Engineering Oral Formulation of Adenosine and *Parabacteroides distasonis* for Suppressing Colorectal Cancer

A thesis submitted by Qiannan Ma

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Abstract

Inflammation in GI tract can cause many kinds of cancers, such as colorectal cancer (CRC). As we can see from the previous research, adenosine is a famous kind of anti-inflammation in GI tract, so increase of adenosine can suppress inflammation and decrease the risk of CRC. *P.dist* (*Parabacteroides distasonis*) is a kind of anaerobic bacteria in gastrointestinal tract. Studies have found that *P.dist* bacteria is always absent in CRC patients. Both live *P.dist* bacteria and *P.dist* bacteria lysate have the function to suppress inflammation. The goal of this research is to investigate the oral administration of adenosine and *P.dist* (either in live bacteria or bacteria lysate) to suppress the inflammation and CRC. There are some barriers for oral delivery such as fast half life of adenosine and intolerance of *P.dist* (both live or lysate), so I want to develop an effective approach to deliver adenosine and *P.dist* orally.

The first chapter mainly focuses on developing an approach to adenosine encapsulation. In this part I use wax, Eudragit S100, and Eudragit L100 to formulate the particle and calculate encapsulation efficiency, stability and release kinetics for the particle in order to show the effectiveness of encapsulation methods.

The second chapter develops an approach to encapsulate live *P.dist* or *P.dist* lysate. In this part, I use Eudragit FS30D to formulate the particle. Since *P.dist* are obligate anaerobes, it is hard to imitate the living environment *in vitro* and so, *E.coli are used* as the model.

Acknowledgements

I am grateful to Professor Qiaobing Xu for his tremendous help during my study at Tufts University, especially in the research aspect. He taught me many methods to complete my research and his attitude for research influence me a lot for my future study and work. Anytime when I need help, he will give me a hand. I also thank my colleagues in Xu's lab, especially Dr. Ming Wang who he taught me how to use the machines in our lab and help me solve many experimental problems. In addition, I want to thank Feng Jia for helping me develop the encapsulation method, which is very important to my research. He provided advice on not only research, but also my daily life. I want to thank committee members, Dr. Asatekin and Dr. Crott. In particular, I want to thank Jimmy Crott and Dr. Gar Yee for their help and contribution on this project. Finally I want to appreciate my family for their support during my master study.

Figures

Tables

Developing approach for encapsulate adenosine for oral delivery.

Background and introduction

Colorectal Cancer (CRC) and Inflammation

Colorectal cancer (CRC) can be caused by many factors, such as smoking, alcohol, old age, family history, inflammation, and has become the third most common cancer in the US[1, 2]. This can be attributed by the lack of symptoms during the early stages of colorectal cancer, which makes it difficult for patients to be aware of the disease[3]. Between 2005-2012, the number of colorectal cancer has increased by 10%[2].

Reason of CRC

There are two main reasons for CRC: one is inflammation [4] and the other one is genetic predisposition[1]. In this research, I will mainly focus on anti-inflammatory agents. Inflammation is closely associated with cancers such as CRC, hepatic cancer, and gastric cancer and so, it is a hallmark for these cancers[5]. Depending on the degree and time the inflammation occurs in intestine or colon, reactive oxygen and nitrogen species (RONS) can be produced which can damage DNA and increase the chances of gene mutation and thus, increase the possibility tumor growth [1]. Previous research shows that some gut microbe[6] and small molecules[7] can be

anti-inflammatory, so we can use them to control CRC with obesity.

Current treatment

Six methods usually used in clinical treatment include surgery, cryosurgery, radiofrequency ablation, chemotherapy, radiation therapy and targeted therapy[8]. People use different methods in different CRC stages.

Therapeutic summary	Stage I ($n = 33$)	Stage II ($n = 69$)	Stage III ($n = 68$)	Stage IV ($n = 72$)
Curative resection or surgical palliation, *n* (%)	28 (85)	69 (100)	66 (94)	39 (54)
Endoscopic resection (tumor confined to polyp)	5 (100)	N/A	N/A	N/A
Received chemotherapy, *n* (%)	0 (0)	23 (34)	54 (80)	50 (70)
Received both surgery and chemotherapy, *n* (%)	0 (0)	23 (34)	54 (80)	27 (38)

N/A = not applicable.
Summary of therapeutic intervention(s) received by patients diagnosed and treated for colon cancer during the study interval.

Figure 1. Summary of treatment methods depending on the disease stage Millas, S.G., et al., *Treatment delays of colon cancer in a safety-net hospital system.* Journal of Surgical Research, 2015. **198**(2): p. 311-316.

New therapy

In this research, I want to encapsulate two kinds of anti-inflammatory agents for oral delivery. From the former report, some microbes play important parts in CRC. For example, *Lactobacillus* strains have the ability to decrease the mutagens so that reduce the risk of CRC[9].

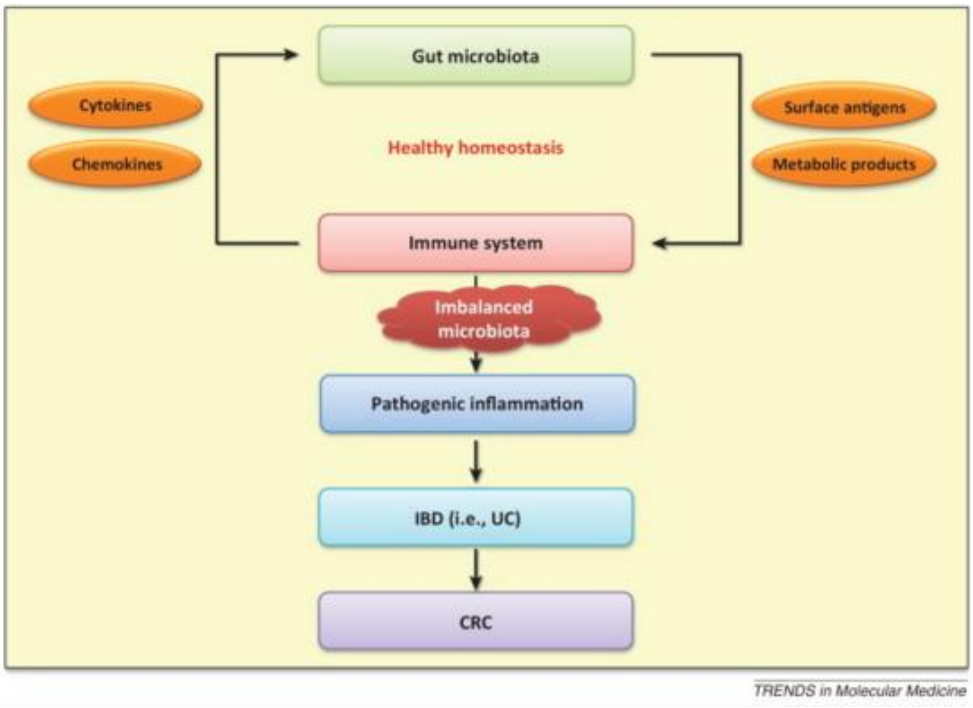

Figure 2. Approach of gut microbe reduce CRC Abbreviations: IBD, inflammatory bowel disease Ambalam, P., et al., *Probiotics, prebiotics and colorectal cancer prevention.* Best Practice & Research Clinical Gastroenterology, 2016. **30**(1): p. 119-131

In the literature, some small molecules also have the function to reduce CRC risk. For example, butyrate can make hyperacetylation of histones by suppressing the enzyme activity of histone deacetylase[10]. According to previous research, the amount of *Parabacteroides distasonis* (*P.dist*) is always much lower in CRC patients and mice[6]. As a kind of anti-inflammatory agent, some researchers want to deliver some components, such as membrane of *P.dist* bacteria orally, to see whether the function still remains[11]. The result shows that both membrane of *P.dist* and *P.dist* bacteria have the ability for anti-inflammation [11]. On the other hand, small molecules also

have the function of anti-inflammation, [12, 13] such as adenosine.

But there are still some barriers for oral delivery of adenosine and *P.dist* in colon. For adenosine, the release speed for small molecules is fast and the half life is short; adenosine will soon be absorbed when they are released outside the particle. So they are unable to reach the colon and properly complete its task. The toxicity of adenosine should also be taken into consider[14]. For *P.dist*, gastric juice is composed of hydrochloride acid and pepsin, so it is a harsh environment for *P.dist* to survive and pepsin will destroy the proteins on *P.dist* membrane. As a result, this decreases its effect for anti-inflammation.

To achieve long-lasting effects for adenosine, we need to increase its release time. A good way to do this is to make the injection into oral administration. Particularly for *P.dist*, we need to deliver them directly to the colon.

Role of adenosine in suppressing inflammation and CRC

Adenosine

Formula of adenosine: $C_{10}H_{13}N_5O_4$

6-amino-9-beta-D-ribofuranosyl-9-H-purine

Molecular weight: 267.241 g/mol

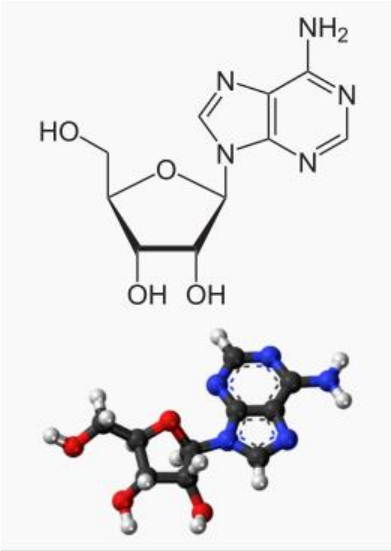

Figure 3. Chemical structural formula for adenosine

Picture from Wikipedia

Adenosine is a kind of white powder that is soluble in water and insoluble in ethanol. It has many functions in the biological process. It can synthesize ATP, ADP and contributes in the energy generation process. Adenosine is a part of cAMP which is called “second messenger” and plays an important part in signal transduction. Adenosine can be used as an antiarrhythmic agent[15]. It also can be used as a stress test method for heart.

Adenosine has some side effects, such as headaches and stomachaches. So it is essential for patients to control the dosage intake of adenosine. There are some contraindications for taking adenosine such as decompensated heart failure, Asthma, and so on[16].

Adenosine is also famous for its anti-inflammatory function [17]. After adenosine is released to the extracellular environment, it will bind to the receptors and enter into the cell membrane [17]. There are four kinds of adenosine receptors: A_1, A_{2A}, A_{2B} and A_3.

A_1 adenosine receptor has functions including, decreasing the intracellular cAMP for signal transduction and increasing the intracellular calcium ion to activate some enzymes for biological processes [18-20]. So far, there is not much evidence showing that A_1 adenosine receptor has a relationship with inflammation.

A_{2A} adenosine receptor has the function to increase cAMP. A_{2A} is widely known for its anti-inflammation function[21]. With the ability to decrease tissue damage and inflammation, the gene of A_{2A} adenosine receptor is widely expressed in cells [19, 22].

With the activation of A_{2B} and A_{3B} adenosine receptor, the adenylate cyclase will be inhibited [23].

Barriers for small molecule oral delivery

Small molecules here are mainly related to the molecules whose molecular weight is under 500Da[24]. With the development of biotechnology, more and more therapeutics medicines are small molecule, such as fluorescein, doxorubicin,

diclofenac sodium and so on[14]. Due to the low absorption, rapid clearance, and toxicity of these small molecules, people want to deliver them orally [25-27]. For GI oral drug delivery, small molecules should be protected from acid environment and pepsin degradation. Preventing the rapid release of small molecules before they reach their functional place is also needed for oral drug delivery.

From previous reports, there are many methods for small molecule oral delivery. For example, they make hydrogel, polymeric lipid nanoparticle, solid lipid nanoparticle, micelles and liposome for small molecule encapsulation[27]. Many materials are also used for oral delivery such as chitosan, PEGylation, Eudragit, alginate, starch or starch derivatives, azoaromatic polymer and so on [28, 29].

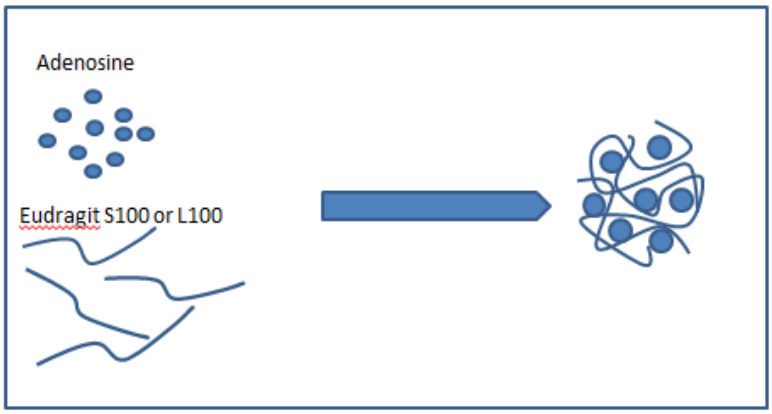

Figure 4. Traditional encapsulation method for adenosine

From figure 4, the traditional method for adenosine is to mix the adenosine and polymer together in organic solvent. After removing the organic solvent, the polymer will then coat the outside of adenosine. Preliminary experiments show that by this method, the release speed of adenosine is very fast. More than 95% of adenosine will

be released within 15 minutes. So I need to improve our method.

Eudragit L100 and Eudragit S100

The polymer I used in my research is Eudragit. Eudragit is a series of polymers produced by Evonik Industries. This series of polymers are all pH sensitive. They dissolve when the pH exceeds a given value and remains insoluble when the pH is lower than that value. This provides protection before the drugs reaches the released place. The specific polymers that I use in the research is Eudragit L100, S100.

Polymer	Availability	Dissolution Properties
Eudragit L100	Powder	Dissolution above pH 6.0
Eudragit S100	Powder	Dissolution above pH 7.0

Table 1. Characters of Eudragit. From http://eudragit.evonik.com/

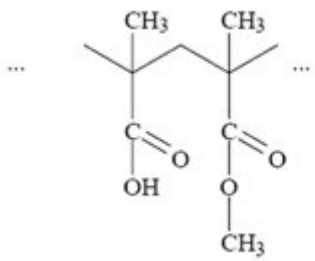

Figure 5.Eudragit L100 chemical structural formulas. Picture from

http://eudragit.evonik.com/

Eudragit L100 is widely used in intestine drug delivery since the intestine pH is 6.8, which is not high enough for Eudragit S100 to dissolve[30]. People used to encapsulate ferulic acid by spray dry using Eudragit L100 as the coating material [31]. Encapsulation of insulin by Eudragit L100 is another important application. Many researchers find that Eudragit L100 encapsulation of insulin can reach high encapsulation efficiency and maintain protein activity for several hours after release at high pH[32].

Eudragit S100 can be used in colon drug delivery since the pH of colon is over 7.

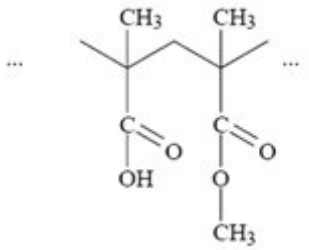

Figure 6. Eudragit S100 chemical structural formulas. Picture from

http://eudragit.evonik.com/

Eudragit S100 can encapsulate zaleplon, a drug that treats insomnia, by spray dry and reaches 91%-95% encapsulation efficiency[30]. People also use Eudragit 100 to encapsulate diphenylhydantoin and glibenclamide with high encapsulation efficiency [33].

Adenosine needs to release into the colon to have function, so Eudragit S100 should be the polymer adenosine encapsulation. But I still encapsulate Eudragit L100 because

I want to test whether the new method for encapsulation could still work if I change materials. Moreover, it can also be used as a model for other small molecules encapsulation for intestine oral delivery.

Wax encapsulation

To deal with the fast release speed of adenosine, I apply wax in the encapsulation procedure.

Existing naturally, wax is a common thing that people used for their daily life. Wax can be found in many places, such as in plants; there are bayberry wax, soy wax, japan wax, in insect, there are beeswax, shellac wax and so on [34].

So far, there are many barriers in oral drug and food encapsulation. For example, the toxicity of the encapsulation material, the storage stability and the release time for the encapsulation [35]. Since wax is insoluble in water and will melt when the temperature is over 40℃, it can be used for drug encapsulation. In addition, the toxicity of wax is low, so it will be very safe for patients to take. Beside non-toxicity, there are many other advantages of using wax as an oral drug encapsulation material, such as it has good biocompatibility [36], its cost of production is low, and the *in vivo* tolerability of wax is high [37]. In literature, people found that wax encapsulation can be storage for weeks with low leakage [34, 38].

Because of its numerous advantages, there are many applications for wax encapsulation of drugs. People used to encapsulate salicylic acid by beeswax. The encapsulation efficiency can reach more than 90% and salicylic acid can be released almost 100% in 20 hours [37]. Wax can also be used in encapsulate flavor molecules, with high encapsulation efficiency and long release time [39].

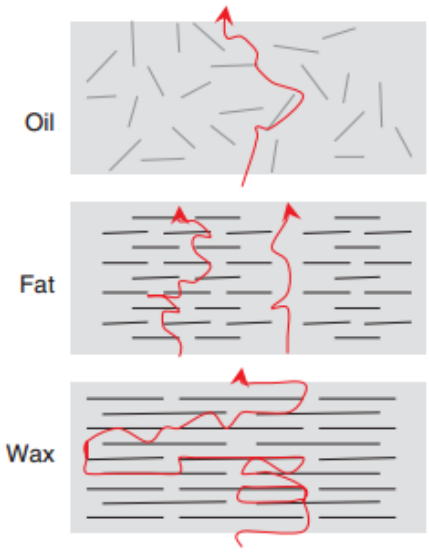

Figure 7. Comparison of wax release effects with other materials. Mellema, M., et al., *Wax encapsulation of water-soluble compounds for application in foods.* Journal of Microencapsulation, 2006. **23**(7): p. 729-740.

From figure 7 we can see that there is another advantage of wax-encapsulation. The structure of wax is very tight and has many complex layers. So adenosine will need more time to release outside the wax and thus, increase the release time.

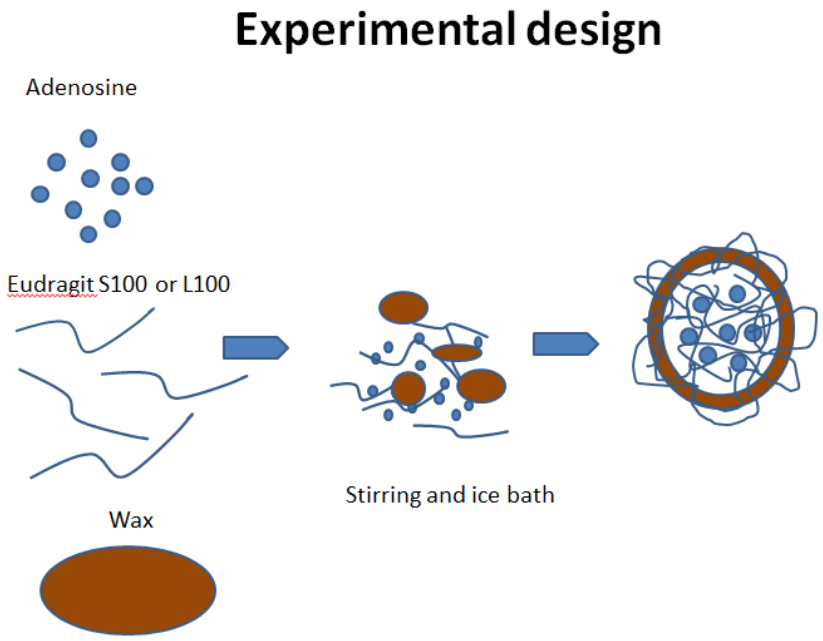

Figure 8. The experimental design for Eudragit-Wax-Adenosine encapsulation

I add melted wax into the encapsulation system and decreased the temperature to formulate the particle. From this, the particle now has an outer wax layer that can decrease the release speed.

The Eudragit is used to control the release place. For example, in acid environment, adenosine can't release outside the Eudragit. The purpose of the wax is to decrease the release speed of adenosine in high pH environment while the Eudragit dissolves.

Materials and Methods

Materials

Drugs and reagent

Adenosine (Sigma-ALDRICH A9251-25G), Eudragit S100 (Evonik industries B100405198), Eudragit L100 (Evonik industries B090803171), Wax, Phosphate buffered saline 10X (Boston Bioproducts BM-220), Dichloromethane (Sigma- ALDRICH 34856), Ethanol (Sigma- ALDRICH 34963), Sodium hydroxide (Fisher Scientific BP359-500), Hydrochloric acid(SIGMA-ALDRICH 320331), Plastic pH indicator strips (Fisher Scientific 13-640-516), Gastric Juice Artificial (Aqua Solutions G1618-1L), Methanol (Sigama-alorich 197337-4L)

Facilities

Zeta Potential Analyzer (ZetaPALS), Nouva Stir Plate (Thermolyne), Branson 5510 Ultrasonic, Balance (Mettler AE100), Lyophilizer (Labconco), Electronic pH meter (Accumet basic AB15 Plus), SpectraMaxM2/M2 Plate reader, Isotemp Waterbath (Fisher scientific), Analog Vortex Mixer (VWR), SEM (EVO MA10)

Methods

Particle preparation

Prepare 1:1 (v/v) Dichloromethane : Ethanol reagent;

Dissolve Eudragit S100 or Eudragit L100 into that reagent with 50mg/mL;

Add adenosine into Eudragit S100 or Eudragit L100 solution at 1:5 w/w (Adenosine : Eudragit S100 or Adenosine : Eudragit L100) with initial weight adenosine 0.5g, Euuragit S100 or Eudragit L100 2.5g;

Mix them well by vortex;

Melt paraffin wax over 40℃;

Add melted wax into Adenosine- Euragit S100 or Adenosine-L100 solution at 1:10 v/v (wax : solvent) while stirring, initial volume for adenosine-Eudragit S100 or adenosine-Eudragit L100 solution is 50mL, and wax is 5mL;

Put the wax-adenosine-Euragit S100 or wax-adenosine-Eudragit L100 sample on ice for 10 minutes to formulate particles;

Stirring overnight to evaporate the solvent and let the sample dry at room temperature;

Wash up by 5mL pH=4 PBS;

Centrifuge for 10 minutes at 10,000 rpm;

Do the wash up step for three times;

Lyophilization.

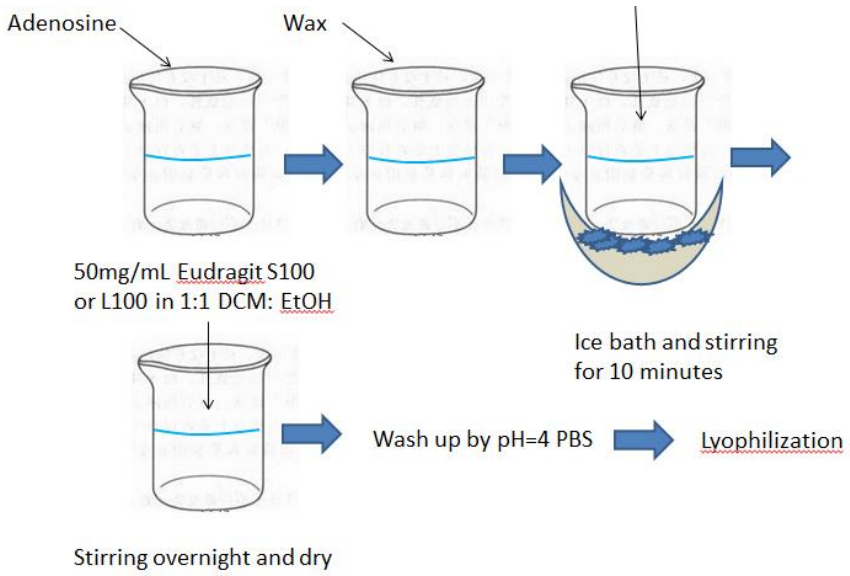

Figure 9. Modified adenosine encapsulation procedure

Particle Size

Use 2mg sample, put it into 1mL methanol then vortex until the sample is dissolved;

Put the sample into DLS and test the size

Morphology

Use SEM to take the picture of Eudragit S100-Wax-Adenosine encapsulation and Eudragit L100-Wax-Adenosine encapsulation.

Encapsulation efficiency

$$\text{Actual Loading} = \frac{Drug\ amount\ in\ sample}{Sample\ weight} \times 100\%$$

Encapsulation Efficiency

$$= \frac{Actual\ drug\ amount\ encapsulated\ in\ sample}{Theoretical\ amount\ of\ drug\ should\ be\ encapsulated} \times 100\%$$

Weigh 5mg sample;

Put the sample into 0.1M NaOH reagent then vortex;

When sample is completely dissolved, dilute the sample for 10 fold;

Test the absorbance at 260nm for concentration of adenosine in sample

Particle stability (Low pH release)

Preheat the gastric juice at 37℃ water bath;

Put the sample into 5mL preheated gastric juice at 37℃ water bath for 30 minutes

Centrifuge at 12,000rcf for 10 minutes;

Dilute the supernatant for 10 fold;

Test the absorbance at 260nm for adenosine concentration in gastric juice

$$Release\ ratio = \frac{Released\ drug\ amount\ in\ gastric\ juice}{Total\ encapsulated\ drug\ amount} \times 100\%$$

Drug release Curve

Prepare pH=7.5 PBS solvent, water bath at 37℃ for preheat;

Use 20mg sample then add 10mL preheated PBS;

Put the sample solvent into 37℃ incubator shaker at 220rpm;

Use 100uL of the sample at t=0.5h, 1h, 2h, 3h, 4h, 5h, 6h;

Add 100uL pH=7.5 PBS back into the sample solvent to keep the volume constant each time after removal of the sample;

Dilute the picked up sample for 10 fold;

Test the absorbance at 260nm for adenosine concentration in gastric juice.

Release ratio:

$$Release\ ratio = \frac{Released\ drug\ amount}{Total\ encapsulated\ drug\ amount} \times 100\%$$

Results and Discussion

Results

Figure 10. Left: Eudragit S100-Wax-Adenosine encapsulation Right: Eudragit L100-Wax-Adenosine encapsulation

Particle size

After testing the Eudragit S100-Wax-Adenosine and Eudragit L100-Wax-Adenosine encapsulation by DLS, the particle is 154945.3 $\pm$ 146807.4nm for Eudragit S100-Wax-Adenosine encapsulation and 106895.85 $\pm$ 28312.6nm for Eudragit L100-Wax-Adenosine encapsulation.

Morphology

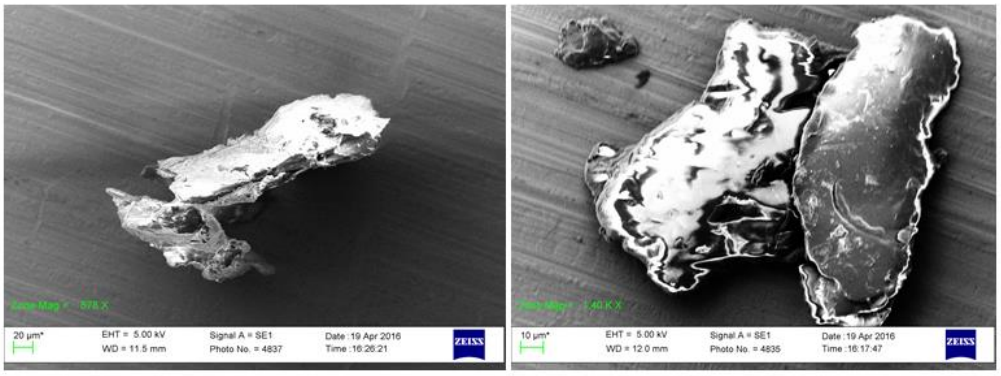

Figure 11. Morphology for Eudragit S100-Wax-Adenosine encapsulation

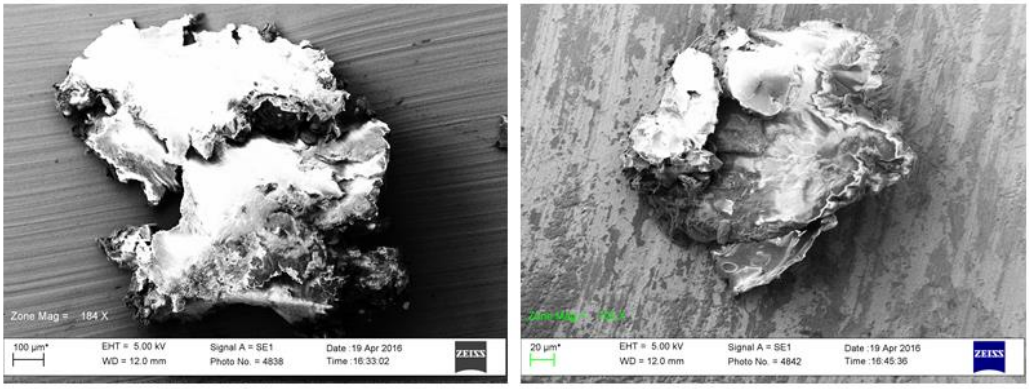

Figure 12. Morphology for Eudragit L100-Wax-Adenosine encapsulation

Encapsulation efficiency

Before I test the encapsulation efficiency, I do a standard curve for adenosine.

X: The absorbance at 260nm for adenosine.

Y: Concentration of adenosine ug/mL

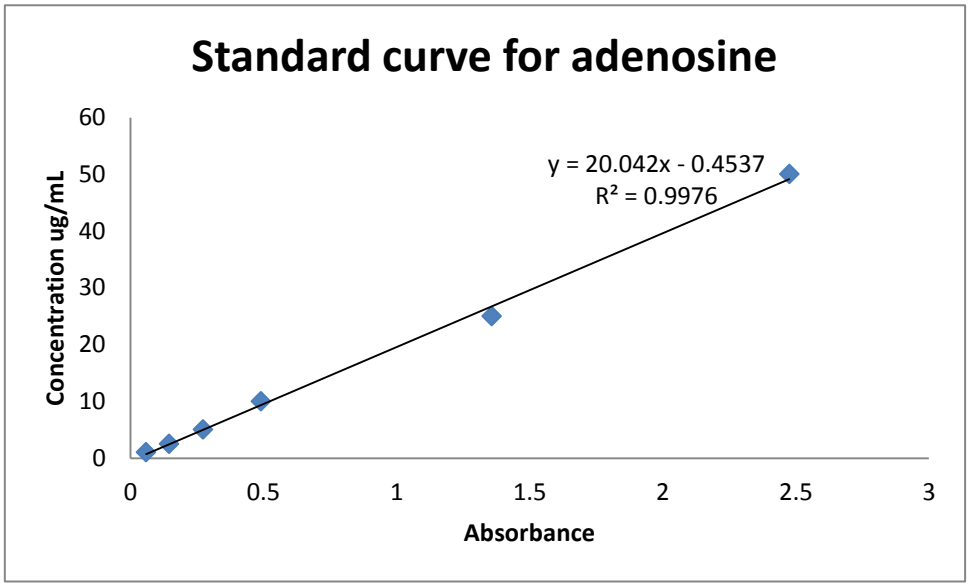

Figure 13. Standard curve for adenosine

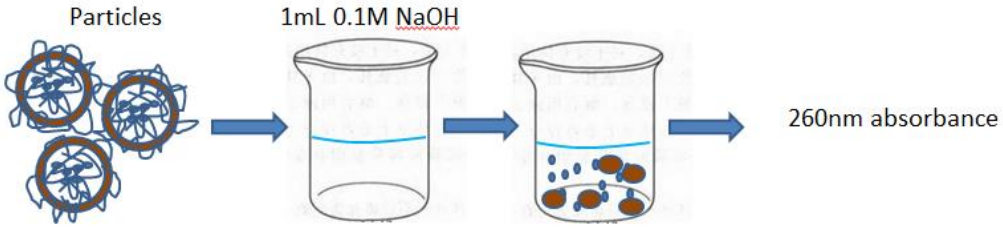

Figure 14. Loading and Encapsulation efficiency test procedure

Since Eudragit S100 and Eudragit L100 can easily dissolve in 0.1M NaOH, all of the encapsulated adenosine will be released after that.

For Eudragit S100-Wax-Adenosine encapsulation I use 5533ug of the sample, dissolve it in 1mL 0.1M NaOH dilute 10 fold, and then test the absorbance at 260nm.

Sample weight: 5533ug

Absorbance: 1.344

Standard curve: y=20.042x-0.4537

Put x=1.344 into standard curve we can get y=26.489 ug/mL;

Since I diluted my sample for 10 folds, the actual adenosine concentration in my sample is 26.489 x 10=264.89 ug/mL;

Total loading amount of adenosine in 1mL 0.1M NaOH= 264.89 ug/mL x 1mL=264.89ug;

$$\text{Actual Loading} = \frac{Drug\ amount\ in\ sample}{Sample\ weight} \times 100\%$$

$$Actual\ Loading = \frac{264.89\ ug}{5533\ ug} \times 100\% = 4.78\%$$

Total weight of the sample in this system is 6.1345g;

So the total loading adenosine is 6.1345x10^3mg x 4.78%=293.22mg;

The total adenosine amount that I put into the system initially is 0.5g;

$$\text{Encapsulation Efficiency} = \frac{Actual\ drug\ amount\ encapsulated\ in\ sample}{Theoretical\ amount\ of\ drug\ should\ be\ encapsulated} \times 100\%$$

$$Encapsulation\ efficiency = \frac{293.22\ mg}{500\ mg} \times 100\% = 58.54\%$$

For Eudragit L100-Wax-Adenosine encapsulation I use 4222.6 ug of the sample, dissolve it in 1mL 0.1M NaOH dilute 10 fold then test the absorbance at 260nm.

Sample weight: 4222.6ug

Absorbance: 1.306

Standard curve: y=20.042x-0.4537

Put x=1.344 into standard curve we can get y=25.7278 ug/mL;

Since I diluted my sample for 10 folds, the actual adenosine concentration in my sample is 26.489 x 10=257.278 ug/mL;

Total loading amount of adenosine in 1mL 0.1M NaOH= 257.278 ug/mL x 1mL=257.278 ug;

$$\text{Actual Loading} = \frac{Drug\ amount\ in\ sample}{Sample\ weight} \times 100\%$$

$$Actual\ Loading = \frac{257.278\ ug}{4222.59\ ug} \times 100\% = 6.092\%$$

Total weight of the sample in this system is 4.2876g;

So the total loading adenosine is 4.2876×10^3mg x 6.092%=261.2mg;

The total adenosine amount that I put into the system initially is 0.5g;

$$\text{Encapsulation Efficiency} = \frac{Actual\ drug\ amount\ encapsulated\ in\ sample}{Theoretical\ amount\ of\ drug\ should\ be\ encapsulated} \times 100\%$$

$$Encapsulation\ efficiency = \frac{261.2\ mg}{500\ mg} \times 100\% = 52.24\%$$

Particle	Actual loading efficiency	Encapsulation efficiency
Eudragit S100-Wax-Adenosine	4.78% ± 0.87%	58.64% ± 10.67%
Eudragit L100-Wax-Adenosine	6.092% ± 1.18%	52.24% ± 10.1%

Table 2. Actual loading efficiency and encapsulation efficiency for Eudragit S100-Wax-Adenosine and Eudragit L100-Wax-Adenosine encapsulation

Figure 15. Paraffin Wax used for encapsulation

Adenosine release in gastric juice

Before the particles arrive at the colon, they need to pass through the stomach where the pH is about 1.2 and contains pepsin. So I want to know whether the encapsulation particles can slow down the passive diffusion of adenosine at low pH and pepsin existence.

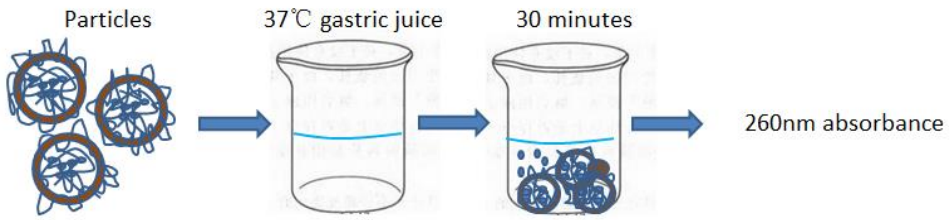

Figure 16. Gastric juice stability for Eudragit-Wax-Adenosine encapsulation

For Eudragit S100-Wax-Adenosine encapsulation, I immerse 16100 ug sample into 5mL 37℃ gastric juice for 30 minutes and dilute the supernatant for 10 folds, testing the absorbance at 260nm.

Sample add into gastric juice: 16100 ug

Loading efficiency: 4.78%

The encapsulated adenosine amount in sample is 16133 x 4.78% =771.19 ug;

Absorbance of supernatant: 0.164;

Standard curve: y=20.042x-0.4537;

So y= 2.833 ug/mL

Since the sample is diluted for 10 folds, the actual concentration of adenosine in supernatant is 2.833ug/mL x 10=28.33ug/mL

So in 5mL gastric juice, the total amount of adenosine is

28.33 ug/mL x5 mL=141.6594 ug;

$$Release\ ratio = \frac{Released\ drug\ amount\ in\ gastric\ juice}{Total\ encapsulated\ drug\ amount} \times 100\%$$

$$Release\ ratio = \frac{141.6594\ ug}{771.19\ ug} \times 100\% = 18.37\%$$

For Eudragit L100-Wax-Adenosine encapsulation, I immerse 15700 ug sample into 5mL 37℃ gastric juice for 30 minutes and use supernatant dilute 10 folds, testing the absorbance at 260nm.

Sample add into gastric juice: 15700 ug

Loading efficiency: 6.092%

The encapsulated adenosine amount in sample is 15700 x 6.092% =956.13 ug;

Absorbance of supernatant: 0.168;

Standard curve: y=20.042x-0.4537;

So y= 2.92 ug/mL

Since the sample is diluted for 10 folds, the actual concentration of adenosine in supernatant is 2.92ug/mL x 10=29.2 ug/mL

So in 5mL gastric juice, the total amount of adenosine is

29.2 ug/mL x5 mL=146.0018 ug;

$$Release\ ratio = \frac{Released\ drug\ amount\ in\ gastric\ juice}{Total\ encapsulated\ drug\ amount} \times 100\%$$

$$Release\ ratio = \frac{146.0018\ ug}{956.13\ ug} \times 100\% = 15.27\%$$

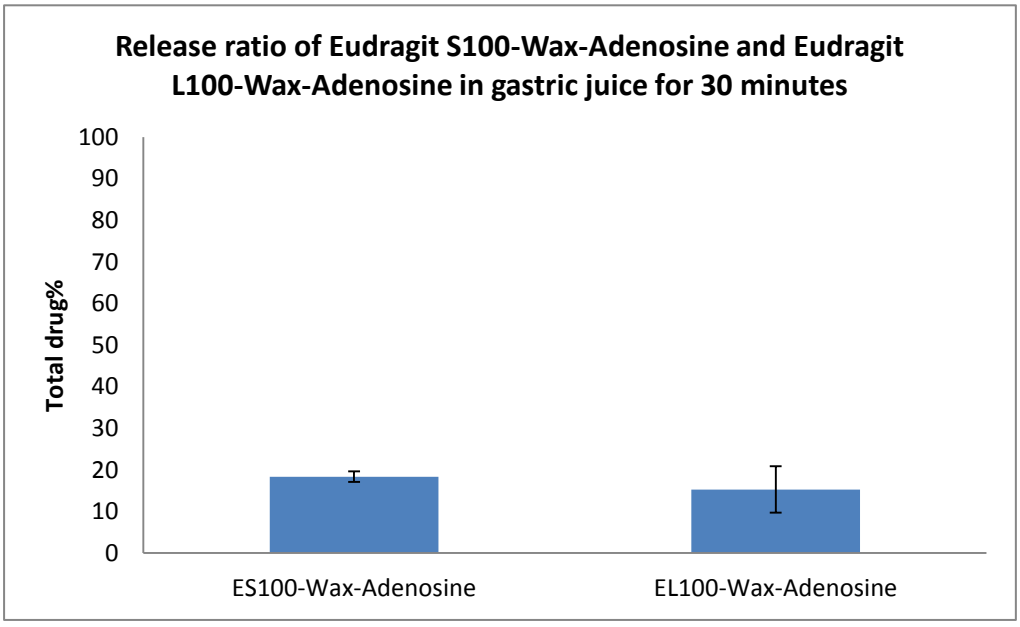

Figure 17. Release ratio of Eudragit S100-Wax-Adenosine and Eudragit

L100-Wax-Adenosine in gastric juice for 30 minutes

Particle	Release ratio after 30 min gastric juice
Eudragit S100-Wax-Adenosine	18.37% ± 1.3%
Eudragit L100-Wax-Adenosine	15.27% ± 5.58%

Table 3. Release ratio for Eudragit S100-Wax-Adenosine and Eudragit L100-Wax-Adenosine encapsulation in gastric juice for 30 minutes.

Drug release curve

Human colon pH is about 7.5. The colon pH environment is imitated to test the Eudragit S100–Wax-Adenosine and Eudragit L100-Wax-Adenosine dug release kinetics and draw the release curve.

For Eudagit S100-Wax-Adenosine encapsulation, I use 25405 ug of the sample, add 10 mL pH=7.5 PBS, 37℃ 220 rpm shake. Pick up 100 uL sample at 0.5h, 1h, 2h, 3h, 4h, 5h and 6h, add back the same volume of PBS to the sample. Then dilute the sample for 10 folds and test at 260nm.

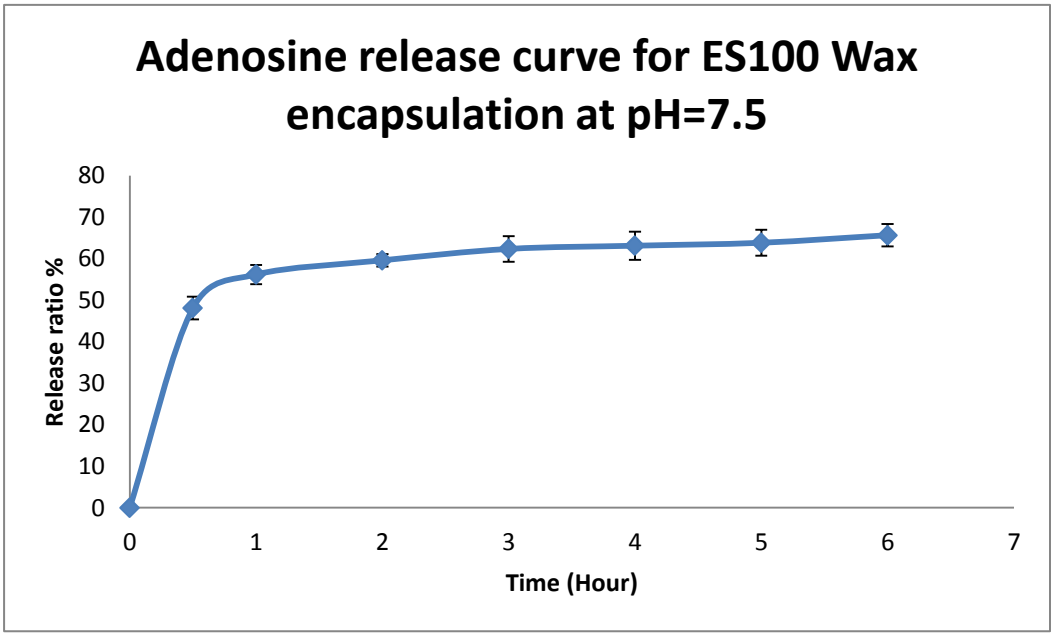

Figure 18. The release curve for Eudragit S100-Wax-Adenosine encapsulation at pH=7.5 for 6 hours

To calculate the final release ratio for Eudragit S100-Wax-Adenosine:

Sample weight: 25405 ug;

Loading efficiency: 4.78%

Loading adenosine in the sample is 25405 ug x 4.78% =1214.365 ug;

After 6 hour release, the absorbance is 0.42;

Standard curve: y=20.042x-0.4537;

So y=7.9706 ug/mL;

Since it was diluted for 10 fold, the actual concentration of the sample is

10 x 7.9706 ug/mL= 79.706 ug/mL;

Total release amount of adenosine in 10 mL PBS is 10 x 79.706 ug/mL=797.06 ug;

$$Release\ ratio = \frac{Released\ drug\ amount}{Total\ encapsulated\ drug\ amount} \times 100\%$$

$$Release\ ratio = \frac{797.06\ ug}{1214.365\ ug} \times 100\% = 65.53\%$$

Time	Release ratio
0	0
0.5	48.09% ± 2.73%
1	56.17% ± 2.29%
2	59.58% ± 1.49%
3	62.34% ± 3.07%
4	63.11% ± 3.41%
5	63.82% ± 3.11%
6	65.64% ± 2.68%

Table 4. Release ratio for Eudragit S100-Wax-Adenosine encapsulation in 6 hours pH=7.5 PBS

For Eudagit L100-Wax-Adenosine encapsulation, I use 26233 ug of the sample, add 10 mL pH=7.5 PBS, 37℃ 220 rpm shake. Pick up 100 uL sample at 0.5h, 1h, 2h, 3h, 4h, 5h and 6h, add back the same volume of PBS to the sample. Then dilute the sample for 10 folds and test at 260nm.

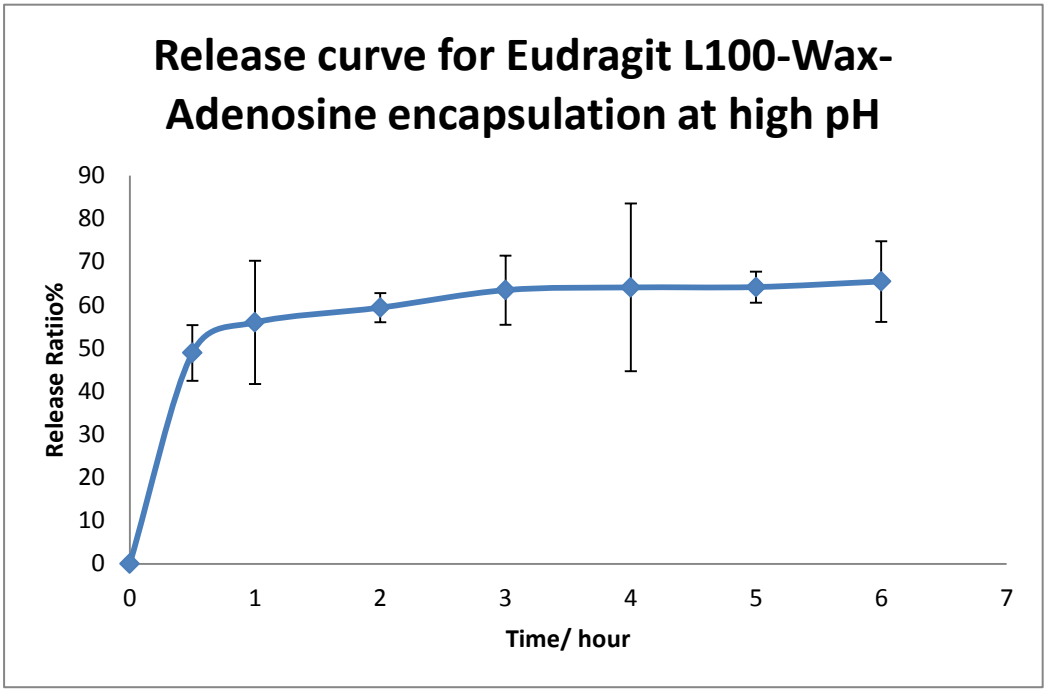

Figure 19. The release curve for Eudragit L100-Wax-Adenosine encapsulation at pH=7.5 for 6 hours

To calculate the final release ratio of

Eudragit S100-Wax-Adenosine:

Sample weight: 26233 ug;

Loading efficiency: 6.092%

Loading adenosine in the sample is 26233 ug x 6.092% =1597.6 ug;

After 6 hour release, the absorbance is 0.544;

Standard curve: y=20.042x-0.4537;

So y=10.45583 ug/mL;

Since it was diluted for 10 fold, the actual concentration of the sample is

10 x 7.9706 ug/mL= 104.5583 ug/mL;

Total release amount of adenosine in 10 mL PBS is 10 x 104.5883 ug/mL=1045.883 ug;

$$Release\ ratio = \frac{Released\ drug\ amount}{Total\ encapsulated\ drug\ amount} \times 100\%$$

$$Release\ ratio = \frac{1045.883\ ug}{1597.6\ ug} \times 100\% = 65.44\%$$

Time	Release Ratio
0	0
0.5	48.89% ± 6.44%
1	55.95% ± 14.29%
2	59.38% ± 3.39%
3	63.44% ± 8.00%
4	64.07% ± 19.46%
5	64.15% ± 3.59%
6	65.45% ± 9.35%

Table 5. Release ratio for Eudragit L100-Wax-Adenosine encapsulation in 6 hours pH=7.5 PBS

Discussion

From DLS data, the particle size for Eudragit S100-Wax-Adenosine and Eudragit L100-Wax-Adenosine encapsulation is 154.945±146.807um and 106.896±28.313 um that closely matches the SEM picture of the morphology. This proves that I

successfully formulated the particle.

For gastric juice released for 30 minutes, from the data we can see that when encapsulated by Eudragit S100 or Eudragit L100 and wax, the release speed of the particle decreases a lot. Thus, coating with these materials make the particle stable in the acid environment and protects them from deterioration by pepsin. The particles keep more than 80% of the adenosine after they pass though the stomach, allowing greater amount of adenosine to reach the colon.

Rapid diffusion speed is always a barrier for oral delivery of small molecule. By the encapsulation method mentioned here, we can see that in the first two hours, the particle can release about 60% of adenosine. In the last 4 hours, the release speed is low and increases to an approximately 65% release ratio. So the method decreases the diffusion speed of adenosine in a great degree and finally reaches good release ratio.

Since dissolution pH of Eudragit S100 is 7, it can be used for colon delivery. In contrast, Eudragit L100, has a dissolution pH of 6, so it will dissolve in the intestine. The results show that the particle size, loading and encapsulation efficiency, stability in gastric juice and release kinetics are good for Eudragit L100 encapsulation. So this method can also be used for some other materials for the oral delivery of small molecules.

Conclusion

In this research, I developed a method for encapsulation of small molecule (adenosine), by Eudragit S100 or L100 and wax for treatment of CRC. Since adenosine is now known as an anti-inflammatory, the increase of its endogenous and extracellular concentration can suppress inflammation and decrease the risk of CRC [40]. The Eudragit S100-Wax-Adenosine encapsulation can have good stability in gastric juice for 30 minutes, which means the coating can protect the adenosine from fast release into gastric juice. The encapsulation efficiency is more than 50% and the release ratio can reach more than 60% after 6 hours release in alkaline environment. So this is an effective formulation for adenosine oral delivery in order to treat CRC.

Future perspective

Fast diffusion speed and short half life are barriers for adenosine oral delivery. In the future work we can pay more attention to increasing the encapsulation efficiency and stability of adenosine to extend the release time. For example, we can add some surfactants to make the particle more stable or we can change the ratio of adenosine to polymer and wax. But we have to control the toxicity of the reagent used during the encapsulation in order to minimize the harm for patients' health. This method also works for encapsulation by Eudrait L100, so we can use this method to encapsulate some other small molecule for intestine oral drug delivery. The Eudragit-Wax-Adenosine encapsulation also needs to be tested *in vivo*.

Developing approach for encapsulate live *P. dist* or bacterial lysate for oral delivery

Background and Introduction

P.dist bacteria (*Parabacteroides distasonis*)

Even though the environment in gastrointestinal tract is complex, more than 90% of the intestinal microbiota are obligate anaerobes [11]. Literature shows that the bacteria environment is strongly linked with host immune system [11]. When host immune system is dysfunctional, the intestinal microbiota will also change and sometimes will lead to inflammation [41-43].

Parabacteroides distasonis (*P.dist*) is a kind of anaerobic bacteria commonly found in the gastrointestinal tract. It has been suggested that administering live *P.dist* or bacteria lysate into the colon area could suppress the inflammation and lower the risk of CRC. There are three possible reasons for this phenomenon. First, the membrane of *P.dist* can change the environment into anti-inflammatory mode by regulating the production of cytokine at gut. Second, the membrane of *P.dist* has proved that it can maintain a stable bacteria environment in the gastrointestinal tract and thus, reduces the risk of inflammation. Third, the membrane of *P.dist* can trigger some immune system response [11]. Macrophages functions are included in this procedure[44, 45]. From literature, the direct change of both *P.dist* and live *P.dist* membranes is correlated with the amount of TNF-α, a kind of production of macrophage [11]. T_{regs} (T

regulatory cell) also has a relationship with *P.dist* and live *P.dist* membranes towards the regulation of anti-inflammation [46, 47].

The stomach provides a harsh environment for various biologics, including live bacteria and biologics derived from bacteria lysate. The hypothesis here is administration of *P.dist* either live or cell lysate into colon can minimize CRC. *P.dist* are aerobic bacteria which can't survive *in vitro*. If I use live *P.dist* for the encapsulation, the techniques will be very complex. I encapsulate live *E.coli* as a model for *P.dist* and finally find a way to delivery live *P.dist* in order to anti-inflammation.

Eudragit FS30D

Eudragit FS30D, 30% aqueous dispersion, also a kind of pH sensitive material can dissolve when pH above 7.

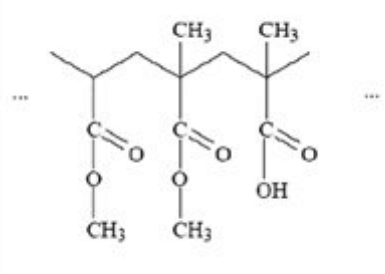

Figure 20. Eudragit FS30D chemical structural formulas Picture from

http://eudragit.evonik.com/

Some people use it as the outer layer for double layer emulsion. For example, they use Eudragit RL+RS as the inner layer for encapsulation of diclofenac sodium and Eudragit FS30D as the outer layer to protect the drug at the place which pH between Eudragit FS30D and Eudragit RL+RS dissolution pH [48]. In other research, people use Eudragit FS30D to encapsulate meloxicam and can reach 100% release in pH above 7.4 release PBS[49].

Single Emulsion

Single emulsion involves one liquid/gas phase dispersing into another immiscible liquid phase [50, 51]. There are three main kinds of single emulsion: water-in –oil single emulsion, oil-in-water single emulsion and multiple emulsion [50]. In this research, I use water-in-oil single emulsion to encapsulate the bacteria lysate and live bacteria. There are many types of oil that can be used as the outside oil phase such as vegetable oil, mineral oil and so on [52]. One issue with the water-in-oil single emulsion is that the particle sometimes may not be very stable after emulsification. The water-in-oil single emulsion can be classified into two kinds: stable emulsion and unstable emulsion [50].

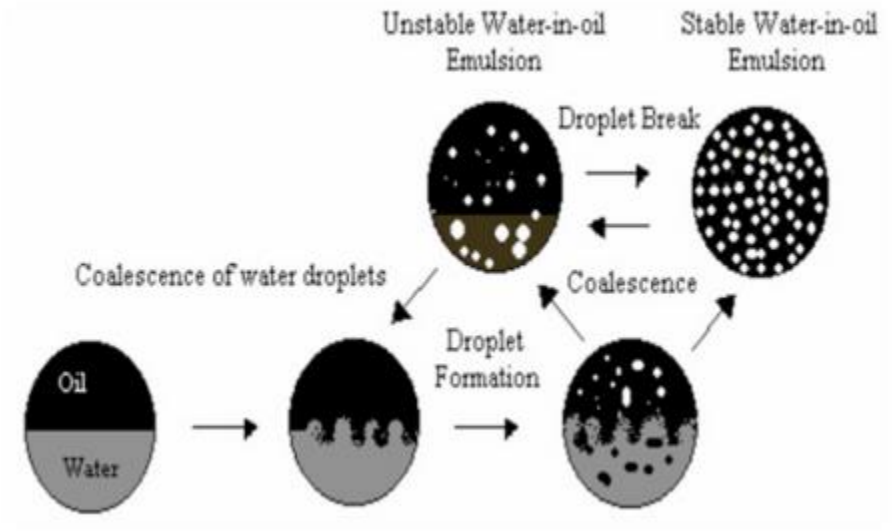

Figure 21. Water-in-oil single emulsion formulation Wong, S.F., J.S. Lim, and S.S. Dol, *Crude oil emulsion: A review on formation, classification and stability of water-in-oil emulsions.* Journal of Petroleum Science and Engineering, 2015. **135**: p. 498-504.

To solve this problem, people usually use some surfactants to stabilize the particle of W/O single emulsion. For example catanionic surfactants can stabilize the W/O emulsion [52]. Some other chemicals can also be used as the surfactant for W/O single emulsion, such as span 60 and span 80 [53].

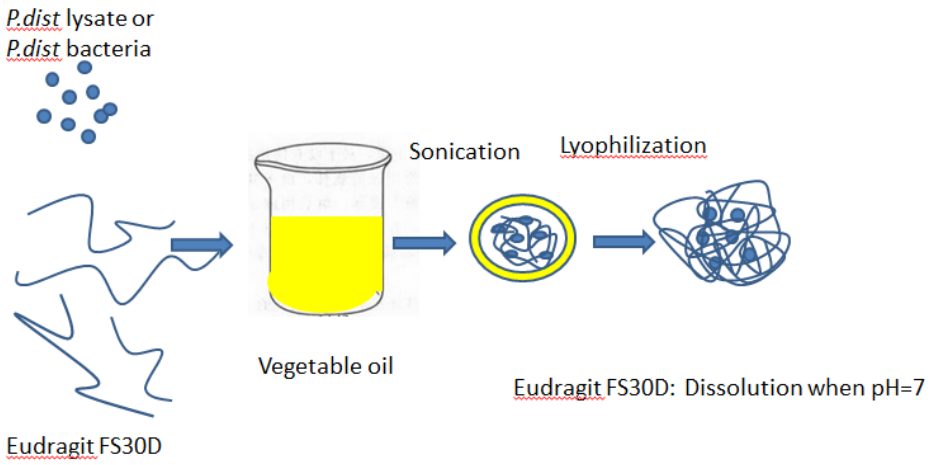

Figure 22. Experimental design for P.dist lysate and live bacteria

In this research I use Eudragit FS30D live bacteria or *P.dist* lysate as the inner water phase and vegetable oil as the extern oil phase to do the water-in-oil single emulsion. The reason that I change the polymer is because I use vegetable oil to formulate the particle. However, vegetable oil can't evaporate and we need water-soluble material to encapsulate the membrane.

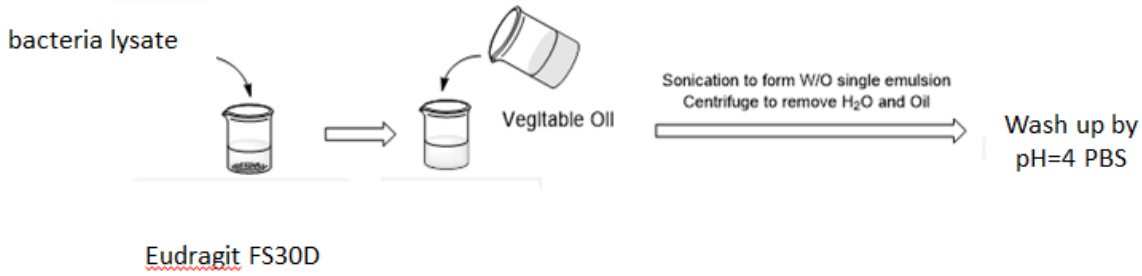

Figure 23. Emulsion procedure for Eudragit FS30D-bacteria lyste encapsulation

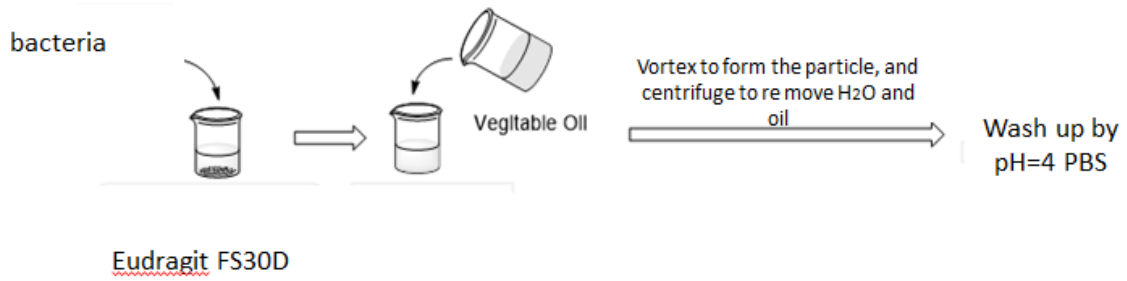

Figure 24. Emulsion procedure for Eudragit FS30D-*E.coli* encapsulation

Figure 23 shows the Eudragit FS30D-lysate encapsulation procedure. By vortex and sonication the particles are formulated. Many often use lyophilization or spray dry to obtain powder like particles [54, 55]. In this research I use lyophilization to remove the water. Figure 24 shows the emulsion procedure for *E.coli* encapsulation. I only use vortex to form the particle and omit the lyophilization step since sonication and lyophilization will kill the bacteria.

Materials and Methods

Materials

Drugs and reagent

Phosphate buffered saline 10X (Boston Bioproducts BM-220), Sodium hydroxide (Fisher Scientific BP359-500), Hydrochloric acid(SIGMA-ALDRICH 320331), Vegetable oil (Golden Choice 48OL), Agar (Fisher scientific BP1423-500), LB Broth Miller (Fisher scientific BP1462-500), Glass beads solid 3mm (Fisher scientific 11-312A), Pierce BCA Protein Assay Kit (Thermo scientific 23227), Plastic pH indicator strips (Fisher Scientific 13-640-516), Gastric Juice Artificial (Aqua Solutions G1618-1L)

Facilities

Zeta Potential Analyzer (ZetaPALS), Branson 5510 Ultrasonic, Balance (Mettler AE100), Forma Direct Heat CO_2 Incubator (Thermo Scientific), Tissue culture plate 96 wells (Corning Incorporated 353072), Centrifuge (Eppendorf 5840 R), Incubator Shaker (New burnswick scientific Co.LNC), Lyophilizer (Labconco), Electronic pH meter (Accumet basic AB15 Plus), SpectraMaxM2/M2 Plate reader, Isotemp Waterbath (Fisher scientific), Analog Vortex Mixer (VWR)

Methods

P.dist membrane encapsulation

Encapsulation preparation

Weigh 15mg *P.dist* membrane, dissolve them in 1mL distilled water;

Add 30% Eudragit FS30D at weight ratio 1:3 (membrane: Eudragit FS30D);

Mix them together by vortex for 1minutes (this solvent called inner water phase);

Drop by drop add the inner water phase into vegetable oil with the volume ratio 1:4 (inner water phase : oil phase);

Vortex the sample for 1 minute;

Sonication the sample for 1 minute;

Centrifuge at 12,000 rcf for 10 minutes;

Discard the supernatant, then wash up the sample by pH=4 PBS;

Centrifuge at 12,000 rcf for 10 minutes then discard the supernatant;

Was up for three times by pH=4 PBS;

Freeze the sample at -20℃ fridge;

Lyophilization.

Particle size

Add 2mg sample into 1mL distilled water then vortex until the sample dissolved;

Put the sample into DLS and test the size

Morphology

Use SEM to take the picture of Eudragit FS30D-Membrane encapsulation.

Particle stability

Preheat the HCl at 37℃ water bath;

Put the sample into 5mL preheated gastric juice at 37℃ water bath for 30 minutes

Centrifuge at 12,000rcf for 10 minutes;

Use 100uL supernatant then test the BCA assay

Encapsulation efficiency

The calculation formula is the same with the adenosine encapsulation

Weigh 5mg sample;

Put the sample into 0.1M NaOH reagent then vortex;

When sample all dissolve use 100uL for test of BCA assay

Membrane release kinetics

Prepare pH=7.5 PBS solvent, water bath at 37℃ for preheat;

Use 20mg sample then add 5mL preheated PBS;

Put the particle sample solvent into 37℃ incubator shaker at 220rpm;

Use 100uL of the sample at t=0.5h, 1h, 2h, 3h, 4h, 5h, 6h;

Add 100uL pH=7.5 PBS back into the sample solvent to keep the volume constant each

time after removal of the sample;

Test the BCA assay for all the samples.

BCA assay

Prepare for work reagent, mix reagent A and reagent B with ratio 50 : 1 v/v;

Add 25uL sample into 200uL work reagent then incubate at 37℃ for 30 minutes;

Put the sample at room temperature until it cool down;

Test the absorbance at 562nm to get the concentration.

E.coli* bacterial encapsulation model for *P.dist

Encapsulation preparation

Get the CAS-9 *E.coli* from -80℃ fridge;

Awaken the *E.coli* bacteria then cultivate in Lb medium 37℃ incubator shaker over night;

Test the original OD_{600} value;

Prepare 2% Eudragit FS30D solvent in LB medium;

Put 1mL of the original *E.coli* in 3mL 2% Eudragit-LB medium solvent;

Mix them together by vortex for 1 minute (this called inner water phase);

Using 2 μm filter to deal with the vegetable oil;

Drop by drop add the inner water phase into vegetable oil with the ratio 1:4 v/v, that is 16mL vegetable oil;

Vortex for 2minutes;

Centrifuge at 8,000 rcf for 10 minutes;

Discard the supernatant;

Wash up by 5mL pH=4 PBS then centrifuge at 8,000 rcf for 10 minutes then discard the supernatant;

Particle size

Add 2mg sample into 1mL distilled water then vortex until the sample dissolved;

Put the sample into DLS and test the size

Morphology

Use SEM to take the picture of Eudragit *FS30D*-E.coli encapsulation.

Test of encapsulation efficiency

Add 50mg of the sample into 1mL 0.1M NaOH, then vortex for 1 minute until the particle dissolve;

Test the OD_{600} of the sample

Release ratio and livability of Eudragit FS30D-*E.coli* encapsulation

Preheat the gastric juice at 37℃;

Add 100mg of the sample into 5mL preheated gastric juice;

Water bath at 37℃ for 30 minutes;

Centrifuge at 8,000 rcf for 10 minutes then discard the supernatant;

Add 5mL pH=7.5 medium into the sample;

Put the sample into 4℃ incubator shaker for 6 hours;

Get 100uL from the released solvent gradient dilute the sample by 10, 10^2, 10^3 fold;

Do the plate culture overnight at 37℃;

Counting the colonies on the plate and taking the picture.

Results and Discussion

P.dist lysate encapsulation

Results

Figure 25. Eudragit FS30D-membrane encapsulation

Particle size

After adding the Eudragit FS30D-membrane encapsulation into 1mL distilled water, then vortex and sonication to disperse the particle. Use DLS to test particle size, the size is 907.47±265.23nm.

Morphology

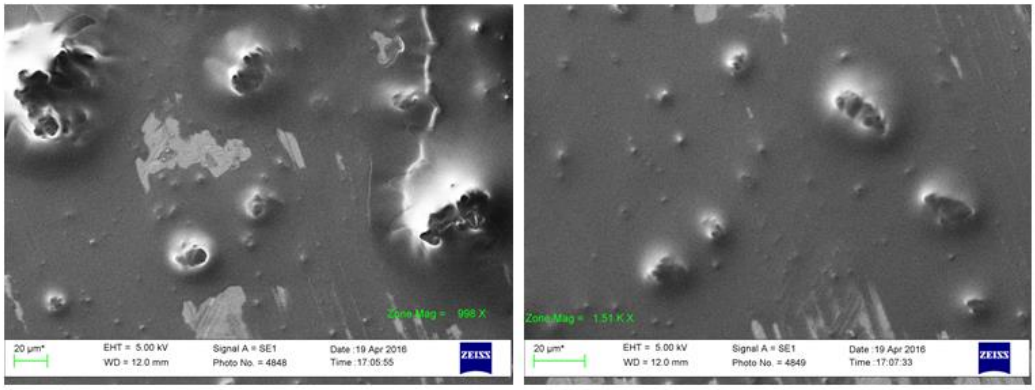

Figure 26. Morphology for Eudragit FS30D-membrane encapsulation

From figure 26 since the particle size is about 1 um from DLS, the small little spheres may be the particles. The large spheres may be the conjunction.

Loading and Encapsulation Efficiency

Before calculate the loading and encapsulation efficiency, I do a standard curve for *P.dist* membrane. Since cell membrane is composed of proteins and lipid bilayer, so I test the concentration of protein to indicate the membrane concentration by BCA assay.

X: Absorbance of membrane at 562 nm;

Y: Concentration of membrane ug/mL;

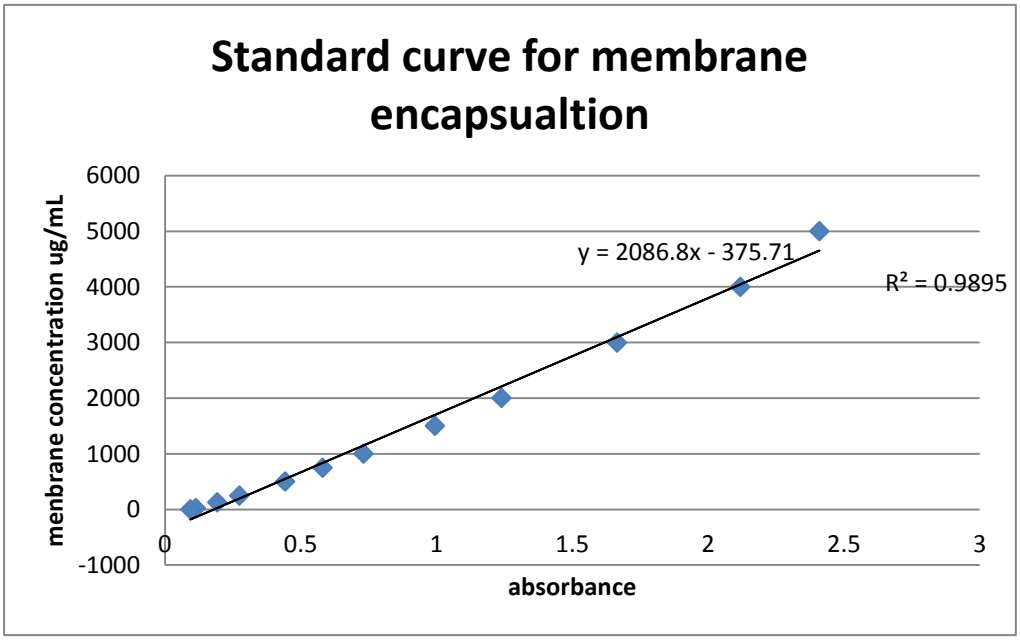

Figure 27. Standard curve for *P.dist* membrane

To test the loading and encapsulation efficiency, I use 8400 ug of the sample. Put them into 1mL 0.1M NaOH, vortex until the sample all dissolve. Then do BCA assay to test the concentration.

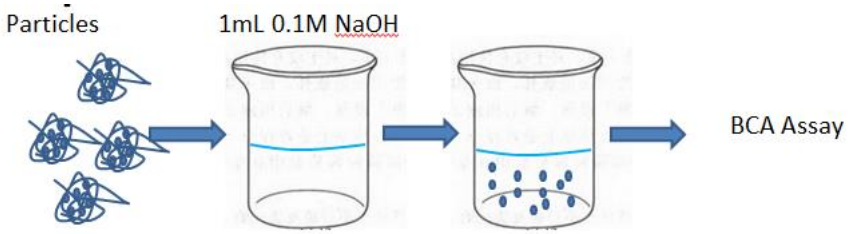

Figure 28. Loading and encapsulation efficiency test for *P.dist* membrane encapsualtion

Sample weight: 8400 ug;

Standard curve for *P.dist* membrane: y=2086.8x-375.71

The absorbance of the sample: 0.2933;

Put absorbance into standard curve: y=236.418 ug/mL;

P.dist membrane amount in 1mL NaOH: 236.418 ug/mL x 1mL = 236.418 ug;

$$\text{Actual Loading} = \frac{Drug\ amount\ in\ sample}{Sample\ weight} \times 100\%$$

$$Actual\ Loading = \frac{236.418\ ug}{8400\ ug} \times 100\% = 2.81\%$$

The total sample weight for the system: 431mg;

Total encapsulated membrane amount is: 431mg x 2.81%=12.13 mg;

Total initial amount of membrane put into system: 15mg;

$$\text{Encapsulation Efficiency} = \frac{Actual\ drug\ amount\ encapsulated\ in\ sample}{Theoretical\ amount\ of\ drug\ should\ be\ encapsulated} \times 100\%$$

$$Encapsulation\ efficiency = \frac{12.13\ mg}{15\ mg} \times 100\% = 80.87\%$$

Particle	Loading efficiency	Encapsulation efficiency
Eudragit FS30D-membrane	2.81% $\pm$ 0.146%	80.87% $\pm$ 4.18%

Table 6. Loading and encapsulation efficiency for Eudragit FS30D-membrane encapsulation

Release Ratio at Low pH

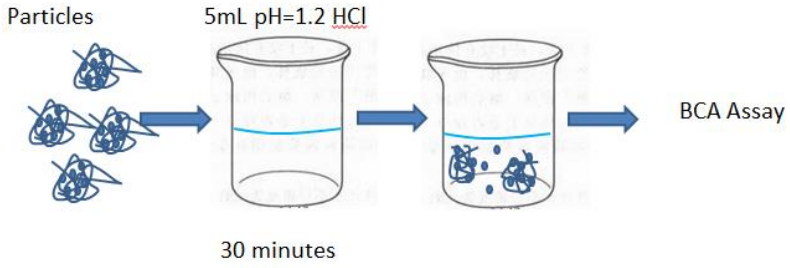

Figure 29. Acid environment stability for *P.dist* membrane encapsulation

To test whether the formulation can slow down the passive diffusion of membrane and provide protection, I put 24000 ug sample into 5mL pH=1.2 HCl. Since gastric juice contains a lot of pepsin, I will use BCA to calculate the concentration of the membrane as pepsin will introduce tremendous noise into my sample. Here I only use HCl to imitate the acid environment. Immerse the sample into HCl at 37℃ for 30 minutes, then centrifuge, pick up the supernatant to test the BCA assay.

Sample weight: 24000ug;

Absorbance of supernatant: 0.183;

Standard curve for *P.dist* membrane: y=2086.8x-375.71

Put absorbance into the standard curve: y=6.1744 ug/mL;

Membrane release into 5mL gastric juice is: 5 mL x 6.1744 ug/mL=30.872 ug;

Loading efficiency: 2.81%

Total drug loading in sample: 24000 ug x 2.81%= 679.2 ug;

$$Release\ ratio = \frac{Released\ drug\ amount\ in\ HCl}{Total\ encapsulated\ drug\ amount} \times 100\%$$

$$Release\ ratio = \frac{30.872\ ug}{679.2\ ug} \times 100\% = 4.55\%$$

Particle	Release ratio in low pH
Eudragit FS30D-membrane	4.55% ± 3.07%

Table 7. Release ratio for Eudragit FS30D-membrane at pH=1.2 HCl release ratio

Release curve at alkaline environment

Human colon pH is about 7.5. The colon pH environment is imitated to test the Eudragit FS30D-membrane dug release kinetics and draw the release curve. I prepare 24000 ug of the sample and add 5 mL pH=7.5 PBS, 37℃ 220 rpm shake. Get 100 uL sample at 0.5h, 1h, 2h, 3h, 4h, 5h, 6h and add back the same volume of PBS to the sample. Then do the BCA assay.

To calculate the final release ratio for Eudragit FS30D-membane encapsulation:

Sample weight: 24000 ug;

Loading efficiency: 2.81%

Loading adenosine in the sample is 24000 ug x 2.81% =679.2 ug;

After 6 hour release, the absorbance is 0.2374;

Standard curve: y=2086.8x-375.71;

So y=119.8345 ug/mL;

Total released membrane amount in 5 mL PBS is: 119.8345 ug/mL x 5mL=599.1772 ug

$$Release\ ratio = \frac{Released\ drug\ amount}{Total\ encapsulated\ drug\ amount} \times 100\%$$

$$Release\ ratio = \frac{599.1772\ ug}{679.2\ ug} \times 100\% = 88.21\%$$

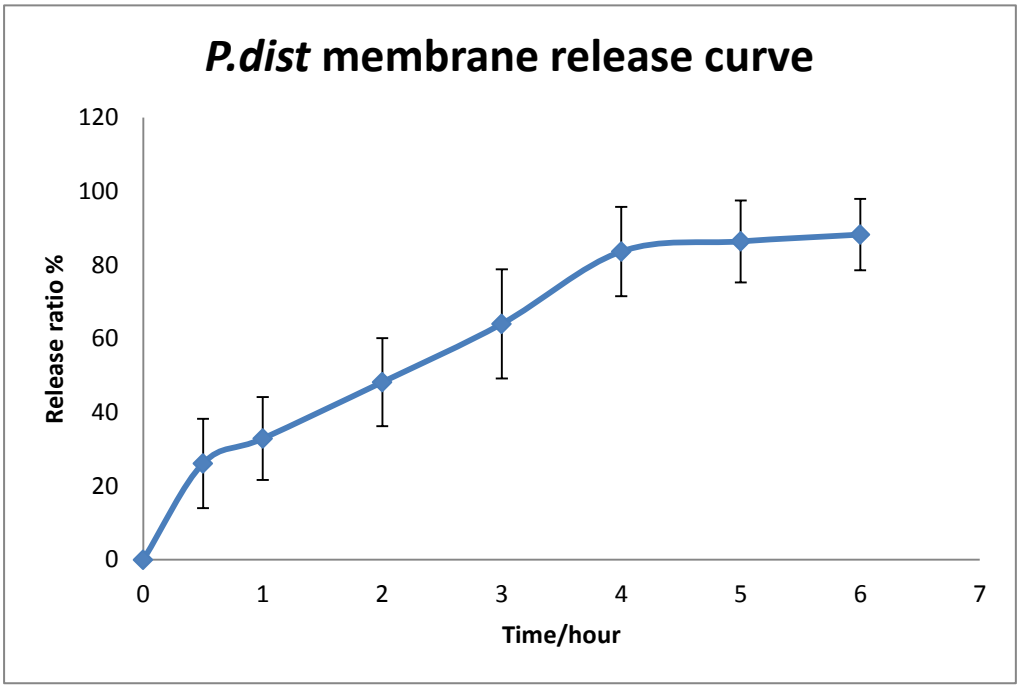

Figure 30. Release curve for Eudragit FS30D-membrane in pH=7.5 PBS for 6 hours

Time	Release ratio
0	0
0.5	26.13% ± 12.12%
1	32.86% ± 11.23%
2	48.17% ± 11.93%
3	63.99% ± 14.79%
4	83.62% ± 12.13%
5	86.37% ± 11.13%
6	88.21% ± 9.69%

Table 8. Release ratio for Eudragit FS30D-membrane encapsulation in pH=7.5 PBS for 6

hours.

Discussion

The DLS data shows that the particle size for Eudragit FS30D-membrane is about 1um, so in the SEM picture, the small spheres may be the particles. The larger spheres may be the conjunction.

The loading efficiency is about 2.8%, which may be because oil remains in our sample. Since vegetable oil is very sticky, it is impossible for us to completely remove. Vegetable oil is non-toxic so it is no worry for the patients to take. Even though the loading efficiency is low, the encapsulation efficiency is about 80%. That means the loss is low and we can decrease the expense of the formulation.
When I immerse the particle into pH=1.2 HCl for 30 minutes, it only releases about 4% of the membrane. The particle is very stable in the acid environment.

For the release kinetics, the membrane can maintain a stable and fast release speed in the first 4 hours. In the last two hours, the release ratio is low and reaches about 88%.

E.coli as the model for P.dist live bacteria encapsulation

Results

Particle size

After adding the Eudragit FS30D-*E.coli* sample into 1 mL distilled water, we can get the particle size by DLS. The size is 15.677±10.657 um.

Morphology

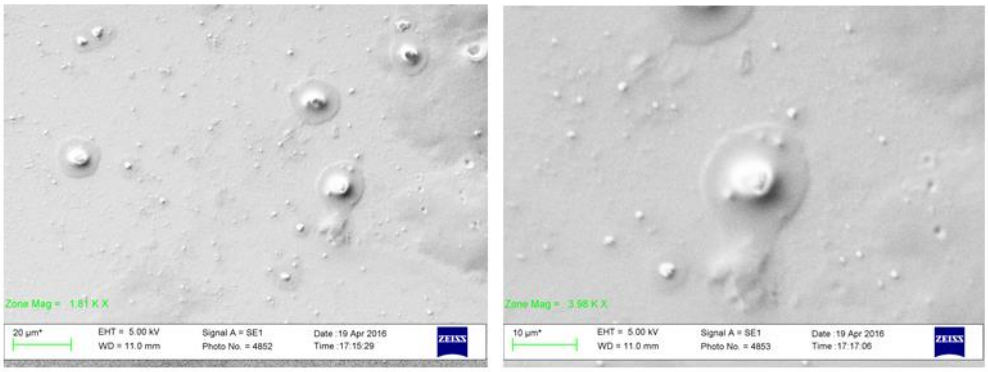

Figure 31. Morphology for Eudragit FS30D-*E.coli* encapsulation by SEM

The size of the particle closely matches the picture of SEM, so the large sphere is the particle.

Loading and Encapsulation Efficiency for Eudragit FS30D-*E.coli* encapsulation

Before I calculate the loading and encapsulation efficiency of the formulation, I do a standard curve for *E.coli* concentration and OD600 absorbance.

X: OD_{600} value;

Y: Bacteria concentration $x10^8$ CFU/mL;

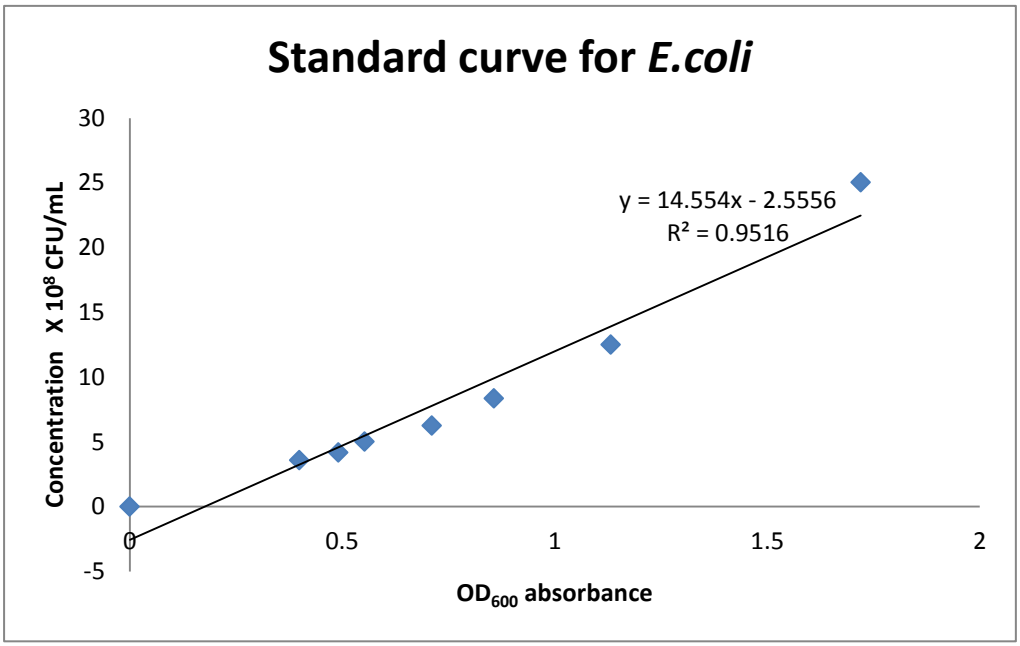

Figure 32. Standard curve for *E.coli* concentration

The initial OD*600* value for the bacteria that I put into the system is: 1.72

The standard curve: y=14.554x – 2.5556;

Put the OD_{600} value into the standard curve we can get the initial *E.coli* concentration:

y= 22.52 x 10^8 CFU/mL;

Total volume of bacteria that I put into the system is 1mL, so the total amount of *E.coli* that I put into the system is: 22.52 x 10^8 CFU/mL x 1mL= 22.52 x 10^8 CFU;

To get the loading and encapsulation efficiency, I use52mg of the sample and add them into 1mL 0.1M NaOH vortex them test the OD_{600} value.

The OD_{600} value is: 0.402;

Put the OD_{600} value into the standard curve: y=3.295 x 10^8 CFU/mL;

Total bacteria amount in 1mL NaOH is: 3.295 x 10^8 CFU/mL x 1mL =3.295 x 10^8 CFU;

$$Loading\ Efficiency = \frac{Encapsulated\ bacterial\ amount\ in\ sample}{Sample\ weight} \times 100\%$$

$Loading\ Efficiency$ =((3.295 × 10^8 CFU)/(0.052g))× 100% =63.37 x 10^8 CFU/g;

Total sample weight is: 317mg;

Total encapsulated bacteria amount: 0.317g x 63.37 x 10^8 CFU/g=20.09 x 10^8 CFU;

$$Encapsulation\ Efficiency = \frac{Total\ encapsulated\ bacteria\ amount}{Initial\ bacteria\ amount} \times 100\%$$

Encapsulation Efficiency = ((20.09 x 10^8 CFU)/(22.52 x 10^8 CFU))× 100% = 89.2%

Actual Loading efficiency	63.37 x 10^8 CFU/g ± 1.56 x 10^8 CFU/g
Encapsulation efficiency	89.2% ± 2.2%

Table 9. Loading and Encapsulation efficiency for Eudragit FS30D-*E.coli* encapsulation

Viability and Live bacteria loading

To test the viability of the sample, I use 105mg of the sample immerse it into 5mL gastric juice at 37℃ for 30 minutes then centrifuge and discard the supernatant. Release the sample in 5mL pH=7.5 medium for 6 hours, test the OD_{600} value and plate culture.

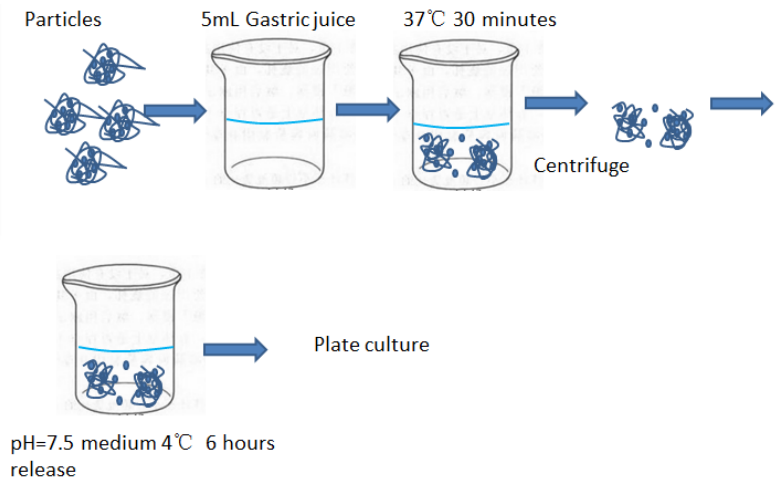

Figure 33. Viability and live bacteria loading for *E.coli* encapsulation

Sample weight: 105 mg;

After 6 hours OD_{600} is: 0.253;

Standard curve: y=14.554x – 2.5556

Put the OD600 value into standard curve: y=1.127 x 10^8 CFU/mL;

Released bacteria amount in 5 mL pH=7.5 medium:

5mL x 1.127 x 10^8 CFU/mL= 5.6328 x 10^8 CFU

Loading efficiency: 63.37 x 10^8 CFU/g

Total encapsulated bacteria amount is: 0.105g x 63.37 x 10^8 CFU/g= 6.6539 x 10^8 CFU

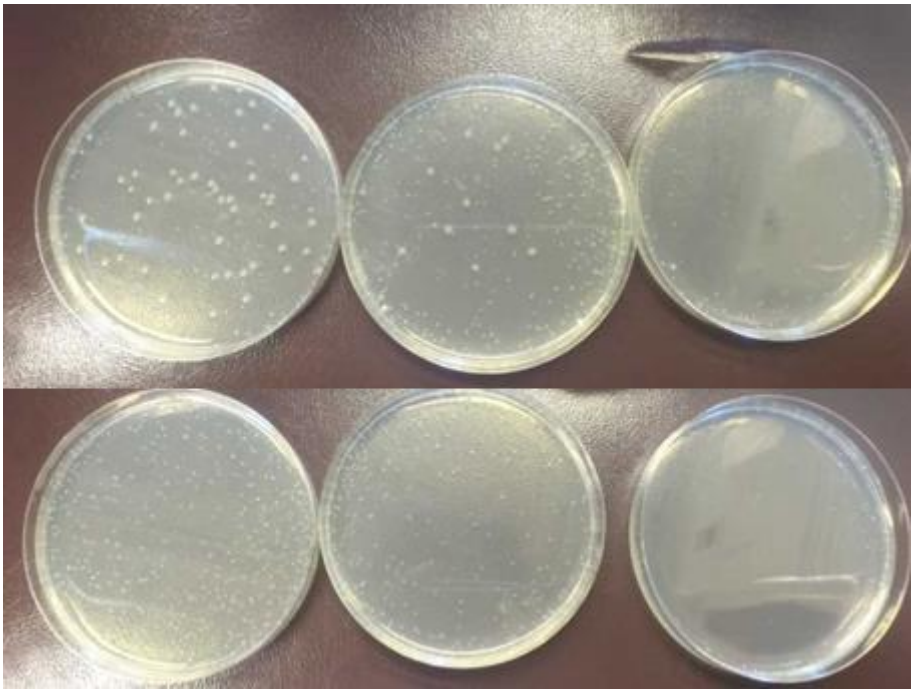

Figure 34. Plate culture result for released bacteria after 6 hours pH=7.5 medium at 4℃. First row is the Eudragit FS30D-*E.coli* encapsulation diluted to 10 folds, 100 folds and 1000 folds; Second row is the using medium instead of Eudragit FS30D to finish the encapsulation procedure and diluted to 10folds, 100 folds and 1000folds.

I pick up 100 uL of the released sample plate culture in 37℃ CO_2 incubator overnight and count the colonies. The blank group, which uses medium instead of Eudragit FS30D during the particle preparation procedure, can't survive after 30 minutes gastric juice and 6 hours pH=7.5 release. But the Eudragit FS30D coating *E.coli* can survive according to figure 34.

The number of colony in 100 uL released sample is $(5.19 \pm 1.47) \times 10^2$ CFU;

Since the total released sample is 5mL, so the total released bacteria amount is:

5.19×10^2 CFU x 5000uL ÷ 100uL=2.595×10^4 CFU;

$$Viability = \frac{Live\ bacteria\ amount}{Total\ encapsulated\ bacteria\ amount} \times 100\%$$

Viability=(2.595 x10^4 CFU)/(6.6539 x 10^8 CFU)=0.0039%

$$Live\ bacteria\ loading = \frac{Live\ bacteria\ amount}{Sample\ weight} \times 100\%$$

Live bacteria loading=(2.595 x10^4 CFU)/0.105g= 24.71 x10^4 CFU/g

Livability After Release	0.0039% ± 0.001%
Live Bacteria Loading	(24.71 ± 6.72) x10^4 CFU/g

Table 10. Viability and live bacteria loading after 6 hours release in pH=7.5 medium

Discussion

The particle size tested by DLS is consistent with the SEM photo. For the loading and encapsulation efficiency experiments, both of them are high but the final viability is low. After the bacteria goes through the encapsulation procedure, immersing into gastric juice for half an hour and release in pH=7.5 for 6 hours, many of them die. OD_{600} test method can test all the bacteria in the system no matter they are alive or not. So the high loading and encapsulation efficiency doesn't necessarily correlate to high viability. I released the bacteria in 4℃ for 6 hours because I wanted to get the actual bacteria amount in my sample. If I released the sample in 37℃, the bacteria will grow at that temperature and bring large noise into our experiment. After 6 hours release in pH=7.5 medium for both Eudragit FS30D-*E.coli* formulation and

non-Eudragit FS30D existence blank group, the plate culture result shows that Eudragit FS0D has the function to protect the *E.coli* during immersion into gastric juice and release for 6 hours in pH=7.5 PBS. Even though the viability is low, the live bacteria loading is about (24.71 ± 6.72) x10^4 CFU/g. There are still bacteria alive in the drug. When we take the particles, after the live bacteria are released at the colon, they may grow to high concentrations and have the function to suppress inflammation.

Conclusion

For *P.dist* membrane encapsulation by Eudragit FS30D, as the result of the vegetable oil remains in the formulation, the actual loading efficiency is low but the encapsulation efficiency is more than 80%. This means there is not very much loss during the encapsulation procedure. In addition, the particle can reach about 88% release ratio after 6 hours release in pH=7.5 PBS. The Eudragit FS30D encapsulation can also protect the particle from acid environment since the release ratio at low pH is about 4.55%. Thus, the Eudragit FS30D-*P.dist* membrane encapsulation is effective for oral delivery.

For *E.coli* model of *P.dist* encapsulation, according to the data of DLS, our particle size is about 15 um and consistent with the SEM picture. The loading efficiency of Eudragit FS30D-*E.coli* is 63.37 x 10^8 CFU/g, and after 6 hours release at pH=7.5 medium, the bacteria can release about 84.5%. But the dead bacteria can introduce large noise into the experiment, so the live bacteria after 6 hours release is much lower than the total encapsulated bacteria amount. The loading is 24.71 x10^4 CFU/g and viability after release is 0.0039%. Even though the viability is low, *E.coli* can survive after immersing into gastric juice and release at pH=7.5 medium by Eudragit FS30D protection. Since the bacteria will grow when it reaches its functional place, they may still have the ability to reach a high concentration even when the initial live bacteria amount is not

high. So the Eudragit FS30D-*E.coli* encapsulation is an effective method for live bacteria oral delivery.

Future Perspective

In this part I encapsulate *P.dist* membrane and get satisfying encapsulation efficiency, release ratio and acid environment stability. I also encapsulate live bacteria *E.coli* as the *P.dist* model. Even though the viability is low, the bacteria can survive after immersing into gastric juice and 6 hours' release at pH=7.5 medium. For the future work, we should test the anti-inflammatory function for encapsulated *P.dist* membrane in mice and patient model. However, the live bacteria encapsulation method still requires improvement to increase the viability. The goal in the future is to use *E.coli* encapsulation method to encapsulate live *P.dist* and test its function *in vivo*.

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