SYNTHESIS, CHARACTERISATION AND EVALUATION OF BIOCOMPATIBLE DISULPHIDE CROSS-LINKED SODIUM ALGINATE DERIVATIVE NANOPARTICLES FOR COLON TARGETED DRUG DELIVERY

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by

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µL	Microlitre
µM	Micrometre
^{1}H-NMR	Proton nuclear magnetic resonance
ADME	Administration, distribution, metabolism and excretion
ANOVA	One-way analysis of variance
CFU/mL	Colony forming unit/mililitre
-CHO	Aldehyde group
COOH	Carboxyl group
CRL 1790	Human normal colon cell line
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DO	Degree of oxidation
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EPR	Enhanced permeability and retention effect
ESCA	Electron Spectroscopy for Chemical Analysis
eV	Binding energy
FBS	Fetal Bovine Serum
FeSEM	Field Emission scanning electron microscopy
FT-IR	Fourier transform infrared spectroscopy
G	l-gluronic acid
G2 phase	Growth phase
GI tract	Gastrointestinal tract
GRAS	Generally regarded as safe

GSH	Glutathione
H_2O	Water
HCl	Hydrochloric acid
HSD	Honestly Significant Difference
HT-29	Human colon cancer adenocarcinoma cell line
IC_{50}	Half maximal inhibitory concentration
KCl	Potassium chloride
LbL	Layer by layer
M	d-mannuronic acid
M phase	Mitotic phase
m^2	Meter square
MD	Mean diameter
mL	Mililitre
MTT	3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide
mV	milliVolt
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometre
OD	Optical densities
-OH group	Hydroxyl group
PAA	Poly(acrylic acid)
PAH	Poly(allylamine hydrochloride
PBS	Phosphate buffer saline
PCX	Paclitaxel

PDI	Polydispersity index
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PEMs	Polyelectrolytes multilayers
PSS	Poly(styrene sulfonate)
PSSCMA	Poly (4-styrenesulfonic acid-co-maleic acid) sodium salt
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SEM	Scanning electron microscopy
SH	Thiol group
S-S	Disulphide bond
TEM	Transmission electron microscopy
TNB^{2-}	Dianion 2-nitro-5-thiobenzoic acid
UV-Vis	Ultraviolet-visible spectrophotometer
XPS	X-ray Photoelectron Spectroscopy

SINTESIS, PENCIRIAN DAN PENILAIAN BIOSERASI NANOPARTIKEL TERBITAN NATRIUM ALGINAT DISULFIDA BERANGKAI SILANG UNTUK PENYAMPAIAN DRUG KE KOLON

ABSTRAK

Penyampaian drug bersaiz nano khusus kepada kolon ialah sistem yang mencabar bagi sesuatu drug untuk sampai ke tapak sasaran kolon tanpa sebarang pembebasan di perut dan usus kecil. Penggunaan lapisan-demi-lapisan (LbL) pada salutan di permukaan nanopartikel telah meningkatkan penyampaian drug yang ketidaklarutan air secara oral ke kolon. Dalam kajian ini, kami mensasarkan untuk menghasilkan natrium alginat (SA) berangkai-silang disulfida berasaskan sisteamina dengan fabrikasi (LbL) untuk memperbaiki penyampaian paklitasel ke sel-sel kanser kolon secara kemoterapi oral. Sisteamina dikonjugasikan dengan tulang belakang SA yang teroksida untuk membentuk teras penswapasangan nanosfera berangkai-silang disulfida. Lima formulasi terbentuk, P1DL-P5DL dan disahkan oleh analisis Raman. P3DL telah dipilih untuk muatan paklitasel dan fabrikasi (LbL) dengan poli(alilamina hidroklorida) (PAH) dan poli (asid 4-stirenasulfonik asid-ko-maleik) garam natirum (PSSCMA) setelah menunjukkan keputusan yang memberangsangkan dari kajian pencirian dan pembebasan drug. Nanosfera P3DL muatan paklitasel yang difabrikasi, P3DL/PAH/PSSCMA telah menunjukkan kecekapan enkapsulasi sebanyak 77.1 % dengan kumulatif pelepasan drug sebanyak 45.1 %. Analisa sebaran cahaya dinamik (DLS) menunjukkan nilai pada 173.6 ± 2.5 nm dengan indeks kepoliserakan pada 0.394 ± 0.105 dan potensi zeta pada -58.5 mV. Analisa TEM dan SEM menunjukkan nanopartikel dalam bentuk sfera. Dalam kajian kepekaan pH (pH 1 hingga 7),

peningkatan saiz sebanyak 102.2 % menunjukkan bahawa peralihan P3/PAH/PSSCMA adalah bergantung kepada pH. Sementara itu, saiz P3/PAH/PSSCMA meningkat sebanyak 33.0 % dalam kajian pengurangan-responsif selepas diinkubasi dalam 10 mM glutationa (hari ke-7). HT-29 menunjukkan daya hidup yang tinggi (86.7 %) selepas dirawat oleh nanosfera pada kepekatan 50 μg/mL dan dianggap sebagai mengalakkan pembawa nano dalam penyampaian drug ke kolon. Lebih daripada 70 .0 % nanosfera terfabrikasi telah diserap masuk ke dalam sel-sel HT-29, dan ini menunjukkan proses kemasukan selular ke dalam sel-sel kanser telah berjaya. Oleh itu, natrium alginat berangkai-silang disulfida terfabrikasi ini boleh dianggap sebagai pembawa nano yang berpotensi untuk penyampaian kemoterapeutik khusus ke kanser kolon.

SYNTHESIS, CHARACTERISATION AND EVALUATION OF BIOCOMPATIBLE DISULPHIDE CROSS-LINKED SODIUM ALGINATE DERIVATIVE NANOPARTICLES FOR COLON TARGETED DRUG DELIVERY

ABSTRACT

Colon specific nano-drug delivery is a challenging system for a drug to reach the colon target site without any release along the stomach and small intestines. The application of layer-by-layer (LbL) on nanoparticles surface coating has been applied to improve the colon targeted oral drug delivery of insoluble drugs. Here, we aimed to formulate a self-assembly cysteamine-based disulphide cross-linked sodium alginate (SA) (with LbL) to improve the delivery of paclitaxel to colonic cancer cells to promote oral chemotherapy. Cysteamine was conjugated to the backbone of oxidised SA to form a core of self-assembly disulphide cross-linked nanospheres. Five formulations were formed, P1DL-P5DL and verified by Raman analysis. P3DL was chosen for paclitaxel loading and fabricated (LbL) with poly(allylamine hydrochloride) (PAH) and poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (PSSCMA) after showing promising results from the characterisation and drug release studies. P3DL fabricated paclitaxel loaded nanospheres, P3DL/PAH/PSSCMA exhibited an encapsulation efficiency of 77.1 % with cumulative drug release of 45.1 %. Dynamic Light Scattering (DLS) analysis was reported at 173.6 ± 2.5 nm with polydispersity index of 0.394 ± 0.105 and zeta potential of -58.5 mV. TEM and SEM analyses exhibited spherical shapes of nanoparticles. P3DL/PAH/PSSCMA was pH-

dependent swelling transition from the pH sensitivity study (pH 1 to 7, increase of 102.2 %). Meanwhile, the size of the nanospheres increased 33.0 % in reduction-responsive study after incubating in 10 mM glutathione (day 7). The HT-29 cells showed high viabilities (86.7 %) after been treated with the nanospheres at 50 µg/mL.and therefore, it was regarded as promising nanocarrier in colon drug delivery. More than 70.0 % of the fabricated nanospheres were uptaken in the HT-29 cells, thus, suggesting successful cellular internalisation process in the cancer cells. Therefore, this fabricated disulphide cross-linked sodium alginate nanospheres may be considered as a potential nanocarrier for colon cancer targeted chemotherapeutic drug delivery.

CHAPTER ONE

INTRODUCTION

1.1 Research background

In the recent years, many researchers have shown interest in the development and innovation of colon targeted drug delivery system. The colon drug delivery system provides potential solutions for many diseases such as inflammatory bowel diseases, specifically Crohn's disease, ulcerative colitis and colorectal cancer (Sreelatha and Brahma, 2013). In addition, it provides a wide range of potential treatment for systemic diseases and applications in the delivery of peptides and vaccines into the human body (Saphier *et al.,* 2012). Colon drug delivery system facilitates the amount of drugs that are able to be delivered to the colon disease region successfully by minimising any adverse effects. This system gives high retention time for drugs to localise in the colon which would improve the absorption of the drugs into the human body (Sreelatha and Brahma, 2013). For colon drug delivery, oral route is preferable and most convenient route of administration due to its flexibility (Deng *et al.,* 2015) and improvement of patient's compliance (Sosnik, 2014).

The different pH values along the gastrointestinal tract (GI tract) is one of the greatest obstacles for drugs to reach the target sites accurately (Chang *et al.*, 2012 ; Huang *et al.*, 2015). Many strategies related to colon specific drug delivery were introduced, ie. covalent linkage between drugs and polymers (pro-drug), pH, time and microbial responsive strategies (Tiwari *et al.*, 2010), osmotic pressure controlled drug delivery

system and also the use of multiparticulate systems such as microspheres and nanospheres (Agarwal *et al.,* 2015).

The human colon contains abundance of bacterial populations that are able to produce reducing environment for many chemical bonds like azo, nitro and disulphide bonds (Saphier *et al.,* 2012). The polymers with disulphide bonds are widely investigated due to their stability in extracellular fluids compared to intracellular fluids with glutathione (GSH). The GSH functions as reducing atmosphere for disulphide linkages (Chang *et al.,* 2012). In cancer cells, the concentration of GSH is four times higher than normal cells. For this reason, the introduction of disulphide cross-link in polymers could be a viable alternative strategy in colon cancer drug delivery studies (Gao *et al.,* 2014).

To date, there are varieties of nano carriers used in drug delivery system. Polysaccharide-based nanoparticles such as alginate play a vital role due to the qualities and favourable results as carrier for several anticancer drugs (Martinez *et al.,* 2012). Originated from brown algae, alginate consists of d-mannuronic acid (M) and l-gluronic acid (G) residues and bonded linearly by 1, 4-glycosidic linkages (Zhao *et al.,* 2012). It is biodegradable, biocompatible, non-toxic, (Chang *et al.,* 2012) and possess mucoadhesive properties (Hauptstein *et al.,* 2015).

Nanotechnology has also become a promising tool in improving therapeutic outcomes by focusing the delivery of drugs to the target sites to minimise drug accumulation at non-specific sites (Wang *et al.,* 2010). New system and technologies are required to

be developed and explored in overcoming current limitations in colon targeted drug delivery system. The therapeutic efficacy of the drug carriers could be improved and the undesirable adverse effects could be minimised *via* modification of the nanoparticles' features (Lim *et al.,* 2013).

In this research, a novel biodegradable nanosphere derived from thiolated sodium alginate was synthesised to improve the delivery of the hydrophobic drug, paclitaxel to colonic cancer cells. Sodium alginate was first oxidised using sodium periodate prior to addition of cysteamine hydrochloride to modify the backbone of the sodium alginate. The disulphide cross-linked nanospheres were then produced by self-assembly technique *via* the oxidation of thiol groups from cysteamine. Layer by layer (LbL) surface modification was conducted using polyelectrolytes; poly (allylamine hydrochloride) (PAH) and poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (PSSCMA) to control the drug release of paclitaxel. The characterisations, pH sensitivity, reduction response, *in vitro* drug release, cytotoxicity and cellular uptake for paclitaxel loaded nanospheres were further investigated. In this study, pH sensitivity and reduction responsive environments play a major role to ensure the release of drug at the cancer target site.

1.2 Objective

The main objective of this research is to develop a novel biodegradable thiolated sodium alginate derived nanospheres with surface modification to improve the delivery and release of paclitaxel to the targeted site of colonic cancer cells.

The sub-objectives are:

a) To synthesise thiolated sodium alginate by applying different ratios of oxidised sodium alginate and cysteamine hydrochloride.
b) To assess and characterise the self-assembly of thiolated sodium alginate into nanospheres.
c) To fabricate the nanospheres with Layer by layer (LbL) assembly technique using synthetic polyelectrolytes: poly (allylamine hydrochloride) (PAH) and poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (PSSCMA).
d) To determine the drug release of paclitaxel, cytotoxicity profile and cellular uptake of the nanospheres in colon cancer cells (HT-29).

CHAPTER TWO

LITERATURE REVIEW

2.1 Drug delivery

Drug delivery has received great attention nowadays due to its benefits in cancer therapy treatment. Cancer is recognised as one of the leading causes of death around the world. This issue has been declared as one of the public health problems due to the outrageous number of patients increased day by day (Jain *et al.,* 2016). Therefore, many researchers have conducted studies on the drug delivery systems that could be helpful for the development of innovative targeted therapies (Iglesias *et al.*, 2012).

The main purpose of drug delivery technology is to ensure therapeutic amount of drug is able to be delivered at the target site within the specific time. However, almost all chemotherapy drugs do not have this property (Wang *et al.*, 2010). Due to this, significant efforts are needed to improve the delivery of anticancer drugs to the tumour cells (Iglesias *et al.*, 2012) and reduce undesirable side effects to other non-targeted tissues or cells in the human body (Cortez *et al.*, 2006; Sreelatha and Brahma, 2013; Deng *et al.*, 2015). Many approaches have been reported to discover the best formulation to enhance the drug uptake by cancer cells. Therefore, it is really crucial to choose the right carrier for drug delivery system in order to ensure the pharmacological and pharmacokinetics of the selected drugs could be improved as compared to the 'free' drug alone (Hauptstein *et al.*, 2015).

The colon specific drug delivery system is highly useful for the treatment of local and systemic diseases. Examples of local targeting diseases are Crohn's disease, ulcerative colitis, irritable bowel syndrome, colorectal cancer and constipation. For systemic diseases, the delivery of peptides and vaccines such as insulin and typhoid are able to treat diabetic and typhoid fever respectively (Singh and Khanna, 2012; Sreelatha and Brahma, 2013). In colon cancer drug delivery, the drug should be protected until it reaches the target site. This means the drug release should neither occur in stomach nor small intestine. Further this, the drug should be degraded at the dissolution sites and be absorbed once it reaches the colon (Prasanth *et al*., 2012).

There are two main routes of administration for colonic delivery; oral and direct (rectal) administration. However, for direct administration, only small amount of drug is able to reach the target site due to the presence of splenic flexure at transverse colon (Tiwari *et al.*, 2010). Moreover, this method is not convenient and may not be acceptable by the patients (Lau and Lim, 2016). In cancer treatment, most patients prefer oral drug administration (Singh and Khanna, 2012; Anitha *et al.*, 2014; Lau and Lim, 2016) compared to parenteral administration (intravenous and intramuscular) (Deng *et al.*, 2015). This is because oral administration does not bring any harm or pain to the tissue during injection process and require less supervision, subsequently increases patients' compliance and decreases the cost in hospital (Fox *et al.*, 2015).

2.2 Colon cancer

In 2015, colon or colorectal cancer is the third most leading causes of mortality among cancer patients worldwide after lung and female breast cancer. It has been reported that 1.36 million new incident cases with more than half a million deaths every year. More than 55% cases are diagnosed in developed regions and the highest rates are in Australia and New Zealand with elderly over 69 years old the main target (Lin *et al.,* 2015).

Colon cancer cases have been reported in the United States since early 1940 (Siegel *et al.,* 2014). Based on the research conducted by American Cancer Society, the number of new cases would increase up to 27 million in 2050 (Pourjavadi *et al.,* 2014). Colon cancer cells are able to grow in the human body over 10 to 15 years. This cancer is often initiated from a group of benign adenomas, noncancerous colonic polyps. About 10% of these adenomas will develop and change into cancer cells caused by mutation factors in tumour suppressors, apoptotic genes and also oncogenes. Many colon cancer survivors have to cope with long term sequence of treatments due to the possibility of relapse (Lin *et al.*, 2015).

There are several ways to treat colon cancer such as surgery, radiation therapy, chemotherapy and radiofrequency ablation. Chemotherapy is usually used as an immediate treatment alone or together with surgery or radiotherapy. Surgical resection with chemotherapy utilises either drug or combination of drugs are suitable for the current treatment based on the stage of the disease (Anitha *et al.,* 2014). Nevertheless,

only 30% of the cases give good response in this treatment. Most treatments failed due to the development of tumour resistance cells and inability of drugs to distinguish between the tumour and normal cells within the body (Iglesias *et al.*, 2012). As a result, this condition affects the healthy cells and develop undesirable side effects due to the high toxicity possessed by anticancer drugs (Jain *et al.*, 2016). Some of the adverse effects from this treatment are neutropenia, anaemia, hand-foot syndrome, diarrhoea, gastrointestinal toxicity, mucositis, nausea, vomiting, fatigue, haematological disorder and liver toxicity (Lin *et al.*, 2015).

2.3 Anatomy of colon

The gastrointestinal tract (GI tract) consists of several parts of organ from mouth to anus (Figure 2.1). The digestive system is responsible in taking food, digesting it to gain energy and nutrients and also eliminating waste through faeces. There are two major parts of lower abdomen in digestive system namely stomach and intestine. The intestine consists of small and large intestine (Sreelatha and Brahma, 2013).

The small intestine divides into duodenum, jejunum and ileum which function to absorb nutrients and minerals from food. The colon is located at the distal end of the GI tract and comprised of caecum, ascending, transverse, descending segments and sigmoid region (Sreelatha and Brahma, 2013). This part plays a major role in extracting water and salt from solid wastes, storing wastes, absorbing some vitamins and an ideal site for microflora aided in fermentation process (Cummings and MacFarlane, 1991; Sreelatha and Brahma, 2013). The small intestines have higher

absorption capacity with 6.0 m length and ~120 m^2 surface area as compared to colon (1.5 m length and 0.3 m^2 surface area). Therefore, this unique physiological properties of the GI tract has become the major challenge for researchers to ensure the specific drug reaches the target site without any premature drug release either in stomach or small intestine (Chang *et al.*, 2012). The summary of physiological properties for GI tract are stated in Table 2.1 (Prasanth *et al.*, 2012 ; Lu *et al.*, 2016).

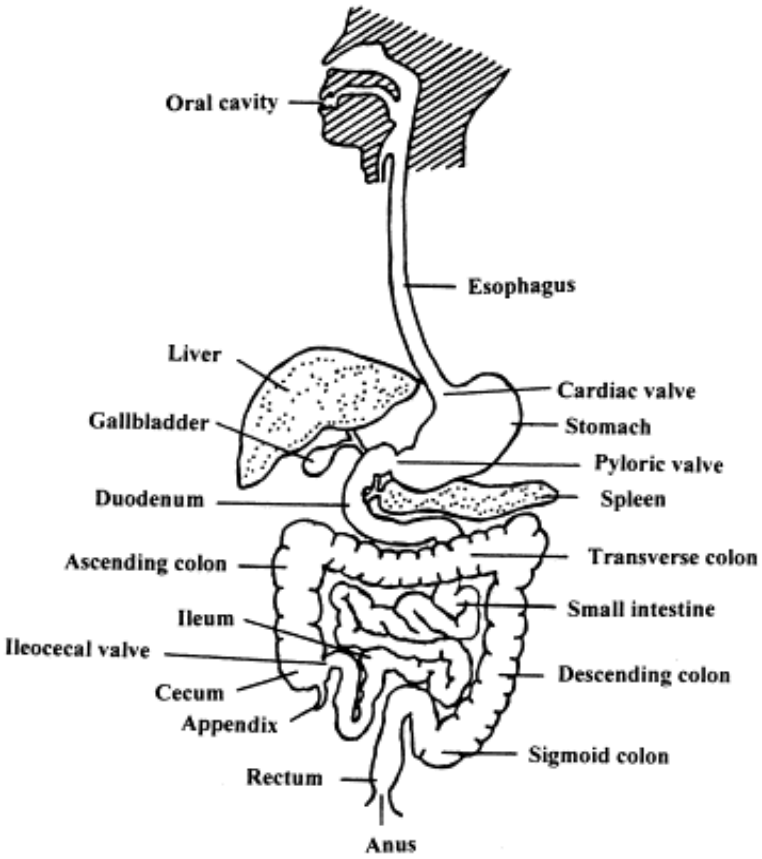

Figure 2.1: The gastrointestinal tract (GI tract) of human (Yousef *et al.*, 2012).

Table 2.1: The physiological properties of the GI tract.

Organ	pH	Transit Time	Bacteria Count CFU/mL
Stomach	1.0-3.5	>3	10^2-10^4
Small intestine	6.5-7.4	3-4	10^3-10^4
Large intestine	6.0-8.0	>20	10^{11}-10^{12}

However, there are several factors that influence and affect the physiological pH in the gastrointestinal fluid such as the food intake and the disease, for example, in colon cancerous environment, the pH might become slightly acidic with values between 4.0 to 5.0 (Gao et *al.*, 2014). In addition, at the colon entrance, pH would drop due to the presence of short chain fatty acids arising from bacterial fermentation of polysaccharides. There are more than 400 distinct species of microflora colonic bacteria that are able to produce large amount of lactic acid contributing to the decrease of pH in the colon (pH 5.0) (Prasanth *et al.*, 2012).

2.4 Paclitaxel as chemotherapeutic drug

Paclitaxel (PCX) is one of the chemotherapeutic drugs that has been widely used to treat various types of cancers such as colon (Wang *et al.*, 2010), head and neck cancers, multiple myeloma, melanoma and Kaposi's sarcoma (Smitha *et al.*, 2013), ovarian cancer, advanced breast cancer and non-small-cell lung cancer (Szczepanowicz *et al.*, 2016). PCX is derived from the taxane group and isolated from the bark of *Taxus brevifolia*. The mechanism of action for this antimitotic drug is to disrupt the

microtubules system in the cell cycle during the late of G2 and M phase by inhibiting the cell from undergo replication process (Danhier *et al.,* 2009). PCX will bind specifically at the β tubulin, a subunit of microtubule that causes total disruption in cell division process and leads to cell death (Kumari *et al.,* 2010).

To date, there are only a few formulations of PCX available in the market, eg. the paclitaxel albumin-bound nanoparticles, approved by the Food and Drug Administration (FDA) for the treatment of breast and lung cancers (Ma and Mumper, 2013). The limitation of the PCX formulation is due to poor water solubility, which is approximately less than 1 µg/mL (Koo *et al.,* 2013). PCX is solubilised with the mixture of Cremophor EL and dehydrated ethanol (1:1) v/v in intravenous administration to improve the bioavailability of PCX (Wang *et al.,* 2010 ; Koo *et al.,* 2013). However, few adverse effects were reported by the patients including hypersensitivity (Szczepanowicz *et al.,* 2016), neurotoxicity (Koo *et al.,* 2013), nephrotoxicity, vasodilatation on vascular muscle, laboured breathing, lethargy and hypotension (Danhier *et al.,* 2009).

Due to this, it is very crucial to find solution to overcome the poor water solubility of PCX and its non-specific mechanism of attacking both normal and cancer cells (Smitha *et al.,* 2013). These problems could be solved through the formulation of a carrier or vehicle, which delivers the PCX into the cancer cells. The development of drug carrier formulation is important to optimise the PCX antitumour activity and minimise its adverse effects (Szczepanowicz *et al.,* 2016). The encapsulation of PCX in the nanoparticles, made from biodegradable and non-toxic elements may protect the

human body from any side effects and toxicity caused by the drug. (Ma and Mumper, 2013).

2.5 Nanoparticles in drug delivery

The nanotechnology system for drug delivery research was explored in the late 1970s (Lim *et al.*, 2013). This system has been broadly used in various applications like fibre, textiles, agriculture, forensic science, space and medical therapeutics (Kumari *et al*,. 2010) due to distinctive properties that meet good criteria for many applications and the needs from the market (Rao and Geckeler, 2011). Nanoparticles are defined as solid and colloidal particles between the range from 10 to 1000 nm (Rao and Geckeler, 2011; Iglesias *et al.,* 2012) which offer many advantages in drug delivery compared to conventional approaches. Currently, numerous biodegradable polymers have been developed as nanocarriers to deliver the therapeutic of various water soluble, insoluble medicinal drugs and bioactive molecules. Nanocarriers are able to solubilise hydrophobic anticancer drugs by integrating with different water soluble compounds other than toxic organic solvent (Zhao *et al*., 2015 ; Liu *et al.*, 2016).

Nanoparticles are categorised as nanospheres or nanocapsules based on their structure. The matrix for nanospheres is entirely mass and solid. The drug molecules are usually adsorbed on the surface or encapsulated within the particles. Meanwhile, nanocapsules contain a vesicular system that acts as a reservoir to entrap drugs or other substances in the centre. The drugs are restrained in a cavity at the core of the particles and surrounded by a solid material shell (Rao and Geckeler, 2011). Both of these distinct

architectures have their own benefits as nanocarriers in delivering bioactive compounds to the target site in human body (Zhao *et al.*, 2015).

The main aim for synthesising the nanoparticles is to create safe and effective drug carriers that deliver drugs more precisely to the tumour cells and maintain a therapeutic concentration over an extended period (Iglesias Teij *et al.*, 2012; Lim et *al.*, 2013). The selection of materials in the formulation of nanoparticles becomes a crucial factor in determining the success of delivering drugs to the colon target site (Soni *et al.*, 2010). In addition, the nanoparticles must be able to protect the drugs from premature degradation due to the interaction with different challenging biological conditions caused by enzymes and harsh pH environments in the GI tract to accomplish intracellular penetration at the specific site (Kumari *et al.*, 2010). Therefore, the surface modification of the nanoparticles with distinct physicochemical properties is important in order to facilitate the nanoparticles penetrate into the mucus barrier at colon cancer site (Lu *et al.*, 2016).

The strategy based on enhanced permeability and retention effect (EPR) phenomenon is widely exploited for passive delivery of nanoparticles into cancer target site. The first phenomenon was reported in 1986 involving new vasculatures formation at the cancer site and directly stimulated the extravasation and permeability of nanoparticles to the cancer cells (Jain *et al.*, 2016 ; Lu *et al.*, 2016). In addition, the lack of lymphatic drainage at the cancer site enables accumulation and retention condition for nanoparticles (Szczepanowicz *et al.*, 2016). For colon cancer, the cancer cells will invade and destroy the several layers of colon including mucosa, submucosa,

muscularis and serosa. Therefore, the EPR effects will enable the nanoparticles to retain at the cancer site (Lu *et al.,* 2016). Furthermore, the small diameter of the nanoparticles create more vulnerable condition for nanoparticles to accumulate at the inflamed or cancer colonic region due to pathophysiological changes in cancer cells like increase in mucus production, mucosal surface alterations crypt distortions and ulcers. These conditions occurred with the companion of a disrupted intestinal barrier and infiltration of immune related cells such as macrophages, lymphocytes and dendritic cells (Beloqui *et al.,* 2014).

2.5.1 Surface modification of nanoparticles

There are abundant of developed nanoparticles formulations in drug delivery research. Various new formulations (particle size, permeability and release profile) of the selected polymers are enhanced to ensure their compatibility meet with the specific needs. However, these approaches are still facing some limitations. Therefore, surface modification technique is introduced to enhance the function of the nanoparticles by presenting a new targeting group on its surface. This is important for an effective cancer treatment because the drugs must precisely reach at the cancer cells and retain in a therapeutic concentration over an extended period of time (Lim *et al.*, 2013).

Polyelectrolytes multilayers (PEMs) consist of different deposition charged layers onto the surface of the spherical core carrier. This sequential adsorption of polyelectrolytes has become one of the most versatile methods of forming nanocarriers (Szczepanowicz *et al.,* 2016). There are ionic interactions formed between these two oppositely charged polyelectrolytes in an aqueous solution (Deng *et al.,* 2015). This system was introduced in 1991 by applying various modification theories such as

electrostatic interactions, hydrogen bonding, hydrophobic interactions, covalent bonding, and corresponding based pairing (Wang *et al.*, 2007).

The PEMs system is purposely constructed as a diffusion barrier to the selected encapsulated drug and further control the drug release with desired rate and period at a specific time (Nugraha *et al.*, 2014; Zhou *et al.*, 2014). This technology has been applied to oral drug delivery in improving the features of selected polymers to provide immediate respond towards the surrounding environment and increase drug uptake at the target site (Fox *et al.,* 2015). This system can be used to design for both reservoir and matrix nanoparticles drug delivery system which provides an ability to increase duration of time for polyelectrolyte nanoparticles remain in the blood circulation (Szczepanowicz *et al*., 2016). It also facilitates EPR phenomenon based on passive diffusion to the interstitial cancer target site with the nano-size ~100 nm particles (Ramasamy *et al.*, 2014).

There are several polyelectrolytes used in the surface modification of nanoparticles. The examples for standard synthetic pairs of polycations and polyanions are poly(allylamine hydrochloride) (PAH) and poly(acrylic acid) (PAA) (Guo *et al.,* 2015); polyethyleneimine (PEI) and poly(styrene sulfonate) (PSS) (Warszynski *et al.,* 2015). Both cations (positive) and anions (negative) surface charge of the nanoparticles show their own distinctive characteristics in improving the drug delivery treatment. For instance, the positively surface charged nanoparticles adhere to inflamed tissue in bowel diseases more easily due to the negative charge on the

intestinal mucosa surface. Meanwhile, the negatively surface charged nanoparticles are able to inter-diffuse among the mucus network due to less electrostatic interaction formed between them. Therefore, the nanoparticles would preferentially adhere to the inflamed colon *via* electrostatic interaction with the presence of positively charged proteins (cytokines) in colorectal cancer site (Lu *et al.,* 2016).

2.6 Polysaccharides in colon drug delivery

Natural polymers especially polysaccharides have been chosen as nanocarrier for colon drug delivery due to the benefits (Iglesias *et al.,* 2012). Most of the polysaccharides are known as "generally regarded as safe" (GRAS). These polymers are found originated from plants (guar gum, inulin and pectin) while chitosan, chondroitin sulphate are derived from animals (Tiwari *et al.,* 2010). The alginate and dextran are originated from algae and microbes respectively (Philip and Philip, 2010).

Polysaccharides contain reactive groups such as carboxyl, hydroxyl and amino groups, making it suitable to undergo multiple modifications for drug delivery study (Zhao *et al.,* 2015). Modified polysaccharides possess excellent characteristics in biodegradability, biocompatibility (Philip and Philip, 2010 ; Tiwari *et al.,* 2010) high stability, low toxicity, non-immunogenicity (Zhao *et al.,* 2015) and better mucoadhesive property (Yaser *et al.,* 2016). Further this, the polymers also play a major role in food industries (Kaur *et al.,* 2012) due to their abilities as binders, thickeners, emulsifiers and gelling agents (Sharma and Ahuja, 2011).

The process of fixing two surfaces is known as adhesion. Mucoadhesion is referring to adhesion that occurs in mucosal membrane (Sharma and Ahuja, 2011). Since 1980s, mucoadhesive polysaccharides are known as potential excipients to prolong the duration of contact time by increasing the drug concentration at the absorption site to improve the cellular uptake (Hauptstein *et al.,* 2015) on all the mucosal membrane surfaces (gastrointestinal, nasal, pulmonary, ocular, buccal, rectal and vaginal mucosa) (Hauptstein *et al.,* 2013). The mucus gel, also known as mucin in mucosal membrane consists of 95% water and 5% mucus glycoproteins. The mucus layer protects the epithelial cells surface against infections. In addition, it also acts as a selective physical barrier between the plasma membrane of the epithelial cells with the environments (Davidovich-pinhas and Bianco-peled, 2011).

As mentioned earlier, the increase of residence time for drugs to contact with mucosa membrane would improve the absorption of drugs at the target site (Singh and Khanna, 2012). The polymers and mucin interaction is basically based on the formation of non-covalent bonds such as hydrogen bonds, van der Waal's forces, and ionic interactions. These interactions are categorised as having weak adhesion properties. Therefore, to improve the adhesive interaction, the conjugation of polymers with other functional groups are further investigated (Sharma and Ahuja, 2011).

2.6.1 Alginate

Alginate (Figure 2.2) is a natural polysaccharide obtained from the cell wall of brown algae seaweed of *Macrocystis pyrifera*, *Laminaria hyperborea*, *Laminaria digitata, Laminaria japonica, Sargassum vulgare* (Masuelli and Illanes, 2014) and *Ascophyllum nodosum*. There are also alginates originated from bacteria such as *Azotobacter* and *Pseudomonas* species, however, they are very limited and only suitable for small scale research studies (Goh *et al.*, 2012). The alginate is widely used in textile, food, paper and cosmetics (Masuelli and Illanes, 2014). This polymer functions as an emulsion thickener, carrier matrix, wound dressing and agent against heartburn and gastric reflux in pharmaceutical and biomedical (Hauptstein *et al.*, 2015).

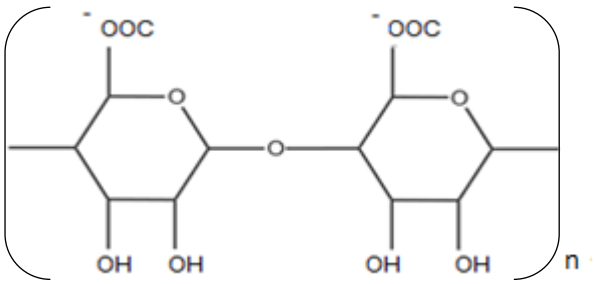

Figure 2.2: The structure of alginate.

In 1980, the first alginate particles were developed for drug encapsulation and many studies have been conducted on drug delivery since then (Paques *et al.*, 2014). Alginate is good in biocompatibility, higher degree of aqueous solubility, non-immunogenicity (Zhao *et al.*, 2015), low toxicity and possible for chemical modification. The mild gelatine characteristic of alginate forms when the alginate binds selectively with

certain multivalent cations such as sodium, potassium (Sosnik, 2014), calcium, barium and strontium (Zhao *et al.,* 2012). In addition, the presence of calcium ions in alginate polymer helps to form nanoparticles (Pawar and Edgar, 2012).

Alginate consists of linear β-d-mannuronic acid (M block) and α-l-gluronic acid (G block) which are combined to form 1,4-glycosidic linkages (Chang *et al.,* 2012). The M block segments show linear and flexible conformation whereas the G block segments act diversely by giving rise to glycosidic linkages and assist in steric hindrance around the carboxyl group. Therefore, the G block segments provide folded and rigid structural conditions of the alginate molecular chains (Yang *et al.,* 2011). The source and methodology used for algae extraction cause the diverge ratio between both subunits in biosynthesis of alginates (Bubenikova *et al.,* 2012 ; Zhao *et al.,* 2012).

The calcium alginate showed promising result in drug delivery with more than 90% of 5-fluorouracil was released at the colon target site (Agarwal *et al.,* 2015). As an anionic polymer, alginate has the capability to form hydrogen bonds with mucin type of glycoprotein (mucus layer of epithelial cells) from carboxyl and hydroxyl groups in the alginate. This interaction has made alginate as a popular choice for biotechnological and pharmaceutical applications (Davidovich-pinhas and Bianco-peled, 2011).

2.6.2 Structural modifications of alginate

The alginate consists of numerous free hydroxyl and carboxyl groups distributed along the backbone of its polymer chain. These functional groups make alginate to be vulnerable to various chemical modifications (Masuelli and Illanes, 2014). The properties of alginates are improved by modifying the chemical moiety for solubility, hydrophobicity, physicochemical and biological characteristics. There are several reported methodologies involved in alginate synthesis such as oxidation, sulfation, esterification, amidation or grafting methods. All of the alginate derivatives have good potential in the biomedical fields (Yang *et al.*, 2011).

The important points in designing alginate derivatives are solubility, reactivity and characterisation. In its native form, alginate is insoluble with mixed salt of the cations in seawater (Masuelli and Illanes, 2014). The choice of solvents in extraction process determines the chemical modification in aqueous, organic or mixed aqueous-organic media. The degree of solubility for alginate brings an impact to derivative substitution pattern (Pawar and Edgar, 2012). The reactivity of alginate is based on chemical modification of hydroxyl or carboxyl functional group. For hydroxyl group (OH), there are two positions available for chemical modification to occur; C-2 or C-3 and at C-6 for carboxyl group (COOH) (Pawar and Edgar, 2012). The hydrophilic and hydrophobic properties of alginate also could be adjusted by protonation and deprotonation of carboxyl groups (Yang *et al.*, 2011). The range and patterns of β-d-mannuronic acid (M) and α-l-guluronic acid (G) ratios affect the characteristics too

(Pawar and Edgar, 2012). The standard ratio between M and G block is 2:1 where the M block is hydrophilic while G block is hydrophobic (Masuelli and Illanes, 2014).

2.7 Disulphide cross-linked in polysaccharide polymers

Disulphide bond is formed from the coupling of two or more thiols. To date, disulphide polymers have received much interest in the study related to colon drug delivery due to its stability (Zhao *et al.,* 2015) and resistant in the upper GI tract but susceptible to degrade at colon region due to low reduction potential environment (Lau and Lim, 2016). Furthermore, the higher level of GSH as reducing agent in cancer cells also provides a conducive environment for the degradation of disulphide cross-linked polymers (Chang *et al.,* 2012 ; Gao *et al.,* 2014 ; Gao *et al.,* 2017).

The chemically modified polymers with sulfhydryl (thiol) groups are considered to have higher adhesive property compared to mucoadhesive polymers (Davidovich-pinhas *et al.,* 2009). It is due to the formation of disulphide bonds through covalent linking between the thiolated polymers (thiomers) with the cysteine subdomains of mucus glycoproteins at mucus membrane (Kast *et al.,* 2001; Davidovich-pinhas and Bianco-peled, 2011). The formation of covalent bond may improve the 'Pharmaceutical Glue' in delivery of drug to the target site (Jindal *et al.,* 2010). As a result, this condition reduces the dose of drugs used as it localises the polymers to the target site (Sharma and Ahuja, 2011). It is also believed to prolong the residence time for drug polymer formulations to assemble at the mucosal surface (Davidovich-pinhas and Bianco-peled, 2011) and therefore, enhances the cellular permeation at the target

site (Palmberger *et al.,* 2015). For this reason, further studies are needed to explore the advantages of thiomers in modifying the polysaccharides as the selective carrier in drug delivery system.

2.8 Strategies of disulphide cross-linked polymers in colon drug delivery

Based on the distinctive physiological properties of the colon, many strategies have been developed to fit the colon targeted drug delivery. Several approaches have been reported include pH-responsive, time-dependent, pressure dependent, bacterial degradation and reduction response drug delivery system (Chang *et al.*, 2012). All these approaches have good potential in delivering the selected drugs to the target site. Furthermore, optimisation of the physicochemical properties like size, surface charges, and the conjugation to some ligands binding to the receptors on the target cells are the additional factors that contribute to the accomplishment of this strategy (Koo *et al.,* 2013).

2.8.1 pH-responsive system

In the recent years, more research have been conducted on the pH-responsive polymers in drug delivery due to changing pH values in distinctive regions of the GIT (Kocak *et al.,* 2017). The pH in the colon cancer cells is lower compared to the normal and healthy cells. According to Gao *et al.* (2014), the environment in cancerous endosomes and lysosomes are slightly acidic (pH 5.0 to 6.5 and pH 4.5 to 5.0 respectively) compared to the environment in the normal cells. The slightly acidic environment in

the colon cancer cells is an ideal condition to stimulate the selective release of anti-cancer drugs.

The concept of pH responsive delivery was designed based on the ionisable transitions property of the nanocarrier to give a good respond towards its environment (Beloqui *et al.,* 2014 ; Kocak *et al.,* 2017). The pH responsive nanocarriers are also able to control the drug release by retaining the ionisable groups like carboxylic and amino groups. Weak and stable interactions are formed within the structure of the carrier. Weak interaction refer to the hydrogen bond and electrostatic interactions between the molecules in the nanocarries. This interaction is easily broken and causes serious drug leakage in the circulation. On the other hand, the stable interaction is formed by chemical cross linked which is difficult to get a good responsive degree due to its immovability. However, this interaction shows poor selectivity to tumour cells (Gao *et al.,* 2014).

Previous study stated that both calcium alginate and carboxymethyl cellulose hold unique properties in having anionic surface charge due to the presence of carboxylic groups. This condition allows both polymers to shrink and swell after exposing to acidic and basic environment. Both of these polymers showed good sensitivity towards different pH environments and this criteria meet the requirement in the delivery of drug through the GI tract (Agarwal *et al.,* 2015). In addition, Gao *et al.,* (2014) suggested that the disulphide cross-linked in carboxymethyl cellulose shows a good potential in controlling the methotrexate release profile with the presence of gluthathione, a reducing agent in cancer cells.

2.8.2 Bacterial degradation system

Colon is a suitable place for anaerobic bacteria such as *Bacteroides, Bifidobacteria, Eubacteria, Clostridia, Enterococci, Enterobacteria* and *Ruminococcus*. These microflora bacteria obtain their nutrient from the fermentation of undigested food in the small intestine (Tiwari *et al.*, 2010). The polysaccharides get a lot of attention for colon targeted drug delivery because of their ability to degrade by colonic microflora into simple saccharides. These biodegradable polymers are able to activate the microflora anaerobic systems to break the glycosidic linkages in the polymers (Cummings and Macfarlane, 1991; Smitha *et al.*, 2013).

The microbially triggered strategy has been reported as one of the popular methods in colon targeted delivery (Tiwari *et al.*, 2010). High number of bacteria populations in the human colon are able to create a reductive environment for chemical bonds like azo (Prasanth *et al.*, 2012) and disulphide bonds (Saphier *et al.*, 2012; Lau and Lim, 2016). Further this, the abundance of enzymes (glucoronidase, xylosidase, arabinosidase, galactosidase, nitroreductase, azareducatase, deaminase and urea dehydroxylase) produced by the normal microflora in the colon are able to assist the bacterial degradation for colon targeted drug delivery (Prasanth *et al.*, 2012).

The tricarballylic acid based disulphide cross-linked polymer is able to retain and maintain in harsh GI tract conditions. This study demonstrated high thiol concentrations present in colon simulated media from the disintegration of branched disulphide cross-linked polymers by *Bacteroides fragilis.* This finding concludes that

the thiol-based polymer is able to withstand the GI tract and only can be cleaved in reducible environment at the colon with the presence of bacteria (Lau and Lim, 2016).

2.8.3 Reduction responsive system

The reduction responsive stimuli has gained much attention as smart drug delivery in nano platform for colon drug delivery system (Liu *et al.*, 2016). Besides the different pH in the GI tract, there is significant reductive potential between intracellular and extracellular of the cancer site. Glutathione (GSH) is an important reducing agent in the human body (Zhao *et al.*, 2015). The concentration of GSH in the cytoplasm (intracellular) and plasma (extracellular) are 1-10 mM and 10 μM respectively (Huang *et al.*, 2015). The concentration of GSH in the cancer cells is 100 to 500 fold higher than the normal cells. Therefore, by utilising the difference between the normal and pathological site, the selected carrier would be able to control the release of drug at the specific target site (Huang *et al.*, 2013; Gao *et al.*, 2014; Liu *et al.*, 2016).

Nearly 99% of all bacteria species are found in the proximal small intestine to distal colon (Tiwari *et al.*, 2010 ; Sreelatha and Brahma, 2013). The expression of total metabolic and enzyme activity changes the pH in GI tract. The reduction potentials of the proximal and distal small intestines are between −67 mV and −196 mV respectively whereas −415 mV at the ascending colon showing the lowest redox potential for colon. The disulphide cross-linked polymers are referred to reduction sensitive polymers because they could be reversibly cleaved by low reduction potential in the colon (Lau and Lim, 2016) with the standard reduction potential of ~250 mV for disulphide bonds (Chang *et al.*, 2012).