Mahidol University

รายงานวิจัยฉบับสมบูรณ์

โครงการพอลิเมอร์จากเดกซ์แทรนสำหรับนำส่งไบโอแอคทีฟเปปไทด์

โดย ดร.นิสา ปฏิการมณฑล

ธันวาคม 2560

สัญญาเลขที่ TRG5880150

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ดร.นิสา ปฏิการมณฑล **มหาวิทยาลัยมหิดล**

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

Abstract

Project Code: TRG5880150

Project Title: Dextran-based polymeric particle for bioactive peptides delivery

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Nanoparticles have become one of the important materials in food, pharmaceutical and medical perspectives. For pharmaceutical application, nanoparticle can be used as a protective system for drug delivery. Along with other natural materials, Dextran (Dex) is recognized for its biodegradable, biocompatible, and low cytotoxic properties. However, dextran requires some modification to alter its hydrophobicity for nanoparticle formation. Long alkyl chain esters of vinyl laurate (VL) and vinyl decanoate (VD) were added to dextran by esterification reaction to obtain amphiphilic dextran. Both dextran with vinyl laurate (Dex-VL) and dextran with vinyl decanoate (Dex-VD) are used to form nanoparticle and study their characteristics. The nanoparticle formation was using Nanoprecipitation with Solvent evaporation, successfully formation technique for Dex-VD, and Dialysis technique for Dex-VL. The size of nanoparticle was determined using dynamic light scattering (DLS) while the morphology was observed under Transmission Electron Microscopy (TEM). The results show that Dex-VL and Dex-VD nanoparticles can be synthesized in spherical shape with size of 196.10 ± 20.41 nm and 195.76 ± 31.18 nm in diameter, respectively. The effect on cell viability of Dex-VL and Dex-VD nanoparticles were investigated in HT-29 and HCT 116, human colorectal adenocarcinoma cell lines using MTT assay. The study found that Dex-VL and Dex-VD have cytoxicity effect to both cells. The maximum dose treated without cytotoxic effect were 0.05 mg/ml in HT-29 and <0.05 mg/ml in HCT 116. The information obtained from this work are these dextran-vinyl ester group nanoparticles required further development for using as an effective drug carrier.

Keywords : dextran, nanoparticle, cytotoxicity

บทคัดย่อ

รหัสโครงการ: TRG5880150

ชื่อโครงการ: พอลิเมอร์จากเดกซ์แทรนสำหรับนำส่งไบโอแอคทีฟเปปไทด์

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อนุภาคนาโน เริ่มเข้ามามีบทบาทในการเป็นวัสดุสำหรับอาหาร ยา และการแพทย์ ซึ่งอนุภาคนาโนสามารถใช้ในด้านเภสัชกรรม โดยเฉพาะการใช้เป็นวัสดุสำหรับห่อหุ้ม และนำส่งยาได้ ทั้งนี้เดกซ์แทรนซึ่งเป็นหนึ่งในวัสดุที่พบได้ในธรรมชาติ ถือเป็นวัสดุที่มีคุณสมบัติย่อยสลายได้ในทางชีวภาพ มีความเข้ากันได้ทางชีวภาพ และมีความเป็นพิษน้อยมากเมื่อใช้กับเซลล์ อย่างไรก็ตาม เดกซ์แทรนยังต้องถูกดัดแปลงให้มีคุณสมบัติไม่ชอบน้ำเพื่อให้มีความเหมาะสมกับการรวมตัวเป็นอนุภาคนาโน ไวนิลลอเรท และไวนิลเดคาโนเอท ซึ่งเป็นกลุ่มของสารแอลคิลเอสเตอร์ที่มีสายยาว ได้ถูกเติมลงบนเดกซ์แทรนให้มีคุณสมบัติแอมฟิฟิลิกโดยใช้ปฏิกิริยาเอสเตอริฟิเคชั่น เดกซ์แทรนที่เติมด้วยไวนิลลอเรทและไวนิลเดคาโนเอทได้ถูกนำมาพัฒนาเป็นอนุภาคนาโน และทำการศึกษาคุณสมบัติเบื้องต้น การสังเคราะห์อนุภาคนาโน สำหรับเดกซ์แทนที่เติมไวนิลเดคาโนเอท ทำได้โดยใช้วิธีตกตะกอนนาโน ร่วมกับการระเหยสารละลาย และสำหรับเดกซ์แทนที่เติมไวนิลลอเรท ทำได้โดยใช้วิธีการแพร่ผ่านเยื่อเลือกผ่าน อนุภาคนาโนที่ได้ถูกนำมาวัดขนาดโดยใช้เทคนิคการวัดการกระเพื่อมของความเข้มแสง และศึกษารูปร่างของอนุภาคนาโนภายใต้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน จากการศึกษาพบว่าอนุภาคนาโนจากเดกซ์แทรนมีลักษณะเป็นทรงกลม และมีเส้นผ่านศูนย์กลางประมาณ 196.10 ± 20.41 นาโนเมตร และ 195.76 ± 31.18 นาโนเมตร สำหรับเดกซ์แทนที่เติมไวนิลลอเรท และไวนิลเดคาโนเอท ตามลำดับ นอกจากนี้ยังได้ทำการทดสอบความเป็นพิษของอนุภาคนาโนต่อเซลล์ของสัตว์เลี้ยงลูกด้วยนมสองชนิด ได้แก่ HT-29 และ HCT 116 ซึ่งเป็นเซลล์ไลน์ของมะเร็งลำไส้ใหญ่ โดยใช้วิธีทดสอบ MTT ซึ่งพบว่าอนุภาคนาโนทั้งสองชนิดที่เตรียมได้ ยังคงมีความเป็นพิษต่อเซลล์ที่ทดสอบ ซึ่งทำให้ได้ข้อสรุปว่าอนุภาคนาโนที่ได้ อาจยังต้องพัฒนาเพื่อให้มีคุณสมบัติที่เหมาะสมต่อไปสำหรับใช้เป็นวัสดุสำหรับนำส่งยา

คำสำคัญ : เดกซ์แทรน, อนุภาคนาโน, ความเป็นพิษต่อเซลล์

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Introduction

Bioactive peptides are small of polypeptides composed of 2-50 amino acids, obtained from chemical synthesis process from amino acids, or from proteolysis process from protein by enzymatic digestion or in food processing. These peptides are able to enhance the biological function in human body. These biomolecules can act as drug-like or hormone-like substance, with various type of activities such as anti-oxidative, anti-angiogenesis, anti-microbial, etc.

Consumption of bioactive peptides can be benefit for health in medical or nutritional aspects, however, direct eating of these biomolecules might not be appropriated as they can be digested by proteolytic enzyme in the body, leading to reduction of their activity. In addition, even our body can generate bioactive peptides from protein digestion in gastrointestinal tract, but generation of these biomolecules is involved with many factors including pH, type of enzymes, the precursor protein. Therefore, it cannot be ensured that the type and amount bioactive peptides are sufficient for improvement the biological function.

Nanoparticles play important roles in various applications especially in medical, pharmaceutical and food industries. Mainly use in medical application, several attempts have been made to invent nanoparticles as a drug carrier in drug delivery system to treat disease such as cancer, neurodegenerative disease and ocular disease.

Dextran is one of the natural materials that use widely to form nanoparticle (Martínez et al., 2012; Murthy, 2007). Dextran consist of glucose monomer as a subunits linked with α-1,6 glycosidic bonds for the backbone and α-1,3 glycosidic bonds for the branching. Due to the biocompatibility and biodegradability, it can be applied as intravenous solutions, parental nutrition and antithrombotic. Dextran can be synthesized by several bacterial strains including *Leuconostoc mesenteroides, Streptococcus sp., Lactobacilli sp., Weissella sp.* and also yeast such as *Candida cylindracia* (Zhang, Yang, Zhu, Zhang, & Liu, 2015). However, dextran is naturally hydrophilic, so modification to get amphiphilic in order to become self-assembly nanoparticle is required. The modification has been successfully reported by addition of ester group using lipase-catalysed transesterification.

Improvement of bioactive peptides stability is required to maintain their function in human body. Among many options, nanoparticle can be used to form a protective shield for those bioactive peptides, which could enhance activeness of those biomolecules. Therefore, this research will focus on the study of dextran-based particle to encapsulate the bioactive peptides, in order to protect them from the enzymatic degradation and maintain their activities after being orally uptake in the body.

Aim of the research

Research hypothesis: dextran-based nanoparticle can be developed and applied as a bioactive peptide carrier with low cytotoxicity.

Objectives

- to investigate the appropriate condition and methodology for preparation of dextran-based nanoparticle
- to study the property of dextran-based nanoparticle
- to observe the cytotoxicity of dextran-based nanoparticle on mammalian cell line

Literature Review

1. Bioactive peptides

Bioactive peptides are the specific peptides with biological activities that provide benefit for health. They can be defined as 'peptides with hormone- or drug-like activity that eventually regulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses' (Fitzgerald & Murray, 2006). The first bioactive peptide reported in 1950 is the casein phosphopeptide which can bind to calcium and enhance the bone formation in rachitic infants (Mellander, 1950). Later on, many bioactive peptides have been studied and identified in various sources.

1.1. Characteristics of bioactive peptides

The property of bioactive peptides depends on the amino acid sequence of the polypeptide (as shown in *Figure 1)*. Small peptide (less 5 amino acid residue) is normally water-soluble. The solubility of peptides is correlated to the amount of hydrophobic residue in the peptides. Peptides with less than 50% hydrophobic residue is assumed to be dissolved in aqueous solution. Peptides with 50-75% hydrophobic residue become partially soluble or insoluble in water. Peptides with higher than 75% hydrophobic residue is considered as insoluble peptide and can be precipitated in water.

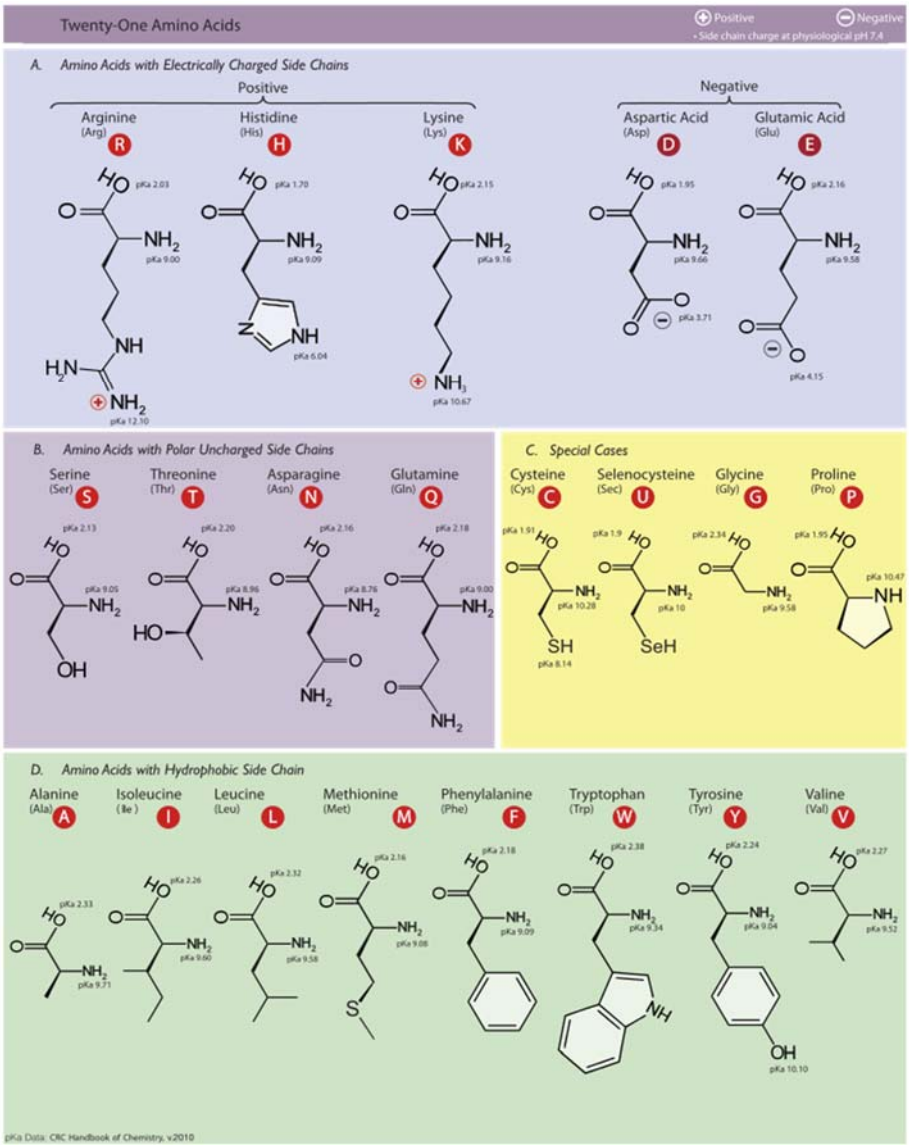

Figure 1: List of 21 amino acids (Haynes & Lide, 2010)

1.2. Types of bioactive peptides and their applications

Various properties such as anti-oxidant, anti-angiogenesis, anti-microbial, or anti-cancer, were found in these protein-derived biomolecules. Researches in this field are mostly based on the identification and characterisation of the bioactive peptides, some examples are listed in *Table 1*.

Table 1: Examples of peptides of different bioactivities from various sources. This table was adapted from (Agyei & Danquah, 2011).

Source	Identified peptide/amino acids composition	Bioactivity	References
Chicken muscle	Leu-Pro-Lys	ACE inhibitory	(Murray & FitzGerald, 2007)
α-zein (maize endosperm)	Leu-Arg-Pro	ACE inhibitory	(Murray & FitzGerald, 2007)
α-casein f(104–109) (bovine milk)	Tyr-Lys-Val-Pro-Glu-Leu	ACE inhibitory	(Murray & FitzGerald, 2007)
Shellfish (*Mytilus coruscus*)	Ala-Phe-Asn-Ile-His-Asn-Arg-Asn-Leu-Leu	Anti-cancer	(E. K. Kim et al., 2012)
American lobster (*Homarus americanus*)	Gln-Tyr-Gly-Asn-Leu-Leu-Ser- Leu-Leu-Asn-Gly-Tyr-Arg	Anti-microbial	(Battison, Summerfield, & Patrzykat, 2008)
Lysozyme (egg)	Lysozyme f(98–112) Ile-Val-Ser-Asp-Gly-Asn-Gly- Met-Asp-Ala-Trp-Val-Ala-Trp-Arg	Anti-microbial	(Exposito & Recio, 2006)
Hoki frame protein (fish)	Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn	Anti-oxidant	(S. Y. Kim, Je, & Kim, 2007)
Algae proteins (*Chlorella vulgarian* 87/1)	Uncharacterized peptides of molecular weight 2–5 kDa	Anti-thrombotic	(Humberto et al., 2009)
10–5 kDa hydrolysates of oyster	Leu, Glu, Asp, Phe, Tyr, Ile, Gly	Anti-viral	(Zeng et al., 2008)
β-lactoglobulin (bovine milk)	Ile-Ile-Ala-Glu-Lys	Hypocholesterolemic	(Hartmann & Meisel, 2007)
Soy hydrolysates	Low molecular weight cationic peptides	Immunomodulatory	(Kong, Guo, Hua, Cao, & Zhang, 2008)
Para-**K**-casein (bovine milk)	Phe-Phe-Ser-Asp-Lys f(17–21)	Immunomodulatory	(Gill, Doull, Rutherfurd, & Cross, 2000a)

Source	Identified peptide/amino acids composition	Bioactivity	References
Hemoagglutinative protein (mushroom — *Auricularia polytricha*)	Uncharacterized peptides of molecular weight 13.4 kDa	Immunomodulatory	(Sheu, Chien, Chien, Chen, & Chin, 2004)
K-casein (bovine and human milk)	**K**-casein (f106–116) Met-Ala-Ile-Pro-Pro-Lys-Lys-Asp- Gln-Asp-Lys	Immunomodulatory	(Hartmann & Meisel, 2007)
Rice albumin (rice)	Oryzatensin Gly-Tyr-Pro-Met-Tyr-Pro-Leu- Arg	Opioid antagonist and immunomodulatory	(Hartmann & Meisel, 2007)

1.3. Source of bioactive peptides

Bioactive peptides are naturally generated by enzymatic degradation of precursor proteins in the gastrointestinal tract. They can be produced in the fermentation process by enzyme or acidic hydrolysis as well. Food rich in protein is the best source for bioactive peptides, as peptide can be prepared directly by digesting with enzyme. Milk is the most common source for food-derived bioactive peptides (Meisel, 1997). In addition, bioactive peptides were isolated and identified in other sources such as cheese (Roudot-Algaron, Bars, Kerhoas, Einhorn, & Gripon, 1994; Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000; T. K. Singh, Fox, & Healy, 1997), fish muscle (Matsui et al., 1993; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011), soy bean (B. P. Singh, Vij, & Hati, 2014), seaweed (Jimenez-Escrig, Gomez-Ordonez, & Ruperez, 2011), and many more.

Microbial proteolytic enzyme has been applied to the bioactive peptide production. Lactic Acid Bacteria (LAB), the group of bacterial that has been widely used in diary fermentation process, also play an important role in bioactive peptide production. This group of bacteria provides a good source of bioactive peptides because microbial proteases provide more randomly hydrolysing activity than that of human proteases, resulting in high amount of free amino acids and bioactive peptides (Y. W. Park, 2009).

Acids have been used in acid hydrolysis to cleave protein into small peptides, according to the ability to hydrolyse specifically at aspartic acid residue (Smith, 1996). Weak acids, such as formic acid (pKa 3.75) and acetic acid (pKa 4.76) (Haynes & Lide, 2010) were used at high temperature (Gobom, Mirgorodskaya, Nordhoff, Hojrup, & Roepstorff, 1999) to enhance the reaction and shorten the period of digestion. The method was established in several applications including protein identification and sample preparation in mass spectrometry (Gobom et al., 1999).

1.4. Problems with the oral uptake of bioactive peptides

Oral administration is the most convenient route for introduction of drugs or other compounds into the body, also it provides the systematic effect for the treatment with low cost. However, this treatment method can result in the destruction of those compounds. In this case, degradation of bioactive peptide

can occur when these molecules enter to the gastrointestinal tract. As bioactive peptides are linked with the peptide bonds, enzymatic cleavage by protease together with the acidic condition in the digestive tract (as shown in *Table 2*) can deteriorate these molecules, resulting in loss of their bioactivity. Therefore, enhancement for the stability of these molecules are required.

Table 2: List of the organs involved in the gastrointestinal tract with retention time, pH conditions, and gastrointestinal juices. Information obtained from (Fallingborg et al., 1989)

Part	Time	pH	Secretion
Mouth	Mins	6.0-7.0	α-amylase, potassium and bicarbonate ions, mucus, lysozyme.
Oesophagus	3 secs		Mucous
Stomach	4 hrs	1.8-3.5	Hydrochloric acid, pepsinogen, intrinsic factor, and mucous.
Pancreas			(1) trypsin and chymotrypsin, carboxypolypeptidase, (2) pancreatic amylase, (3) pancreatic lipase, cholesterol esterase, phospholipase
Liver			Bile salts, bilirubin, cholesterol, fatty acids, lecithin, Na^+, K^+, Ca^{2+}, Cl^-, HCO_3^-
Small intestine	7.5-8.0 hrs		several peptidases
Duodenum		6.0-6.4	sucrase, maltase, iso- maltase, and lactase intestinal lipase
Jejunum and ileum		7.4	mucous
Large intestine	10 hrs		mucous with bicarbonate ions
Caecum		5.7-5.9	
Appendix			
Colon	17.2-17.5 hrs		
Rectum	Few mins	6.5-6.7	

1.5. Improvement of bioactive peptides for oral administration

Chemically modified bioactive peptides have been developed for altering the stability and bioavailability of the molecules. Substitution of unnatural amino acids and modification of the peptide bond, or 'peptidomimetics' (Liskamp, Rijkers, Kruijtzer, & Kemmink, 2011), and other modifications have been investigated to promote the peptide stability (as shown in *Figure 2*). Cyclisation, cyclic peptides synthesis, (Lambert, Mitchell, & Roberts, 2001) have been reported to decrease a degree of proteolysis.

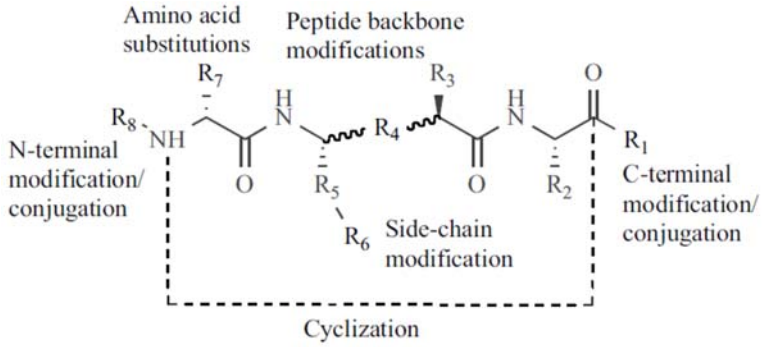

Figure 2: Examples of chemical modification of peptides (Goodwin, Simerska, & Toth, 2012). Possible modification side chain to lipids, sugars, acetyls, polymers (R1, R8); peptidomimetic (R4); methylation, acetylation, hydroxylation (R6); D-conformation (R7).

1.6. Peptides delivery

Short peptides are able to penetrate through the cell membrane with their cationic property on the molecules. Therefore, improvement of the peptide delivery system has been conducted with the use of peptides as an enhancer for the delivery system. Short peptides are attached to the surface of the carrier, resulting in increasing of delivered particles loaded with drugs or other molecules, not to delivery peptide themselves. Those studies are including peptide-linked polymer for gene carrier (Layek & Singh, 2013), for vaccine carrier (Sakuma et al., 2012); peptide-linked lipid (Asai et al., 2014; Gurnev, Yang, Melikov, Chernomordik, & Bezrukov, 2013) ; or peptide-based hydrogel (Tang, Miller, & Saiani, 2014). Not all peptides are able to enter to the cells, therefore development for delivering peptide into the cells is required. Use of polymeric particles have been conducted, for example, use of chitosan/tripolyphosphate microspheres to deliver growth factor peptides (Niu et al., 2014), anti-hepten antibody-conjugated peptide (S. Park et al., 2014).

2. Dextran

Dextran is a homopolysaccharide made up of glucose subunit which connected to each other with the α-1,6 glycosidic bonds for the backbone and the α-1,3 glycosidic bonds for the branching as shown in *Figure 3*. Its basic molecular formula is $H(C_6H_{10}O_5)_xOH$, where x stands for the number of glucose subunit. Dextran is the natural polymer that can be produced by several bacterial strains including *Leuconostoc mesenteroides, Streptococcus sp., Lactobacilli sp., Weissella sp.* and also yeast such as *Candida cylindracia* (Kaewprapan, Inprakhon, Marie, & Durand, 2012; Siddiqui, Aman, Silipo, Ul Qader, & Molinaro, 2014; Zhang et al., 2015).

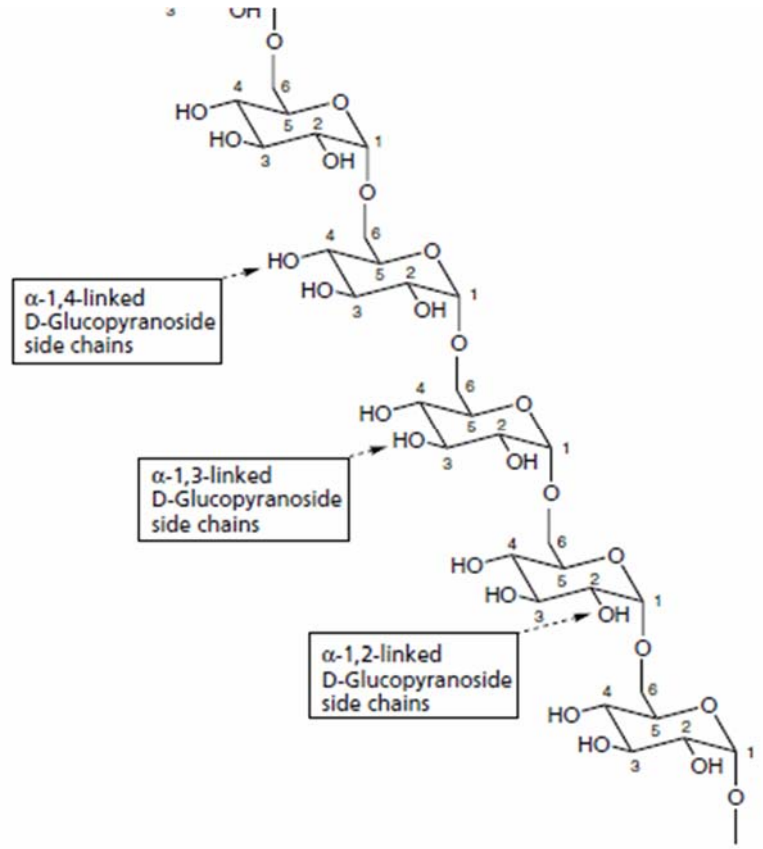

Figure 3: Dextran structure

Dextran is a polar molecule, with molecule weight ranging from 1,000 to 2,000,000 Daltons. The characteristics of dextran is depended on the molecular weight. Dextran fractions are easily soluble in water appear as clear solution. Dextran solution act as Newtonian fluids. Moreover, Solution viscosity rely on concentration, temperature and molecular weight (Vicki, 2008). It is a biodegradable and biocompatibility material. There are numerous uses in medical applications as ophthalmic solution, blood cell separation, Intravenous solutions, parental nutrition and antithrombotic. Also, dextran is applied in patients with hypovolemia. Nowadays, dextran is used in pharmaceutical as a drug delivery by coating other material for prevent digestion in human body.

According to Dextran composition, there are plenty of hydroxyl groups so this leads to inducing substitution reaction with hydrophobic groups. Modified dextran by dextran modification will have amphiphilic properties which suitable to apply in synthesis of nanoparticles used in drug delivery system, aqueous formulations (Kaewprapan, Tuchinda, Marie, Durand, & Inprakhon, 2007) and hydrogel polymer formation. The nanoparticle from amphiphilc dextran modified by vinyl decanoate has been successfully formed using nanoprecipitation method (K. Kaewprapan et al., 2012), which cannot be applied with the one modified by vinyl laurate. Therefore, the dialysis technique has to be introduced in order to find the suitable method for dextran-vinyl laurate nanoparticle formation. In addition, the effect on cell viability of both of the dextran modified with vinyl decanote and vinyl laurate have to be studied to confirm its toxicity on the cell.

3. Nanoparticles

3.1. Self-assembled polymeric carrier

Polymers can be chemically synthesised, therefore, their molecular weight, size, charge, pKa or hydrophobicity can be variated according to the user requirement. With this tuneable property, the formation of polymeric particles can be done. In order to generate the self-assembled structure allowing the entrapment or encapsulation of the small molecules inside their bodies, amphiphilic property (contain hydrophobic and hydrophilic parts) is required. Block copolymers composed of two or more different polymers connected with covalent bonds can offer this property by linking one hydrophilic polymer with another hydrophobic polymer. Two most common shapes of self-assembled polymeric particles in delivery system are 'micelle' and 'polymeric vesicle' (*Figure 4*). These polymeric particle can be used as carriers for different types of molecules, such as fluorescent probes, drugs, proteins, and oligonucleotides

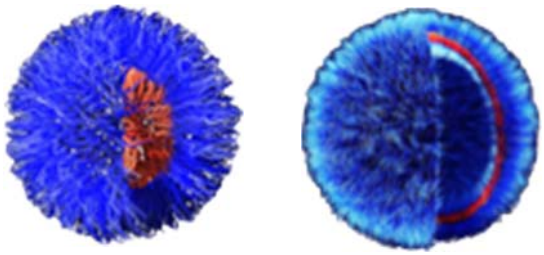

Figure 4: Two different morphologies of polymeric particles. Polymeric micelle (left) and polymeric vesicle (right), with the hydrophobic (in red) and hydrophilic (in blue) parts. (Pictures courtesy of Silvia Bianco)

An example of widely used block copolymer is the poly(ethyleneglycol)-poly (*D,L*-lactic-co-glycolic acid) (PEG-PLGA) block copolymers. PLGA is a copolymer of lactic acid and glycol acid, with biodegradable and biocompatible property that has been approved in therapeutic use by FDA. PEG is the polyethylene glycol which can be referred to as polyethylene oxide (PEO). PEG is water soluble with low cytotoxicity and reduction in immunogenicity (Abuchowski, van Es, Palczuk, & Davis, 1977). This block copolymer has been extensively used as delivery system, such as doxorubicin-conjugated micelles (Yoo & Park, 2001), Docetaxel and ^{14}C-paclitaxel-loaded nanoparticles (Cheng et al., 2007), insulin-loaded micelles (Ashjari, Khoee, Mahdavian, & Rahmatolahzadeh, 2012).

Natural polymer, dextran, have been studied as a promising carrier for drug delivery according to its biocompatibility. Chemical and enzymatic modification of dextran have been performed to change its hydrophobicity to enable its property for nanoparticles formation. Recently, the method of dextran modification has been established (Kaewprapan et al., 2007; Kaewprapan et al., 2011) and dextran-based nanoparticles have been developed and studied through lipase-catalyzed transesterification method to adding the vinyl ester group onto the dextran backbone inorder to alter the hydrophobicity of the substance (K. Kaewprapan et al., 2012).

3.2. Nanoparticle formation methods

The preparation of nanoparticle depends on the characteristic of solution by using the principle of accumulation. Before synthesizing nanoparticle, Dextran has to be modified to have amphiphilic property. When nanoparticles are forming, hydrophobic group will be in the core and hydrophilic dextran will be outside.

3.2.1. Nanoprecipitation

Nanoprecipitation is based on the displacement of semi-polar solvent to allow nanoparticle formation. First step is to dissolve polymer in polar solvent. Second step is to replace polar solvent with semi-polar solvent the facial interaction of polymer will occur. Nanoparticles are received from this facial interaction. From the previous study, nanoparticles from dextran-modified with vinyl decanoate (Dex-VD), have been successfully formed using nanoprecipitation method. Also, study about factors that affect nanoparticle formation such as degree of substitution (DS), polymer concentration and structure of dextran have been investigated. The hydrophilic surface layer in nanoparticle is related to an increase the self-generated nanoparticle formation (Kothari, Tingirikari, & Goyal, 2015).

3.2.2. Dialysis

This method is use the difference of particle size to pass through dialysis membrane bag against distilled water (as shown in *Figure 5*). Water will pass through into dialysis membrane bag that contain mixture solution. Solvent will come out from the membrane. Then chemicals can contact each other and form nanoparticle.

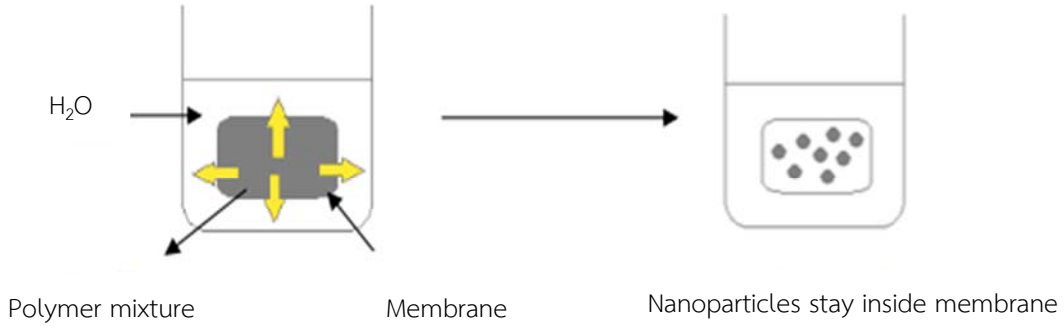

Figure 5: Dialysis nanoparticle formation method.

3.2.3. Solvent evaporation

Dissolve dextran polymer in volatile and insoluble solution then vanish the solution by evaporator (as shown in *Figure 6*). Particle will present in the solution. Make sure that the solvent boiling

point is lower than boiling point of water so the solvent will evaporate before water. When the solution is concentrated, nanoparticles will form easier.

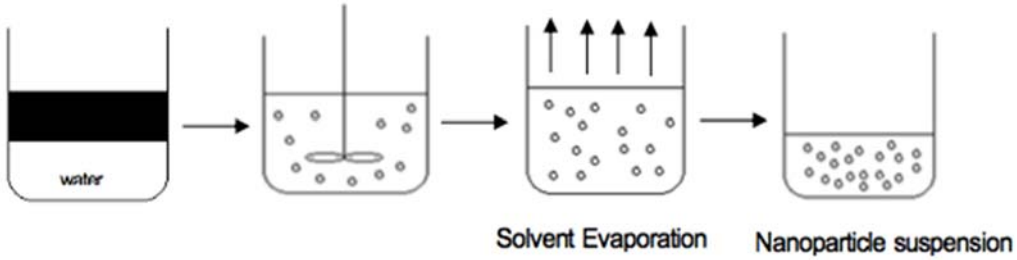

Figure 6: Solvent Evaporation method.

3.2.4. Emulsion-Solvent Evaporation Method

Preparation of a mixture by use principle of emulsion to homogenize the solution (as shown in *Figure 7*). After that stir the solution under high pressure condition and evaporate the polymer solvent. Particles will colloidal dispersion in solution. When liquid phase is vaporized, it is easier to form nanoparticles.

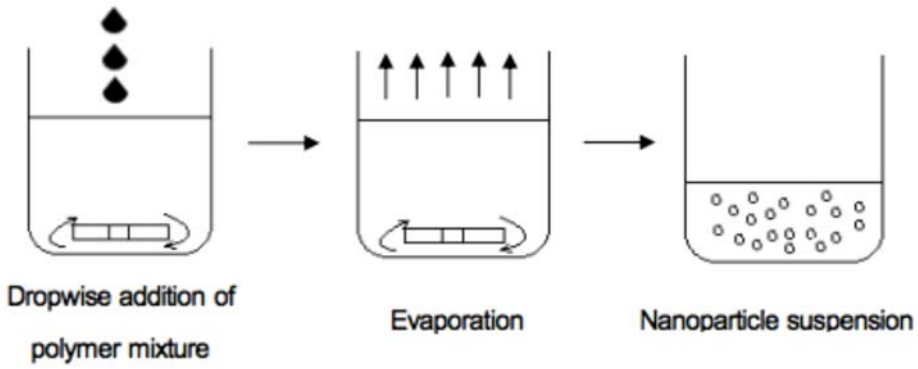

Figure 7: Emulsion-Solvent Evaporation method.

3.3. Factors involved in nanoparticle formation

3.3.1. Degree of substitution (DS)

Degree of substitution describes average amount of vinyl decanoate or vinyl laurate that are attach to the dextran backbone. Size of nanoparticle is increased at low degree of substitution because of there are less interactions between hydrophobic group leading to loose hydrophobic core whereas high degree of substitution has numerous of interaction induce compact hydrophobic core of nanoparticle formation.

3.3.2. Critical aggregation concentration (CAC)

The critical aggregation concentration indicates the minimum concentration of surfactants which micelles start to form and additional surfactants added to the system go to micelles. Pyrene is used as a

probe for detecting the micelles formation as it is highly sensitive to polarity environment. When nanoparticles are forming, pyrene will press into nanoparticle to hydrophobic part. This leads to the change of fluorescent intensity which tell the state of nanoparticle. Pyrene is one of several methods for determining the critical aggregation concentration (F. Gu, B. Z. Li, H. P. Xia, B. Adhikari, & Q. Y. Gao, 2015)

3.3.3. Modified starch concentration

Modified starch concentration influences on the size distribution of nanoparticle. Increasing of concentration causes higher viscosity in solution and the accumulation of modified starch occurs. This leads to formation of the larger size of nanoparticle.

3.3.4. Initial water content

In nanoparticle formation using dialysis method, initial water content is the important factor that affect the nanoparticle size. Adding initial water content before dialysis will slow down dialysis speed. Nanoparticles will be self-assembled with dense and compact. Moreover, the Z-average will become smaller (F. Gu et al., 2015).

3.3.5. Types of polymer

Apart from several factors mentioned previously, responsive property of the polymer is another factor involved in nanoparticle formation technique. Polymer with special properties such as thermo-, pH-responsive have been developed to enhance their ability in nanoparticle formation and release of encapsulated materials.

3.3.6. Thermo-responsive polymers

Changing of temperature leads to changing of the microstructure of the polymer. An important feature that indicate polymer behavior is the critical solution temperature. Polymeric solution at lower critical solution temperature (LCST) or transition phase will become insoluble increasing of temperature. From this information, lower critical solution temperature (LCST) of polymer can be used in drug delivery. The polymer mix with therapeutic agents at liquid state (temperature below LCST or transition temperature) be able to inject into the damaged area in human body. When temperature is increased, the polymeric system will turn into gel and releasing the drug (Hugo Almeida, 2012).

3.3.7. pH-responsive polymers

In human body, there are various pH in gastrointestinal tract, target tissue, wound and cancer cell. For intracellular uptake, molecules get into cell via endocytosis through pinocytosis or receptor-mediated endocytosis. The molecules are trapped in the endosome and moves toward to lysosome. After internalization, pH in endosome drops from 6.2 to 5.0 which cause the change of proton concentration which release molecules into cytosol (Schmaljohann, 2006).

4. Cell Viability

4.1. MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay is the colorimetric assay that used widely in academic research. This assay is used for measuring the activities of mitochondrial enzymes that can reduce the MTT-substrate to the formazan as shown in *Figure 8*. After incubation with MTT substrate for 2-4 hour, the mitochondrial dehydrogenase enzyme in viable cells will convert yellowish MTT substrate into a purple colored formazan that can be measured at absorbance 570 nm by spectrophotometer. A reference wavelength of 630 nm is used to subtract interference from cells. The quantity of formazan is directly proportional to number of viable cells that survived from research treatment condition. The MTT-assay can be used to determine the cytotoxicity effects of compounds. The advantages of MTT-assay are it is standard-operated method, rapid testing and developed as non-radioactive alternative. The others cell –based assay are Resazurin assay, MTS assay and ATP assay for example (Tim Mosmann, 1983).

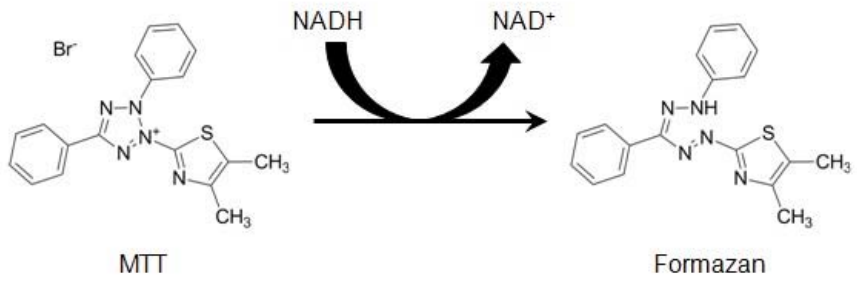

Figure 8: Structures of MTT and colored formazan product (Tim Mosmann, 1983)

4.2. Cytotoxicity of Dextran

In previous study, dextran was confirmed it has no effect on the viability of cells. HEK-293, INT-407 and HT-29 cells had been tested by dextran derived from *L. mesenteroids* treatment. The data showed even at high concentration of dextran (1000 µg/ml) there's still no significant effect on viable cells observed after 36 h (*Figure 9*). Also, the percentage between dextran-treated cells and untreated cells was similar at all concentration (50-1000 µg/ml). This results from paper demonstrated that dextran polymer alone is non-toxic, biocompatible and safe to use (Kothari et al., 2015).

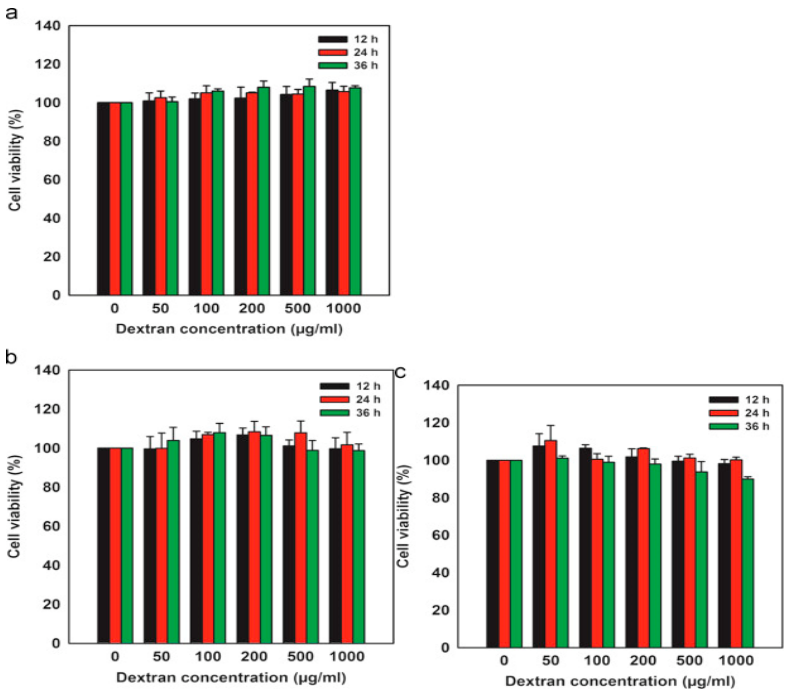

Figure 9: The effect of dextran (0–1000 µg/ml) on the viability of (a) HEK-293, (b) INT-407 and (c) HT-29 cells over a period of 12–36 h of incubation. Data are expressed as the mean±SD of three experiments (Kothari et al., 2015)

The other paper (Cees J. De Groot et al., 2001) shows dextran T-40 produced from *Leuconostoc mesenteriods* polymer at 100 mg/ml had lower cell proliferation inhibition index (%) to human fibroblast culture for 72 h compared to others polymer such as latex rubber, methacrylated dextran (dex-MA), and hydroxyethyl methacrylated dextran (dex-HEMA) as in *Figure* 10. Dextran exhibited a relative inhibition of 25± 7% which is less than growth inhibition per cell cycle (cell cycling time of 24 h). The results presented in this study demonstrated that dextran hydrogels have good biocompatibility *in vitro*.

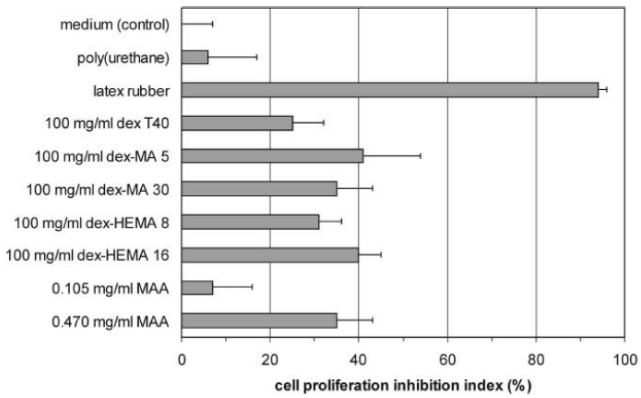

Figure 10: The cell proliferation inhibition index (mean%$SD) after 72 h for the polymers dextran, methacrylated dextran (dex-MA), and hydroxyethyl methacrylated dextran (dex-HEMA) with different DS, and for the degradation product methacrylic acid (MAA). (Cees J. De Groot et al., 2001).

Nevertheless, dextran can be used for forming oxide nanoparticles for biomedical application in site-specific drug delivery part. In this study, dextran coated ferrite nanoparticles (DFNPs) in nano-size (<25 nm) was tested the cytotoxicity potential with A545, human lung carcinoma cell lines, by MTT-assay. The results of MTT-assay show concentration dependent cytotoxicity effect of DFNPs to this cell line at 24, 36 and 72 hr of treatment. In *Figure 11* showed that cell viability was decreased under 80% at 1,000 µg/ml of treatment in 24 hr, 800 µg/ml in 48hr and 600 µg/ml in 72 hr. It can be indicated that difference of treatment duration effected to viability of cells as well as nanoparticles concentration (Reshma VG, 2016).

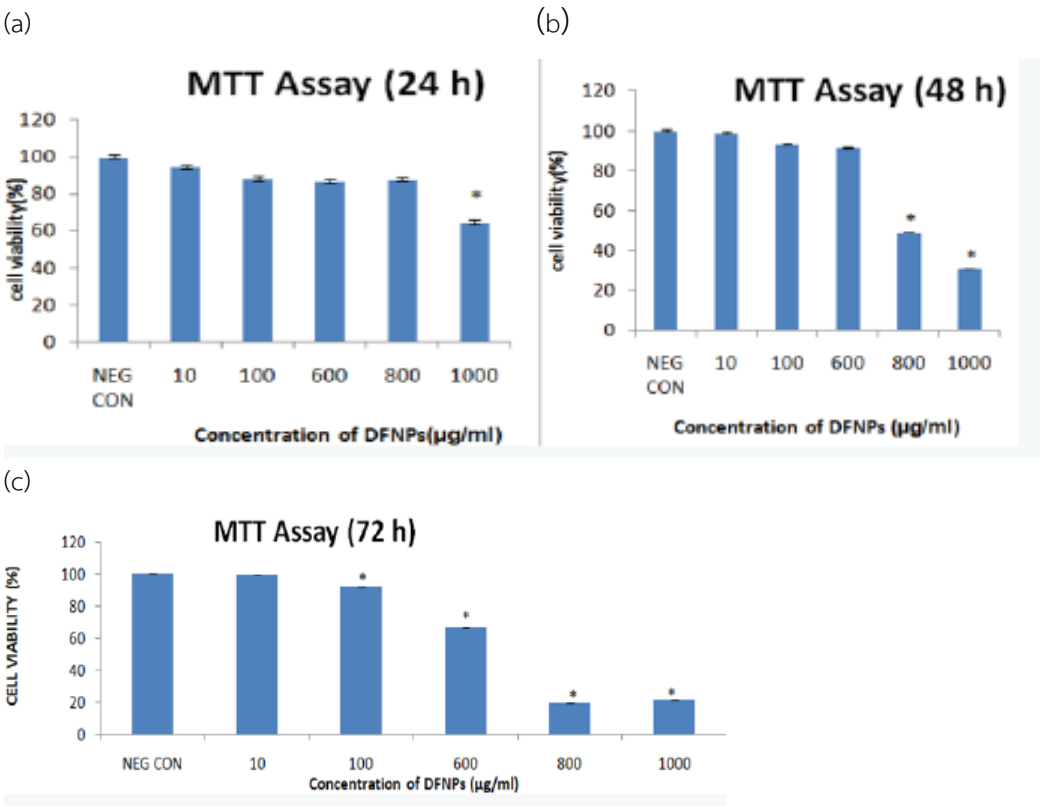

Figure 11: MTT assay showing cytotoxic effect of DFNPs on A549 cells after (a) 24, (b) 48, (c) 72hr of incubation. Cells were incubated with different concentrations of DFNPs. Measured mitochondrial activity represented as percentage with respect to control. The data expressed as mean ± SD. Asterisk above columns denotes statistically significant difference when compared to the control group (P <0.05) (Reshma VG, 2016).

Materials and methods

1. Materials

Dextran T40 with average molecular weight of 40,000 g/mol synthesized by Leuconostoc mesenteriodes was purchased from Pharmacia (Uppsala, Sweden). Tetrahydrofuran was purchased from Carlo erba Reagemt (Italy). Lipase AY (EC 3.1.1.3) from Candida rugosa was obtained from Amano Enzyme Co. (Nagoya, Japan). Vinyl decanoate (VD), vinyl laurate (VL), 18-crown-6, and dimethyl sulfoxide (DMSO, CHROMASOV®) was purchased from Sigma-Aldrich (St. Louis, US and Steinheim, Germany). The dialysis membrane with MWCO of 6-8000 was purchased from Spectrum Laboratories Inc. (CA, USA). Thiazolyl Blue tetrazolium bromide (MTT) Powder was obtained from AlfaAeser®(Yantai, China). D-MEM (High glucose) with L-glutamine and Phenol Red, Trypsin-EDTA in Hank's salts was purchased from Millipore. Fetal Bovine Serum was purchased from Gibco. Spectra/Por® membrane, with molecular weight cut-off 3500 g mol^{-1} was obtained from Spectrumlabs, US.

2. Methods

2.1. Modified dextran with lipase

Modified dextran was prepared by transesterification using lipase according to the previous method (Kaewprapan et al., 2011) The pH-adjusted lipase with additional 18-crown ether was prepared as described previously (Kaewprapan et al., 2007) to enhance the transesterification reaction in organic solvent. Briefly, the reaction was done by preparing 5 mg/ml of dextran T40 in DMSO, following by addition of vinyl ester at different ratio. The mixer was stirred at 50°c for 5 min prior to an addition of lipase enzyme. The mixer was left for 48 h, before removal of excess vinyl ester by dialysis in 85% ethanol for 2 days, with 65% ethanol for 2 days, and distilled water for 2 days. The modified dextran sample was then lyophilized using freeze dryer in order to obtain the solid substance for further experiment.

2.2. Observing the degree of substitution

^{1}H-NMR spectra of modified dextran samples were recorded with a Bruker AVANCE 400 spectrometer (400 MHz) in DMSO-d6. The signal of each samples were shown in *Table 3*. The degree of substitution was calculated based on previous study as shown in following equation.

$$\%DS = \left[\frac{\frac{I_1}{3}}{\frac{I_1}{3}+\left(\frac{I_2-I_1}{4}\right)}\right] \times 100$$

where I_1 is the signal obtained at 0.84 ppm responsible for the three protons of methyl group ($-CH_3$) on vinyl decanoate and vinyl laurate; I_2 is referred to the signal at 4.51-4.91 ppm which is obtained from the 9 protons of anomeric carbon (OH4, O-H3, O-H2, และ H1) ของ dextran.

Table 3: The postition of δ, ppm of native dextran (Dex-T40), vinyl decanoate (VD), vinyl laurate (VL), and modified dextran (Dex-VD, Dex-VL).

Position	CH=CH2	O-H4	O-H3	H1	O-H2	H6	H5	H6	H3	H2	H4	-CH2-C=O	-CH2-CH2-C=(	-CH2-, 8H	-CH3
ppm	7.64-4.52	4.91	4.86	4.66	4.51	3.73	3.61	3.49	3.41	3.20	3.15	2.29	1.25	1.23	0.84
Dex-T40	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	-	-	-
VD	✓	-	-	-	-	-	-	-	-	-	-	✓	✓	✓	✓
VL	✓	-	-	-	-	-	-	-	-	-	-	✓	✓	✓	✓
Dex-VD	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Dex-VL	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

2.3. Dextran-based nanoparticle formation

Dex-VD nanoparticle was prepared as described in previous study (K. Kaewprapan et al., 2012). Briefly, 12.5 mg of Dex-VD sample was dissolved in 2.5 ml of THF/water mixture (90/10, v/v). The sample was mixed by vortexing until it becomes homogeneous prior to the ultrasonicating for 3 minutes. The solution was added drop-wise into 5.0 ml of distilled water under vigorous magnetic stirring. Nanoparticle suspensions were obtained after evaporation at 37ºC until THF disappear.

Dex-VL nanoparticle formation was done by dialysis method according to the previous report (Hornig & Heinze, 2007). Dex-VL sample was added into 5.0 ml DMSO and stirred using magnetic stirrer until completely dissolved. The sample was transferred to a dialysis membrane with molecular weight

cut-off of 6-8000 and dialyzed against distilled water for 3 days. The water was removed and changed once a day.

2.4. Size distribution measurement by Dynamic light scattering

Size distribution (Z-average size) and polydispersity index (PDI) of Dex-VL and Dex-VD nanoparticles were determined in filtered distilled water using a dynamic light scattering particle size analyzer (Malvern Zeta Nanosizer ZS, Malvern Instruments Ltd). Samples were carried out at 25ºC with scattering angle fixed at 90º.

2.5. Morphological analysis of nanoparticle by Transmission electron microscopy

The Dex-VL nanoparticle samples were prepared on a carbon-coated copper grid and negatively stained with uranyl acetate solution (2.0 wt%) (Chiang et al., 2012). The TEM images were obtained from a Hitachi HT7700 microscope operating at an accelerating voltage of 120 kV.

2.6. HT-29 and HCT-116 Cell culture

In this study, HT-29 and HCT 116 human intestinal cell lines obtained from ATCC® (Kettering Cancer Center with American Type Culture Collection) are used for cell viability assay and drug testing. HT-29 and HCT-116 were passaged at cell density of $1x10^6$ cells in T-25 cm^2 flask and grown in DMEM medium with 4.5 g/L glucose, L-Glutamine (Sigma-Aldrich, UK) supplemented with 10% Fetal Bovine Serum (FBS), 1% (v/v) 100x penicillin-streptomycin (Millipore) and 1% (v/v) of 100 mM Na-pyruvate (Millipore) as complete culture medium. Both cell lines were incubated at 37°C with 5%CO_2. The media changing is required in the day after thawed frozen cells and every two or three days after. The subculture procedure is needed when the cell density reach 80% confluent (at a cell concentration between 8×10^4 to 5×10^5 cell/cm^2) then the cells were washed with PBS to remove the remaining culture medium before trypsinised with 0.25% Trypsin- EDTA in Hank's salts solution (Millipore) and incubated for 10 minutes. The cells were centrifuged at 500 RCF for 3 minutes then only cell pellet was kept for further step. The seed cell density was $5x10^5$ cells per well in 24 well-plates calculated by standard cell counting assay using Hemacytometer. Population doubling time of HT-29 and HCT 116 cell lines are approximately 23 h and 21 h, respectively.

2.7. Dextran-based nanoparticles treatment

Before proceeding MTT-assay, HT-29 and HCT 116 cells were seeded in 24 well-plate at a cell density 5×10^5 cells/cm^2 per well depending on their growth in experimental condition. After cell reached to about 60% (1×10^6 cells/cm^2), the cells were treated with dextran at a different concentration of 0.5, 1,

5, 10 and 50 mg/ml, dextran-based nanoparticles at a different concentration of 0.05, 0.1, 0.2, 0.5 and 1 mg/ml for 24 hr. The highest dextran-based nanoparticle treatment concentration (10 mg/ml) was prepared by concentrating the nanoparticle solution with Rotary Evaporator (Rotavaper R-210, Buchi, Switzerland). The mixture at lower concentration were prepared by dissolving with filtered distilled water. To reach final concentration, 100 µl treatment solution was mixed with 900 µl culture medium before added to cells. The control experiment is untreated cells, only normal culture medium added. The positive control is vinyl-decanoate or vinyl-laurate treatment at concentration 19.64 mg/ml and 5.792 mg/ml, which is the approximate concentrations that vinyl ester groups were found in firstly prepared nanoparticles, which death of cells were expected. Negative control is filtered distilled water treatment. Incubation time duration is set at 24 hr.

2.8. Cell viability Test by MTT-assay

In this study, cytotoxicity of nanoparticles solution on HT-29 and HCT 116 cell lines were evaluated using MTT. After treatment, the media in each well was removed and the cells were washed by PBS. Afterward, 500 µl of MTT solution (0.5 mg/ml) was added to each well and the cells were incubated for 30 minutes at 37°C and 5% CO_2. MTT solution in each well was decanted and replaced with 350 µl of acidified isopropanol to dissolve the formazan product. The 150 µl of formazan solution of each treatment was transferred into a new 96 well-plates (2 replicates). The samples were then analyzed by using a Multiskan™ GO Microplate Spectrophotometer (Thermofisher Life Science) to measure the absorbance at a wavelength of 570 nm and a reference wavelength is 630 nm to subtract interference. The quantity of formazan reflects the mitochondrial reductase activity in the cell, which assume to be directly proportional to number of viable cells that survived from experimental conditions. Cell viability (%) was calculated by following equation (T. Mosmann, 1983).

$$\text{Cell viability (\%)} = (Nt/Nc) \times 100$$

; Where Nt is absorbance of cells treated with amphiphilic dextran-based nanoparticles and Nc is absorbance of untreated cells (C. J. De Groot et al., 2001).

2.9. Statistical analysis

The statistical data analysis for this thesis was perfomed using Dunnett's test, ANOVA (the Real Statistics Resource Pack software (Release 4.3), Charles Zaiontz, www.real-statistic.com). The significant difference between control and treated cells was statistically analyzed by *t* test at 95% confidence interval ($p < 0.05$)

Results and Discussion

1. Transesterification of dextran and vinyl laurate to obtain amphiphilic dextran

Dextran T40 was modified with vinyl laurate with different ratio between dextran and vinyl laurate. Lipase catalyzed the transesterification between dextran and vinyl laurate according to proposed reaction shown in Figure 12. The modification of dextran was confirmed by ^{1}H-NMR spectroscopy, presented in Figure 2. The degree of substitution (%DS) of each condition was calculated based on the previous study (Kaewprapan et al., 2007) and presented as shown in *Table 4*. As expected, the pH-imprinted lipase with 18-crown-ether is the most effective catalyst in this experimental condition and the results were similar to the previous study(Kaewprapan et al., 2007). In addition, the degree of substitution of modified dextran was not only correlated to the amount of lipase but also depend on vinyl laurate available in the reaction. According to the data, the condition used in sample 8 and 6 provides the highest degree of substitution of about 72% and 67%, respectively. It is also interesting that in this experiment, the amount of vinyl laurate added into the reaction affect the transesterification more than that of lipase. For example, when compared with sample 5, the degree of substitution was increased for 18% in sample 6, whereas only 13% in sample 7.

Table 4: Condition used in transesterification for modification of dextran with vinyl laurate..

Sample no.	Type of lipase	lipase (mg)	DexT40 : VL[a]	%DS
1	No lipase	-	1 : 2	1.05
2	No lipase	-	1 : 4	4.77
3	pH-adjusted lipase	26	1 : 2	25.76
4	pH-adjusted lipase	26	1 : 4	30.53
5	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	26	1 : 2	49.97
6	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	26	1 : 4	67.73
7	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	52	1 : 2	63.70
8	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	52	1 : 4	71.55

[a]DexT40:VL is the ratio between weight of dextran T40 to vinyl laurate.

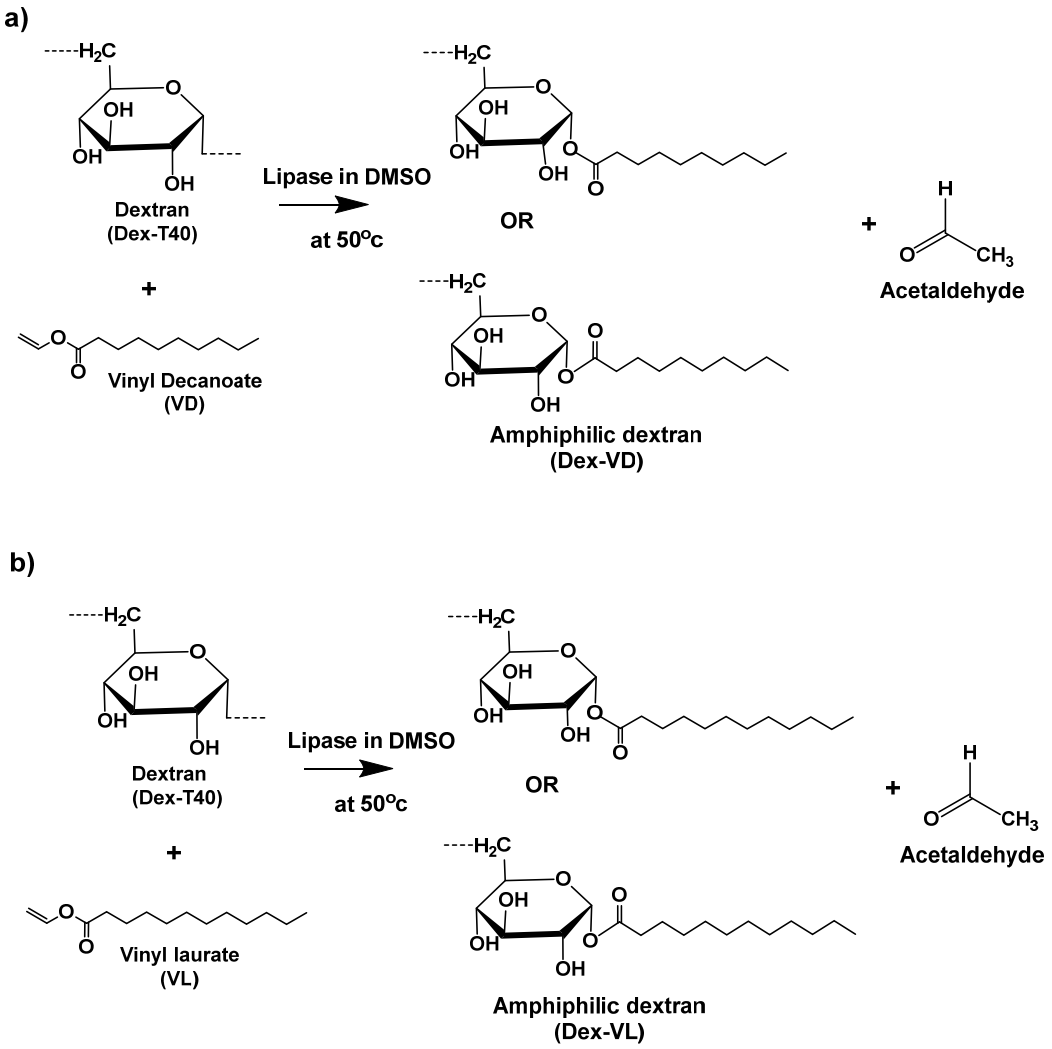

Figure 12: Proposed transesterification of Dextran T40 and vinyl decanoate (a) and vinyl laurate (b). The reaction was performed in DMSO at 50°c with lipase AY as a catalyst.

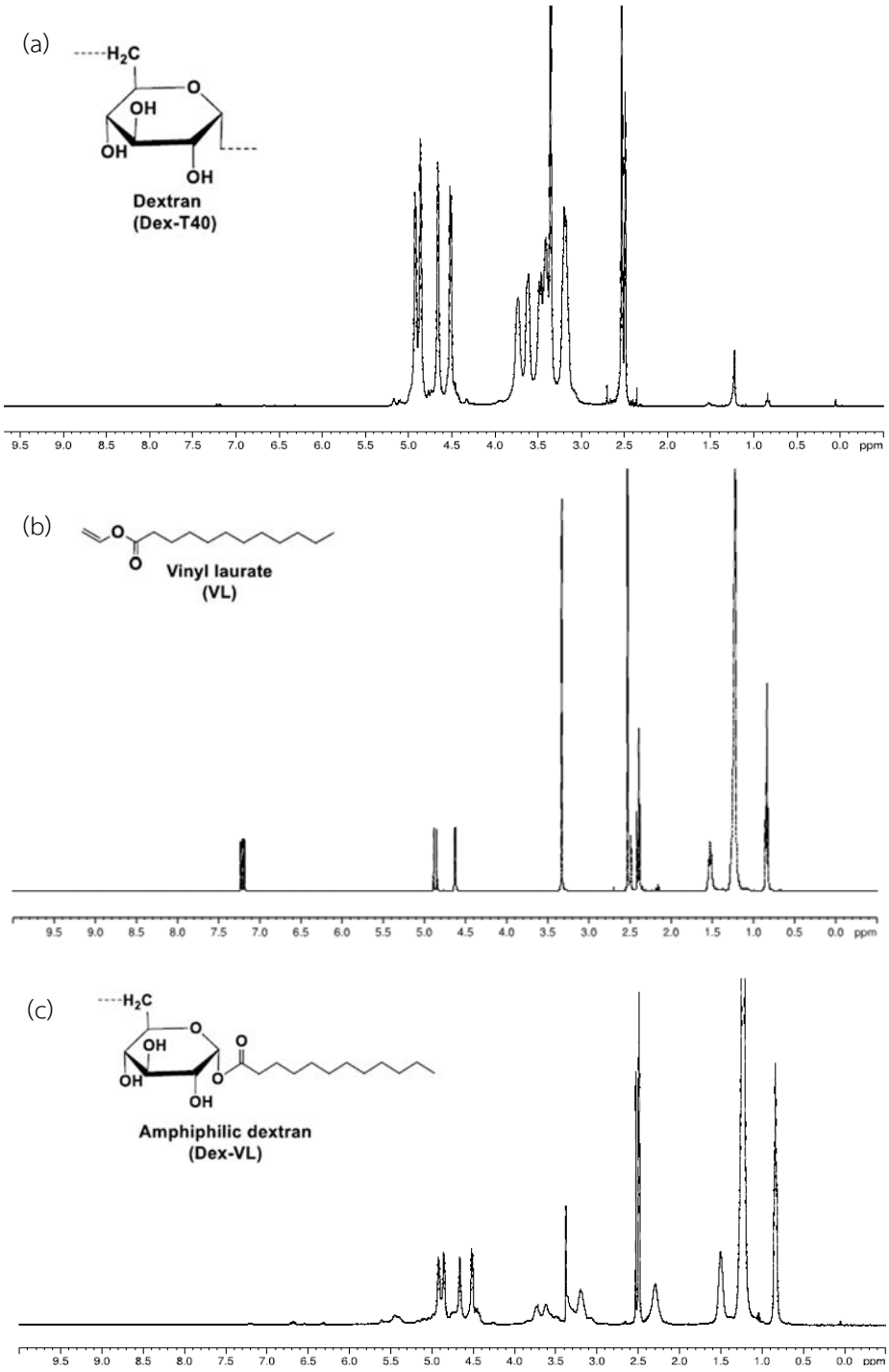

Figure 13: 1H-NMR spectra (400 MHz) in DMSO-d6 of Dextran T40 (a), Vinyl laurate (b), and Dex-VL (sample 8, see Table 1) (c).

2. Solubility of Dex-VL

The solubility of Dex-VL obtained from previous experiment was investigated for the selection of suitable solvent for particle formation using solvent evaporation technique. Several organic solvents

including ethanol, methanol, ethyl acetate, tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), and H_2O were used for testing the solubility of modified dextran. THF was selected for dextran modified with vinyl decanoate (Dex-VD) in previous study (Kaewprapan, Baros, Marie, Inprakhon, & Durand, 2012). The result shown in *Table 5* suggested that the solubility of Dex-VL is different from Dex-VD. Dex-VL is less soluble in THF and other organic solvents than Dex-VD due to the nature of its additional group of vinyl ester, vinyl laurate contain longer carbon chain resulting in less water soluble property. According to the solubility of Dex-VL, the nanoparticle formation protocol of Dex-VD cannot be applied. The nanoparticle formation using dialysis have to be developed to synthesize Dex-VL nanoparticle.

Table 5: Solubility of Dex-VD and Dex-VL in various organic solvents (at concentration of 1 mg/ml)

Solvent (polarity) / Material	THF (4.0)	Acetone (5.1)	Acetonitrile (5.8)	Ethanol (4.4)	Chloroform (4.1)	DMSO (7.2)	Water (10.2)
Dex-T40 (%DS = 0)	i	i	i	i	i	s	s
Dex-VD (%DS = 90)	s	i	i	i	i	s	i
Dex-VL (%DS = 70)	i	i	i	i	i	s	i

* i = insoluble, s = soluble

3. Nanoparticle preparation

The results were divided into 2 parts. Which are Dex-VD nanoparticle formation using nanoprecipitation method and Dex-VL nanoparticle formation using dialysis method.

3.1. Dex-VD nanoparticle formation using nanoprecipitation method

The formation of Dex-VD was performed to bulk the quantity of Dex-VD for cell viability assay. In order to obtain the Dex-VD nanoparticles with diameter ranging from 100-200 nm, the nanoparticle formation method had developed according to the previous report (K. Kaewprapan et al., 2012). The final Dex-VD nanoparticle samples were in acceptable average size of 195.76 ± 31.18 nm.

3.1.1. Effect of polymer concentration on the nanoparticle formation

As shown in *Table 6*, increasing of Dex-VD concentration from 2 mg/ml to 5 mg/ml gave size distribution of nanoparticle from 252.26 to 356.20 nm and PDI from 0.164 to 0.327. This results confirm that Dex-VD at concentration 5 mg/ml is the most suitable concentration that bring about the smallest size of nanoparticle. Actually, these results is opposite to the result in the previous study (Sharma, Madan, & Lin, 2016), which found that increase in the polymer concentration leads to enhancement of the viscosity in organic solvent. The more viscosity might block the breaking down of droplets therefore

bigger size of nanoparticle occurs. The difference of the result may come from the stirrer speed and blending of the mixture as well as the nature of the polymer.

Table 6: Effect of polymer concentration on the particle size and polydispersity index (PDI) of Dex-VD nanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	17	2 mg/ml	5	2.5	288.56	0.287
2	17	4 mg/ml	10	2.5	356.20	0.327
3	17	5 mg/ml	12.5	2.5	252.26	0.164

3.1.2. Effect of stirring speed

The different stirring speed for nanoparticle formation has been studied, as provided in *Table 7*. The results show the mean size distribution of Dex-VD nanoparticle tend to be smaller as the speed increase. *Table 7* shows the mean size distribution of Dex-VD nanoparticle 886.23, 782.6 and 219.73 at low speed, middle speed and high speed, respectively.

Table 7: Effect of stirring speed on the particle size and polydispersity index (PDI) of Dex-VD nanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	16	Low speed	6.25	1.25	886.23	0.329
2	16	Middle speed	6.25	1.25	782.60	0.402
3	16	High speed	6.25	1.25	219.73	0.148

3.1.3. Effect of ultrasonication

Ultrasonication was used in this experiment to increase the dispersion of particle in solution after vortexing. Sample was sonicated in ultrasonicator for 3 minutes. If sonicate too long, agglomeration of nanoparticles may occur (Dan Li, 2006). The results show in the *Table 8* suggest that size distribution of Dex-VD nanoparticle with ultrasonication step is smaller than that without ultrasonication. This ultrasonication step helps breaking up aggregation particle suspended in liquid as described elsewhere (Gedanken, 2004). In addition, there should be study more about optimal time period that disperse particles properly in solution.

Table 8: Effect of ultrasonication on the particle size and polydispersity index (PDI) of Dex-VD nanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	20	Without sonicate	6.25	1.25	423.60	0.440
2	20	Sonicate 3 min	6.25	1.25	374.63	0.326

3.1.4. Effect of the mixture volume

As listed in *Table* 9, this study found that Dex-VD and THF at ratio 6.25:1.25 gave the mean size 238.67 and 258 nm respectively while Dex-VD and THF at ratio at 12.5:2.5 gave Z-average size of 300.73 nm. The larger size occurred at 12.5:2.5 ratio because this ratio contains larger amount of solution which caused not thoroughly mixing condition made poorer formation of small nanoparticles size.

Table 9: Effect of the mixture volume on the particle size and polydispersity index (PDI) of Dex-VDnanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	16	microstirrer	6.25	1.25	238.67	0.290
2	16	microstirrer	6.25	1.25	258.00	0.282
3	16	microstirrer	12.5	2.5	300.73	0.329

3.1.5. Effect of initial Water content

The results in *Table 10* showed that different initial water content of 5 ml, 6, and 10 ml gave the diffferent Z-average size of 251.4, 213.07, and 246.07 nm, respectively. This might be that the initial water prevents aggregation of the particles in THF solution. In this experiment, initial water content 6 ml is the optimize volume that gave the smallest size distribution.

Table 10: Effect of initial water content on the particle size and polydispersity index (PDI) of Dex-VD nanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	16	5 ml	12.5	2.5	251.4	0.266
2	16	6 ml	12.5	2.5	213.07	0.178
3	16	10 ml	12.5	2.5	246.07	0.210

3.1.6. Optimal condition for Dex-VD nanoparticle formation

The following table (*Table 11*) shows the Dex-VD nanoparticle prepared by optimized method based on the previous experiment (with high speed stirring with sonicated for 3 minutes, with initial water content of 5 ml) which provides acceptable average size distribution of 195.75 ± 31.18 nm with polydispersity index (PDI) of 0.229. Those samples were selected for cell viability assay in further experiment. The size distribution of Dex-VD nanoparticles were plotted as shown in Figure 14.

Table 11: Size distribution of Dex-VD nanoparticle

Exp	Sample	Material		Size distribution	
		Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	20	12.5	2.5	210.93	0.351
2	16	12.5	2.5	194.70	0.213
3	16	12.5	2.5	217.53	0.181
4	16	12.5	2.5	215.40	0.266
5	16	12.5	2.5	246.07	0.210
6	16	12.5	2.5	213.07	0.178
7	16	12.5	2.5	188.40	0.322
8	20	12.5	2.5	227.53	0.386
9	23	12.5	2.5	159.67	0.097
10	20	12.5	2.5	182.07	0.270
11	23	12.5	2.5	161.17	0.115
12	23	12.5	2.5	132.53	0.169
Average size (nm)				195.75 ± 31.18	0.229

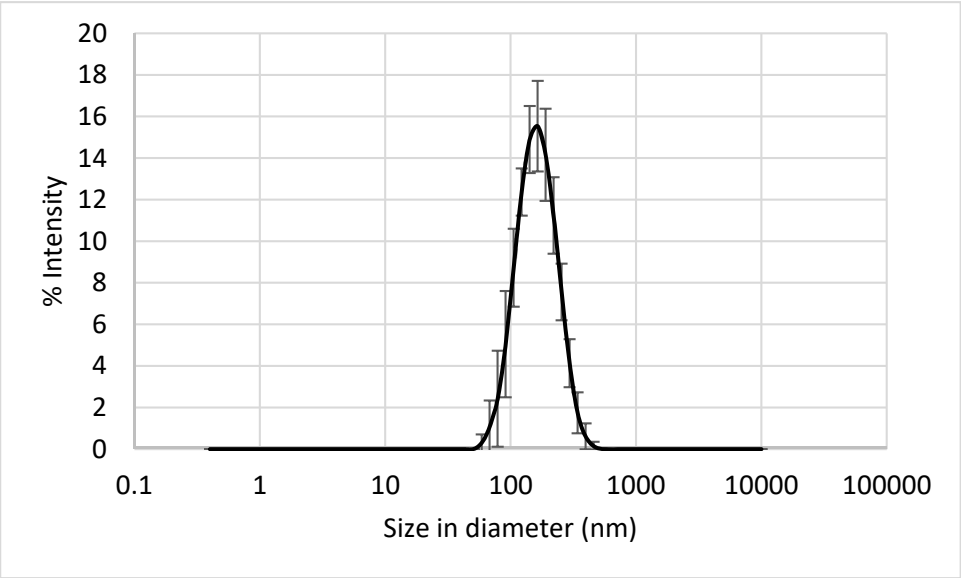

Figure 14: Size distribution of Dex-VD nanoparticles obtained by optimized nanoprecipitation method.

3.2. Dex-VL nanoparticle formation using dialysis method

As shown in previous experiment, the solubility of Dex-VL is different from Dex-VD as vinyl laurate cannot be dissolved in THF. Moreover, only DMSO that can be used to dissolve Dex-VL but the removal by evaporation cannot be applied due to the boiling point is higher than water. To get rid of DMSO, dialysis method was used in this study. The procedure had been developed until the diameter of spherical shaped Dex-VL nanoparticle is in 100-200 nm.

3.2.1. Effect of polymer concentration on the nanoparticle formation

To study the effect Dex-VL concentration, Dex-VL 2-5 mg/ml was mixed together with DMSO, prior to addition of initial water content of 5 ml before dialysis. Dex-VL concentration at 5 mg/ml provides the best result in forming smallest particle size, as shown in *Table 12*. This result is different from previous study (F. Gu, B.-Z. Li, H. Xia, B. Adhikari, & Q. Gao, 2015) that low concentration influence the formation of smaller nanoparticles. However, different type of polymer was used in this experiment.

Table 12: Effect of polymer concentration on the particle size and polydispersity index (PDI) of Dex-VL nanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	8	2 mg/ml	5	2.5	264.03	0.571
2	8	4 mg/ml	10	2.5	216.83	0.527
3	8	5 mg/ml	12.5	2.5	184.83	0.443

3.2.2. Effect of the mixture volume

Table 13 shows the result of size distribution and PDI of Dex-VL nanoparticle. The study found that larger mixing volume of Dex-VL and DMSO provides less Z-average size of Dex-VL nanoparticle. The mixture volume does not affect dialysis speed so it does not afeect to the size distribution of nanoparticle (Sharma et al., 2016).

Table 13: Effect of mixture volume on the particle size and polydispersity index (PDI) of Dex-VL nanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	21	Different ratio	6.25	1.25	297.7	0.195
2	21		12.5	2.5	228.6	0.074

3.2.3. Effect of initial water content

In order to study the effect of initial water content on the size of nanoparticle, 0-10 ml distilled water was added into Dex-VL-DMSO solution before dialysis. The average size and PDI of nanoparticle is presented in *Table 14*. The table shows that initial water content of 10 ml provides the smallest size of Dex-VL nanoparticle. Previous study suggested that the initial water content is one of the important factor that affect the particle size (Feng Gu et al., 2015) as water disturbs dialysis speed and causes more compact nanoparticle formation.

Table 14: Effect of initial water content on the particle size and polydispersity index (PDI) of Dex-VL nanoparticles.

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	21	Initial water content 0 ml	12.5	2.5	649.16	0.183
2	21	Initial water content 5 ml	12.5	2.5	303.33	0.182
3	21	Initial water content 10 ml	12.5	2.5	263.93	0.165

3.2.4. Optimal condition for Dex-VL nanoparticle formation

The following table shows the Dex-VL nanoparticles prepared by method optimized based on dialysis technique with high speed stirring, with sonicated for 3 minutes, with initial water content of 5 ml, which provides acceptable average size distribution of 196.10 ± 20.41 nm with polydispersity index (PDI) of 0.270. Those sample were selected for cell viability assay in further experiment.

Table 15: Size distribution of Dex-VL nanoparticle

Exp	Sample	Condition	Material		Size distribution	
			Dex-VD (mg)	THF (ml)	Z-average (nm)	PDI
1	8	4 mg/ml	10	2.5	216.83	0.532
2	8	6 mg/ml	6.25	2.5	184.83	0.571
3	21	5 mg/ml	12.5	2.5	263.93	0.165
4			12.5	2.5	228.60	0.527
5	22		6.25	2.5	218.00	0.310
6			12.5	2.5	218.40	0.108
7			12.5	2.5	216.27	0.157
8			6.25	1.25	201.13	0.385
9			12.5	2.5	190.70	0.195
10			12.5	2.5	170.63	0.108
11			12.5	2.5	168.33	0.111
12			12.5	2.5	178.10	0.277
13			12.5	2.5	181.77	0.122
Average size (nm)				196.10± 20.41		0.270

Dex-VL sample no.8 and no.21 gave the acceptable size range but with high values of PDI, suggesting broad size range. The study found that degree of substitution of Dex-VL sample plays important role in Dex-VL nanoparticle formation, Numerous interaction between Dextran and vinyl laurate lead to forming dense hydrophobic core of nanoparticle (K. Kaewprapan et al., 2012). As the mean size distribution and PDI of Dex-VL sample no.22 gave more suitable results. In the future study, the degree of substitution and critical aggregation concentration of Dex-VL should be investigated to identify the material characteristic. The size distribution of Dex-VD nanoparticles were plotted as shown in Figure 15.

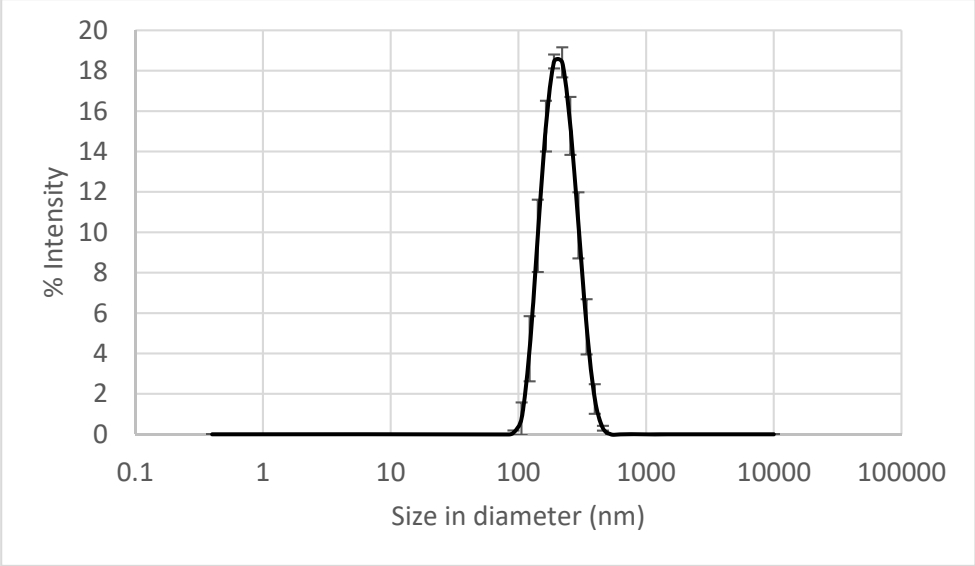

Figure 15: Size distribution of Dex-VL nanoparticle obtained with the optimized dialysis method.

4. Morphological structure of nanoparticle observed by TEM

Dextran-based nanoparticles morphology were shown in Figure 16. Dex-VD and Dex-VL samples had size around 150-200 nm with spherical shape. The concentrataed Dex-VL sample done by Rotary Evaporater still be in spherical shape with desirable size range.

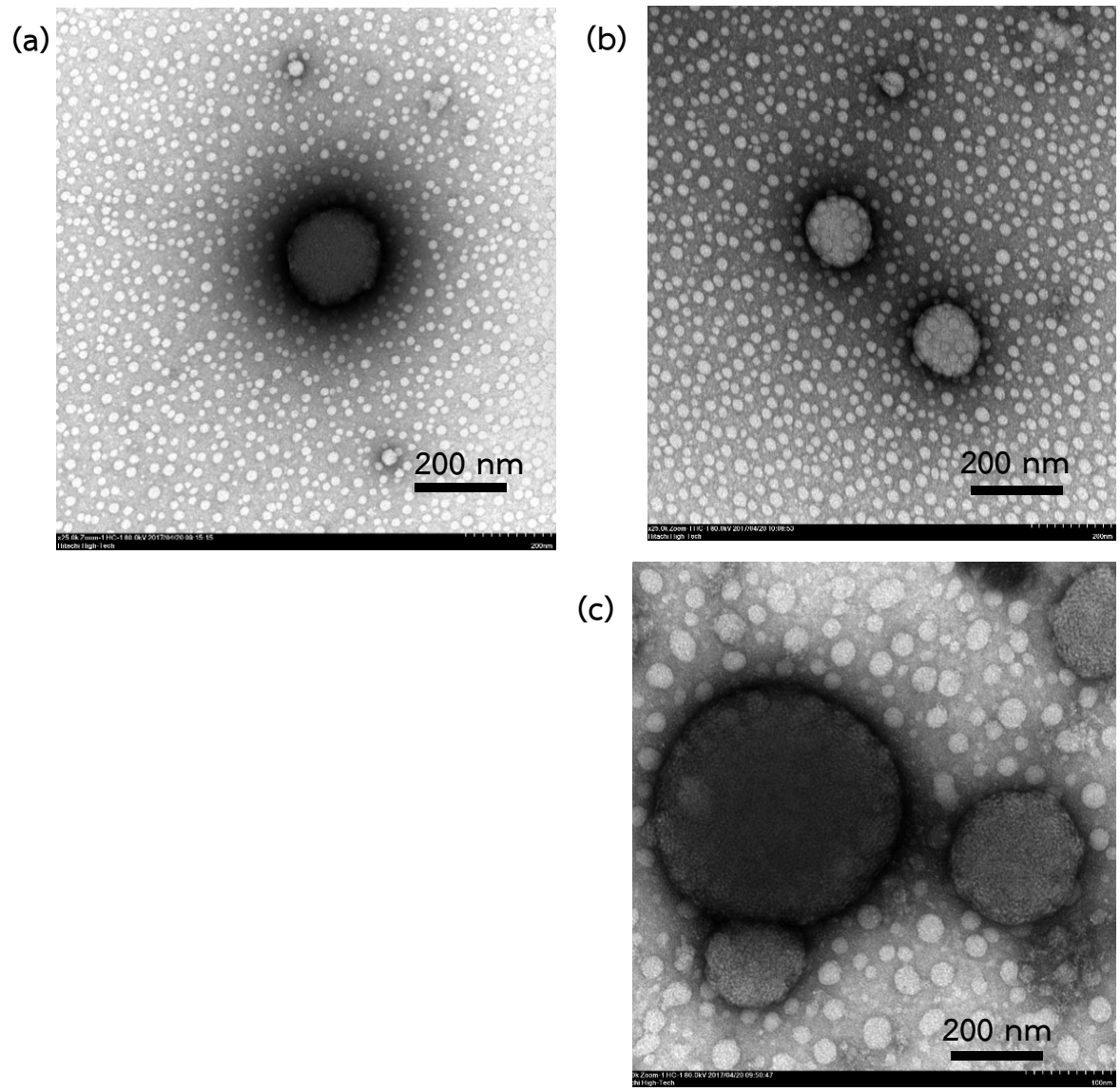

Figure 16: Morphology of Dextran-based nanoparticles (a) Dex-VD (25000X 180000V) (b) Dex-VL (25000X 180000) (c) Dex-VL after concentrated 10000X 180000V

5. The effect of Dextran-based nanoparticles treatment

The cell viability of HT-29 and HCT 116 cells were affected the most by dextran-vinyl laurate nanoparticle (Dex-VL), following by dextan-vinyl decanoate nanoparticles (Dex-VD) and not significantly affected by dextran solution treatment. The data revealed that the higher concentrations of treatment, the lower cell viability were observed, especially in HCT 116 cells.

5.1. Dextran treatment

The percentage of cell viability of dextran treatment at 0.5, 1, 5, 10, 50 mg/ml were 90.04%, 89.82%, 87.21%, 72.39% in HT-29 cells and were 60.14%, 55.59%, 72.34%, 49.68% and 44.23% in HCT 116 cells, as shown in Figure 17. These results suggest that HT-29 were not significantly affected by dextran T-40 from *Leuconostoc mesenteriods* strain except in higher concentration (10-50 mg/ml) compared to positive control experiment, cells treated with DMSO. In contrary, HCT 116 shows lower percentage of cell viability. The maximum dose of dextran that remained in acceptable range of cytotoxicity were 5 mg/ml for HT-29 which is much higher than HCT116 (<05 mg/ml). HCT 116 cells were more sensitive to dextran T-40. The morphology of treated HCT 116 cells remained in good-shaped and not totally be destroyed by dextran treatment even with high concentration (data not shown). These results were comparable to the previous study (Kothari et al., 2015) which confirmed that dextran from *Leuconostoc mesenteriods* is biocompatible, biodegradable and non-toxic to human intestine cell lines.

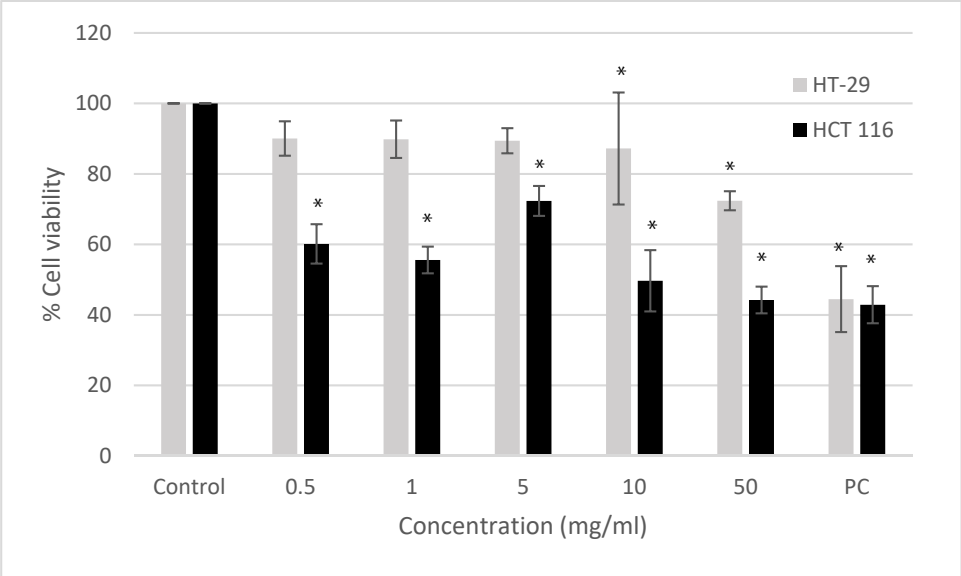

Figure 17: The effect of dextran on cell viability of mammalian cell lines. *The dextran was incubated with HT-29 (grey) and HCT116 (black) for 24 hours. Data are shown as the mean ±SD of the three experiments. Asterisk above columns denoted statistical difference in comparison to the control experiment (n = 3, p < 0.05).*

5.2. Dextran-Vinyl decanoate treatment (Dex-VD)

The results of cell viability from Dex-VD treatments showed in Figure 18. The data revealed that both cell lines were more affected by higher treatment concentrations as in dextran treatment. The percentage of cell viability affected by Dex-VD treatment at 0.05, 0.1, 0.2, 0.5, 1 mg/ml were 78.18%, 76.10%, 79.64% and 72.69% in HT-29 and were 45.58%, 47.7%, 58.86%, 39.10% and 10.02% in HCT 116 cells. The negative control already exhibited moderate cytotoxic effect at 85.37%, and 66.0% cell viability in HT-29 and HCT 116 cell lines, respectively. To conclude, the maximum dose of Dex-VD that still remained in in acceptable range to HT-29 and HCT 116 cells were approximately 0.2 mg/ml and <0.05 mg/ml, respectively. The results show that HCT 116 was more sensitive to Dex-VD than HT-29.

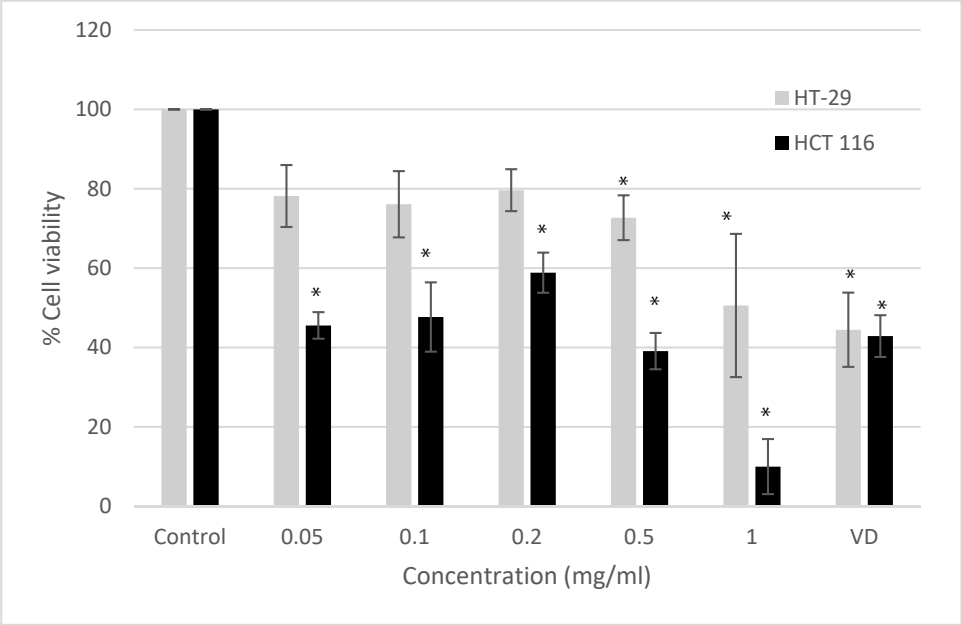

Figure 18: The effect of Dex-VD nanoparticles on cell viability of mammalian cell lines. *The Dex-VD nanoparticles were incubated with HT-29 (grey) and HCT116 (black) for 24 hours. Data are shown as the mean ±SD of the three experiments. Asterisk above columns denoted statistical difference in comparison to the control experiment (n=3, p<0.05).*

5.3. Dextran-Vinyl Laurate treatment (Dex-VL)

Dex-VL nanoparticle treatment exhibited lower cell viability to both cell lines in higher concentration as shown Figure 19. The data suggest that Dex-VL provides linear cell viability percentages of HT-29 cell line from effects of serial treatment concentration. The percentage of cell viability at 0.05, 0.1, 0.2, 0.5, 1 mg/ml of Dex-VL treatment in HT-29 and HCT 116 cell lines were 84.44%, 69.05%, 66.42%, 34.86% and 60.0%, 54.70%, 58.46%, 48.56% and 35.79%, respectively. While the negative controls show higher percentage of cell viability of 87.0% for HT-29 and 77.87% for HCT 116. The maximum dose of

Dex-VL nanoparticles that does not affect to cell viability of HT-29 and HCT 116 cells were <0.05 mg/ml. HCT 116 was more sensitive to Dex-VL in the same way as dextran treatment.

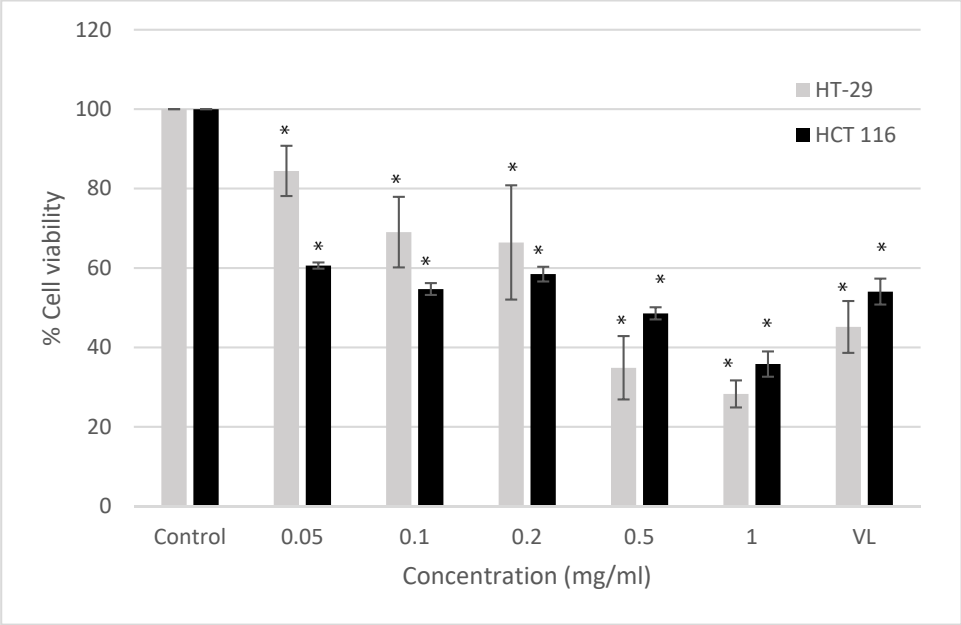

Figure 19: The effect of Dex-VL nanoparticles on cell viability of mammalian cell lines. *The Dex-VL nanoparticles were incubated with HT-29 (grey) and HCT116 (black) for 24 hours. Data are shown as the mean ±SD of the three experiments. Asterisk above columns denoted statistical difference in comparison to the control experiment (n=3, p<0.05).*

The positive control, Vinyl decanoate and Vinyl laurate, shows low cell viability as expected Figure 18Figure 19. The cell viability was at 50.60% for HT-29 and 10.02% for HCT 116 cells treated with vinyl decanote, and 45.14% for HT-29 and 54.05% for HCT 116 cells treated with vinyl laurate. The morphology of HT-29 and HCT 116 cells were changed which may cause by VL and VD treatment (data not shown). These data exploited that these Vinyl ester groups might be the cause that made dextran-based nanoparticles have cytotoxic effects to human intestinal cancer cell lines HT-29 and HCT 116. These results referred to HCT 116 characteristic which is more sensitive to anti-cancer treatment compared to HT-29 (Goodspeed, Heiser, Gray, & Costello, 2016).

For future experiments, better choices of choosing water should be considered in nanoparticle formation steps and cell culture since growth of human tumor cell lines could be inhibited by distilled water according to the hypotonic condition (Mercill, Jones, & Harbell, 1985). The positive control was not performed in every experiment because first we only aimed to test the cytotoxic effect of Vinyl-ester in maximum amount that appeared in nanoparticles products.

Conclusion and Future work

From all of this work we can conclude that;

- Amphiphilic dextran of Dex-VL can be synthesized using lipase-catalyzed transesterification method.
- Dex-VL nanoparticle can be formed using Dialysis method. The average size was 196.10 ± 20.41 nm with 0.270 PDI value which is in desirable size range of 100-200 nm with spherical shape. Dex-VD nanoparticle, bulking formed by repeat Nanoprecipitation and Solvent evaporation method, had average size 195.76 ± 31.18 nm.
- Dextran-based nanoparticle with Vinyl ester group as acyl donor have cytotoxicity effect to cell viability of HT-29 and HCT 116, human colon adenocacinoma cell lines. The maximum dose of Dex-VD and Dex-VL nanoparticles were 0.2 mg/ml and 0.05 mg/ml in HT-29 and both <0.05 mg/ml in HCT 116 cell. The positive control, Vinyl decanoate and Vinyl Laurate, showed cytotoxic effect to both cells lines unlike Dextran. Dextran had maximum dose for treatment at 5 mg/ml in HT-29 which confirmed its biocompatibility nature.

Future work

The future work should be focused on optimization of Dialysis method and testing more cell viability assay of involved components in nanoparticle formation and cell culture to find out the true reason behind the cytotoxic effect of nanoparticles. The other suggests are optimization of nanoparticle centrifugation test, nanoparticle concentration measurement, DS measurement of different samples by H^1NMR, Fluorescence method using Pyrene as probe for critical aggregation concentration and more of encapsulation test.

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Appendix

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

การเสนอผลงานในที่ประชุมวิชาการ (Poster presentation and Book of Abstracts)

- **Nisa Patikarnmonthon**, Supawadee Sangpao, Ariya Naneto, Pranee Inprakhon, Watanalai Panbangred. *Preparation and nanoparticle formation of amphiphilic dextran modified by lipase*. The 28th Annual Meeting of the Thai Society for Biotechnology and International Conference. 28-30 November 2016 The Empress Hotel, Chiang Mai, Thailand

โครงการพิเศษของนักศึกษาระดับปริญญาตรี

- นางสาวอริยา เณรโต SCBT5405295 / ปริญญาตรี / “Altering hydrophobicity of dextran with enzymatic reaction” / สำเร็จการศึกษา
- นางสาวศุภวดี สังข์เภา SCBT 5505214 / ปริญญาตรี / “Study of the dextran-based nanoparticle formation and stability under biological condition”/ สำเร็จการศึกษา
- นางสาวภาวิดา ณ นคร SCBT 5605183 และนางสาวภิญญาพัชญ์ แสงแก้ว SCBT 5605184 / ปริญญาตรี / “Development of amphiphilic dextran-based nanoparticle and its effect on cell viability”/ สำเร็จการศึกษา