SYNTHESIS AND CHARACTERISATION OF PEG-PLGA NANOPARTICLES FOR GERANIIN ISOLATED FROM *Phyllanthus watsonii* (DUKUNG ANAK) EXTRACT

SREEGAYATHRI JAIRAMAN

UNIVERSITI SAINS MALAYSIA

2018

SYNTHESIS AND CHARACTERISATION OF PEG-PLGA NANOPARTICLES FOR GERANIIN ISOLATED FROM *Phyllanthus watsonii* (DUKUNG ANAK) EXTRACT

by

SREEGAYATHRI JAIRAMAN

Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

August 2018

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, Dr Siti Hawa Ngalim for the continuos support of my Master study and for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better supervisor for my Master study. Beside my supervisor, I would also like to thank my co-supervisors Dr Lim Vuanghao and Dr Yit Lung Khung for their insightful comments and encouragement. Although, there were many hurdles faced along the journey to complete this research work but yet Im glad I have learnt lots of new skills. I would like to thank god for giving me strength throughout my research to complete.

I am feeling grateful to have received My Masters Scholarship and I thank USM for giving me USM fellowship scheme. My studies completion would have not been possible without this financial support.

Our heartiest thanks to MARDI organization and USM for funding the project. Special thanks to the scientific officers, lab assistants of Cluster Integerative and Regenerative Medicine Laboratory, who had given good cooperation and assistance throughout this project.

Last but not least, Im grateful to my father Mr Jairaman and my mother Mrs Paramesvari for their moral support and emotional support to complete the studies and in my life in general. Without their support, it is definitely would have been possible to conduct this research and complete my studies. Not forgetting all my friends who have been helping me a lot along the way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PEG-PLGA	Poly(ethylene glycol) methyl ether-block- poly (lactide-co-glycolide)
PDI	Polydispersity index
SDS	Sodium dodecyl sulphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PBS	Phosphate buffered saline
EMEM	Eagle's modified eagle medium
FBS	Fetal bovine serum
DPBS	Duelbecco's phosphate buffered saline
PFA	Paraformaldehyde
DAPI	4',6-diamidino-2-phenylindol
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPS	Mononuclear Phagocyte System
TEM	Transmission electron microscope
FESEM	Field emission scanning electron microscope
DLS	Dynamic Light Scaterring

SINTESIS DAN PENCIRIAN PEG-PLGA NANOPARTIKEL BAGI GERANIIN PENCILAN DARIPADA EKSTRAK *Phyllanthus watsonii* (DUKUNG ANAK)

ABSTRAK

Semakin banyak penyampaian drug pada masa kini menggunakan teknologi perubatan nano. Kecekapan penyampaian drug ke tapak sasaran dalam badan adalah bergantung kepada faktor fiziko-kimia pembawa drug tersebut. *Phyllanthus watsonii*, yang dikenali sebagai dukung anak boleh didapati di hutan tropika di Malaysia. Ekstrak tumbuhan ini mempunyai kesan anti-hipertensi, anti-virus dan anti-diabetis. Geraniin yang digunakan dalam kajian ini merupakan sebatian aktif utama daripada ekstrak *P.watsonii* yang diperolehi daripada Dr. Indu S Jaganath dari Institut Penyelidikan dan Pembangunan Pertanian Malaysia (MARDI). Walau bagaimanapun, ciri bioavailabiliti fitokimia semasa penghantaran drug sering terhalang oleh degradasi fitokimia yang dipengaruhi oleh aktiviti pH dan tindakan enzim dalam salur gastrousus sebelum ke sistem peredaran darah. Oleh itu, kajian ini bertujuan untuk 1. Mensintesis nanopartikel berasaskan poli (etilenaglikol)-poli (laktik-ko-glikolikasid) (PLGA-PEG) berkapsulkan geraniin yang berdiameter kurang daripada 200 nm dengan menggunakan kaedah emulsi berganda 2. Mencirikan sifat fizikokimia dan morfologi PEG-PLGA nanopartikel, 3. Menimbang geraniin yang telah dikapsulkan dalam PEG-PLGA nanopartikel 4. Menentukan kestabilan *in vitro* dan kesan sitotoksik PEG-PLGA nanopartikel dan PEG-PLGA nanopartikel dikapsulkan dengan geraniin, 5. Menentukan kesan organisasi selullar terhadap pengambilan sellular. Nanopartikel polimer (PEG-PLGA) telah disintesis menggunakan kaedah emulsi berganda. Ujian pelepasan drug secara *in vitro*

digunakan untuk menentukan pembebasan sebatian aktif geraniin dari nanopartikel dalam persekitaran terkawal. CRL1790 (juga dikenali sebagai CCD 841 CoN) yang merupakan sel epitelia kolon manusia normal digunakan sebagai model sel. Ujian kedayahidupan sel dilakukan untuk menentukan kepekatan optimum nanopartikel yang mempengaruhi kemandirian sel. Pemerhatian terhadap perlekatan sel dan pengambilan selular dilakukan untuk mengkaji ciri-ciri sel pada permukaan mikrobentuk dan laluan endositosis yang lebih diutamakan untuk induksi nanopartikel. Nanopartikel PEG-PLGA (NP) menunjukkan diameter 102.7 ± 12.36 nm dengan nilai indeks kepoliserakan (PDI) 0.298 ± 0.024. Nilai potensi zeta PEG-PLGA nanopartikel ialah -39.1 ± 1.05 mV. Di samping itu, diameter PEG-PLGA nanopartikel yang dikapsulkan dengan geraniin ialah 134.2 ± 1.45 nm dengan nilai PDI 0.241 ± 0.01. Nilai potensi zeta nanopartikel ini ialah - 40.8 mV ± 0.90 mV. Kajian pelepasan drug *in vitro* menunjukkan bahawa nanopartikel berkapsul geraniin dilepaskan dalam masa 3 jam. Rawatan dengan 1, 5, 10, 25 dan 50 ug/mL nanopartikel kosong dan nanopartikel muatan geraniin menunjukkan tiadanya kesan yang ketara ke atas kemandirian sel. Lebih daripada 50% sel masih hidup selepas rawatan dengan kepekatan tertinggi nanopartikel kosong dan nanopartikel muatan geraniin. Imej konfokal diperoleh untuk mengkaji kesan pengambilan selular ke atas permukaan mikrobentuk. Pengambilan selular yang lebih tinggi, dengan nilai kolokalisasi pada 0.123 ± 0.006 direkodkan dalam busur silang (corak asimetri) manakala aktiviti pengambilan selular yang lebih rendah dengan nilai kolokalisasi 0.07 ± 0.001, 0.102 ± 0.008, 0.119 ± 0.01 masing-masing diperhatikan dalam cakera, corak simetri I dan Y. Kesimpulannya, nanopartikel PLGA-PEG yang dihasilkan adalah sesuai secara fiziko-kimia untuk mengkaji pengambilan selular CRL 1790 berhubung dengan pelekatan sel dalam ruang

terkurung. Demikian juga, PEG-PLGA yang dikapsulkan dengan geraniin menunjukkan ciri-ciri fiziko-kimia kognat tanpa kesan ketoksikan yang ketara ke atas kemandirian sel CRL 1790.

SYNTHESIS AND CHARACTERISATION OF

PEG-PLGA NANOPARTICLES FOR GERANIIN ISOLATED FROM

***Phyllanthus watsonii* (DUKUNG ANAK) EXTRACT**

ABSTRACT

A growing number of drug delivery nowadays utilises nanomedicine technology. The efficiency of drug delivery to the target site in the body depends on the physico-chemical factors of the drug carrier. *Phyllanthus watsonii*, which is locally known as dukung anak, is found in tropical forests in Malaysia. Extracts from the plants have anti-hypertensive, anti-viral and anti-diabetes properties. Geraniin, as the major active compound from *P.watsonii* extract was utilized in the study which is given by Dr. Indu S Jaganath from Malaysia Agricultural Research and Development Institute (MARDI). However, the bioavailability of the phytochemicals during drug delivery are often hampered from the degradation of bare phytochemicals by the pH and enzymatic actions in the gastrointestinal tract before transcending into the circulatory system. Thus, this research aims to 1. Synthesize Poly(ethyleneglycol)-poly(lactic-co-glycolicacid) (PLGA-PEG) based nanoparticles encapsulated with geraniin which are less than 200 nm in diameter using double emulsion method, 2. Characterize the physicochemical properties and morphology of PEG-PLGA nanoparticles, 3. Measure the amount of geraniin encapsulated in the PEG-PLGA nanoparticles, 4. Identify the *in vitro* stability and cytotoxicity of PEG-PLGA nanoparticles and PEG-PLGA nanoparticles encapsulated with geraniin on normal colon human epithelial cell line, 5. Determine the effect of cellular organization on cellular uptake. Polymeric nanoparticles (PEG-PLGA) were synthesized using double emulsion method. *In vitro* drug release test was employed

to determine the release of active compound, geraniin from the nanoparticles in a controlled environment. CRL1790 (also known as, CCD 841 CoN) which is normal colon human epithelial cell line was used as the cell model. Cell viability test was done to determine the optimal concentration of PEG-PLGA nanoparticles affecting cell viability. Observation on effect of cellular organization on cellular uptake was done to study cell characteristics on micropattern surfaces and endocytosis pathway preferred upon induction of the nanoparticles. PEG-PLGA nanoparticles (NP) had diameter of 102.7 ± 12.36 nm with a polydispersity index (PDI) value of 0.298 ± 0.024. The zeta potential value of the PEG-PLGA nanoparticles was -39.1± 1.05 mV. In addition, the diameter of geraniin encapsulated nanoparticles were 134.2 ± 1.45 nm with PDI value of 0.241 ± 0.01. The zeta potential value of these nanoparticles is - 40.8 mV ± 0.90 mV. *In vitro* drug release study shows that, the geraniin encapsulated nanoparticles were released within 3 hours of time. Treatment with 1, 5, 10, 25 and 50 ug/ml blank nanoparticles and nanoparticles encapsulated with geraniin showed less detrimental effect on cell viability. More than 50 % of cells were still viable following treatment with the highest concentration of both blank and nanoparticles encapsulated with geraniin. It was assumed that this PEG-PLGA nanoparticles and PEG-PLGA nanoparticles encapsulated with geraniin are at functional dosage up to 10 ug/mL. Confocal images were obtained to examine the effect of cellular organization on cellular uptake on micropattern surfaces. Higher cellular uptake, with a colocalisation value of 0.123 ± 0.006 was recorded in the crossbow (asymmetrical pattern) whereas lower cellular uptake activity with the colocalisation values of 0.07 ± 0.001, 0.102 ± 0.008, 0.119 ± 0.01 was observed in the disk, I and Y symmetrical patterns, respectively. In conclusion, PLGA-PEG nanoparticles produced were physico-chemically suitable

for studying CRL 1790 cellular uptake in relation to the cell adhesion within confined spaces. Likewise, geraniin containing PEG-PLGA nanoparticles show cognate physico-chemical properties without remarkable toxicity effects on the viability of CRL 1790 cells.

CHAPTER 1

INTRODUCTION

Nanotechnology is defined as the technology which involved in the synthesis and characterization of materials on nanometer scale. Application of nanotechnology in treating and diagnosing a disease is defined as nanomedicine (Pautler and Brenner, 2010). Implementation of nanotechnology in the medical field has offered numerous possibilities to enhance the efficacy of drug substances for a better treatment of the diseases. Active ingredients from plant sources have been widely used. However, the effectiveness of the drugs is yet to be understood.

Tropical countries like Malaysia have abundant supplies of local plants that have lots of medicinal values. One type of local plants that have constituents that are ideal for anti-diabetic, anti-viral and anti-cancer properties is *Phyllanthus* species (locally known as dukung anak) (Tang et al., 2010). It is ideal to embark in nanotechnology to improve our nutraceutical industry by using this phytochemicals as a model for the benefit of community healthcare. The drug substances can be sent to the target site with the help of nanoparticles. Moreover, it is important to have a general understanding on the biological fate of the nanomaterials after its administration in the body. Nanomaterials have to encounter biological barriers before reaching the target site. Thus, it is better to study the endocytosis mechanism of the nanomaterials for an efficient therapeutic application.

Problem Statement : Despite numerous discoveries of neutraceuticals as alternative medicine, a common problem that curtails the efficacy of phytochemicals is its poor

bioavailability in the biological system. Bare phytochemicals are easily degraded by the pH and enzymatic actions before transcend into the circulatory system. Physicochemical properties of nanoparticles such as size, surface charge and shapes influence the ability of particles to overcome the biological barriers. Therefore, it is interesting to study how does the properties of nanoparticles appear to influence the cellular uptake. Nanoparticles which are more than 200 nm are rapidly cleared from the body (Alexis et al., 2008a). The problem lies in the complex behaviour of cellular, enzymatic and filtering activities in the gastrointestinal (GI) system. How do the properties of nanoparticles affecting cellular uptake activities still remain poorly understood. Hence, size controlled PEG-PLGA nanoparticles need to be synthesized and I am interested to know about the interaction of nanoparticles with gastrointestinal tract cells. Thus, I am going to study on how does the structure of the cell organization actually can influence the cellular uptake pathway. Normal colon human epithelial cells were used as the model of gastrointestinal tract cells.

Aim : The aim of this project is to develop PEG-PLGA nanoparticles and to study how do the properties of nanoparticles affect the cell model of intestinal tract biochemically and microscopically. Additionally, geraniin (from *Phyllanthus watsonii*) was used as a drug model and encapsulated into the nanoparticle. Phyllanthus species plants have exerted anti-cancer properties and also well known for their pharmacological properties in traditional and modern medicines (Eldeen et al., 2011). Geraniin as the major bioactive compound in *Phyllathus watsonii* plant has the ability to function as an anti-oxidant (Palanisamy et al., 2011). Thus, encapsulation of bioactive compounds in the nanoparticles might improve the bioavailability.

The objectives of this study as listed below,

1) To synthesize the PEG-PLGA nanoparticles which are less than 200 nm in size using double emulsion method.

   Poly(lactic-co-glycolic acid)-polyethylene glycol (PEG-PLGA) polymer is chosen since it is an approved polymer by the United States Food and Drug Administration. (Makadia and Siegel, 2011). Double emulsion technique will be utilized for producing nanoparticles down to 200 nm in diameter to encapsulate the phytochemicals.

2) To characterize the physicochemical properties and morphology of PEG-PLGA nanoparticles.

   Characterization of nanoparticles such as particle size, charge and morphology will be studied.

3) To measure the amount of geraniin encapsulated in PEG-PLGA nanoparticles.

   High performance liquid chromatography (HPLC) analysis was done to determine the amount of extracts encapsulated in the nanoparticles.

4) To identify the *In vitro* stability and cytotoxicity of PEG-PLGA nanoparticles and PEG-PLGA nanoparticles encapsulated with geraniin on normal colon human epithelial cell.

   An *In vitro* drug release test will be employed to show the release rate of geraniin from the nanoparticles into phosphate buffered saline. Moreover, cell viability test will be done using normal colon epithelial cell line to determine the optimal level of the safety nanoparticles.

5) To determine the effect of cellular organization on the cellular uptake of nanoparticles

Micropatterned surfaces (CYTOOchip) will be used to study the effect of cellular organization on the cellular uptake using confocal microscope. It is an immunofluorescence analysis. This chip is arrayed with different standard micropattern designs. Flourescent-tagged antibodies will be used to study the localization of proteins involved in the cellular uptake in the epithelial cells. Confocal microscopy study on the nanoparticles *in vitro* will open up our insight into how cells reorganize their cellular membrane and cytoskeleton when in contact with nanoscale particles and adhesive cues (the micropatterns).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to nanomedicine

Nanotechnology plays a vital role in research fields nowadays and most importantly in medical and pharmaceutical fields. Nanomedicine is a combination of nanotechnology and medical which its applications offers lots possibilities in healthcare such as in drug delivery, in vivo imaging, tissue regeneration and biosensors. For an example, in drug delivery, nanoscale carriers are being used to enhance the therapeutic efficacy of the drugs. However, for in vivo imaging magnetic nanoparticles, quantum dots are used to track the progression of disease and it is also helpful in detecting early stage of disease (Shi et al., 2010).

Furthermore, modification of nanoscale carriers with ligands or nanostructured scaffolds act as promising strategy to achieve targeted drug delivery and to improve treatment efficacy. Bisphosphanates (BP) has been widely used for treatment of bone disease by encapsulating into nanoparticles. In addition to this, nanostructured scaffolds promotes bone regeneration (Gu et al., 2013). Nanomedicine has the potential to solve the health issues in terms of fighting chronic diseases. For an example, traditional treatment such as chemotheraphy is not able to differentiate the cancerous and healthy cells. So, it affects both of the cells. Many research efforts were taken to produce nano-enabled drugs for an efficient cancer treatment with fewer side effects (Ferrari, 2005).

The advancement of nanotechnology could give a significant impact in medical and pharmaceutical field. However, researchers also have a keen eye on the growing concern among the public regarding the potential health benefits and effects associated with the nanomaterials. For this, researches need to be conducted continuously and challenges are also need to be addressed before implementing this nanomedicine in daily clinical practice. Eventhough, the nanomaterials has its own unique features which makes them as an attractive candidate in drug delivery, considerable efforts have to be taken to do deeper research about the safety implications of exposure to nanomaterials. There are certain factors such as biological fate, half life and circulation time of the nanoparticles need to be addressed for an effective and safer treatment outcome. So that, applications of nanotechnology could pave the way to turn these advanced drug delivery systems into clinical effectiveness in the future (Pautler and Brenner, 2010).

2.2 Types of nano drug delivery carriers

There are myriad of drug delivery carriers which give great potential in the field of drug delivery. They are in the forefront of progress in drug delivery system. Examples of drug delivery carriers are liposomes, quantum dots, dendrimers, solid lipid particles, and polymeric nanoparticles. Liposomes are colloidal carriers which are formed when phospholipids are dispersed in an aqueous media. They forms like a vesicular structure encloses an aqueous environment bounded by bilayer of lipids. Liposomes can be used to encapsulate both hydrophilic and lipophilic drugs since it has phospholipid bilayers and also aqueous compartment within. Encapsulation efficiency of hydrophilic drug into liposomes can be low due to the drug leakage in the external aqueous media. Lipid membrane composition, phospholipid chain length,

particle size, charge and liposomes preparation methods have effect on the encapsulation efficiency (Eloy et al., 2014). Advantages of using liposomes are, the carriers are biocompatible, biodegradable, versatile and it can achieve targeted drug delivery. However, the stability of the liposomes had been the bottleneck in the drug delivery system. Later on, this stability and drug loading problems were solved with different formulations of liposomes such as inclusion of cholesterol in the liposome bilayer, developing cholesterol free peglyated liposomes. PEG has stealth properties where it can extend the circulation time of the drug delivery carriers by repelling opsonin proteins from attaching on the layer of carriers (Chaudhury et al., 2012).

Quantum dots, which are synthesized of semiconductor materials, are useful for fluorescent based medical diagnostic and for conjugation with targeted biomolecules. Quantum dots have notable characteristics compared with fluorescent proteins such as resistance against photobleaching, size tunable light emission and improved signal brightness (Qi and Gao, 2008). Because of the aforementioned optical properties, application of quantum dots is helpful in tracking the nanocarriers at cellular level by maintaining the overall characteristics of the nanocarriers (Probst et al., 2013). However, quantum dots application in healthcare filed are limited in terms of reproducibility and the toxicity issues of the materials (Azzazy et al., 2007).

Dendrimers, are spherical, macromolecular compounds and highly branched macromolecules with a size range from 1 nm to 100 nm. Configuration of dendrimers consist of three domains such as internal core, dendrons and surface active groups. They are able to carry multiple active and different target biomolecules on the branches and embedding the hydrophobic drugs within the

dendrimer scaffold. Application of dendrimers such as polyamidoamine (PAMAM) in cancer theraphy has been beneficial compared to the conventional anticancer agents which carry lots of drawback such as poor membrane permeability, low aqueous stability and rapid clearance rate of the drugs (Jia et al., 2011). On the downside, the branches of the dendrimer affects the rate of the drug release (Bao et al., 2013). In addition to that, the properties of dendrimer such as size, charge and the core material influences the cytotoxicity of the dendrimers.

Solid lipid particles are alternative carriers for liposomes. Solid particles are made up of triglycerides and their size range from 50 nm to 1000 nm. These colloidal carriers consist of hydrophobic core with a monolayer of phospholipid coating. Solid lipid particles were used in topical medication such as in acne treatment. Toxicity level of the solid lipid particles depends on the concentration of the particles (Severino et al., 2014). On the other hand, one of the disadvantage of using this solid lipid particles is it has low capacity of loading the water soluble drugs (Shah et al., 2012).

2.2.1 Polymeric nanoparticles

Nanoparticles are the particles with a solid structure usually made up of synthethic and natural polymers with a size of preferably smaller than 500 nm (Rizvi and Saleh, 2018). Nanoparticles have been widely explored and extensively studied in medical field for theraupeutic drug delivery, medical diagnostics such as detecting the disease at early stage with the help of molecular probes to visualize the biological processes at molecular level. Unlike the liposomes, polymeric micelles are synthethic amphipiles that self assembled in single layer to coat the drug content (Xu et al., 2013). Moreover, nanoparticles were also exploited for imaging diseased cells. For

an example, fluorescent silica nanoparticles, magnetic iron oxide nanoparticles have garnered the interest in biomedical imaging such as in cancer imaging (Laurent et al., 2010).

In general, polymeric nanoparticles ideally and structurally need to fit the requirement for having optimal biodegradability, biocompatibility, for providing better pharmacokinetics profile, less toxic properties to promote them as the potential carrier to encapsulate theraupetic agents. The diameter of the nanoparticles can be down sized when using the polymeric micelles. In this regard, the size of these polymeric nanoparticles have marked the effect on the biological fate of nanoparticles in human body. Nanoparticles can be easily accumulated in tumor tissues of their ability in crossing the biological barrier compared to microparticles and also other drug delivery carriers. In pharmaceutical industry, delivering drugs to the specific site of the diseases tissues been one of the major drawback. Drug can be delivered via passive and active targeting. Passive targeting is where the circulating nanoparticles in blood accumulate at tumor tissues because of the leaky tumor vasculature. Active targeting can be achieved by surface modification of nanoparticles with antibody, ligands, and aptamers. It can promotes the site specific targeting.

Surface modification of nanoparticles is required to suit different applications in nanotechnology field. For an example, in drug delivery, nanoparticles have to escape from the mononuclear phagocytic system (MPS) to reach the target site. MPS system consist of phagocytic cells such as macrophages, neutrophils and monocytes. This is one of the body's defence system. These phagocytic cells will ingest any foreign

substances which are entering the body. Opsonins are protein components which are always present in the blood circulation. They will go and bind on the viruses, bacteria, any foreign substances and induce the phagocytosis process. Opsonin proteins tend to adsorb more on the surface of the hydrophobic particles. Opsonization process must be blocked in order to increase the blood circulation half life of the nanoparticles. Thus, it can reach the target site easily. For this, surface properties of the nanoparticles plays an important role in the opsonisation process. So, it is better for the nanoparticle to have hydrophilic layer and it creates stealth properties for the nanoparticles. Coating of hydrophilic layer on the nanoparticles will repel the opsonin proteins from attaching on the particles via steric repulsion force.

Many polymers such as polyethylene glycol (PEG), poloxamer, poloxamine, dextran and polyvinyl polymers, polysaccharides, polyacrylamide, were tried in previous studies to confer the stealth properties for the nanoparticles. However, polyethylene glycol (PEG) is the polymeric material which has been widely used to impart the stealth characteristics for the nanoparticles. They are highly hydrophilic and can block the hydrophobic and electrostatic interaction which helps the opsonins to bind to the particle surfaces. Moreover, PEG is neutrally charged. Polymeric nanoparticles without any surface modification are poorly soluble in water and tend to aggregate. To prevent this issue, nanoparticles surfaces usually coated with PEG, which is hydrophilic and also biocompatible (Chen et al., 2010). Furthermore, when the nanoparticles provide sustained drug release within the target site, it actually enhances the bioavailability of the drug. Moreover, when the paclitaxel loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles were introduced in Hela cells to

test on the antiproliferative effects, it shows an enhanced apoptosis of the cell. It was suggested that this is possible because of the sustained release of drugs in the cancer cells (Parveen et al., 2012). However, for non-biodegradable nanoparticles, it will be either removed by renal system or sequestered in MPS organs such as liver and spleen. It is depends on the molecular weight and relative size of the particles (Owens and Peppas, 2006). There are lot of synthethic polymers were studied for nanomedicine application. PEG and PLGA were mostly studied polymer due to its biodegradability and biocompatibility properties. Variety of drug formulations have been made using the PEG-PLGA block copolymer. PEG-PLGA block copolymer becoming prominent for nanoparticle formulation since it can be easily synthesized, its ability of self assembly and the well known stealth behaviour of PEG (Paka and Ramassamy, 2017) (Rafiei and Haddadi, 2017). The stealth property of PEG reduces the adhesion of opsonin proteins on the nanoparticles. The hydrophilicity of PEG prevents aggregation of nanoparticles by forming hydrogen bonds with the solvent and it increases the steric distance between nanoparticles (Stiufiuc et al., 2013). Furthermore, PEG-PLGA nanoparticles can degraded into non-toxic substances and eliminated from the body. Figure 2.1 below shows the structure of PEG-PLGA polymer.

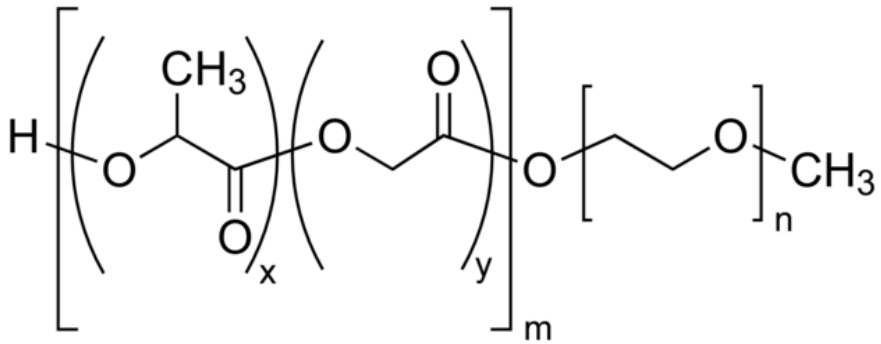

Figure 2.1: Structure of PEG-PLGA polymer, *Adapted from* Sigma Aldrich

The bonds present in this PEG-PLGA polymer are carboxylic acid, ether, ester, methyl and carbonyl groups. Combination of polar and non-polar bonds makes the structure amphiphilic in nature.

Oral delivery is a favourable option and less invasive than other means for medicinal delivery into the body. The drugs via oral route will go to stomach, intestinal lumen, and finally will reach the intestinal epithelium. Oral route is easy for administration without the need to swallow. Oral route improves patient compliance. In addition to that, production cost for oral dosage forms tend to be cheaper compared to other type of dosage forms. The oral administration of chemotheraphy is shown to have less side effects due to favourable pharmacokinetics (Pfeiffer et al., 2006). Larger surface area of gastrointestinal (GI) tract can enhance the nanoparticle uptake (Gamboa and Leong, 2013). Drugs can be formulated in a way as to protect them from digestive enzymes.

2.3 Encapsulation of plant extract into nanoparticles

2.3.1 *Phyllanthus watsonii*

Tropical countries like Malaysia have abundance of local plants that are not well-described, in terms of the phytochemical constituents and their potential use in healthcare. The source of phytochemicals in Malaysia is limitless- due to our rich rainforest and tropical fruits we have. Major classes of bioactive compounds such as alkaloids, flavonoids, lignans, phenols and tannin have been identified from *Phyllathus* species (Bagalkotkar et al., 2007). One type of local plants that have been shown *via* metabolomics study to have constituents that are ideal for anti-diabetic, anti-viral and anti-cancer properties is *Phyllanthus* species (locally known as dukung

anak) (Sarin et al., 2014). The genus *Phyllanthus* has approximately 1000 species distributed worldwide. Lots of it have been used for the treatment of a variety of ailments (Ramasamy et al., 2013). The bioactive compounds from *Phyllathus* species could slow down the growth of various cancers cells like lung, liver, breast and colon cancer (Sarin et al., 2014). *Phyllanthus watsonii*, especially, have shown interesting finding in anti-cancer properties above the other *Phyllanthus* species tested (Tang et al., 2010). The phytochemicals that have been screened to contribute in the observation are geraniin; corilagin; rutin; 1,6-digalloylglucopyronoside; trigalloylglucopyronoside; tocopherol and β-sitosterols (Jia et al., 2013), (Jasuja et al., 2012), (Toda et al., 2001), (Pazdro and Burgess, 2010) and (Loizou et al., 2010). These phytochemicals have global action on homeostasis by regulating carbohydrate and lipid metabolism, and oxidative stress (Yadav et al., 2011). Geraniin is a bioactive compound which has been isolated from *Phyllathus watsonii*. Geraniin belongs to hydrolysable tannin group and it has multiple pharmaceutical properties. Geraniin has been shown to have antioxidant, antiviral and hepatoprotective property (Sarin et al., 2014) . Geraniin decreases carbohydrate metabolism via inhibiting α-glucosidase, α-amylase, aldol reductase and production of advanced glycation end product (Palanisamy et al., 2011). These bioactive properties were the reason for choosing this active compound, geraniin to be encapsulated in the polymeric nanoparticle. Figure 2.2 belows shows the structure of geraniin. The multiple presence of hydroxyl groups make the geraniin to be hydrophilic in nature.

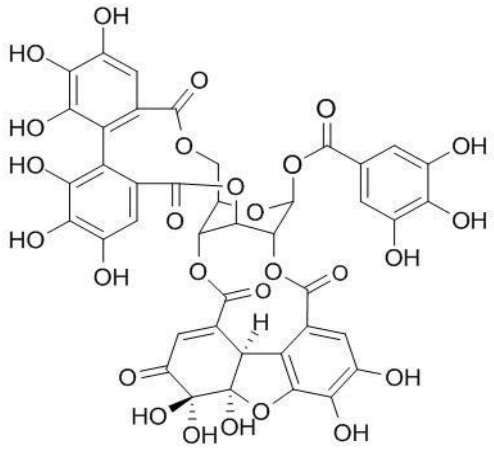

Figure 2.2 Structure of geraniin, *Adapted from* Chemfaces

2.4 Encapsulation techniques of nanoparticles

2.4.1 Double emulsion method

Nanotechnology is being applied in various fields such as agriculture, electronics, textiles and most prominently in medical field. There are several feasible techniques for the preparation of nanoparticles in the field of drug delivery. It is either by polymerization of monomer or from dispersion of pre-formed polymers. Examples of some techniques which are being used in the nanomedicine field are solvent evaporation, salting out, dialysis , nanoprecipitation and double emulsion. There are several number of methods for encapsulating hydrophilic drugs/phytochemicals into the nanoparticles.

However, one of the common method which has been developed to encapsulate hydrophilic drug is solvent evaporation method. This method involves with two emulsions. There are two types of emulsion, single emulsion: oil to water (O/W) and double emulsion: water to oil to water (W/O/W). Double emulsion method is an effective method for encapsulating hydrophilic drugs. It starts with desolubilization of polymer into an organic solvent. The hydrophilic drug will be dissolved using

water. The drug suspension will be added into the polymer solution containing organic solvent with the presence of surfactant. Then, this emulsion will be dispersed into a continuos stirring phase. As the solvent evaporates, nanoparticle suspension forms and diffuses into continuos stirring phase. Solidified nanoparticles can be collected by centrifugation after several times of washing. Finally the product is lyophilized. In single emulsion, polymer is dissolved into organic phase that is emulsified with a surfactant and hydrophobic drug are added directly to the organic phase (oil phase) . Whereas, for a hydrophilic drug it need to be dissolved in water and emulsified with polymer solution with a presence of surfactant. The high solubility of hydrophilic drug in the water will eventually leads to low encapsulation efficiency. However, studies have reported that double emulsion is the suitable method to encapsulate hydrophilic drug (Giri et al., 2013) and (Zhang et al., 2011). Furthermore, calcium phosphate has shown a positive impact in increasing the encapsulation efficiency of hydrophilic drugs (Dordelmann et al., 2014).

2.5 Physicochemical properties of nanoparticles

2.5.1 Size of the nanoparticles

Nanoparticle size has been known to be one of the key parameter of the cellular uptake pathways. It actually influences the ability of the particles to overcome the biological barriers. It has been reported in numerous studies that, particles which are more than 200 nm expelled rapidly from blood circulation. Most of the particles which are more than 200 nm would be sequestered in liver and spleen. However, particles which are lesser than 150 nm have showed an increased uptake activity (Blanco et al., 2015). Nanoparticles across the barriers depending on its size, nanoparticles which are less than 50 nm in size will be transported in a paracellular

pathway. Moreover, nanoparticles which are less than 500 nm in size will be using endocytic pathway. However, nanoparticles which are lesser than 2000 nm in size would be adsorbed by the M cells of the Peyer's patches (Florence et al., 1995).

Polymeric nanoparticles with diameter of 150 nm exhibited higher cellular uptake compared to larger size of particles such as 300 nm and 500 nm. Phagocytic cells such as macrophages favoured the uptake of larger particles. Biodistribution of nanoparticles in tumor cells was examined as well. Nanoparticles which are smaller in size resulted in a higher accumulation in tumor tissues (He et al., 2010). Nanoparticle size is playing a major role in determining the in vivo functions of the particles. In addition, effect of particle size on cellular uptake of polystyrene nanoparticles for drug delivery across the physiological drug barrier was also investigated using kidney epithelial cells and human colon carcinoma cell line. Higher cellular uptake efficiency of both the cells were observed for the 100 – 200 nm size nanoparticles compared to those with sizes of 25, 50 and 500 nm. Possible explanation for this could be, particles which are very smaller in size (< 100 nm), would be lacking in surface energy for bending in the endocytosis process. However larger particles have to be engulfed by phagocytic cells (Kulkarni and Feng, 2013).

Apart from influencing the cellular uptake efficiency, size of the nanoparticles may also induces cytotoxicity of the nanomaterials. In this regard, many studies have been done to describe the correlation of cytotoxic effect and size. However, this may vary for phagocytic and non phagocytic cells. For non phagocytic cells, smaller size of particles (< 100 nm) showed higher cytotoxicity. Moreover, they engulf particles within a size range between 100 and 200 nm. In contrast to this, for phagocytic cells, microparticles are causing damage to cells compared to nanoparticles. They ingest

particles between 2 and 3 um to a higher extent (Frohlich, 2012). Eventhough, reactive oxygen species (ROS) play a key role in cells signalling and homeostatis but overproduction of ROS leads to damage of DNA, protein and etc (Fu et al., 2014). Nanomaterials which are in extremely smaller size can easily traverse the biological barriers and it can induces the generation of ROS. This leads to consequent damage to the cells (Fu et al., 2014a).The surface area of the particles increases when the size of the particles increases. Accumulation of the particles in the body without proper excretion can cause toxicity (Blanco et al., 2015). For an instance, nanoparticles which are more than 6 nm in diameter tend to accumulate in liver and spleen since it cannot be eliminated by the kidneys. This can leads to toxicity (Longmire et al., 2008). Therefore, size of nanomaterial is an important determinant of pharmacokinetics.

2.5.2 Charge of the nanoparticles

There are two types of transport system such as passive and active transport where the nanoparticles use to enter into the cells. Mostly, nanoparticles enter the cells by endocytosis, a type of active transport. One of the parameter such as surface charge of the nanoparticles can be considered as an important determinant which affects the cellular uptake of the particles. Generally, positively charged particles are taken up better by the cells. The head groups of phospholipids of plasma membrane are negatively charged. Thus, positively charged particles can easily adhere on the membrane due to the electrostatic attraction between the positive and negative surfaces. Polymeric nanoparticles with higher positive and negative charges exhibited higher phagocytic uptake. The cellular uptake activity depends on the surface charges of the particles (He et al., 2010). Non-phagocytic cells interacts with

positively charged particles more compared to negatively charged particles. Whereas, macrophages ingest anionic particles, probably because of the ingestion of bacterias which carry negative charges. The transport routes of substances into the cells classified into four routes such as clathrin mediated endocytosis, caveolae mediated endocytosis, macropinocytosis and clathrin-independent and caveolae independent pathways. But, charges of the particles play no role in determining the endocytic uptake mechanism. Furthermore, some findings have shown that positively and negatively charged chitosan and poly (lactic acid) particles were taken up by clathrin mediated uptake pathway (Fröhlich, 2012). Moreover, correlation between cellular uptake efficiency and zeta potential of B-lactoglobulin nanoparticles was investigated in a study. It showed that cellular uptake of these anionic particles are higher when the zeta potential value is increased (Ha et al., 2015). Further study was carried out using different nanoparticle such as chitosan nanoparticle to evaluate the surface charges role to evaluate the efficiency of cellular uptake.

Positively, negatively, and neutrally charged nanoparticles were compared to see their performance on cellular uptake. It was reported that, positively charged chitosan nanoparticles exhibited higher cellular uptake among all the other nanoparticles. Eventhough, this cationic particles show pronounced effect on the cellular uptake efficiency, it was found that negatively charged particles are better in diffusing deeper into the tissues (Yue et al., 2011). Therefore, they might be a suitable carrier for delivering the drugs which needs to be present in the blood circulation for a longer time (Kim et al., 2010). Thus, charge of the nanoparticles is one of the important physicochemical characteristics of nanoparticles. Surface charge can affect the nanoparticles in terms of their function in drug delivery.

2.5.3 Shape of the nanoparticles

Shape of the nanoparticles seem to have a vital role for an effective cellular uptake. Designing a suitable shape of the nanoparticles have been an important criteria for a targeted drug delivery. Different shapes of the nanoparticles pose different rate of internalization (Salatin et al., 2015). For an example, elongated nanoparticles showed higher efficiency than spherical shaped nanoparticles. The possible reason could be because the spherical shape nanoparticles expose limited binding sites to the cell surface receptors. Whereas, the elongated shape of the nanoparticles with high aspect ratio have multivalent interaction with cell surface receptors (Dasgupta et al., 2014). Elongated shape of the particles have larger surface areas that is in contact with cells. This can leads to more effective cell adhesion.

In other study, particles were designed with varying shapes and size using particle replication in non-wetting template (PRINT). It was found that, both cylindrical and cubic shaped nanoparticles were internalized by Hela cells. However, high aspect ratio rod like particles showed four times greater uptake than low aspect ratio particles. This could be attributed to the interaction between the particles and cells. High aspect ratio particles have an enormous total surface area in contact with cell membrane (Gratton et al., 2008). There are reasons for why does the shape of the nanoparticles affect their internalization behaviour are, because different shape of the nanoparticles exhibit different surface area to volume ratios and different surface curvature. Four shapes of the nanoparticles such as cubic, rod, disk like and sphere were compared to study their effect on cellular uptake. In this review, it was reported that spherical shape of nanoparticles exhibited higher rate of internalization followed by the other shapes of the nanoparticles. Spherical shape nanoparticles exhibit fast

internalization rate in the cells compare to any other morphologies like rod, cube and disc (Hao et al., 2012). The possible explanation is, this sphere nanoparticles need to overcome least bending energy when it comes in contact with cell membrane for the membrane invagination. It is reducing the energy barrier caused by membrane bending. Thus, it can be rapidly internalized by the cells. Sphere nanoparticles are the homogenous shape that cannot suffer from complicated rotation issues (Li et al., 2015).

Moreover, in other study it was found that nanoparticle geometry and their surface orientation actually influences the mode of cellular uptake. Several endocytic inhibitors were used to study the uptake mechanism of different shaped particles. The clathrin mediated inhibitors have reduced the amount of spherical uptake of nanoparticles whereas phagocytic inhibitors have reduced the worm like nanoparticles uptake. However, many aspects need to be taken into consideration for designing a nanocarrier (Herd et al., 2013). The importance of nanoparticles morphology on cellular uptake mechanism was further proven with a comparison between sharp edged and spherical shaped nanoparticles. Nanoparticles with sharp edges tend to break the endosomal membrane easily and escape to cytoplasm. They remain in cytoplasm for long hours without being expelled out. Whereas, spherical shaped nanoparticles can reside in the endosomes until it matures to late endosomes (Chu et al., 2014). Eventhough, the shape of the nanoparticles dictate their capability for an uptake, detailed studies still need to be carried out in the future to have better understanding between the interactions of proteins on the cell membrane and the nanoparticles.

2.6 Types of microscopes in nanomedical research

Microscopy analysis is one of an important characterization analysis for imaging the nanomaterials to obtain the quantitative measures of particle, size distribution and morphology. Different microscopes are being used for various reasons in different fields to visualize different physical characteristics of the samples.

Microscopes can be classified based on their physical principle to obtain the image. It can be divided into two categories such as light or compound microscope and electron microscope. Microscopes such as simple microscope, compound microscope and stereo microscope and confocal microscopes are the examples of light microscope. Light microscopes generating images with a simple of principle of light and lens. The light source illuminates the object while the lens magnifies it. Simple microscope uses single lens for magnifying the sample (Dresser, 2001). Eventhough, the principle of compound microscope is the same as the simple microscope, but it differs by having two different optical parts such as eye piece and objective lens for magnifying the objects. This microscope can be used to view the smaller cell structure which cannot be seen at lower level of magnification. Stereo microscope provides three dimensional (3D) images since it has lens in different angles. Confocal microscope is a microscope where it is being widely used in biology field. It scans the sample in depth and layer by layer (Kulkarni and Shaw, 2016).

Some examples of the electron microscope are transmission electron microscope (tem), scanning electron microscope (sem), scanning transmission electron microscope (stem), scanning tunnelling microscope (stm) and atomic force microscope (afm). Electron microscope operates on the different principle from the

light microscope. It uses beam of electrons as the illuminating source unlike the light microscope in which light waves are used to make an image. It is mostly used to study the external surface of the sample, cross section view of the sample and ultra thin organisms. Whereas, light microscope is used to study the detailed internal structure of the sample (Ostovic et al., 2013). How does each microscope works, their own advantages and limitations and how is it being used for characterization analysis of a particle were discussed in the section below.

2.6.1 Transmission Electron Microscope (TEM)

Transmission electron microscope is used to study the biological specimens from cells to macromolecules (Thompson et al., 2016). It visualizes object using thin beam of rapidly moving electrons which interferes with that specimen sample. The electrons have much shorter wavelength (100,000 x) than the visible light. Thus, they can easily pass through the sample. The scattered electrons from the specimen will be detected by the detector. Now we can have a look at the working mechanism of the transmission electron microscope (TEM). The filament presents in the electron gun, is the one emits the electrons. The filament wire is heated by the current in order to emit the electrons. The emission of the electrons from this transmission electron microscope called as thermionic emission. Emitted electrons travel through the vacuum tube. This vacuum tube should not have any air particles, because it could interfere with the movement of electrons. Electromagnetic lens which present along the vacuum tube act as anode. Since it is positively charged, the electrons can migrate toward the direction of magnetic lens. These magnetic lens are made up of many magnetic coils. When there are more magnetic coils, more magnetic field will be generated (Thompson et al., 2016). These magnetic fields help the electrons to

move in a fast speed in the vacuum tube and hits the specimen (Thompson et al., 2016). Electrons will be scattered as it hits the specimen. The electrons which has been transmitted through the thin slice of the specimen carries the information about the structure of the specimen. However, this information is not enough to be recognized by the charged couple device (CCD). This signal will be amplified with the help of the certain type of other lenses such as projector lenses and then it will be projected to the CCD. Eventually, we can see the structure of the ultra thin specimen as it will projected on the computer screen (Thompson et al., 2016).

Application of TEM is in a wide range of fields such as nanotechnology, life sciences, biology, medical and material research. TEM images allow us to view the sample at molecular level. It can provide information on the compositional of the structure. Therefore, researchers can analyze the structure and texture of the materials clearly (Malatesta, 2016). Moreover, TEM provides information on the interaction between the nanoparticles in the cells (Malatesta, 2016). Furthermore, TEM technique is also useful to study the morphological defects in sperm. It gives information about the internal structures of the sperm (Moretti et al., 2016). TEM has been a useful technique for the analysis of cellular components such as cytoskeleton, membrane systems and cilia (Winey et al., 2014). Particle size distribution can be obtained by using TEM technique. TEM provides two dimensional images of the particles and it can be used to produce particle size distribution curve (Rice et al., 2013).

2.6.2 Field emission scanning electron microscope (FESEM)

Field emission scanning electron microscope analysis will be usually employed to study the topographic details on the surface of specimen. The electrons scans the surface of a sample whereas in TEM, the electrons are transmitted through the sample. FESEM images focus on the surface of the specimen whereas in TEM, it provide internal composition details of the structure.

This microscope uses different type of electron emission source to release the electrons from the filament. This filament is placed at high electrical potential gradient and strong electric field is used to extract the electrons from the filament presents in the emission gun. This field emission source do not heat the filament. Once the emission gun releases the electrons, the positively charged anode will attract those electrons to travel down the vacuum chamber. This anode act as electrostatic lenses which focus the beam into small initial cross over. Objective lens and condenser lens are present along the vacuum system of the FESEM machine. Condenser lens narrow down the beam of electrons which bombard the specimen. It controls the amount of demagnification. Objective lens is the final lens in the column. It actually deflects the electron beam and focus the thin beam onto the specimen. . When the electron beam hits the specimen, it interacts with the material in a way that triggers the emission of secondary electrons and backscattered electrons. Emission of backscattered electrons will be more when the atomic mass of an element is higher and the beam current is increasing. Backscattered electrons have energy higher than 50 eV. Whereas, secondary electrons have low energy. This FESEM machine is actually equipped with backscattered and secondary electrons detector. When this