosan-Functionalized Graphene Oxide in Drug Delivery ...	37

2.7	Carboxymethylcellulose and Carboxymethylcellulose-Functionalized Graphene Oxide in Drug Delivery	40
2.8	Chitosan, Carboxymethylcellulose and Graphene Oxide in Bladder.....	42
	References	46
Chapter 3* Experimental Methodology.....		73
3.1	Rationale for selection.....	74
3.2	Materials.....	77
3.3	Graphene Oxide Sonication	78
3.4	Synthesis of Chitosan Functionalized Graphene Oxide.....	78
3.5	Synthesis of Carboxymethylcellulose-Functionalized Graphene Oxide.....	79
3.6	Characterization of Nanocarriers	80
3.7	Dispersion Stability Study.....	81
3.8	Cell Viability Assay for Cytocompatibility	82
3.9	Drug Loading and Release Capability	83
3.10	In vitro Mucoadhesion Study by Mucin Particle Method	85
3.11	Ex-Vivo Porcine Bladder Experimentation	85
	References	90
Chapter 4* Synthesis of Polysaccharide-Functionalized Graphene Oxide		95
4.1	Introduction	96
4.2	Materials and Methods	98
4.2.1	Materials	98
4.2.2	Preparation of graphene oxide	99
4.2.3	Chitosan functionalization of graphene oxide	99
4.2.4	Carboxymethylcellulose functionalization of graphene oxide	101
4.3	Results and Discussion.....	102
4.3.1	Graphene oxide selection and characterization.....	102
4.3.2	Effect of chitosan density on properties of functionalized graphene oxide.	107
4.3.3	Chitosan grafting and functionalization studies.....	114
4.3.4	Carboxymethylcellulose grafting and functionalization studies.....	125
4.3.5	Mucoadhesive property of polysaccharide-functionalized graphene oxide.	133

4.4	Summary	136
	References	138
 Chapter 5* Polysaccharide-Functionalized Graphene Oxide Stability, Cytocompatibility and Drug Delivery 147		
5.1	Introduction	148
5.2	Materials and Methods	149
5.2.1	Materials	150
5.2.2	Methods	150
5.3	Results and Discussion.....	151
5.3.1	Dispersion stability in physiological condition	151
5.3.2	Elucidation of the mucoadhesion mechanisms	154
5.3.3	Cytocompatibility of polysaccharide-functionalized graphene oxide	157
5.3.4	Drug loading and release capability of polysaccharide-functionalized graphene oxide.....	160
5.4	Summary	163
	References	165
 Chapter 6* Interaction of Polysaccharide-Functionalized Graphene Oxide with Simulated Intravesical Conditions 173		
6.1	Introduction	174
6.2	Materials and Methods	177
6.2.1	Materials	177
6.2.2	Analysis of stability in simulated urine conditions.....	177
6.2.3	Pig Bladder Mucoadhesion Study.....	177
6.3	Results and Discussion.....	177
6.3.1	Dispersion stability in disease urine variant	178
6.3.2	Fluorescence mucoadhesion study of nanocarriers.....	181
6.3.3	Retention study of nanocarriers on bladder mucosa	184
6.3.4	Effect of nanocarriers on bladder surface morphology.....	187
6.4	Summary	190
	References	192

Chapter 7* Conclusions and Recommendations.....	198
7.1 Conclusion.....	199
7.2 Recommendations for Future Studies	200
7.2.1 Expansion of <i>in vitro</i> and <i>in vivo</i> study	200
7.2.2 Synergistic drug delivery	203
7.2.3 Co-delivery of therapy genes	203
7.2.4 Future Outlook	204
References	208
APPENDIX A	I
APPENDIX B	VIII
APPENDIX C	XI

Table Captions

Table 4.1 Specification of graphene oxide purchased for pilot study. Information is accurate at the time of purchase; however some products has stopped production or had a change of specifications at the time this document is prepared.	98
Table 4.2 Weight ratios of chitosan to graphene oxide used in functionalization synthesis to investigate the effect of grafting degree to the properties of chitosan functionalized graphene oxide.	100
Table 4.3 Weights of chitosan and graphene oxide in five combinations for selection of optimal method.	101
Table 4.4 Weights of adipic acid dihydrazide and sodium carboxymethylcellulose in six combinations for selection of optimal method.....	102
Table 4.5 Percentage of functionalized chitosan on graphene oxide estimated based of residues from thermal decomposition of CS-sGO samples at 700°C.	112
Table 4.6 Estimated weight percentages of chitosan and graphene oxide in sample CS-sGO1, CS-sGO2, CS-sGO3, CS-sGO4 and CS-sGO5.	121
Table 4.7 Estimated weight percentages of carboxymethylcellulose and graphene oxide in sample CMC-sGO1, CS-sGO2, CS-sGO3, CS-sGO4 and CS-sGO5.	131
Table 5.1 Drug loading capacity and loading efficiency of sGO, CS-sGO, and CMC-sGO.	161
Table 6.1 Volumes of artificial urine irrigation required for complete removal of sGO, CS-sGO, and CMC-sGO from the surface of bladder mucosa.	187

Figure Captions

- Figure 2.1** Structure of human bladder epithelium layer. Reprinted with permission from Tyagi et al.⁴⁴. Copyright (2006) American Chemical Society..... 17
- Figure 2.2** Defining properties for intravesical drug delivery systems for enhanced drug permeability and prolonged drug residence. (Mice model illustration adapted with permission from Mugabe et al.⁴⁴. Copyright (2011) American Chemical Society.) 19
- Figure 2.3** Drug delivery systems developed for intravesical treatment of bladder cancer and the limitations..... 28
- Figure 2.4** Chemotherapeutic drugs commonly used in intravesical administration of bladder cancer and the corresponding mechanism of drug loading on graphene oxide as a nanocarrier. 37
- Figure 3.1** Schematic showing preparation of drug-loaded CS-sGO and CMC-sGO. 75
- Figure 3.2** Experimental set up for artificial urine irrigation of porcine bladder tissues by a syringe pump..... 87
- Figure 4.1** Particle size changes of graphene oxide samples with increasing probe sonication duration. The inset table lists the linear fitting for time estimation required to achieve a particle size of 200nm via DLS. 103
- Figure 4.2** Particle size and zeta potential at varied pH environment of graphene oxide samples cGO(A), gGO(C), sGO(E), and chitosan functionalized graphene oxide samples CS-cGO(B), CS-gGO(D), CS-sGO(F). Table in (G) summarized the particle size, polydispersity and zeta potential values of graphene oxides and functionalized graphene oxide samples..... 104
- Figure 4.3** FTIR absorbance spectra showing characteristic peaks for functionalized cGO(A), gGO(B), and sGO(C)..... 105
- Figure 4.4** Thermal decomposition curves of CS-cGO(A), CS-gGO(B), and CS-sGO(C) in comparison to chitosan, cGO, gGO, and sGO..... 106
- Figure 4.5** Changes to pH stability in terms of particle size of chitosan functionalized graphene oxide samples synthesized at weight ratios of 1:1, 2:1, 3:1, 4:1, and 5:1 in

comparison to sGO(A), and the changes to synthesized particle sizes and zeta potentials with increasing weight ratios(B). Figure 4.5(C) shows the trend in particle size and zeta potential of CS-sGO 5:1 across different pH values and (D) lists the particle size, polydispersity index and zeta potential values for all samples.	109
Figure 4.6 TEM images of CS-sGO 1:1 to CS-sGO 5:1 from (A) to (E).	110
Figure 4.7 FTIR spectra for CS-sGO samples synthesized from varying weight ratio showing presence of similar peaks.	111
Figure 4.8 TGA curves showing percentage weight loss from thermal decomposition in nitrogen atmosphere.	112
Figure 4.9 Dispersion stability in pH 3,5,6,7,8, and 10 (left to right) of CS-sGO 1:1 to 5:1 (A-E) 3 hours and one day after preparation. Images in (F-H) are dispersions in physiological media of cell culture media DMEM, phosphate buffered saline PBS, and artificial urine AU for CS-sGO 3:1(F), CS-sGO 4:1(G), and CS-sGO 5:1(H) in physiological media of cell culture media DMEM, phosphate buffered saline PBS, and artificial urine AU (left to right).	113
Figure 4.10 Changes in particle size and zeta potential of sample CS-sGO5 at varying pH plotted as mean $\pm$ standard deviation from three measurements of three batch samples. The mean particle size is 297 ± 19 nm.	115
Figure 4.11 TEM images for CS-sGO1 (A), CS-sGO2 (B), CS-sGO3 (C), CS-sGO4 (D), and CS-sGO5 (E) showing particle size range of 200-500nm.	117
Figure 4.12 Comparison of particle sizes across different pH for Group A synthesized at fixed weight ratio but reaction volumes (A), and Group B synthesized at increasing weight ratio at fixed chitosan concentration (B).	117
Figure 4.13 FTIR absorbance spectra for graphene oxide, chitosan, and chitosan-functionalized graphene oxide, CS-GO5.	119
Figure 4.14 FTIR absorbance spectra for all chitosan-functionalized graphene oxide samples showing negligible deviations between the samples.	119
Figure 4.15 TGA curves showing weight per temperature change of graphene oxide, chitosan, and CS-sGO5.	120
Figure 4.16 Comparison of TGA curves between samples in Group A (A), and Group B (B) of chitosan-functionalized graphene oxides.	122

Figure 4.17 Images showing dispersions in various pH, in the order of pH3, pH5, pH6, pH7, pH8, and pH10 for samples CS-sGO1 (A), CS-sGO2 (B), CS-sGO3 (C), CS-sGO4 (D), and CS-GO5 (E) taken after 3hours, 1day, 3 days, 1 week and 1 month.....	124
Figure 4.18 Images showing dispersions in physiological media in the order of DMEM, PBS and artificial urine for CS-sGO1 (A), CS-sGO2 (B), CS-sGO3 (C), CS-sGO4 (D), and CS-GO5 (E) taken after 3hours, 1day, 3 days, 1 week and 1 month.	124
Figure 4.19 Particle sizes of carboxymethylcellulose functionalized graphene oxide at varying pH and the tabulated particle size, polydispersity index, and zeta potential values of each sample as synthesized.....	127
Figure 4.20 Changes in particle size and zeta potential of sample CMC-sGO3 at varying pH plotted as mean $\pm$ standard deviation from three measurements of three batch samples. The mean particle size is $297\pm 53\text{nm}$	127
Figure 4.21 TEM images for CMC-sGO1 to CMC-sGO6 in the order(A-E).....	128
Figure 4.22 FTIR absorbance spectra for graphene oxide, hydrazide functionalized graphene oxide, sodium carboxymethylcellulose and carboxymethylcellulose-functionalized graphene oxides.....	129
Figure 4.23 FTIR absorbance spectra for all carboxymethylcellulose-functionalized graphene oxide samples showing negligible deviations between the samples.	130
Figure 4.24 TGA curves showing weight per temperature change of graphene oxide, sodium carboxymethylcellulose, hydrazide-functionalized graphene oxide and CMC-sGO3.	131
Figure 4.25 Images showing dispersions in various pH, in the order of pH3, pH5, pH6, pH7, pH8, and pH10 for samples CMC-sGO1 to CMC-sGO6 (A-F) taken after 3hours, 1day, 3 days, 1 week and 1 month.	132
Figure 4.26 Images showing dispersions in physiological media in the order of DMEM, PBS and artificial urine for CMC-sGO3 (A), and CMC-sGO4 (B) taken after 3hours, 1day, 3 days, 1 week and 1 month.	133
Figure 4.27 Outcome of mucin-particle method showing changes in particle size after interaction with mucin	134
Figure 4.28 Outcome of mucin-particle method showing zeta potential of samples before and after interaction with mucin.	135

- Figure 5.1** Digital images and particle sizes of sGO, CS-sGO, and CMC-sGO 3hours, 1 day and 3 days after addition into DMEM (A,B), pH5 phosphate buffer (C,D), pH7.4 phosphate buffered saline (E,F) ,and pH6.3 artificial urine (G,H) as compared to original conditions at 0hr..... 153
- Figure 5.2** Particle size and zeta potential measurement of mucin interaction with CS, CMC, sGO, CS-sGO, and CMC-sGO with deionized water, pH4.5 acetate buffer, pH7.4 phosphate buffered saline and pH6.3 artificial urine. 155
- Figure 5.3** Cell viability of L929 24hours and 72hours after incubation with sGO, CS-sGO, and CMC-sGO at concentration of (A) 10 μ g/ml, (B) 50 μ g/ml, and (C) 100 μ g/ml, and (D) morphology of L929 cells treated with medium only as control and 100 μ g/ml of sGO, CS-sGO, and CMC-sGO. Significant difference from control is determined using one-way ANOVA with Bonferroni test, * representing $p < 0.05$ 158
- Figure 5. 4** (A) Loading capacity and efficiency of CPT on CS-sGO with increasing CPT:CS-sGO weight ratio, (B)UV-vis absorbance spectra for CPT loaded samples in comparison to original samples with inset calibration curve of CPT, and (C-E) CPT release profiles in PB, PBS and AU..... 163
- Figure 6.1** Blue columns representing zeta potential values of CS-sGO(A), and CMC-sGO(B) in acidified and alkalized artificial urine in comparison to pH-adjusted trend from Chapter 4.3.3 and Chapter 4.3.4. Insets show respective images taken of sample one day after preparation, prior to measurement of zeta potential values. 178
- Figure 6.2** Comparison of particle sizes (A), zeta potential (B) of CS-sGO and CMC-sGO in aqueous dispersion DI, artificial urine AU and disease simulated artificial urine AU'. CS-sGO in AU' appeared to retain the dispersion stability in (C) but aggregation of CMC-sGO in AU' was observed visually in (D)..... 180
- Figure 6.3** (A)Loading capacity and efficiency of sodium fluorescein on CS-sGO, and (B) size variation of CS-sGO-FL with different mixing weight ratios. 182
- Figure 6.4** (A-D) Fluorescence microscopy images of porcine bladder incubated with NaFL, sGO-FL, CS-sGO-FL, and CMC-sGO-FL, (F) Fluorescence spectra of samples showing blue-shift of emission peaks. 183

- Figure 6.5** Representative microscope images showing mucoadhesion and retention of sGO, CS-sGO, and CMC-sGO on porcine bladder mucosa after irrigation with pre-determined artificial urine volumes. AU represents control bladder tissues incubated and irrigated solely with artificial urine. Scale bar denotes 1mm. 185
- Figure 6.6** Surface plots of microscope images taken with peaks showing presence of sGO, CS-sGO and CMC-sGO. Absence of peaks is direct representation of complete removal of nanoparticles from the surface of porcine bladder mucosa. 186
- Figure 6.7** SEM images showing morphological changes of pig bladder mucosa surface after two-hour incubation with AU(A), sGO(B), CS-sGO(C), and CMC-sGO(D) at accelerating voltage of 15kV and 100x magnification. 189
- Figure 6.8** SEM images showing morphological changes of pig bladder mucosa surface after two-hour incubation with AU(A), sGO(B), CS-sGO(C), and CMC-sGO(D) at accelerating voltage of 15kV and 1000x magnification. 190

Abbreviations

GO	Graphene Oxide
sGO	Graphene Oxide (Sigma Aldrich source)
CS	Chitosan
CMC	Carboxymethylcellulose
NaCMC	Sodium carboxymethylcellulose
CS-sGO	Chitosan functionalized graphene oxide
CMC-sGO	Carboxymethylcellulose functionalized graphene oxide
ADH	Adipic acid dihydrazide
sGO-ADH	Hydrazide conjugated graphene oxide
CPT	Camptothecin
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PB	Phosphate buffer (pH5)
PBS	Phosphate buffered saline
AU	Artificial Urine
HA	Hyaluronic Acid
HSA	Human Serum Albumin
FL	Fluorescein
NaFL	Sodium fluorescein
FTIR	Fourier Transform Infrared Spectroscopy
TGA	Thermogravimetric Analysis
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
ANOVA	One-way Analysis of Variance

Chapter 1* Introduction

The treatment of bladder cancer, although follows well-established management strategy, are still hampered by tumor recurrence after treatment. The limitations and challenges of bladder cancer chemotherapy is outlined to facilitate design of drug delivery system specific to intravesical application. Functionalized graphene oxide is proposed to possess great potential in successfully transporting drug molecule across the permeation restrictive bladder urothelium and improve drug efficacy. The present study investigates the feasibility of the concept with hypotheses, objectives, and key findings summarized within the chapter.

1.1 Background

Urinary bladder cancer ranks the seventh most common cancer globally.¹ Though the intravesical treatment strategy provides a direct contact between therapeutic agents with disease sites, the effectiveness is still lacking. By direct administration of drug formulation into the bladder via a catheter, the efficacy of drug is at the mercy of the natural functions of bladder. The bladder vesical empties periodically every one to two hours, while in the intervening time there is a constant production and filling of the urine. This causes intravesical drug to be susceptible to dilution and subsequent elimination with the first urine voiding. In the clinical setting, the constraint is countered by repeated administration, prompting patients inconvenience and adverse reactions. Therefore, in the drive to increase drug efficacy in intravesical environment, research have focused on developing drug delivery systems that enable prolonged drug residence time within the bladder cavity and increased permeability of bladder wall during intravesical procedure.

Notably, various forms of drug delivery systems have emerged, mainly liposomes, micelles, nanospheres, nanocapsules, hydrogel systems, nanoparticles (cationic, mesoporous, or polymeric) and some novel combination systems. These systems generally display improved loaded drug efficacy at killing bladder cancer cells compared to introducing drugs alone into the bladder. The encapsulation of drugs in the drug delivery systems also provide a certain degree of protection from the bladder environment, allowing lower dosage of drugs to achieve similar level of therapeutic effects. Others interact with the bladder wall by attractive forces to withstand elimination with urine voiding. Among these, mucoadhesive drug delivery system has emerged as a promising solution to the shortcomings of current intravesical treatments. Mucoadhesive formulations prolong drug retention by adhering to the bladder mucosa and endure wash out by urination. This ultimately enhance drugs bioavailability and effectively reduce instillation frequency and dose required for therapeutic potency. Pairing with permeation-enhancing or cancer cell targeting biomaterials effectively augments the localized drug concentration and hence the therapeutic effects. Developed mucoadhesive drug delivery systems for intravesical use generally comprise of hydrogels and nanoparticles functionalized with polymers of muco-

adhering nature. Hydrogels are essentially plagued by low drug loading capacity and burst releases, while the nanoparticles have underlying issues with synthesis scaling up as well as aggregation.

Protection of cargo drug from degradation and perturbation from surrounding environment as well as controlled release at the targeted sites form the main mission for a drug delivery system. The behavior of nanomaterials introduced into biological environment is greatly influenced by its physicochemical properties. Specifically, the shape, size, surface morphology, hydrophobicity, type of functionalization, colloidal stability, and the tendency to form protein corona changes the biological response.² These are critical factors governing the reliability of an engaged drug delivery system. Often, the incorporation of therapeutic agents as cargo involved direct conjugation to the body of drug delivery systems, as are the cases for most of the currently reported intravesical drug delivery systems. This results in structural modification which could potentially affect the therapeutic efficacy, of which graphene oxide-based drug delivery system appears to hold the ideal solution.

Graphene oxide (GO) is a two-dimensional material that has been brought into the limelight in recent years due to its coveted properties in the biomedical field. The material has high surface area, two surfaces available for reactions, as well as good mechanical and chemical stability.³ Of note, drug loading to graphene oxide is achievable via physical attractions, i.e. π - π stacking, hydrogen bonding, and hydrophobic interactions. Prior publications have even reported pH-controllable drug releases and loaded drug efficacy that is much superior than free drugs.⁴ In addition, the variety of oxygen-containing functional groups on its basal plane confer stability of graphene oxide for aqueous dispersion. Due to these properties, functionalized graphene oxide is presently an emerging research frontier in drug delivery application of cancer, with evidence of common chemotherapeutic drugs as cargo, such as doxorubicin, paclitaxel, docetaxel, camptothecin, and others. Meticulous planning of biomaterials functionalization to graphene oxide facilitates the birth of drug delivery systems with specialized functionality, from additional properties to targeting activity, multi-cargo loading and adaptable stability.⁵

Attempting to bring over the advantages of graphene oxide as a drug delivery system to application in an intravesical setting, the feasibility of the concept is investigated. Well-established in biomedical field, the cationic chitosan and anionic carboxymethylcellulose are selected as functionalizing polymers due to their biocompatibility, mucoadhesive properties and prominence in bladder drug delivery.⁶ The differing ionic charge of both polymers provide parallel analysis of the effects incurred on properties of synthesized drug delivery system. Finally, the high surface-to-volume ratio of graphene oxide is an attribute associated with improved intravesical drug absorption.⁷⁻⁸ The proposed polysaccharide drug delivery systems shall bring significant contribution to chemotherapy treatment of bladder cancer in the form of stability, sustained drug delivery and high retention on bladder mucosa.

1.2 Hypothesis/Problem Statement

Chitosan and carboxymethylcellulose functionalized graphene oxide, although reported, has not been studied for intravesical bladder application. The polysaccharides, chitosan and carboxymethylcellulose, on the other hand, are polymers that has been introduced either as governing intravesical drug delivery system or as solvent for bladder complications. Specifically, both are mucoadhesive with studies establishing affinity for the bladder mucosa, and chitosan is known to increase bladder permeability for therapy transport. Coupled with the outlined advantages of graphene oxide in drug delivery, the prevailing challenges of intravesical drug delivery system such as instability and low drug loading efficiency could be tackled.

Preceding studies for chitosan functionalized graphene oxide⁹⁻¹¹ and carboxymethylcellulose functionalized graphene oxide¹² placed emphasis on the delivery capability of therapeutic agents, but none investigated the mucoadhesive property conferred by the polysaccharides to graphene oxide. Correspondingly, there were limited information on the effect of functionalization degree on overall characteristics and physiological responses in simulated environment. However, since both polysaccharides

are known mucoadhesive polymers, the functionalized product should retain the same property.

Overall, it is hypothesized that chitosan and carboxymethylcellulose functionalization of graphene oxide can effectively improve the biocompatibility and stability of the nanoparticle in intravesical bladder environment, facilitating effective drug delivery. Profiting from the functionalization, the functionalized graphene oxide would exhibit mucoadhesive property, allowing adherence and prolonged retention on bladder surface upon administration. Hence, chitosan-functionalized graphene oxide and carboxymethylcellulose-functionalized graphene oxide would be stable mucoadhesive drug delivery systems in intravesical conditions.

1.3 Objectives and Scope

The project aims at investigating the potential of chitosan and carboxymethylcellulose functionalized graphene oxide for drug delivery application for bladder cancer. For application in bladder, key factors to consider includes stability, biocompatibility and safety. As an intravesical drug delivery system, the desired qualities have been identified to be mucoadhesive for retention against urine voiding, protection of drugs from urine dilution, high drug loading and sustained drug release. These characteristics are studied and the role of polysaccharides functionalization in the respective interactions in investigated conditions are analyzed.

The instability of graphene oxide in protein rich and high salt content solutions has been widely demonstrated.¹³ As the urine contents and pH values of bladder cancer inflicted patients are varied in individuals, the stability of functionalized graphene oxide are vital for efficient drug delivery. Therefore, the dispersion stability in a variety of simulated physiological media and pH conditions are studied for a minimum of four days. The performance of bladder retention ability, drug loading and release potential are evaluated against existing reported systems as validation of intravesical drug delivery feasibility. Finally, the cytocompatibility of functionalized graphene oxide as well as effect on bladder

surfaces are studied primarily to verify the safety to living cells.

The experimental section shall take reference from the work of Bao et al.⁹ and Rao et al.¹² with adequate refining where deemed required. Briefly, graphene oxide is grafted with chitosan and carboxymethylcellulose at varied grafting and the relationship between grafting degree and property change is established. Selection of one from each group of study based on stability criteria shall advance on the application-specific properties studies. Predominantly, the biocompatibility of the nanocarriers is of utmost importance preceding all others. Once established, the primary function of the nanocarrier as drug delivery system are investigated. A hydrophobic chemotherapeutic drug is selected for loading onto the nanocarriers and release profile are examined. The selection of hydrophobic drug over the hydrophilic counterpart is based upon the fundamentally more challenging transport of insoluble drugs within the bladder environment. Finally, the retention capability on bladder mucosa shall be tested on *ex-vivo* porcine bladders to confirm *in-vitro* elucidation of mucoadhesive property. The effect of introducing graphene oxide-based nanomaterials to bladder surface morphology is concurrently explored to ensure controllable local toxicity from the material.

1.4 Dissertation Overview

The thesis addresses the syntheses of chitosan and carboxymethylcellulose functionalized graphene oxide, optimization for size and particle stability, characterizations, determination of biocompatibility, drug loading and release capability, as well as intravesical behavior and mucosal retention. The current chapter provided an overview of study motivation, hypotheses, objectives, and research significance. Chapter 2 shall give an introductory prelude to bladder cancer and the current clinical treatment regimes, followed by the challenges centering on treatment efficacy of intravesical chemotherapy. Preceding published works of drug delivery systems targeting bladder cancer are reviewed in detail. Finally, graphene oxide, chitosan, and carboxymethylcellulose, the characteristics relevant to drug delivery and application in bladder field are summarized.

Chapter 3 elaborates on the rationale and principles behind the methodology employed within this work. In Chapter 4, synthesis optimization is conducted to obtain varying functionalization degree of the polysaccharides. Emphasis is placed on the particle size, general stability in physiological media and mucoadhesive property. Selection of one of each polysaccharide functionalized graphene oxides are analyzed in subsequent studies in Chapter 5 and 6. The principle functions of nanocarriers as drug delivery systems are explored in Chapter 5, where in depth study of particle stability is executed, followed by fundamental biocompatibility study with L929 cell lines, as well as drug loading and release capability in three physiological conditions. The evaluation of mucoadhesive property is also conducted for elucidation of the interaction mechanism. The responses of synthesized nanocarriers to simulated intravesical conditions are examined in Chapter 6. Artificial urine is adjusted to mimic disease urine by adding components pivotal to particle stability and observed for perturbations. Mucoadhesion retention is investigated on *ex-vivo* porcine bladders, and the morphological changes to bladder surfaces examined. Finally, concluding remarks in Chapter 7 are supplemented by future outlook and outstanding analyses in realizing actual implementation in intravesical drug delivery.

1.5 Findings and Outcomes

This research led to several novel outcomes as follows:

1. Controlling the degree of functionalization of chitosan and carboxymethylcellulose facilitates modification of pH stability range of functionalized graphene oxide.
2. Chitosan and carboxymethylcellulose confers graphene oxide with mucoadhesive property sufficient for a crude estimation of 20 urine voiding.
3. Graphene oxide induces extensive exfoliation of bladder superficial layer, but the extend can be alleviated with chitosan and carboxymethylcellulose functionalization.

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Chapter 2* Literature Review

The chemotherapeutic treatment of bladder cancer is limited by the inherent watertight nature of the human bladder as well as constant urine generation and short interval between voiding. A few strategies have been employed to improve this drawback by increasing drugs permeability at the bladder mucosa and prolonging the residence time within bladder. Mostly, mucoadhesive polymers and materials that disrupt bladder integrity is manipulated for this purpose. With the aim of using functionalized graphene oxide as drug delivery systems to achieve treatment efficiency, the properties of graphene oxide and its functionalized forms as well as reported applications in drug delivery were outlined. This includes the polymers, chitosan and carboxymethylcellulose studied for graphene oxide functionalization in this project. Finally, precursory relevance of chitosan, carboxymethylcellulose and graphene oxide application in bladder environment is discussed.

2.1 Bladder Cancer and the Chemotherapy Treatment

Based on the global cancer statistics 2018¹, bladder cancer is ranked the tenth most common form of cancer worldwide. Being the sixth most common type of cancer among men, the incidence and mortality rate is four times more than women. Including both sexes, the overall number of new cases is the seventh highest, with thirteenth highest mortality rate among all cancers. Of most concern is the fact that based on this statistics, the incidence and mortality by bladder cancer is the highest in Asia.² While in the United States projection, it is estimated that 81,400 newly diagnosed cases and 17,980 patients death will occur in 2020.³

Among newly diagnosed cases, approximately 75% are Non-Muscle Invasive Bladder Cancer (NMIBC).⁴ Removal of diseased tissues via transurethral resection of visible tumours is insufficient as recurrence cases of up to 70% has been reported with 25% progressing to muscle invading disease state.⁵ Oral or systemic administration of therapeutic agents are ineffective as only a small fraction will reach the bladder, while the large doses required are mainly metabolized or biodistributed to other regions of the body causing undesirable side effects.⁶ To maximize drugs dose at immediate vicinity of the urothelium, intravesical drug delivery was introduced. Delivery of drug formulations into the bladder vesical via a catheter enables localized immediate exposure to affected sites, effectively optimizing the drug dosage for therapeutic efficacy and reducing side effects.⁷

The current gold standard for treatment of NMIBC is a transurethral resection (TUR) followed by the instillation of Bacillus Calmette-Guerin (BCG).⁸ BCG is a live attenuation of mycobacterium bovis, which is a vaccine developed for tuberculosis (TB) disease. First introduced by Morales, the application of intravesical BCG for superficial bladder cancer was FDA approved since 1990 and remained the most widely implemented clinical therapy.⁹ Among the few effective intravesical therapies for NMIBC, BCG is the sole treatment that is capable of decreasing progression risk against transurethral resection.¹⁰⁻¹¹ Still, recurrence rate is high at approximately 40% cases within 2 years while intolerance and resistance to BCG treatment of certain patients are still significant.¹² In fact, the adverse

effects such as BCG-induced cystitis, haematuria and toxicity in response of BCG is reported to be on the higher end among other intravesical treatments¹³, occurring in as high as 75.2% of 487 patients followed in a phase III trial¹⁴. Meanwhile, dysuria and urinary frequency is as common as 90%.¹⁵ Correspondingly, intravesical chemotherapy is among the alternative regimens used as adjuvant and salvage therapy for cases of adverse reactions or unresponsive treatment.¹⁶

Intravesical chemotherapy involves instillation of anti-cancer drug into the bladder cavity via a catheter inserted through the urethra. This method is effective for NMIBC treatment as there is a limited host systemic exposure while providing localized high concentration of therapeutic agents facilitating greater exposure of therapeutic agents to diseased tissues. The common chemotherapeutic drugs used for intravesical treatment of bladder cancer are mitomycin C (MMC), gemcitabine (GEM), thiotepa (TT), the taxanes and anthracyclines as detailed in Table 2.1.¹⁶⁻¹⁸ Among these the taxanes have high potential in intravesical treatment due to high penetration into bladder tissues which can achieve a rate that is approximately 20-folds higher than mitomycin C and doxorubicin (DOX).¹⁹ However, the non-water soluble taxanes are particularly difficult to administer. Commercial form of paclitaxel, Taxol contains Cremophor and ethanol to solubilize the drug which reduces paclitaxel penetration into the urothelium.²⁰ Commercial formulations of docetaxel (DTX) are prepared in Tween 80 and ethanol. Like Taxol, Taxotere form micelles during administration, directly decreasing uptake efficiency.

Use of thiotepa and cisplatin (CDDP) is generally less practised. Thiotepa was the first intravesical treatment introduced for superficial bladder cancer, but has been gradually phased out due to high level of toxicity reactions, systemic absorption and insignificant advantage against control.^{15, 21} Cisplatin, although proven effective for treatment of bladder cancer, is limited by the high incidence of renal failure in bladder cancer patients.²² Instead, the weaker derivative carboplatin (CP) is employed. MMC is statistically more effective than thiotepa in eradicating transitional tumor and is the most widely used chemotherapy for low-risk tumors.²³ Due to a high molecular weight, the systemic absorption and toxicity is relatively low for MMC but local side effects can be as high as 41% among treated

patients.¹⁵ Doxorubicin is inferior to MMC in terms of treatment efficacy.²⁴ Epirubicin (EPI) and valrubicin (VLB) stemmed from doxorubicin, but with reduced toxicity. A randomised trial revealed no difference in treatment efficacy among thiotepa, doxorubicin, and cisplatin.²⁵ On the other hand, gemcitabine has been introduced as intravesical treatment fairly recently. Phase III randomized trial indicated on par efficacy between gemcitabine with cisplatin treatment versus methotrexate, vinblastine, doxorubicin, and cisplatin for bladder cancer patients. The treatment of gemcitabine with cisplatin generally had better tolerability among patients with better faring in terms of weight, performance, and fatigue.²⁶⁻²⁷ Table 2.1 summarizes the clinical dosages, treatment regimens and commercial forms available for each of the intravesical chemotherapeutic drugs.

Meta-analysis involving 2548 patients on immediate intravesical chemotherapy post-transurethral resection procedures verified the effectiveness of the method in reducing NMIBC recurrences with adverse effects.²⁸ Among the drugs tested were epirubicin, peplomycin, thiotepa, MMC, pirarubicin, and gemcitabine. In fact, multi-drug regime with MMC, doxorubicin, and cisplatin was reported to have similar recurrence rate to BCG but with less side effects.²⁹ However, intravesical chemotherapy is generally not adopted by practising urologist due to the cost, potential side effects, and administration inconvenience involving frequent and repetitive treatments .²⁸

Essentially, a higher concentration of drug instillation drives the increase of recurrence-free rate³⁰, also accounting for 100% increase in drug delivery.³¹ However the higher concentration introduces greater adverse reactions from the patients, reducing patients compliance. Furthermore, to date the clinical trials for intravesical chemotherapy has small success in long term efficacy without recurrence. Clinical trials for gemcitabine and docetaxel reported relatively small percentages (20-30%) for complete durable response that remained disease free.³²⁻³³ The cause of limited efficacy is related to insolubility of hydrophobic drugs, poor exposure of drugs to cancer cells, obstruction by the bladder construct such as impermeable mucosa layer, as well as constant dilution and discharge by urine voiding. Due to inefficient drug delivery to disease tissues, the high recurrence rate often mandates lifetime surveillance, positioning bladder cancer as the fifth most expensive

cancer to treat when considering the total medical care expenditures.²⁴ As such, a great amount of interest has been placed in researching strategies to achieve highest localized drug concentration at cancer site with controllable exposure dosage and duration with minimal systemic and local toxicity to ultimately enhance treatment efficacy and reduce tumor recurrence. Notably, development of new drug delivery systems that enhance drug absorption across bladder urothelium, prolong drug residence duration, improve drug therapeutic response and reduce toxicity has high prospective in improving chemotherapeutic treatment efficacy of NMIBC and reducing recurrence rate by 20-40%.³⁴

Table 2.1 Intravesical chemotherapy treatments, the mechanisms of action, clinical dosage, instillation frequency, and respective commercial trade names.

Drug	Mechanism of action	Dosage	Commercial forms
Mitomycin C	Inhibits DNA synthesis through induction of cross internal links	40mg in 20-40ml sterile water administered weekly for 6-8 weeks ^{17 21, 35}	Mutamycin [®]
Gemcitabine	Inhibits thymidylate synthetase, blocks DNA synthesis and subsequent cell death	Max. 2000mg (total 40mg/ml in 50ml volume) ³⁶⁻³⁷	Gemzar [®]
Paclitaxel	Inhibits tubulin disassembly and stops cell division	(nab-paclitaxel) 500 mg/100 ml administered in 6 weekly followed by 6 months monthly maintenance ³⁸	Taxol [®]

Docetaxel	Inhibits tubulin disassembly and stops cell division	5-75 mg in 100 mL normal saline (phase I trial) ³⁹	Taxotere [®]
Doxorubicin	Inhibits Topoisomerase II activity	30-100mg with weekly or 3 weekly schedule ⁴⁰	Adriamycin [®] , Rubex [®]
Epirubicin	Inhibits Topoisomerase II and DNA helicase activity	30-80 mg in 50 mL normal saline ¹⁸	Pharmorubicin [®]
Valrubicin	Inhibits incorporation of nucleosides into nucleic acids, causing chromosomal damage and arrests G2 cell cycle	800mg in 75ml normal saline weekly for 6 weeks ^{35, 41}	Valstar [®]
Thiotepa	Inhibits DNA replication by alkylating and crosslinking with DNA	30 mg thiotepa in 15-50 mL sterile water or saline ⁴⁰⁻⁴¹	Thioplex [®]
Cisplatin	Inhibit DNA replication and transcription by formation of DNA adduct	50-150mg in 1mg/ml weekly ⁴²	Platinol [®] , Platamine [®]

2.2 Human Bladder and its Resistance to Therapeutic Drugs

Forming the blood-urine barrier of the human bladder is the urothelium as depicted in Figure 2.1. This layer relies on the bladder permeability barrier (BPB) to prevent reabsorption of waste back into systemic circulation. The apical layer of umbrella cells consists of densely packed rigid plaques formed from uroplakins which create a watertight barrier with the help of tight junctions between the apical surfaces. Furthermore, the hydrophilic glycosaminoglycan (GAG) layer of mucin on the surfaces forms a stationary aqueous film that barricades foreign particles from adhering and reaching the underlying

tight junctions and cell membranes.⁴³⁻⁴⁴ These intrinsic natures of the bladder cavity surface inevitably create challenges for intravesically-introduced therapeutic agents to be absorbed. In addition, instillation of drugs is subjected to discharge within one to two hours as the bladder capacity is limited. Residence time will shorten further should the instilled drug formulation be irritative, sensitizing the bladder detrusor muscle.

Figure 2.1 Structure of human bladder epithelium layer. Reprinted with permission from Tyagi et al.⁴⁴. Copyright (2006) American Chemical Society.

2.3 Current Strategies for Enhancing Treatment Efficiency

Although intravesical instillation of drugs overcomes the limitation encountered via conventional oral therapy, the method introduces the shortcomings of constant drug dilution by urine and low permeability across the urothelial tight junctions, necessitating repeated catheterization for treatment efficacy. Drugs absorption across the urothelium is dependent on five factors, mainly molecular weight of drug, surface modification of urothelial surface, formulation pH, drug concentration and duration of drug exposure at targeted site.⁴⁵ Two main approaches being adopted to increase the efficacy of intravesical-administered chemo drugs are by increasing the permeability across the bladder wall and prolonging the drug residence within the bladder cavity. These strategies target the

reduction of treatment frequency as well as achieving equivalent therapeutic action with lower drugs concentration.

The GAG layer, more commonly referred as the mucus layer, consists of dense domains of negatively charged carboxyl or sulfate moieties which is separated by cysteine-rich hydrophobic regions. The pore size of the mucus layer is approximately 300nm, and molecules with a smaller size are theorized to diffuse through based on the principle of Fick's first law.^{31, 46} A particle size in the size range of 200-400nm has been reported to enhance permeation and diffusional transport into bladder urothelium.⁴⁷⁻⁴⁸ Meanwhile, drugs that are lipophilic, weakly acidic or neutral can penetrate the bladder urothelium more easily than the hydrophilic counterparts.⁴⁹⁻⁵⁰ The action of some drugs such as MMC, EPI, and DOX are limited by the acidity of urine, necessitating urine alkalization prior to treatments.^{31, 47} The degradation and stability of some drugs will also be perturbed by pH variations¹⁵, not to mention that pH range of urine lies between 4.5 to 8.^{6, 51} Correspondingly, solvent for intravesical drug formulation favors distilled water over saline solutions due to better efficiency.^{15, 52-53} Still, delivery of water soluble drugs are also confined by the tendency for quick release upon contact with urine.⁵⁴ Although the effects of these hindrance to treatment efficacy are small compared to bladder permeability and short intravesical residence, it will be advantageous to be taken into consideration in the development of drug delivery systems. Designing according to size, surface charge and pH stability as the basis would maximize the therapeutic activity of the drugs. Additionally, as a drug delivery system, the ability to maximize loading, protect cargo drugs, retain drug potency with targeted functionality and minimal systemic toxicity is vital as well.

To summarize, an ideal intravesical drug delivery system should possess the following properties: a permeation enhancing size of 200-400nm, stability in pH values of 4.5-8, high intravesical retention assisted by mucoadhesive materials, high drug loading and controlled release, and most importantly the biocompatibility of employed materials as outlined in Figure 2.2. However, a substantial number of published research works build on biased emphasis on the outlined properties, but rarely on an all-encompassing system, as will be discussed in Section 2.3.1 and 2.3.2.

Figure 2.2 Defining properties for intravesical drug delivery systems for enhanced drug permeability and prolonged drug residence. (Mice model illustration adapted with permission from Mugabe et al.⁴⁴. Copyright (2011) American Chemical Society.)

To date, a few intravesical drug delivery systems are in the progress of clinical trials. The most successful development will be albumin-bound nanoparticle for paclitaxel encapsulation that has currently undergone phase II trial for superficial bladder cancer treatment after BCG treatment failure, showing minimal toxicity and low progression rate of the disease.³⁸ The nanoparticle was already previously approved for treatment of metastatic breast cancer.⁵⁵ TC-3 sterile hydrogel developed by UroGen Pharma's (previously known as TheraCoat Ltd.) is being evaluated as MMC drug delivery system for cases of recurrent NMIBC patients.⁵⁶ GemRIS device by Taris Biomedical, utilized a self-coiling, semipermeable silicon tube that pumps gemcitabine in a controlled manner over a 2 weeks period. The physical device is generally well-tolerated in the phase I safety assessment.⁵⁷ These precedent drug delivery systems are established based on maximizing the intravesical drug response, such as increasing drug solubility and hence availability for cancer elimination based on maximum potency, cargo protection, and retention beyond

urine voiding for prolonged therapeutic activity.

2.3.1 Enhancing bladder permeability to drugs

In enhancing the permeability of the bladder surface, materials used for drug delivery systems play an important role. Researchers have utilized materials that are biocompatible and capable of increasing permeability across the urothelium layer. Chitosan has been known to increase the paracellular route by disrupting intracellular tight junctions and has been widely utilised in drugs encapsulation due to its biocompatibility and biodegradability to normal body constituents.⁵⁸ Kos et al. reported a time and concentration dependent increase in pig urinary bladder permeability resulted from urothelial cells desquamation. This finding is supported by Veranič et al. who showed that the bladder epithelium regenerated within an hour by differentiation of new superficial layers after chitosan-induced desquamation.⁵⁹

The presence of permeation enhancer within the drug delivery system potentiates drug accumulation at urothelium. DMSO has been known to improve the absorption of drugs.⁶⁰⁻⁶¹ Chen et al. proved that addition of DMSO into a standard formulation of paclitaxel in Cremophor and ethanol results in disruption of drug entrapment within Cremophor micelles as well as enhanced drug absorption into the bladder.⁶² Nanoemulsion developed from soybean and Brij 98 as emulsifier incorporated α -Terpineol as permeation enhancer. The resulting emulsion performed with 2.4 ~ 3.3-fold enhancement of bladder permeability and sustained release of encapsulated cisplatin showing higher cytotoxicity activity than free drug.⁶³

Incorporation of cationic components into a drug delivery system enables electrostatic interaction with the anionic GAG layer, promoting permeability of the urothelium. Amine-terminated polyglycerols were synthesized and used to load the hydrophobic drug docetaxel, proving a higher accumulation in bladder tissue as compared to Taxotere formulation or non-amidated polyglycerol nanoparticle.⁶⁴⁻⁶⁵ It is inferred that the enhanced docetaxel uptake into pig's bladder was due to the exfoliation of bladder wall by the

cationic polyglycerols which caused the temporary elimination of urothelial permeability barrier. The amine group on the hydrophobically derivatized hyperbranched polyglycerols also rendered the nanoparticulate mucoadhesive, which further increased the drug uptake in mouse bladder tissues.⁶⁶ Incorporation of cationic 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) onto methoxypoly (ethylene glycol) (MPEG-PLA) nanoparticles by Jin et al. exhibited a sustained release profile of loaded doxorubicin with a higher cellular uptake and tissue absorption than pure MPEG-PLA nanoparticles.⁶⁷ Further, the permeation-enhancing polysaccharide chitosan acts as a cationic molecule with the protonation of its amine groups, enabling electrostatic interactions with the negatively charged sialic acid moieties on the mucosa mucin layers. Chitosan coated poly- ϵ -caprolactone (CS-PCL) nanoparticles transporting MMC showed the longest survival rate among treatment groups due to localisation and accumulation in the bladder tissue as assisted by the cationic coated surface. The treatment groups included instillation of aqueous MMC solution, pure CS-PCL nanoparticles, chitosan-MMC formulation and chitosan coated CS-PCL-MMC nanoparticles. Within the same study, it was also observed that the survivability of bladder tumour bearing rats treated with chitosan nanoparticles surpasses that of CS-PCL-MMC nanoparticles, further validating the inherent antitumor property of chitosan.⁶⁸

Increasing the drug concentration can be a direct method to enhance intravesical drug permeability since the relationship between therapeutic response and drug concentration is proportional.^{15, 69} In the context of a drug delivery system, a higher drug loading capacity and subsequent controlled release will benefit the overall functionality immensely. Polypeptide nanogel of poly(lysine)-poly(l-phenylalanine-co-l-cystine), (PLL-P(LP-co-LC) reported a drug loading capacity as high as 30.6wt% with 88.2wt% efficiency of 10-Hydroxycamptothecin (HCPT).⁷⁰ The positively charged PLL enhanced attraction to bladder mucosa, and subsequent mucus penetration result in long periods of consistently high drug concentration within the bladder. Meanwhile, the characteristics of the nanogel facilitated T24 human bladder cells cytotoxicity and tumor growth inhibition that is 3.4-folds superior to free HCPT. The reported accumulation in other organs *in vivo* in rat model were at insignificant levels. In a separate study, paclitaxel was loaded in PLZ4 micelle

formulation at a 2-3fold higher dosage than the maximum tolerated dose in clinical usage. No heightened cytotoxicity was detected and cellular internalization was enhanced due to targeting potential of PLZ4.⁷¹

Hydrophobic drug has higher permeability due to affinity for cell membrane, however insolubility in water necessitates the use of solvents that causes bladder intolerance. Development of nanoparticle to mitigate insolubility of hydrophobic drugs can therefore improve drug permeability in bladder. Encapsulation of the hydrophobic deguelin in cation DOTAP and monomethoxy poly(ethylene glycol)- poly(ϵ -caprolactone) (MPEG-PCL) hybrid nanoparticles (DMP) that was further incorporated into Pluronic F127 thermosensitive hydrogel significantly increased the drug concentration as well as extended the intravesical residence time within the bladder.⁷² The presence of DOTAP conferred the system with stronger cytotoxicity potential due to cation-assisted cellular uptake.

2.3.2 Prolonging drugs residence time within bladder

Encapsulated drug administered into the bladder cavity delays the time progressive dissolution of drugs in urine, which allows a controlled drug release and long residence duration within the bladder cavity at the same time reducing symptoms of bladder irritation from instantaneous high drug dose contact. Exploiting the self-assembling nature of micelles in aqueous solutions to encapsulate drugs within the hydrophobic core, Chen et al. has reported a doxorubicin nanocarrier system utilizing triblock copolymers of poly(ϵ -caprolactone)-block-poly(glycidyl methacrylate)-block-poly(poly(ethylene glycol)methyl ether methacrylate) (PCL-b-PGMA-b-P(PEGMA)) with a fluorescent group. The PCL-b-PGMA-b-P(PEGMA) loaded doxorubicin has a 70% loading efficiency with slow release over a period of three days in artificial urine, showing high antitumor activity against UMUC3 cells.⁷³ The ability of reverse thermosensitive hydrogels to form gel at body temperature while maintaining a liquid state at room temperature has the potential to increase the dwell time of encapsulated drugs introduced intravesically by remaining within the bladder cavity even during urine voiding. This will decrease the administration

frequency of drugs because the dose required for effective antitumor capacity has been reduced. The VesiGel™ encapsulates high doses of MMC within a hydrogel matrix, effectively increasing drug dwell time to 6-8 hours while promoting adhesion to bladder epithelium. The hydrogel dissolves completely at the end of drug release and is removed together with urine voiding. Preclinical results showed a higher level of MMC detected within bladder tissues as compared to same dosage of aqueous MMC instillation, proving competency as drug carrier.⁷⁴ Gelatin hydrogel developed for intravesical delivery of epirubicin presented similar behaviours with effective penetration of epirubicin into the bladder basal cells whereas aqueous epirubicin instillation was only capable of reaching the umbrella cells level. This is associated with the formation of dense hydrogel coating at the urothelium surface which facilitated intensified drug absorption across the urothelial layers.⁷⁵

Mucoadhesive nanocarriers adhere to the bladder mucosa by interaction with the mucin surface, forming an almost immediate localised attachment to targeted site upon instillation. The presence of carboxyl, hydroxyl, amino, sulfate groups in charged polymers with high molecular weight and flexible chains typically form mucoadhesion with mucosal surfaces through electrostatic attraction, hydrogen bonding, hydrophobic effects and chain entanglement.⁷⁶⁻⁷⁷ Adhesion of drug carriers to the bladder wall provides a high drug concentration gradient in the immediate vicinity of the urothelium and prolongs the dwell time.⁷⁸ This assures a sustained and continuous delivery of drugs without the risk of washout with urine voiding, effectively decreasing the instillation dose and frequency while eliminating the need for repeated catheterization. Mucoadhesive materials are usually hydrophilic polymers with hydroxyl, carboxyl and amine groups such as chitosan, polycarbophil, polyacrylates, hydroxypropylcellulose, sodium alginate, thiomers and others. Thiolated polymers are capable of forming strong mucoadhesion by covalent disulfide bonding with biological membranes, in addition to having permeation-enhancing properties.⁷⁹ Zhang et al. developed a novel mesoporous silica nanoparticle system with thiol group functionalisation which exhibited significantly higher mucoadhesivity compared to parallelly-studied hydroxyl- and amino-functionalised nanoparticles.⁸⁰ Lu et al.⁸¹ developed a chitosan-polymethacrylic acid nanocapsule via electrostatic interaction

for the co-delivery of peptide-modified cisplatin and doxorubicin. Evaluation of nanocapsule mucoadhesive property *in-vitro* and *ex-vivo* showed that chitosan in the outer layer of nanocapsule contributed to increased adherence to bladder wall and sustained release profile. It is also found that chitosan facilitates the escape of nanocapsule from endosomes allowing entrance to cytosol and delivery of cargo drugs to the cell nucleus. A separate study by Ay Şenyiğit et al. revealed an improved intravesical residence time when gemcitabine HCl-loaded chitosan-thioglycolic acid nanoparticles were dispersed in a chitosan gel reporting 33.4% release within 4 hours which is sustained up to 24 hours.⁸² Amide-functionalized polyacrylamide developed as a nanogel delivery system for docetaxel exhibited a sustained release for over 9 days with high mucoadhesivity and cancer killing capability equivalent to free docetaxel formulation.⁸³

In vivo evaluation of intravesical administration of PNIPAM hydrogel exhibited sevenfold enhanced drug concentration at the bladder wall of Female Sprague-Dawley rats. After grafting with hyaluronic acid (HA) and gelation to form thermosensitive copolymer hydrogel (HPNG), the bladder accumulation of drugs was two-fold from cisplatin in double distilled water.⁸⁴ Although sustained release was maintained after an initial burst release, the release profile was reportedly affected by urine dilution. GuhaSarkar and co-researchers⁸⁵ reported a urine-triggered liposome-in-gel composite system capable of delivering both hydrophobic and hydrophilic drugs. The biocompatible fluidizing unsaturated soya phosphatidylcholine liposomes drug encapsulating system were incorporated into a gellan hydrogel capable of enhancing adhesion to the bladder mucosa mucin layer. Gelation of gellan upon coming into contact with ions present in urine allows adherence of liposomal carrier at the bladder urothelium for at least 24 hours, with fluorescence dye penetration up to the lamina propria layer and a 7 days retention of drug concentration within the urothelium.⁸⁵

2.3.3 Alternative approaches

Assisted drug delivery via physical means has also emerged in order increase therapeutic efficacy for the treatment of bladder cancer. Externally applied magnetic field, heat-

induced hyperthermia as well as iontophoresis have been investigated with favourable outcomes. Use of magnetic targeted carriers (MTC) has been investigated for site-specific delivery of intravesical chemotherapeutic drugs based on the concept of attraction between incorporated superparamagnetic or ferromagnetic particles to an externally applied magnetic field. This allows highly specific localisation at diseased tissues facilitating higher drug absorption and magnetic field dependent dwell time when applied in intravesical chemotherapy. The suitability of MTC for the intravesical administration of doxorubicin has been demonstrated in swine bladder with a reportedly higher drug penetration into the epithelium layer and accumulation of drug-loaded MTC at targeted site. In the study, doxorubicin was adsorbed on the activated carbon component of the MTC while metallic iron of the composite particle reacts to the applied 5kG magnetic field for a 30-min period with a projected distance of 5 to 15cm from skin surface. Microscopic evaluation of bladder tissues after instillation showed minor cell sloughing which is correlated with the size and number of MTCs.⁸⁶ Huang's group encapsulated superparamagnetic iron oxide nanoparticles (SPION) into polymeric amphiphilic poly(ϵ -caprolactone)-b-poly(propargyl methacrylate-click-mercaptosuccinic acid-co-poly(ethylene glycol) methyl ether methacrylate) (PCL-b-P(PMA-click-MSA-co-PEGMA)) nanoparticles for intravesical delivery of cisplatin via conjugation on the pendant dicarboxylic groups of the hydrophilic shell. In-vitro cytotoxicity study of superparamagnetic particles showed no significant toxicity towards UMUC3 bladder cancer cells with above 90% cell viability. Further, as SPIONs have the unique property of generating heat under the influence of an external alternating magnetic field, these nanoparticle have the potential to be used for chemohyperthermia application.⁸⁷

Chemohyperthermia or Thermo-chemotherapy refers to the application of radio-frequency energy for introduction of heat into intravesical drug within the bladder. Hyperthermia has been exploited in oncology for cancer-killing as it interferes with cell functions at the molecular level by inhibiting DNA replication, RNA and protein synthesis. Local hyperthermia introduced over a temperature range of 40-43°C with intravesical chemotherapy can also accelerate the action of the drug while simultaneously improving drug penetration.⁸⁸⁻⁸⁹ On the other hand, electromotive drug administration (EMDA) or

iontophoresis based on the principle of electro-osmosis and electrophoration utilizes low electric current to boost the movement of drug molecules across the urothelium, effectively increasing the transport rate and subsequently antitumor capability.⁹⁰ A comparison study was done on 80 patients suffering from recurrent low stage and low grade superficial bladder cancer where patients are separately administered MMC with microwave-induced hyperthermia, MMC with EMDA, and solely MMC formulation respectively. The complete response rate on marker lesion was higher for MMC with hyperthermia, seconded by MMC with EMDA therapy. The study concluded the feasibility and safety of assisted chemotherapy with hyperthermia and EMDA, albeit with a slightly higher local toxicity perceived for the hyperthermia method.⁹¹ Nevertheless, these two methods are currently still not widely used in NMIBC treatment as neither are approved for use in the United States while both are considered as experimental according to the European Association of Urology Guideline.⁹² As such, further investigation is required to guarantee the safety and efficacy of these methods.

2.3.4 Challenges and outlook for intravesical drug delivery

Drug delivery systems in the micro- or nano- order has been demonstrated to potentially improve chemotherapy treatment of NMIBC. The development of intravesical drug delivery systems are abundant and many more are not mentioned in this summary. The approach implemented are similar, aiming for increased drug permeation or prolong drug residence duration, or both. Local administration via the intravesical route ensured reduction of systemic absorption to a minimum, effectively decreasing adverse effects stemming from reaction to chemotherapeutic dose. Protection of drug from degradation and loss of stability from bladder environment essentially maximize the therapeutic efficacy, allowing reduction of drug dose to achieve equivalent potency.

Mucoadhesive drug delivery system appear to be highly robust for the future of intravesical drug delivery, ascribed to the potential in adhering to the urothelium upon instillation, prolonging retention of localized drug dosage, improving uptake of payload and final elimination without urine obstruction. The enhanced bioavailability and pharmacological

potentials will ultimately reduce administration frequency, simplifying administration procedures and increasing patient compliance. Accounting for the high toxicity of chemotherapeutic drugs with non-specific killing of both healthy and cancer cells, the use of targeting ligands with direct zeroing on disease cells are advantageous in increasing cancer-killing efficiency.

Nevertheless, a few limitations remain to be addressed prior to successful clinical translation. In order to achieve increased urothelial drug permeation, surfactants are still being incorporated into drug delivery system. However, the use of surfactant has been associated with bladder irritation and damage to the urothelium, and the search for milder permeation enhancers are pertinent. Figure 2.3 outlines the limitations existing in the different types of drug delivery systems presently researched for intravesical treatment of bladder cancer. Thermosensitive hydrogels have been extensively studied as effective drug delivery system for bladder cancer due to the ability to gel and adhere to the bladder to increase drug residence time. The drug delivery system however, remained elusive of human trial due to the technicality of achieving uniform thin layer within bladder in addition to maintaining mechanical strength against urine flow and bladder stretching. Furthermore, hydrogels are highly prone to degradation and risk of urinary obstruction exists due to the high viscosity. The drug release profile on the other hand, tends to have an initial burst release and easily affected by urine dilution. Micelles and liposomes, though reported great potential, are restricted by complex fabrication process and low loading capacity. Micro- and nanoparticles including mesoporous particles generally perform well in enhancing drug delivery efficacy. The smaller dimension of nanoparticles especially, facilitates cellular uptake but the feature comes at the price of particle stability, being especially prone to aggregation which limits drug loading and causes burst release. There is also a concern of toxicity due to systemic accumulation and conjugation of biomaterials as well as targeting moieties is relied heavily to mitigate the issue. Finally, successful synthesis of this group of drug delivery systems necessitate small scale execution, which eliminates the possibility of mass production therefore increasing cost.

Figure 2.3 Drug delivery systems developed for intravesical treatment of bladder cancer and the limitations.

The benefits of drug delivery system assisted intravesical chemotherapy for bladder cancer are indisputable. However, realization for lab-to-bedside translation entails careful consideration of form stability, definite deterrent from systemic absorption and toxicity to healthy cells, optimal drug loading and sustained releases, as well as ease of mass production.

2.4 Graphene Oxide and Functionalized Graphene Oxide: The Properties

Graphene Oxide (GO) nanosheets have in recent years emerged as a novel carbon-based material owing to the oxygen-containing functional moieties on basal graphene plane that grant colloidal stability and capability for molecular conjugation. GO represents an excellent platform for various applications due to the facile fabrication in bulk quantity, susceptibility to chemical modification and reduction reactions as well as high solubility in common solvents, especially water. Its unique two-dimensional planar structure enables reaction at both sides of the plane, large surface area, mechanical and chemical stability, and most importantly good biocompatibility.⁹³ The functional groups on graphene oxide consisting of hydroxyl, epoxide and carboxyl groups facilitate interactions with polymers

and drugs via covalent bonding, π - π stacking, electrostatic interaction, and hydrogen bonding. The carboxyl groups located at the periphery afford GO a pH-dependent negative surface charge as well as colloidal stability.⁹⁴ Due to these polar groups, GO is dispersible in water and organic solvents, such as ethylene glycol, DMF, NMP and THF.⁹⁵

Graphene oxide consists of monolayer or minimal layers of graphite oxide formed by exfoliation process. The synthesis of graphite oxide involves the oxidation of graphite flakes using strong oxidizing agents and concentrated acids, as detailed by Brodie⁹⁶, Staudenmaier⁹⁷, and Hummers⁹⁸. Brodie and Staudenmaier used a combination of concentrated sulfuric acid and nitric acid with potassium chlorate to achieve graphite oxidation. The Hummers method, which is to date the most widely used synthesis method, involves treatment of graphite with potassium permanganate (KMnO_4) and sulfuric acid (H_2SO_4). Subsequent methods reported are mostly refinement or modified versions of the Hummers method.⁹⁹⁻¹⁰⁰ The resulting graphite oxide is subsequently exfoliated either mechanically or via sonication with stirring in water or organic stirring. The sonication process facilitates formation of GO monolayers in the size range of several hundred nanometers, albeit resulting in a greater size variance.

GO behaves as an amphiphilic material due to its intrinsically unique structure. The basal plane of GO consists of free surface π electrons that are hydrophobic in nature. At the same time, GO structure has a range of characteristic functional groups that contribute to its hydrophilic nature. However, the mechanical, electrical, and optical properties that are the characteristics of its parent compound graphene have been significantly subdued. The optical transmittance, charge carrier concentration and room temperature mobility are negligible in GO due to disruption from functional groups and defects.¹⁰¹ Young's modulus is reduced by approximately 5-folds and fracture strength is in order of 120MPa ¹⁰² as compared to the high value of 130GPa ¹⁰³ for graphene. Similarly, the thermal conductivity and electrical conductivity are also significantly less at 2000W/mK ¹⁰⁴ and 10^{-1}S/cm ¹⁰⁵ respectively. This indicates that GO is almost insulating in nature, but the mechanical and electronic properties can be tuned via control of the oxidation process. GO has a water contact angle of 30.7° ¹⁰⁶ and reportedly forms stable dispersion in water at concentrations

ranging from 1 mg mL^{-1} up to 4 mg mL^{-1} .¹⁰⁷ Nevertheless, graphene oxide aggregates in salt solutions and aqueous medium rich in protein such as cell medium and serum.¹⁰⁸ To overcome the inhibition to functionality and stability within a physiological environment due to charge screening effect, surface modification of graphene oxide by functionalization has been found to be an effective approach.¹⁰⁹

Chemical functionalization of GO refers to the addition of other functional groups via covalent or non-covalent interactions with existing functional moieties on the GO sheets. Essentially, functionalized graphene oxide introduces new desirable properties specific to applications, aside from enhancing the cellular toxicity.¹¹⁰⁻¹¹¹ PEG functionalized hydroxylated graphene was non-toxic for up to $250\mu\text{g/ml}$ as compared to the bare counterpart that showed dose-dependent toxicity at $50\mu\text{g/ml}$.¹¹² Covalent functionalization is possible with polymers such as polyethylene glycol, poly(vinyl alcohol) (PVA), polyethylenimine (PEI), amphiphilic copolymers, folic acids, chitosan, dendrimers, etc. Meanwhile, non-covalent functionalization occurs via π - π stacking, Van der Waals forces, electrostatic interactions, and hydrogen bonds.

The carboxylic acid groups at the periphery of GO sheets form covalent bonds with nucleophilic species such as amines and hydroxyls via amidation or esterification process following activation via activating agents. The common carboxyl activating agents used are 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)¹¹³⁻¹¹⁴, thionyl chloride (SOCl_2)¹¹⁵⁻¹¹⁶, and 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)¹¹⁷⁻¹¹⁸. Introduction of amine groups has afforded extended application of GO in various fields and substituted long, aliphatic amine groups are capable of increasing dispersibility of modified GO in organic solvents.¹¹⁹ Similarly, the amine-functionalized carboxyl group also enables grafting of polymers via an atomic transfer radical polymerization (ATRP) process using an initiator. In a grafting-from process, the initiator binds to the terminal group and hydroxyl groups on GO basal plane, allowing subsequent growth of polymers from the surface.¹²⁰ Conversely, polymer chains can be attached on carbodiimide-activated carboxyl groups via a graft-on approach with amidation or esterification process similar to the above-mentioned reactions with amines and

hydroxyls. Addition of isocyanate derivatives has also been shown to form functionalized GO with good dispersibility in polar aprotic organic solvents resulting from amide and carbamate ester groups produced.

The epoxy groups on the basal planes can undergo ring opening reactions such as amine nucleophilic attack on the α -carbon. Superhydrophobic GO film was successfully synthesized via nucleophilic substitution reaction with octadecylamine.¹²¹ Thiol-functionalized GO (GO-SH) by ring-opening mechanism was induced with potassium thioacetate as a source of sulfur nucleophile. The resulting thiol-functionalized GO exhibited high affinity for deposition of gold nanoparticles and facilitates further functionalization with butyl bromide generating GO-SBu with enhanced thermal stability.¹²²

The most common type of non-covalent functionalization for graphene oxide is via π - π stacking. Due to the aromatic nature of the basal plane, attraction of compounds with aromatic rings is possible. This interaction has been manipulated for the attachment of various aromatic materials, finding applications especially in the field of biomedicine for drug delivery and biomolecule detections. The interaction via π - π stacking could reach a capacity as high as 200% by mass ratio.⁹³

Attributed to the ability of graphene oxide and its functionalized forms in attracting a plethora of biomolecules, from drugs, DNA, genes, proteins, antibodies, and targeting molecules, great research focus has been placed in exploring the material for drug delivery applications. The allure of targeted drug delivery, synergistic drug loading of multi-drug systems or co-loading with therapeutic genes have driven the direction of studies for cancer therapies in recent publications. The following three sections (Section 2.5-2.7) discuss the pertinent findings of graphene oxide and functionalized graphene oxide as cancer drug delivery system with focus placed on chemotherapeutic drugs specific to bladder cancer treatments as cargo. The key ideas theorized for the great drug loading capacity and general enhancement of cancer-killing capability are outlined as well.

2.5 Graphene Oxide in Drug Delivery

Spontaneity of graphene oxide-based drug delivery system as compared to the graphene-based counterpart for membrane cell penetration has been demonstrated by Hashemzadeh et al. in a recent publication.¹²³ Graphene oxide manipulation into a single monolayer sheet constitutes a large surface area with every atom exposed, enabling relatively higher drug loading capacity. Therefore, for the purpose of drug delivery, the number of graphitic layers play an important role in available surface area for bio-interactions, especially cell penetration and uptake. The particle dimension will also determine the biological fate, specifically the successful transport, degradation, and clearance, as well as the overall toxicity.¹²⁴ Russier and co-workers demonstrated the impact of graphene oxide size in cell viability, reactive oxygen species (ROS) generation, and metabolism rate.¹²⁵ Graphene oxide is also prone to formation of protein corona which predominantly determine the route of cell internalization by size dependency.¹²⁶ Large particles are internalized via phagocytosis while clathrin-mediated endocytosis is employed for smaller particles.¹²⁷ A smaller dimension will also diminish the accumulation in liver and spleen significantly.¹²⁸ As such, monolayer graphene oxide of smaller size is related to greater biocompatibility. Notwithstanding the surface chemistry to be contributed by functionalized moiety will also play an important role.

As a drug carrier, targeted loading, delivery, and release of therapeutic agent at the site of action is of essence. Cargo release from graphene oxide-based carriers are generally instigated by change in physicochemical properties of the environment.¹²⁹ Chemotherapeutic drugs are usually loaded via non-covalent bonding with the graphene oxide plane while the more hydrophilic edges provide a foundation for functionalization and act to maintain colloidal stability by contributing a negative surface charge.¹³⁰ Drug loading via π - π stacking interactions facilitates slower and sustained release as compared to conventional drug release by matrix diffusion, as the π bonds restrict the tendency for quick release in a medium.¹³¹⁻¹³² For some drugs susceptible to hydrogen bonding, the additional forces at work promote pH-triggered drug releases from ionization of interacting groups. For instances, the interaction of DOX to GO is supported by both π - π interactions

and hydrogen bonds between amino, hydroxyl and carboxyl moieties. Both DOX loading and release exhibited pH dependency, with greatest loading up to 2.35mg/mg in neutral condition and maximum release at acidic pH.¹³³ This is a desirable attribute as the tumor environment are reportedly acidic. Additionally, drug loading via physisorption prevents chemical modification of molecular structure, allowing retainment of biological activity, as demonstrated by DFT calculations.¹³⁴⁻¹³⁵

Functionalized graphene oxide first came into the limelight as a potential drug delivery system with the successful functionalization of graphene oxide with branched polyethylene glycol (PEG). Noncovalent π - π stacking loading of hydrophobic camptothecin (CPT) showed a high therapeutic efficacy as compared to existing CPT-derived FDA-approved drug.¹⁰⁸ Since then, there have been intensive research works focusing on application of graphene oxide as a drug delivery vehicle via functionalization. Functionalization of graphene oxide with biocompatible agents has been linked to diminished drug toxicity towards normal healthy cells¹³⁶ and enhanced dissolution of insoluble anti-cancer drugs.¹³⁷ Meanwhile, increase in apoptotic potential is a generally reported outcome of drug delivery via functionalized graphene oxide. Poly(lactide)-poly(ethylene glycol) (PLA-PEG) copolymer functionalized graphene oxide and PEG functionalized graphene oxide for the delivery of paclitaxel has both been reported to show relative stability and biocompatibility in physiological solution. Xu's group¹³⁸⁻¹³⁹ demonstrated that hydrophobic drug paclitaxel can be attached via adsorption on the GO basal plane as well as via covalent bonding to the PEG chain. The paclitaxel loaded on PEG chain showed only slight decrease in loading capacity as compared to the case where paclitaxel is attached to GO via non-covalent bonding. The different drug loading methods did not have any effect on the efficacy of paclitaxel in *in vitro* cancer cell killing.¹³⁸⁻¹³⁹ *In vivo* investigation of paclitaxel conjugated PEG-GO using B16 melanoma cancer bearing C57 mice model exhibited comparably better tumor-suppressing efficacy and cellular uptake than commercial form of paclitaxel, Taxol. In addition, no signs of toxicity or tissue damage were detected for 30 days after intravenous administration of paclitaxel loaded PEG-GO.¹⁴⁰ The study also suggested that lower dosage of GO-PEG-PTX might achieve Taxol-equivalent anticancer efficacy. While PEG functionalized graphene oxide exhibited a higher cytotoxicity to cancer cells as

compared to free paclitaxel, the PLA-PEG graphene oxide carrier proved to be less effective.^{139, 141} The reported lower cytotoxicity when compared to freely administered drugs could be explained by a partial incomplete release of drug from nanocarrier. The same conclusion was reached in the cytotoxicity evaluation of doxorubicin hydrochloride (DOX-HCl) loaded on adipic acid dihydrazide functionalized graphene oxide carrier with covalently conjugated sodium alginate.¹⁴²

Efficacy of graphene oxide and functionalized graphene oxide for enhancing drug delivery is also demonstrated for other chemotherapeutic drugs. Polyamidoamide-functionalized graphene oxide loaded with carboplatin showed enhanced drug efficiency of up to 4 times the capability of pure carboplatin when tested on HeLa human cervical carcinoma cell lines and human mesenchymal stem cells (hMSCs). The introduction of PAMAM increased the biocompatibility of graphene oxide by assisting cell penetration and internalization.¹⁴³ Graphene oxide nanoplatelet has been speculated to improve cellular uptake resulting in enhanced toxicity of loaded cisplatin on A549 cells.¹⁴⁴ Modification of ciprofloxacin (CIP) with graphene oxide reported complete mortality in human bladder carcinoma line T24 and highest increase in cytotoxicity to renal adenocarcinoma 786-0 cells.¹⁴⁵ Loading of methotrexate on dopamine conjugated nanographene oxide enhanced internalization into dopamine-receptor positive MCF7 human breast adenocarcinoma cell line surpassing that of free methotrexate and dopamine-receptor negative HEK-293 cells. The release mechanism was postulated to be based on Fickian diffusion at pH7.4, where diffusion of dissolution medium into drug carrier induces deprotonation of methotrexate amine groups while parallelly generating electrostatic repulsion between the drug and carrier.¹⁴⁶

Functionalized graphene oxide nanocarrier is capable of synergistic loading of mixed drugs, targeted delivery by conjugated ligand specific to cell receptors, and loading of therapeutic genes. Co-loading of cisplatin (Pt) and DOX on pegylated graphene oxide (pGO) reported doubling of anti-tumor potential compared to singly loaded pGO and tumor inhibition exceeding the efficacy of free drugs. The authors established that pGO was able to reduce the toxicity of drugs towards normal cells.¹⁴⁷ Targeting ligands such as hyaluronic acid (HA) targeting cancer cells overexpressing CD44 receptors¹⁴⁸, lactobionic acid (LA)

binding to overexpressed asialoglycoprotein receptors (ASGPRs)¹⁴⁹ and transferrin (Tf) acting on transferrin receptors¹⁵⁰ has been proven to be effective against specific cancer cells with overexpression of receiving receptors. HA functionalized nanographene oxide (NGO-HA) rapidly delivered epirubicin to B16F1 mouse melanoma cells by HA receptor mediated endocytosis, with anti-tumor effect surpassing that of free epirubicin. In addition, the NGO-HA had insignificant toxicity up to a concentration of 500 μ g/ml and epirubicin release was pH dependent.¹⁶ Graphene oxide functionalized with sulfonic acid group and conjugated with folic acid was used to co-load doxorubicin and camptothecin with relatively higher cytotoxicity than when the drugs are individually loaded. The graphene oxide nanocarrier exhibited specific cytotoxicity to the breast cancer cell line, Michigan Cancer Foundation-7 (MCF-7), which has folic acid receptors as compared to the devoid A5249 cells.¹⁵¹ Folic acid was also conjugated onto superparamagnetic GO- Fe₃O₄ nanoparticle to achieve dual-targeting functionality for delivery of doxorubicin hydrochloride.¹⁵²

Similarly, improved antitumor efficacy was achieved for targeted docetaxel loaded transferrin/poly(allylamine hydrochloride)(PAH) functionalized graphene oxide nanocarrier as compared to parallelly investigated non-targeting carrier with free drug formulation.¹⁵⁰ The surface modification with PEI endowed a graphene oxide carrier with the ability to load fluorescein isothiocyanate and PEG-linked lactobionic acid targeting cancer cells overexpressing asialoglycoprotein (ASGPR) receptors. With subsequent loading of doxorubicin by π - π stacking, the multi-functionalized nanocarrier showed specificity without compromising the antitumour capability.¹⁵³ Independently, Bcl-2 protein targeting short interfering RNA (siRNA) was successfully loaded onto cationic polyethylenimine-functionalized graphene oxide (PEI-GO) via electrostatic adsorption process. Simultaneous loading of doxorubicin by π - π interactions onto the PEI-GO however, showed contrasting result with no significant synergistic effect on cancer cells killing. It was suggested that sequential separate delivery of siRNA loaded PEI-GO and doxorubicin loaded PEI-GO could lead to a better synergistic anticancer capability.¹⁵⁴ Yang et al. conjugated PEGylated nanographene oxide with antibody C225 for inhibition of epidermal growth factor receptor (EGFR), and concurrently load epirubicin (EPI) for

targeted drug delivery. Photothermal therapy was subsequently achieved via NIR light irradiation. The combined therapy was successful in reducing EGFR expression and removed tumor completely without regrowth in 50 days, achieving 100% survival rate in animal model.¹⁵⁵

The feasibility of π -loading doxorubicin, epirubicin, paclitaxel, docetaxel, methotrexate, and carboplatin has been demonstrated by different research groups as summarized above. These chemotherapeutic drugs are commonly used in the treatment of bladder cancer. Aside from these, the widely used intravesical treatment included Mitomycin C, gemcitabine, and cisplatin. Although interaction via π - π stacking is ineffective, the presence of amine groups allowed covalent bonding with carboxyl groups on graphene oxide surface. Drugs loaded via this method may play a part in reducing cytotoxicity effect and reduce release rate of the drugs akin to the mechanism in carboplatin.¹⁵⁶ The potency of cisplatin were not affected by the covalent attachment to PEGylated graphene oxide, in fact high level of cell apoptosis and necrosis was reported for the treated MCF-7 cells and CAL-27 oral adenosquamous carcinoma cells.¹⁵⁷ The feasibility of covalent functionalization of Mitomycin C on graphene oxide was evidence by reported utilization in biosensing. Mitomycin C covalently linked to carboxyl groups of graphene oxide via its amine groups form the electrode in biosensing the interaction with calf thymus double-stranded (dsDNA) to study the signal enhancement by graphene oxide.¹⁵⁸ Due to the presence of free amine groups on methotrexate, covalent functionalization by amidation is also possible and has been reported for study with MCF7 human breast adenocarcinoma cell line.¹³²

The mechanisms involved in loading of common bladder cancer specific chemotherapeutic drugs on graphene oxide-based drug delivery systems are as summarized in Figure 2.4. Generally, published works on graphene oxide-based nanocarriers report enhanced drug efficacy as compared to the free forms. Increase in drug potential especially in cancer-killing capability potentiates achieving same therapeutic effect at reduced dosage, which ultimately reduces the undesirable side effects caused by drugs administration.

Figure 2.4 Chemotherapeutic drugs commonly used in intravesical administration of bladder cancer and the corresponding mechanism of drug loading on graphene oxide as a nanocarrier.

2.6 Chitosan and Chitosan-Functionalized Graphene Oxide in Drug Delivery

The polycation chitosan has an extensive background in drug delivery, pursued for the plethora of desirable properties such as controlled release of anionic drugs from formed matrix, mucoadhesive and permeation enhancing property, in situ gelling potential, as well as gene transfections efficiency.¹⁵⁹ Due to these properties, development of drug delivery systems are vast, including for oral, ocular, nasal, buccal, parenteral, vagina, and others.

Chitosan is a natural polysaccharide that is derived from chitin obtained from exoskeleton of crustaceans and insects. The polymer is aqueous soluble in acidic pH lower than pH6, therefore, it is weakly basic and easily form salts with inorganic and organic acids via its protonated amine groups.¹⁶⁰ Chitosan is generally graded based on the molecular weight and degree of deacetylation. Highly deacetylated chitosan is highly flexible, enhances cell adhesion and has low inflammatory response¹⁶¹⁻¹⁶³, but is associated with greater toxicity.¹⁶⁴⁻¹⁶⁷ Lower deacetylation degree however, is associated with higher permeability

enhancing capability due to lower positive charge density and steric effect from the acetyl groups.¹⁶⁸ In recent years there has been an increased interest for application of chitosan in pharmaceutical and biomedical field due to its properties of being biocompatible, non-toxic, antimicrobial, hemostatic, anticholesteremic and biodegradable to normal body constituents.¹⁶⁵ Chitosan is highly suitable for biomedical applications with these innate properties in addition to having antitumor activity, low immunogenicity and high absorption capacity.¹⁶⁹⁻¹⁷⁰ These features have led to extensive studies into development of chitosan-based therapeutic systems in the form of nanoparticles especially for drug delivery and controlled drug release. As mentioned in Section 2.3.2 above, the mucoadhesive property of chitosan is greatly coveted for drug delivery to mucus-infused sites. Nevertheless, the strength of mucoadhesion of chitosan is considered weak even with high molecular weight when compared to other reported mucoadhesive polymers such as polycarbophil, carbomer and hyaluronic acid.¹⁷¹

Use of chitosan in cancer therapeutics is not new, acting as capsules, hydrogels, coating and grafting material innovated for delivery of anti-cancer drugs, genes, immunological agents and peptides. Incorporation of chitosan in drug delivery systems generally controls the rate of drug release. For this purpose, chitosan is particularly effective when used for delivery of anionic drugs. Naproxen loaded on a chitosan drug carrier matrix showed slower and sustained drug release.¹⁷² As a hydrogel, chitosan allowed a certain degree of site-specific targeting by undergoing a sol-gel transition with trigger from the change of pH or temperature.¹⁷³ For example, Gupta et al. developed a polyacrylic acid and chitosan hydrogel systems that remain in aqueous state at pH 6 but transitions to a viscous gel phase at physiological pH of 7.4.¹⁷⁴

The role of chitosan in enhancing drug absorptions via permeation-enhancing properties was demonstrated with enhanced insulin transport and intestinal absorption through the paracellular pathway using bioadhesive polysaccharide chitosan nanoparticles.¹⁷⁵ In fact, this property can be enhanced 30-folds upon thiolation of chitosan.^{159, 176} The presence of chitosan induces short period of disruption to the tight junction integrity of Caco-2 human

colorectal adenocarcinoma cells, measured as transepithelial electrical resistance (TEER) which was recoverable within a few hours.¹⁷⁷⁻¹⁷⁹

The highly desirable properties of chitosan are exploited to endow similar properties in chitosan functionalized graphene oxide. Whether by covalent functionalization^{113, 180-181}, non-covalent coating¹⁸²⁻¹⁸⁵, layer-by-layer synthesis¹⁸⁶, or as nanocomposite¹⁸⁷⁻¹⁸⁹, chitosan incorporation into a graphene oxide system has been proven to improve its biocompatibility, dispersion stability, pH-sensitive drug release as well as cancer-killing capability. Furthermore, chitosan functionalization has been found to diminish apoptosis death induced by graphene oxide.¹⁹⁰ When tested against L929 mouse fibroblast cells, no significant cytotoxicity was detected for graphene/chitosan composite film, with only a delay in cell attachment at the highest graphene concentration of 0.6wt%. Potential hemolytic activity by graphene oxide was substantially dwindled with electrostatic adsorption of chitosan.¹⁹¹

In 2010, Rana et al.¹⁸¹ and Bao et al.¹¹³ separately synthesized chitosan-functionalized graphene oxide and established the potential of chitosan-functionalized graphene oxides as a novel material for biological and medical applications. Incorporation of chitosan into graphene oxide enhanced the stability of GO in aqueous medium due to the hydrophilic and cationic characteristic of chitosan. The functionalization of graphene oxide with a chitosan derivative, carboxymethyl chitosan, improved water dispersibility and stability of graphene oxide in physiological medium after modification.^{148-149, 192} The positive potential of chitosan enables effortless electrostatic interaction with anion systems, especially the negatively-charged cell membranes.¹⁹³ Chitosan incorporation into graphene oxide systems has been proven to diminish the cytotoxicity effect and in certain cases, enhances the cell proliferation.¹⁹⁰ CPT loaded chitosan functionalized graphene oxide reported greater stability and maximum inhibition of MCF-7 cell growth.¹⁹⁴ When tested alongside graphene oxide and reduced graphene oxide, chitosan coated graphene oxide induces the least degree of damage to erythrocyte membrane due to shielding of electrostatic interaction between graphene oxide and membrane.¹⁹¹ Notably for drug release, chitosan and graphene oxide complement each other, in that graphene oxide alleviates the tendency

to burst release of chitosan based nanocarriers, while chitosan facilitates controlled release in graphene oxide loaded drugs.¹⁹⁵⁻¹⁹⁷ The pH-responsiveness of chitosan due to its protonation mechanism facilitates better cumulative drug release. Model drug folic acid loaded on chitosan- hydroxylethyl cellulose functionalized graphene oxide nanohybrid (rGO-CS-HEC) revealed 27% more release than when chitosan is absent within the nanohybrid complex, suggesting that chitosan enhanced the release efficiency of loaded drug.¹⁹⁸ In a fascinating manipulation of electrostatic affinity between chitosan and graphene oxide, Zhao and colleagues¹⁹⁹ managed to develop a pH-sensitive charge reversible self-assembly drug delivery system. While in circulation, the system carries a negative charge which inverts upon arrival at the acidic tumor microenvironment. The establishment of these characteristics further enforce the prospect of the chitosan-graphene oxide as a nanocarrier system for drug delivery.

The multi-functionality of chitosan functionalized graphene oxide nanocarrier is validated with reported stable co-loading of chemotherapeutic drug camptothecin and plasmid DNA. The drug loaded nanocarrier showed a higher cytotoxicity than pure CPT in HepG2 and HeLa cell lines with reasonable in vitro transfection efficiency.¹¹³ Likewise, trimethyl chitosan functionalized graphene oxide showed potential for drug and gene delivery with achieved doxorubicin loading of 30.9% and pDNA condensation with 31.1% accumulation release.²⁰⁰ That aside, the capability of chitosan functionalized graphene oxide in protecting immobilized protein from enzymatic reaction²⁰¹ and functioning as magnetic targeted photodynamic therapy was also reported.²⁰²

2.7 Carboxymethylcellulose and Carboxymethylcellulose-Functionalized Graphene Oxide in Drug Delivery

The polysaccharide carboxymethylcellulose (CMC) or its sodium salt, NaCMC is widely used in the food and cosmetic industry as additive, thickener, emulsion stabilizer, viscosity modifier and agent of water-retention.²⁰³ The water soluble derivative of cellulose forms part of the primary structure of plant cell walls, with the solubility controllable with degree of substitution.²⁰⁴ At high level of substitution, the hydrophilicity and viscosity of NaCMC

increases due to a decrease in inter-chain cross-linking.²⁰⁵⁻²⁰⁸ Available in high purity commercially at a low cost, the anionic material has unparalleled properties that is well-suited for drug delivery.

NaCMC is associated with greater stability in an environment subjected to pH and ionic strength variations. The viscosity of the material stayed almost constant when pH is altered from values of 4 to 9. As such, the factor influencing viscosity is mostly the polymer concentration.²⁰⁹ NaCMC has also been reported to be mucoadhesive, although not as extensive as chitosan.²⁰⁹⁻²¹⁰ Theoretically, the COO⁻ groups of CMC contribute to mucoadhesion through hydrogen bonds and van der Waals forces with the mucin oligosaccharide side chains. Gurny and colleagues demonstrated a higher adhesion strength for NaCMC with linear conformation due to a longer penetration length for interpenetrative entanglement with mucus.²¹¹ Meanwhile, mucoadhesion study of carboxymethylcellulose with ex-vivo pig tongue displayed prolonged mucoadhesive retention.⁵¹

In pharmaceutical area, CMC is added to drugs for extension of expiration period. A few FDA approved application of CMC for parenteral use in drug products included Sandostatin, Sandalog, and Vivitrol.²¹²⁻²¹³ CMC is reportedly bio-eliminable in these products. The material is also claimed to be resorbable when introduced into living organism, more easily so at low degree of polymerization and high carboxylic substitution.²¹⁴ Inspired by the prospects of CMC in biomedical application, there were a few reported studies that manipulated the properties of CMC to complement drug delivery systems.

Cellulose/carboxymethyl cellulose hydrogel and chitosan/carboxymethyl cellulose microcapsule showed great potential as protein drug delivery system while double cross-linked carboxymethylchitosan/carboxymethylcellulose hydrogel showed biocompatibility and non-toxicity in 3T3 cells.²¹⁵ Combining carboxymethylcellulose and polyethylene glycol as a copolymer coating for nanostructured lipid carriers result in stabilization and significant suppression of burst release of docetaxel at pH7.4. The coating also enhanced the in vivo anti-tumor effect in A549-bearing nude mice, due to longer circulation time and

subsequently greater accumulation in tumors.²¹⁶ Hydroxycamptothecin (HCPT) encapsulated within carboxymethylcellulose conjugated with PEG, folate and betulinic acid (BA) in an approach to co-load two anticancer drugs successfully enhanced tumor inhibition exceeding that of pristine drugs and reduced therapeutic adverse effects. The amphiphilic polymeric nanoparticle has extended circulation half-time as a contribution from the stable backbone of carboxymethylcellulose.²¹⁷ Similarly, carboxymethylcellulose nanoparticle loaded with doxorubicin and endostar reported longer systemic circulation, lower toxicity and enhanced antitumor properties.²¹⁸ Acetylated carboxymethylcellulose was used to covalently conjugate docetaxel to improve hydrophobic drug coupling and solvent stability. The resultant PEG-conjugated nanoparticle, named Cellax in the publication, exhibited cytotoxic effects greater than free drug in vitro.²¹² Other examples of CMC-conjugated drugs also showed tremendous tumor accumulation.²¹⁹

Combining carboxymethylcellulose with graphene oxide for drug delivery system has not been extensively researched. The few reported applications involving the two materials typically employ graphene oxide incorporation to increase the mechanical strength of carboxymethylcellulose hydrogel²²⁰⁻²²¹, while chemical conjugation of polymer to graphene oxide sheets are aimed to enhance overall biocompatibility and pH sensitivity of the drug delivery system^{148, 222}. pH sensitivity of CMC facilitated selective higher release of doxorubicin at pH6.4 from CMC-GO composite hydrogel bead.²²¹ Meanwhile, no obvious toxicity were detected when 100µg/ml carboxymethylcellulose modified graphene oxide (GO-CMC) was introduced to in NIH-3T3 mouse embryonic fibroblast cells and HeLa cells. The authors²²² observed mitigation effect of loaded doxorubicin cytotoxicity towards NIH-3T3 cells but unperturbed efficacy towards HeLa cells.

2.8 Chitosan, Carboxymethylcellulose and Graphene Oxide in Bladder

Chitosan has a reputed presence in the delivery of therapeutic biomolecules for bladder complications due to its intrinsic properties that enhance bladder permeability resulting in controllable and sustained delivery of therapeutic agents. Chitosan is reported to increase permeability across bladder by approximately 2.8times.^{78, 168} A molecular weight of 30-

400kDa and 86% deacetylation degree generated rapid decrease in transepithelial resistance which is related to loosening of tight junctions and increase in permeability.²²³⁻²²⁴ N-sulfonato-N,O-carboxymethylchitosan (SNOCC) encapsulated with 5-aminosalicylic acid (5-ASA) used for treatment of interstitial cystitis in rat bladder effectively reduced inflammation and urinary frequency due to enhanced permeation contributed by chitosan.²²⁵

Investigation of rod-shaped paclitaxel/chitosan nanosuspension in mouse bladder cancer model exhibited good mucosa-adhesion, low toxicity with limited side-effects, and sustained drug release for up to 10 days.²²⁶ Treatment of interstitial cystitis in rat by combination of sulphated chitosan derivative and anti-inflammatory drug results in reduction in inflammation with potency superior to commercial medicine.²²⁵ An antibiotic therapy incorporated with 30-400kDa chitosan completely eliminated UroPathogenic Escherichia coli (UPEC) from infected urinary tract without recurrence or lasting damage to urothelium. In fact, bladder integrity was restored rapidly.²²⁷ Chitosan was found to improve antitumor immune reaction of magnetic carrier used in bladder imparted by the biocompatibility nature of the polymer.⁸⁰ Chitosan functionalization of poly(lactic-co-glycolic acid) (PLGA) nanoparticles enhanced intravesical tumor uptake in mouse by 10-folds compared to unmodified PLGA nanoparticles.²²⁸ At pH6.1, chitosan exhibits high toxicity towards RT112 bladder cancer cells even at low concentration of 100 μ M, causing cell apoptosis by nuclear fragmentation and chromatin condensation.²²⁹ A treatment regime based on interleukin 12 (IL-12) with chitosan glutamate (molecular weight 200-600kDa, 75-90% deacetylated) was able to eliminate 90% of bladder tumours in animal studies, even triggering memory response preventing future recurrence.²³⁰⁻²³²

In contrast, the anionic carboxymethylcellulose (CMC) is commonly used as a solvent or excipient for drug formulations injected into bladder for disease treatments.^{86, 233-235} Use for development of drug delivery system in bladder however, has been rare although there were studies reporting the mucoadhesive properties in bladder.²³⁶⁻²³⁸ The development of silver nanoparticle containing carboxymethylcellulose hydrogel composite exhibited high antibacterial activity when tested against a variety of urinary tract infection pathogens.²³⁹

Oxidized carboxymethylcellulose was used as a crosslinking agent replacement to reduce the cytotoxicity and immune response generated by glutaraldehyde in bladder acellular matrix in addition to slowing down deterioration rate.²⁴⁰ Incidentally, carboxymethylcellulose application has reported enhanced tumour inhibition exceeding that of pristine drugs, extended systemic circulation, and reduced toxicity.^{212, 217-218}

Graphene oxide, on the other hand has not been widely used for intravesical bladder application. The few bladder related research involved bladder cancer imaging, photodynamic therapy, and external study for potential biosensing and tissue scaffoldings. Reduced graphene oxide functionalized with tetraethylene pentaamine (rGO-TEPA) was used for signal amplification and immobilization of antibodies conjugated AuPdPt nanoparticles in the development of electrochemical sensor for bladder cancer biomarker, nuclear matrix protein 22 (NMP22). Detection of other bladder cancer biomarkers such as apolipoprotein AII protein (APOA2)²⁴¹ and telomerase activity²⁴² was also reported. PEG modified nanographene oxides capsulated aggregation-induced emission (AIE) nanoparticles achieved efficient internalization and successful photodynamic-induced cancer cells death in UMUC3 tumor xenograft mice models.²⁴³ Urinary bladder implantation of scaffolds fabricated using graphene oxide/ 6 ARM-PEG-amine/poly (ϵ -caprolactone) film facilitated complete repair of lesion in rat urinary bladder with high adhesion and proliferation of adipose-derived stem cells.²⁴⁴

At the point when this document is being prepared, a study has been published discussing the novelty of graphene oxide and its functionalized derivative in NMIBC treatment.²⁴⁵ The group reported co-administration of covalently loaded doxorubicin on carboxylated graphene oxide, GO-CO-DOX and interfering RNA complexed polyethyleneimine conjugated graphene oxide, GO-PEG-PEI/siRNA to bladder cancer induced rats. The study demonstrated the promising treatment of bladder cancer from a 60% normal diagnosis after the administration of nanoparticles. Cytotoxicity evaluation via human bladder carcinoma 5637 cells (5637 ATCC/HTB-9) exhibited comparable toxicity to free DOX of the same concentration. The report essentially established the potential and relevance of graphene oxide and functionalized forms as part of the bladder cancer

treatment. The same group outlined the potential of graphene oxide scaffolds for stem cells application of urothelial surgeries.¹²⁴

Nonetheless, the actual implementation of graphene oxide as a prospective material in bladder environment is still limited by the drawbacks mentioned in Section 2.4. At the top of the list is the stability of nanocarriers in physiological conditions, which brings attention to the aim of this project. The use of chitosan and carboxymethylcellulose to cover the shortfall of graphene oxide, especially to confer stability in bladder conditions are pertinent considering the aforementioned prior successful intervention of the polymers in associated applications. Furthermore, both polymers are reported to be mucoadhesive, which is a value-adding property for increasing residence time of targeted drugs intravesically. The physiological stability of mucoadhesive nanoparticles intended for intravesical application has not been extensively studied, especially for disease condition. The novelty of functionalized graphene oxide, though reported, did not focus on the nanoparticle stability nor was the direction of study placed on mucoadhesive retention in bladder. As such, relevant studies are vital as the property of nanoparticles substantially path the way for operational functionality in actual application, which should not be the outstanding hurdle at the finalizing stage of execution.

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Chapter 3* Experimental Methodology

There exists a set of properties that an ideal drug delivery system should comply for functional efficacy, which includes suitable size, stability, biocompatibility, high drug loading and good release profile. Selection of graphene oxide that stemmed from a variety of synthesis process with different functional groups density, as well as polysaccharides of appropriate molecular weight and polymeric characteristics determine the physiological behavior. The optimization of nanocarriers synthesis procedures and characterization techniques selection are outlined in the current chapter. Evaluation of desired properties for intravesical environment is also conducted in a series of in vitro and ex-vivo simulated conditions.

3.1 Rationale for selection

Mucoadhesive drug delivery systems are usually synthesized from hydrophilic polymers. Mucoadhesion arise from interaction with mucin glycoproteins via electrostatic or hydrophobic interactions, hydrogen bonding and physical forces.¹⁻² A mucoadhesive polymer should have the characteristics of high molecular weight, long and flexible chains, high viscosity and other properties that are dependent on usage conditions.³ Hydroxyl, carboxyl and amine groups that are commonly found in hydrophilic polymers has been associated with good mucoadhesion properties.⁴⁻⁵ Chitosan and carboxymethylcellulose are selected for functionalization of graphene oxide due to their biocompatibility and the extensively researched credibility in biomedical field and drug delivery. More importantly, both polysaccharides have prior background in intravesical drug delivery, good mucoadhesive property as well as association with enhanced permeability. The pH sensitivity of chitosan and carboxymethylcellulose can ideally expand the pH solubility of the otherwise aggregative graphene oxide.

The suitability of chitosan for drug delivery system is highly dependent on its molecular weight, degree of deacetylation and viscosity. As mentioned in literature review, higher deacetylation degree correlates with larger range of pH stability and lower inflammatory responses. Inversely, lower deacetylation degree is associated with lower toxicity and higher permeability enhancing capability.⁶ Polymers with molecular weight above 100kDa is recognized as having mucoadhesive property via interpenetration and mucin entanglement with mucosa layer.⁷⁻⁸ Too high a molecular weight however, would inhibit bioadhesion due to extensive entanglements of long chains.⁵ Chitosan with higher molecular weight of 150kDa has greater interaction with mucin as compared to the lower molecular weight.⁹ Hence, to achieve a balance between desired properties, chitosan with molecular weight averaging at 120kDa and 75-85% deacetylated was selected for the study. Meanwhile, sodium carboxymethylcellulose with molecular weight of 250kDa was selected for the study as NaCMC of lower molecular weight has been associated with weak mechanical integrity and negligible mucoadhesive property.

Conjugation of chitosan to graphene oxide would be via amidation reaction between carboxyl group from graphene oxide and amine groups of chitosan initiated by carbodiimide. To facilitate similar reaction with carboxymethylcellulose, graphene oxide is first functionalized with hydrazide groups before amidation with carboxyl groups of the polymer. The synthesis process is as outlined in Figure 3.1.

Figure 3.1 Schematic showing preparation of drug-loaded CS-sGO and CMC-sGO.

The capability of graphene oxide and modified graphene oxide as carriers for drugs via π - π stacking has been widely demonstrated as per literature review in Chapter 2.5. Furthermore, majority of drugs used for chemotherapy treatment of bladder cancer can be loaded via this method on graphene oxide. In view of this, a model drug with π -loading capability is selected as representation of drug loading and release capability of current system and taken as proof of concept. The molecular weight of camptothecin is 348.35 g/mol, which lies in the average range of sizes for the general anti-cancer drugs. Furthermore, camptothecin has limited solubility and considering the comparatively greater success of water-soluble drugs introduced intravesically, selecting CPT as a model drug in current study allows visualization of nanocarriers suitability for transporting drugs with limited solubility. Incidentally, insoluble drugs have always been associated with

greater therapeutic efficacy but successful delivery was heavily constrained by the inherent limited solubility.¹⁰

Van der Waals forces are the main source of aggregation for graphene oxide sheets, and the main instigation are pH changes in media.¹¹ The stability in a variety of physiological conditions also plays a governing role in the interaction with biological systems *in vivo*. Depending on the particle size, shape, surface charge, dispersity, aggregative form, sedimentation, hydrophobic segments, adsorbed molecules, the operational functionality may deviate greatly from the idealized design. The effect includes variation in *in vivo* biodistribution, toxicity and clearance mechanism, drug loading, release as well as the degree of targeting potential.¹² Hence, the stability of nanoparticles in pH and physiological media are crucial as a successful drug delivery system. Particle size measurement is utilized as indicative signs of aggregation and instability in dispersing media. It is recognized that DLS using Zetasizer is not the perfect size determinant for graphene family suspensions due to the highly anisotropic nature of the two-dimensional sheets.¹³ Nevertheless, the method can still sufficiently display differences in terms of comparative trend and is adopted for analysis of particle size changes as a function of pH and buffer compositions. The morphology of functionalized graphene oxide is further analysed through TEM, where images offer information on size, shape, surface morphology and aggregative state. TEM is the most straightforward method as dispersion form of nanoparticles can be dropped and dried on copper grids for visualization.

The mucin particle method is used as an *in vitro* indicator of mucoadhesion property. The method defines the existence of mucodhesion mechanism from increase in particle size and zeta potential. Commercial mucin from porcine stomach was used in the study due to the high results reproducibility and minimal experimental batch variations of commercial mucins.¹⁴⁻¹⁵ Although the obtained mucin is originated from the stomach instead of bladder, for the purpose of determining mucoadhesive properties, mechanism elucidation and comparison with existing research data, it is sufficiently effective in providing a preliminary information on the interactions with mucoadhesive materials. The conformational changes from pH triggers and interactions with polysaccharides can be

accurately depicted by partially purified porcine gastric mucin.¹⁶

As a preliminary cytocompatibility test, L929 murine fibroblast cells were selected. Per suggested by ASTM F813-20 and ISO 10993-5, L929 cell culture with new materials or formulations meant for medical application may be used as an *in vitro* cytotoxicity potential evaluator. The isolated pig bladder has been commonly used for analysis of bladder urothelium interactions with external agents. The pig bladder is analogous to human bladder in terms of physiological conditions, responses to external stimuli and capacity.¹⁷⁻¹⁸ Furthermore, barrier functions and cellular ultrastructure of pig bladder were preserved for up to 6 hours after excised, allowing accurate analysis of urothelial responses.¹⁹⁻²⁰ Hence, mucoadhesion of nanoparticles to bladder urothelium and the morphological changes were conducted via *ex-vivo* porcine bladders for better visualization of potential interactions *in vivo*.

3.2 Materials

Graphene oxide were sourced from five different suppliers, with detailed specifications outlined in Chapter 4. All chemicals and reagents were obtained from Sigma-Aldrich unless stated otherwise. For cell culture, L929 murine fibroblasts were from ATTC, with culture medium and supplementary reagents purchased from Gibco BRL (Gaithersburg, MD). Procurement of porcine bladders for *ex-vivo* studies was via local abattoir ((Buroh Lane Abattoir, SG). Reagents used were of analytical grade and used as received. All aqueous solutions were prepared with ultra-pure water (>18 M Ω) from a Milli-Q Plus system (Millipore). To avoid experimental variation originating from undetectable degradation of polymers, the nanoparticles were not stored more than one month, and new batches were synthesized when necessary. pH measurements when taken, are accepted within a deviation of $\pm 2\text{mV}$.

Where used, buffer solutions were prepared based on the protocol stated. DMEM medium was prepared with 10% FBS and 1% penicillin streptomycin. Phosphate buffered saline (PBS) was prepared from tablets to a concentration of 10mM at pH7.4. pH5 phosphate

buffer was adjusted from PBS by addition of phosphoric acid. Artificial Urine (AU) at pH6.2 was formulated based on AU-Siriraj²¹ by mixing salts of urea (200mM), uric acid (1mM), creatinine (4mM), sodium citrate (5mM), sodium chloride (54mM), potassium chloride(30mM), ammonium chloride (15mM), calcium chloride (3mM), magnesium sulfate (2mM), sodium bicarbonate (2mM), sodium oxalate (0.1mM), sodium sulfate (9mM), monosodium phosphate (3.6mM), and disodium phosphate (0.4mM) in DI water. pH4.5 acetate buffer solution was prepared by mixing adequate volumes of acetic acid with sodium acetate trihydrate solution at 0.1M. The concentration was further diluted as required. Acetic acid was diluted by volume from glacial acid solution.

3.3 Graphene Oxide Sonication

For the purpose of biomedical applications, GO of smaller dimensions is desired due to higher aqueous stability attributed to higher densities of ionised carboxyl groups²² and least damage to cellular membrane²³. Reduction of particle size of purchased graphene oxide were achieved via probe sonication of the aqueous dispersions. However, the method introduces new concerns as longer sonication results in increased structural defects and reduced aspect ratios that were linked to cell toxicity.²⁴ Therefore, a balance between minimal sonication duration and greatest particle size reduction is required.

10ml graphene oxide samples at concentration of 0.2mg/ml were first probe sonicated (QSonica Q125, 50% amplitude, 4s on 2s off) using a $\frac{1}{8}$ inches probe tip. The sonication was conducted for two hours and at every 30minutes intervals, particle size and polydispersity (PDI) are measured using dynamic light scattering. From linear fitting of the relational curve between particle size and sonication duration, the time required to reach particle size of 200nm can be estimated. A final graphene oxide is selected based on shortest sonication duration to achieve smallest particle size.

3.4 Synthesis of Chitosan Functionalized Graphene Oxide

Preparation of chitosan-functionalized graphene oxide was adapted from the protocol previously developed by the lab group.²⁵ Briefly, chitosan was conjugated to graphene oxide sheets via amidation process in the presence of EDC, NHS and MES buffer. Graphene oxide dispersion (2mg/ml) were probe sonicated (QSonica Q125, 50% amplitude, 4s on 2s off) in an ice bath to the desired starting size. Chitosan was dissolved in 2.5ml MES buffer (0.1M, pH5 adjusted) and mixed overnight. Next, GO was added to chitosan solution dropwise and sonicated for 1 hour. EDC (42mg) and NHS (60mg) were added into the flask and mixed for 20mins before bath sonicating for 6 hours. The reaction was then left stirring overnight for 16 hours. 0.2 μ m nylon microporous membrane was used to filter out unreacted CS with copious amount of acetic acid (0.1M) and DI water. The residue is then re-dispersed and dialyzed (MWCO=300kDa) against DI water for 3 days. Weights and volumes of chitosan and graphene oxide were modified throughout the project and are listed in the respective chapters.

3.5 Synthesis of Carboxymethylcellulose-Functionalized Graphene Oxide

GO of set size was prepared as mentioned above. Synthesis of carboxymethylcellulose functionalized graphene oxide was modified based on the method in literature.²⁶ EDC (42mg) and NHS (60mg) were first dissolved in 1.25ml DI water and immediately added into stirring GO. After allowing EDC-NHS reaction activation for 15mins, adipic acid dihydrazide (ADH) were added to the reaction mixture. The mixture was stirred overnight with occasional shaking to prevent exclusion of precipitates. Next, the synthesized hydrazide-terminated graphene oxide is filtered through 0.45 μ m nylon microporous membrane and dialyzed (MWCO=300kDa) against DI water for 3 days to remove unreacted materials. The product was then re-dispersed to a concentration of 1.25mg/ml and 2ml was added dropwise into pre-prepared 2.5ml sodium carboxymethylcellulose in MES buffer (0.1M, pH6 adjusted). The mixture was bath sonicated for 1 hour before addition of EDC (42mg) and NHS (60mg), mixing for 20mins, 2 hours bath sonication and stirring overnight. The synthesized carboxymethylcellulose-functionalized graphene oxide was filtered using 0.2 μ m nylon microporous membrane and finally dialyzed (MWCO=300kDa) against DI water for 3 days. The ratio of acid adipic dihydrazide to

sodium carboxymethylcellulose were modified for optimization study and a final synthesis ratio is selected based on desired properties, detailed in Chapter 4.

3.6 Characterization of Nanocarriers

Nanoparticles were freeze-dried prior to analysis via FTIR and TGA. FTIR spectra were obtained from samples prepared in KBr pellets via Nicolet 6700 FT-IR Spectrometer recording 32 scans at 4cm^{-1} resolution in the $500\text{-}4000\text{cm}^{-1}$ region. Obtaining absorbance peaks from FTIR scans, the chemical composition of a material can be determined from the characteristic peaks that represents functional groups present. More importantly, the key identification of amide I absorbance peak in the range of $1650\text{ -}1690\text{ cm}^{-1}$ allows verification of successful covalent conjugation of chitosan and dihydrazide groups to graphene oxide²⁷⁻²⁹. The appearance of distinct spectra correlated to chitosan and carboxymethylcellulose such as the glycosidic linkages and primary alcoholic groups in functionalized graphene oxide samples also signify successful functionalization.

Thermogravimetric analysis (TGA) was conducted with TGA Q500 TA Instruments Thermogravimetric Analyzer at a heating rate of $10^{\circ}\text{C min}^{-1}$ from $30\text{ to }800^{\circ}\text{C}$ in nitrogen atmosphere of 60mL min^{-1} . Identifying the temperature point where either graphene oxide, polysaccharides or functionalized graphene oxide achieves complete decomposition, the grafted polymer percentage can be estimated by simple weight-fraction calculation method. Supposing the fraction of graphene oxide is “x” and polymer fraction is “y” in functionalized graphene oxide, “a”, “b” and “c” as fraction weight loss of graphene oxide, polymer and functionalized graphene oxide at selected temperature, the following equations apply if no other impurities are present:

$$x + y = 1 \quad \text{Eq 3.1}$$

$$ax + b(1 - x) = c \quad \text{Eq 3.2}$$

For Transmission Electron Microscopy (TEM) analysis, 0.05mg/ml nanoparticles dispersion in DI water were first bath sonicated for 15mins to achieve homogenous dispersion. Dispersions were then dripped on 300-mesh carbon-coated copper grids, dried in desiccator and examined with JEOL JEM-2010 operated at 200kV. The samples were observed at suitable magnifications and 3 images were recorded at different regions for each sample.

DLS measurements of particle size, polydispersity, and zeta potential was acquired via Malvern Zetasizer Nano instrument. Three measurements at 25°C were recorded for each sample at 0.2mg/ml and averaged for data analysis.

3.7 Dispersion Stability Study

The dispersion stability of nanoparticles is important for formulations to be introduced *in vivo*. Aggregation, flocculation, foreign material adsorption and subsequent change in surface charge will critically affect the planned transport path and biological responses. For one, the intravesical route involves exposure to urine which consists of a variety of components, including ionic salts, proteins, blood, urothelium and cell fragments, not to mention that urine pH varies widely based on individuals³⁰. Hence, a simple test of dispersion stability in a variety of simulated environment were conducted. Changes in particle size over few days were taken as signs of aggregation and hence instability. Zeta potential values can also give insight to the general stability of the nanoparticle system. Based on universal guidelines, stable colloidal suspensions have a zeta potential value of 30mV and above. Signs of agglomeration or sedimentation visible visually after a 48 hours period can also be associated as colloidal instability.³¹ Understandably, hydrodynamic diameter measured by DLS method is not real representation of the dimensions of non-spherical graphene oxide and its derivatives. Instead, it is used as a qualitative assessment of the changes in nanoparticles dispersion state over time.

0.2mg/ml sample dispersions were prepared in different pH solutions, where pH values were adjusted with CH₃COOH (0.1M) or NaOH (0.1M). The dispersions were bath

sonicated for 15mins and left overnight. Particle size, polydispersity, and zeta potential of the samples were then measured via Dynamic Light Scattering (DLS). The samples were kept for 30 days at room temperature to observe any changes to the sample in pH solution. Digital images were recorded 3 hours, 1 day, 3days, 1 week and 1 month after preparation.

Similarly, 0.5mg/ml aqueous dispersion of nanoparticle were added to equal volume of physiological media and mock intravesical urine conditions and studied for short term dispersion stability based on application duration. Samples were monitored at room temperature over one week for signs of aggregation and sedimentation. For DLS size measurement, samples were diluted to 0.2mg/ml concentration for consistency. Digital images were recorded 3 hours, 1 day, 3days, and 1 week after preparation.

Sample used for DLS measurements were disposed after measurement to prevent potential aggregation instigation from contaminants. For the benefit of comparison, all concentrations within the study is quoted based on graphene oxide content instead of conjugated variations while assuming negligible loss via filtration and dialysis process.

3.8 Cell Viability Assay for Cytocompatibility

Cell Counting Kit-8(cck-8) is used for cytotoxicity evaluation. The working principle of the kit is based on total metabolic activity of cells, deriving toxicity from decrease in metabolic activity.

The cell culture step involved seeding L929 murine fibroblast into 48-well plate at cell density of 1×10^5 per well. The cells were cultured in DMEM supplemented by 10% fetal bovine serum, 100units/ml penicillin and 100 μ g/ml streptomycin at 37°C under 5% CO₂ humidified atmosphere. After 24 hours incubation, the media were aspirated, and replaced with media containing samples in test concentrations of (10, 50, and 100 μ g/ml) respectively. Cells cultured solely with culture medium were taken as control. After 24- and 72-hours incubation, the cells were washed with PBS and 30 μ l of CCK-8 solution was added to each well. Further incubation of 2 hours is followed by evaluation of optical

intensity of each well with a microplate reader at 450nm. The cell viability was expressed as a percentage of $(OD_{\text{test}} - OD_{\text{test blank}}) / (OD_{\text{control}} - OD_{\text{blank}})$, where OD_{test} refers to optical density of cells treated with samples, $OD_{\text{test blank}}$ as treatment wells without cells, OD_{control} representing that of negative control and OD_{blank} from void wells. Percentage cell viability was reported as mean $\pm$ standard deviation and analysed using one-way analysis of variance and post-hoc Bonferroni test, with $p < 0.05$ depicting significant difference from control.

Graphene oxide interference of cck-8 readings has been reported whereby fluorescence quenching interferes with optical density reading accuracy and reagent absorption limits the formazan quantity.³² The light absorption of colored graphene oxide can also cause reduction of light intensity as measured by plate reader. As control measures, post treated cells were washed with PBS prior to cck-8 incubation and additional test blank wells were prepared. The test blank wells were wells not seeded with cells but treated with media containing nanoparticle dispersions. This is to ensure that the possibility of leftover graphene oxide materials even after the washing step is accounted for.

3.9 Drug Loading and Release Capability

CPT is fluorescent in nature and has an emission peak at approximately 440nm.³³ The UV absorbance peak is at 369nm while graphene oxide showed characteristic peak in the range of 220-230nm. As graphene oxide is known to cause fluorescence quenching, using UV-vis absorbance instead of fluorescence intensity would be a more controllable and straightforward way to access the presence of CPT. As a coloured sample, graphene oxide has absorbance values within the whole UV-vis region. Hence, prior calibration of graphene oxide absorbance intensity in accordance to the concentrations is necessary.

To obtain calibration curves of both CPT and GO, the UV absorbance for concentrations of 1,2,4,5,10,20,50 μ g/ml were recorded via Shimadzu UV-2600 spectrophotometer. Graphene oxide samples were sonicated for 15 minutes prior to absorbance measurement, while CPT samples were prepared the day before and left stirring until measurement were taken. Calibration curve is obtained based on the Lambert-Beer Law by plotting absorbance

intensity at characteristic peak of CPT (369nm) against predetermined concentrations. In view that there is overlapping of absorbance from both materials at 369nm, calibration curve of graphene oxide is obtained from intensities at 800nm since CPT records zero absorbance. For a given CPT-loaded graphene oxide sample, the calibration curve at 800nm is used to estimate the concentration of graphene oxide present and the corresponding absorbance intensity contributed by graphene oxide is calculated from graphene oxide calibration curve at 369nm. Subsequent deduction of absorbance from CPT-loaded graphene oxide returns the absorbance from CPT and hence, the CPT concentration present can be obtained from CPT calibration curve at 369nm. The final weight of CPT present is derived from total volume of CPT-loaded graphene oxide. Similar procedures entail for polysaccharide functionalized graphene oxide.

Camptothecin loading on chitosan-functionalized graphene oxide in varying weight ratio was first conducted to determine the optimal drug loading. Chitosan-functionalized graphene oxide was selected instead of graphene oxide to account of possible inhibition by large polymeric chains to physisorption of drug molecules. 50 μ l CPT dissolved in DMSO was added dropwise to 0.5ml functionalized graphene oxide with concentration of 0.5mg/ml. The mixture was left stirring overnight in the dark. Next, the loaded nanocarriers were extracted by repeated centrifugation at 4000rpm until no white precipitate was observed. The supernatant was then dialyzed (MWCO 10kDa) against water. The weights of loaded CPT were derived from UV-vis absorbance intensities at wavelength of 369nm after deducting the absorbance of functionalized graphene oxide and referring to calibration curve. Percentage of CPT loading capacities were calculated as a percentage of CPT weight loaded over particle weight and loading efficiencies from the percentage of CPT weight loaded over initial CPT weight.

The nature of release media will have a significant influence on drug release, even within similar pH conditions.³⁴ For the ease of comparison with preceding studies, drug releases in pH5 phosphate buffer and pH7.4 phosphate buffered saline were investigated. Meanwhile, considering the continuous urine generation in bladder, drug release in pH 6.3 artificial urine was also studied. Drug loading for release studies was implemented based

on selected weight ratio. 1ml CPT in DMSO was mixed with 6ml sample volume at concentration of 0.5mg/ml. Drug loading capacities and efficiencies were calculated. For drug release study, 2ml of CPT loaded samples were inserted into individual dialysis bags (MWCO 10kDa) and dialyzed against 20ml of release buffers at 37°C with 100rpm shaking. At selected time interval, the entire release buffer is replaced and 2ml is retained for UV-vis spectrometer quantitative analysis. Amount of CPT released are determined from calibration curve via UV-vis absorbance intensities at 369nm.

3.10 In vitro Mucoadhesion Study by Mucin Particle Method

Mucoadhesiveness of functionalized graphene oxide samples was primarily evaluated based on the mucin-particle method.^{9, 35-36} Mucin was suspended in hydration buffers at a concentration of 0.6% w/v. The suspension was incubated overnight after gently stirred for 3 hours. Next, the suspension was probe sonicated for 30 mins followed by centrifugation at 4000rpm for 30mins. The supernatant was verified to be having a size of less than 200nm before being used for mucoadhesive test. The mucin suspension was added to equal volume of samples (0.5mg/ml) and incubated for 1 hour. Particle size and zeta potential were subsequently recorded. Mucin interaction with chitosan in 0.1% acetic acid solution (10mg/ml), sodium carboxymethylcellulose dissolved in water (10mg/ml) and GO nanocolloids (0.5mg/ml) were also tested as control. To ensure homogeneous solution, the polymers were added gradually into stirring solution at 300rpm and allowed to stir overnight before using in study. For each polymer, three different polymer solutions were prepared and mixed with mucin to yield three different mixtures. Results were quoted as mean $\pm$ standard deviation.

3.11 Ex-Vivo Porcine Bladder Experimentation

Evaluation of particle mucoadhesion to porcine bladder tissues was adapted from a method reported by Mun and coworkers.³⁷ Based on the published method, fluorescence intensity of fluorescently-labelled nanoparticles were used as quantitative assessment of presence of attached nanoparticles on bladder mucosa. Graphene oxide and functionalized graphene

oxides are labelled with sodium fluorescein (NaFL) by π - π interaction for the purpose of fluorescence microscopy. Chitosan functionalized graphene oxide is firstly mixed with sodium fluorescein in different weight ratio (GO: NaFL) to identify the optimal for efficient labelling. A final weight ratio is selected for subsequent fluorescent-tagging based on highest NaFL loading efficiency, calculation of which is consistent with that outlined in Section 3.9 for drug loading. Covalent conjugation of sodium fluorescein to chitosan was also conducted with the same weight ratio to serve as positive control. Successful fluorescein attachment is verified by characteristic peaks of NaFL at 488nm using UV-vis spectrometer.

Porcine bladders were transported on ice from local abattoir and used immediately upon arrival in the lab. Meanwhile, unused bladders were stored at 4°C. Bladder tissues sized approximately 3cm x 3cm sections were cut from mid-lateral region and placed on individual petri dish with mucosal surface facing upward. All tissue samples were rinsed with 3ml of artificial urine. The fluorescein-labelled samples were re-dispersed in water at concentration of 5mg/ml and 0.3ml were pipetted onto mucosal surface. Sodium fluorescein dissolved in water at 5mg/ml was also tested to investigate affinity of sodium fluorescein to bladder mucosa. The samples were incubated at 37°C for 2 hours and fluorescence of samples was investigated at 3 different locations via Avantes Spectrofluorometer at 4x magnification and 11.4ms exposure. Images were recorded by Nikon DS-Ri2 camera employing “FITC” filter. Following that, samples were irrigated 3 times with 10ml artificial urine at the rate of 5ml/min using syringe pump. The experimental set up is as per Figure 3.2 where tubing outlet from syringe pump is not further than 2cm from tissue surface and tissue is angled at approximately 15° to guide irrigation flow. Experiment was conducted in triplicates and fluorescence intensity recorded as mean value normalized against initial fluorescence before artificial urine irrigation.

Figure 3.2 Experimental set up for artificial urine irrigation of porcine bladder tissues by a syringe pump.

The procedures in fluorescence study were modified to better facilitate the study for complete removal of muco-adhered materials. Experimentation is done in three groups of replicates, with each test group having tissue cut from the same bladder but incubated with different nanoparticles. Microscopic images as a group were recorded within 2 minutes at irrigation time intervals, amounting to approximately 3 hours used for each group experimentation excluding incubation period. To eliminate the effect that waiting period between irrigation may have on the mucoadhesion strength, irrigation order is rotated between samples with different sequence of rotation across test groups.

All tissue samples were rinsed with 3ml of artificial urine. sGO, CS-sGO and CMC-sGO were re-dispersed in water at concentration of 5mg/ml and 0.3ml were pipetted onto mucosal surface. Bladder tissues treated with artificial urine were taken as control. The samples were incubated at 37°C for 2 hours and imaging of samples was conducted at 3 different locations with Olympus SZX7 stereo microscope at 1x magnification and 250ms

exposure. Following that, samples were irrigated with 0, 10, 20, 30, 50, 100, 150, 200ml artificial urine at the rate of 5ml/min using syringe pump. Experiment was conducted in triplicates and the total volume of artificial urine required for complete removal of muco-adhered nanoparticles was noted.

The correlation to actual urine volume or number of urine voiding required for complete removal of nanocarriers can be estimated by accounting for total area of spherical bladder and urine production capability. The software Image J was used to generate surface plots of the images to facilitate visualization of nanoparticles presence. As the surface plot is a three-dimensional plot of the grayscale intensities of image pixels, the peaks are direct representation of dark regions and irrefutably confirms the presence of graphene oxide-based nanoparticles.

Although the normal bladder capacity is in the range of 400-600ml, the urge for urination occurs when the bladder is 150-300ml full and volume of voided urine is averaged at 350ml.^{18, 38-39} Similarly, the average voided urine volume for pigs is within the same range and the pig bladder has been reported to respond in a similar manner with human bladder to external stimuli.¹⁷ Hence, the actual urine volume and duration required for complete nanoparticles removal from a whole bladder can be estimated by simple ratio of total bladder volume to the area of experimental tissue, A_{total}/A_{expt} . A_{total} is calculated from bladder volume of 350cm³ while assuming a spherical bladder shape. Assuming there is negligible washing effect from the speed of urine irrigation, the overall total urine volume for complete nanocarriers wash-off from whole bladder, V_{total} can be estimated from multiplying the ratio to urine volume for complete wash-off from the study, V_{expt} as detailed in Equation 3.3. Here, average volume of voided urine is taken as average volume of bladder. Knowing normal average produced urine for human, R_u to be 1.5litres/ day⁴⁰⁻⁴¹, the days required for complete removal of nanocarriers from bladder, d_{total} is estimated to be approximately 4.7days from equation 3.4. Finally, the number of urine voiding before complete removal of nanocarriers, N_{void} can be estimated by dividing total wash-off urine volume, V_{total} with the volume of voided urine, V_{void} as outlined in equation 3.5.

$$V_{total} = \frac{A_{total}}{A_{expt}} \times V_{expt} \quad Eq. 3.3$$

$$d_{total} = \frac{V_{total}}{R_u} \quad Eq. 3.4$$

$$N_{void} = \frac{V_{total}}{V_{void}} \quad Eq. 3.5$$

The effect on the urothelial morphology from an agent introduced intravesically is of particular interest especially when desquamation of superficial cells has been associated with increased permeability⁴², as well as undesirable adverse effects when induced overboard³⁹. As such, bladder tissues incubated with nanoparticles were preserved and observed via scanning electron microscopy (SEM).

Similar to mucoadhesion study, pig bladder tissue of size 3x3cm is washed with artificial urine before two hours incubation with 1mg/ml aqueous dispersion of graphene oxide and functionalized graphene oxide. Tissue pieces rinsed with solely artificial urine are used as control. The incubated tissue samples are washed with 50ml artificial urine followed by surface preservation via immersion in 4% glutaraldehyde in PBS for 30 minutes at 4°C. The tissues were then dehydrated for 15 minutes in each concentration of ethanol from 30 to 100% and finally freeze-dried for more than 48hours. Morphology of the tissue samples were subsequently examined via JEOL JSM-5600LV SEM at 15kV accelerating voltage after platinum sputter coating. Images were recorded at three different locations of each samples and experiment was performed in sets of three from different bladders.

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Chapter 4* Synthesis of Polysaccharide-Functionalized Graphene Oxide

Dispersion stability in physiological media and at specific pH range has been one of the main factors limiting applicability of nanoparticles in vivo. Polymer functionalization of graphene oxide has been widely executed to achieve stability in simulated biological conditions. Nevertheless, the relationship between the composition and corresponding effect on improved dispersibility has not been clearly defined. This chapter identifies the contribution of grafting degree of polysaccharides in enhancing the properties of graphene oxide, with emphasis on physiological stability and mucoadhesion property. Generally, higher degree of polymer grafting is found to be associated with improved dispersibility pH and physiological conditions as well as enhanced mucoadhesion property.

4.1 Introduction

Drug delivery system with high surface-to-volume ratio is associated with improved intravesical drug absorption.¹⁻² The two-dimensional graphene oxide, therefore, comes into the picture with promising improvement in drug transport to targeted sites. The limiting factor for the aqueous stable material, however, is the aggregative nature in salt solutions and aqueous medium rich in protein such as cell medium and serum.³⁻⁴ To overcome the inhibition to functionality and stability within a bladder environment due to charge screening effect, surface modification of graphene oxide by functionalization is crucial.

The polysaccharides, chitosan and carboxymethylcellulose are of particular interest as conjugates for graphene oxide in the development of intravesical drug delivery system. Chitosan especially, increases drug permeability across the bladder mucosa⁵, and confers graphene oxide with pH-sensitive drug release⁶ and diminishes cytotoxicity effect.⁷ Both polymers are not foreign in intravesical formulations, whether as drugs excipient or as part of the components in drug delivery systems. Furthermore, covalent functionalization to carboxyl groups of graphene oxide can be achieved easily via carbodiimide reactions.

It is critical to understand the effect of polymer conjugation degree on the final properties of functionalized graphene oxide. For that, the starting material, graphene oxide is important since the maximum viable degree of functionalization will depend on the density of carboxyl groups. The graphene oxide particle size will also be a controlling factor on the final measurements of the functionalized form. Hence, selection of graphene oxide is based upon the smallest particle size and highest degree of functionalization achievable.

As a fundamental step in nanoparticle synthesis, the size, degree of functionalization and the corresponding stability in pH and physiological conditions are used as determinants of suitability. For biocompatibility, a size of 300-500nm for graphene oxide is recommended⁸, while circa 350nm graphene oxide induces less inflammatory responses.⁸⁻⁹ Meanwhile, drug carriers in the size range of 200-500nm are better able in enhancing diffusion across mucin mesh for mucoadhesive drug transport. The opposing effect may apply for particle

sizes falling outside the range.¹⁰ Furthermore, particle size that is smaller than 400nm is considered suitable for permeation-enhancing into bladder cancer tissues.¹¹ Graphene oxide of smaller flake size also had less tendency for interparticle attraction and aggregation.¹² On that note, a drug carrier sized approximately 200-350nm appears to be prospective biocompatible, permeation-enhancing, mucoadhesive efficient drug delivery system.

The pH of urine can vary from 4.5 to 8 for healthy individuals as well as in diseased conditions.¹³⁻¹⁴ In addition, the stability of bladder cancer chemotherapeutic drugs are pH-dependent. Doxorubicin stays stable in acidic environment while an alkaline pH is more suitable for thiotepa and epirubicin. Mitomycin C though, has a larger pH stability range of pH5-8 which complements the pH environment of urine in bladder.¹⁵ Correspondingly, drug delivery system with stability in larger range of pH is idealistic. Stability in physiological media, especially artificial urine is vital as well, considering that maintenance of drug carrier in its unmodified form *in vivo* is crucial in drug delivery efficacy.

Mucoadhesion is a value-adding property for intravesical drug delivery systems and is usually viable with the presence of hydrophilic groups such as carboxyl, hydroxyl, sulphate, amine and amide groups.¹⁶⁻¹⁹ Over the decades, researchers have manipulated mucoadhesive polymers to introduce mucoadhesive capability into biomedical applications, with special emphasis on drug delivery systems. Targeted drug delivery into mucosal regions such as ocular site, nasal and oral cavity, gastrointestinal tract, bladder and vagina lumen was achievable where local adhesion of drug carriers at specific areas allows prolonged and controlled drug delivery²⁰⁻²¹. Since both chitosan and carboxymethylcellulose are mucoadhesive polymers, the functionalized graphene oxide will show promises of localized sustained drug delivery instigated by mucoadhesion. The concentration of the polymers play an important role, where overly high and low concentrations result in unfavorable decrease in affinity for mucus.¹⁹ Therefore, quantifying mucoadhesive strength is essential to determine the influence of polymers concentration on mucoadhesion property of functionalized graphene oxide.

The study involves functionalization of selected commercially available graphene oxide nanocolloids with polysaccharides in different synthesis ratios to achieve different degree of grafting. The synthesized nanoparticles were characterized via DLS, TEM, FTIR and TGA to verify successful functionalization, percentage chitosan grafting, particle size and corresponding zeta potential. Subsequently, the effects of grafting percentage on the dispersibility in different pH solutions and physiological media as well as mucoadhesive characteristics were evaluated.

4.2 Materials and Methods

4.2.1 Materials

Graphene oxide were sourced from different suppliers, with specifications as outlined in Table 4.1.

Table 4.1 Specification of graphene oxide purchased for pilot study. Information is accurate at the time of purchase; however some products has stopped production or had a change of specifications at the time this document is prepared.

Sample	Product name	Supplier	Specification size (nm)
GO	Graphene oxide flakes	XFNano Materials Tech Co.,Ltd	500-5000nm
cGO	COOH-functionalized graphene oxide	XFNano Materials Tech Co.,Ltd	500-5000nm
gGO	Nano graphene oxide powder	Graphene Supermarket, Graphene Laboratories, Inc	90nm
kGO	Graphite oxide, KNG-21	Knano Graphene Technology Co., Ltd	1-100µm

sGO	Graphene oxide nanocolloids, 2mg/ml	Sigma Aldrich	Information unavailable
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Low molecular weight chitosan (CS, MW 50-190kDa, 75-85% deacetylated) and sodium carboxymethylcellulose (NaCMC, MW~250kDa, degree of substitution 0.5) were purchased from Sigma Aldrich. Chemicals used for synthesis and characterization included 2-(N-Morpholino)ethanesulfonic acid (MES, $\geq 99\%$), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC $\cdot$ HCl, $\geq 99\%$), N-hydroxysuccinimide (NHS, 98%), adipic acid dihydrazide (ADH, $\geq 98\%$), phosphate buffered saline tablets (pH7.4), mucin from porcine stomach (type III, bound sialic acid 0.5-1.5%, partially purified powder) and potassium bromide (KBr, $\geq 99\%$, FTIR grade). Dulbecco's modified eagle medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were purchased from Gibco BRL (Gaithersburg, MD). All other laboratory chemicals and reagents used for experimental study were sourced from Aldrich and used as received.

4.2.2 Preparation of graphene oxide

All samples in Table 4.1 were prepared with the same procedures, starting with overnight stirring at the speed of 1000rpm, before probe sonication at a concentration of 0.2mg/ml as per Chapter 3.3. The particle sizes of each samples are measured by Zetasizer at every 30 minutes interval for a duration of two hours. For all samples, the relationship between particle size and sonication duration is assumed to be linear. Extrapolation of the linear curves allow an estimation of the total duration required to achieve a particle size of 200nm. Three graphene oxide samples that returned shortest estimated time is selected for chitosan functionalization and particle stability in physiological conditions are studied. Chitosan functionalization of graphene oxide at this stage followed the protocol reported by Bao and group.²² Selection of one graphene oxide is based on highest chitosan functionalization degree.

4.2.3 Chitosan functionalization of graphene oxide

Following selection of graphene oxide, subsequent synthesis for varied chitosan to graphene oxide weight ratio were executed as outlined in Chapter 3.4, consisting of two parts. Firstly, the effect of chitosan density on the properties of functionalized graphene oxide was explored. Graphene oxide with a starting size of $200\pm 10\text{nm}$ was functionalized with chitosan based on the weight ratios (CS:sGO) tabulated in Table 4.2.

Table 4.2 Weight ratios of chitosan to graphene oxide used in functionalization synthesis to investigate the effect of grafting degree to the properties of chitosan functionalized graphene oxide.

Sample	Weight ratio (CS:sGO)	Weight of chitosan in 2.5ml acetic acid (mg)	Volume of 2mg/ml graphene oxide (ml)	Weight of graphene oxide (mg)
CS-sGO 1:1	1:1	5	2	5
CS-sGO 2:1	2:1	10	2	5
CS-sGO 3:1	3:1	15	2	5
CS-sGO 4:1	4:1	20	2	5
CS-sGO 5:1	5:1	25	2	5

The second part involved extended functionalization study with a starting graphene oxide size of $175\pm 5\text{nm}$. Table 4.3 showed the details of functionalization with five combinations. The rationale behind this secondary study is attributed to maximum solubility limit of chitosan and maximum concentration of starting graphene oxide. From the synthesis protocols, chitosan at 10mg/ml in 2.5ml acetic acid was observed to be the limit of solubility, as some flakes of undissolved polymer were seen. Meanwhile, graphene oxide was purchased as a 2mg/ml nanocolloid dispersion, hence, greater amount of graphene oxide required for synthesis cannot be achieved if volume is a controlled variable. To study effects of chitosan to graphene oxide weight ratio beyond 1:5, volume of graphene oxide is decreased while keeping other parameters constant. The effect of reducing graphene oxide volume on the synthesis process was also analyzed by fixing the weight ratio at 5:1 for all graphene oxide concentrations used.

Table 4.3 Weights of chitosan and graphene oxide in five combinations for selection of optimal method.

Sample	Weight ratio (CS:sGO)	Weight of chitosan in 2.5ml acetic acid (mg)	Volume of 2mg/ml graphene oxide (ml)	Weight of graphene oxide (mg)
CS-sGO1	5:1	25	2.5	5
CS-sGO2	5:1	18.75	1.875	3.75
CS-sGO3	5:1	12.75	1.25	2.5
CS-sGO4	6.67:1	25	1.875	3.75
CS-sGO5	10:1	25	1.25	2.5

Synthesized chitosan functionalized graphene oxides were characterized via DLS, TEM, FTIR and TGA. Dispersion stability in pH solution, DMEM, PBS, and AU were investigated, and the respective mucoadhesion properties compared using the mucin particle method in Chapter 3.10 with Mili-Q water as mucin hydration buffer.

4.2.4 Carboxymethylcellulose functionalization of graphene oxide

Carboxymethylcellulose functionalization of graphene oxide was executed with 1.25ml graphene oxide of concentration 2mg/ml and size of 175 ± 5 nm based on Chapter 3.5. Adipic acid dihydrazide was used as linker between the carboxyl groups of graphene oxide and carboxymethylcellulose. The amount of linker used in synthesis will play a role in the totality of ADH conjugation to graphene oxide. Hence, the weight ratio of linker ADH to polymer NaCMC was modified based on Table 4.4.

Similarly, carboxymethylcellulose functionalized graphene oxides were characterized via DLS, TEM, FTIR and TGA. Dispersion stability in pH solution, DMEM, PBS, and AU as well as mucoadhesion properties were investigated.

Table 4.4 Weights of adipic acid dihydrazide and sodium carboxymethylcellulose in six combinations for selection of optimal method.

Sample	Weight ratio (ADH:CMC)	Weight of ADH (mg)	Weight of NaCMC in 2.5ml MES buffer (ml)
CMC-sGO1	1:2.5	10	25
CMC-sGO2	1:4:1	35.7	25
CMC-sGO3	3.5:1	87.1	25
CMC-sGO4	3.8:1	87.1	23
CMC-sGO5	4.4:1	87.1	20
CMC-sGO6	8.7:1	87.1	10

4.3 Results and Discussion

4.3.1 Graphene oxide selection and characterization

After probe sonication of the five graphene oxides for three hours with 30 minutes interval, the linear fitting of the changes in particle size with increasing sonication duration is plotted in Figure 4.1. Using linear fit equation derived from the relation plot, an extrapolation of the sonication time required to achieve a particle size of 200nm is estimated. FTIR absorbance spectra in Appendix Figure A.1 showed that all graphene oxide samples have similar functional groups. Thereafter, three graphene oxides, cGO, gGO, sGO were selected for subsequent study based on less sonication time required. kGO was excluded due to the observed poor aqueous stability of the dispersion. (Refer Appendix Figure A.2)

As the particle sizes were still relatively large after sonicating based on estimated time in extrapolation relation, the sonication time was extended. Referring to Figure 4.2G, a size of 165.8 ± 4.6 nm was achieved for sGO after a 3 hour sonication period. After sonication for 12 hours, cGO achieved a size of 361.3 ± 83.6 nm. Meanwhile, gGO achieved a similar size i.e. 345 ± 13.9 nm after sonication for 17 hours. There were little size reduction efficiency with greater sonication, therefore the sonication times used for cGO, gGO, and sGO were not extended further and characterization studies were proceeded at this stage although particle size of 200nm has not been reached for sample cGO and gGO. In Figure

4.2(A,B), both cGO and gGO have similar size trend with pH changes, exhibiting aggregation at acidic and alkaline conditions. sGO in Figure 4.2C, however has good stability at pH7-10, with zeta potential values maintained at approximately -40mV. The more negative zeta potential value of sGO as compared to cGO and gGO samples signifies greater density of carboxyl groups that deprotonates and repels the sheets for greater colloidal stability. Deprotonation of the carboxyl groups on graphene oxide occurs from pH2 to pH7 and the corresponding stabilization effect intensifies at higher pH values.²³⁻²⁴ The instability in acidic conditions is attributed to the increased hydrophobicity due to neutralization of repulsion forces by protonation of hydroxyl and carboxyl groups.²⁴⁻²⁶ Larger dimension will also result in greater hydrophobic regions as charge density decrease, causing aggregation at lower pH.²⁷

Figure 4.1 Particle size changes of graphene oxide samples with increasing probe sonication duration. The inset table lists the linear fitting for time estimation required to achieve a particle size of 200nm via DLS.

Figure 4.2(B,D,F) plots the pH stability of chitosan functionalized graphene oxide. After chitosan functionalization, the zeta potential values inverted to positive values in increasing order of CS-sGO>CS-gGO>CS-cGO. The trend could imply higher degree of chitosan functionalized graphene oxide for CS-sGO. The particle sizes for aqueous dispersions of CS-cGO, CS-gGO, and CS-sGO were much higher than in pH6 and pH8 conditions. This

is due to the limited aqueous solubility of conjugated chitosan that borders at pH6, resulting in aggregation of particles.²⁸ Meanwhile, stabilization by hydroxyl groups and graphene lattice could be at work at pH8.²⁹ Overall, CS-cGO, CS-gGO and CS-sGO exhibited similar trend for particle size and zeta potential values across different pH conditions.

Figure 4.2 Particle size and zeta potential at varied pH environment of graphene oxide samples cGO(A), gGO(C), sGO(E), and chitosan functionalized graphene oxide samples CS-cGO(B), CS-gGO(D), CS-sGO(F). Table in (G) summarized the particle size, polydispersity and zeta potential values of graphene oxides and functionalized graphene oxide samples.

From Figure 4.3, the graphene oxide samples showed characteristic FTIR peaks corresponding to the myriad of functional groups present, specifically, peaks at 1709-1722 cm^{-1} due to stretching vibration from C=O, O-H bending at 1363-1396 cm^{-1} , C-O stretching at 1225-1248 cm^{-1} and 1057-1061 cm^{-1} , and C-O-C peaks appearing at 843-847 cm^{-1} . The broad peaks between 3000-3500 cm^{-1} is attributed to stretching vibrations of hydroxyl groups.³⁰⁻³³ cGO appeared to not contain any epoxy groups while gGO has a weak peak at 1794 cm^{-1} associated with acid halide, suggesting presence of impurities from the manufacturing process.

Figure 4.3 FTIR absorbance spectra showing characteristic peaks for functionalized cGO(A), gGO(B), and sGO(C).

TGA decomposition curves were as plotted in Figure 4.4. Loss of graphene oxide mass at temperature below 100 $^{\circ}\text{C}$ occurs due to water evaporation from π -stacked lattice while significant weight loss before 200 $^{\circ}\text{C}$ is associated with pyrolysis of labile oxygen-containing functional groups.^{32, 34} The second drop in weight loss starting at approximately 500-550 $^{\circ}\text{C}$ is attributed to loss of graphene oxide carbon skeleton integrity.³⁵ Interestingly for cGO in Figure 4.4A, the thermal decomposition at this temperature range was not significantly great. This phenomenon indicates that the absence of epoxy groups on graphene oxide improved its thermal stability due to reduced defects on the graphitic lattice. The onset of rapid weight loss for chitosan occurs around 270 $^{\circ}\text{C}$, which represents the decomposition of the polymeric chain and pyranose ring.³⁶⁻³⁷ At the end of TGA study, the remaining residue is approximately 29.4%, 26.0%, and 30.5% for CS-cGO, CS-gGO, and

CS-sGO respectively. The weight losses are analysed at 600°C as per the method by Bao et al²² where grafted polymer on CS-gGO and CS-sGO is estimated as 78% and 81% respectively. Estimation of chitosan density on CS-cGO was not possible as the sample underwent greater thermal composition than either of CS or cGO. This could be attributed to increase in defects on graphene oxide surfaces as covalent functionalization occurs. Therefore, percentage polymer conjugated is a mere estimation and cannot to be taken as direct representation of functionalization degree.

Figure 4.4 Thermal decomposition curves of CS-cGO(A), CS-gGO(B), and CS-sGO(C) in comparison to chitosan, cGO, gGO, and sGO.

Analysing the properties of the functionalized graphene oxide samples, sGO was ultimately selected based on least sonication duration to achieve smallest particle size with lower polydispersity. sGO and CS-sGO also had greater dispersion stability, which was apparent from the greater absolute values of zeta potential. Although prolonged sonication does reduce the particle size and polydispersity index values, breaking of graphene oxide to smaller sizes by ultrasonication leads to reduction due to exposure of poorly oxidized interlayers as well as gradual loss of carboxyl and carbonyl groups.^{29, 38-39} Not to mention, extensive sonication does not yield uniform size distribution.⁴⁰ Furthermore, graphene oxide of smaller dimensions has higher density of ionized -COOH groups and are hence more hydrophilic and colloidally stable.⁴⁰ Study by Huang and co-workers has also linked a highly charged graphene oxide of smaller dimension with greater compatibility with biomolecular interaction and sensing³⁹ Therefore, selection of process with minimal sonication will limit changes to graphene oxide oxidation level, giving rise to better

biocompatibility and biological responses.

4.3.2 Effect of chitosan density on properties of functionalized graphene oxide

Upon selection of sGO for subsequent study, further optimisation of the synthesis process was done with the aim of achieving smaller particle size and consistent conjugation. Chitosan was conjugated to sGO in different weight ratios (CS:sGO = 1:1, 2:1, 3:1, 4:1, 5:1) at reduced volume to investigate the effect on final particle size and pH stability. The sizes of CS-sGO aqueous and pH dispersions are detailed in Figure 4.5. Particle size and zeta potential generally increases with increasing synthesis ratio, as shown in Figure 4.5(B,D). More positive zeta potential value for CS-sGO samples is indicative of higher density of conjugated chitosan.⁴¹ With higher synthesis ratio, and presumably higher percentage of chitosan grafted, the particle stability at pH5, pH6 and pH8 are improved, with significant decrease in aggregation degree indicated by insignificant increase in particle size.

Graphene oxide nanocolloids exhibited greater particle sizes at pH5 and 8 due to aggregation as shown in Figure 4.5A. The degree of aggregation was reduced by sonication based on Appendix Figure A.3, where there was apparent stabilization at pH5 and pH8 as compared to un-sonicated samples. When studied in relation to un-sonicated graphene oxide, the pH stability of sonicated sGO was greater at alkaline pH, reflected in the more negative zeta potential values. This is in agreement with the conception that sonicated graphene oxide has higher density of oxygenated species, especially ionized carboxylic groups that is present due to the enhanced edge-to-area ratio of smaller graphene oxide sheets.^{27, 40, 42} The trend shown for change in particle size across pH for sGO deviated from Chapter 4.3.1 due to batch variation of purchased graphene oxide nanocolloids.

The particle size and zeta potential changes of sample CS-sGO 5:1 is as shown in Figure 4.5C. Across pH 5 to pH8, the particle size was relatively constant ranging from 400 to 465nm. The particle sizes of all samples are confirmed via TEM in Figure 4.6, with sizes generally ranging from 200-500nm. Size variations observed across samples in Figure 4.6(A-E) are not representative of the effect of synthesis ratio, instead it is a general size

variation of the samples due to the sonication process as samples are used after sonication without segregation by size. It is interesting to note that the sample stayed stable at pH8 when the zeta potential is nearing the isoelectric point. While zeta potential of $\pm 30\text{mV}$ has been associated with colloidal stability, the prediction is based solely on stabilization by electrostatic repulsion without accounting for van der Waals attractions.⁴³⁻⁴⁴ The increase in chitosan density on the surface of graphene oxide could have contributed to steric stabilization⁴⁵⁻⁴⁷ while stability before pH6 is attributed to the good solubility of chitosan at acidic pH.²⁸ CS-sGO 5:1 was synthesized thrice and plotted in Appendix Figure A.4 for verification of repeatability. The trend for size and zeta potential across different pH remained consistent.

Figure 4.5 Changes to pH stability in terms of particle size of chitosan functionalized graphene oxide samples synthesized at weight ratios of 1:1, 2:1, 3:1, 4:1, and 5:1 in comparison to sGO(A), and the changes to synthesized particle sizes and zeta potentials with increasing weight ratios(B). Figure 4.5(C) shows the trend in particle size and zeta potential of CS-sGO 5:1 across different pH values and (D) lists the particle size, polydispersity index and zeta potential values for all samples.

Figure 4.6 TEM images of CS-sGO 1:1 to CS-sGO 5:1 from (A) to (E).

FTIR analysis was conducted to confirm successful conjugation of CS to sGO. The FTIR spectra of all CS-sGOs outlined in Figure 4.7 indicated successful chitosan conjugation, except for CS-sGO 1:1. Again, the absorbance at approximately 1556 cm^{-1} and 1653 cm^{-1} confirms the existence of amide N-H bending and C=O stretching^{24, 51} while the spectra at 2883 cm^{-1} is attributed to C-H symmetric and asymmetric stretching that are inherent to polysaccharides.³⁷ Sample CS-sGO 1:1 did not show distinct peaks for 1653 cm^{-1} and 2883 cm^{-1} , suggesting negligible chitosan functionalization. Absorbance peak at 1153 cm^{-1} meanwhile, is associated with C-O-C in glycosidic linkage of chitosan.⁴⁸⁻⁴⁹

Figure 4.7 FTIR spectra for CS-sGO samples synthesized from varying weight ratio showing presence of similar peaks.

Chemical composition of formed CS-sGO samples was estimated from TGA thermal decomposition. Figure 4.8 showed the thermal decomposition curve of CS-sGO 5:1, while comparison with other samples plotted in Appendix Figure A.5. sGO was found to have undergone complete decomposition at 700°C. Hence, based on the residual weight of samples at 700°C, the estimated percentage of functionalized chitosan is as per summarized in Table 4.5. Not surprisingly, increasing weight ratio of chitosan to graphene oxide during synthesis generally increase the degree of chitosan functionalization on graphene oxide surface. The sample CS-sGO 1:1 and Cs-sGO 2:1 was not in conformation to this trend due to insufficient chitosan present in the reaction for full coverage of graphene oxide functionalization. This led to uneven functionalization of chitosan on graphene oxide surface, causing chitosan density to differ from sheet to sheet.

Figure 4.8 TGA curves showing percentage weight loss from thermal decomposition in nitrogen atmosphere.

Table 4.5 Percentage of functionalized chitosan on graphene oxide estimated based of residues from thermal decomposition of CS-sGO samples at 700°C.

Sample	Residual weight fraction of CS	Residual weight fraction of sGO	Residual weight fraction of CS-sGO	Estimated percentage functionalized chitosan (%)
CS-sGO 1:1	0.7348	0.9501	0.8926	26.7
CS-sGO 2:1	0.7348	0.9501	0.9038	21.5
CS-sGO 3:1	0.7348	0.9501	0.8836	20.1
CS-sGO 4:1	0.7348	0.9501	0.9067	30.9
CS-sGO 5:1	0.7348	0.9501	0.8718	36.4

Images of the fabricated CS-sGO in pH solutions and physiological media were taken 3 hours after preparation and after standing for one day as arranged in Figure 4.9. All samples

aggregated at pH 10 almost immediately. The images correlated well with particle size measurements, with aggregation and sedimentation detectable visually for measured increase in size. With the exception of CS-sGO 1:1 and CS-sGO 2:1, the samples were observed to be homogeneously dispersed from pH3 to pH8 three hours after preparation. The stability of CS-sGO 3:1 in pH8 however, was not sustainable as sedimentation was visible the next day. CS-sGO 4:1 and CS-sGO 5:1 exhibited good stability in pH range 3-8 with some sediments observed for pH8. It is obvious that chitosan functionalization improved pH stability of graphene oxide, as pH-adjusted graphene oxide aggregated extensively in Appendix Figure A.6.

Figure 4.9 Dispersion stability in pH 3,5,6,7,8, and 10 (left to right) of CS-sGO 1:1 to 5:1 (A-E) 3 hours and one day after preparation. Images in (F-H) are dispersions in physiological media of cell culture media DMEM, phosphate buffered saline PBS, and artificial urine AU for CS-sGO 3:1(F), CS-sGO 4:1(G), and CS-sGO 5:1(H) in physiological media of cell culture media DMEM, phosphate buffered saline PBS, and artificial urine AU (left to right).

Subsequently, dispersion stability in physiological media for samples CS-sGO 3:1 to 5:1 was investigated. Three hours after preparation, all samples appeared to be homogeneously dispersed in cell culture medium (DMEM), phosphate buffered saline (PBS), and artificial

urine (AU). For both CS-sGO 3:1 and CS-sGO 4:1, the dispersion stability in all physiological media disappeared within a day. The stability in both PBS and AU appeared to be dependent on the density of conjugated chitosan, and hence increased with increasing weight ratio. Interestingly, the dispersion stability of graphene oxide in cell culture medium diminished after chitosan functionalization. Based on Appendix Figure A.6, sGO aggregated in PBS and AU but is relatively well-dispersed in DMEM.

In summary, increased addition of chitosan contributes to pH stability of CS-sGO especially from pH 5-8 as well as in PBS and AU which are key conditions for planned application. CS-sGO5:1 with approximately 36% conjugated chitosan has very promising property, but with a particle size that exceeded 200-350nm desired for good biocompatibility and permeability as analysed in Introduction. Therefore, the following section explores the approach to achieve a particle size that falls within the range

4.3.3 Chitosan grafting and functionalization studies

To achieve a smaller particle size based on chitosan to graphene oxide weight ratio of 5:1, the starting size of sGO was reduced from 200nm to 175nm. Due to the limitations set by solubility limit of chitosan and fixed concentration of purchased sGO, investigation of functionalization using weight ratio greater than 5:1 is executed by reducing the volume of sGO added to the reaction. The detailed changes made to synthesis of chitosan functionalized graphene oxide has already been tabulated in Table 4.3. For ease of analysis, the chitosan functionalized graphene oxides were categorized into two groups. Group A consisting of CS-sGO1, CS-sGO2, and CS-sGO3 synthesized with weight ratio of 5:1 but different starting volume of sGO while Group B encompassed CS-sGO1, CS-sGO4, and CS-sGO5 that represented increasing weight ratios achieved via decreasing starting volume of sGO.

Figure 4.10 represents the change in pH stability after chitosan functionalization of graphene oxide for sample CS-GO5. The data for other CS-GO samples are included in Appendix Figure A.7 as all exhibited similar trend in particle size and zeta potential at

different pH solutions. As can be derived from Figure 4.10, the particle size is maintained at approximately 200-400nm from pH3 to pH8. The dispersion stability trend is very similar to CS-sGO 5:1 in Section 4.3.2, but with less deviation in particles size as pH changes. The greater dispersion stability at pH3 implied electrostatic repulsion of protonated amino groups from a greater density of conjugated chitosan. The gradual deprotonation of the amino groups between pH5-7 is equated with coulombic charges from deprotonated carboxyl groups and steric stabilization from extended conformation of chitosan chains.⁵⁰⁻⁵¹ Above pH 8 however, hydrophobic attractions and hydrogen-bonding interactions ensue, leading to destabilization of the dispersion and hence aggregation of CS-GO particles.⁵²⁻⁵³ The functionalization of chitosan on graphene oxide is essentially a play of the ratio of hydrophilic and hydrophobic components on the particle surface. The proportion of each part on a functionalized graphene oxide sheet ultimately determines the behaviour in pH stability.

Figure 4.10 Changes in particle size and zeta potential of sample CS-sGO5 at varying pH plotted as mean $\pm$ standard deviation from three measurements of three batch samples. The mean particle size is 297 ± 19 nm.

The synthesized CS-sGO samples were of size $364.6 \pm 1.2 \text{ nm}$, $308.8 \pm 4.3 \text{ nm}$, $213.6 \pm 3.0 \text{ nm}$, $345.3 \pm 6.6 \text{ nm}$, and 272.2 nm in that order. As the particle size measured using DLS were not accurate representation of the anisotropic and light interfering^{43, 54} graphene oxide and CS-sGOs, TEM imaging were also conducted to confirm size range of synthesized CS-sGOs, as per Figure 4.11. The particle sizes according to TEM images were in the range of 200-500nm. As shown in Figure 4.12A, the particle sizes decreased for aqueous dispersion when volume of starting sGO is decreased. Similar trend is also observed when the same sGO volumes are used to synthesize CS-sGO in Group B at fixed chitosan concentration of 25mg in 2.5ml. At these conditions, the weight ratios for CS-sGO1, CS-sGO4, and CS-sGO5 (Group B) translated to 1:5, 1:6.67 and 1:10 respectively. Figure 4.12B comparing the three CS-sGO samples showed that CS-sGO5 has the smallest particle size from pH6 to pH10 as compared to the other samples. The size of CS-sGO5 at pH10 was approximately 811nm as compared to other samples that exceeded $1 \mu\text{m}$ due to extensive aggregation. It can be inferred that greater weight ratio between graphene oxide nanocolloids and chitosan solution synthesized in minimal reaction volume results in a product with reduced dimensions and enhanced dispersion stability, as demonstrated by CS-sGO5. In addition, smaller particle sizes were also achieved at the lowest reaction volume, as can be seen from the sizes of CS-sGO3 and CS-sGO5 at total reaction volume of 3.75ml as compared to 4.375ml for CS-sGO1 and 5ml for both CS-sGO2 and CS-sGO4.

Figure 4.11 TEM images for CS-sGO1 (A), CS-sGO2 (B), CS-sGO3 (C), CS-sGO4 (D), and CS-sGO5 (E) showing particle size range of 200-500nm.

Figure 4.12 Comparison of particle sizes across different pH for Group A synthesized at fixed weight ratio but reaction volumes (A), and Group B synthesized at increasing weight ratio at fixed chitosan concentration (B).

FTIR transmission spectra of sGO nanosheets in Figure 4.13 showed the characteristic peaks corresponding to the stretching vibration of functional groups hydroxyl -OH group ($3200\text{--}3500\text{cm}^{-1}$ and 1227cm^{-1}), carboxyl -COOH group (1726cm^{-1}), carbonyl C=O group (1616cm^{-1}) and epoxy C-O-C group (1057cm^{-1}).^{30-31, 33, 55} Comparing the sGO spectra with

that of CS-sGO5, CS-sGO5 spectra showed disappearance of the carboxyl group peak at 1726 cm^{-1} , signifying complete participation of carboxyl groups in chitosan functionalization. Moreover, the N-H bending and C-N stretching of secondary amide represented by the peak at 1554 cm^{-1} ^{30, 48} showed a shift from the equivalent amino group (-NH₂) peak in the pure CS transmission spectra, which was at 1593 cm^{-1} . The obvious shift to a lower wavenumber corresponds to hydrogen bonding and electrostatic interactions of CS with sGO and therefore, confirmed newly-formed amide bonds between GO and CS.^{53, 56-58}

The characteristic signals matched to aliphatic C-H symmetrical and asymmetrical stretching in chitosan were transferred to CS-sGO spectra at 2882 cm^{-1} and 1317 cm^{-1} .^{37, 52, 59} Similarly, the bands of CS at 1154 cm^{-1} and 898 cm^{-1} related to C-O-C in glycosidic linkage were conveyed in the CS-sGO spectra at 1153 cm^{-1} and 897 cm^{-1} respectively.⁴⁸⁻⁴⁹ The slight shifts after CS functionalization on GO suggested interactions involving the functional groups.⁴⁸ Figure 4.14 consolidated FTIR spectra for all CS-sGO samples showing indisputable similarity with negligible shift in peaks. Hence, it can be deduced that all CS-sGO samples were successfully conjugated with chitosan.

FTIR spectrum for sonicated graphene oxide was also observed to differ slightly from the un-sonicated sample as portrayed in Figure A.8 in Appendix. The apparent deviations lie in the blue shift of hydroxyl and carbonyl groups corresponding peaks to higher wavenumber. Since carbonyl groups are inherently polar, the shift to higher wavenumber after GO sonication could be associated with disruption of the polar attractions between carbonyl groups of adjacent graphene oxide sheets. In addition, inter- and intra-layer hydrogen bonding between carbonyl, hydroxyl, and epoxide groups of GO sheets as well as with water molecules may also have been broken.⁶⁰⁻⁶¹ This correlates well with the exfoliation effect of sonication that enables formation of single sheets graphene oxide as hydrogen bonding are a part of the interaction that contributes to multi-layer stacking. Combined with the effects from elimination of hydroxyl groups and subsequently carbonyl groups from graphene oxide framework due to sonication, the contribution to hydrogen bonds network would have decreased.⁶²

Figure 4.13 FTIR absorbance spectra for graphene oxide, chitosan, and chitosan-functionalized graphene oxide, CS-GO5.

Figure 4.14 FTIR absorbance spectra for all chitosan-functionalized graphene oxide samples showing negligible deviations between the samples.

TGA decomposition curves for sGO, CS, and CS-sGO5 were as plotted in Figure 4.15. From the curves, graphene oxide loses mass quickly even below 100°C, and the rate is accelerated at approximately 170-220°C. The thermal stability of sonicated graphene oxide was found to have deteriorated significantly as compared to un-sonicated samples (refer Appendix Figure A.8), with complete decomposition at 700°C. Overall, chitosan-functionalized graphene oxide displayed improved thermal stability due to the influence of chitosan below 300°C and graphene oxide for the temperature range of 300-570°C. Nevertheless, CS-sGO5 exhibited a higher rate of weight loss when temperature exceeds approximately 570°C, matching thermal decomposition trend of graphene oxide. Using thermal compositional residues at 700°C in accordance with complete decomposition of sGO, the weight percentages of each component for all CS-sGO samples were estimated and tabulated in Table 4.6.

Figure 4.15 TGA curves showing weight per temperature change of graphene oxide, chitosan, and CS-sGO5.

Table 4.6 Estimated weight percentages of chitosan and graphene oxide in sample CS-sGO1, CS-sGO2, CS-sGO3, CS-sGO4 and CS-sGO5.

Sample	Weight ratio (CS:sGO)	Weight percentage of CS (%)	Weight percentage of sGO (%)
CS-sGO1	5:1	70.6	29.4
CS-sGO2	5:1	19.6	80.4
CS-sGO3	5:1	1.5	98.5
CS-sGO4	6.67:1	11.5	88.5
CS-sGO5	10:1	52.4	47.6

Based on the tabulated compositions, for the same weight ratio of 5:1, the conjugated chitosan is higher for CS-sGO1 with the highest starting sGO volume. This is in conformation with collision theory whereby a higher concentration of reactants will increase the frequency of collision and hence probability of reaction to occur. Incidentally, the polymer exclusion effect would have worked in the same favorable direction. For CS-sGO1, the concentration of graphene oxide per reaction volume was 1mg/ml which was higher than 0.857mg/ml and 0.667mg/ml for CS-sGO2 and CS-sGO3 respectively. Meanwhile, the chitosan concentrations per reaction volume were 5 mg/ml, 4.286 mg/ml, and 3.333 mg/ml for the samples. Overall, the concentration per reaction volume for both graphene oxide and chitosan are the highest for CS-sGO1. The density of chitosan conjugated on CS-sGO3 and Cs-sGO4 was relatively scarce, with only estimated 1.5% and 11.5% chitosan present. The scarcity of chitosan also materialized as poor dispersion stability at pH8 as shown in Figure 4.12, further enforcing the theory that steric stabilization is at work at high density of conjugated chitosan. The reduction in functionalized chitosan signified the divergence of the reaction from optimal conditions.

Figure 4.16A illustrated the TGA curves for Group A functionalized graphene oxides synthesized with fixed weight ratios while Figure 4.16B showed the curves for Group B functionalized graphene oxides. The thermal stability of CS-sGO had a direct relation to the percentage of conjugated chitosan, with higher conjugation degree having better thermal stability at high temperatures above 600°C, as can be seen for CS-sGO1 curve. It

is also noted that CS-sGO2 and CS-sGO3 underwent complete decomposition at a lower temperature of 650-700°C, hence taking a compositional analysis at temperature of 700°C may not be an accurate representation of the actual value. However, the estimation is taken at 700°C for all samples for consistency and comparison accuracy of the study.

Figure 4.16 Comparison of TGA curves between samples in Group A (A), and Group B (B) of chitosan-functionalized graphene oxides.

After taking DLS measurements, the samples in pH solutions, arranged in Figure 4.17, were observed for a month. As per reflected in particle size measurement, sample CS-sGO3 and CS-sGO5 exhibited lesser degree of aggregation in alkaline pH, portrayed in terms of smaller particle size. Visual observation of samples three hours and a day after pH adjustment showed quick sedimentation of dispersion in pH10, predictably at slower rate for samples CS-sGO3 and Cs-sGO5. Three days later, there was obvious sediments for all samples in pH8 solution due to the influence of conjugated chitosan. Within one week, even the samples in DI water dispersion showed signs of sedimentation. At the end of the one-month observation, samples CS-sGO3 and CS-sGO5 appeared to have undergone fewer changes, with minimal sedimentation. This phenomenon could be associated with the particle size effect where smaller particle sizes has better dispersion stability, since CS-sGO3 and CS-sGO5 has relatively smaller sizes in comparison to the other samples.⁶³ Chitosan conjugation on graphene oxide noticeably improved dispersion stability in pH environment as compared to graphene oxide dispersion that is only stable in DI water. (Refer Appendix Figure A.6 for pH dispersions of sGO). This advantage is more apparent at acidic pH due to electrostatic repulsion from amino groups protonation. At higher pH

however, there is intermolecular hydrogen bond attraction between neighbouring chitosan, shifting the dispersion stability towards a tendency to aggregate.⁵⁴

Observation of CS-sGO dispersion stability in DMEM, PBS and artificial urine after 3 hours following preparation, 1 day, 1 week and 1 month later were shown in Figure 4.18. The stability was much improved in contrast to that of graphene oxide in Appendix Figure A.6. Surprisingly, dispersion stability was not retained long, with obvious sediment detected within 1-3 days. As DMEM is comprised of a variety of components including amino acids, vitamins, and salts; the stabilization effect contributed by graphene oxide oxygenated groups and chitosan could be removed, causing charge screenings and loss of surface functionality.⁶⁴ Additionally, chitosan binds with protein via combination of electrostatics and hydrophobic interactions.⁶⁵ Sure enough, zeta potential measurement taken for sample CS-sGO5 after 3 hours in DMEM yielded a value of $-0.73 \pm 0.56 \text{ mV}$ despite having retained particle size at $299.5 \pm 2.7 \text{ nm}$. The zeta potential value verified that the CS-sGO samples do not possess colloidal stability in DMEM, resulting in gradual settling of the particles within 1-3 days. Based on the settling rates in Figure 4.18, sample CS-sGO2 and CS-sGO3 seemed to have greater stability in DMEM. It is speculated that increasing population of chitosan on CS-sGO surface generated electrostatic repulsion and steric stabilization effect in DMEM up till an inversion point where charge screening held a more dominant effect.

Figure 4.17 Images showing dispersions in various pH, in the order of pH3, pH5, pH6, pH7, pH8, and pH10 for samples CS-sGO1 (A), CS-sGO2 (B), CS-sGO3 (C), CS-sGO4 (D), and CS-sGO5 (E) taken after 3hours, 1day, 3 days, 1 week and 1 month.

Figure 4.18 Images showing dispersions in physiological media in the order of DMEM, PBS and artificial urine for CS-sGO1 (A), CS-sGO2 (B), CS-sGO3 (C), CS-sGO4 (D), and CS-sGO5 (E) taken after 3hours, 1day, 3 days, 1 week and 1 month.

Contrary to the better dispersibility in DMEM for less-conjugated CS-sGO, the inverse could be derived for the case of phosphate buffered saline and artificial urine. At day 3, there were undisputedly less sediment for sample CS-sGO1 and CS-sGO5 which

corresponded to higher degree of conjugation. It was also interesting to note that CS-sGO showed positive stability in artificial urine. This could be due to the presence of urea, which has been known to disrupt intramolecular hydrogen bonding and hydrophobic effects of chitosan, resulting in extended chitosan conformation.⁶⁶ The stability of CS-sGO in phosphate buffered saline are mainly an effect of pH, rather than ionic strength, as PBS has a molarity of 10mM and pH value of 7.4. Based on the previous zeta measurements at pH7 and pH8, the particles are relatively stable between these pH range. Hence, the stability in PBS is comparatively more superior than in other media, with minimal aggregation following long periods of incubation. Meanwhile, the effect of ionic strength dominated in artificial urine which is within the safe pH region of 6.3. Artificial urine is comprised of a mixture of multiple salts, which effectively increased the ionic strength of the solution. Although the contribution of protonated amine groups from chitosan is still considerable at pH 6.3, a high ionic strength will cause a neutralization effect that cancels out the electrostatic repulsion.⁶⁶ Therefore, CS-sGO1 and CS-sGO5 with higher chitosan grafting degree displayed greater dispersibility in artificial urine. The higher proportion of available protonated amine groups created an excess of protonated form even after charge neutralization from the salts in artificial urine. Nevertheless, dispersion stability in different media is a combined effect of surface chemistry, concentration of both dispersant and particle, pH, temperature and shear rate.⁶⁴ In fact in this context, even the starting material of the particle itself played an important role, considering that both graphene oxide and chitosan possessed a variety of functional groups. The molecular weight, degree of deacetylation and substitution level of chitosan are also accountable for any changes in dispersion stability of the functionalized graphene oxide.^{45, 67}

4.3.4 Carboxymethylcellulose grafting and functionalization studies

Following the graphene oxide 175nm starting size and volume of 1.25ml from chitosan functionalization studies in Section 4.3.3, carboxymethylcellulose functionalization of graphene oxide was executed based on the designed ratio of adipic acid dihydrazide to carboxymethylcellulose. Figure 4.19 outlined the pH stability and the as synthesized particle size, polydispersity, and zeta potential of the CMC-sGO. All samples have absolute

zeta potential values exceeding 30mV, signifying stable colloidal stability. However, CMC-sGO2, CMC-sGO5 and CMC-sGO6 exhibited particle sizes nearing 1 μ m range although having narrow polydispersity profiles. The particle size of these samples at pH3 was also not measurable by the Zetasizer due to extensive aggregation. From the six samples, only CMC-sGO3 had the dimension falling in the desired size range. The particle size and zeta potential of CMC-sGO remained relatively constant across changing pH as per Figure 4.20. Interestingly, CMC-sGO4 was having a broad pH stability that included all tested pH, with no significant changes in size from pH3 to pH10. This could be a direct influence of conjugated carboxymethylcellulose as the polymer is stable in the pH range of 2 to 10.⁶⁸ In addition, the zeta potential was lower for both CMC-sGO3 and CMC-sGO4, suggesting presence of free NH₂ groups as the functional group carries positive zeta potential.⁶⁹⁻⁷⁰ The presence of free amine groups unconjugated to CMC may have also caused the dispersion stability due to deprotonation at low pH.⁷¹

Referring to TEM images recorded in Figure 4.21, CMC-sGO1, CMC-sGO2, CMC-sGO5, and CMC-sGO6 appeared to be aggregated. There were interparticle attraction even at low concentration of 0.05mg/ml used for TEM samples preparation. It can be concluded that the density of both CMC and ADH as linker between sGO and CMC are crucial in final colloidal stability of CMC-sGO due to the tendency for interparticle attraction forces depending on the functional groups present.

Figure 4.19 Particle sizes of carboxymethylcellulose functionalized graphene oxide at varying pH and the tabulated particle size, polydispersity index, and zeta potential values of each sample as synthesized.

Figure 4.20 Changes in particle size and zeta potential of sample CMC-sGO3 at varying pH plotted as mean ± standard deviation from three measurements of three batch samples. The mean particle size is 297±53nm.

Figure 4.21 TEM images for CMC-sGO1 to CMC-sGO6 in the order(A-E).

FTIR absorbance peaks of CMC-sGO and sGO-ADH in Figure 4.22 confirmed the presence of both adipic acid dihydrazide and carboxymethylcellulose conjugation on sGO. Introduction of adipic acid dihydrazide linker to sGO showed complete occupation of carboxyl groups and a spike in absorbance peak representing carboxylate and amino groups at 1600cm^{-1} and 1640cm^{-1} .^{69, 7269} Even after further reaction with CMC, the amino group peak remained strong, specifying the presence of excess free -NH_2 groups that remained unreacted with CMC. The peak at 1490cm^{-1} attributed to amide II bond of hydrazide was present in CMC-sGO3 and CMC-sGO4, implying presence of free -NH_2 groups.⁷³ All CMC-sGO samples exhibited duplication of spectra peaks from CMC, as shown in Figure 4.23. Specifically, peaks 3210cm^{-1} , 2840cm^{-1} , 1420cm^{-1} , 1370cm^{-1} , and 1060cm^{-1} corresponds to stretching vibration of hydroxyl group, C-H group from -CH_2 of carboxymethylcellulose, asymmetric and symmetric vibration of carboxylates, and vibrational stretching of $\text{CH}_2\text{-O-CH}_2$ bonds, respectively.^{69, 74-75} The absorption band at 1260cm^{-1} refers to C-O stretching of alcohol with slight shifting from the peaks in sGO and CMC due to an overlapping effect and interparticle hydrogen bonds. The absorbance

spectra for CMC-sGO1 and CMC-sGO2 are plotted with the corresponding sGO-ADH1 and sGO-ADH2 in Appendix Figure A.9. The trend in sGO-ADH1 and sGO-ADH2 did not differ much from that of sGO-ADH3 except for a slight shifting of peaks to lower wavenumber, indicating changes in interparticle attractive forces from the different density of functional groups.

Figure 4.22 FTIR absorbance spectra for graphene oxide, hydrazide functionalized graphene oxide, sodium carboxymethylcellulose and carboxymethylcellulose-functionalized graphene oxides.

Figure 4.23 FTIR absorbance spectra for all carboxymethylcellulose-functionalized graphene oxide samples showing negligible deviations between the samples.

TGA decomposition curves for sGO, GO-ADH, CMC and CMC-sGO3 were as plotted in Figure 4.24. The TGA curves for other samples are included in Appendix Figure A.10 as reference. The decomposition curve of sGO-ADH followed that of sGO before 500°C but had slower weight loss from 500-700°C. Sodium carboxymethylcellulose has a very similar thermal decomposition behaviour with chitosan. The onset of rapid weight loss occurs around 270°C due to decomposition of the polymeric chain and pyranose ring.³⁶⁻³⁷ The weight loss trend of CMC-sGO follows closely to that of CMC between 300 to 550°C, further verifying the presence of conjugated CMC. Taking reference of the temperature for complete decomposition of sGO at 700°C, the weight losses of sGO, CMC, and CMC-sGO is analyzed and the grafted polymer CMC-sGO is estimated in Table 4.7. The presence of adipic acid dihydrazide was excluded from the estimation calculation assuming negligible effect from cross-linker in overall thermal decomposition weight loss in CMC-sGO. Analysing the percentage functionalized carboxymethylcellulose with synthesized size and dispersion stability, it can be inferred that dispersion stability for CMC-sGO exists when there is a population of free -NH₂ groups and conjugated polymer. Hence, the key lies in

striking a balance in ensuring even carboxymethylcellulose functionalization among all graphene oxide sheets after populating -NH_2 groups on the surface.

Figure 4.24 TGA curves showing weight per temperature change of graphene oxide, sodium carboxymethylcellulose, hydrazide-functionalized graphene oxide and CMC-sGO3.

Table 4.7 Estimated weight percentages of carboxymethylcellulose and graphene oxide in sample CMC-sGO1, CS-sGO2, CS-sGO3, CS-sGO4 and CS-sGO5.

Sample	Weight ratio (ADH:CMC)	Weight percentage of CMC (%)	Weight percentage of sGO (%)
CMC-sGO1	1:2.5	11.4	88.6
CMC-sGO2	1:4:1	54.9	45.1
CMC-sGO3	3.5:1	22.0	78.0
CMC-sGO4	3.8:1	8.1	91.9
CMC-sGO5	4.4:1	3.5	96.5
CMC-sGO6	8.7:1	10.2	89.8

Images of the CMC-sGO in pH solutions and physiological media were taken 3 hours after preparation and after standing for one day as shown in Figure 4.25. In contrast to CS-sGO, the samples have better stability in alkaline instead of acidic condition, with most samples aggregating within one day in pH3. CMC-sGO3 and CMC-sGO4 exhibited prolonged stability in all tested pH up to one week with small scale sedimentation. In correlation to measured particle size at varying pH, CMC-sGO4 was found to be stable in all pH, showing good dispersion up till 30 days. Similar to chitosan functionalization, carboxymethylcellulose functionalization improved pH stability of graphene oxide substantially.

Figure 4.25 Images showing dispersions in various pH, in the order of pH3, pH5, pH6, pH7, pH8, and pH10 for samples CMC-sGO1 to CMC-sGO6 (A-F) taken after 3hours, 1day, 3 days, 1 week and 1 month.

The dispersion stability of CMC-sGO3 and CMC-sGO4 in DMEM, PBS and AU was observed over the course of one month. Other samples of CMC-sGO were not studied due to their relatively large sizes. Based on Figure 4.26, CMC-sGO3 appeared to retain good dispersion stability in all physiological media up to one week, while CMC-sGO4 started to show considerable sedimentation in DMEM and PBS within three days. Overall, CMC-sGO exhibited best stability in artificial urine, which most probably is contributed by pH

stability and steric hindrance from functionalized carboxymethylcellulose. The mechanisms will be discussed in further details in Chapter 5.

Figure 4.26 Images showing dispersions in physiological media in the order of DMEM, PBS and artificial urine for CMC-sGO3 (A), and CMC-sGO4 (B) taken after 3hours, 1day, 3 days, 1 week and 1 month.

4.3.5 Mucoadhesive property of polysaccharide-functionalized graphene oxide

The mucoadhesive property of the CS-sGO and CMC-sGO was tested via the mucin-particle method. The mucin-particle method identifies the mucoadhesive properties of particles by detecting the change of particle size and zeta potential after contact with mucin. Polymer mucoadhesion to mucin modifies the surface properties of mucin, leading to a change in zeta potential. The level of zeta potential variation however, is influenced by polymer concentration, molecular weight as well as affinity with mucin.⁷⁶ When zeta potential values remain unchanged, the mucoadhesive affinity is defined as poor.⁴¹ Changes to particle size are mainly an indication of aggregation triggered from interaction with mucin.

The mucoadhesion test was investigated for samples CS-sGO1, CS-sGO2, CS-sGO3, CS-sGO4, CS-sGO5, CMC-sGO3, and CMC-sGO4. Other samples of carboxymethylcellulose-functionalized graphene oxide were not included due to the large particle sizes. Preliminary test on samples from Section 4.3.2 showed that the extend of increase for particle size and zeta potential values after mixing with mucin in DI water is correlated to the percentage of chitosan present. The increase in particle size was negligible

for functionalized graphene oxide that do not exceed a certain degree of chitosan conjugation. (Refer Appendix Table A.1 for preliminary data of mucin particle method in DI water) Hence, the mucoadhesive test is conducted with mucin in DI water to simultaneously confirm the density of mucoadhesive polymers present to facilitate selection of the final CS-sGO and CMC-sGO

The change in particle size after introducing nanoparticle samples into mucin in DI in Figure 4.27 showed general increase except for sGO and CMC-sGO4. The negligible size increase in sGO is expected as it was hypothesized that graphene oxide does not possess mucoadhesion property. The unresponsiveness of CMC-sGO4 to mucin could be due to hindrance from hydrogen bonding of free $-NH_2$ groups. Interparticle hydrogen bonding would have removed participation of carboxymethylcellulose and $-NH_2$ groups from interacting with mucin.

Figure 4.27 Outcome of mucin-particle method showing changes in particle size after interaction with mucin

Figure 4.28 plots the original zeta potentials of samples adjacent to the values after interacting with mucin. The samples exhibited marked change in zeta potential after mucin interaction, except for CMC-sGO4. Notably, the change in zeta potential for sodium carboxymethylcellulose were minimal and the great size increase measured in Figure 4.27 was attributed to the size of polymer network in water. This further reinforced the conception that the action of hydrogen bonding suppressed the originally weakly adhesive carboxymethylcellulose from performing. On the other hand, sGO sample exhibited great increase in zeta potential. Again, this is associated with the effect of hydrogen bonding and hydrophobic interactions between similar functional groups on graphene oxide and mucin, such as hydroxyl and carboxylic groups.⁷⁷⁻⁷⁸ This also implied that graphene oxide does have affinity to mucin, but may not be strong enough to be mucoadhesive.

Figure 4.28 Outcome of mucin-particle method showing zeta potential of samples before and after interaction with mucin.

Mucoadhesive interaction between chitosan and mucin has been reported to be largely governed by electrostatic attraction when in acidic media due to the effect of chitosan

protonation.⁷⁹ Hence, it is assumed that since mucin-particle method is conducted in DI water, there is negligible protonation of chitosan amino groups and the mucoadhesive mechanism is mainly attributed to hydrogen bonding and hydrophobic attractions.⁸⁰ All chitosan functionalized graphene oxide samples exhibited good mucoadhesive properties in terms of high increase in particle size and change in zeta potential values. As per analysis from Appendix Figure A.9, high density of functionalized graphene oxide results in a smaller zeta potential value after mucin interaction. Incidentally, CS-sGO1 and CS-sGO5 with greater percentage of functionalized chitosan returned a smaller zeta potential compared to the rest. In view of the mucoadhesion behavior portrayed by chitosan-functionalized graphene oxide, careful tuning of grafted chitosan will realize the potential for mucoadhesive applications in a variety of context. As it happens, for optimal bioadhesion there exists an effective concentration where mucoadhesion strength diminishes below and above the value.¹⁶ At highly concentrated solution, the polymer will be in coiled configuration due to solvent deficit, resulting in less available chain length for interpenetration mechanism of mucoadhesion. Therefore, the proportion of active bioadhesive component present is critical to good mucoadhesion properties.

4.4 Summary

The optimized synthesis method for both chitosan and carboxymethylcellulose functionalized graphene oxide has been established. The stability in pH and physiological conditions as well as mucoadhesive property of synthesized CS-sGO and CMC-sGO is heavily influenced by the degree of polymer functionalization. Controlling the degree of functionalization is achievable via modulation of the proportion of graphene oxide, linker and polymers within reaction. Effectively, the higher the concentration of both graphene oxide and polymer present in the synthesis, the greater the degree of functionalization and correspondingly, the greater the particle stability and mucoadhesion stability. The dispersion of CS-sGO in cell culture medium however, is the only exception whereby higher degree of functionalization result in decrease in stability. From the study, CS-sGO5 and CMC-sGO3 with satisfactory particle size exhibited good mucoadhesive property, and great dispersion stability in wide range of pH and physiological conditions. CS-sGO5 and

CMC-sGO3 are therefore selected for subsequent study and will be denoted as CS-sGO and CMC-sGO from the next chapter onwards.

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Chapter 5* Polysaccharide-Functionalized Graphene Oxide Stability, Cytocompatibility and Drug Delivery

The behavior and interaction with intended environment are crucial for the optimized functional performance of a drug carrier. Investigating the responses in vitro provides an insight of what could transpire in actual setting. Hence, extended study of dispersion stability and mucoadhesion property in a few simulated environments is explored. To ensure safe induction to living tissues, cytocompatibility of synthesized chitosan functionalized graphene oxide (CS-sGO) and carboxymethylcellulose functionalized graphene oxide (CMC-sGO) is evaluated in vitro with L929 mouse fibroblast cell line. Finally, the functional competency of the nanocarriers, i.e. the drug loading and release capability is assessed. The stable and mucoadhesive nanoparticles loaded the model drug, camptothecin (CPT) effectively with controlled releases in physiological environment. Both particles demonstrated minimal cytotoxicity effect up to a concentration of 50 µg/ml.

5.1 Introduction

The pH-sensitivity of chitosan and carboxymethylcellulose has been exploited greatly in the drive to fabricate pH responsive drug carriers.¹⁻³ The amino groups of chitosan deprotonates at pH lower than pH6⁴ while above pH 2 carboxylic groups on carboxymethylcellulose deprotonates^{1,5}. These pH-triggered change in conformation of polymeric drugs facilitates protection of encapsulated drug molecules and subsequent release based on pH environment of targeted site. In the previous chapter, the pH stability of synthesized chitosan and carboxymethylcellulose functionalized graphene oxide has been demonstrated. Both displayed stability in the pH window from pH5 to pH8, which is the expected range in normal bladder environment. To establish the nanoparticles as drug delivery systems, the drug loading and release capability is primary. Secondly, the drug carriers must not be harmful to living cells.

Graphene oxide introduction to cells is related to size and dose dependent oxidative stress that decreases cell viability.⁶⁻¹¹ Functionalized graphene oxide, on the other hand, generally reports improved biocompatibility compared to the starting material, graphene oxide.¹²⁻¹³ The association with hydrophilic polymers leads to improved cell adhesion and proliferation.¹¹ Enhanced cell viability of chitosan grafted graphene oxide is attributed to occupancy of graphene oxide active edges accountable for damaging cellular membrane.¹⁴ The cell adhesion, proliferation and growth of osteoblasts on chitosan functionalized graphene oxide scaffold was also found to be superior to chitosan scaffold.¹⁵ Meanwhile, L929 cells proliferation were enhanced in esterified sodium carboxymethylcellulose hydrogel, suggesting the advantageous role of carboxymethylcellulose in cellular growth.⁵ As such, chitosan and carboxymethylcellulose functionalization is expected to improve the cytocompatibility of graphene oxide.

Basing upon literature, graphene oxide has a high loading capacity of drugs mainly via the π - π stacking attraction and supplemented by hydrogen bonding. Drug loading via π - π stacking interactions contributes to slower and sustained release as compared to

conventional drug release by matrix diffusion, as the π bonds restrict the tendency for quick release in a medium.¹⁶ In the treatment of bladder cancer, lipophilic drugs are postulated as having greater effectiveness in intravesical therapy due to the affinity to urothelium which enhances penetration and tissue localization.¹⁷ However, the efficient delivery of these drugs has always been a challenge due to the limited solubility. Hence, in the interest of concurrently exploring the potential of the nanoparticles in improving drug delivery of lipophilic drugs, the water insoluble camptothecin is selected as model drug to assess the drug loading and release capability of CS-sGO and CMC-sGO.

Analysis of mucoadhesion strength is strongly influenced by the pH and type of hydration buffer used. When detachment forces of polymeric film from pig bladder is determined with pH4.5 phosphate buffer, equivalent mucoadhesion strength was derived for both chitosan and carboxymethylcellulose films.¹⁸ Conducting similar study with pH7.4 Tyrode buffer however, returned a conflicting conclusion with chitosan films showing higher detachment forces.¹⁹ The degree of ionisation and subsequent interaction of mucoadhesive polymers with mucus therefore, varies with pH environment and presence of ionising components. With this factor in consideration, mucin particle study is further expanded to include mucin in different physiological buffers, including artificial urine.

On that note, although dispersion stability of CS-sGO and CMC-sGO in physiological media was investigated for one month, the study was by visual observation. The evaluation of cytocompatibility, drug release and mucoadhesion in a variety of buffers all involved prolonged immersion in media of up to four days. Hence, to avoid experimental misinterpretation arising from undetected aggregation of nanoparticles, the particle size, polydispersity, and zeta potential of nanoparticles in physiological media is monitored prior to the execution of other studies. The actual particulate state in the different environment can thus be gauged, allowing predictions of responses in biological environments.

5.2 Materials and Methods

5.2.1 Materials

(S)-(+)-Camptothecin powder ($\geq 90\%$), phosphate buffered saline tablets (pH7.4) and mucin from porcine stomach (type III, bound sialic acid 0.5-1.5%, partially purified powder) were obtained from Aldrich. L929 murine fibroblast cell lines were procured from ATCC. Dulbecco's modified eagle medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were purchased from Gibco BRL (Gaithersburg, MD). Other laboratory chemicals and reagents used for experimental study were purchased from Aldrich and used as received. Mili-Q ultrapure water ($>18\text{M}\Omega$) is used for preparation of all buffers.

5.2.2 Methods

CS-sGO is prepared based on the synthesis method of CS-sGO5 in Chapter 4.3.3. 1.25ml sonicated graphene oxide sized approximately $170\pm 10\text{nm}$ were reacted with 25mg chitosan in pH5 adjusted MES buffer, assisted by EDC and NHS. Synthesis of CMC-sGO is based on the methods outlined for CMC-sGO3 in Chapter 4.3.4. 1.25ml of sonicated graphene oxide were first conjugated with 87mg adipic acid dihydrazide before amidation reaction with 25mg sodium carboxymethylcellulose in pH6 adjusted MES buffer. The synthesized size of CS-sGO and CMC-sGO are $297\pm 19\text{nm}$ and $297\pm 53\text{nm}$ respectively.

Dispersions stability study was conducted as detailed in Chapter 3.7. sGO, CS-sGO and CMC-sGO dispersions (0.5mg/ml) were added to equal volume of DMEM, PB, PBS and AU. The change in size, polydispersity index, and zeta potential were monitored across 4 days and physical observations recorded as digital images. Tested sGO was pre-sonicated to approximately $170\pm 10\text{nm}$.

Mucin-particle method was analyzed with 0.6% w/v mucin suspended in 10mM acetate buffer solution (AB), Mili-Q water (DI), phosphate buffered solution (PBS) and artificial urine (AU) as per Chapter 3.10.

Cell viability assay and drug loading and release study of sGO, CS-sGO and CMC-sGO take reference from Chapter 3.8 and Chapter 3.9, respectively.

5.3 Results and Discussion

5.3.1 Dispersion stability in physiological condition

The stability of CS-sGO and CMC-sGO in physiological media was investigated over a 4-day period, considering the interaction with associated media that will be used in the consecutive studies. The study was conducted at room temperature to allow direct comparison with the results from pH stability study in Section 4.3.3 and 4.3.4 for determination of aggregation trigger by either pH condition or media constituents. CS-sGO and CMC-sGO is added into cell culture media (DMEM), pH5 phosphate buffer (PB), pH7.4 phosphate buffered saline (PBS) and pH 6.3 artificial urine (AU). The mixtures are observed for 4 days and DLS measurements of particle size were taken 3hours, 1 day and 3 days after addition as outlined in Figure 5.1. Generally, graphene oxide aggregation is induced in solutions with high salt and protein content due to screening of electrostatic charges and non-specific binding of protein on surface.²⁰⁻²² Graphene oxide was found to aggregate almost instantly upon introduction into physiological media, except for DMEM. With the exemption of pH7.4 PBS, CS-sGO were relatively stable in testing media. CS-sGO exhibited substantial increase in particle size after one day in PBS. This is correlated to surface charge neutralization by salt ions.²² It can be concluded that CS-sGO were able to retain its size in physiological media in the order of DMEM>PB>AU>PBS. The pH of media appears to contribute to the dispersion stability, as the order of dispersion stability is in parallel to increasing pH values. In addition, the pH value of 7.4 for PBS falls outside the range for chitosan solubility which leads to chains contraction and aggregation. CMC-sGO however, appears to have satisfactory stability in all physiological media, with gradual increase in particle size over time. Based on the change in particle size, the stability can be defined in decreasing order of PB> PBS> DMEM>AU. The stability of CMC-sGO in physiological media is mostly a consequence of steric hindrance from free -NH₂ ends

and long CMC chains. Typically, ionization of carboxyl group in CMC that occurs from pH1.2 to pH7.4 promotes electrostatic repulsion and hence, enhances the dispersion stability.^{2, 23} The stabilization effect however is suppressed in the presence of low molecular weight ions, resulting in electrostatic shielding and less expanded conformation of CMC chains²⁴, explaining the perceived instability in artificial urine.

The aggregation induced by physiological media is correlated to the oxidation degree of graphene oxide. Greater oxidation results in higher percentage of anionic moiety that confer superior dispersion stability, even for higher molarity phosphate buffered saline and 10% FBS in MEM cell culture medium.²⁵ Hence, it is inferred that the atypical dispersion stability in PBS of sGO is also related to the oxidation degree of the native product. As it happens, the observed change in pH stability as graphene oxide underwent slight reduction via sonication process (Appendix Figure A.3), verified the role of oxygenated functional groups densities in overall dispersion stability behaviour. In contrast, phase separation of CS-sGO in cell culture medium was observed within one day. The instability of CS-sGO in DMEM is confirmed via polydispersity index plotted in Appendix Figure B.1(A) that increases with time. Bridging flocculation by chitosan could be at play here resulting from charge neutralization and electrostatic screening effect with negatively charged proteins.²⁶ Furthermore, the zeta potential of CS-sGO was close to isoelectric point in DMEM. (Refer Appendix Table B.1) The great decrease in zeta potential values of CS-sGO in DMEM as compared to CMC-sGO also implied greater level of FBS protein absorption.²⁷ As for negatively charged CMC-sGO, protein absorption would be non-specific, hence conferring better colloidal stability.²⁶

Figure 5.1 Digital images and particle sizes of sGO, CS-sGO, and CMC-sGO 3hours, 1 day and 3 days after addition into DMEM (A,B), pH5 phosphate buffer (C,D), pH7.4 phosphate buffered saline (E,F) ,and pH6.3 artificial urine (G,H) as compared to original conditions at 0hr.

CS-sGO and CMC-sGO dispersion in AU showed a gradual increase in particle size with time from Figure 5.1H, and a time dependent decrease in zeta potential as tabulated in Appendix Table B.1. Generally, the absolute value of zeta potential decreases after

addition of physiological media due to reduction of free surface charge from the interaction with salts and proteins in media, the effect being particularly significant with high valency ions.²⁸ Accordingly, the polydispersity index changes in Appendix Figure B.1 also showed deviations after one day. It can hence be concluded that CS-sGO and CMC-sGO has temporal stability in artificial urine and hence predictably in actual bladder environment. However, the perturbation to stability is gradual and therefore should not pose an immediate functional disturbance to the nanoparticles upon intravesical introduction. As per consistency in measured nanoparticles sizes when stable in tested physiological media, the zeta potential values remained almost unchanged with minimal deviation from the values of original aqueous dispersion for the samples that retained colloidal stability.

5.3.2 Elucidation of the mucoadhesion mechanisms

Mucoadhesion occurs mainly via electrostatic attractions, hydrogen bonding, Van der Waals and hydrophobic forces.^{18, 29} In consideration of the effects of hydration buffers, the pH, and test volume used on mucoadhesion, mucin-particle study was conducted in 4 different hydration buffers. Preincubation of sGO, CS-sGO, and CMC-sGO in acetate buffer was executed to confirm no contribution to aggregation of the nanoparticles. (Refer Appendix Table B.2 for particle size of nanoparticles in hydration buffers) The previous study of dispersion stability has already established insignificant effect of PBS and AU on change in particle size within three hours, except for sGO in AU. Therefore, excluding interactions of sGO with mucin in AU, the particle size changes of other samples are direct effects of mucin interactions.

In Figure 5.2, all samples exhibited increase in size after introduction to mucin in buffers, with sGO and CMC-sGO having minimum particle size change. This is in line with hypothesis where sGO is not expected to have any mucoadhesion properties. The deviation in size will be primarily caused by dispersion instability in buffer, leading to aggregation, as per media dispersion stability section above. The almost negligible size increase of CMC-sGO in all buffers however, is a little surprising, considering that pure

CMC performed appreciably with detectable increase in particle size in all buffers. This observation is speculated to be caused by a smaller density of CMC molecules present on CMC-sGO, resulting in ineffective detection of mucoadhesion property. Lehr and co-workers³⁰ has also reported lack of mucoadhesive property from carboxymethylcellulose when detachment force of polymeric film from pig intestinal mucosa was investigated. Other research cited same charge repulsion as the negligible interaction of CMC with mucin.³¹

Figure 5.2 Particle size and zeta potential measurement of mucin interaction with CS, CMC, sGO, CS-sGO, and CMC-sGO with deionized water, pH4.5 acetate buffer, pH7.4 phosphate buffered saline and pH6.3 artificial urine.

Examining changes in zeta potential gained much clearer comparisons. There were significant changes in zeta potentials except for sGO in all buffers and CS-sGO in artificial urine. Mucin interaction in water portrayed interactions mostly via hydrophobic attractions and hydrogen bonding.³²⁻³³ CMC and CMC-sGO showed changes to zeta potential, confirming the role of the forces in instigating mucoadhesion. The analysis for CS and CS-sGO mucoadhesion in DI however, is limited by aqueous solubility. In acetate buffer solution at pH4.5, mucin is fully hydrated and protonated

chitosan will be in extended conformation, hence the electrostatic attraction effect dominates. On the contrary, hydrophobic attraction will be at work for CMC in compact coil arrangement.²³ Sure enough, CS and CS-sGO had the greatest change in zeta potential from a negative value to positive value. CMC and CMC-sGO also showed substantial decrease in zeta potential. Due to the presence of free -NH₂ groups in the synthesized CMC-sGO, electrostatic attraction is also a mucoadhesion contributor in acetate buffer.

The effect of pH on mucoadhesion is evaluated by using PBS as hydration buffer. In PBS, mucoadhesion may not be of significance due to insolubility of chitosan. However, CMC deprotonates in alkaline environment resulting in electrostatic repulsion between polymers. This in turn hinders mucin interactions via electrostatic route, but interaction via other methods can occur. In addition, mucin also holds a negative charge, adding to the repulsion forces. Hence the only possible interaction in PBS will be by hydrophobic forces. Thus, it can be concluded that hydrophobic forces do contribute to mucoadhesion of CMC and in turn CMC-sGO too, as decrease of zeta potential values were detected. The presence of urea and sodium chloride in artificial urine can effectively hinder electrostatic interactions²⁹, hence mucoadhesion will be mainly instigated by hydrogen bonding and hydrophobic interaction. For CS and CS-sGO, the charge screening effect facilitates the detection of mucoadhesion via hydrogen bonding and hydrophobic forces. The minimal change observed suggests that the contribution by these two forces are small. Considering that both mucin and CMC are negatively charged, attraction forces due to hydrogen bonding needs to be strong to overcome the electrostatic repulsions for mucoadhesion to occur. Presence of salts will subdue electrostatic repulsion effect, allowing hydrogen bonds to have greater effect in interacting with mucin particles. Hence for CMC and CMC-sGO, the interaction indicated by change in zeta potential is associated with mucoadhesion by hydrogen bonding.

Summarising the results from zeta potential changes, mucoadhesion property of chitosan and accordingly CS-sGO, is mainly by electrostatic attraction, supported by hydrogen bonds and hydrophobic interactions. This is in line with literature, where electrostatic

forces is driven by protonated amines, hydrophobic interactions via acetyl group, and hydrogen bonding occurs between hydroxyl and amine groups.^{29, 34} CMC and CMC-sGO on the other hand interacts with mucin predominantly via hydrogen bonding between carboxylic group with oligosaccharide chains of mucin³⁵, and least by electrostatic forces and hydrophobic forces^{18, 36}. The correlation of zeta potential to mucoadhesive strength was described to be relative to the negativity of zeta potential. The more negative zeta potential results in greater repulsion forces with negatively charged glycoaminoglycans, hence the weaker the mucoadhesion force.³⁷⁻³⁸ Seeing that the zeta potential of CMC-sGO was at -30.1 ± 2.2 mV as opposed to the positive value of CS-sGO, it can be concluded that the mucoadhesion strength of CMC-sGO is inferior to CS-sGO. A study of microsphere incorporated separately with chitosan, sodium carboxymethylcellulose and polycarboxophil and the respective mucoadhesive drug release in pig bladder, agreed that chitosan has greater mucoadhesion interaction than sodium carboxymethylcellulose.¹⁹ Other studies have also indicated weak synergism with mucin³⁹, as well as transient and low strength adhesion to mucus.^{30, 40} Additional, the influence of polymer concentration present should not be ignored.

5.3.3 Cytocompatibility of polysaccharide-functionalized graphene oxide

L929 mouse fibroblast cells were used to evaluate the cytocompatibility of sGO, CS-sGO, and CMC-sGO to normal healthy cells. Treatment of cells to the nanoparticles in increasing concentration up to 100 µg/ml did not display any remarkable changes to cellular morphology as summarized in Appendix Figure B.2. (Refer Appendix Figure B.2 for images captured at day1 and day3 at varied treatment concentrations) The cellular morphology at treatment concentration of 100 µg/ml as compared to control (culture medium only) after 3 days incubation is outlined in Figure 5.3. Dark silhouettes representing nanoparticles sediments can be visually detected in images captured for cell sample treated with CS-sGO in Figure 5.3D. This is in line with dispersions stability study in Section 5.3.1 that revealed CS-sGO instability and sediment from DMEM within one day. Similar observation was not found for cell samples treated with sGO

and CMC-sGO, signifying stable particle dispersion in DMEM as well-dispersed graphene oxide dispersion are not discernible via light microscopy when unstained.⁴¹

Introduction of sGO, CS-sGO and CMC-sGO concomitantly induced a change in cell morphology into more elongated structures with long protrusions, which has been attributed to cell proliferation and migration.⁴² Similar morphology was also reported for primary bone marrow derived macrophages (BMDMs)⁴³ and mouse myoblasts (C2C12)⁴⁴ where graphene oxide treated cells causes elongated protrusions. It was suggested that the morphological change was induced by wrinkled surface nanotopography of the graphene oxide.⁴⁴ Another group suggested the role of pendant hydroxyl groups on graphene oxide acting as cell adhesion sites.¹⁵ The elongated morphology became more apparent for higher concentration of all samples on Day 3. Investigation of the cellular morphology in Figure 5.3D showed extend of protrusion elongation in the order of CMC-sGO>sGO>CS-sGO.

Figure 5.3 Cell viability of L929 24hours and 72hours after incubation with sGO, CS-sGO, and CMC-sGO at concentration of (A) 10µg/ml, (B) 50µg/ml, and (C) 100µg/ml, and (D) morphology of L929 cells treated with medium only as control and 100µg/ml of sGO, CS-sGO, and CMC-sGO. Significant difference from control is determined using one-way ANOVA with Bonferroni test, * representing $p < 0.05$.

Figure 5.3(A-C) outlined the cell viability at 24 hours and 72 hours interval after incubation with varying concentration of sGO, CS-sGO and CMC-sGO. At a concentration of 10 μ g/ml, sGO and CMC-sGO caused a minimal decrease in cell viability at day 1 at which the cells recovered 2 days later. Chitosan functionalized graphene oxide did not cause any perturbation to cell viability at this concentration. sGO at a concentration of 50 μ g/ml however displayed a substantial increase in cell viability at day 1 that is accompanied by reduced cell viability at day 3. This is associated with the increased metabolic activity that is caused by oxidative stress induced by graphene oxide. Since the CCK-8 assay derives cell viability via cells metabolic activity, the increase in metabolic activity due to ROS generation will cause a misrepresentation of viability data. Lammel and group has reported similar elevated fluorescence intensity via alamarBlue assay and associated the increase in metabolic activity to intracellular ROS generation from mitochondrial membrane depolarization⁴⁵ As it happens, great increase of ROS level was reported when cells were treated with graphene oxide at concentration above 50mg/ml.^{7, 46} Interestingly, 50 μ g/ml of CS-sGO exhibited a slight increase in cell viability after 72hours where cell viability equivalent to control was observed at Day 1. CMC-sGO however, cause a decrease in cell viability 3 days after treatment at this concentration. At 100 μ g/ml, all samples displayed signs of oxidative stress 24hours after incubation in the form of high levels of metabolic activity, with CS-sGO and CMC-sGO treatment showing recovered cell viability after 72hours. These results are in agreement with literature where no significant effect on cell viability was detected for GO tested on various cell lines up to 20-30 μ g/ml.⁴⁷⁻⁴⁹ Cytotoxicity effect of graphene oxide on human fibroblast cells (HDF) showed cytotoxicity at concentration above 50 μ g/ml.⁵⁰ At 24hrs, A549 treated with graphene oxide nanosheets showed decreased cell viability of approximately 20%, which closely matched with plotted data. The decrease of cell viability is associated with interrupted cell cycles resulting in lower proliferation rates. At 100 μ g/ml, folic acid-chitosan functionalized graphene oxide and pegylated nanographene oxide caused only slight decrease in cell viability.⁵¹⁻⁵³

In summary, CS-sGO did not have any cytotoxicity effect up to a concentration of 50 μ g/ml. At 100 μ g/ml, CS-sGO started to induce oxidative stress to L929 cells in the

first 24 hours but the effect did not cause any disruption to cell viability at day 3. CMC-sGO started showing time-dependent decrease in cell viability at concentration of 50 µg/ml with recoverable oxidative stress effect at higher concentration. In contrast, sGO started to exhibit oxidative stress disruption to cell viability at concentration of 50 µg/ml. These results validated that functionalization mitigated the cytotoxicity of graphene oxide, with chitosan outdoing carboxymethylcellulose. The underperforming CMC-sGO could be associated with the comparatively less percentage of conjugated carboxymethylcellulose on graphene oxide as well as the influence of free -NH₂ groups present. The presence of amine groups on aminated graphene oxide has been associated with cell deformation and loss of cellular compartment due to high attraction to cell membranes.⁵⁴

5.3.4 Drug loading and release capability of polysaccharide-functionalized graphene oxide

Loading capacity and efficiency of camptothecin on CS-sGO at various CS-sGO:CPT ratio is as illustrated in Figure 5.4A. The weight ratio of 1:1 is selected for subsequent drug loading due to the highest loading capacity achieved at acceptable loading efficiency. UV absorbance of samples at 369 nm is taken as percentage of CPT loaded based on pre-obtained calibration curve and subtraction of background absorbance contributed by graphene oxide. Figure 5.4B shows the UV absorbance spectra of the samples with obvious peak at the wavelength of 369 nm for CPT, sGO-CPT, CS-sGO-CPT and CMC-sGO-CPT, confirming successful loading of CPT. CPT loaded graphene oxide sheets report an averaged drug loading capacity of 18% with loading efficiency of 6.3%. Both CS-sGO and CMC-sGO was able to load a higher percentage of CPT with loading capacity of 27% and 35% while loading efficiency was 14.5% and 26.2%, respectively. The loading capacity is higher than other similarly CPT loading on graphene oxide-based nanocarriers which reported 15.6% for poly(2-(diethylamino) ethylmethacrylate functionalized graphene oxide (GO-PDEA) and 20% for cited chitosan-functionalized graphene oxide (GO-CS). When compared with other bladder cancer drug delivery systems, the loading capacities of Cs-sGO and CMC-sGO lies in

the higher end as most has a value below 20%, with a rare few reporting high loading capacity of above 50%.⁵⁵⁻⁵⁷

Table 5.1 Drug loading capacity and loading efficiency of sGO, CS-sGO, and CMC-sGO.

Sample	Loading Capacity (%)	Loading Efficiency (%)
sGO-CPT	18	6.3
CS-sGO-CPT	27	14.5
CMC-sGO-CPT	35	26.2

CPT releases in physiological media pH5.5 phosphate buffer (Figure 5.4C), pH7.4 phosphate buffered saline (Figure 5.4D) and pH6.3 artificial urine (Figure 5.4E) were studied for sGO-CPT, CS-sGO-CPT, and CMC-sGO-CPT. All samples released CPT rapidly in the first 10 hours, before slowing down and reaching a plateau after 24hours. sGO-CPT exhibited drug release rate in increasing order of PB>AU>PBS, which is in the order of increasing pH. Mechanism of drug release for graphene oxide absorbed drugs is directly linked to the protonation of functionalized polymer and drug molecules. The weakening of π interactions and hydrogen bonding causes displacement and subsequent release of drugs.⁵⁸ Relatively faster and higher cumulative drug release of graphene oxide-based nanocarrier in acidic pH has conventionally been reported as a consequence of weakening of pi-pi stacking interaction and disruption of hydrogen bonding between drug and carrier.^{21, 59-61} The phenomenon is even observed in similar CPT loaded functionalized graphene oxide.⁶²⁻⁶³ In addition, CPT has greater solubility at acidic pH due to nitrogen protonation of its ring structure.²¹ The CMC and CS conjugated graphene oxide in this study however, deviated from the pH-dependent drug release paradigm. Similar trend has been reported for non-covalently loaded paclitaxel on chitosan with increasing cumulative drug release as pH of release medium increases.⁶⁴ It is hence possible that CPT was loaded on both graphene oxide and polymer surfaces although Karnati and co-workers reported drug binding preference for graphitic region rather than chitosan.⁵⁸

Interestingly, CS-sGO-CPT has the highest rate and cumulative release in artificial urine, but otherwise showed similar release profile to sGO and CMC-sGO in PB and PBS. Drug release profile of CMC-sGO did not show a drastic increase in lower pH, but cumulative release followed the order of PBS>PB>AU. This could be related to the compact coils conformation of CMC in acidic conditions which may hinder smooth drug release.⁶⁵ Furthermore, the presence of free NH₂ groups that were unbonded to carboxymethylcellulose in protonated configuration could be another contributing barricade of drug passage to release media. Overall, the rate of release is slowest for sGO-CPT and CMC-sGO-CPT in PB and AU.

Comparing the releases of the three nanoparticles across the three media, percentage releases of CS-sGO-CPT are highest for all media after 72 hours release, with approximately 40% for both pH5.5 phosphate buffer and pH7.4 phosphate buffered saline, and 75% in pH6.3 artificial urine. In all media, percentage drug release is similar for CMC-sGO-CPT and sGO-CPT, with values between 30-35%. The divergence of drug release behaviour of CS-sGO-CPT could be attributed to the presence of hydrogen bond breaker, urea in artificial urine that is linked to disruption of hydrophobic interactions as well as structural stability in biopolymers. Urea addition broadens the pH solubility window by acting as co-solvent to chitosan via hydrogen bonding.⁴ This could result in extended conformation of chitosan chains that cleared up the route for drug release into media.

Overall, the drug release profiles were not very significantly different across the different release media. Except for CS-sGO-CPT drug release in artificial urine, the samples did not reach cumulative drug release beyond 50% at the end of 72hours. The interaction forces between drug and sGO appeared to be stronger than perturbation by pH media for releases. This again, reinforced the theory that CPT hydrogen bonding exists with the chitosan chains on CS-sGO, and perturbation from urea allowed substantial release of CPT linked to chitosan. The influence of physical obstruction from long polymeric chains may have played a significant role in inhibiting drug release as well.⁶⁶

Figure 5. 4 (A) Loading capacity and efficiency of CPT on CS-sGO with increasing CPT:CS-sGO weight ratio, (B)UV-vis absorbance spectra for CPT loaded samples in comparison to original samples with inset calibration curve of CPT, and (C-E) CPT release profiles in PB, PBS and AU.

5.4 Summary

Chitosan and carboxymethylcellulose functionalization of graphene oxide exhibited marked improvement in terms of stability in physiological conditions. Apart from CS-sGO in DMEM and phosphate buffered saline, both CS-sGO and CMC-sGO have prolonged stability in tested physiological media. Nonetheless, CS-sGO is still able to

retain reasonable stability for up till one day in DMEM and phosphate buffered saline, which is sufficient for cell culture study and robust mucoadhesion mechanism. Compared to CMC-sGO, CS-sGO was better able to maintain size stability in artificial urine with lower degree of particle size variation. CS-sGO exhibited greater mucoadhesion strength than CMC-sGO, due to electrostatic attractions as dominating force interacting with mucosa. In contrast, CMC-sGO loaded higher capacity of model drug CPT as compared to CS-sGO but was lacking in terms of release profile, especially in artificial urine. Finally, cytocompatibility study established minimal decrease of cell viability at CS-sGO and CMC-sGO concentrations up to 50 μ g/ml. Conclusively, the feasibility of chitosan and carboxymethylcellulose functionalized graphene oxide as mucoadhesive drug delivery system for potential bladder application has been demonstrated. Basing upon mucoadhesive strength, drug release capability and stability in artificial urine, CS-sGO appeared to be better suited for intravesical usage. The following chapter expands further on the behavioural adaptation of nanocarriers in simulated intravesical conditions.

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Chapter 6* Interaction of Polysaccharide-Functionalized Graphene Oxide with Simulated Intravesical Conditions

The ideal mucoadhesive drug delivery system for intravesical application should adhere rapidly to bladder mucosa, remain attached after urine voiding, while allowing unobstructed release and penetration of drug to the targeted site with minimal damage to the urothelium in the process. This context however, comes with the condition that the mucoadhesive nanoparticles should firstly retain its stability long enough for adhesion to occur and the adhesion should not be permanent. Therefore, the stability in simulated disease urine, mucoadhesion to ex-vivo porcine bladder and the subsequent effect on morphology is investigated. CS-sGO and CMC-sGO exhibited comparable mucoadhesion to porcine bladder tissues. On the other hand, the bladder morphological changes induced by CMC-sGO were much more extensive while the performance in disease urine variant was lacking.

6.1 Introduction

The physiological condition of urine differs dynamically for patients afflicted with bladder cancer. In addition to changes in pH, the urine constituents diverge greatly. Specifically, urine of bladder cancer patients have elevated level of proteins, bladder tumor antigens, survivin, hyaluronic acid fragments, and fibrin degradation product.¹⁻⁵ Serum amyloid A4 protein, SAA4 was increased 4-17-fold in bladder cancer patients^{1, 6}, while nuclear matrix proteins, NMP are elevated up to 25times¹⁻³. For hyaluronic acid, the increase was 4-9folds than normal levels in urine.⁷⁻⁸ Elevated albumin-to-creatinine ratio has also been associated with bladder cancer, an albumin (mcg/L) to creatinine (mg/L) ratio (ACR) of 30-300 and beyond 300 are considered albuminuria and linked to urinary endothelial dysfunction.⁹⁻¹⁰

Protein absorption or more specifically disruption of nanoparticle stability and functionality is a concern. The concept is analogous to the protein corona effect. In the protein corona postulation, absorption of biomolecules on nanomaterials when in physiological environment alters the biological identity of the material. The wrapping of proteins triggers an irreversible change in physicochemical properties and functional efficacy of nanoparticles, leading to uncertain fate of the altered form. Protein corona constituents and extent of effect on nanoparticles vary across patients, even between those with same disease and lifestyle, resulting in performance difference for immune response, cellular uptake, biodistribution, toxicity, degradation and clearance.¹¹⁻¹² Hyaluronic acid is a glycosaminoglycan forming part of the urothelium GAG layer^{1, 13}, while albumin is a type of globular protein found in blood which becomes detectable in urine when the kidney malfunctions¹⁴. High levels of both hyaluronic acid and albumin are associated with various other types of cancer such as colon, breast, lung, and kidney.^{2, 9} Considering that there is a higher tendency for protein and glycosaminoglycan moiety to disrupt nanocarrier dispersion stability than other compounds present in urine, hyaluronic acid (HA) and human serum albumin (HAS) is selected to further investigate the stability of nanocarriers.

The study of intravesical responses using *ex-vivo* pig bladders as a model is pertinent due to close resemblance with human bladder. The capacity, average voided urine volume and

physiological characteristics are equivalent, facilitating correlation between the two systems.¹⁵⁻¹⁶ In previous chapters, the mucin-particle method is utilized to assess the mucoadhesion property of the nanoparticles due to the convenience and repeatability of the method. However, the fact that the used mucin originates from porcine stomach would have been a source of disparity. The mucoadhesion on *ex-vivo* pig bladder tissue is therefore investigated to further justify the previous evaluations.

Water and mucin form more than 99% of the mucus of bladder mucosal layer.^{17, 30} The mucus are gel-like attributed to intermolecular entanglements and non-covalent interactions of the mucins. Numerous theories have been associated with the mechanism of mucoadhesion¹⁷, which summarises to the ability of polymer to wet and penetrate the mucus network to form strong electrostatic and hydrogen bonding with mucin.¹⁸⁻¹⁹ Interactions with the mucus layer include hydrogen bonding between sialic acids and carboxylate or sulfate groups, cysteine amino acids hydrophobic interaction, and entanglement with long polymeric chains.²⁰ Ideally, a mucoadhesive drug delivery system should adhere rapidly to mucosal layer without alteration of its physical property, enhance drug penetration to targeted site, cause minimal obstruction to drug release with ample period of retention and capable of total removal after drug release.¹⁸ The polymer characteristics that are related to these criteria are hydrophilicity, negative charge potential, and possessing hydrogen bonding groups.¹⁹ Both chitosan and carboxymethylcellulose satisfied more than one of these rules, suggesting good retention behaviours on *ex-vivo* porcine bladder.

The normal turnover rate of the protective superficial urothelial cells are relatively slow at 6-7 months.²¹ In order to achieve greater drugs permeability across the urothelium, the removal of superficial cells are sometimes instigated. The use of desquamation inducers such as cyclophosphamide²², 12-o-tetradecanoylphorbol-13-acetate (TPA)²³, sodium saccharin²⁴, or hyperthermic shock²⁵ have been used. Chemical enhancers such as DMSO and Tween 80 with chemotherapeutic drugs during treatment is also widely practised for insoluble drugs especially for taxols. However, there exists the risk of inflammatory response, uncontrollable desquamation and transitioning to other complication such as

hyperplasia, incontinence, increase in urgency and urination frequency as well as pain during voiding.²⁶⁻²⁷ As the goal for intravesical drug delivery systems is to improve permeability by disruption of tight junction with minimal damage to bladder urothelium, the extent of urothelial damage caused by introduction of nanocarriers is a determining factor for suitability.

Chitosan has been highly cited as permeation enhancer for urinary bladder wall.²⁸⁻²⁹ Concentration as low as 0.0005% w/v is sufficient to enhance model drug permeability of isolated pig urinary bladder.²⁹ The concentration required to achieve significant effect of permeability however, is accompanied by superficial cells desquamation or necrosis. Nevertheless, the time and concentration dependent desquamation of urothelial cells is followed by rapid regeneration of the urothelium in *ex vivo* and *in vivo* conditions.²⁹ Complete exfoliation of rat bladder umbrella cells occurred within 20mins of being introduced to 0.005% w/v chitosan hydrochloride (86%DA, 30-400kDa) but recovery of transepithelial integrity can be achieved within 60-200mins upon removal of desquamation agent.³⁰⁻³¹ Intravesical chitosan introduction under these conditions are considered safe as only weak inflammatory response and temporary urothelial damages existed in experimental animals.³¹ Hence, reducing exposure time and concentration can significantly limit urothelium damage and shorten urothelium recovery time. Permeation enhancing effect of sodium carboxymethylcellulose was established by Clausen and Bernkop-Schnürch where 1% w/v was associated with 1.3-fold increased transport of sodium fluorescein across intestinal mucosa of guinea pigs. The contributor for increased permeability was linked to opening of tight junction as a consequence of calcium ion depletion by NaCMC.³² In view that permeating enhancement is often an aftermath of bladder wall desquamation of superficial cells, investigation into the effect of nanocarriers on urothelium morphology is crucial to facilitate design for targeted exfoliation and controlled damage within the duration of interaction. Thus, the morphological changes after incubation of *ex-vivo* pig bladder tissues with nanocarriers are investigated.

Overall, this chapter focuses on the behaviour and interaction of nanoparticles in simulated disease condition and intravesical setting. The set of studies facilitate visualization of

possible responses that may occur *in vivo*, providing important details for design consideration prior to *in vivo* studies.

6.2 Materials and Methods

6.2.1 Materials

Albumin from human serum (HSA), hyaluronic acid sodium salt (HA, MW 70,000-90,000) and other chemicals mentioned were procured from Aldrich. The porcine bladders were purchased fresh from local abattoir (Buroh Lane Abattoir, SG).

6.2.2 Analysis of stability in simulated urine conditions

The pH stability of nanocarriers in previous chapters were investigated by addition of acetic acid and sodium hydroxide. However, this method does not account for the effect of salt components present in actual urine. To investigate the stability of nanocarriers in urine of varying pH, artificial urine of pH6.3 is acidified by addition of ammonium chloride and alkalized by sodium bicarbonate. After 15mins sonication and standing overnight, the change in zeta potentials as compared to acetic acid and sodium hydroxide adjusted dispersions were measured with DLS and analysed.

Simulated disease urine variant, AU' was prepared by adding 1.36mg HSA and 0.02mg HA into 10ml of pH6.3 artificial urine. After stirring overnight, CS-sGO and CMC-sGO were dispersed in AU' at a concentration of 0.2mg/ml and sonicated for 15mins. DLS measurement were taken for particle size and zeta potential after standing overnight.

6.2.3 Pig Bladder Mucoadhesion Study

The mucoadhesion study and subsequent morphological analysis via SEM is as detailed in Chapter 3.11.

6.3 Results and Discussion

6.3.1 Dispersion stability in disease urine variant

Dispersion stability of nanocarriers in acidified and alkalized artificial urine were maintained as shown in images inset in Figure 6.1 taken 24 hours after dispersion and before DLS measurement. There were no significant changes detected in particle size, whether in terms of deviation from original size or difference from pH adjusted dispersions in Chapter 4, as tabulated in Appendix Table C.1. However, there was substantial difference in zeta potentials measured. Acidification by addition of ammonium chloride reached saturation at pH5.5 while alkaline urine at pH8 was achieved by sodium bicarbonate. For chitosan functionalized graphene oxide, both acidic and alkaline artificial urine resulted in decreased zeta potential. In contrast, zeta potential increases towards positive value for carboxymethylcellulose functionalized graphene oxide. Salting out effect and higher ionic strength of adjusted artificial urine reduced the electric double layer of dispersion causing shifting of zeta potential towards isoelectric point. Essentially, the nanocarriers have lower stability in adjusted urine but dispersion stability is still maintained, which reaffirmed the stability of CS-sGO and CMC-sGO in pH variations.

Figure 6.1 Blue columns representing zeta potential values of CS-sGO(A), and CMC-sGO(B) in acidified and alkalized artificial urine in comparison to pH-adjusted trend from Chapter 4.3.3 and Chapter 4.3.4. Insets show respective images taken of sample one day after preparation, prior to measurement of zeta potential values.

Since intermediate-sized HA in the range of 10-100kDa is detected in both low grade bladder cancer and high grade bladder cancer, hyaluronic acid of size 70-90kDa is selected for this study.⁸ The amount of HA added to simulated disease urine AU' is estimated based on a healthy concentration of 48.7 ± 32.3 pmol/mg creatinine³³, with a 9-fold increase as per literature⁸. For HSA, the amount added is based on albuminuria levels at 300 ratio to creatinine.⁹

At physiological pH, GO is negatively charged and its functional group and basal graphene structure promotes interaction via hydrogen bonding, electrostatic attraction, hydrophobic van der Waals as well as π - π interactions with serum protein and hyaluronic acid.³⁴⁻³⁵ The presence of calcium ions has also been reported to contribute to HSA absorption via charge neutralization and bridging effect.³⁶ The same philosophy applies to functionalized graphene oxide, CS-sGO and CMC-sGO as well. From the DLS measurements in Figure 6.2A, particle size did not undergo significant changes from dispersion in standard artificial urine, AU. However, when observing dispersion of CMC-sGO in simulated disease urine, aggregation of nanoparticles can be seen clearly as shown in Figure 6.2D.

Protein corona on graphene oxide manifests as increase in zeta potential due to surface protein absorption with less effect detected in particle size. Any increase in size is associated with aggregation due to loss of electrostatic repulsion between graphene oxide.³⁷ This explained the unaltered particle size of CS-sGO dispersion while aggregation of CMC-sGO can be attributed to complete loss of electrostatic repulsion upon excessive HSA and HA absorption.

When dispersed in AU', the zeta potential of both CS-sGO and CMC-sGO moved towards isoelectric point. This change in zeta potential is a definite indication of protein absorption and is in direct correlation with amount accumulated.³⁸ Based on this, the protein adsorption on CMC-sGO is presumably greater than that of CS-sGO. It is interesting to note that dispersion stability was maintained when the zeta potential of CS-sGO in AU and AU' outlined in Figure 6.2B were bordering the isoelectric point. This signify that dispersion stability of CS-sGO is not completely reliant on electrostatic repulsion. Both

HA and HSA are negatively charged and is expected to form attractive interactions with chitosan in CS-sGO. The absence of aggregation from loss of electrostatic repulsion reasserted the presence of other forces sustaining dispersion stability. The extended conformation of HSA in acidic condition could have formed steric repulsion with other CS-sGO absorbed HSA, conferring dispersion stability despite low zeta potential.³⁶ Finally, the inclination of hyaluronic acid as a natural polysaccharide to form steric repulsion with chitosan and carboxymethylcellulose would have inhibited absorption, causing less effect on dispersion stability.³⁹

Figure 6.2 Comparison of particle sizes (A), zeta potential (B) of CS-sGO and CMC-sGO in aqueous dispersion DI, artificial urine AU and disease simulated artificial urine AU'. CS-sGO in AU' appeared to retain the dispersion stability in (C) but aggregation of CMC-sGO in AU' was observed visually in (D).

HSA is simulated to adsorb on graphene surface in as short as 200ns, with net charges falling in the range of -0.24 to -0.67 depending on the density of hydroxyl groups present.⁴⁰ The adsorption of human serum albumin is revealed to be inversely correlated to availability of hydroxyl groups on graphene.⁴⁰ Hydrophilicity disfavored HSA adsorption, and interaction of HSA proteins appears to be triggered by exposure of hydrophobic residues as the proteins flatten when coming into contact with graphene. The study by Lu and researchers also proposed the significant role of hydroxyl groups density in mitigating the protein denaturation by graphene materials.⁴⁰ This suggests that reduction of absorption degree can be modulated by altering the hydroxyl groups density or hydrophilicity of nanoparticles.

6.3.2 Fluorescence mucoadhesion study of nanocarriers

Although studying mucoadhesion via the mucin-particle method allowed the determination of the forces at play in initiating mucin interaction, the results could not be quantitatively taken as direct representation of mucoadhesion strength. In addition, it is also impossible to distinguish aggregation due to mucin-particle interaction from normal particle aggregation. Hence, the mucoadhesion of fluorescent-labelled particles on *ex-vivo* porcine bladder tissues is assessed to complement the results from mucin-particle method. For this purpose, sGO, CS-sGO, and CMC-sGO were labelled with sodium fluorescein by π - π interaction for the purpose of fluorescence microscopy. CS-sGO is firstly mixed with sodium fluorescein in different weight ratio (sGO: NaFL) to identify the optimal for efficient labelling. From Figure 6.3A, it can be discerned that loading capacity increases with increasing weight ratio while the opposite applied for loading efficiency. Moreover, large particles were found to form at high weight ratio, and subsequent size measurement via DLS showed exponential rise in particle size with increase in weight ratio as summarized in Figure 6.3B. Thus, the weight ratio of 5:1 is selected for subsequent fluorescent-tagging based on highest NaFL loading efficiency. Covalent conjugation of sodium fluorescein to chitosan was also conducted with the same weight ratio. UV absorbance spectra of final fluorescein-tagged samples is plotted in Appendix Figure C.1

the characteristic absorbance of sodium fluorescein. The sodium fluorescein loading capacity and efficiency is calculated based on UV absorbance intensities at the wavelength of 488nm and tabulated in Appendix Table C.2.

Figure 6.3 (A) Loading capacity and efficiency of sodium fluorescein on CS-sGO, and (B) size variation of CS-sGO-FL with different mixing weight ratios.

Intending to define mucoadhesion strength from fluorescence intensity of fluorescein-tagged nanoparticles remaining on pig bladder mucosal surface after artificial urine irrigation as per the work of Mun and colleagues⁴¹, the fluorescein-tagged samples were redispersed at 5mg/ml for porcine bladder mucoadhesion study. However, the fluorescence intensity of bladder samples incubated with fluorescein-labelled nanoparticles, sGO-FL, CS-sGO-FL, and CMC-sGO-FL in Appendix Figure C.3 did not show a decreasing trend after washing off with artificial urine. Instead, there was a general increase of fluorescence intensity. Therefore, quantification of mucoadhesion strength via the decrease in fluorescence intensity is not applicable for the tested nanoparticles. Nevertheless, a few conclusions can be derived from the fluorescent images captured before and after three washes of 10ml artificial urine. Firstly, sGO possesses minimal or negligible mucoadhesive property with almost complete wash off after urine irrigation in Figure 6.4B. Secondly, physical attachment of biomolecules to functionalized graphene oxide does not interrupt mucoadhesion mechanism of the nanoparticles. As displayed in Figure 6.4 (C-D), CS-sGO and CMC-sGO were still visibly attached to bladder mucosa after three artificial urine

washing. The relatively high population of dark materials representing CS-sGO and CMC-sGO after urine wash off verified the mucoadhesive property of both chitosan and carboxymethylcellulose functionalized graphene oxide. Third, bladder urothelial delivery of biomolecules or drugs loaded via π - π interactions or hydrogen bonding is verified. The fluorescence was still strongly detected on bladder tissues after almost complete sGO wash off, substantiating the capability for drug release for likewise loaded drugs. Kolawole and coworkers using NaFL as model drug has also demonstrated the successful drug delivery using similar *ex-vivo* pig bladder mucoadhesive study.⁴²

Figure 6.4 (A-D) Fluorescence microscopy images of porcine bladder incubated with NaFL, sGO-FL, CS-sGO-FL, and CMC-sGO-FL, (F) Fluorescence spectra of samples showing blue-shift of emission peaks.

The fluorescence spectra were plotted as per Figure 6.4E showing blue shift of fluorescence peak with reference to sodium fluorescein for all samples. The trend is linked to the covalent functionalization and graphene oxide quenching through π -electrons energy transfer.⁴³⁻⁴⁴ As a result, the fluorescence intensity measurement method from literature^{41, 45} cannot be taken as direct representative of particle retention on mucosa. Therefore, the study is modified to deducing mucosa retention of sGO-FL, CS-sGO-FL and CMC-sGO by visual observation of dark regions in the following section.

6.3.3 Retention study of nanocarriers on bladder mucosa

The dark-coloured sGO, CS-sGO, and CMC-sGO facilitated the direct visualization of their presence through microscope. During urine washing, it was observed that sGO was detached and washed off in huge aggregates whereas CS-sGO and CMC-sGO were in smaller pieces. After washing with 20ml of artificial urine, there was a large region of tissue surface completely cleared of sGO. On the contrary, particles of CS-sGO and CMC-sGO were fairly dispersed on the tissue surface. Therefore, sGO was surmised to have minimal or negligible mucoadhesion property. sGO sheets appeared to be stacking on top of one another, resulting in complete dark regions covering mucosa surface. Meanwhile from Figure 6.5, CS-sGO and CMC-sGO on tissue surfaces comprised of thin layers with darker areas representing greater mucoadhesion. Both nanocarriers were cleared from bladder surface after 200ml urine irrigation. Surface plots of the images taken shown in Figure 6.6 confirmed the complete removal of CS-sGO and CMC-sGO from the surface of bladder mucosa. Mun and co-workers reported 89ml of urine washing required to remove 50% of chitosan adhered on bladder surface.⁴¹ This amounts to 178ml for complete removal, which conforms well with CS-sGO.

Figure 6.5 Representative microscope images showing mucoadhesion and retention of sGO, CS-sGO, and CMC-sGO on porcine bladder mucosa after irrigation with pre-determined artificial urine volumes. AU represents control bladder tissues incubated and irrigated solely with artificial urine. Scale bar denotes 1mm.

Based on the method outlined in Section 3.11, it is estimated that 4.7days is required for complete removal of nanocarriers from bladder mucosal surface. Meanwhile, the amount of urine voiding for complete removal from bladder mucosa was calculated to be approximately 20, signifying a 20-fold enhancement of loaded drug retention as compared to current drugs instillation that rarely stay beyond the first voiding.⁴⁶ The actual time required *in vivo* for complete wash off of adhered nanocarriers however, may be at a faster rate due nanocarrier wash-off contribution from bladder filling, urine immersion effect and volume motion. The reported retention is also expected to be an overestimation than actual condition in human bladder due a difference in mucus thickness.⁴⁷

Figure 6.6 Surface plots of microscope images taken with peaks showing presence of sGO, CS-sGO and CMC-sGO. Absence of peaks is a direct representation of complete removal of nanoparticles from the surface of porcine bladder mucosa.

Table 1 summarized the total volume of artificial urine required for complete removal of muco-adhered nanoparticles. Interestingly, the volume of artificial urine required to remove both CS-sGO and CMC-sGO were similar, implying equivalent mucoadhesion strength which is in contradiction to prediction from mucin-particle method. This could be due to the reduced mucoadhesion effect of chitosan by introduction of cations from repeated artificial urine irrigation, resulting in lower mucoadhesion strength portrayed. The presence of cations in artificial urine has been associated with shielding of carboxylic and sulphate groups of urothelial glycosaminoglycans from participating in electrostatic attraction with amino groups of chitosan.⁴⁸ Kolawole and co-workers also agreed that the envisaged chitosan interaction with sialic acid-rich mucosal surface was impeded by counter ions from artificial urine used for wash-out studies similar to current study.⁴²

Considering that the percentage grafted polymer is higher for chitosan than carboxymethylcellulose, it can be concluded that the limiting effect on mucoadhesion is significant since *in-vitro* study predicted greater mucoadhesive strength of chitosan as compared to CMC. Ratio of grafted polysaccharides is approximately two to one to achieve a comparable mucoadhesion between the two nanocarriers. In addition, the pH of artificial urine used to precondition the bladder tissues that was bordering the limit of solubility of chitosan may also be a contributing factor. The compositional difference between porcine bladder mucus layer and mucin from porcine stomach in mucin particle method cannot be ignored as well.⁴⁹ Finally, the substantiated inferior mucosa adhesion and weak force of detachment from few *in-vitro* studies⁵⁰⁻⁵¹, is indicative of the prominent role played by free -NH₂ groups in mucoadhesion retention of CMC-sGO. The introduction of amine groups has previously been shown to increase the mucoadhesion strength of gelatin microspheres, with the effect attributed to greater chain flexibility and configuration favorable to mucoadhesion.⁵²

Table 6.1 Volumes of artificial urine irrigation required for complete removal of sGO, CS-sGO, and CMC-sGO from the surface of bladder mucosa.

Sample	Artificial urine volumes for total wash off (ml)
sGO	30
CS-sGO	200
CMC-sGO	200

6.3.4 Effect of nanocarriers on bladder surface morphology

Bladder urothelium treated with sGO, CS-sGO and CMC-sGO in Figure 6.7(B-D) exhibited areas with cracks and deep fissures. Poinard and coworkers associated similar morphological disruptions with material-specific strong electrostatic or hydrophobic interactions with mucus layer and possible incompatibility with bladder tissue while Lu et

al. related the observations to initial signs of exfoliation.⁵³⁻⁵⁴ As cracks are not observed on bladder tissue treated with artificial urine, the discussed damage would have been contributed by graphene oxide. Based on the relatively less extensive cracks observed for CS-sGO and CMC-sGO, polysaccharide functionalization appeared to have reduced the morphological damage caused by graphene oxide.

At higher magnification in Figure 6.8, there was obvious exfoliation and loosening of urothelial tight junction for sGO-treated tissues while patchy removal of umbrella cells were observed when treated with CMC-sGO. For control tissue interacted with artificial urine only, intact superficial cells with tight junctions can be discerned. Interestingly, CS-sGO displayed the least degree of observable exfoliation, having surface morphology analogous to control. Considering the recognised desquamation effect of chitosan as well as the induced exfoliation effect by graphene oxide observed in current study, higher degree of urothelial damage would be expected. The contradicting observation could be attributed to the relatively small percentage of chitosan present in CS-sGO as well as non-acidic nature of aqueous dispersion, causing reduced desquamation effect contributed by chitosan. Furthermore, the result also demonstrated the effect of chitosan functionalization in abating the urothelial integrity disruptions caused by graphene oxide. The extensive damage caused by graphene oxide incubation on bladder urothelium could be associated with the impenetrable nature on sGO layers. As shown in mucoadhesion study in Section 6.3.3, graphene oxide sheets were inclined to form stacked layers on bladder surface at high concentration. This phenomenon is presumed to have interrupted the supply of air to superficial cells, causing accelerated cell exfoliation and death.

Figure 6.7 SEM images showing morphological changes of pig bladder mucosa surface after two-hour incubation with AU(A), sGO(B), CS-sGO(C), and CMC-sGO(D) at accelerating voltage of 15kV and 100x magnification.

Urothelium exfoliation is common when foreign substances are introduced into the bladder. In fact, chemotherapeutic drugs introduced intravesically reported extensive exfoliation effect that led to good tissue penetration of the drugs. This is especially apparent for amine-containing drugs such as mitomycin C, doxorubicin and gemcitabine, suggesting the prospect of mucosa cationic interaction as exfoliation initiator.⁵⁵ The presumably permeation enhancing and exfoliation effect attribution to amine groups has also been described by other researchers working on drug carriers with amine groups, for instances, amine functionalized polyglycerols⁵⁶ and amine-functionalized polyacrylamide nanogels⁵³. Pandey's group reported a proportional relation between exfoliation degree and density of amine groups.⁵⁶ Poly-L-lysine with greater amount of amine groups too, showed greater desquamation effect on bladder mucosa.⁵⁷ This suggests that exfoliation and permeation enhancing capability of chitosan is also attributed to the amine group. Thus, the reduced exfoliation effect of current study would be a direct consequence of NH_2 depletion from amidation conjugation with graphene oxide. On that note, the extensive exfoliation in CMC-sGO sample would have been mainly contributed by unconjugated free amine groups on CMC-sGO. Seeing that exfoliation is correlated to improved drug permeability, CMC-

sGO is anticipated to greatly improve permeability from the obvious exfoliation in Figure 6.8D.

Considering the almost immediate differentiation of superficial cells after an injury^{30-31, 58}, it can be concluded that functionalized graphene oxide is a viable intravesical drug delivery system as the urothelial damage caused by 1mg/ml of nanocarriers were not as extensive as that generated by 1mg/ml of chemotherapeutic drugs reported in literature.⁵⁵

Figure 6.8 SEM images showing morphological changes of pig bladder mucosa surface after two-hour incubation with AU(A), sGO(B), CS-sGO(C), and CMC-sGO(D) at accelerating voltage of 15kV and 1000x magnification.

6.4 Summary

The pH stability of CS-sGO and CMC-sGO were again verified via acidified and alkalized artificial urine. However, when tested in artificial urine modified to mimic disease condition, aggregation of CMC-sGO was visible after one day while CS-sGO maintained homogeneous dispersion. Fluorescence detected on bladder mucosa after removal of fluorescein π -loaded graphene oxide suggested the potential success of biomolecules release at the nanoparticle-mucosa interface. Meanwhile, mucoadhesion study with porcine

bladder revealed comparable mucoadhesion strength between CS-sGO and CMC-sGO. From the morphological study, the bladder urothelium is much more tolerable to CS-sGO as compared to CMC-sGO and sGO. Exfoliation of superficial cells was present only to a small extent, however, the effect of reduced exfoliation on permeation remains to be investigated. In conclusion, CS-sGO and CMC-sGO possess great potential as mucoadhesive drug delivery systems in bladder with stability in simulated bladder environment, mucoadhesion capability, and prolonged retention for up to 20 urine voiding. The same of which, could not be said for conventional intravesical treatments that are removed on first voiding.

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Chapter 7* Conclusions and Recommendations

The feasibility of chitosan and carboxymethylcellulose functionalized graphene oxide as nanocarriers for chemotherapy of bladder cancer has been demonstrated. Notably, the nanocarriers possess dispersion stability in urine variants, biocompatible up to a concentration of 50µg/ml, mucoadhesive with bladder retention up to 20 urine voiding, relatively high drug loading capacity and controlled releases. Adhesion to bladder mucosa induces minimal morphological change and is manageable via higher polymer incorporation. Nonetheless, many more research works are imperative to warrant the competence of the nanocarriers in actual human bladder settings. The succeeding works and outlook are briefly enumerated.

7.1 Conclusion

The nanocarriers, chitosan functionalized graphene oxide and carboxymethylcellulose graphene oxide were successfully synthesized with slight modification of cited literature to attain particle sizes that are adequately suited for intended application in terms of biocompatibility and permeation enhancement. The grafting degrees of chitosan and carboxymethylcellulose are 54% and 22% respectively, which was demonstrated to be adequate in conferring graphene oxide with enhanced biocompatibility, dispersion stability in high salt and protein solutions, as well as good mucoadhesive property. Testing with L929 mouse fibroblasts cell line revealed cytocompatibility up to a concentration of 50 $\mu\text{g/ml}$ for both nanocarriers.

Particle sizes remained stable in a series of physiological media, including cell culture media, artificial urine for a minimum of four days. pH stability was achieved in the range of pH5 to 8, which conformed well with urine pH values. Due to generally acidic disease urine and pH sensitivity of chemotherapeutic drugs¹, alkalization is often done prior to intravesical instillation.² The reported nanocarriers which are pH stable would be able to protect loaded drugs from destabilizing environments at the same time simplifying administration procedures.

Drug loading capacity of both nanocarriers were consistent with functionalized graphene oxide nanocarriers in literature at 27wt% and 35wt%, however loading efficiencies were low at 14.5% and 26.2% respectively. Release of model drug, CPT exhibited restrictive profile where plateau appeared to be reached at approximately 30-40% cumulative release. CPT release from CS-sGO in artificial urine was the only exception, releasing about 80% load at 22hours time point. It remains to be discovered if less release in AU may be more beneficial in terms of less loss to urine, *in vivo* testing would be pertinent for actual visualization and verification of release performance.

Investigation of the intravesical response of nanocarriers were conducted by testing dispersion stability in mock disease urine prepared by incorporation of high levels of HA

and HSA, as well as study with *ex vivo* porcine bladders. Chitosan functionalized graphene oxide were able to sustain its dispersion stability in mock disease urine while carboxymethylcellulose functionalized graphene oxide fell short by showing aggregation within a day. Nevertheless, the CMC-sGO dispersion was still sufficiently homogenous when particle size was measured three hours after sample preparation, which is sufficient time for intravesical mucoadhesion to occur. Mucoadhesion of both nanocarriers to *ex-vivo* porcine bladders had high level of retention, with complete removal achieved after 200ml of urine washing, which matches up to a total of 20 urine voiding based on normal bladder statistics of human. Conventionally, intravesical administered drugs are unable to stay beyond the first urine voiding, resulting in repeated administration to achieve therapeutic efficacy. Therefore, the long retention would facilitate greater administration efficacy by eliminating the need for instillation frequency. In addition, with a bladder retention of up to 20 urine voiding and release plateau reached within 22hours, the degree of retention would have been sufficient for complete drug release to occur basing upon average of 7 urine voiding in 24 hours.

Both nanocarriers induced minimal morphological changes to bladder surfaces after two hours incubation. CMC-sGO exhibited greater level of exfoliation as compared to CS-sGO, which is associated with lesser degree of CMC functionalization and presence of amino groups. As such, the exfoliation degree is easily manageable by manipulating polymer grafting.

Taking into account all desired properties outlined for bladder application, CS-sGO performed slightly better than CMC-sGO. In terms of dispersion stability in urine variants, CS-sGO was more consistent in measured particle size. Although CMC-sGO loads higher percentage of drugs than CS-sGO, the release in artificial urine is much limited. Regardless, both nanocarriers showed great potential as drug delivery systems for bladder cancer, but with numerous works still warranted to verify functionality and safety of the nanoparticles.

7.2 Recommendations for Future Studies

7.2.1 Expansion of *in vitro* and *in vivo* study

As summarized above, the drug loading efficiencies reported in the work is relatively low and are most likely a deterrent caused by long polymeric chains and loading in neutral conditions. Studies have demonstrated the possibility of increasing drug loading efficiency by adjusting pH values of loading conditions. Therefore, the drug loading methodology of nanocarriers are still lacking in depth. The study conducted in this work however, established proof-of-concept that the synthesized nanocarriers have good drug loading capability. After all, literature has established the prospect of functionalized graphene oxide for delivery of common chemotherapeutic drugs used for bladder cancer, and drug loading protocol for each of the drug should be optimized in future studies since certain drugs have higher affinity to graphene oxide surface absorption than others.³⁻⁴ The desired drug loading ultimately, will depend on design for optimal dose for the drugs individually.

Ideally, continuous availability of drug within the bladder is achieved by protection from urine pH, uniform distribution and instantaneous adherence of drug carriers to the urothelium, as well as controllable permeation-enhancing desquamation of the superficial cells. Implication of *in vitro* drug release trend will depend highly on whether the drug is released on contact with urothelium, into the urine or after cell internalization. This will in turn have great significance on toxicity profile of the nanocarriers.

Although urothelial exfoliation is common even in chemotherapeutic drugs treatments, careful design of present drug carrier system entails such that desquamation is regenerative and limited to only upper cellular layers. Predominantly, *in vivo* study is essential to investigate the time and concentration dependent desquamation, such that an optimal treatment concentration and duration correlated with swift recovery of bladder integrity can be established. In this work, a time study for removal of muco-adhered nanoparticles were not done to avoid error caused by possible biological degeneration of the mucosa mucus layer over time. Instead, immediate artificial urine irrigation was done after imaging, whereas in actual condition 2 to 3 hours is required before urine voiding. Furthermore, urine filling would have been gradual over the timeframe. Henceforth, drug loading and dosage can be designed based on effective therapeutic window without causing disruption of

bladder functions and with minimal irritation symptoms.

For controlled release applications, mucoadhesion may be a contradictory barrier to drug penetration due to high viscosity of interacting layers or impenetrable chain entanglement networks. On the other hand, high drug loading could also cause change in configurations of drug carriers, effectively modifying the mucoadhesive strength. Susceptibility of protein corona formation, especially when contacting patient urine high in protein content with traces of blood would also determine the biological response of the nanocarriers. Furthermore, it takes less than thirty seconds for protein corona to develop on nanoparticles coming into contact with cell culture medium or human plasma⁵, and has been shown to inhibit drug release from nanoparticles⁶⁻⁷. For these circumstances, investigation of drug penetrative depth in *in-vivo* bladders are crucial to verify the enhancement of localized drug concentration and residence time. Effectiveness in cancer-killing and tumor elimination capability are also critical analyses to be examined with bladder cancer cell lines and orthotopic animal models.

Most importantly, the biocompatibility and cytotoxicity effects should not be taken lightly. The toxicity of the graphene family *in vitro* and *in vivo*, the mechanism and causing factors is summarized in a few reviews.⁸⁻¹⁰ Notably, varied viability response was reported for different cell lines, however the researchers unanimously agreed that reduced cytotoxicity and occasionally divergence of nano-biointeractions are detected for functionalized graphene oxide.¹¹⁻¹² There were also contradictory insignificant toxicity response from human macrophages as compared to the murine source.¹¹ Mechanism of toxicity for graphene oxide is postulated to be mainly due to oxidative stress, cell damage from shape edges of graphene sheets, hydrophobic modification of cell membrane lipid and its inherent physicochemical properties.¹¹ Unfortunately, cell oxidative stress was not part of this study. It is therefore still unclear if this aspect of GO is a drawback or as some papers suggested, a desirable, anticancer trait. Needless to say, there is an urgency for internalization study with bladder cells for detection of cell apoptosis, necrosis response, and oxidative stress.

There were also reports of thrombogenicity¹³ and immune response¹⁴ triggered by

graphene oxide. Hence, local toxicity must be well defined to ensure no leakage to systemic circulation through damaged urothelium or leaky cells. Typically, graphene oxide modification results in change of tumor-targeting efficiency, systemic uptake, and organ accumulations. Systemic entry of graphene oxide in mice has been demonstrated to accumulate by size in liver and lungs.¹⁵ Although minimal systemic absorption is expected to occur in intravesical drug delivery, study of *in vivo* local and systemic toxicity should not be neglected. Intravesical introduction of drug loaded nanocarriers to animal bladders should be followed by histological and hematological analysis to monitor potential signs of toxicity.

7.2.2 Synergistic drug delivery

As reviewed in Chapter 2.5, several studies have reported greater cancer-killing efficiencies when more than one chemotherapeutic drug were loaded by graphene oxide-based nanocarriers. The dual delivery strategy typically display cytotoxicity surpassing that of free drugs or even individually loaded drugs on the same nanocarrier.^{3, 16} The drug adsorption on graphene oxide was even demonstrated to increase when two drugs are loaded together, suggesting improved adsorption kinetics.³ Therefore, consideration of multi-drug delivery for intravesical treatment of bladder cancer has great prospective since a number of clinical trials has also established the synergistic effect of multi-drug treatments regiments.¹⁷⁻¹⁸

7.2.3 Co-delivery of therapy genes

Gene delivery in the therapeutic context involves the transport of genomic material into the cell to repair or replace defective gene to trigger a positive therapeutic effect. Because of the strong negative charges of free RNA or DNA rendering cellular uptake difficult, gene therapy for cancer treatment using cationic materials to condense DNA for transport has been widely studied.

The concept of gene delivery via functionalized graphene oxide is relatively new with few published works presently. Functionalization of graphene oxide nanosheets with cationic conjugates adds the capability to condense DNA, which leads to the potential function for gene delivery. When complexed on graphene lattice, DNA is also believed to be protected against nucleases and degradation.¹⁹⁻²¹ Previous work of the group reported the capability of chitosan-functionalized GO (CS-GO) in condensing plasmid DNA to form small and stable complexes, exhibiting feasibility of CS-GO for simultaneous drug and gene therapeutic applications. Chitosan is capable of condensing DNA into complexes that are smaller than 100nm in defined conditions.²² Complexes formed provide protection from degradation before reaching a targeted site. It was also observed that strong electrostatic interactions between pDNA and the CS-GO carrier resulted in a round nanoparticle profile instead of the usual angular shape of GO and functionalized GO.²³ Similarly, synergistic delivery of chemotherapeutic drug and siRNA was demonstrated with chitosan oligosaccharide-folic acid conjugated GO (GO-FACO+), affording high doxorubicin drug loading and efficient transfection of MDR1 siRNA to doxorubicin chloride resistant MCF-7 human breast cancer cells. The cellular uptake of GO-FACO+ loaded DNA by MCF-7 cells was comparatively higher than GO-CO+ (without FA conjugation) loaded DNA and even free DNA. Cytotoxicity of DOX was also significantly enhanced with decreased IC50 values compared to free DOX administration.²⁴

The combination of chemotherapy with gene therapy has been widely studied with the aim of increasing the efficacy of cancer treatment. Therefore, the co-loading of two therapeutic forms also warrant investigation as gene therapy has also been demonstrated to be effective in treating bladder cancer, of note are p53²⁵⁻²⁶, interleukin-12²⁷⁻²⁸, and IFN- α ²⁹.

7.2.4 Outlook

Functionalized graphene oxide has been demonstrated to be a viable novel approach to enhance intravesical drug delivery for bladder cancer. However, as with the general introduction of graphene material into the biomedical field, there are yet many questions left unanswered before real-world application can be proceeded.

Firstly, graphene oxide is intrinsically heterogeneous.³⁰ The functionalization of graphene oxide is therefore subjected to inconsistencies. In the current study, variations between different batches of synthesis as well as from different group of purchased graphene oxide were observed. Characterization of physicochemical properties graphene oxide and derivatives are greatly crippled by lack of a standardized fabrication method that can produce consistency in surface chemistry and functional group density, which has an important definition in terms of toxicological profile. As a result, comparison between similar research works are simply ineffective. Hence, there is an urgent need for standardization of synthesis protocol for high quality graphene oxide with controllable oxidation degree and functional groups distribution in fulfilment of biomedical applications.

The major delimiting factor in biomedical implementation of graphene materials lies in the biocompatibility, toxicological and oxidative stress profile. The associated toxicity appears to be an influence of a combination of parameters including lateral dimensions, surface charge and chemistry, functionalization, number of layers, hydrophobicity, concentration, and others. While a significant amount of work published indicated toxicity of graphene materials, the results are hardly conclusive, seeming to differ from cell lines to cell lines and even enhancing cell proliferations for some. At present, the unanimous verdict was the fact that toxicity scales down with smaller dosages, positive surface charge, and higher density of carboxyl groups. Therefore, until the toxicological profile of graphene based nanocarriers are validated, or at least controlled, these nanocarriers would stay in the laboratory setting. In the meantime, design of graphene oxide-based drug delivery systems may require adjustment of treatment dosages while keeping nanocarriers within a defined safe concentration.

As drug loading to functionalized graphene oxide depends on physical means, the drug release will be mainly by diffusion and is reliant on perturbation to the non-covalent forces attaching drug molecules to the carrier. Controlled release by drug diffusion is then, further affected by the strength of interaction, drug molecular weight and surface chemistry of carrier.⁴ Therefore, the release profiles from functionalized graphene oxide will vary with

the type of drugs loaded, the available functional groups that are present for interaction with carrier as well as having the tendency to protonate. These factors would also indirectly modulate the speed of release, and there is a real possibility of insufficient drug molecules delivered to targeted cells. Admittedly, drug release in actual condition may differ considerably from *in vitro* conditions. For in *in vitro*, environment and variables are well controlled, which is not the case *in vivo*. In addition to disease conditions that vary widely between individuals, cellular interactions are also difficult to be anticipated. Controllable cellular uptake is an intricate balance of GO morphology such as the size, shape, and sharp edges, surface charge and functional groups, dispersion state and degree of aggregation, stacking layers, and presence of foreign material in addition to synthesis process.³¹ In cell culture, experimental conditions such as concentration, exposure time, type of cells, interaction with culture medium and assay used are also influential factors. The speed of drug internalization by cells versus the release into urine will affect the actual amount of drugs that ultimately reach targeted site, not to mention the possibility of efficacy disruption from the different interactions the drug carriers will have to undergo in the meantime. The formulation design therefore, plays an important role and should be given priority after establishing physicochemical properties of the nanocarrier. In this context, the incorporation of targeting ligands for markers overexpressed in tumors would be an added advantage for precise internalization.

The tendency of protein corona formation for nanoparticles has been one of the emerging research frontier since its identification as the main source of functional failure for internalized nanoparticles. As aforementioned, the urine stored in the bladder of afflicted patients are typically high in protein, blood, and consists of unbalanced constituents of salts, hence functionalized graphene oxide introduced intravesically has a high probability of forming protein coronas. Evaluation of the degree of protein adsorption, the subsequent degree of change to the physicochemical characteristics of nanocarriers, the effect on functionality disruptions, and potential for adverse effect should be performed. These information can then be manipulated for design innovation.

The degradation mechanism of graphene oxide also remains to be elucidated. Although it

has been demonstrated that graphene oxide is biodegradable by human myeloperoxidase, eosinophil peroxidase, lactoperoxidase, hemoglobin and xanthine oxidase by generation of free radicals^{24, 32-33}, effect of functionalization on degradation profile and speed is still not well-founded. On that note, the long-term stability of grafted polymers should also be considered.

Although the concern for systemic toxicity is greatly diminished for intravesical treatment of bladder cancer, drug delivery in the bladder environment possesses a different set of challenges. Development of drug delivery systems aims at increasing drug permeability and prolong drug availability even after urination. From the standpoint of clinical therapy, these effectively reduce drug dosage and administration frequency, which simplifies treatment procedures and improves patient compliance. The enhanced drug retention and efficacy have been demonstrated in published studies, but translation to clinical application is still very much out of reach. In cancer research, approximately only 0.7% researched drug delivery systems from the past 10 years was successful in delivering payload to a solid tumor, with limitations coming from mass production, cost, toxicity, safety profile and deviation of actual results from experimentation.³⁴⁻³⁵ Without a doubt, nanotechnology holds great prospective in the future of intravesical drug delivery systems, however caution should be enacted when filling the gaps between design and reality.

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APPENDIX A

Additional information on synthesis characterizations.

Figure A.1 FTIR spectra of graphene oxide samples showing absorbance peaks corresponding to similar functional groups present.

Figure A.2 Graphene oxide sample, kGO showing instability in aqueous dispersion after standing overnight.

Figure A.3 Particle size and zeta potential of (A)graphene oxide, (B)sonicated graphene oxide in different pH solutions. Table in (C) lists the particle size, polydispersity and zeta potential values of samples as mean±standard deviation of three measurements.

Figure A.4 Change of particles size and zeta potential values across pH3-10, data are reported as mean ± standard deviation for 3 measurements from 3 batches of synthesis for CS-sGO 5:1

Figure A.5 TGA curves of CS-sGO synthesized at increasing chitosan to graphene oxide weight ratio 1:1 to 5:1.

Figure A.6 (A) pH stability of sGO in the order of pH3, 5, 6, 7, 8, 10 after 3 hours and 3 days, and (B) GO stability in DMEM, PBS and AU after 3 hours and 3 days.

Figure A.7 Change of particle size and zeta potential with pH variation for CS-sGO1 to CS-sGO5 (A-E), and table detailing the values for particle size, polydispersity index, and zeta potential of the samples (F).

Figure A.8 Comparison of FTIR absorbance spectra between graphene oxide and sonicated graphene oxide

Figure A.9 FTIR Spectra for CMC-sGO1 and CMC-sGO2 synthesized from sGO-ADH1 and sGO-ADH2 respectively. The two hydrazide functionalized graphene oxide differs in terms of initial adipic acid dihydrazide added to graphene oxide nanocolloids. The FTIR spectra for sGO-ADH does not differ significantly, except for a slight shifting of peaks to lower wavelength.

Figure A. 10 TGA thermal decomposition curve for CMC-sGO1 to CMC-sGO6 in the order of (A-F). The decomposition temperature generally decreases with decrease in functionalized carboxymethylcellulose and increase in free amino groups.

Table A 1 Tabulated changes in particle size and zeta potential when samples are tested with mucin in deionised water for mucoadhesive determination from mucin-particle method. The changes in size and zeta potential showed a direct correlation to the percentage of conjugated chitosan.

Sample	Estimated percentage chitosan (%)	Original particle size (nm)	Particle size after mucin interaction (nm)	Original zeta potential (mV)	Zeta potential after mucin interaction (mV)
CS-sGO 1:1	26.7*	258.4±1.4	373.6±12	16.4±0.1	-17.9±0.15
CS-sGO 2:1	21.5*	307.4±2.5	302.3±2.8	19.4±0.5	-17.7±0.12
CS-sGO 3:1	20.1	344.6±1.6	575.1±23	21.7±0.4	-16.6±0.45
CS-sGO 4:1	30.9	374.0±4.8	1906±61	26.8±0.5	-15.2±1.1
CS-sGO 5:1	36.4	463.2±2.6	2222±476	19.5±0.5	-15.2±0.55

* Chitosan functionalization was incomplete and uneven, hence the estimated percentage of chitosan conjugated is not accurate for these samples.

APPENDIX B

Additional information supplementing studies on stability and cytocompatibility.

Figure B.1 Changes in polydispersity index across 4 days of sGO, CS-sGO and CMC-sGO dispersion in DMEM(A), PB(B), PBS(C), and AU(D). Great changes in polydispersity index are indications of aggregation occurring within dispersion.

Table B.1 Zeta potential of sample dispersions in physiological media recorded three hours, one day and three days after preparation.

Sample	Dispersed Medium	Zeta Potential (mV)			
		original	3 hour	1 day	3 days
sGO	DMEM	-35.2 ± 5.3	-11.1 ± 3.1	-13.2 ± 0.8	-8.7 ± 0.3
	PB		-19.8 ± 0.9	-	-
	PBS		-21.0 ± 2.2	-	-
	AU		-22.3 ± 1.1	-	-
CS-sGO	DMEM	24.0 ± 2.7	-1.4 ± 0.7	-1.5 ± 0.5	-1.9 ± 2.6
	PB		16.0 ± 0.9	15.5 ± 1.1	15.7 ± 2.1
	PBS		4.8 ± 0.3	5.0 ± 0.8	5.2 ± 0.4
	AU		4.9 ± 5.1	1.4 ± 1.8	-0.9 ± 0.5
CMC-sGO	DMEM	-30.1 ± 2.0	-14.3 ± 2.2	-19.3 ± 2.6	-11.0 ± 3.4
	PB		-23.4 ± 1.5	-22.9 ± 1.2	-24.3 ± 1.2
	PBS		-23.4 ± 0.7	-23.8 ± 1.2	-24.0 ± 1.6
	AU		-23.6 ± 0.1	-15.3 ± 2.2	-9.5 ± 1.0

* Dubelcco's Modified Eagle Medium is represented as DMEM, pH5 phosphate buffer as PB, PBS for phosphate buffered saline and AU as artificial urine.

Table B.2 Particle size, zeta potential and polydispersity index of sGO, CS-sGO, and CMC-sGO dispersions in pH4.5 acetate buffer solution.

Sample	Particle size (nm)	Zeta potential (mV)	Polydispersity Index
sGO	$185.5 \pm 1.8^*$	-21.7 ± 0.9	0.2423
CS-sGO	327.6 ± 6.8	19.1 ± 1.3	0.1876
CMC-sGO	448.7 ± 4.8	-30.2 ± 1.0	0.2093

Figure B.2 Morphology of L929 cells treated with different concentration of (A) sGO, (B) CS-sGO, (C) CMC-sGO in comparison to control.

APPENDIX C

Additional information supplementing studies on intravesical interactions.

Table C.1 Comparison of particle sizes from pH adjusted dispersion in Chapter 4 against acidified and alkalized artificial urine. The particle sizes did not show significant changes between the two conditions at similar pH.

Sample	Original particle size (nm)	pH adjusted dispersion		Acidified/alkalized artificial urine	
		pH5	pH8	pH5.5	pH8
CS-sGO	297.0±19.0	239.8±28.0	296.0±16.0	324.2±5.2	329.4±2.6
CMC-sGO	297.2±52.5	313.4±45.1	351.5±92.7	375.1±5.9	367.4±10.5

Figure C.1 UV absorbance spectra of fluorescein-labelled samples showing peak at wavelength 488nm

Table C.2 Loading capacities and efficiencies of CS-FL, sGO-FL, CS-sGO-FL, and CMC-sGO-FL

Sample	Loading capacity (%)	Loading efficiency (%)
CS	1.5	37.5
sGO-FL	4.8	17.3
CS-sGO-FL	8.2	36.3
CMC-sGO-FL	2.5	12.5

Figure C.2 Fluorescence intensity of pig bladder mucosa surface incubated with sodium fluorescein (NaFL), fluorescein conjugated chitosan (CS-FL), fluorescein-labelled graphene oxide and functionalized graphene oxide (sGO-FL, CS-sGO-FL, and CMC-sGO-FL) before and after 3 washes of 10ml artificial urine. sGO-FL, CS-sGO-FL, and CMC-sGO-FL failed to portray quantitatively, the nanoparticles wash-off with decrease in fluorescence intensity, hence the data is unable to provide meaningful interpretations.