PH-SENSITIVE AND TARGETED PLGA-BASED DRUG DELIVERY TO COLORECTAL CANCER

By

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B. E and M. E

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University
January, 2012
I am the author of the thesis entitled

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ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my supervisor, Professor Lingxue Kong, for his discussions critical to the progress of the research, for the help he gives to tackle all scientific challenges, for support and encouragement during my studies here. I appreciate his care and efforts to ensure my professional development and my successful integration here. All these were extremely essential for the completion of this dissertation. I consider myself fortunate that he taught me critical scientific thinking and that he was willing to share with me his scientific visions of the doing research and paper writing. I have learnt a lot from him, how to become a wise scientist, to be an excellent researcher.

Special thanks go to Dr. Jane Dai, Dr. Mary She, Dr. Weimin Gao, Dr. Puwang Li and Ms. Juan Zhang for the discussion and suggestions during the work. I am also grateful to all of my colleagues for giving me a helping hand during this whole process. I would like to give thanks to Prof. Zeng and Mr. Lijue Chen at Huazhong University of Science and Technology for their help to do the in-vitro drug release experiments.

I am also grateful to Prof. Zheng Peng and Prof. Maofang Huang for their hosting of me to do half years’ joint research experiments in the Agricultural Product Processing Research Institute, Chinese Academy of Tropical Agricultural Science, China.

My deep gratitude goes to the technician Ms. Marion Wright for her day-to-day support in the laboratory work and for managing the ordering of chemical reagents and facilities.

Last, but not least, I would like to thank my parents, my family and my friends for their encouragement, unconditional love and support throughout these years, for their patience in listening to my complaints and frustrations over the last
three years. With their encouragement, I finally passed through the most challenging period.
ABSTRACT

Drug delivery systems using biodegradable and biocompatible nanoparticles offer great potential applications for the treatment of colorectal cancer. However, the current drug delivery systems have serious drawbacks and have difficulties in delivering the therapeutic drugs to the specific site. Moreover, many drugs can be lost in the upper gastrointestinal tract in the oral delivery route. The strategies used to address these challenges were to design the drug delivery systems functionalized targeting molecule folic acid (FA) and encapsulate the drugs loaded nanoparticles into the pH sensitive Eudragit S100 microspheres. In this way, the novel drug delivery systems not only maintain the original properties such as sustained and delayed drug release profile but render new and improved functionalizations. Therefore, overall, this thesis focuses on five main areas: fabrication of 5-fluorouracil (5-FU) loaded PLGA nanoparticles with enhanced drug loading and small particle size; chemical modification of PLGA nanoparticles surface by cancer specific targeting molecule folic acid (FA); microencapsulation of 5-FU loaded PLGA nanoparticles for the oral delivery to colon area; the PLGA surface functionalization by chitosan (CS); and plasma treatment for enhancement of hydrophilicity and functional groups on the PLGA surface for further utilizations.

Firstly, nanoparticles for delivery of therapeutic drugs always suffer from low drug loading and high nanoparticles size. A drug delivery system with enhanced drug loading and maintaining small particle size was designed to deliver the anticancer agents to colon area with high effectiveness and efficiency. In the studies, 5-FU was successfully incorporated into the PLGA matrix by modified double emulsion and solvent evaporation method (W_1/O/W_2). The initial drug feeding, volume ratio of outer water phase to the organic phase, pH value of outer water phase and PVA concentration were all optimized to achieve the enhanced drug loading, encapsulation efficiency and small particle size. After optimization, a drug loading of 5.8%, encapsulation efficiency of 28.6% and nanoparticles size of 197.8 nm was obtained by the
optimized fabrication parameters. The drugs 5-FU in PLGA polymeric nanoparticles were confirmed in an amorphous or molecular dispersion state by XRD and DSC characterization. Moreover, the drugs and polymer matrix have only physical interaction and no chemical one from the FTIR results. The \textit{in vitro} cumulative drug release profile showed an initial burst drug release and followed by a slow and sustained drug release over an extended period of more than 120 hours. The results obtained in this study suggest that the fabrication of this kind of drug delivery system is feasible and the prepared systems have great potential for the practical application for treatment of colorectal cancers.

Targeted drug delivery systems overcome traditional systems' limitations such as non-selectivity and less affinity. In this study, the colorectal cancer cell specific molecule folic acid was selected as the targeting ligand to incorporate into the PLGA backbone, in order to facilitate the drug delivery system to the designated sites. Folic acid conjugated PLGA was synthesized via the reagent 1, 3-diaminopropane which has the amine groups on both sides. During the synthesis, the kind of solvents and reagent amount were found to have great impact on the successful preparations. Remnant PVA on the particle surfaces cannot be neglected as properties of the nanoparticles were highly affected by the residues. The composition and structure of the synthesized PLGA-1, 3-diaminopropane-folic acid copolymer was detected and verified by $^1$H NMR. \textit{In vitro} cell experiments were confirmed the efficacy of the prepared systems. Therefore, this chapter provided a feasible scheme to fabricate folic acid functionalized drug delivery carriers. And the unique properties of the 5-FU loaded PLGA-1, 3-diaminopropane-folic acid nanoparticles make them promising systems for the targeted delivery of therapeutic drugs to colorectal cancers.

The 5-FU loaded PLGA nanoparticles suffer from great drug loss in the upper gastrointestinal tract in the oral delivery route. Before the drug delivery systems were delivered to the colon area, it has to sustain the harsh and complex pH conditions. Therefore, the well-designed drug carriers have to be developed to transport the nano-drug delivery system and prevent them from releasing in the unnecessary parts. Microencapsulation of drug loaded
nanoparticles through coating layers of Eudragit S100 microspheres was prepared by oil-in-oil technique (O/O), in order to achieve pH sensitive functions. Microspheres fabricated by different parameters were all exhibited spherical shapes from the SEM images. The probe sonication times have effect on the microspheres sizes, encapsulation efficiency and zeta potential. Microsphere sizes and drug release profiles are variable based on the surfactants used in the fabrications. Limited drug release was tested under the pH 7.4 drug dissolution environment. Therefore, the microspheres Eudragit S100 can keep the drug loaded nanoparticles intact before drug carrier reach the colon area.

Although PLGA is a biodegradable and biocompatible material and has been used in many areas, its extended usage is always hampered due to few functional groups on the PLGA surface. The incorporation of chitosan (CS) onto PLGA surface can not only increases the reactive functional amine groups (NH₂), but also promotes the drug carrier affinity to the cancerous cells. Therefore, in this research, the biocompatible material chitosan (CS) was coated onto the drug loaded PLGA nanoparticles in two different methods, one is physical adsorption and the other is chemical binding. Zeta potentials changed from the negative charge to positive one after coating chitosan. Moreover, the particles sizes increased after formation of PLGA/CS blends. FTIR and XPS spectra confirmed the existence of the chitosan on the PLGA nanoparticles surface. The drug release from PLGA/CS nanoparticles showed burst drug release profiles and attained higher cumulative drug release. The additions of chitosan onto PLGA nanoparticles further explore the utilization of PLGA drug delivery carriers.

Finally, the PLGA surface modified by plasma technology was studied in chapter 6. PLGA’s limited functional group in the polymeric chain and hydrophobic surface always restrict its widely usage. Plasma technology has the advantages of easy operation, surface only treatment and solvent free make it a very promising technique to do the PLGA surface modification. In the studies, PLGA surfaces were modified by oxygen gas plasma in continuous mode and pulsed mode. Different plasma treatment powers and times were
applied to investigate the surface hydrophilicity by contact angle measurement, surface morphology by SEM images and functional groups immobilization by XPS. The results exhibited that different treatment parameters have a great impact on the surface hydrophilicity. Contact angles are dependent on the treating mode, time and power. SEM images showed the morphologies are relied on the treating energies. The amount of ester bonds derived from XPS calculation exhibited a great enhancement after the plasma treatment. The results from this study suggest that more functional groups expressing on the PLGA surface and PLGA surface hydrophilicity can be achieved by plasma treatment, which will make the PLGA surface easy manage and conjugation to targeting molecules.

In summary, the present work has successfully developed an oral drug delivery system for the therapy of colorectal cancer. Enhanced drug loading and encapsulation efficiency drug delivery carriers with small particle size were achieved. The system exhibited a sustained and prolonged drug release profile. The targeting molecule directed the drug carriers to go to cancerous sites. pH-sensitive Eudragit S100 coating well-protected the system from releasing in the upper gastrointestinal tract. Chitosan and plasma technology treating PLGA surface will make PLGA based product more versatile. Therefore, the prepared drug delivery system and related modifications will be very promising for the targeted drug delivery systems.
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<th>Description</th>
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<tbody>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluouracil</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>FDA</td>
<td>American Food and Drug Administration</td>
</tr>
<tr>
<td>FR</td>
<td>Folate receptor</td>
</tr>
<tr>
<td>FA</td>
<td>Folic acid</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention effect</td>
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<tr>
<td>PLA</td>
<td>Poly (lactic acid)</td>
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<td>PLGA</td>
<td>Poly (lactic acid-co-glycolic acid)</td>
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<tr>
<td>PEG</td>
<td>Poly (ethylene glycol)</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
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<tr>
<td>PCL</td>
<td>Poly (caprolactone)</td>
</tr>
<tr>
<td>EA</td>
<td>Ethyl acetate</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>TPGS</td>
<td>D-α-tocopheryl polyethylene glycol 100 succinate vitamin E</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>CS</td>
<td>Chitosan</td>
</tr>
<tr>
<td>EDC·HCl</td>
<td>N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride</td>
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<tr>
<td>NHS</td>
<td>N-hydroxy-succinimide</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>DL</td>
<td>Drug loading</td>
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<td>EE</td>
<td>Encapsulation efficiency</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>UV-Vis</td>
<td>Ultraviolet visible</td>
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<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<tr>
<td>XRD</td>
<td>Powder X-Ray Diffraction</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectrometer</td>
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<tr>
<td>¹H NMR</td>
<td>¹H nuclear magnetic resonance</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticles</td>
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<tr>
<td>MS</td>
<td>Microspheres</td>
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<td>W₁/O/W₂</td>
<td>Double emulsion and solvent evaporation</td>
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<td>O/O</td>
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<td>TDL</td>
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<td>Pulsed mode</td>
</tr>
<tr>
<td>PTFE</td>
<td>Poly (tetrafluoroethylene)</td>
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<td>RF</td>
<td>Radio frequency</td>
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1 LITERATURE REVIEW

1.1 Introduction

Colorectal cancer (CRC), a term used to refer to cancers that affect the colon and rectum, is the second most common cancer in both men and women in Australia, affecting 1 in 12 Australians in their lifetime [1]. Every year, approximately 140,000 new cases of colorectal cancer are diagnosed and 560,000 people die of this disease worldwide [2]. The colorectal cancers appear, develop and spread gradually. Correspondingly, the selection of treatments depends on the staging of the cancers and individual medical conditions. Surgery is still the primary method for treatment of early stage colorectal cancer. Chemotherapy and radiation therapy as adjunctive therapies are used before and after the surgery [1].

Currently, most of chemotherapies are administered by intravenous injection. Intravenous therapy is giving the chemotherapeutic agents directly into the vein, which is the fastest way to deliver anticancer drugs and medications throughout the body. But this method always suffers from a risk of infection as the bacteria are easily introduced into blood through the break of the skin. Moreover, the intravenous therapies are not effective to treat the cancers in colon area. The drugs circulated in the blood vessel kill not only the cancerous cells but also the healthy ones, which results in a very high side-effect. Furthermore, the therapeutic drugs usually have a short half life time and rapid plasma clearance. Therefore, most of the drugs lost without any effect to the cancer cells.

Using oral drug delivery systems to transport the therapeutic drugs from gastrointestinal tract to the designated area greatly advance the treatment and overcome the traditional treatment side-effect. Compared to previous treatments, oral drug delivery systems have many advantages, such as less pain, easy operation. The patients only need to take the drugs by mouth and don’t have to endure the pains from the break of skin. Also, there is no necessity to
ask the nurses to do the injections. The oral drug delivery systems can achieve the sustained drug release using the biodegradable encapsulation materials. This also enables the drug delivery carriers to be compatible with human body.

There are many different biodegradable and biocompatible materials for application of drug delivery system. They are selected based on the degradation rate of encapsulation materials and required drug release profiles. Poly (lactide-co-glycolide) (PLGA) is a commonly used biomaterial and has been approved by American Food and Drug Administration (FDA) for clinical usage, which facilitates the translation of the products into practical applications. The degradation rate of PLGA can be regulated by ratio of two monomers in the polymer chains. But drug loaded PLGA cannot resist the degradation in the upper gastrointestinal tract and the system are not able to recognize the colon targeting points.

To functionalize the drug loaded PLGA system can not only maintain the original properties but render new functions. Microencapsulation of drug delivery carriers by pH sensitive materials can assist the carriers to pass the harsh environment and keep the systems intact. The incorporation of targeting molecule onto the systems will direct the carriers to the specific targeting site. However, the PLGA has few functional groups in the polymer chain, which leads to the difficulty of conjugation between PLGA and targeting molecules.

Using diversity of techniques and chemicals to modify PLGA surface for further reactions have been widely investigated. By coating chitosan onto PLGA surface, large amount of amine groups can be expressed. Plasma technology is also a versatile method to treat the sample surface. It can not only immobilize the functional groups onto the biomaterials, but also change the hydrophilicity of the samples surface. This is quite beneficial for the enhancement of hydrophilic drug loading.

Overall, in this literature review, some fundamental information on the colon and colon cancer are described. The treatment of colon cancer and commonly used chemotherapeutic drugs are also briefly reviewed. Different encapsulation
materials with biocompatibility and biodegradability are summarized and kinds of targeting molecules are analyzed. Techniques for material modifications and varieties of drug encapsulation methods are depicted. Finally, key parameters of drug dosages are fully discussed.

1.2 Colon and colon cancer

1.2.1 Background

Colon is located in the final part of the digestive system in human body. And it is also the last portion of the human gastrointestinal (GI) tract. Normally it is about 1.5 meters long and 63.5 mms in diameter. It starts from the small intestine and ends in the rectum. In general, the colon consists of four sections [3]: the ascending colon, the transverse colon, the descending colon, and the sigmoid colon. The large intestine is formed by colon, cecum, and rectum. The colon position and its structure are described in Fig. 1.1.

![Colon position and Structure](image)

Figure 1.1 Colon position and Structure [4]
Colon has three major functions that make important contributions to human’s health and well-being. It absorbs water, essential vitamins and minerals from the digested food. It can eliminate the solid wastes from the human body, including toxins and harmful bacteria as well as indigestible materials. Furthermore, it also serves as a site in which the unabsorbed materials ferment with the aid of flora.

Like all other organs in human body, the colon comprises many types of cells. Usually, normal cells in the body can grow, divide and die in an orderly fashion. Cancer or abnormal cells appear due to the damage of DNAs. DNA exists in every cell and controls all of the cells’ activities. For most of the time when there is something wrong with DNA, the cells will either die or are able to restore. In cancer cells, the damaged DNA is not repaired, resulting in the growth and division of the cells to form a mass of malignant tissues.

The incidence of colorectal cancer is quite different from region to region in the world. It is noticed that the disease is especially common in most of the developed and industrialized countries including North America, Western Europe, Australia and New Zealand. The high incidence of colorectal cancer in these areas might be due to high rate of red meat consumption [5]. Generally, about 8.5% of all new cases of diagnosed cancers are colorectal cancers throughout the world [6], which lead to 140,000 new cases of colorectal cancer and 556,000 cases of deaths worldwide every year [2], making colorectal cancer as the second most common cause of death from cancer and third most common malignancy in the world [7].

It was found that the people who come from countries at lower risk of colorectal cancer seem to develop high risk of this cancer when they immigrate to other countries. This can be inferred that lifestyle and behaviour play a very important role in the occurrence of this disease. Although there are varieties of reasons to cause the colorectal cancer, some risk factors that affect people to have the high potential to suffer from this disease usually include [8]:
CHAPTER ONE

- Age older than 50 years: Young people might have the colorectal cancer, but the possibility for a person older than 50 increases dramatically. It was reported that over 90% people who are diagnosed with colorectal cancer are older than 50 years.

- A history of colorectal adenomas: If a patient has a history of colorectal adenomas, he or she has higher chances to get this cancer again. Even the colorectal cancer was completely removed by surgery, it can be found in other parts of the colon area.

- A history of uterine, ovarian and breast cancer: Research discovers that women who have a history of uterine, ovarian and breast cancer have a high risk of developing colorectal cancer.

- Familial history of colorectal cancer or adenomas: One person’s parents, children and brother or sister who had colorectal cancer may increase the chance for that person to have the colorectal cancer. A family history of hereditary polyp syndrome increases the risk of suffering from colorectal cancer among the whole family members.

- Diet: A diet which is very high in fat and calories and low in fibre may be associated to high risk of developing colorectal cancer.

1.2.2 Stages of colorectal cancer

For most of the cases, colorectal cancers develop very slowly, which takes a time range of several years. In fact, before a real cancer appears, the growth of tissue or tumour starts from a non-cancerous polyp with a mushroom-like shape which usually grows on the lining of colon. The image of polyps and the position they grow in the colon are showed in Fig. 1.2. One kind of polyps called adenomatous polyp usually results in colorectal cancers over time. Once cancer develops within a polyp, it can start to grow into the colon wall eventually. After that, the cancer cells may grow into lymph vessels or even
blood vessels. At the stage of spreading into blood vessels, the cancer cells can travel and appear in distant parts of the human body. This process of spread is regarded as metastasis [9]. Different stages of colorectal cancers are described in Fig. 1.3 and detailed information is provided below.

Figure 1.2 Polyps in colon

Stage 0

This is the earliest stage of colorectal cancer. The cancer only involves the lining or mucosa of the colon or rectum and is confined to polyp(s) (tissue bulging from the surface of an organ). When the polyps are removed during a colonoscopy, the chance of them progressing to later stages of cancer is eliminated.

Stage 1

At this stage, the polyp has progressed to a tumour, and extends into the wall of the colon or rectum when colon cancer involves more than just the inner lining of the colon. Treatment can include surgery to remove the section of the colon that is cancerous. This type of surgery is called a resection. The healthy, non-
cancerous sections of the colon are reconnected again. The five-year survival rate is 95%.

Stage II

This stage of colorectal cancer is when the cancer has spread beyond the colon to the tissue surrounding the colon but has not spread to lymph nodes. Cancer spreading in this manner from one part of the body to another is called metastasis. A resection surgery may also be used to treat this stage of cancer. The five-year survival rate drops dramatically to 60%.

Stage III

Cancer that has spread outside the colon and on to the lymph nodes in the area surrounding the colon is known as Stage III. In this stage, the cancer has not spread to other organs in the body, and treatment is more aggressive. Surgical resection of the colon, chemotherapy, and other medical therapies may be necessary. The five-year survival rate is 35 to 60%.

Stage IV

In this stage, the cancer had spread to other organs in the body such as the lungs or liver. In addition to a surgical resection and chemotherapy, radiation treatment, surgery to remove other affected parts of the body may be necessary. At this stage, there is only a 3% chance of reaching the five-year survival time.

Recurrent Cancer

Cancer that comes back again after treatment, either in the colon, or in some other part of the body, is called recurrent cancer. Even after successful treatment of colon cancer, regular check-ups are necessary to find any recurrent cancer early.

Therefore, the early detection and cure of colorectal cancer are essential to a successful treatment. When the stages III and IV are reached, it will have less possibility to kill the cancer cells completely.
1.3 Treatment of colorectal cancer

There are different methods for the treatment of colorectal cancers based on the stages of the cancers. Surgery is still the primary method for the treatment. The chemotherapy and radiation therapy are used as adjunctive therapies before and after the surgery, based on individual patient’s condition and other medical factors.

Surgical removal is limited to kill the cancer cells which have already spread. It was found that large percentage of colorectal cancers have spread to the nearby organ or lymph node when the colorectal cancers are diagnosed [11]. Therefore, the combination of different treatment methods is required and necessary.
There are many differences between the two adjunctive therapies, chemotherapy and radiation therapy. Chemotherapy can be applied for all kinds of cancers, while the radiation therapy is only suitable for a limited amount of cancers. Radiation therapy makes use of radio isotope to impose action on the tumour while a certain drug or a combination of various drugs are used in the chemotherapy method.

Radiation therapy is an important cancer treatment method and is normally used for reducing the tumour size. As the tumours grow larger, they reach some parts of body and give pressure to the contact part such as nerves and organs, which results in the pain and pressure to the patients. Radiation therapy by shrinking the tumour alleviates the pains. For some cases, the radiation therapy causes the redness of the skin. Therefore, how to take steps to protect the skin before the treatment is a concern to the patients.

Intravenous chemotherapy is another systemic therapy which uses anticancer drugs that enter the blood stream to destroy cancer cells in the body [12]. It is commonly applied to treat the patients who have advanced cancers. If administered before surgery, chemotherapy is also called neoadjuvant therapy, in order to shrink a large tumour. If the chemotherapy is carried on after the surgery, it is called adjuvant therapy. The purpose of the adjuvant therapy is to kill any remnant cancer cells and at the same time prevent cancers from returning to the colon or other organs.

The chemotherapeutic drugs oxaliplatin (Eloxatin®), 5-fluorouracil (5-FU) and leucovorin (Wellcovorin®) are potent treatments for the colorectal cancer, whose structures are described in Fig. 1.4. Fluorouracil is one of the most widely used components of chemotherapy regimens for colorectal cancer, and nowadays, the standard regimen is an intravenous bolus injection of 5-fluorouracil [13]. 5-Fluorouracil is a pyrimidine analogue and can inhibit RNA function. Moreover, it can prevent the processing and synthesis of thymidylate and is normally administered parenterally, as the absorption of 5-FU is unpredictable and incomplete after ingestion [14]. One of the recent chemotherapeutic regimens is combination usage of oxaliplatin and 5-FU,
followed by administration of leucovorin, which was confirmed to be suitable for the metastatic carcinoma of the colon [15]. In 2004, American Food and Drug Administration (FDA) authorized two new drug therapies, i.e., bevacizumab (Avastin®) and cetuximab (Erbitux®); the former can block the blood vessels growing to the cancer cells [16], while the latter can affect the hormone-like factors which boost cancer cell growth [17]. When using the therapeutic drugs, more attention has to be paid. Some chemotherapy drugs affect the nervous system, which cause the numbness and tingling pain in the hands and feet [18]. Other side effects of chemotherapy include mouth sores, vomiting and nauseating feeling, thus some anti-suffering drugs such as Kytril and Zofran can be employed to stop the vomiting and nausea [19].

Although most of these chemotherapeutic drugs are currently administered by intravenous injections which are giving the chemotherapeutic agents directly into the vein and lead to the anticancer drugs and medications throughout the body in a short time, sometimes this method results in a risk of infection due to the introduction of bacteria from the break of skin [20]. Furthermore, chemotherapeutic drugs in the blood kill not only the malignant cells but also normal cells and tissues, thus causing serious side-effects. The therapeutic drugs circulated also suffer from a short half-life time and rapid plasma clearance, which causes the drug lost without taking any effect.

Therefore, it is essential to develop novel and effective methods to overcome these problems. Using drug delivery systems, especially oral drug delivery carriers, to transport the therapeutic drugs to the targeted areas aroused wide interest [21]. Compared to the current treatment methods, the oral drug delivery systems possess many advantages, such as less pain and easy operation. Using the oral drug delivery administration, the patients only need to take the drugs from mouth and don’t have to endure the pains from the break of skin. Moreover, the patients can take the drug carriers orally and there is no need to ask professional persons to do the injections or radiation therapy.

Polymer based oral drug delivery systems with biodegradability and biocompatibility are widely needed, for the therapeutic drugs loaded carriers
can not only retain the drug therapeutic effect but also achieve the sustained and controlled drug release by degradation rate of encapsulation materials [22]. This also enables the drug delivery system to be compatible with the human body. Therefore, it is necessary to have full investigation of the commonly used biodegradable and biocompatible materials.

![Oxaliplatin](image1)

**Oxaliplatin**

![5-FU](image2)

**5-FU**

![Leucovorin](image3)

**Leucovorin**

Figure 1.4 Structure of Oxaliplatin, 5-FU and Leucovorin
1.4 Biodegradable polymers for drug delivery carriers

Polymers used for drug delivery carriers can be roughly classified into biodegradable and non-biodegradable ones. The biodegradable polymers are more widely investigated than the non-biodegradable ones as the latter ones need further treatment and retrieval after administration of the drug carriers. While biodegradable polymers result in hydrolysis in the human body and yield monomers that are able to be absorbed by organisms or are the by-products of cellular metabolism [23]. The hydrolysis of biodegradable polymers derives from the bond cleavage in the long polymer chains in certain \( pH \) values environments [24]. Here some of the most popular biodegradable polymers for drug delivery carriers are fully discussed.

1.4.1 Poly (lactic acid) (PLA)/Poly (lactic acid-co-glycolic acid) (PLGA)

Homopolymer poly (lactic acid) (PLA) and random copolymer poly (lactic acid-co-glycolic acid) (PLGA) are the most widely used and studied biodegradable materials, both in the research and in the practical applications. They have been approved by American Food and Drug Administration (FDA) for clinical usage, which means the products are easily to be translated into practical utilizations. Both PLGA and PLA are belonged to polyesters, which can be prepared from ring-opening polymerization of the corresponding cyclic lactone monomer [25].

Due to PLGA and PLA’s biodegradability and biocompatibility, PLGA and PLA have been used in many areas, such as suture, bone plates, extended drug release and abdominal mesh [26]. Fig. 1.5 and Fig. 1.6 describe the schematic hydrolysis of poly (lactic acid) and poly (lactide-co-glycolide) into monomer units, respectively. Ester bonds in the polymer backbones degrade chemically through hydrolysis. The lactic acid and glycolic acid, which are the degradation products, are non-toxic and water soluble products which further metabolize to carbon dioxide and water [27].
Based on these biodegradable properties, numerous systems utilize PLGA and PLA to fabricate the drug loaded microspheres and nanoparticles system, in order to achieve the sustained drug release and extend a long time’s drug duration [28]. The different PLGA and PLA properties such as lactide/glycolide ratio, molecular weight, capped end PLGA or uncapped one have great influence on the drug release. Also, the surface of the uncapped PLGA and PLA which have carboxylic group at the end of the polymer can be easily modified. PLGA and PLA conjugated to some targeting molecules [29] to form targeted drug delivery carriers maintain these original properties and render new and improved targeting functions. Moreover, when therapeutic drugs are encapsulated into the PLGA and PLA nanoparticles, how to achieve enhanced drug loading [30], encapsulation efficiency [31] and optimal particles size [32] are key issues to be considered.

1.4.2 Poly (ethylene glycol) (PEG)

Poly (ethylene glycol) (PEG) is a biodegradable, hydrophilic and biocompatible material. It exhibits excellent properties and has been approved by FDA for practical medical consumption. Usually two kinds of PEG, the methoxyl-PEG and uncapped PEG, are widely used in the chemical reactions (Fig. 1.7). At high molecular weights, poly (ethylene glycol) (PEG) is also called poly (ethylene oxide) (PEO). PEG has the hydroxyl end groups (OH) and ether linkages in the backbone. There are a variety of different routes for PEG molecules to be added onto the drug delivery systems, including blending during copolymer fabrication [33] and covalent bonding [34].
Figure 1.5 Schematic hydrolysis of poly (lactic acid) into monomer units

Figure 1.6 Schematic hydrolysis of poly (lactide-co-glycolide) into monomer units
PEG can be attached at the end groups of other polymers and molecules, in order to facilitate the reaction of base materials with other reagents. As part of a drug delivery carrier, it usually combines with PLA and PLGA. PEG facilitates the terminal functionalization and easy chemical conjugations with PLGA/PLA. For example, the bi-functional NH$_2$-PEG-NH$_2$ was used to connect the PLGA or PLA to some drug targeting molecule such as folic acid [35] as it is challenging to combine the PLGA/PLA directly to the folic acid due to few functional groups on the PLGA/PLA surface. PEG as a bridge to connect the encapsulation materials and functional moieties greatly enhances the conjugation rate and stability [36].

Furthermore, the PEG block in the drug delivery vehicle is able to extend the drug delivery residence time in the human body. The immune system in the organism can recognize the drug delivery carriers as alien objects from the bodies, thus it rapidly clears up the carriers in the liver or in the spleen [37]. PEG is able to escape reticuloendothelial system (RES) uptake of drug delivery carriers [38]. Therefore, the modification of PEG onto the drug carrier surface largely prevents this rapid clearance [39]. Moghimi et al [40] reported that particles with PEG decoration extend the half life time up to 45 hours. Also, pharmacokinetics was carried out to compare PLGA nanoparticles and PLGA-PEG nanoparticles [41]. For PLGA nanoparticles, high doses resulted in decrease of particles clearance, indicating that PLGA nanoparticles are dose dependent. While on the other hand, PLGA-mPEG nanoparticles didn’t exhibit
this phenomenon and display a much longer residence time than the PLGA nanoparticles.

PEG is beneficial for the entrapment of the therapeutic drugs into the drug delivery carriers. PLGA-PEG and PLA-PEG copolymer systems possess surfactant characteristics, for PEG block is hydrophilic and both of PLGA and PLA are hydrophobic. Therefore, when the therapeutic drugs are hydrophilic, double emulsion and solvent evaporation methods are normally used to encapsulate the drugs into hydrophobic polymers such as PLGA and PLA. It is found that encapsulation of drugs into PLGA-PEG and PLA-PEG are more efficient than that with PLGA and PLGA as more drugs can be entrapped due to the affinity of the drugs and materials [42].

1.4.3 Poly (caprolactone) (PCL)

Poly (caprolactone) (PCL) is a biodegradable and non-toxic polyester. Like PLGA, PLA and PEG, it has also proved to be biocompatible [43]. It is fabricated by ring opening polymerization of ε-caprolactone, using catalyst stannous octanoate. The reaction scheme is described in Fig. 1.8.

Due to PCL's high percent crystallinity, it showed a very slow degradation rate as compared to commonly used biodegradable encapsulation materials PLGA and PLA [44]. Usually the amorphous parts within a semi-crystalline polymer degrade earlier than the crystalline region, resulting in an alteration in the drug release profiles [45]. Therefore, the polymers with a high percent crystallinity are less water permeability and thus degrade at a slow rate. As PCL degrades slowly, it has been incorporated into other biodegradable polymers and used as blends. To meet the need of specific degradation kinetics, combinations of polymers allow the users to tailor drug encapsulation materials. The PCL/F68 implant for the delivery of levonorgestrel (LNG) exhibited a long term and sustained drug release for 2 years from the in vitro and in vivo drug release study [46]. The preparation of copolymer PLA/PCL reduced the crystallinity
by increasing the lactide content, which led to a relatively fast degradation rate and met the practical usage [44].

![Polymerization Diagram](image)

Figure 1.8 Ring opening polymerization of ε-caprolactone to polycaprolactone

Currently, all the biomaterials listed are widely investigated. The sustained and prolonged drug release profiles for oral drug delivery carriers can be obtained by the degradation of these biodegradable materials. In the practical applications, PLGA gets most attention from the researchers. As biodegradable and biocompatible material PLGA has been approved by American Food and Drug Administration (FDA) for practical medical usage, so it facilitates the translation of prepared products into practical applications [47]. And PLGA can be classified by ratio of monomers in the polymer chains and its molecule weights. The drug release rate can be well-designed by the custom-made parameters of PLGA. A review of PLGA in variety of aspects will be helpful for the understanding of drug carriers, properties of dosage and drug release profiles.

### 1.5 PLGA’s preparation, properties and applications

#### 1.5.1 Methods for PLGA preparation

Poly (lactic-co-glycolic acid) (PLGA) copolymers are fabricated on the basis of random ring-opening copolymerization of two different monomers [48], the cyclic dimers (1, 4-dioxane-2, 5-diones) of glycolic acid and lactic acid. The poly (lactide-co-glycolide) synthetic scheme is described in Fig. 1.9. Normally, monomer D, L-lactic acid is recrystallised in ethyl acetate (EA) at room
temperature until the racemic mixture melting point reached 124-126 °C and the glycolic acid (m.p. 88-90 °C) is following added. 0.1% (w/w) of stannous octoate and 0.01% (w/w) of lauryl alcohol are commonly used as catalyst and chain regulator in this preparation, respectively. During the polymerization, lactic acid and glycolic acid as successive units are connected together to form PLGA via ester linkage. Finally a linear, amorphous aliphatic polyester product is obtained [49]. The reactions are usually conducted in long necked glass flasks. In order to control temperature, an oil bath is used. The as-prepared copolymers are extracted by first dissolving in solvent chloroform, followed by precipitation in methanol.

1.5.2 Physicochemical properties

There are different forms of PLGA, which are determined by the initial feeding of monomers’ ratio in the polymerization. Frequently used PLGA 50:50 indicates a PLGA copolymer whose composition in the whole chain is 50% lactic acid (LA) and 50% glycolic acid (GA). While many other forms of PLGA, such as PLGA 85:15 [50], PLGA 75:25 [50] and PLGA 65:35 [51] are also popular in use.

Table 1.1 and Table 1.2 summarize the chemical properties of different PLGA and physical properties of different PLGA, respectively [52]. From Table 1.1, all PLGAs are in amorphous state rather than crystalline one. All of them have glass transition temperatures in the range of 45-55 °C. Different from the monomer formulation into homo-polymers of polylactide and polyglycolide which exhibited poor solubilities in solvents, copolymer PLGA can be dissolved in a large number of common solvents [53], such dichloromethane, ethyl acetate, chloroform, hexafluoropropanol, tetrahydrofuran and acetone. The approximate resorptions between different PLGA vary a lot. As the amount of glycolic acid decreases from 50% to 15%, the approximate resorption time ranges from 1-2 months to 5-6 months.
Table 1.2 exhibits the physical properties of different PLGA including density, tensile strength, elongation and modulus. The different parameters of PLGA don’t change when the ratio of lactic acid and glycolic acid alters. Only the density shows a slight difference. So the physical properties have little relationship to the PLGA specific components. But it was reported that molecular weight of PLGA affects its strength [54] and the ability to form drug delivery systems.

### 1.5.3 PLGA applications

PLGA has been used in various areas due to its versatility. It is a biodegradable and biocompatible material, which has been approved by the American Food and Drug Administration (FDA) for clinical use [47]. For PLGA hydrolyzes in the human body and yields lactic acid and glycolic acid which are usually the by-products of cellular metabolism, it has been used for the production of many biomedical devices.
### Table 1.1 Chemical properties of different PLGA

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Inherent Viscosity (dL/g)</th>
<th>Crystalline</th>
<th>Glass Transition temp (°C)</th>
<th>Solubility</th>
<th>Approx. Resorption (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 50:50</td>
<td>0.55-0.75</td>
<td>Amorphous</td>
<td>45-50</td>
<td>*123456</td>
<td>1-2</td>
</tr>
<tr>
<td>PLGA 65:35</td>
<td>0.55-0.75</td>
<td>Amorphous</td>
<td>45-50</td>
<td>*123456</td>
<td>3-4</td>
</tr>
<tr>
<td>PLGA 75:25</td>
<td>0.55-0.75</td>
<td>Amorphous</td>
<td>50-55</td>
<td>*123456</td>
<td>4-5</td>
</tr>
<tr>
<td>PLGA 85:15</td>
<td>0.55-0.75</td>
<td>Amorphous</td>
<td>50-55</td>
<td>*123456</td>
<td>5-6</td>
</tr>
</tbody>
</table>

*Solvents
1 = acetone  2 = tetrahydrofuran  3 = hexafluoroisopropanol  4 = chloroform  
5 = ethyl acetate  6 = dichloromethane

### Table 1.2 Physical properties of different PLGA

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Density (g/mL)</th>
<th>Tensile Strength</th>
<th>Elongation (%)</th>
<th>Modulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 50:50</td>
<td>1.34</td>
<td>6000-8000</td>
<td>3-10</td>
<td>2-4x10⁵</td>
</tr>
<tr>
<td>PLGA 65:35</td>
<td>1.30</td>
<td>6000-8000</td>
<td>3-10</td>
<td>2-4x10⁵</td>
</tr>
<tr>
<td>PLGA 75:25</td>
<td>1.30</td>
<td>6000-8000</td>
<td>3-10</td>
<td>2-4x10⁵</td>
</tr>
<tr>
<td>PLGA 85:15</td>
<td>1.27</td>
<td>6000-8000</td>
<td>3-10</td>
<td>2-4x10⁵</td>
</tr>
</tbody>
</table>

The selection of the PLGA is largely dependent on the purpose to be used. Based on different PLGA molecular weights, the degradation rate varies significantly. Normally, low molecular weight of PLGA degrades slower than lower molecular one [55]. Also, for the different components of PLGA, if the amount of glycolide increases, the polymer degradation rate increases.
1.5.3.1 Drug delivery carrier

PLGA composed of polylactic acid and polyglycolic acid has been widely applied in drug delivery system [56]. Due to PLGA’s excellent biocompatibility, it has become one of the most widely used biomaterials and has been commercialized for different kinds of drug delivery systems, in the form of nanoparticles, microspheres, pellets, blends and films [57]. PLGA drug delivery carriers have been used for the delivery of various drugs including 5-FU [58], doxorubicin [59], paclitaxel [50], cisplatin [60], and ciprofloxacin [61]. Different fabrication methods can be used for the formation of PLGA nanoparticles or microspheres, which will be discussed in the next section.

![Degradation mechanism of PLGA nanoparticles](image)

Figure 1.10 Degradation mechanism of PLGA nanoparticles
(a) bulk erosion, (b) surface erosion

In aqueous solution, the PLGA drug delivery carriers mainly experience homogenous and bulk erosion (Fig. 1.10a) although they also undergo surface erosion sometimes (Fig. 1.10b). And drug release usually exhibits a three-phase release profile; diffusion and erosion are the two major processes (Fig.
1.11). Therefore, sustained drug release is achieved by controlling the swelling and erosion of the PLGA polymer.

In the bulk degradation, ester bonds result in cleavages in the random chain homogeneously throughout the PLGA polymer matrix [22]. PLGA hydrolyses to yield lactic acid and glycolic acid monomers, which are normally the by-products of cellular metabolism. Drug release from the PLGA drug delivery system is dependent on the PLGA degradation, which is thus decided by the characteristic of PLGA polymers. PLGA’s molecular weight, monomer lactide/glycolide ratio and capped/uncapped terminal functional group have a great impact on the drug release from the carriers. Ravivarapu et al. [62] reported that low molecular weight PLGA leads to a more rapid drug release and faster polymer degradation. Due to lactide’s hydrophobicity, an increase in the lactide composition in the copolymer slows down the polymer dissolution rate and thus leads to a prolonged drug release [63]. The uncapped PLGA has the carboxylic groups at the end of the polymers, which is more hydrophilic than the capped one. Uncapped PLGA also has higher hydrolysis rate [64]. PLGA degradation rate is inversely to the PLGA crystallinity, as aqueous solution cannot easily penetrate the crystalline areas [54]. Therefore, PLGA with low crystalline degrades faster than the high crystalline one. Moreover, the high molecular weight PLGA polymer degrades slower than the PLGA composed of low molecular weight polymer chains.

Some other factors such as particles size, particles surface morphology and shape of the nanoparticles also have a great impact on the PLGA degradation. A high ratio of surface area to volume facilitates the aqueous solution to penetrate the PLGA matrix, which significantly enhances the polymer degradation.

1.5.3.2 Surgical sutures

Surgical suture as a medical device can be used for wound closure after a surgery. The suture can also combine the separated body tissues together when
the operations are finished. The ideal suture should be inert in biology and should not be involved in any tissue reactions. PLGA has the following characteristics to make the suture:

- PLGA doesn’t support bacterial growth
- Biodegraded after serving the functions
- Possess high tensile strength
- Easy to sterilize

![Graph showing cumulative release over time]

**Figure 1.11 A typical three-phrase drug release profile**

From above, the PLGA sutures have not only the characteristics of high strength and but also dissolubility in the body fluids. To meet the mechanical and degradation criteria as suture materials, usually there is a high percentage of GA, one of the monomers in PLGA.

**1.5.3.3 Scaffold**

One kind of scaffold is using the biodegradable polymer which can stimulate the isolated cells to regenerate tissues with specific shapes and size. PLGA has
been widely used to make scaffolds for both *in vitro* and *in vivo* tissue regeneration models in vascular smooth muscle tissue [65], bone [66] and cartilage [67] for many years. PLGA is an ideal material for tissue engineering because of its following unique characteristics:

- PLGA surface is suitable for cell attachment, proliferation, differentiation and growth
- Neither PLGA nor its degradation products are toxic
- PLGA scaffold can provide high porosity, up to 90% for the cell-PLGA interaction and cell seeding or growth
- PLGA scaffold can be resorbed after serving as the template for the regenerating tissue
- PLGA scaffold degradation rate can be adjustable and controllable to match the tissue or cell growth based the molecular weight of PLGA

All of these properties are very important for the cell distribution and regeneration throughout the scaffold device. Various processing methods have been developed to fabricate the three dimensional porous PLGA scaffolds, including solvent casting and particulate leaching [68], emulsion freeze-drying [69], gas foaming [70], phase separation [71] and combination of the above methods [72]. Among them, the solvent casting and particulate leaching method is currently widely used. The process is mainly about PLGA dissolving into organic solvent which contains the leachable porogen. Then the porous PLGA scaffold is obtained by the solvents and porogens removal. Different porogens, type of solvents and feeding ratio of PLGA to porogen result in various scaffold architectures [73], in order to meet different requirements and needs.

### 1.5.3.4 Electrospun fibers

Electrospinning has become a very popular technology in the last 10 years. It can produce polymer fibers with diameters between 3 nm and 5 μm [74]. The electronspun PLGA is a kind of ultrafine polymer fibre which has the property
of high specific surface area and length to diameter ratios. These unique characteristics are quite useful in many areas and applications including filtration membranes, artificial blood vessels, and catalytic nanofibers [75].

Schematic diagram of electrospinning facility is described in Fig. 1.12. From the diagram, after PLGA polymers are dissolved into some organic solution, they are stored in a syringe with a millimetre size capillary. Under the syringe pump, the polymer solution is ejected from the capillary and at the same time a high electric charge is implemented. A grounded collection drum is used for collecting the fibers.

The morphologies and diameters of fibers produced by this method largely depend on many factors and parameters which include

- PLGA concentration and viscosity
- solvent’s volatility and polarity,
- Humidity and temperature in the laboratory [76]
- Voltage of the power
- and the specific processing parameters

Figure 1.12 Schematic diagram of electrospinning apparatus [77]
CHAPTER ONE

From above, overall information of PLGA on preparation, physicochemical properties and application has been fully discussed. Normally the oral drug carriers using PLGA as encapsulation materials are in form of nanoparticles or microspheres. Different encapsulation methods are adopted based on the interaction between PLGA and therapeutic drugs to be loaded. Here comprehensive details of different fabrication methods for PLGA oral drug delivery carriers are discussed.

1.6 Fabrication methods for PLGA drug delivery carriers

Commonly used methods for PLGA nanoparticles synthesis consist of top-down and bottom-up methods. The top-down techniques usually employ as-prepared polymers to synthesize the nanoparticles [78], such as emulsion diffusion, salting out, nanoprecipitation method, and emulsion evaporation; while the bottom-up methods start from a monomer [78]. The precipitation polymerization, emulsion and microemulsion polymerization and interfacial polymerization are the specific forms. Here details are discussed on different types of top-down techniques to form the PLGA nano-drug delivery carriers. In general, all of these methods are composed of two major steps. They share the first step which is to prepare an emulsified system. The nanoparticles are hardened and formed in the second step, which is varied for different methods.

1.6.1 Emulsion diffusion method

There are single emulsion and double emulsion systems in this fabrication method. Single emulsion encapsulation method is conducted for the formulation of oil soluble substances (hydrophobic) [79]; while double emulsion is adopted by entrapment of hydrophilic chemicals [80].

Fabrication parameters in the emulsion diffusion method have a great impact on the results. One of the requirements for emulsion diffusion method is the selection of an organic phase (oil phase) which must be partially miscible in aqueous phase. The most important fabrication step is solvent diffusion, in
which the organic phase diffuses from the oil phase to outer water phase and the formed particles become hardened. The selection of the surfactants in the outer water phase is also crucial to the successful fabrication. Different kinds of surfactants, such as non-ionic surfactant polyvinyl alcohol (PVA) [81], anionic surfactant sodium dodecyl sulphate (SDS) [78] and cationic surfactant didodecyl dimethyl ammonium bromide (DMAB) [82] are commonly applied based on emulsion systems. Different surfactants can induce particles in different sizes [83]. Budhian et. al. [84] reported that when DMAB was used as surfactant for the fabrication of PLGA nanoparticles, smaller particles were fabricated than the ones prepared by using PVA as the surfactant. Another popular stabilizer for PLGA nanoparticles fabrication is amphiphilic d-α-tocopheryl polyethylene glycol 100 succinate vitamin E (TPGS) [85, 86] as TPGS has very high emulsion efficiency and can also enhance the cellular adhesion. The amount of TPGS used as surfactant usually can be as low as 0.015% (w/v).

The amount of surfactant used has an effect on the properties of the nanoparticles. Low concentration of surfactants usually leads to a high polydispersity and particle aggregation [87]. However, if excessive surfactants are used, the drug loading can decrease due to the strong interaction between the drugs and surfactants. Therefore, the suitable concentration of surfactant is the key to successful fabrication. Another method to form the mono-dispersed emulsion is using the probe sonicator to impose high energy in the formed emulsion [88]. The selection of specific sonicator mode, time and power is essential to formation of emulsions.

1.6.2 Salting out method

Salting out is another method for the PLGA nanoparticles fabrication. Firstly the PLGA is dissolved into the organic solutions (oil phase) which are usually water-miscible. Typical solvents are tetrahydrofuran (THF) and acetone. Then the oil phase is emulsified in an aqueous phase, under strong shearing force by overhead mechanical stirrer. The aqueous phase consists of the surfactant and
salts of high concentration. The salts should not be soluble in the organic solvent. Typically, the most commonly used salts are magnesium chloride hexahydrate with a concentration of 60% (w/w) [89] or magnesium acetate tetrahydrate which is normally used with a ratio of 1:3 (polymer to salt) [90]. The obvious difference between the emulsion diffusion method and salting out method is that there is no solvent diffusion step for the latter one as the existence of salts. In order to decrease the ionic strength in the salt, the pure water is added into the formed O/W emulsion under magnetic stirrer. At the same time, the hydrophilic organic solvents migrate from the oil phase to aqueous phase, which results in the formation of the nanoparticles. Finally, the salting out agent is eliminated by centrifugation and the samples are purified.

1.6.3 Nanoprecipitation method

Nanoprecipitation is also called solvent displacement method, which was first developed and introduced by Fessi's group [91]. The principle of this fabrication method is known as Marangoni effect [92]. Formation of the nanoparticles is instantaneous and needs only one step so it has the advantage of rapid and easy operation. Also, the nanoprecipitation method can get a narrow particle size distribution because of the absence of shearing stress. This method is used mostly for hydrophobic drug entrapment [93], but it is also employed sometimes to incorporate hydrophilic drugs [94]. Polymer and drug are dissolved in an organic solvent which is water miscible, for example acetone or methanol. The solution is then added into an aqueous solution which contains surfactant in a drop-wise manner. Through rapid solvent diffusion, the nanoparticles are formed immediately. After that, the solvents are removed under reduced pressure.

1.6.4 Emulsion evaporation method

Emulsion evaporation has been used for a long time to form polymeric nanoparticles from as-prepared polymers. The method is based on the emulsification of polymer organic solution into a water phase, followed by
organic solvent evaporation. The polymer is first dissolved in a suitable solvent. Ethyl acetate, chloroform and methylene chloride are commonly used as the solvent. The organic phase is poured into the continuous phase (aqueous phase) in which a surfactant is dissolved to impart stability to the emulsion. Emulsification is carried out under high-shear force to reduce the size of the emulsion droplet. And this process will be largely dependent on the final particle size. After the formation of emulsification, the system evaporates the organic solvent under vacuum, which leads to polymer precipitation and nanoparticle formation.

From this section, the fabrication methods including emulsion diffusion, salting out, nanoprecipitation and emulsion evaporation for PLGA oral drug delivery carriers are described. Drugs loaded PLGA nanoparticles or microspheres can be obtained by using these techniques. Although the PLGA based drug delivery carriers can get the sustained and prolonged drug release profiles through the degradation of PLGA matrix, the systems have difficulties in achieving the functionalization, thus their wide usages are always hampered. In order to maximize the efficacy of oral drug delivery carriers, multi-functions such as drug targeting and pH-sensitivity have to be achieved. For the oral drug delivery systems were designed, these properties can maximize the utilizations of PLGA based drug delivery systems. In this way, the drug carriers can keep intact in the upper-gastrointestinal tract, accumulate in the specific areas and release the drugs pertinently. Therefore, different skills for modifications of PLGA drug delivery carriers are described in next section.

1.7 Techniques for PLGA modification

Although PLGA is a biodegradable and biocompatible material and has the potential to be used in many areas, its practical utilization is always restricted due to its lack of suitable functional groups on the surface [95]. Therefore, a variety of attempts for physical and chemical PLGA surface modification have been made to meet the specific requirements. This mainly includes the
enhancement of hydrophilicity for drug entrapment and conjugation of specific functional group for certain functionalization.

1.7.1 Improved hydrophilicity

PLGA is a naturally hydrophobic material, like most of the biodegradable polyester. Its hydrophobic index is depended on the ratio between the amount of two monomers LA and GA.

Poor hydrophilicity always limits PLGA in the practical drug formulations, especially for the entrapment of hydrophilic drugs. Also, the hydrophobic drug carriers are recognized as a foreign substance by the body. When administered, the drug carriers with hydrophobic surface are surrounded by mononuclear phagocytic system (MPS), which can absorb the carries, especially in the liver. Therefore, one of the purposes for the surface modification with hydrophilic components is to make the carriers unrecognizable by the MPS.

To enhance the hydrophilicity and other physicochemical properties, poly (ethylene glycol) (PEG) has been conjugated onto PLGA. PEG is a biocompatible, non-toxic and water soluble polymer. Block copolymers composed of PLGA segment and PEG segment bring a large interest in recent year due to the copolymer's biodegradability, biocompatibility and tailer-made properties [96]. A number of drug formulations including micro/nano particles were achieved by using PLGA-PEG and PLGA-mPEG [42]. It was also found that the dissolution rate of PLGA-PEG copolymers is much higher than that of unmodified PLGA due to enhancement of hydrophilicity [97]. Natural polysaccharide chitin was investigated to form chitin/PLGA blends [98]. Chitin is more hydrophilic than PLGA. There are β-glycosidic bonds between D-acetylglucosamine units where the cleavage leads to the chitin degradation. It was reported that the drug release from the chitin/PLGA blending was influenced by the hydrophilicity enhancement [98]. The D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) has been used as an emulsifier and
segment of drug delivery vehicles for many years [99]. The PLGA/TPGS nanoparticles were found a high encapsulation efficiency [100].

1.7.2 Chitosan functionalization

In order to improve the functionality of PLGA surface, a conjugate can be synthesized by grafting chitosan on PLGA via the amino groups. Chitosan functionalization on PLGA surface explores deeper the utilization of this polymer. The surface modification of chitosan can also increase the particles surface zeta potential, which is beneficial for the in vitro cytotoxic effects. Chitosan (CS) is a natural cationic polymer which has been widely used in pharmaceutical area. It has a very good biocompatibility, biodegradability and nontoxicity. Therefore, using chitosan and its derivatives to prepare nanoparticles or coated onto nanoparticle surface has been widely investigated in recent years [101]. Chitosan nanoparticles surfaces have a relatively high zeta potential, which adheres strongly to the cells as cancer cells generally have a negative charge on their surface [102]. As NH₂ groups are existed in each unit of chitosan, PLGA is grafted onto the chain randomly. Therefore, the crystallinity of chitosan is largely affected. Chitosan also acts as the bridge to form a PLGA based triblock polymers. Alginate-chitosan-PLGA composite microspheres containing Hepatitis B vaccine were prepared by Liang’s group [103]. This system showed improvement of encapsulated protein stability, drug release amounts enhancement and high antibody levels [103].

1.7.3 Targeting functionalization

The concept of selective drug delivery system was introduced by Paul Erhlich 100 years ago [104]. The “Magic Bullet” proposed has great affinity and specificity to the cells, tissue and organs in the human body [105]. Targeting functionalization of PLGA surface enables the drugs delivered to the designated area to target specifically the cancerous cells. Therefore, the targeted drug delivery carriers reach the cancer sites with specificity and affinity, while they have less side-effect to the healthy tissues. The
transportation of the drugs to the designated areas can be mainly achieved in two ways: active and passive targeting (Fig. 1.13), while each of them can be divided into several sub-categories.

![Diagram of Drug Targeting]

Figure 1.13 Classification of targeting drug delivery system

### 1.7.3.1 Active targeting

Active targeting is achieved by target specific ligands which usually actively incorporate into drug carriers. The ligands attached on the drug delivery carrier direct the systems to cells which have the receptors. In general, the active ligands can be divided into three types: ligand-receptor, antigen-antibody and aptamer.

> **Ligand and receptor interaction**

Ligand and receptor interaction can be classified based on the receptors used. Among them, folate receptor (FR) is one of the most popular ones. The folate receptor is a highly specific cancer cell marker that is overexpressed on many tumor tissues. At the same time, this kind of receptor is absent and less-
expressed in most normal cells and thus is widely used for drug targeting purpose [35]. Using this property, the folate conjugate drug delivery systems exhibit high affinity which enables them to combine with folate receptor rapidly and become internalized through an endocytic process. Liu et al. [106] have synthesized a block copolymer poly (N-isopropylacrylamide-co-N, N-dimethylacrylamide-co-2-aminoethyl methacrylate)-b-poly (10-undecenoic acid) (P(NIPAAm-co-DMAAm-co-AMA)-bPUA) and then the folic acid (FA) was incorporated onto the hydrophilic block via the amine group. The anticancer drug doxorubicin (DOX) was entrapped into the as-prepared products. The results showed that the IC$_{50}$ of DOX loaded folate conjugated nanoparticles against 4T1 and KB cells with folate receptor overexpressed were much lower than that of DOX loaded unmodified nanoparticles. In another study by Zhang et al. [86], folate conjugated poly (lactide-co-glycolide)-vitamin E TPGS nanoparticles of DOX were synthesized. The result showed that TPGS-folic acid cellular uptake was 1.7 and 1.5 times higher in C6 and MCF 7 cells, respectively, as compared with nanoparticles without TPGS-folic acid component, showing the improvement in efficacy and effectiveness.

Moreover, the carbohydrate on the cell surface can be employed as the receptor. The carbohydrates affect the cancerous cells interaction with the extracellular matrix during growth and metastatic spread [9]. The interactions can be mediated by lectin-carbohydrate interaction, in which the nanoparticles containing lectin moieties are directed to certain carbohydrate [107]. There are different kinds of lectins and the suitable selections of lectins facilitate the drug delivery carrier to the specific cells [107].

➢ Antigen and antibody interaction

Antibody based cancer treatment depends on the highly specific targeting of antigens on the cell surface which are restricted to the tumor site. The first introduction of monoclonal antibody (mAb) technology was in the 1970s [108]. The over-expressing cancer-specific antigens have become the key index for
developing different drug delivery systems. The drug delivery carriers by targeting cancer-specific antigens gather at the tumour site, which is beneficial for the diagnostic imaging and cancer treatment [109]. Kirpotin et al [110] conjugated the anti-HER2 mAb fragments to liposome-rafted PEG chain, which was targeted to human epidermal growth factor receptor 2 (HER2). The results showed that the intracellular drug delivery mAb-mediated endocytosis leaded to the anti-HER2 immunoliposomes. HuA33 mAb targeted to EGF receptor was investigated by the group of Caruso [109], which exhibited a rapid antibody localization to tumor deposits and achieved minimal toxicity. Their findings also show that layer-by-layer (LbL) particles biofunctionalized with huA33 mAb have the potential for targeting colorectal cancer with high specificity and important prerequisite for effective colloidal delivery systems.

➢ Aptamer

Aptamers belong to DNA or RNA oligonucleotides, which are able to bind to the target antigens with specificity and affinity. The action is similar to the interaction between antibody and antigen [111]. Although antibodies provide targeting options, aptamers exhibit advantages over antibodies as they can be engineered completely in a test tube and are rapidly produced by chemical synthesis [112]. Moreover, due to the small size and similar structures to the endogenous molecules, aptamers are easy to penetrate into the tissue [111]. Farokhzad et al. (2006a) [47] in a recent research have developed docetaxel (Dtxl) loaded nanoparticles using biocompatible and biodegradable PLGA-block-PEG (PLGA-b-PEG) copolymer. After that, the surface of the nanoparticles was functionalized with A10 2'-fluoropyrimidine RNA aptamers which are able to recognize the extracellular domain of the prostate-specific membrane antigen (PSMA). Their findings showed significant enhanced in-vitro cytotoxicity of modified nanoparticles when compared with non-targeted nanoparticles with no PSMA aptamer. Furthermore, after injection of Dtxl-NP-Apt nanoparticles, the tumor in five of seven LNCaP xenograft nude mice was dramatically reduced and a 100% survival rate was presented. However, for the mice treated with Dtxl loaded nanoparticles or free drug Dtxl, the survival rate
is significantly lower. Therefore, the results in these studies indicated that the application of nanoparticle-aptamer bioconjugates for active targeting is promising. Moreover, how the aptamer modified PLGA-PEG nanoparticles are distributed in LNCap (PSMA+) xenograft mouse model of prostate cancer was investigated [47]. Results found that nanoparticles with surface functionalization of A10 PSMA aptamer obviously facilitate the targeted transportation of nanoparticles to cancer cells.

1.7.3.2 Passive targeting

Passive targeting is composed of localized delivery and enhanced permeability and retention effect (EPR) and can be achieved by taking advantage of the distinct pathophysiological features of a tumor tissue and its permeability [113]. The cancerous cells grow rapidly due to rapid vascularization, which results in a leaky and defective structure, and some of them are localized. These properties are able to facilitate the toxic therapeutic drugs to access the tumor sites.

» Localized delivery

In order to avoid the systemic circulation, the localized deliveries of drugs aim to direct the drugs directly to the tumor site [114]. Normally, the approaches such as intravenous injection and oral drug delivery require high concentration of anticancer agents, which always results in various side-effects and is not always feasible in the practical applications. Therefore, localized deliveries of drugs as a targeting approach were widely investigated [115]. Through the intratumoral administration, localized drug delivery’s effectiveness has been conducted and tested [116]. The administration of mitomycin by localized delivery leads to an enhanced drug concentration around the cancerous cells and toxicity to the normal cells obviously decreased [117]. As prostate tumors are localized, Sahoo et al [118] found that direct intra-tumoral injection of paclitaxel loaded biodegradable nanoparticles was more effective and practical than the systemic drug therapy. However, some of the cancerous cells are
metastatic and tumors are not in localization, therefore, this will limit the use of localized delivery the drug [119].

**Enhanced permeability and retention (EPR) effect**

Many polymeric nanoparticles exhibit enhanced permeability and retention (EPR) effect (Fig. 1.14), which was first introduced by Maeda [120]. The extravasation of drug loaded nanoparticles was prevented due to normal tissue vasculature lined by tight endothelial cells (Fig. 1.14a). Drugs are easily accumulated in the tumor tissue with hyperpermeable vasculature (Fig. 1.14b) [120]. The EPR effect makes the extravasation of circulating polymer nanoparticles in the tumor interstitium and raises the chemotherapeutic agents concentration in the tumor tissue [121]. A lack of lymphatic drainage leads to drug accumulation in the tumor tissue [122]. So if a chemotherapeutic drug is encapsulated into a suitable polymer, then such a system has great potential to enhance the concentration of anti-cancer agent within the cancer cells. For the capillary endothelium in the cancerous cells exhibits more disorderly, its permeability to the macromolecules is higher than the one in normal cells [123], which results in circulating polymeric nanoparticles taking effect within the cancer cells. Due to these properties, the concentration of drug carriers within the cancer site attains a level 10 to 100 times higher than that from the dosage of free drugs [124].

### 1.7.4 pH sensitive coating

A pH sensitive coating can protect the drug loaded PLGA nanoparticles from releasing in the oral delivery route. As many drug delivery carriers are administered by oral, it would be more promising if the drug carriers are pH-sensitive as the gastrointestinal tract which the drug delivery carriers pass is a multi-pH values environment. The properties of water-insolubility at low pH and water-solubility at high pH polymers are of great interest in recent years. The release rate of the drugs or when the drugs release can be controlled and
Figure 1.14 Enhanced permeability and retention effect (a) The extravasation of drug loaded nanoparticles was prevented due to normal tissue vasculature lined by tight endothelial cells (b) Drugs are easily accumulated in the tumor tissue with hyperpermeable vasculature [120]
triggered by the pH values of the environments. However, the drug release mechanisms from the core-coated microspheres are not fully understood yet [125]. Many complicated process, such as water imbibition, drug diffusion and dissolution through the intact microspheres are all included. For example, it was reported that the chemotherapeutic drug phenylpropanolamine hydrochloride release from ethylcellulose coated microspheres was manipulated not only by the drug dissolution but also an osmotic effect [126]. Also, the cracks derived from hydrostatic pressure in different coated microspheres drug delivery system were comprehensively reviewed [127]. Eudragit S100 is another popular pH sensitive polymer and widely used for coating the PLGA nanoparticles. There are carboxyl group and ester group in the polymer structure of Eudragit S100 and the ratio is 1:2. It is due to the carboxylic acid group which make the polymer pH sensitive and insoluble under pH 7.

1.7.5 Plasma treatment

Modification by plasma technology can be divided into two categories: one is gas plasma treatment and the other is plasma polymerization. Plasma treatment is very convenient for the material surface modification [128]. It can treat the biomaterials with complex shape and surface. As this technology can introduce the reactive groups or chains onto the material surface very easily, it is commonly applied for the enhancement of cell affinity [128]. For gas plasma treatment, different gases, such as oxygen, ammonia, combination of nitrogen and hydrogen, were used for different functional groups immobilization, including amine, hydrogen and carboxylic groups.

The modification of polymer surfaces by plasma polymerization is substrate independent. Plasma polymerization involves a first substrate fragmentation process, followed by a subsequent deposition of organic monomers. The schematic diagram of plasma polymerization facility is showed in Fig. 1.15.
Due to the deficiency of suitable functional groups on their surface, conventional PLGA nanoparticles lack the possibility of specialized targeting or biomimetic purposes. The surface property of nanoparticles is important for the performance of nanoparticles in vivo. Such plasma modifications will greatly improve the effectiveness of nanoparticles delivery system and cell affinity. Lee et al. investigated the PLGA surface modification by plasma treatment, in order to induce the cell affinity on the polymer surface [129]. The tests showed that cell proliferation was significantly enhanced in the human dermal fibroblast (HDF) attachment for experimental group after 6 days of incubation due to the improved hydrophilicity of PLGA film by plasma treatment. Huang’s group reported using oxygen plasma for PLGA surface modification for the immobilization of laminin [130]. The results showed that the proportion of laminin incorporated on PLGA surface by plasma treatment is much higher than that by conventional chemical methods. And plasma treatment can induce the PLGA surface more hydrophilic. Hasirci et al. [131] applied oxygen plasma treatment for the PLGA surface modification and found the PLGA water contact angle decreased from 67° to 38° after the treatment.

Different from the gas plasma treatment, plasma polymerization coats the organic monomer onto the substrate rather than co-valently binding various functional groups onto the polymer surface. Currently one of the commonly
used monomers for the PLGA plasma polymerization is monomer allylamine. In a study by Modo [132], polymerized allylamine treated PLGA scaffold particles as a structural support for the neural cells in the brain. The results indicated that the plasma treated scaffolds ensure the cell grafting and improve the recovery of damaged brain cells. Heptylamine is another monomer for plasma polymerization as it mainly incorporates the amine groups on the PLGA surface and has been confirmed to be a useful medium for subsequent biomolecules grafting [133]. Djordjevic et al. demonstrated that the samples with heptylamine plasma polymerization achieved three-dimensional functionalization [133].

Different methods on PLGA modifications were discussed in this section. An enhanced entrapment of hydrophilic drugs can be achieved after improved hydrophilicity of PLGA. The pH-sensitive coating on PLGA nanoparticles surface can assist the oral drug delivery carriers to pass the upper gastrointestinal tract. The functionalization of PLGA by chitosan and plasma technology makes the PLGA active surface. Therefore, a well-formulated PLGA based oral drug delivery system can be obtained using these methods. However, there are some key parameters which have great impact on the performance of oral drug delivery carriers. Clear understanding and optimization of the parameters are beneficial for the experimental design and the practical administration of the oral drug carriers.

1.8 Key parameters of drug dosage

Well-formulated oral drug delivery systems for administration to the patients are crucial for the successful cancer treatments. Every aspect in the ingredients of a product should be carefully considered in the formulation of a drug product. This mainly consists of chemical, physical and biological properties, and also includes the compatibility between these properties.
1.8.1 Particle size

Generally, it is widely accepted that small particles have an enhanced ability to reach their targeted cancer sites [32]. After the administration of oral drug delivery systems, the particles with size around 20-30 nm are easily eliminated by renal excretion [134, 135]. Larger particles can be rapidly taken up by the mononuclear phagocytic system (MPS) cells present in the liver and spleen [136]. Organs in different parts of the human body have affinity to certain size of the particles. Nanoparticles of 150-300 nm are found mainly in liver and spleen [136], whereas particles of 30-150 nm are located in the heart, kidney and stomach [137]. From the previous published results, the “ideal” size requirement for nanoparticles developed for cancer treatment are between 70 and 200 nm [138]. To fabricate the drug loaded particles with specific size range can accumulate them in the different sites [32].

Particle size and size distribution have a great impact on the physicochemical properties of drug carriers [83]. The drug dissolution rate, encapsulated drug uniformity and stability are all related to the particle size. It is necessary to figure out the relationship between the particle size and formulation efficacy. One of the interests is the effect of particle size on the oral adsorption profiles. If the drugs are encapsulated in large particles and have poor solubility, it may lead to poor absorption [139]. Drugs loaded in smaller particles often results in better dissolution during the administration. The drug uniformity in the dosage is also dependent on the particle size and distribution of therapeutic drugs throughout the formulation [140]. When the particle size of carriers is uniform, there is less tendency of the particles to separate.

1.8.2 Polymorphism

The form of drugs in the encapsulation materials is another important factor in the drug formulation [141]. The drugs can be either in crystal or amorphous state in the polymer matrix. Different polymorphic forms can result in various physicochemical properties, such as solubility and melting point [142]. It is
known that the energy which the drugs release from crystal encapsulation materials is higher than that from amorphous or noncrystalline ones [141]. Therefore, the amorphous drug delivery carriers are more soluble than the crystal one.

So the physical stabilization of amorphous form can be generated intimate contact between the amorphous drugs and stabilizer by creating drug-stabilizer dispersion [143, 144]. The use of such dispersions, particularly with polymers, to intentionally enhance drug solubility has been known for many years. And practical formulations which achieve facile low-solubility drug dissolution and supersaturation have recently been described [141]. The stabilized amorphous forms can also be developed for intentional bioavailability improvement [141]. The use of such form is to provide a dosage form which is bioequivalent to the stable drug crystalline forms.

1.8.3 Solubility

Drugs' solubility in the aqueous solution is another important physicochemical property [145]. A balance between hydrophilicity and hydrophobicity is important for the aqueous dissolution and absorption through the biological lipid membranes. A drug should have the aqueous solubility to some degree in order to achieve a high therapeutic efficacy, for drugs exert the therapeutic effect in aqueous solution in the human systemic circulation [146]. Some drug like erythromycins show an erratic absorption and usually have to be modified to become more soluble [147]. This modification usually can be accomplished based on the chemical structure and kind of drugs. For example, a higher solubility of the drugs can be obtained by modifying the drugs into ester or salt [148]. Another strategy to enhance the solubility is adjusting the pH value of the aqueous solution [149]. However, changing the pH value of non-electrolytes has no effect on their solubility, such as prednisone and dextrose. In this case, it is necessary to use the co-solvents or solid dispersion to improve the aqueous solubility of these non-electrolytic drugs [150].
1.8.4 Dissolution

In the aqueous solution, the drug dissolution and the time it takes to dissolve are rate-limiting step in the process of drug adsorption [151]. The dissolution rate of the drugs can be achieved by reducing particles size which can be equivalently considered as the increase of the surface area [152]. Another effective way is to increase the drug solubility in the diffusion phase. In this situation, if pH value increases, there is a higher dissolution rate due to the improvement in solubility [153].

Oral drug delivery carriers for the therapy of colon cancer have to experience the harsh gastro-intestinal tract [154]. The pH values in different gastrointestinal tracts are shown to increase gradually from the stomach (\( pH \ 1-3 \)), Duodenum (\( pH \ 4-5 \)), small intestine (\( pH \ 6.5-7 \)) to colon (from \( pH \) onwards \( pH \ 7.0-7.8 \)) [155], as exhibited in Fig. 1.16. And a drug dosage can take from 4 hours to more than 8 hours to reach the colon area if administered orally. The well design drug delivery system can avoid the carriers from dissolution in the front parts of the tract [156].

Figure 1.16 Drug carriers in the gastrointestinal tract
1.9 Summary

In this literature review, systematic and comprehensive information was provided on the colon cancer, commonly used materials for drug delivery, biodegradable and biocompatible material PLGA and its usage, different drug delivery carrier fabrication methods and PLGA surface modification strategies. The relevant literatures to this research were fully explored. This part includes the research background and the work done by other people. It is expected that these basic information and concept reviewed in this chapter are beneficial for the understanding of the future chapters.

Chapter 2 introduced the materials and solvents used in the research, nano-drug delivery carrier fabrication methods and characterization techniques. The operation procedures of in vitro drug releases from the nanoparticles and microsphere in various PBS solutions were fully discussed. Cell culture and tests including the drug uptake and MTT assay were given in detail in this chapter.

Chapter 3 focuses on the fabrication of 5-FU loaded PLGA nanoparticles using double emulsion and solvent evaporation method (w/o/w). The effect of preparation parameters, such as the initial drug feeding, high volume ratio of outer water phase to organic phase, adjusting the pH value of outer phase and surfactant concentration on the drug loading, encapsulation efficiency, nanoparticle size and the state of the drugs in nanoparticle matrix were fully investigated. The drug release in PBS solution (pH 7.4) was also tested.

Chapter 4 explores the fabrication of PLGA conjugated targeting molecule folic acid (FA). The reaction scheme and mechanism were discussed in details. The effect of a number of preparation parameters including the organic solvents and surfactants are described. In vitro drug release, cell targeting, cell uptake and MTT assay were investigated to compare between the treated and untreated samples.
Chapter 5 explores the microencapsulating the prepared PLGA nanoparticles into pH sensitive microspheres Eudragit S100 using oil in oil method and PLGA surface modification by chitosan using adsorption and covalent binding techniques. The fabrication parameters in microencapsulation were fully optimized and the cross-section images were presented to illustrate the inner part of the microspheres. The surface charges between the PLGA and chitosan modified PLGA nanoparticles were compared.

Chapter 6 outlines the PLGA surface modification by gas plasma treatment and plasma polymerization of different monomers. Different plasma parameters including the power, continuous wave (CW) mode, pulse mode, processing time and vacuum degree were optimized to achieve the high amount of carboxylic group on the PLGA surface. Various characterization methods such as water contact angles measurement, X-ray Photoelectron Spectroscopy (XPS), Scanning Electron Microscopy (SEM), etc were used to compare samples’ properties before and after the plasma treatment.

Chapter 7 summarizes the conclusions obtained through this research work and contain suggestions for future research in these areas.
2 MATERIALS, PREPARATION AND CHARACTERIZATION

2.1 Introduction

This chapter describes the materials, fabrication process, and facilities used for material characterization in this thesis. First, all the chemicals and reagents are listed, including the main chemical structures. Then, methods for the formulation of drug delivery carriers are outlined, mainly on the fabrication of 5-FU loaded PLGA nanoparticles. Furthermore, the process for conducting the drug release from nanoparticles and microspheres are described. Finally, the facilities used to characterize the materials including particle size, zeta potential, and surface morphology, physicochemical state of the drugs within the polymer matrix, material surface composition and hydrophilicity/hydrophobicity are all discussed.

2.2 Materials

PLGA-COOH 50/50 with an average molecular weight of 15k was purchased from Ji’nan Daigang Biological Co. Ltd (Shandong, China). The drug 5-fluorouracil (5-FU), emulsification surfactant tocopheryl polyethylene glycol succinate (TPGS) and polyvinyl alcohol (PVA) with 86.7%-88.7% hydrolysis degree and molecular weight 31k, 1, 3-diaminopropane 99%, N-hydroxy-succinimide (NHS), Coumarin-6 (98%, Mw 350.44), folic acid (FA), boric acid, iodine and potassium iodide were ordered from Sigma-Aldrich, Australia. The organic solvents dichloromethane (DCM), acetone and ethyl acetate (EA), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC-HCl) were ordered from Fluka, Australia. Dimethylsulfoxide (DMSO) was obtained from Ajax Finechem Pty Ltd. N, N-Dimethylformamide (DMF) and diethyl ether were ordered from Chem Supply Pty Ltd. Eudragit S100 was obtained from You Pu Hui Co. Ltd (Shenzhen, China).
The HT-29 cell line was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Jiehui Biotechnology Company (Wuhan, China), fetal calf serum was sourced from Sijiqing Biotechnology company (Hangzhou, China), and RPMI 1640 medium was bought from Sigma.

All chemicals used in the experiments were analytical grade and used without further purification. The structures of the main chemicals are described in Fig. 2.1. 5-FU was selected as model therapeutic drugs, as it has been used for chemotherapy of colorectal cancer for over 50 years and is still the major anticancer agent in the practical applications. PLGA-COOH (50/50) with molecular weight 15k was chosen as the model encapsulation material. The reason for that is the uncapped end PLGA is more hydrophilic and thus easy to entrap the hydrophilic therapeutic drugs. Another reason is that the carboxylic group at the end of polymeric chain facilitates the PLGA surface modifications.

2.3 Formulation of nano-drug delivery carrier

5-FU loaded PLGA nanoparticles were fabricated by a modified W₁/O/W₂ multiple emulsion and solvent evaporation technique (Fig. 2.2) [42]. Briefly, 100 mg of PLGA was dissolved in 3 ml methylene chloride (DCM). 11 mg of 5-FU was dissolved into a water solution to get the inner aqueous phase of 0.75 ml. Into the organic phase (O), aqueous drug solution (W₁) was emulsified using probe sonicator (Shengxi instrument company, Shanghai, China) for 2 minutes with a 30% of amplitude to form W₁/O emulsion. The first emulsion was incorporated into an aqueous phase containing surfactant polyvinyl alcohol (PVA) or tocopheryl polyethylene glycol succinate (TPGS) (external phase/W₂) and sonicated for 1 minute. The resulting W₁/O/W₂ emulsion was stirred with a magnetic stirrer for 5 hours to allow the solvent evaporation and particle hardening. The nanoparticles were then separated by ultra-centrifugation at 12k rpm for 20 minutes and washed for three times using distilled water, in order to remove the 5-FU as well as the residual surfactant on the surface of particles.
5-Fluorouracil

PLGA-COOH

Eudragit S100

Chitosan

Coumarin 6

Folic acid

Figure 2.1 Structures of main chemicals
The washing solution was eliminated with a further centrifugation. Finally, the nanoparticles were collected from a freeze-dryer and preserved in a desiccator for characterization and evaluation.

![Fabrication process of 5-FU loaded PLGA NPs](image)

**Figure 2.2 Fabrication process of 5-FU loaded PLGA NPs**

### 2.4 *In vitro* drug release from PLGA nanoparticles and Eudragit S100 microspheres

The main parts, which the drug carriers would pass in human gastrointestinal tract (GIT), and the pH conditions and time required to travel in the GIT are described in Table 2.1.

The release of 5-FU from PLGA nanoparticles and Eudragit S100 microspheres was performed in a pH progression medium simulating the conditions of different parts of a real gastrointestinal tract. In the release study, two buffer solutions - HCl buffer and phosphate buffered saline (PBS) - were selected. The pH of the medium increased gradually, from pH 1.2 (HCl buffer) for the first hour’s drug release, pH 4.5 (PBS) for the next 2 hours, pH 6.8 (PBS) for the following 2 hours and pH 7.4 (PBS) until the end of the test.
There are a number of distinct steps in the release test procedure [157]. First, the as-prepared microspheres or nanoparticles were placed in a dialysis bag whose molecular weight cut-off is 8,000 Da. The tests were performed in a constant temperature shower mixer at 100 rpm, 37°C and 50 ml dissolution solution and measured in corresponding buffer solutions at different pH values. After a fixed time interval, 3.5 ml of the solution was taken out and diluted to 30-fold volume. Then the UV absorbance at 265 nm was tested. After that, 3.5 ml fresh buffer solution was added into the release medium, in order to maintain a constant solution volume. After n times of sampling, the drugs which have been released can be calculated as \( C = C_n \times 50 + (C_1 + C_2 + \ldots + C_{n-1}) \times 3.5 \), where \( C_i \) is concentration of the solution at \( n \) time.

Table 2.1 Dissolution condition and transit time [158]

<table>
<thead>
<tr>
<th>Part of simulated GIT</th>
<th>pH value</th>
<th>Transit time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>stomach</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Duodenum</td>
<td>4.5</td>
<td>2</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>6.8</td>
<td>2</td>
</tr>
<tr>
<td>Colon and rectum</td>
<td>7.4</td>
<td>---</td>
</tr>
</tbody>
</table>

2.5 *In vitro* cell culture and tests

2.5.1 Cell culture

Human colorectal adenocarcinoma cell line HT-29 were cultivated in RPMI1640 culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C, 5% CO₂ and 95% relative humidity in an incubator. After 24 h incubation, the cells were in exponential growth phase.
2.5.2 In vitro cell viability

Cell viability was evaluated using the MTT assay, similar to what previously described by Zhang and Feng [159]. Briefly, HT-29 cells in exponential growth phase were digested by 0.25% trypsin and were seeded in 96-well plates (Cellstar, Greiner Bio-One, Germany) at the density of $1 \times 10^4$ viable cells per well and incubated for 24 h to allow cell attachment. The cells were incubated with 5-FU, 5-FU loaded PLGA non-targeted nanoparticles and 5-FU loaded PLGA-1, 3-diaminopropane-FA targeted nanoparticles suspension at a concentration ranging from 1 to 50 µg/ml for 48 hours. Then 20 µl MTT (5 mg/ml) was added to each well. After incubation for 4 h, the culture solution was centrifuged and the supernatant was removed. The formazan crystals in every well were dissolved in 150 µl DMSO and optical density value (OD value) at 492 nm was detected with enzyme mark instrument. Cell viability can be calculated using the following Equation.

$$\text{Cell viability} \, \% = \frac{\text{Int}_1}{\text{Int}_2} \times 100$$ (1)

where $\text{Int}_1$ is the optical density of cells incubated with nanoparticles samples and $\text{Int}_2$ is the optical density of cells incubated with the culture medium (negative control sample). Moreover, $\text{IC}_{50}$, the sample concentration at which the drug dosage can inhibit 50% cell growths, was calculated by the curve fitting of the cell viability data, in comparison with that of the control samples.

2.5.3 In vitro cellular specific targeting

The HT-29 cells in exponential growth phase were digested by 0.25% trypsin and the cell concentration was adjusted into $5 \times 10^4$ cells/ml using folate-free RPMI1640 culture medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were seeded into a 6-well plastic plate with 18 mm $\times$ 18 mm cover slips. After incubation for 24 h, HT-29 cells were exposed to coumarin-6 loaded PLGA or PLGA-1, 3-diaminopropane-folic acid
nanoparticles with or without the 1 mM free folic acid. After 2h incubation at 37°C, PBS solution was used to wash the cells for six times. The cover slips were put on slides. Finally, the distribution of coumarin-6 loaded nanoparticles in the cells was characterized under fluorescent microscope (IX71, Olympus, Japan).

2.6 Physicochemical properties characterization

2.6.1 Drug Loading (DL) and Encapsulation Efficiency (EE)

To determine the 5-FU drug entrapped in PLGA nanoparticles, an indirect method was carried out by measuring the drugs that were not encapsulated. The prepared nanoparticle solution was centrifuged at 12k rpm for 20 minutes. Then the supernatant was collected and tested by ultraviolet-visible (UV-Vis) spectrometer at a wavelength of 265 nm. The drug loading (DL) and encapsulation efficiency (EE) were calculated with the following Equations [160]:

\[
\text{Drug Loading (DL)(%)} = \frac{A - B}{C} \times 100
\]  

\[
\text{Encapsulation Efficiency (EE)(%)} = \frac{A - B}{A} \times 100
\]

where \( A \) is total feeding drugs; \( B \) is the drug in the supernatant solution after centrifugation; and \( C \) is the weight of prepared nanoparticles. During the test, all measurements were made in triplicate and the mean values are shown in the results.
2.6.2 Differential Scanning Calorimetry (DSC)

DSC is very helpful in the studies of thermal properties of particles, showing both qualitative and quantitative information on the physicochemical state of drugs in the particles [161]. The DSC was performed using Perkin-Elmer Diamond Instrument. Samples were carefully weighted (5-10 mg) and placed in the sealed aluminium pans. Indium was used to calibrate the equipment and the measurements are carried out under atmosphere of helium gas. The samples were scanned from 0°C to 300°C at a rate of 10°C/min.

2.6.3 Powder X-Ray Diffraction (XRD)

Powder X-Ray Diffraction (XRD) was conducted to determine materials’ crystallinity. In the experiments, XRD patterns were recorded through a Philips X’Pert PRO MRD XL powder X-ray diffractometer. The specific diameters on operation of this machine are using Cu Kα radiation, a voltage of 40 kV and a current of 30 mA. The measurements were performed in the range of 5° - 80° (2θ) and step size is 0.01°.

2.6.4 The Fourier Transform Infrared Spectroscopy (FTIR)

The Bruker Vetex-70 FTIR spectrometer was used for testing samples’ FTIR spectra. FTIR is used to characterize the chemical structures of materials and the interaction between the polymer and the drugs. The FTIR experiments were conducted using KBr disk method. Prepared samples were added to KBr powder and the two powders were mixed and grounded evenly to make a KBr disk. The disks were kept in a vacuum oven at 40 °C before the FTIR test, in order to make sure that the disks are dry enough. Average 32 scans of spectra were recorded in the standard wavenumber range of 400-4,000 cm⁻¹ at a resolution of 4 cm⁻¹. A DigiTech DLATGS detector with integrated preamplifier is adopted to equip the spectrometer. The standard beam splitter was a Ge-based coating on KBR and the scanner velocity is 6 Hz.
2.6.5 UV-Vis spectrophotometry

UV-Vis optical absorbance was measured at different wavelength using Cary 300 UV-Vis spectrophotometer (Varian, Australia). The selection of wavelength range was based on the materials characterized. The solutions are diluted to certain fold to meet the test requirement.

2.6.6 $^1$H NMR spectroscopy

Polymers were dissolved in deuterated chloroform ($\text{CDCl}_3$) (5-10 mg/ml) and placed in a glass NMR tube. The composition of each polymer was determined using JEOL JNM-GX 270 MHz NMR spectrometer, using TMS as an internal standard. Samples were analysed for the presence of any intermediary products and to quantify the conjugation extent.

2.7 Material surface characterization

2.7.1 Surface morphology

The shape and surface morphology of the nanoparticles and microspheres were characterized by using a Zeiss Supra 55 VP FEG scanning electron microscope (Zeiss SMT, Germany). The steps to prepare SEM samples are as follows. The freeze dried samples were sprayed on the surface of conductive adhesive and coated with gold using Emitech SC7620 Sputter Coater in a high vacuum evaporator. The gold-coated samples were firstly scanned under an ordinary microscope and detailed morphologies were scanned with the scanning electron microscopy. The samples were viewed at 3-5 kV electron beam.
2.7.2 Particle size and zeta potential

Particle size and zeta potential measurements were performed with a Malvern DLS spectrometer (Zetasizer Nano ZS). The spectrometer is equipped with a He-Ne laser and the digital correlator inside has a wavelength of 633 nm. All the measurements were carried out at room temperature, with a detection angle of 173°. Before the measurement, the solution was carefully diluted to a proper concentration.

2.7.3 X-ray photoelectron spectroscopy (XPS)

The XPS (VG310F scanning Auger Microprobe) was used to investigate the surface composition of the materials and nanoparticles. The XPS microprobe has an Al anode. For the X-ray source, 15 kV accelerating voltage and 20 mA emission current were applied. Hemispherical analyser was used to record the spectra at 100 eV pass energy for survey spectra, while high resolution peak analysis was conducted by 20 eV passing energy. The elemental composition of carbon (C) and oxygen (O) were obtained by survey scans. The C1s binding energies of the samples were established by charge shifting the lowest binding energy peak of C1s to 285.0 eV.

2.7.4 Contact angle measurement

Contact angle measurements were performed to examine the surface wettability using KSV CAM100 contact angle. Distilled water was used for the droplet with 180 pixels in size. Three different points of each specimen were tested and mean value was calculated. Wettability time was also calculated simultaneously using KSV CAM100 software. Based on the different rate of material absorption, the interval of the frames captured by camera was varied from 33 ms to 1 second. For all PLGA samples, frame interval was set at 33 ms due to the fast rate of absorption. All readings were performed within 2 hours of material processing.
CHAPTER THREE

3 DEVELOPMENT OF ENHANCED DRUG LOADING PLGA NANOPARTICLES

3.1 Introduction

The well-designed drug delivery vehicles using biodegradable and biocompatible polymers for the treatment of colorectal cancer have attracted increasing attention, as this kind of carriers provide a sustained and controlled drug release and reduce side-effects [109]. Furthermore, colonic drug delivery is convenient to be administered and painless to the patients compared to the traditional treatments, like surgery and radiation therapy [162]. Among these drug delivery systems, the formulation of nanoparticles (NP) or microspheres (MS) has been one of the most promising technologies. However, for the nanoparticle drug carriers, low drug loading and large particle size have always been big concerns in the pharmaceutical area. Therefore, designing a new drug delivery vehicle that carries enhanced drug formulation while at the same time maintains suitable particles size will significantly forward the utilization of the therapeutic drugs.

The drug delivery vehicles particularly in the form of nanoparticles [159, 163, 164] with high drug loading and suitable particle size are a potential practical administration of therapeutic drugs for the treatment of colorectal cancer. The nanoparticles can provide a sustained drug release and due to enhanced permeability and retention (EPR) effect, the nanoparticles of small size are much easier to penetrate into cancerous cells [165], although achieving low drug loading but maintaining large particles size have been very challenging during the particle fabrication [166, 167]. Therefore, for effective administration of nanoencapsulated therapeutic cancer drugs to colon, the nanoparticles must have a high drug loading for sustained release and small particle size to achieve the passive targeting.
Hence, this chapter was designed to optimize the fabrication parameters to enhance the drug loading and encapsulation efficiency for nanoparticle drug delivery carrier while at the same time maintaining small particle size. The physicochemical state of drugs in the nanoparticles matrix was investigated. Fabrication scheme was deliberately planned for encapsulation of therapeutic drug into nanoparticles. In the experiments, hydrophobic poly (lactide-co-glycolide) (PLGA) and hydrophilic 5-fluorouracil (5-FU) were used as the model encapsulation material and model drug, respectively in the nanofabrication process. The probe sonicator and surfactant were used in the first and second emulsion. Drug release was tested to characterize the 5-FU release profile from the PLGA polymeric matrix.

3.2 Challenges in this study

The first challenge of delivering nanoparticles to colorectal cancer cells is to encapsulate sufficient therapeutic drugs into nanoparticles, especially when encapsulating the hydrophilic drugs such as the popular drug 5-fluorouracil (5-FU) into the hydrophobic materials. During the formulation of drug loaded nanoparticles, drug loading has always been a major challenge. In fact, maintaining a high drug loading is important for drug administration as the number of drug administration for the patients could be effectively minimized [168]. The intrinsic difference of chemical properties of drugs and encapsulation materials causes less affinity to each other. When trying to encapsulate the hydrophilic therapeutic agents into hydrophobic materials, great repulsion takes effect. Therefore, a variety of methods to improve drug loading for drug delivery carriers have been developed. Most of them are concentrated on the modification of the therapeutic drugs and decoration of the encapsulation materials. For instance, 1-alkylcarbonyloxymethyl is the prodrug of hydrophilic drug 5-fluorouracil (5-FU). McCarron and Hall [169] substituted the prodrugs for the commonly ordinary drugs in the fabrication process, which resulted in a dramatic increase in drug loading from 3.68% to 47.23%. Zhang and Feng [159] conjugated the hydrophilic groups of tocopheryl polyethylene glycol succinate (TPGS) onto the hydrophobic materials PLA/PLGA and
synthesized a copolymer, in order to enhance the hydrophilicity of the drug carrier and affinity to the hydrophilic encapsulated drugs.

However, in practical preparation, the fabrication parameters also have great impact on the drug loading. And double emulsion and solvent evaporation method (W₁/O/W₂) is one of the most popular methods in the pharmaceutical research [88]. During the fabrication, when the first emulsion transfers to the second one, the drugs in the inner water solution can easily migrate to the outer aqueous phase. And some other parameters, like the initial drug feeding, pH value of the solution influence the results a lot, which could be optimized to get high drug loading.

Another major challenge, however, is to prepare the nanoparticles of a small size, as the particle size is an important index for the adhesion and interaction with the cancerous cells [83]. It was reported that nanoparticles with a size between 70 and 200 nm have more affinity to the biological cells [32]. Also the particle size has a great influence on the circulation half life time [170]. The large particles can be rapidly taken up by the mononuclear phagocytic system (MPS) cells which are present in the liver, spleen and bone marrow. Basic principle of controlling nanoparticle size in the emulsion system is using the external energy such as probe sonicator or surfactant to provide a shearing force as any alternation in the material fabrication parameters can reduce the shearing force and thus result in a large nanoparticle size. Mainardes and Evangelista [171] adopted a microtip probe sonicator set at 55 W of energy output for 1 min to produce the oil-in-water emulsion when preparing the praziquantel loaded PLGA nanoparticles, and the particles prepared by all fabrication parameters were found in nano-size with a nanoparticle size ranging from 100 nm to 400 nm. Poly (vinyl alcohol) (PVA) was also used in the emulsion formation and results showed that the surfactant PVA concentration has great impact on the particle size [164]. However, in most of the current research, only one of the shearing forces probe sonicator and surfactant was adopted. The combination usage of sonication and surfactant can make full use of the advantage on both sides to achieve the small particle size. However, the reduction in the size of nanoparticles can heavily compromise the other
important properties of drug delivery, such as the drug loading and encapsulation efficiency [172]. The optimization of different fabrication parameters to get overall quality is necessary.

3.3 Nano-fabrication parameters on drug loading

As described in Chapter 2, 5-FU as the model therapeutic drugs, PLGA as the model encapsulation material and double emulsion and solvent evaporation method were adopted to fabricate the 5-FU loaded PLGA nanoparticles.

3.3.1 Effect of theoretical drug loading of 5-FU

As 5-FU is a very expensive anticancerous drug, achieving a high encapsulation efficiency will lead to less waste of the drug as more drug will be encapsulated. Also, if low drug DL in the drug carrier, patients who take the drugs have to increase the times and frequency of drug administration, in order to keep enough drug concentration in the body. So it is important to optimise the experimental conditions to achieve a high actual drug loading and encapsulation efficiency.

The practical drug loading in the nanoparticles will depend on the theoretical drug loading (TDL) and synthesis conditions. Four theoretical drug loadings 5%, 10%, 15% and 20% (w/w) were selected to examine the theoretical drug loading effects on practical drug loading (DL) and encapsulation efficiency (EE) of the nanoparticles. All other fabrication parameters were followed by the preparation procedures.

An increase in theoretical drug loading (TDL) has a notable effect on the actual drug loading, encapsulation efficiency and particles size of the nanoparticles (Table 3.1). As the TDL increases from 5% to 20%, the particle size increases gradually from 189.2 nm to 233.6 nm, respectively, due to the increase in actual drug content in the nanoparticles [168]. The particles fabricated with the double emulsion and solvent evaporation method are in nano scale and have a
relatively low polydispersity (PDI<0.19), which means a narrow particle size distribution.

Table 3.1 Effect of theoretical drug loading on the properties of the particles

<table>
<thead>
<tr>
<th>Theoretical drug loading (TDL %)</th>
<th>Size (nm)</th>
<th>Polydispersity (PDI)</th>
<th>DL (%)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>189.2 ± 1.5</td>
<td>0.190</td>
<td>2.4 ± 0.32</td>
<td>38.37 ± 2.4</td>
</tr>
<tr>
<td>10%</td>
<td>205.4 ± 3.3</td>
<td>0.153</td>
<td>4.9 ± 0.28</td>
<td>29.69 ± 3.8</td>
</tr>
<tr>
<td>15%</td>
<td>214.5 ± 2.5</td>
<td>0.124</td>
<td>6.3 ± 0.41</td>
<td>19.20 ± 2.7</td>
</tr>
<tr>
<td>20%</td>
<td>233.6 ± 5.2</td>
<td>0.165</td>
<td>6.8 ± 0.35</td>
<td>17.13 ± 2.1</td>
</tr>
</tbody>
</table>

When the theoretical drug loading is low, it contributes significantly to the actual drug loading and encapsulation efficiency. The DL ranges from 2.4% to 6.8% and increases almost proportionally when TDL is lower than 15% (Table 3.1). However, further increase from 15% to 20% in TDL results in only a very small increase in DL, indicating that there is a saturated drug loading for PLGA and 5-FU drug delivery carrier.

However, as observed from Table 3.1, an improvement in DL could heavily compromise the EE, particularly when the TDL is higher than 15% in which only less than 19.2% of 5-FU is encapsulated into the nanoparticles. The stability of first emulsion was reduced when initial drug feeding increased, which results in more drug loss in the fabrication process and high manufacturing cost. So the compromise scheme should be retrieved. That is to get a relatively high drug loading and encapsulation efficiency. The experiment has found an optimal formulation of TDL 10% where a balance of relative high drug loading and encapsulation efficiency is reached.
3.3.2 Volume ratio of outer water phase to organic phase on the drug loading

In the W\textsubscript{1}/O/W\textsubscript{2} fabrication process, organic phase is the dispersing phase while the outer water phase is the continuous phase that is used for hardening the nanoparticles and promoting the organic solvent to be evaporated [173, 174]. In this work, the DCM was used as the organic solvent, as it has the properties of polarity, less toxicity and low boiling point. And its solubility in water is 13 g/l at 20°C.

A higher volume ratio of outer water phase to the organic phase improves the encapsulation as both the actual drug loading and the encapsulation efficiency increase with the volume ratio (Fig. 3.1). The surfaces of the nanoparticles prepared at a high volume ratio are also much smoother when compared to that prepared at a low ratio from the morphological examination with SEM.

![Figure 3.1 Volume ratio of the outer water phase to organic phase on drug loading and encapsulation efficiency](image)

The double emulsion process determines that a high volume of outer water phase is required. After the formation of the double emulsion, the drugs in the
first emulsion could travel to outer water phase due to its hydrophilic property since it has to be agitated for several hours to eliminate the organic solvent. If there is a small amount of outer water phase, the DCM could not be easily diffused, released and then dissolved into water. A high volume of outer water phase could speed up the solidification at a high volume ratio.

However, nanoparticles prepared with a high volume ratio of outer water phase to the first emulsion are larger in size (Table 3.2) with a particle size ranging from 185.1 nm to 296.8 nm for volume ratio from 30 to 100. The increase in particle size was probably attributed to the reduction of shearing force during the formation of second emulsion and homogenization process as a large outer water phase may decrease the sonication efficiency when the probe sonicator was performed during the first emulsion into the second one.

Table 3.2 Effect of volume ratio on the properties of nanoparticles

<table>
<thead>
<tr>
<th>Volume ratio</th>
<th>Particle size (nm ± SD)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>185.1 ± 2.1</td>
<td>0.109</td>
</tr>
<tr>
<td>50</td>
<td>203.4 ± 1.8</td>
<td>0.175</td>
</tr>
<tr>
<td>80</td>
<td>274.3 ± 3.6</td>
<td>0.154</td>
</tr>
<tr>
<td>100</td>
<td>296.8 ± 4.9</td>
<td>0.131</td>
</tr>
</tbody>
</table>

When the nanoparticles are smaller than 200 nm, they are more likely to penetrate and accumulate in the cancerous cells [32]. In this particle size range, the drug delivery vehicle could enter the tumour area based on the enhanced permeability and retention effect (EPR effect) [175]. By considering the particle size (Table 3.2) and DL and EE (Fig. 3.1), the volume ratio of outer water phase to organic phase of 50 is the optimum as the size of the nanoparticles is around 200 nm while maintaining a relatively high drug loading and encapsulation efficiency.
3.3.3 pH value of outer aqueous phase

The solubility of drugs in solution is normally pH dependent and the drugs can be easily diffused into the outer water phase in nanoparticle hardening process. When a drug is dissolved into a solution whose pH is at the drug’s isoelectric point (pI value), minimum amount of the drugs would be dissolved [149]. Therefore, adjusting the pH value of the outer water phase to its isoelectric point is a potential method to increase the drug loading. For therapeutic drug 5-FU, the isoelectric point is 8.02 and the nanoparticles prepared at pH value 8.0 of outer water phase exhibited a higher drug loading and a higher encapsulation efficiency when compared to other pH values (Fig. 3.2) as great repulsion is applied in the process and less drug appetency to the water occurred, which prevented the drug from releasing from the first emulsion to the second one.

![Figure 3.2 Influence of pH value on drug loading and encapsulation efficiency](image-url)
3.3.4 Surfactant PVA concentration

The PVA are located between the oil phase and the water phase to stabilize the formed nano-emulsion and prevent the aggregation. The nanoparticles were formed with the help of PVA to stabilize the emulsion solvent and droplets formation. Different PVA concentrations were used in the sonication and homogenization process, in order to study the external aqueous phase on the nanoparticles properties. 0.2%, 0.6% and 1.2% (w/v) concentration PVA were selected to do the experiments.

The prepared nanoparticles with a high PVA concentration exhibited a slightly larger particle size than the one with a low concentration. The larger particle size of nanoparticles with higher surfactant is probably due to the adsorption of surfactant to the particle surface. Although repeated washings were conducted, some of the adsorbed PVA were not washed away.

From Table 3.3, when the PVA concentration increases to 1.2% (w/v), the drug loading reduces dramatically. This is probably ascribed to the residue PVA on the particle surface. Based on the Equation 2 in Chapter 2, if the residue existed in the final product, the addition of the PVA weight was ignored, which result in the calculation of a lower drug loading.

Table 3.3 Influence of PVA concentration on particle size and drug loading

<table>
<thead>
<tr>
<th>Concentration$^a$</th>
<th>Particle size (nm)</th>
<th>PDI (poly-dispersity)</th>
<th>DL (%)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2%</td>
<td>185 ± 2.6</td>
<td>0.163</td>
<td>5.2 ± 0.32</td>
<td>22.3 ± 2.4</td>
</tr>
<tr>
<td>0.6%</td>
<td>204 ± 1.9</td>
<td>0.110</td>
<td>5.6 ± 0.28</td>
<td>25.8 ± 4.6</td>
</tr>
<tr>
<td>1.2%</td>
<td>237 ± 2.1</td>
<td>0.145</td>
<td>4.2 ± 0.51</td>
<td>19.6 ± 2.9</td>
</tr>
</tbody>
</table>

$^a$PVA concentration (w/v)
3.4 Characterization of the optimized nanoparticles

In light of the results derived from several fabrication parameters, the parameters effects are interactive. A compromise has to be made to get a better combined result of drug loading, encapsulation efficiency and nanoparticle size. Based on the optimized fabrication parameters, the nanoparticles were fabricated at theoretical drug loading of 10%, volume ratio of outer water phase to the organic phase of 50, pH value of the outer water phase of 8.0. The drug loading and encapsulation efficiency of the nanoparticles is 5.8% and 28.6%, respectively.

3.4.1 DSC analysis

When the drugs are encapsulated into nanoparticles, they could be in the polymer matrix either in a crystalline or amorphous state. And if the drugs are present in the nanoparticles in a molecular dispersion or solid solution state, the results will not get a detectable endotherm [176].

In the present research, the DSC thermograms of pure PLGA, pure 5-FU, physical mixture of PLGA and 5-FU and 5-FU loaded PLGA nanoparticles were tested (Fig. 3.3). For pure 5-FU, there is a melting endothermic peak at 286.4 °C, which corresponds to its melting point. The melting endotherm of physical mixture of PLGA and 5-FU was found at 282.4 °C, in which the peak of 5-FU shifts a little due to the mixing with PLGA. However, no peak was detected at the range from 200 °C to 300 °C for 5-FU loaded nanoparticles, which showed that 5-FU was formulated in the PLGA polymeric nanoparticles in an amorphous or molecular dispersion, or a solid solution state. The glass transition temperature of PLGA doesn’t alter greatly, which shows that this kind of fabrication doesn’t change the thermal properties of the encapsulation materials. Similar results were reported previously [85] where paclitaxel loaded PLGA nanoparticles were prepared.
3.4.2 XRD and FTIR analysis

XRD was used to determine crystallinity and polymorphic forms of the components. XRD spectra of 5-FU, PLGA and 5-FU loaded PLGA nanoparticles are shown in Fig. 3.4. 5-FU exhibited several characteristic intense peaks at $2\theta = 16.2^\circ$, $18.9^\circ$, $21.6^\circ$ and $28.6^\circ$ and $32.9^\circ$. All these peaks are ascribed to its crystalline nature. For PLGA, there were only quite low peaks or nearly no peak in the scanning range, which indicates the amorphous properties of PLGA. Furthermore, the peaks showed in the curve of 5-FU are not recorded in the XRD patterns of 5-FU loaded PLGA nanoparticles. Therefore, the result indicates that the drug 5-FU would be either dispersed in the molecular state or distributed in the amorphous state in PLGA, further confirming the observations from DSC. Similar results have reported when 5-FU was loaded into chitosan drug delivery nanoparticles [177, 178].

Figure 3.3 DSC thermograms of PLGA, 5-FU, physical mixture of PLGA and 5-FU and 5-FU loaded PLGA nanoparticles
Figure 3.4 XRD spectra of 5-FU, PLGA and 5-FU loaded PLGA nanoparticles

The FTIR transmittance spectra of 5-FU, PLGA, the physical mixture of PLGA and 5-FU, and 5-FU loaded PLGA NPs were demonstrated in Fig. 3.5. From the PLGA spectrum, the peaks of C-O-C stretching is at 1088 cm\(^{-1}\), C-H stretching in methyl groups at 1460 cm\(^{-1}\), C=O at 1750 cm\(^{-1}\), CH, CH\(_2\) and CH\(_3\) stretching vibrations between 2850 and 3000 cm\(^{-1}\), and OH stretching around 3500 cm\(^{-1}\). The FTIR spectrum of 5-FU showed many characteristic peaks including CH out of plane deformation at 815 cm\(^{-1}\), CH in plane deformation at 1245 cm\(^{-1}\), C=O stretching at 1716 cm\(^{-1}\), 1657 cm\(^{-1}\) and 1245 cm\(^{-1}\), and C-H stretching around 3000 cm\(^{-1}\). The spectrum of physical mixture of 5-FU and PLGA is the combination of the separated two. There was no significant difference in the spectra of physical mixture of PLGA and 5-FU and 5-FU loaded PLGA NPs. No obvious spectrum shift is observed after the formulation of the 5-FU loaded PLGA NPs, which confirms that the drugs has no chemical interaction with the polymer and were physically dispersed in the polymeric matrix. The drugs keep a good stability during the preparation process.
3.4.3 SEM image of the nanoparticles

The shape and surface morphology of 5-FU loaded PLGA nanoparticles were observed with SEM. The images showed in Fig. 3.6a demonstrate that nanoparticles appear spherical with a relatively mono-dispersed size and non-porous surface. The average particle size is less than 200 nm, which is between 70-200 nm and means it would be easily absorbed by the cancerous cells [32]. The nano Measureur software was used to measure a particle size distribution from SEM and the particle size ranges from 130 nm to 195 nm.

3.4.4 Particle size and zeta potential

DLS is used to futher investigate the size distribution and particle surface charge (Fig. 3.7). From Fig. 3.7a, the Z-average particle size is 197.8 nm,
which is in agreement with the SEM result. It also shows that particles have a small polydispersity.

(a)

(b)

Figure 3.6 SEM image of 5-FU loaded PLGA nanoparticles and size distribution
Zeta potential is the surface charge of the nanoparticles, which has great effect on the stability of the suspended nanoparticles. The nanoparticles in the present study were stable in dispersion state, having a relatively high negative surface charge of -22.5 mV (Fig 3.7b). This could be ascribed as the presence of end carboxyl group in the polymeric nanoparticles surface [179].

3.4.5 Drug release profile of 5-FU loaded PLGA nanoparticles

Fig. 3.8 illustrates the in vitro release profile of 5-FU loaded PLGA nanoparticles, in pH 7.4 PBS buffer solution, by representing the percentage of 5-FU release with respect to the amount of 5-FU drugs encapsulated. The drug dissolution environment pH 7.4 was selected to mimic the colon area. The drug release profile was characterized by an initial and variable rapid release followed by rapid and sustained drug release thereafter. And the whole in vitro drug release profile exhibits a three-phase profile. The first phase is a rapid and burst release in the first 9 hours. This is due to the drugs which were not encapsulated into the PLGA polymeric matrix and absorbed on the nanoparticles surface. Also the small nanoparticle size leads to the high ratio of surface to volume, which has great impact on the burst drug release. The second phase is a period of another 10 hours after the initial burst. The drug release profile exhibited a plateau during this period in which the drug release is mainly characterized by the drug diffusion from the PLGA polymeric matrix and PLGA polymer swelling. The third phase is the constant sustained and delayed drug release over 120 hours. This can be seen that the diffusion of the drugs through the polymer wall as well as its erosion and degradation [180]. Due to the sustained and delayed drug release properties, this drug delivery system offers a great potential for the practical application to the colorectal cancer. For examples, the property can be utilized in the targeted drug delivery system [181], in which the targeting molecules direct the drug carriers to the designated areas and the systems achieve the prolonged drug release in the specific sites.
Figure 3.7 (a) Particle size distribution and (b) zeta potential of 5-FU loaded PLGA nanoparticles
3.5 Conclusions

In this chapter, a drug delivery system with enhanced drug loading and small particle size was designed to deliver the therapeutic drugs to colon area with high effectiveness and efficiency. High drug loading can be achieved by optimizing the nano-fabrication parameters. A drug loading of 5.8% was obtained by moderate initial drug feeding, high volume ratio of outer water phase to organic phase and adjusting the pH value of outer aqueous phase to the isoelectric point of 5-FU. PLGA nanoparticles showed an initial burst release followed by a slow and sustained release over an extended period of over 120 hours. Therefore, this kind of drug delivery system has great potential to transport the therapeutic drugs to the colorectal area.
CHAPTER FOUR

4 TARGETING DRUG DELIVERY FOR ENHANCEMENT OF HT-29 CELL UPTAKE

4.1 Introduction

Drug targeting is a key issue for novel chemotherapy treatment to be effectively achieved. Drugs specifically transported to designated areas can not only fully utilize its cytotoxicity but also avoid its side effect such as non-selectivity. Currently, using the biodegradable and biocompatible polymers as vehicles to deliver the therapeutic drugs has drawn more and more attention, as the system can lead to a sustained and prolonged drug release. The adoption of targeting property in drug delivery carriers will make the system more effective.

Accumulating sufficient anticancer agent in the cancerous area is important for the nano-drug delivery carrier. Passive targeting and active targeting are usually applied to facilitate this process. Passive targeting normally takes advantage of the distinct pathophysiological features of a tumour tissue and makes use of enhanced permeability and retention (EPR) effect [182], but the intratumor overpressure prevents the drugs from penetrating into the neoplastic mass [183]. Active targeting is also widely implemented. For example, Conjugation with targeting molecule for specific recognition is able to achieve the active targeting. In this way, the targeting molecules will direct the drug delivery carrier to specific area. Among them, the anti-epidermal growth factor receptor antibodies [184], aptamer [47] and folic acid [86], acting as the targeting agents to colorectal cancer were widely investigated. To be active-targeting, the identification of biomarkers that can be used to distinguish cancerous cells from normal cells and targeting agents that will find the cancerous cells is the most important step. The folic acid is one of the most promising ones among the current widely used targeting agents, as it could combine the folate receptor with specificity and affinity. Folate receptor (FR) has been known to be overexpressed in several human tumours particularly colorectal cancer, while it is highly restricted and expressed at very low levels.
in normal tissues [35, 185]. This makes folic acid unique to the delivery sites and folate receptor an effective biomarker for the folic acid conjugation targeted drug delivery to the tumours.

The drug delivery carrier in this research was adopted by poly (lactide-co-glycolide) (PLGA). It is a biodegradable and biocompatible material [186] and its safety in clinical use has been well established [47]. Among the therapeutic drugs which have been put into practical use, 5-fluorouracil (5-FU) is one of the most potent and popular ones. Since its introduction in the 1950s, 5-FU still remains to be an effective chemotherapy agent for the treatment of colorectal cancer [187]. The encapsulation of 5-FU into PLGA nanoparticles will reduce the drawback of 5-FU, such as short half life time and rapid plasma clearance. And such a design of folic acid binding 5-FU loaded PLGA drug delivery carrier is a possible means to deliver the 5-FU chemotherapeutic agent to the targeting point.

4.2 Design of novel targeting system

A novel conjugate PLGA-1, 3-diaminopropane-folic acid and its nanoparticles were designed and prepared in this study. The binding of targeting molecule folic acid to PLGA is challenging, partly because there is a lack of functional groups on the surface of PLGA. Although the uncapped PLGA with free carboxyl termini was utilized in the experiments, only one carboxylic group (COOH) unit locates at the end of the polymer. Long chain polymer (such as PLGA)’s coiling and winding might further block the reaction of carboxylic groups with other materials. Therefore, many groups and researchers [59, 188, 189] carried on related reactions using NH$_2$-PEG-NH$_2$ to connect the two. The main reason for using PEG is to avoid reticulo-endothelial system (RES) uptake of the drug delivery carrier [38, 185]. However, the poly (ethylene glycol) (PEG) usually has a relatively long chain and conjugation rate will be lower. Therefore, conjugate of PLGA to folic acid is designed and achieved by the bridge of small chain 1, 3-diaminopropane. The 1, 3-diaminopropane unit is incorporated in many biologically active compounds [190] and has the
applications of various areas including drug delivery carrier. Moreover, the 1, 3-diaminopropane is much cheaper than that of NH₂-PEG-NH₂, which could facilitate mass production in the practical application.

### 4.3 Fabrication of conjugate PLGA-1, 3-diaminopropane-folic acid

Copolymer of the conjugates PLGA-1, 3-diaminopropane-folic acid were fabricated by means of two condensation reactions of PLGA-COOH and 1, 3-diaminopropane, PLGA-1, 3-diaminopropane and folic acid. The overall synthesis mainly consists of the following four steps as shown in detailed reactions scheme in Fig. 4.1.

#### 4.3.1 Activation of carboxylic group of PLGA

1 g PLGA was activated by 80 mg EDC and 60 mg NHS in methylene chloride (DCM) at room temperature under nitrogen atmosphere for 24 hours. The resultant solution was filtered to remove the residual NHS and EDC. After filtration, PLGA-NHS was precipitated by dropping into ice-cold diethyl ether and methanol, followed by drying in a vacuum oven.

#### 4.3.2 Preparation of PLGA-1, 3-Diaminopropane conjugate

The activated PLGA (0.5 g) was first dissolved in 10 ml dimethylformamide (DMF) and then 1 ml 1, 3-diaminopropane was slowly added into the solution in a drop-wise manner. The reaction lasted for 6 hours under nitrogen atmosphere and the resultant solution was precipitated and washed by deionised water. The precipitated product PLGA-1, 3-diaminopropane was filtered and dried.
4.3.3 Activation of folic acid

1 g of folic acid was reacted with 0.9 g of NHS and 0.5 g of EDC in 20 ml of dimethylsulfoxide (DMSO) under nitrogen atmosphere at room temperature for 12 hours. Diethyl ether was added to precipitate the folic acid from the DMSO system.
4.3.4 Conjugate folic acid to PLGA-1, 3-diaminopropane

Folic acid-conjugated copolymer was prepared by reaction of PLGA-1, 3-diaminopropane conjugate with activated folic acid. 500 mg PLGA-1, 3-diaminopropane mixed with activated folic acid and EDC was dissolved in 5 ml dimethylsulfoxide (DMSO). The reaction was carried out at room temperature for 7 hours, followed by mixing with 100 ml cold methanol. The precipitate on the beaker bottom was collected by filtration and dried in vacuum oven. In order to eliminate the unreacted folic acid, the dry samples were then dissolved in DCM. The supernatant was collected by centrifugation and samples were finally dried.

4.4 Preparation of fluorescent loaded nanoparticles

Besides preparation of 5-FU loaded PLGA-1, 3-diaminopropane-FA nanoparticles, in order to compare the targeting effect and visualize the cellular uptake of the nanoparticles, same fabrication procedure was repeated to prepare coumarin-6 loaded PLGA nanoparticles and PLGA-1, 3-diaminopropane-folic acid nanoparticle except 0.05% (w/v) coumarin-6 was encapsulated instead of the drug 5-FU. Coumarin-6 is a fluorescent reagent that was mainly used for nanoparticle tracking purpose. And it is assumed that coumarin-6 is mainly for determining the targeting effectiveness. Formation of the PLGA-1, 3-diaminopropane-folic acid targeting drug delivery system can be described in Fig. 4.2.

4.5 Effect of solvents, reagent and surfactant

4.5.1 Selection of the organic solvents

Organic solvents were used as reaction mediums in the conjugation of PLGA and 1, 3-diaminopropane. For PLGA has a very good solubility in

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Figure 4.2 Schematic formation of the PLGA-1, 3-diaminopropane-folic acid targeting drug delivery system

dichloromethane (DCM), acetone, ethyl acetate (EA), and dimethylformamide (DMF), these solvents were first chosen and used in the sample preparation. All of these solvents are commonly used for dissolving polymers and involved in the related polymeric reactions. When 1, 3-diaminopropane was put into these solvents, it exhibited unexpectedly active properties. Precipitates were found in the solvents of DCM and EA and solvent state changed in the acetone, while no reaction and precipitates were detected in DMF.

DCM is one of the most popular organic solvents in the chemical reaction, for it has the properties of polarity, less toxicity and low boiling point. When it is regarded as a popular solvent to dissolve some other chemicals, it is neglected that it can be used as an alkylating agent. The reaction procedure of DCM with 1, 3-diaminopropane is shown in Fig. 4.3. Flocculent precipitates were found on the bottom of the beaker (Fig. 4.6). There are non-bonding nitrogen’s lone pair electrons in the 1, 3-diaminopropane, which make the nitrogen atom very active. And the bond C-Cl in DCM has a higher polarity than that of C-N, so the tendency for the separation of C-Cl and union of C-N is obvious. Finally, the atoms of carbon and nitrogen formed a new bond C-N and at the same time hydrogen chloride was eliminated.
The reaction of acetone with 1, 3-diaminopropane is illustrated in Fig. 4.4. It is a kind of Schiff's base reaction, which usually takes place between the carbonyl groups and primary amines. The resulted product showed an amber colour (Fig. 4.6). Chronister et al [191] reported the similar reactions and found that protonated Schiff base experienced a blue to purple colour change in the research.

The reaction of ethyl acetate with 1, 3-diaminopropane is demonstrated in Fig. 4.5. EA is one kind of ester and it could be hydrolysis in the acidic or basic solution. The amine group in the 1, 3-diaminopropane is capable of supplying a basic environment to finish the hydrolysis.
Figure 4.5 1, 3-diaminopropane reacts with the EA solvent

Overall, the solvents DCM, acetone and EA as reaction mediums are not suitable for conjugation of PLGA and 1, 3-diaminopropane as all of them are either reactive or not compatible with 1, 3-diaminopropane. DMF has a good miscibility with both 1, 3-diaminopropane and PLGA and cannot react with either of the components in the conjugation of the experiments. From Fig. 4.6, mixed solvent solutions are exhibited a stable state. Therefore, in the experiments, DMF was selected as the organic solvent and the reaction environment.

Figure 4.6 Visual pictures of the solvents with 1, 3-diaminopropane
4.5.2 Feeding amount of 1, 3-diaminopropane

The feeding amount of 1, 3-diaminopropane is vital for the successful fabrication of the conjugate. Experiments were designed that the majority of PLGA-COOH would be involved into the reaction, so the 1, 3-diaminopropane should be excessively fed compared to PLGA-COOH. Two options were selected in the experiments. One is using the large volume of 1, 3-diaminopropane, such as 30 times mole compared to PLGA-COOH; and the other is using small volume of 1, 3-diaminopropane, like 10-fold mole compared to PLGA-COOH. As 1, 3-diaminopropane is a material that is miscible with water and DMF, the remnant 1, 3-diaminopropane could be eliminated by repeated extraction using the separation funnel when purifying the samples. $^1$H NMR was used to analyse the prepared products and characterized results are shown in Fig. 4.7. The hydrogen from different samples was marked in separated symbols. From the proton NMR curves, it is revealed that characteristic peaks of PLGA related materials appear at 1.5, 4.8 and 5.2 ppm (Fig. 4.7 a) when a small volume of 1, 3-diaminopropane is used. All three peaks reflect the internal hydrogen in PLGA and don’t change before and after the reactions [38]. However, when a large amount of 1, 3-diaminopropane is fed, the peak at 4.8 ppm is disappeared (Fig. 4.7 b). This is because the connection of PLGA to 1, 3-diaminopropane has no effect on PLGA backbone. Only the carboxylic group (COOH) located at the end of the PLGA polymer chain is involved in the reactions. The disappearance of one PLGA hydrogen peak is possibly due to excessive 1, 3-diaminopropane as the redundant 1, 3-diaminopropane provides an alkali environment which causes the hydrolysis of PLGA. Therefore, the feeding amount of 1, 3-diaminopropane is crucial to the conjugation and large amount of 1, 3-diaminopropane leads to the decomposition of PLGA.
Figure 4.7 $^1$H NMR results of effect of 1, 3-diaminopropane feeding amount. Characteristic peaks are visible for PLGA (*) and 1, 3-diaminopropane (⊕) (a) Large volume of 1, 3-diaminopropane; (b) Small volume of 1, 3-diaminopropane
4.5.3 Effect of composition

The composition and structure of the synthesised PLGA-1, 3-diaminopropane-FA copolymer was characterised with $^1$H NMR. The as-prepared polymer was first dissolved in deuterated chloroform (CDCl$_3$) and placed in a glass NMR tube. Polymer was analysed on NMR spectrometer using standard proton NMR to verify the conjugation of PLGA-COOH to 1, 3-diaminopropane and PLGA-1, 3-diaminopropane to folic acid. Fig. 4.8a compares the results between the untreated PLGA and PLGA-1, 3-diaminopropane-folic acid prepared by small volume of 1, 3-diaminopropane. The new bonding in the final products implies the successful conjugation. Proton NMR revealed that characteristic peaks of PLGA related materials at 1.5, 4.8 and 5.2 ppm appeared in all types of PLGA related materials (Fig. 4.8 a), which means the PLGA structure doesn’t change during the preparation. This was demonstrated in 4.4.2. From Fig. 4.8b, the signal at 4.21 ppm is from 1, 3-diaminopropane as it can be found in the products of both PLGA-1, 3-diaminopropane-FA and PLGA-1, 3-diaminopropane. Signals at 7.68, 6.84, and 2.59 ppm, are corresponding to folic acid. From this figure, both of the peaks from 1, 3-diaminopropane and folic acid are either quite weak or even are not easily noticeable. The reason for this is that the molecular weight of PLGA takes up large percentage in the prepared conjugates and some of the signal from conjugated 1, 3-diaminopropane and folic acid might be covered by the ones from PLGA. Park [183] and Townsend [38] found similar results when they use $^1$H NMR to characterize the samples prepared from the conjugation of small molecule to high molecular weight PLGA with biotin-binding proteins.

4.5.4 Effect of remnant surfactant PVA on the nanoparticles properties

PVA as an emulsifier is widely used to stabilise the emulsion to form monodispersed nanoparticles. However, the remnant surfactant PVA has a great impact on the properties of the nanoparticles. It always results in wrong
Figure 4.8 $^1$H NMR results of PLGA-1, 3-diaminopropane and PLGA-1, 3-diaminopropane-FA. Characteristic peaks are visible for PLGA (*), 1, 3-diaminopropane (⊕) and FA (Δ). (a) $^1$H NMR characterization of PLGA-1, 3-diaminopropane and PLGA-1, 3-diaminopropane-FA; (b) $^1$H NMR characterization of zoom-in view of PLGA-1, 3-diaminopropane-FA.
calculation of drug loading and encapsulation efficiency [192]. Also it has
great influence on the nanoparticles size and zeta potential. Although repeated
washing is involved in the final fabrication process, the residual PVA
sometimes is not evitable. Usually it forms an interconnected network with the
polymer at the interface [87] and is hard to be removed. To precisely calculate
the fraction of PVA which remains in the prepared nanoparticles is essential to
understand the effect.

The residual PVA on 5-FU loaded PLGA-1, 3-diaminopropene-Folic acid
nanoparticles surface was quantified by a calorimetric method based on the
formation of a colored complex between iodine and PVA’s two adjacent
hydroxyl groups [87]. In brief, lyophilized 5-FU loaded PLGA-1, 3-
diaminopropene-FA nanoparticles were first put into 2 ml of 0.5 mol l\(^{-1}\) sodium
hydroxide and kept in a water bath at 60\(^\circ\)C for 15 mins. To neutralize the
solution, 0.9 ml of 1 mol l\(^{-1}\) hydrochloric acid was added and the volume was
adjusted to 7 ml. Then, 3 ml of 0.65 mol l\(^{-1}\) boric acid together with 0.5 ml of
I\(_2/\)KI solution (0.05 mol l\(^{-1}/0.15\) mol l\(^{-1}\)). The absorbance of the samples was
measured with a UV-Vis spectrophotometer at 690 nm after 15 min of
incubation. A standard curve for PVA was prepared under the same conditions.

Three different PVA concentrations were used in the experiments and their
effects on nanoparticles’ properties are showed in Table 4.1. Nanoparticle sizes
showed in this table were tested by DLS. The amount of residual PVA on the
nanoparticles increases steadily as PVA concentration increase. Specifically,
the amount of remnant PVA ranged from 4.63\% to 9.22\% (w/w) when PVA
concentration in the emulsions changed from 0.5\% to 2\% (w/v). High PVA
concentration in the emulsion results in high residual PVA in the nanoparticles.

When PVA concentrations increases from 0.5 to 1 and then to 2\%, the average
nanoparticles size first decreased from 232 nm to 224 nm and then rises up to
228 nm, while the zeta potential decreases continually, from -22.4 mV to -
20.3 mV and finally to -16.9 mV. The size of the nanoparticles doesn’t change
Table 4.1 PVA concentrations on the properties of 5-FU loaded PLGA-1, 3-diaminopropane-folic acid nanoparticles

<table>
<thead>
<tr>
<th>PVA concentration (% w/v)</th>
<th>Residual PVA (% w/w from nanoparticles)</th>
<th>Nanoparticles size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>4.63 ± 0.33</td>
<td>232 ± 26</td>
<td>-22.4 ± 3.8</td>
</tr>
<tr>
<td>1</td>
<td>7.89 ± 0.89</td>
<td>224 ± 18</td>
<td>-20.3 ± 5.1</td>
</tr>
<tr>
<td>2</td>
<td>9.22 ± 0.56</td>
<td>228 ± 21</td>
<td>-16.9 ± 2.9</td>
</tr>
</tbody>
</table>

significantly with the PVA concentration through the low shearing forces at the lower concentration of surfactant PVA, which indicate that the fabrication process is quite stable. Fig. 4.9 exhibits the visual images of the nanoparticles prepared by different PVA concentrations. It showed that the particles prepared by PVA (2% w/w) have a rough surface with PVA mask on (Fig. 4.9c). For zeta potentials, all the results showed negative charges on the particles surface, due to the presence of end carboxyl group (-COOH) on the polymeric nanoparticles surface [179]. If too much residual PVA on the particles, it would shield the negative charge surface [193] and carboxyl group could be partially covered. Therefore, there is a steady decrease in the zeta potentials when the residual PVA on nanoparticle surface increases.

4.5.5 PVA on drug release profiles

*In vitro* drug releases of 5-FU loaded PLGA-1, 3-diaminopropane-folic acid nanoparticles prepared by 0.5%, 1% and 2% concentrations of PVA are shown in Fig. 4.10. All three drug release profiles exhibit a burst release in a few hours, followed by a sustained and delayed drug release. The initial burst releases were attributed to the 5-FU on the surface that was not encapsulated into the nanoparticles but attached on the particle surface. From the Fig. 4.10, it
Figure 4.9 5-FU loaded PLGA-1, 3-diaminopropane-folic acid nanoparticles prepared by different concentrations PVA (a) 0.5% (w/v); (b) 1% (w/v); (c) 2% (w/v)

is found that the three curves nearly level off after 100 hours’ release and also showed that the nanoparticles prepared by 0.5% PVA get highest cumulative drug release (about 65%); while the ones with 2% PVA have the lowest release (52%). From the discussion above, the remnant PVA on the surface of nanoparticles prepared by 2% PVA is the highest one, so the residual PVA might form a crosslinked membrane that prevents the drugs from releasing from the nanoparticles matrix.
Figure 4.10 PVA concentrations on the \textit{in vitro} drug release profile

\section*{4.6 Physicochemical properties of the nanoparticles}

The physicochemical properties of the nanoparticles are summarised in Table 4.2. It shows that the folic acid conjugated nanoparticles exhibited a larger average particle size than the non-conjugated ones, with a particle size of 198 nm and 224 nm, respectively. The larger particle size is due to the higher drug loading and addition of folic acid to the encapsulation materials.

Zeta potential is also a key factor that influences the nanoparticles suspension stability. High value of zeta potential means high nanoparticles surface charge and great repellent force applied, which prevent the nanoparticles from aggregation \cite{82}. For the PLGA nanoparticles, the zeta potential is -23.6 mV, which could be ascribed as the presence of end carboxyl group in the nanoparticles surface \cite{179}. When folic acid was conjugated to PLGA, the zeta potential changed to -20.3 mV. Therefore the zeta potential does not vary
greatly between the targeted and non-targeted ones, which is consistent with other studies on folate conjugate nanoparticles where doxorubicin (DOX) was used as the model drug and PLGA-PEG-FA was adopted as the encapsulation material [59, 183]. During the synthesis of the conjugates, the carboxyl group on PLGA end was consumed, while the conjugation of folic acid added same amount of carboxyl group after the reactions.

SEM was used to image 5-FU loaded PLGA and PLGA-1, 3-diaminopropane-FA nanoparticles (Fig. 4.11). The nanoparticles show a relatively mono-disperse and smooth surface with an average particles size of around 200 nm, which is an ideal particle size for the drug carrier uptake [32].

<table>
<thead>
<tr>
<th>Sample</th>
<th>Drug loading (%)</th>
<th>Encapsulation efficiency (%)</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA NPs</td>
<td>5.5 ± 0.3</td>
<td>26.3 ± 3.1</td>
<td>198 ± 11</td>
<td>0.108</td>
<td>-23.6 ± 3.8</td>
</tr>
<tr>
<td>PLGA-1, 3-diaminopropene-FA NPs</td>
<td>5.8 ± 0.1</td>
<td>28.9 ± 2.6</td>
<td>224 ± 18</td>
<td>0.236</td>
<td>-20.3 ± 5.1</td>
</tr>
</tbody>
</table>

### 4.7 Folic acid conjugation ratio

The UV absorbance at 365 nm was measured with a UV-Vis spectrophotometer to determine the amount of folic acid conjugated to the copolymer. Serially diluted concentrations of folic acid in DMSO were used to construct a calibration curve. From the UV-Vis measurements, the standard curve formula was $y = -0.02127 + 0.01848 \times (r = 0.99336; \text{axis } y \text{ is absorbance}; \text{axis } x \text{ is concentration of folic acid in } \mu\text{g}\cdot\text{mL}^{-1})$. Certain amount of dried PLGA-1, 3-diaminopropene-folic acid was dissolved into the DMSO and the UV absorbance value was measured to determine the concentration of
Figure 4.11 SEM images of (a) non-targeting nanoparticles and (b) targeting nanoparticles
conjugated folic acid. Thus, on molar ratio basis (mol/mol), the conjugation ratio of folic acid to PLGA-1, 3-diaminopropane was 0.38:1. This result showed an advantage of using 1, 3-diaminopropane to connect the PLGA to targeting molecule folic acid, as the currently commonly used poly (ethylene glycol)-bis-amine as this function [29, 194] is not always as effective as 1, 3-diaminopropane. The 1, 3-diaminopropane’s short chain and biocompatibility greatly facilitate this reaction.

4.8 *In vitro* cytotoxicity of nanoparticles

_in vitro_ cell viability of drug loaded nanoparticles was tested by MTT assay. Fig. 4.12 shows _in vitro_ cytotoxic effect of 5-FU, 5-FU loaded PLGA nanoparticles and 5-FU loaded PLGA-1, 3-diaminopropane-FA nanoparticles for HT-29. The cell viability was calculated using the Equation 1 in Chapter 2. For folic acid targeted nanoparticles, when the dosage concentration increases from 1 µg/ml to 50 µg/ml, the cell viability dropped dramatically from 69.33% to 28.02%, while the reduction in cell viability for 5-FU is just from 80.3% to 37.67%. The results showed that the drug formulated in the PLGA and conjugate PLGA-1, 3-diaminopropane-folic acid nanoparticles exhibits a better performance on getting lower cell viability or equivalent high cytotoxicity than that of pure therapeutic drug 5-FU. Therefore the folic acid in the nanoparticles plays an important role in enhancing the cytotoxic effect. As the pure therapeutic drug 5-FU has a short half-life time (T<sub>1/2</sub>), the drug effect lost very quickly in the administration. The entrapment of 5-FU into PLGA and targeting PLGA nanoparticles could first attach on the cell surface and then achieve a sustained and delayed drug release. In this way, the half-life time of 5-FU was effectively prolonged [195].

Furthermore, from the cells inhibition rate, the IC<sub>50</sub> was calculated and listed in Table 4.3. The IC<sub>50</sub> was calculated as drug dosage concentration which inhibits growth of 50% of cells compared to the control cells. For 5-FU, 5-FU loaded PLGA nanoparticles and 5-FU loaded PLGA-1, 3-diaminopropane-folic acid nanoparticles, IC<sub>50</sub> was 22.91 µg/ml, 14.17 µg/ml and 5.69 µg/ml, respectively.
The drug delivery system made from 5-FU loaded PLGA-1, 3-diaminopropane-folic acid showed the lowest IC$_{50}$, which means only a relative small amount of dosage from this targeting system could kill half amount of the cells, showing a high effectiveness. Therefore, the prepared system using 1,3-diaminopropane to connect the PLGA and folic acid showed high effectiveness and efficiency. The results are also in accordance with the one from the in-vitro cell viability where the cells cultured with 5-FU loaded PLGA-1, 3-diaminopropane-FA nanoparticles showed the lowest cell viability and pure 5-FU samples exhibited the highest cell viability. In order to further confirm whether the targeting molecule can facilitate the drug delivery system to specifically attach the cancer cells, cell targeting experiments using coumarin-6 fluorescent loaded samples were tested.

![Graph showing cytotoxic effect](image)

**Figure 4.12** Cytotoxic effect of 5-FU, 5-FU loaded PLGA and 5-FU loaded PLGA-1, 3-diaminopropane-FA nanoparticles incubated with HT-29
Table 4.3 Cytotoxicity studies of different samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration gradients (µg/ml)</th>
<th>Cells inhibition rate</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Fluorouracil (5-FU)</td>
<td>1</td>
<td>0.1970</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.2388</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.3969</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.5257</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.6233</td>
<td><strong>22.91</strong></td>
</tr>
<tr>
<td>5-FU loaded PLGA nanoparticles</td>
<td>1</td>
<td>0.2128</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.3166</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.4443</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.5851</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.6809</td>
<td><strong>14.17</strong></td>
</tr>
<tr>
<td>5-FU loaded PLGA-1, 3-</td>
<td>1</td>
<td>0.3067</td>
<td></td>
</tr>
<tr>
<td>diaminopropane-folic acid</td>
<td>5</td>
<td>0.4545</td>
<td></td>
</tr>
<tr>
<td>nanoparticles</td>
<td>10</td>
<td>0.6222</td>
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<td></td>
<td>25</td>
<td>0.6530</td>
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<td></td>
<td>50</td>
<td>0.7198</td>
<td><strong>5.69</strong></td>
</tr>
</tbody>
</table>

4.9 *In vitro* specific cell targeting

Fluorescent microscopy images of HT-29 cells incubated with different nanoparticles clearly demonstrates that PLGA-1, 3-diaminopropane-folic acid nanoparticles can attach to the HT-29 cells (Fig. 4.13). Fluorescent invert microscopy was used to record the images. The bright field images are used to display the HT-29 cells of different samples before culture and to compare with the fluorescent invert microscopy images. After 24 hours incubation, green fluorescence around the cells is quite blur and vulnerable with coumarin-6 loaded PLGA nanoparticles (Fig. 4.13a). For coumarin-6 loaded PLGA-1, 3-diaminopropane-folic acid particles, the green fluorescence intensity around the cells is significantly higher (Fig. 4.13c). The green fluorescence emitted
from coumarin-6 was clearly shown around the surface of HT-29 cells, which means folic acid facilitates nanoparticles to specifically target the HT-29 cells. In comparison, there was only little green fluorescence inside the HT-29 cells when incubated with coumarin-6 loaded PLGA-1, 3-diaminopropane-folic acid with free folic acid (Fig. 4.13 e). The reason for that is great competition applied between the free folic acid and the conjugated folic acid. The free folic acid took up most of the folate receptors on the surface of HT-29 cells, which greatly minimizes the internalization of the targeting nanoparticles with folic acid.

MTT assay and *in vitro* specific cellular uptake results clearly show that the incorporation of folic acid in nanoparticles improves the cellular uptake. The drug loaded PLGA-1, 3-diaminopropane-folic acid nanoparticles can be effectively uptake by the cells and used for targeted cancer therapy. The human colon carcinoma HT-29 cells are overexpressing the folate receptors. This kind of receptors showed specificity and affinity to folic acid. The interaction between the folic acid folate receptor on the cancer cells can be described in Fig. 4.14. The folic acid conjugated on the surface of PLGA nanoparticles facilitated the specific uptake of coumarin-6 loaded PLGA-1, 3-diaminopropane-folic acid nanoparticles into HT-29 cells. Therefore, the *in vitro* cell experiments indicate that the folic acid modified nanoparticles can be a feasible way to deliver the therapeutic drugs to cancer cells.
Figure 4.13 Fluorescent microscopy images of HT-29 cells incubated with (a) coumarin-6 loaded PLGA; (c) coumarin-6 loaded PLGA-1, 3-diaminopropane-folic acid; (e) coumarin-6 loaded PLGA-1, 3-diaminopropane-folic acid with free folic acid; (b), (d) and (f) are their corresponding bright field images.
4.10 Conclusions

A novel conjugate PLGA-1, 3-diaminopropane-folic acid for the specific 5-fluorouracil drug delivery carrier was successfully fabricated. Effect of solvents, feeding amount of 1, 3-diaminopropane and PVA surfactant on the reactions were all investigated. Physicochemical properties and in vitro cytotoxicity, cellular uptake and specific targeting between targeting and non-targeting nanoparticles were compared. The enhancement of cellular uptake was gained after the PLGA modified by folic acid. Folic acid conjugated nanoparticles have the ability to specifically and selectively target HT-29 cancerous cells in which folate receptors are overexpressed on the surface. The present formulation can be used as cancer cell specific delivery system for anticancer agent entrapped with PLGA based nanoparticles.
5 MICROENCAPSULATION AND FUNCTIONALISATION

5.1 Introduction

When the designed nanoparticles drug delivery systems are applied to the practical application such as effective oral drug delivery to colorectal cancer, there are many barriers for the systems to conquer. One of the major challenges, however, is to deliver enough therapeutic drugs encapsulated in nanoparticles to the cancerous cells in colonic area. The delivery route is a harsh and complicated environment and encapsulated drugs are easily lost in the various pH environment of gastrointestinal tract [196]. There are many approaches for achieving colonic drug delivery, such as the use of prodrugs [197], time-dependent formulations [198] and pH-sensitive polymer coating [61]. Besides, the use of biodegradable polymer pectin and dextrin for colon targeting are also investigated in the literature [199].

In the practical, some polymeric microspheres have demonstrated strong pH-dependent, which means they only take effect in environments of certain pH value. Eudragit S100, an anionic polymer synthesized from methacrylic acid and methacrylic acid methyl ester, is a commonly used polymer for the fabrication of functionalized microspheres. It is insoluble in acids and pure water and only dissolves into aqueous solution at pH 7 or higher [156, 200], ideal for colonic drug delivery. The drug loaded Eudragit S100 microspheres can effectively prevent the drugs from releasing in the upper gastrointestinal tract, whereas the lack of the functionality such as the passive targeting of cancerous cells affects its application. Microencapsulation of the nanoparticles and coated pH-sensitive Eudragit S100 microspheres will further protect the encapsulated drugs from releasing in the delivery route. Fabrication of nanoparticles and following coating Eudragit S100 microspheres might be the promising method to transport sufficient drugs in the oral drug release system.
While on the other hand, PLGA nanoparticles surface lack the functional groups. PLGA can be divided into capped end PLGA and uncapped PLGA with free carboxyl termini. The uncapped PLGA polymer has only one carboxylic group (COOH) at the end of long chains, which makes PLGA very difficult to react with other chemicals. Due to the deficiency of suitable functional groups on the PLGA surface, conventional PLGA nanoparticles lack the possibility of surface modification for specialized targeting or biomimetic purposes. Such modifications are thought to greatly improve the effectiveness of nanoparticles delivery system.

Chitosan, a naturally occurring linear polysaccharide and amino polysaccharide (poly 1, 4-D-glucoamine), has the biodegradable and biocompatible properties. It can be found in the shells of crustacea, the cuticles of insects and cell walls of some fungi [201]. Chitosan and its derivatives have been widely used in biomedical engineering and formulation of drug delivery systems. For drug delivery carrier purpose, chitosan has been formulated into microspheres, tablets and membranes. As chitosan has a good film forming property, it was used to coat the microspheres including poly (lactic acid)-poly (caprolactone) blend. The prepared microspheres exhibited a very high potential for the targeted delivery of therapeutic agents to treat restenosis [202]. Moreover, some chitosan derivatives like lactosaminated and galactosylated ones were used to conjugated with drugs.

Chitosan as a biomaterial was used in this research for two main reasons. The first one is chitosan has a very high positive zeta potential. This property enables chitosan to have in vitro cytotoxicity against different kinds of human cancer cell lines [102]. Another factor is there are repeating amine (NH₂) units in the chitosan polymer chains, which makes chitosan more easily to involve into the chemical reactions.

Therefore, in this chapter, PLGA nanoparticles surface was modified by pH sensitive Eudragit S100 microspheres, in order to achieve the drugs delivery to colon area and keep the drug carriers intact. Furthermore, PLGA nanoparticles surface was functionalized by chitosan using two different methods, one is
physical adsorption and the other is chemical binding. Different characterization methods were tested to confirm the chitosan on the PLGA nanoparticles surface.

5.2 Microencapsulation with Eudragit S100

5.2.1 Entrapment of 5-FU loaded PLGA nanoparticles into Eudragit S100 microspheres

The core PLGA nanoparticles were coated with pH sensitive polymer Eudragit S100. The fabrication process is an oil-in-oil (O/O) or oil-in-water (O/W) solvent evaporation technique. A mixture of methanol and acetone was used as the oil phase as it dissolves the Eudragit S100 properly but without damaging the PLGA nanoparticles fabricated in Chapters 3 and 4. The microencapsulation process is as follows: core PLGA nanoparticles were dispersed in the Eudragit S100 solution (10% wt/vol) through sonication with a probe sonicator. Then the nanoparticles dispersed in Eudragit S100 solution were emulsified in a liquid paraffin containing 1% vol/vol Span 80 or PVA solutions with a mechanical stirrer. Such an agitation was continued for 5 hours to ensure that all the solvents were evaporated. Encapsulated microspheres were obtained by filtration and washed 3 times with petroleum ether to remove residual liquid paraffin or with water to remove the remnant PVA. The samples were finally dried in a vacuum oven for 24 hours. The schematic process of the drug delivery system with a core-coated structure can be described in Fig. 5.1.

5.2.2 Effect of sonication modes

During the fabrication of the emulsified system, the input and control of separation energy for the emulsion formation are very important. To verify the effect of the sonication on the size of microspheres, encapsulation efficiency and zeta potential, different sonication time from 1, 5, 10, to 15 mins were used
in the experiments. The span 80 was used as the surfactant. All the results are listed in Table 5.1.

![Diagram](image)

**Figure 5.1 Schematic map of core-coated drug delivery carrier formation**

<table>
<thead>
<tr>
<th>Probe sonication time/min</th>
<th>Mean diameters/μm</th>
<th>Encapsulation efficiency/%</th>
<th>Zeta potential/mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85</td>
<td>61.3</td>
<td>-0.2</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>67.8</td>
<td>-1.5</td>
</tr>
<tr>
<td>10</td>
<td>54</td>
<td>59.6</td>
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</tr>
<tr>
<td>15</td>
<td>38</td>
<td>52.1</td>
<td>-6.5</td>
</tr>
</tbody>
</table>

**Table 5.1 Effect of sonication on mean diameters, encapsulation efficiency and zeta potential**

The microspheres fabricated by different sonication time were all exhibited micro-meter size ranges and negative surface charge. The increase of sonication resulted in the reduction of the microsphere diameters, from 85 μm to 38 μm, a decrease of more than 50%. The encapsulation efficiency witnessed a first increase from 61.3% to 67.8%, followed by a steady decrease to 59.6% and 52.1%. And the zeta potential on the microspheres surface
changed dramatically. When the solution was sonicated for 1 min, the surface charge was -0.2 mV; while for 15 mins’ probe sonication, the zeta potential was -6.5 mV.

The stability of the microspheres decreases when using much longer sonication time, which affects the encapsulation efficiency and zeta potential of the microspheres. The PLGA nanoparticles which were encapsulated into the Eudragit S100 polymer matrix can be released or exposed on microsphere surface. This can largely decrease the encapsulation efficiency. Due to the carboxylic group on the PLGA polymer chain, PLGA surface charges have a great influence on the microspheres zeta potential results. The carboxylic groups with negative charge [179] appear on the microsphere surfaces, making the surface charges increase.

5.2.3 Effect of Surfactants on morphology of microspheres

Two different surfactants - span 80 and PVA - were used in this emulsification process. The PVA was dissolved into the deionized water as an aqueous phase and span 80 was mixed with the liquid paraffin as an oil phase. Both PVA and span 80 are non-ionic surfactants.

Eudragit S100 was coated on the PLGA nanoparticles to form microspheres and the ratio of coat to core is 5:1 (w/w). For surfactant span 80, the size of microspheres ranges from 30 µm to 50 µm in a mono-disperse state as observed from SEM images (Fig. 5.2 a, b). The microspheres have rough surfaces and are spherical in shape. From the zoom-in view (Fig. 5.2 c), it clearly shows that there are some very fine particles appeared on the surface of microspheres. These particles could be the un-entrapped PLGA nanoparticles or the surfactant that has not been fully washed off in the final stage of preparation. From the cross-section image (Fig. 5.2 d), some spheres appear in microspheres matrix, with a size much larger than that of the nanoparticles. Probably the nanoparticles become aggregated during the fabrication process.
Figure 5.2 (a) Eudragit S100 microspheres prepared by span 80 surfactant, (b) size distribution, (c) zoom-in view, (d) cross-section image
When the PVA is used as surfactant (Fig. 5.3 a, b) a smooth surface is formed on the microspheres. The sizes of the microspheres are between 50 μm and 90 μm, which is much larger than that of the microspheres prepared by using surfactant span 80. And remnants on the microsphere surface are much less. Moreover, there are many pores on the cross-section images (Fig. 5.3 d), which might be due to the solvent evaporation.

**5.2.4 Drug release from the microspheres**

Four drug dissolution environments, HCl buffer pH value 1.2, PBS pH values 4.5, 6.8 and 7.4 were selected to mimic the real gastrointestinal tract. During the experiments, the microspheres were held in the first three solutions for a fixed period of time (1 h, 2 hrs and 2 hrs, respectively) to represent different transit times in different parts of human body before the carrier reached the colon area. The microspheres were then placed in the PBS solution of pH 7.4.

Two batches of microspheres prepared by using surfactants PVA and span 80 respectively were used to test the drug release. The drug release results show that nearly no drug was released in the first 3 hours at the pH of 1.2 and 4.5 (Fig. 5.4) for both microspheres. This confirms that the Eudragit S100 coated microspheres were pH dependent and the drug loaded nanoparticles were perfectly encapsulated in microspheres matrix. When the environment changes to a pH value of 6.8 that was used to mimic the small intestine, only very limited amount of drugs are released in 2 hours, with about 5.2% cumulative drug release for the microspheres prepared by Span 80 and 5.6% cumulative drug release for the one prepared by PVA. The remnant span 80 on microspheres surface might have contributed to the delay of drug release.
Figure 5.3 (a) Eudragit S100 microspheres prepared by PVA surfactant, (b) size distribution, (c) zoom-in view, (d) cross-section image
The release of 5-FU from the PLGA nanoparticles was then carried out in a PBS solution at pH 7.4 that has a similar environment to colon and rectum. There is a burst release in the first few hours followed by an extended slow drug release of up to 120 hours.

Therefore, there is great opportunity for this unique extended release to be further explored including the controlled and targeted drug delivery by incorporating the targeting agents discussed in Chapters 3 and 4. The drug delivery carriers first recognize the tumor site and attach onto the cells with folic acid [203] and then a sustained and delayed drug release can be achieved that will follow the pattern presented in Fig. 5.4. Due to the swelling and erosion of the PLGA polymer, the release phases are characterized by pore diffusion in the initial phase and polymer erosion and degradation in the second.

Figure 5.4 Dissolution profile of PLGA nanoparticles and Eudragit S 100 microspheres (embedded picture).
phase [180]. In this way, the drug release will be controlled by these changes of the encapsulation materials, which lead to prolong half-life time of the drugs. Therefore, the results offer a great potential for the practical application to the colorectal cancer.

5.3 Chitosan (CS) functionalization

As indicated in the introduction, PLGA's deficient functional groups on the polymer chains restrict it to have the surface modification for specialized targeting and biomimetic purpose. Using biocompatible polymer such as chitosan to functionalize PLGA surface can not only maintain its original property but also render new applications. The repeating amine (NH₂) group in chitosan will make PLGA easy conjugation to targeting molecules. Moreover, the high positive charge of chitosan induces PLGA based drug delivery system to be attached by cancer cells which usually have a negative charge.

5.3.1 Preparing chitosan-coated PLGA encapsulated 5-FU nanoparticles

The surface of the PLGA nanoparticles was modified with chitosan by using two different methods, i.e. physical adsorption and chemical binding. The schematic procedures of these two processes are described in Fig. 5.5 (a) and (b), respectively.

The physical adsorption method is very similar to the double emulsion and solvent evaporation method (W₁/O/W₂) described in Chapter 2. The steps for the fabrication of first emulsion (W₁/O) are the same as the one for the 5-FU loaded PLGA nanoparticles. After that, the first emulsion was added to the outer aqueous phase (W₂) which contains the surfactant and different concentrations of chitosan. Other steps follow these procedures when the pure PLGA nanoparticles were prepared. The surface charge of chitosan is positive
while the PLGA with carboxyl groups have a negative surface charge. So the electrostatic adhesion takes effect in this situation.

For the chemical binding method, after getting the PLGA nanoparticles, they were dispersed in PBS solution (pH 5.0) under bath sonication. N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC-HCl) as activating agent and dehydrating agent were fed into the above solution to activate the carboxyl group of PLGA. Different concentrations of chitosan were added into the obtained solution and reaction lasted for 24 hours. The excess EDC and NHS were eliminated by centrifugation.

5.3.2 Characterization of PLGA, PLGA/CS nanoparticles

Average particle size and zeta potential of the modified and unmodified PLGA nanoparticles are shown in Table 5.1. The un-treated PLGA nanoparticles exhibit a mean diameter of 197.8 nm, which is good particle size to achieve the passive targeting [32]. In contrast, the treated samples all displayed a higher particle size than the ordinary PLGA nanoparticles. It is noticed that more chitosan are electrostatically absorbed or chemical covalently bind on the PLGA surface as the CS/PLGA ratio increases, resulting in a large particle size.

For the nanoparticle surface charge, the untreated PLGA nanoparticles showed a zeta potential of -22.5 mV. The negative charge is due to the carboxylic group (COOH) on the PLGA nanoparticle surface [179]. Obviously, after the chitosan treatment, all samples showed a positive zeta potential. This indicates that chitosan was coated successfully onto the PLGA nanoparticles using both physical and chemical methods and the positive values derive from the amine group in the chitosan structure. The large zeta potential values cause the repulsive force between different particles in a single solution and can stabilize the particle suspension. Furthermore, most of the cancer cell membrane surfaces are negatively charged. The chitosan on the PLGA surface with
positive charge can significantly enhance the interaction between the nanoparticles and cancer cells and also facilitate the cellular uptake.

Figure 5.5 Schematic maps of chitosan modified PLGA nanoparticles (a) by physical adsorption method, (b) chemical binding method
Table 5.2 Nanoparticles characterizations

<table>
<thead>
<tr>
<th></th>
<th>CS/PLGA (w/w)</th>
<th>EE (%)</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure PLGA</td>
<td>0:1</td>
<td>28.6</td>
<td>197.8</td>
<td>-22.5</td>
</tr>
<tr>
<td>Physical adsorption</td>
<td>0.2:1</td>
<td>24.3</td>
<td>233.4</td>
<td>14.38</td>
</tr>
<tr>
<td></td>
<td>0.5:1</td>
<td>21.6</td>
<td>256.1</td>
<td>23.69</td>
</tr>
<tr>
<td></td>
<td>0.8:1</td>
<td>22.9</td>
<td>279.5</td>
<td>24.01</td>
</tr>
<tr>
<td>chemical binding</td>
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<td>22.5</td>
<td>298.3</td>
<td>5.72</td>
</tr>
<tr>
<td></td>
<td>0.5:1</td>
<td>26.7</td>
<td>311.5</td>
<td>22.65</td>
</tr>
<tr>
<td></td>
<td>0.8:1</td>
<td>24.1</td>
<td>335.7</td>
<td>22.01</td>
</tr>
</tbody>
</table>

The morphologies of PLGA/CS nanoparticles prepared by physical and chemical method are characterized. As it is shown in Fig. 5.6, the images from SEM showed that the particles had a spherical shape with a relatively narrow size distribution. The average particle sizes are 200-300 nm and larger than that of the PLGA nanoparticles due to the adsorption and binding of chitosan.

From the encapsulation efficiency (EE) results, the PLGA nanoparticles have an encapsulation efficiency of 28.6%. After coating the chitosan on the PLGA nanoparticles surface, the encapsulation efficiency decreased. It was observed that the higher amount of the chitosan was used in the fabrication, the lower encapsulation efficiency was obtained. The reason for that is both chitosan and therapeutic drugs 5-FU are positively charged. The surface chitosan absorbed the 5-FU to the surface of the nanoparticles rather than the dosage inside, which significantly affected the encapsulation efficiency.
Figure 5.6 SEM images of PLGA/CS prepared by (a) physical adsorption and (b) physical binding
5.3.3 Effect of pH value of the solution

As the surface charge is a very important index for the stabilization of the solution, effect of pH value of the solution on the zeta potential of the PLGA/CS nanoparticles was investigated. The sample fabricated by physical adsorption method and 0.5:1 ratio of chitosan and PLGA was used in this research. At the pH value of 5.0, the zeta potential is 22.65 mV as it is indicated in Table 5.1. It is believed that as the pH value of the outer phase increases, the surface charge of PLGA/CS nanoparticles will increase, as the degree of protonation of amino group in chitosan will be lower [204]. As shown in Fig. 5.7, from the pH of 3 to pH of 9, the zeta potential dropped dramatically, from 52.1 mV to -6.3 mV. When the pH value of solution is higher than 8, the surface charge of the PLGA/CS nanoparticles showed negative values. And it decreased even more as the pH value increased.

Figure 5.7 Effect of pH value on the zeta potential of PLGA/CS nanoparticles
5.3.4 Chemical compositions of the surface

Fig. 5.8 shows the FTIR spectra of different polymeric nanoparticles. From the PLGA spectrum, the peaks of C-O-C stretching is at 1088 cm\(^{-1}\), C-H stretching in methyl groups at 1460 cm\(^{-1}\), C=O at 1750 cm\(^{-1}\), CH, CH\(_2\) and CH\(_3\) stretching vibrations between 2850 and 3000 cm\(^{-1}\), and OH stretching around 3500 cm\(^{-1}\). For chitosan, the intense peaks at 1654 and 1597 cm\(^{-1}\) confirmed the presence of amide I and amide II in the chemical structure of CS. The peak of C-H stretch is at 2900 cm\(^{-1}\), C-H bend at 1360-1440 cm\(^{-1}\). The CS’s peak at 3500 cm\(^{-1}\) corresponds to the N-H stretch which is overlapped with the O-H stretch. CS peaks at 3500 cm\(^{-1}\) and 1654 cm\(^{-1}\) are not seen in the PLGA polymer backbone, while they are clearly shown in the PLGA/CS nanoparticles, indicating that the chitosan has been successfully coated onto the PLGA nanoparticles surface by the two different methods.

![Figure 5.8 FTIR spectra of nanoparticles (a: PLGA nanoparticles; b: Chitosan; c: CS-modified PLGA nanoparticles; d: CS-modified PLGA by covalent binding)](image-url)
In this study, high resolution XPS was also employed to test the surface chemical elements to confirm the chemical composition. XPS can provide the qualitative and quantitative information on different elements on the particles surface and test the chemical compositions on the top layer of the polymer surface.

Since chitosan contains the nitrogen element while no nitrogen element appears in the PLGA polymer backbone, the existence of nitrogen confirmed from XPS of PLGA/CS (Fig. 5.9) indicates the chitosan has successfully coated onto the PLGA surface.

The XPS spectra from the pure PLGA nanoparticles (Fig. 5.9 a) clearly shows that the O1s peak appears at 533 eV and the C1s peak is at 287 eV (Fig. 5.9 a), which confirm the carbon and oxygen elements in the PLGA polymer matrix. The peak around the N1s is not obvious and when the range was zoomed in no peak is showed in this area.

The XPS spectra of PLGA/CS nanoparticles prepared by physical adsorption methods (Fig. 5.9 b) show distinct nitrogen and oxygen peak at 533 and 287 eV, respectively. The zoom-in view of the nitrogen area showed a signal at 400 eV which corresponds to the amino group in chitosan polymer. The results of XPS spectra demonstrate that the chitosan has successfully been coated on the PLGA nanoparticle surface.

5.3.5 *In vitro* drug release profile

*In vitro* drug release curves of 5-FU from the treated and untreated PLGA nanoparticles are shown in Fig. 5.10. All three samples, PLGA nanoparticles, PLGA/CS nanoparticles by physical adsorption and PLGA/CS nanoparticles by chemical binding, show a burst release in the first 10 hours’ release. The chitosan modified PLGA nanoparticles exhibit heavier burst release profile
Figure 5.9 (a) XPS spectra of PLGA and (b) PLGA/CS nanoparticles prepared by physical adsorption method
than the one from untreated PLGA nanoparticles because both the chitosan and the model drug 5-FU are hydrophilic. In the double emulsion and solvent evaporation fabrication process, more drugs are absorbed by chitosan due to drugs transferring from first emulsion to second one and thus appear on the particles surface rather than inside of the nanoparticles. When the nanoparticles are dispersed in the solution to carry out the drug release, the coating of chitosan makes the PBS solution more easily penetrate into the nanoparticles and more drugs are released in the same time range.

![Graph of cumulative drug release](image)

**Figure 5.10** Cumulative drug release from PLGA nanoparticles, PLGA/CS nanoparticles by physical adsorption and PLGA/CS nanoparticles by chemical binding method

### 5.4 Conclusions

In this chapter, the PLGA nanoparticle surfaces were modified by pH sensitive polymer Eudragit S100, in order to assist the nano-oral drug delivery system to pass complex human gastrointestinal tract. Also the PLGA nanoparticle surfaces were functionalized by chitosan. The purpose of utilization chitosan is...
to provide enough easy reactive functional groups on PLGA nanoparticles surface and make PLGA surface a positive to attach the cancer cells which usually have negative charge surfaces.

After microencapsulation of Eudragit S100, the microspheres prepared by surfactant span 80 have a size ranging from 30 μm to 50 μm and the ones prepared by PVA have size between 50 μm and 90 μm. The system was confirmed to greatly prevent the drugs from releasing below the pH 7 in the in vitro drug release test. And only 5.2% and 5.6% cumulative drug releases were tested under the pH 7 solution.

Chitosan was successfully coated to PLGA nanoparticles surface by physical adsorption and chemical binding method, which was confirmed by FTIR and XPS. Chitosan modified PLGA nanoparticles exhibit a positive charge surface. The zeta potential dramatically decreased as pH value increased. The pH values of the solution have great impact on the surface charge of the nanoparticles. The chitosan on PLGA nanoparticles surface affected the drug release profile. The positive charge and hydrophilic property induce the burst drug release and drug release amount.
6 PLASMA MODIFICATION OF PLGA

6.1 Introduction

PLGA is always restricted for wide usage due to there being only very few functional groups [205], which allows only limited reactions with other materials. Furthermore, the poor hydrophilicity and no natural cell recognition site on the surface result in a low efficiency for the drug delivery carrier to target cancerous cells [206]. Therefore, effective modification of PLGA surface will facilitate the conjugation of cancer targeting molecules with PLGA.

In this chapter, the surface modification of PLGA by oxygen plasma treatment was investigated to study the operation parameters on the surface properties of PLGA (50/50), including the hydrophilicity, morphology and chemical composition. During the plasma treatment, the variation of treatment parameters, such as plasma treatment mode, applied power and chamber gas pressure has a significant effect on the chemical structures and compositions of the polymer surface. The studies on these are beneficial for the understanding of the plasma treated PLGA and its usage for targeted drug delivery system.

6.2 Plasma treatment of PLGA surface

Various methods have been carried on to modify PLGA surface to achieve specific functions including hydrophilicity improvement and specific-targeting effect. For drug delivery purpose, as some of the chemotherapeutic drug like 5-fluorouracil (5-FU) is hydrophilic, great repulsion takes effect when trying to encapsulate the drugs into the hydrophobic encapsulation material PLGA. Most of the current work is only concentrated on conjugation of some hydrophilic molecule onto the PLGA surface and functionalization of the PLGA surface by connecting some targeting agent [38, 86]. These currently used strategies are mostly involved in the wet chemistry techniques, in which the hydrophilic component moieties or targeting molecules react with the
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PLGA in the organic solvents. This procedure always leads to numerous residual solvent removals and change of bulk materials properties. Furthermore, lack of functional chemical groups on the PLGA surface results in the difficulty of PLGA surface modification [207] and conjugation ratio of hydrophilic component and targeting molecule is quite low.

Recently, plasma technologies have increasingly been utilized in the modification of nanomaterial surface and morphology. The introduction of plasma technology to surface treatment of biomaterial brings the wide attention [206], as it has the advantages of short reaction time, non-polluting process and providing a wide range of different functional groups onto the material surface. Moreover, it is an effective and efficient way which only affects the near surface of the material and doesn’t change the bulk material properties. Also it can be used to treat the material surface with complex shape and introduce the desired functional groups on the material surface. During the plasma treatment, the excited species, UV light, ions, electrons, and radicals within plasma strongly interact with the material surface by breaking the C=C bonds and creating active sites for binding of functional groups. Therefore, the plasma application for the improvement of PLGA hydrophilicity and reactive groups functionalization will be useful for the further conjugation.

Gas plasma treatment is extensively used for PLGA modification [206]. Different gas plasma can create various reactive sites such as amine group, carboxylic group and sulfonic acid groups on the PLGA surfaces. Among them, oxygen plasma treatment is performed under oxygen gas which is an easily obtained nontoxic gas. By conducting oxygen plasma treatment, some polar groups including the carboxyl, carbonyl, ether, hydroxyl and peroxyl groups which contain oxygen can be introduced onto the material surface [206]. However, the problem is that the modified results will decline with time after plasma treatment, which affects its further application [208]. The density of functional groups on the substrates is subject to ageing and is normally checked by noting changes in water contact angles. An additional surface pre-treatment for the substrate is necessary to decrease the influence the ageing effect and improve the adhesion as well as following functional group immobilization.
Surface pre-treatment of polymeric substrate is a key to the retention of treated polymer surface. This surface pre-treatment can be done by using some gas like nitrogen or argon and it would clean and activate the surface. Surface modification procedure should be carefully selected as polymeric surface got easily over activated, leading to a damaged rather than a modified surface.

Gas plasma treatment can be conducted in continuous wave (CW) mode or pulsed (P) mode. Currently, most of the research is focused on the continuous wave plasma treatment. CW plasma treatment has the higher power, thus it always leads to the cross linked structures on the material surface. The retention of pulsed mode plasma can help preserve the integrity of generated functional groups. Pulse plasma treatment was selected in order to reduce the molecular fragmentation, and to avoid the diversity of functional groups produced by the various chemical reactions in the continuous wave plasma treatment.

6.2.1 Preparation of casted PLGA

In order that the PLGA has more interaction with the plasma gas atmosphere, PLGA was casted onto a thin film which was prepared by pouring 4% (wt) concentration of PLGA solution into a poly (tetrafluoroethylene) (PTFE) mould. The organic solvent was first evaporated in the fume hood, and then the film was removed from the PTFE mould and totally dried in the vacuum oven at the 30°C for 24 hours.

6.2.2 Plasma treatment

In the experiments, the plasma treatments were performed in a custom-built glow discharge quartz reactor which has a radio frequency (RF) generator inside. The discharge chamber is a cylinder with an inner diameter of 15cm and a length of 30cm. There are two processes in the plasma treatment: surface plasma activation and functional group immobilization. Argon and oxygen were used as the plasma gas atmosphere. Specifically, argon plasma is for
etching the surface and making the surface more active, while the oxygen plasma is for generating the functional group carboxyl (COOH) or peroxide (OOH) on the PLGA surface.

During the treatment, samples were placed in plasma source position. Before the chamber was filled with the desired gas, the air in the chamber was evacuated and the pressure was adjusted to zero. In order to protect the materials from cross-linking and destroying, pulse mode type of plasma was implemented. The plasma output power supplies were set at 50 W and 100 W at a frequency of 13.56 MHZ. After plasma treatments were finished and the power was turned off, the treated samples were further kept in the gas atmosphere for another 10-15 mins for stabilization before they were taken out from the chamber.

The whole preparation process including the preparation of casted PLGA and plasma treatment of PLGA is described in Fig. 6.1. Fig. 6.2 shows the schematic diagram of gas plasma treatment facility.

![Figure 6.1 Preparation of surface modification of PLGA films](image)

### 6.3 Contact angle and wettability

The contact angle measurements were performed to check wettability of the samples surface to the water, which was conducted using a KSV CAM 100 contact angle meter. Deionized water was used for the measurement. The interval of the frames captured by camera was 33 ms. For accuracy, the
measurements were repeated 5 times on different parts of a same sample. All the tests were undertaken 2 hours after the plasma treatment.

![Schematic diagram of gas plasma treatment chamber](image)

Figure 6.2 Schematic diagram of gas plasma treatment chamber

Contact angle is an important index for a material surface. The higher the material hydrophilicity is, the lower the water contact angle becomes. When the treated samples are used for hydrophilic drug encapsulation, the hydrophilic PLGA property will enhance the drug loading and encapsulation efficiency. All contact angle measurements are summarized in Table 6.1 and the detailed results are shown in Fig. 6.3. It can be seen that surface hydrophilicity of PLGA was improved by oxygen plasma treatment and such a hydrophilicity enhancement was depended on the treating parameters such as power and treating time. The contact angle of untreated PLGA (control) sample was 76.28°. After the oxygen plasma treatment, all of the contact angles decrease. For the continue wave (CW) plasma treatment, when the power is 50 W, the contact angle decreases gradually as the treatment time increases. The contact angle ranges from 65.33° to 58.11° when the treatment time alters from 3s to 30s. However, further increase of the treatment time to 60s results in only a very small decrease of the contact angle to 57.27°, indicating the saturated functional groups such as hydroxyl (OH), carboxyl (COOH) and carbonyl (C=O) on the PLGA surface.
Table 6.1 Contact angles of different treated PLGA films

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mode</th>
<th>Power (W)</th>
<th>Processing time (s)</th>
<th>Contact angles (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA (control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>76.28</td>
</tr>
<tr>
<td>CW1</td>
<td>CW</td>
<td>50</td>
<td>3</td>
<td>65.33</td>
</tr>
<tr>
<td>CW2</td>
<td>CW</td>
<td>50</td>
<td>5</td>
<td>62.84</td>
</tr>
<tr>
<td>CW3</td>
<td>CW</td>
<td>50</td>
<td>30</td>
<td>58.11</td>
</tr>
<tr>
<td>CW4</td>
<td>CW</td>
<td>50</td>
<td>60</td>
<td>57.27</td>
</tr>
<tr>
<td>CW5</td>
<td>CW</td>
<td>100</td>
<td>30</td>
<td>27.95</td>
</tr>
<tr>
<td>P1</td>
<td>Pulse</td>
<td>50</td>
<td>600</td>
<td>59.87</td>
</tr>
<tr>
<td>P2</td>
<td>Pulse</td>
<td>100</td>
<td>600</td>
<td>52.29</td>
</tr>
<tr>
<td>CWP</td>
<td>CW</td>
<td>50</td>
<td>30</td>
<td>54.86</td>
</tr>
</tbody>
</table>

When the treating time is fixed at 30s but the power changes from 50 W to 100 W, the contact angle decreases dramatically from 58.11° to 27.95°. This means the high power can introduce higher percentage of polar functional groups. The ageing effects after plasma treatments were also studied using these two treatment parameters. From Fig. 6.4, the results showed that the ageing time has great impact on the results. The contact angle for both power levels increases with the ageing times. That means the hydrophilicity on the PLGA surface decreased. The ageing rate in the first 12 hours was very high. After 12 hours, the contact angles levels off and is kept in a relatively stable value at 65.25° and 43.73°, respectively. The results indicate that all the characterizations should be done within a short time of treatment. The retention of the functional groups derive from the gas plasma treatment is a key issue for the further surface conjugation.
Figure 6.3 Contact angle measurements
Figure 6.4 Ageing effect on the contact angles

The duty cycles (D), plasma power (P) and plasma energy (E) are three important parameters in the plasma treatment. They can be calculated using following Equations:

\[
\text{Duty cycle (D)} = \frac{t_{\text{on}}}{t_{\text{on}} + t_{\text{off}}} \\
\overline{P} = P \times D \\
E = \overline{P} \times t
\]  

(4)  
(5)  
(6)

where \( t_{\text{on}} \) and \( t_{\text{off}} \) are the time when the plasma treatment is on and off [209]; Duty cycle is the time that plasma facility spends in an active state; \( \overline{P} \) is the average power and \( P \) is applied power when the plasma is on; \( E \) is the plasma energy.

The samples were also treated using the pulsed mode at the same two plasma energies as the continuous wave plasma treatment for comparison. The pulse plasma treatment conditions are at powers of 50 W and 100 W for 10 mins. From the Equations (4), (5) and (6), their energies are 1500 W's and 3000 W's,
respectively, which are the same as the ones for samples CW3 and CW5 (Table 6.1). In the experiments, the duty cycle was set to 1/20. From the results in Table 6.1, the corresponding treatment by pulse mode is much milder than that by continuous wave treatment. The contact angle from pulsed plasma is higher than that from the continuous wave plasma with same treatment energy.

Also, a continuous wave plasma treatment followed by a pulsed mode treatment was used in this research (CWP samples). The contact angle was found to be 54.86°. The combination of the methods can take the full advantages of the two treatments. It can not only maintain a hydrophilic surface but also incorporate more functional groups. This kind of treatment was also confirmed to generate stable interfaces with high density of functional groups [210].

6.4 Scanning electron microscopy images

The surface morphologies were characterized by scanning electron microscopy (SEM). It can be seen from Fig. 6.5a that the surface of untreated PLGA (control samples) is quite smooth. And the one for the samples treated by continuous wave plasma 50W for 3 seconds (Fig. 6.5b) and 50 W for 5 seconds (Fig. 6.5c) are quite similar to the untreated one. The surfaces haven’t changed much when low energies are imposed.

Prolonged plasma treatments or high plasma energies can result in a rough surface as the plasma can etch and remove the top surface layer [211]. The PLGA surface roughness is another indication of the increase of hydrophilicity. The material surface roughness has a great impact on the surface wettability [212]. P2 samples were treated under the pulsed mode at a power of 100 W for 10 mins and the energy was 3000 W·s. The morphology of P2 samples (Fig. 6.5f) has a much coarser surface than that of CW4 (Fig. 6.5d) and P1 (Fig. 6.5e) samples with lower energies. After the treatment with continuous wave plasma 100 W/30s, which means a 3000 W·s continuous wave plasma energy
Figure 6.5 The SEM images of oxygen plasma treated PLGA under (a) Control (b) CW1 (c) CW2 (d) CW4 (e) P1 (f) P2 (g) CW5

treatment, some parts of the sample surface are burnt. And from the surface morphology of the CW 100 W/30s treated PLGA samples (Fig. 6.5g), great burn and damage appeared, while the one treated by P 100 W/10mins showed a rough surface in Fig. 6.5f. The pulsed plasma treatment can reduce the PLGA surface molecular fragmentation and avoid the damage of the treated surface based on the same energy as the continuous wave plasma. Moreover, the pulsed mode plasma can avoid the diversity of different functional groups [209]. Therefore, it is necessary to control the power and energy in the plasma treatment.
6.5 XPS analysis

In order to compare the compositions and chemical changes before and after plasma treatments, high resolution x-ray photoelectron spectroscopy (XPS) measurements were used to study the PLGA surfaces. Normally the XPS can provide a sampling depth of about 0.8-5 nm.

The positions of different kinds of Cls photoelectrons in PLGA skeleton were showed in Fig. 6.6a. Cls of carbon-hydrogen bonds (C-H) and aliphatic carbon bond (C-C), ether bond (C-O) and ester bonds (C(=O)O, C=O, COOH) were labelled using separated numbers. Three peaks with binding energies of 284.6 eV, 287.5 eV and 289.6 eV were showed in the XPS spectra (Fig. 6.6b). All XPS spectra of treated samples by different parameters are provided in Fig. 6.7. The areas which the curves cover are the amount of the corresponding carbons. Therefore, content percentages of three Cls from different treated samples are calculated and listed in Table 6.2.
Figure 6.7 XPS C1s spectra of different treated PLGA samples
Table 6.2 Fraction of carbon functional groups from high resolution C1s XPS of samples before and after the plasma treatment

<table>
<thead>
<tr>
<th>Samples</th>
<th>284.6 eV; C-H, C-</th>
<th>287.5 eV; C-O, C-N-</th>
<th>289.6 eV; C=O-, -COOH</th>
<th>Oxygen (%)</th>
<th>Carbon (%)</th>
<th>O/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>64.4</td>
<td>20.4</td>
<td>15.3</td>
<td>23.0</td>
<td>77.0</td>
<td>0.3</td>
</tr>
<tr>
<td>CW 50W 30s</td>
<td>28.7</td>
<td>38.2</td>
<td>33.1</td>
<td>40.5</td>
<td>59.5</td>
<td>0.7</td>
</tr>
<tr>
<td>CW 50W 4mins</td>
<td>25.6</td>
<td>40.7</td>
<td>33.7</td>
<td>41.9</td>
<td>58.1</td>
<td>0.9</td>
</tr>
<tr>
<td>CW 100W 30s</td>
<td>28.2</td>
<td>40.0</td>
<td>31.8</td>
<td>40.8</td>
<td>59.2</td>
<td>0.7</td>
</tr>
<tr>
<td>P 50W 10mins</td>
<td>21.9</td>
<td>42.2</td>
<td>35.9</td>
<td>42.5</td>
<td>57.5</td>
<td>0.7</td>
</tr>
<tr>
<td>P 100W 10mins</td>
<td>24.4</td>
<td>42.1</td>
<td>33.5</td>
<td>41.9</td>
<td>58.1</td>
<td>0.7</td>
</tr>
<tr>
<td>CW+P</td>
<td>26.7</td>
<td>39.0</td>
<td>34.3</td>
<td>41.9</td>
<td>58.1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

As can be seen from the ratio of oxygen to carbon (O/C), the oxygen amount increases dramatically after plasma treatments. This indicates a great impact of the oxygen plasma action. Among the ester bonds, the carboxyl groups (COOH) are often used for the further conjugations or reactions. The accurate calculation of the ester bonds is necessary for future applications. From Table 6.2, after the oxygen plasma treatments, the fraction of ester bonds on the
PLGA surfaces changes obviously. For the control sample PLGA, the percentage of ester bonds is only 15.3%. After the plasma treatments, ester bonds on the PLGA surface are more than doubled under all treatment conditions. The highest one from P1 sample was up to 35.9%, which was achieved by the pulsed mode plasma under 50 W for 10 mins treatment. For the sample treated under 100 W for 30s, the amount of ester bond is less than the other treated samples. The reason for that is the prolonged plasma treatment at high energy leads to the cleavage of the ester bonds [206] and the change in ester bonds back to the aliphatic carbon bonds or ether bonds.

### 6.6 Conclusions

In summary, we have demonstrated plasma technology for modification of PLGA surface. It was noted that surface hydrophilicity as indicated by contact angle is dependent on the plasma treating mode, power and time. The contact angle reaches a plateau at 57.27° when the continuous wave plasma treating power is fixed on 50 W and the treating time is 60 s. The highly hydrophilic PLGA can facilitate the hydrophilic drug encapsulation, which improve the drug loading and encapsulation efficiency. Samples treated in continuous wave plasma by the power of 100 W for 30 s exhibit a substantial improvement in hydrophilicity with a contact angle of 27.95°, but the morphology from SEM showed burnt in parts of the samples. The contact angle from the pulsed mode plasma treated samples is larger than that from the continuous wave treated ones at the same treatment energies. SEM images exhibited that the high plasma treatment energies result in rough surface morphologies. XPS spectra showed a significant increase of ester bonds after the plasma treatment. As the reactive functional carboxyl groups are the most important components in the ester bonds, the immobilization of enhanced functional groups onto PLGA will be useful for incorporation of targeting molecules on the drug delivery carriers.
CHAPTER SEVEN

7 CONCLUSIONS AND FUTURE WORK

7.1 Summary

Oral drug delivery systems using nanoparticles have attracted great attention in recent years. This thesis has developed pH-sensitive nanoparticles and targeted nano-drug delivery systems and modified PLGA and PLGA nanoparticle surface in order to immobilize drug targeting molecules immobilization and improve the PLGA surface hydrophilicity.

In Chapter 3, a drug delivery system with enhanced drug loading and small particle size was designed. High drug loading is achieved by optimizing nanofabrication parameters. It was found that nanoparticle size increases gradually as the theoretical drug loading increases. The theoretical drug loading below a concentration of 15% (w/w) contributes proportionally to the actual drug loading and encapsulation efficiency. The drugs become saturated in the dosage when the concentration is higher than 15%.

High volume of outer water phase to organic phase improves the drug loading and results in a large nanoparticle size. Adjusting the pH value of outer water phase to isoelectric point of therapeutic drug 5-FU is beneficial for the enhancement of drug loading. Optimized nanoparticles with a drug loading of 5.8%, encapsulation efficiency of 28.6% and nanoparticle size of 197.8 nm were obtained by using a moderate initial drug feeding, high volume ratio of outer water phase to organic phase and adjusting the pH value of outer aqueous phase to the isoelectric point of 5-FU.

5-FU was formulated in an amorphous or molecular dispersion state in the PLGA polymeric nanoparticles as observed from the XRD results. Drug 5-FU has no chemical interaction with the PLGA polymer and is physically dispersed in the PLGA polymeric matrix PLGA nanoparticles. In vitro drug release experiment showed an initial burst release followed by a slow and
sustained release over an extended period of over 120 hours. Therefore, the prepared drug delivery systems with enhanced drug loading and small particles size have the potential to be utilized in the therapy of colorectal cancers.

In Chapter 4, specific drug delivery to the cancerous cells HT-29 of colorectal cancers is achieved by conjugating folic acid molecules to PLGA nanoparticles. A novel conjugate PLGA-1, 3-diaminopropane-folic acid for the specific 5-fluorouracil drug delivery carrier was successfully fabricated. Solvent dimethylformamide (DMF) is found to be an ideal reaction environment for the conjugation, for all the reactants have good solubility in this solvent and DMF has no reaction with them. Suitable amount of mole ratio of 1, 3-diaminopropane to PLGA is vital for the successful preparation. Excessive 1, 3-diaminopropane results in the hydrolysis of PLGA.

Surfactant PVA is difficult to be removed in the final stage of the preparation and remnant PVA has great impact on the nanoparticle size, zeta potential, the precise calculation of drug loading and encapsulation efficiency and drug release profile of the nanoparticles. The composition and structure of the synthesized PLGA-1, 3-diaminopropane-folic acid copolymer was detected and verified by \textsuperscript{1}H NMR. The sizes of nanoparticle with targeting and non-targeting functions are 224 nm and 198 nm. Targeted and non-targeted nanoparticles sizes were found to be 224 nm and 198 nm, respectively. The nanoparticle surface charge has not changed significantly before and after conjugation as indicated from the zeta potential.

UV-Vis analysis was conducted to investigate the exact amount of targeting molecule folic acid incorporated onto PLGA and it is found that the folic acid conjugation ratio on molar ratio basis was 0.38. \textit{In vitro} cytotoxicity and specific cell targeting exhibited that the modification of PLGA by folic acid can largely enhance the cell uptake and cytotoxic effect. The present formulation can be used as cancer cell specific delivery system for anticancer agent entrapped with PLGA based nanoparticles.
In Chapter 5, the PLGA nanoparticle surfaces were modified by pH sensitive polymer Eudragit S100 or functionalized chitosan. The microencapsulation of nanoparticles is used to protect nano-drug carriers from being released prematurely in the upper gastrointestinal tract. The probe sonication time during the fabrication process has great impact on the mean diameters, encapsulation efficiency and zeta potential. As the probe sonication time increases from 1 min to 15 mins, the microsphere size drops from 85 μm to 38 μm largely due to the increase of shearing force while the encapsulation efficiency decreases from 61.3% to 52.1% and zeta potential changes from -0.2 mV to -6.5 mV.

The average microsphere size prepared by surfactant Span 80 is larger than the one prepared by using PVA surfactant. And more residuals are found on the microspheres prepared by Span 80. The two kinds of microspheres prepared by Span 80 and PVA exhibit pH-dependent property below the pH 6.8 and no drug release is found in the PBS solution. In pH 7.4 drug dissolution environment, 5.2% and 5.6% cumulative drug release were detected from the microspheres prepared by Span 80 and PVA surfactant, respectively.

Chitosan was also used to functionalize the PLGA nanoparticle surface. Chitosan was successfully coated to the PLGA nanoparticles surface by physical adsorption and chemical binding method, which was confirmed by FTIR and XPS. The surface charges of nanoparticles decrease dramatically as pH value of solution increases. After the chitosan is coated on the nanoparticle surface, all of the zeta potentials show positive charges. And the higher the ratio of chitosan feeding, the higher the zeta potential. This shows a distinguished difference from the unmodified PLGA nanoparticles whose surface charge is -22.5 mV.

The modified PLGA nanoparticles sizes are larger than the unmodified ones. The chitosan modification leads to a low drug encapsulation efficiency. The pH values in the solution affect the PLGA/CS surface charges. The chitosan on the PLGA nanoparticle surface affects the drug release profile. The positive charge and hydrophilic property induce the burst drug release and drug release amount.
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The drug release from PLGA/CS nanoparticles shows burst drug release profiles and attains higher cumulative drug release amount due to its hydrophilicity.

In Chapter 6, we have demonstrated that plasma technology can be used for PLGA surface modification to get hydrophilic PLGA surface and the immobilization of more functional groups. It is noted that surface hydrophilicity is dependent on the plasma treating mode, power and time. The contact angle reached a plateau at 57.27° when the continuous wave plasma treating power was at 50 W and the treating time was 60 s. The improved hydrophilicity of PLGA will facilitate the encapsulation of hydrophilic drugs and improve the drug loading and encapsulation efficiency. Samples treated in continuous wave plasma at the power of 100 W for 30 s exhibit a substantial drop of contact angle to 27.95°, but the morphology from SEM showed burnt in parts of the samples. The contact angles of the samples treated with pulsed mode plasma are larger than the ones treated with continuous wave plasma at the same treating energy level.

SEM images exhibit that the high plasma treating energy results in rough surface morphologies. XPS spectra show a significant increase of ester bonds after the plasma treatment. As the reactive functional carboxyl groups are the most important components in the ester bonds, the immobilization of enhanced functional groups onto PLGA will be useful for incorporation of targeting molecules on the drug delivery carriers.

7.2 Future work

As indicated previously, novel drug carriers with targeted drug delivery system are formulated in this research project by fabricating pH-sensitive PLGA nanoparticles. The surfaces of these PLGA nanoparticles are then modified with chitosan and plasma for PLGA nanoparticles surface functionalizations. To further study this drug delivery system, the following work might be investigated.
7.2.1 Targeting therapeutic drugs

In Chapter 4, a drug delivery system with folic acid which is a HT-29 cell-specific targeting molecule was successfully prepared. The design scheme included the formulation of targeting molecule conjugated drug delivery encapsulation materials and the encapsulation of the therapeutic drugs into the prepared polymers. The targeting molecule folic acid will direct the systems to the designated cancer areas. However, great repulsions between the drugs and encapsulation materials usually take effect, which results in the low drug loading and encapsulation efficiency and thus the drug concentrations are very low in the cancerous cells.

Therefore, the incorporation of targeting molecules directly to the therapeutic drugs will definitely enhance chemotherapy effect. But one of the challenges is to ensure the components of drugs have the cytotoxic effect. The conjugation should not be harmful to drug effect.

7.2.2 SEM for the cross-section images

The Eudragit S100 microsphere cross-section images were studied in Chapter 5, in order to characterize the distribution of PLGA nanoparticles in the Eudragit S100 microspheres matrix. Samples were prepared using two methods. One is using ultramicrotome to cut the samples embedded in resins. The other is crushing the brittle samples after freezing the polymeric samples. However, as demonstrated in Chapter 5, it is not easy to distinguish between the microsphere polymeric matrix and encapsulated nanoparticles. The nanoparticle aggregation in the oil-in-oil microspheres formulation is another challenge in the cross-section image characterization. It is better to develop some innovative method to differentiate between Eudragit S100 matrix and PLGA nanoparticles, such as stain one of the components.
7.2.3 Nanoparticle changes in drug release

During the drugs releases from the nanoparticles, the morphologies changed dramatically. In Chapter 3, it showed that drug releases from the nanoparticles matrix experience three distinct phrases composing of rapid and burst drug release, polymer swelling and drugs diffusion from the polymeric matrix, and sustained and delayed drug release as well as erosion and degradation of encapsulation materials. And different morphologies appear in different phrases. The clear pictures of how these nanoparticles change in different phases will be of help to understand the drug release profiles.

Also mathematical modelling can be applied to investigate the drug release and nanoparticle changes, as some of these researches are not easily proved in the experimental work. The modelling can facilitate us to have theoretical understanding and accelerate the applying of the prepared drug release systems for practical usage.

7.2.4 Plasma polymerization treatment

Plasma technology is a very promising method for the modification of the biomaterial surface. Using the plasma polymerization technology, the monomer can be polymerized onto the substrate evenly with high intensity. If the cancer targeting molecules can be immobilized onto the encapsulation materials surfaces using plasma polymerization method, it will avoid the numerous residual solvent removals and loss of the materials and products during the fabrications. Furthermore, the conjugation ratio can be expected to be much higher than the one by traditional wet chemistry methods.

7.2.5 Labelling the carboxylic groups

In Chapter 6, plasma technology with different treatment parameters was used to immobilize reactive functional groups such as carboxyl groups onto the
PLGA surface, in order to facilitate further conjugation of targeting molecules onto the PLGA surface and enhance the conjugation ratio with targeting molecules. However, the XPS spectra for the carboxyl (COOH) groups, carbonyl (C=O) groups and ester (COOR) groups are all in the same region with a binding energy of 289.1 eV. Only the carboxyl groups are active in the chemical reactions and used as the functional group for the conjugations. Furthermore, the ageing effect has a great impact on the treated samples. The functional groups generated by the plasma treatment decrease dramatically as time passes by. Therefore, a chemical derivatization method to be adopted in the experiments should meet the following two criteria: 1) the reagent selectively reacts with the carboxyl group (-COOH); 2) the reagent has specific element that can be easily detected by XPS measurement. In this way, the carboxyl groups will be first uptaken by the selected reagent after the plasma treatment experiment. Then the quantity of the specific elements measured by XPS can be used to determine the amount of carboxyl groups.

### 7.2.6 In-vivo animal test

*In-vivo* animal tests need to be done to confirm the efficacy of the different drug delivery systems prepared in this work, as only *in-vitro* drug release test, *in-vitro* cell uptake and in-vitro cytotoxicity were done in this thesis. This simulation of the gastrointestinal tract and different transition time using different pH value solutions and different incubation times are not enough to get the real pictures of the drug release. More complicated factors such as the enzyme, interaction between different organs have to be considered. So the *in-vivo* animal tests are highly demanded. The shrinkage rate of tumours sizes before and after the administration of dosages can be calculated. This will represent the prepared drug delivery system in the practical applications.
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