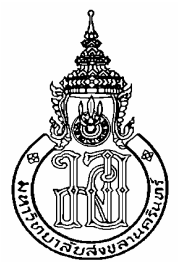

Synthesis of Poly(ethylene) Glycol-5-Amino Salicylic Acid Conjugates for Colonic Specific Drug Delivery

Pathamaporn Sakaopraiphu

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Pharmacy in Pharmaceutical Sciences

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Thesis Title	Synthesis of Poly(ethylene) Glycol-5-Amino Salicylic Acid Conjugates for Colonic Specific Drug Delivery
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ชื่อวิทยานิพนธ์	การสังเคราะห์พอลิเอทิลินกลัยคอล-5-อะมิโนซาลิไซลิก แอซิด คอนจุเกต เพื่อการนำส่งสู่ลำไส้ใหญ่อย่างเฉพาะเจาะจง
ผู้เขียน	นางสาวปฐมาภรณ์ สกาวปรายภู
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บทคัดย่อ

5-อะมิโนซาลิไซลิก แอซิด (5-ASA) ซึ่งเป็นยาในกลุ่มตัวเลือกหลักที่ใช้ในการรักษาอาการอักเสบในบริเวณลำไส้ ได้ถูกพัฒนาให้อยู่ในรูปของพอลิเมอร์ คอนจุเกต เพื่อใช้ในการนำส่งยาไปยังลำไส้ใหญ่อย่างเฉพาะเจาะจง ในการศึกษานี้พอลิเอทิลิน กลัยคอล หรือ PEG (มวลโมเลกุล 5106, 5000, 4000 และ 3677 ดาลตัน) เป็นพอลิเมอร์ที่ละลายน้ำได้ ซึ่งถูกนำมาศึกษาโดยใช้เป็นตัวนำส่งยา พอลิเมอร์ คอนจุเกตที่สังเคราะห์ขึ้นนั้นอยู่ในรูปของสารประกอบเอไมด์และเอสเทอร์ มีจุดประสงค์เพื่อนำส่งตัวยา (5-ASA) ไปยังลำไส้เล็กส่วนปลายและลำไส้ใหญ่ ซึ่งเป็นบริเวณที่มักพบอาการของโรคครอห์นและลำไส้อักเสบ ในการสังเคราะห์ได้เลือกพารา-อะมิโนเบนโซอิก แอซิด (PABA) เป็นตัวเชื่อมต่อระหว่าง PEG กับซาลิไซลิก แอซิด (SA) ซึ่งเป็นสารตั้งต้นในการสังเคราะห์และปลดปล่อยให้ 5-ASA ในลำไส้

เมื่อทำการทดสอบการละลาย พบว่าสารสังเคราะห์ PEG-(PABA-SA) คอนจุเกตที่ได้ สามารถละลายในน้ำได้มากกว่า 5-ASA ถึง 200 เท่า ที่อุณหภูมิ 25 องศาเซลเซียส นอกจากนี้ยังพบว่าคอนจุเกตซึ่งมีพันธะเอสเทอร์ หรือเอไมด์ และพันธะเอโซอยู่ในโครงสร้างทางเคมี มีความคงตัวเมื่อทำการทดสอบในสารละลายซิเตรตบัฟเฟอร์ pH 1.2, ฟอสเฟตบัฟเฟอร์ pH 6.8 และ 7.5 เป็นระยะเวลา 6, 12 และ 24 ชั่วโมงที่อุณหภูมิ 37 องศาเซลเซียส ตามลำดับ ในการทดลองขั้นสุดท้ายได้ทำการทดสอบการปลดปล่อยยา (5-ASA) จากพอลิเมอร์คอนจุเกตในส่วนประกอบในทางเดินอาหารของหนู rat ซึ่งถูกเตรียมในสารละลายซิเตรตบัฟเฟอร์ pH 1.2 (กระเพาะอาหาร), ฟอสเฟตบัฟเฟอร์ pH 6.8 (ลำไส้เล็ก) และฟอสเฟตบัฟเฟอร์ 7.5 (ลำไส้ใหญ่) ซึ่งมีเปปโตนผสมอยู่ร้อยละ 1 เป็นระยะเวลา 6, 12 และ 24 ชั่วโมงที่อุณหภูมิ 37 องศาเซลเซียส ตามลำดับ โดยใช้ HPLC ในการวิเคราะห์ และประเมินผลการทดลอง ซึ่งรูปแบบการปลดปล่อยยาของพอลิเมอร์ คอนจุเกตทุกตัวได้ถูกนำมาเปรียบเทียบกับรูปแบบการปลดปล่อยยาของซัลฟาซาลาซีน (positive compound) พบว่าซัลฟาซาลาซีนสามารถปลดปล่อย 5-ASA ออกมาได้อย่างรวดเร็วในส่วนของลำไส้ใหญ่ภายในเวลา 1 ชั่วโมง ในขณะที่พอลิเมอร์ คอนจุเกตปลดปล่อยยาออกมาอย่างช้าๆ ใน 6 ชั่วโมงแรก และ

สามารถปลดปล่อยยาออกมาได้อย่างต่อเนื่องจนถึงระดับยาสูงสุดภายใน 24 ชั่วโมง นอกจากนี้ยังพบว่าหลังจากการทดสอบการปลดปล่อยยาในลำไส้เล็กเป็นเวลา 12 ชั่วโมง ทั้งพอลิเมอร์ คอนจุเกตและซัลฟาซาลาซีนสามารถปลดปล่อย 5-ASA ออกมาในส่วนของลำไส้เล็กได้บางส่วน แสดงให้เห็นว่าพอลิเมอร์ คอนจุเกตที่ใช้ในการศึกษานี้มีศักยภาพในการนำส่งยาเพื่อรักษาอาการอักเสบในลำไส้เล็กและลำไส้ใหญ่

คำค้น: พอลิเอทิลิน กลัยคอล, 5-อะมิโนซาลิไซลิก แอซิด, พารา-อะมิโนเบนโซอิก แอซิด, การนำส่งยาไปลำไส้ใหญ่

Thesis Title	Synthesis of Poly(ethylene) Glycol-5-Amino Salicylic Acid Conjugates for Colonic Specific Drug Delivery
Author	Miss Pathamaporn Sakaopraiphu
Major Program	Pharmaceutical Sciences
Academic Year	2008

ABSTRACT

For targeting the drug specifically to the colon, the first choice of therapeutic agents, 5-aminosalicylic acid (5-ASA) was developed in polymer conjugated form. Poly(ethylene) glycol (PEG) with molecular weight of 5106, 5000, 4000 and 3677 Da were chosen as water soluble polymers for colonic specific drug delivery. The polymeric conjugates, by both ester and amide bonds were synthesized to accomplish the local delivery of the active compound, 5-ASA, to the terminal ileum (Crohn's disease) and/or the colon (Crohn's disease and ulcerative colitis). *Para*-aminobenzoic acid (PABA) was used as a spacer between the polymer, PEG, and the pro-moeity drug, salicylic acid (SA). The PEG-(PABA-SA) conjugates could be dissolved in distilled water 200 times, compared to 5-ASA at 25^{o}C. The ester, amide and azo bonds containing in the products were stable in citrate buffer pH 1.2, phosphate buffer pH 6.8 and 7.5 after 6, 12 and 24 hours incubation period, respectively, at 37 ^{o}C. The release amounts of the free drug, 5-ASA, from the corresponding products-PEG conjugates in the rats gastrointestinal contents treated with 1% peptone in citrate buffer pH 1.2 (simulated gastric content), phosphate buffer pH 6.8 (simulated intestinal content) and phosphate buffer pH 7.5 (simulated colonic content) for 6, 12 and 24 hours, respectively, at 37 ^{o}C, were evaluated by validated high performance liquid chromatography (HPLC). The drug release profiles of the conjugates were compared to the drug release profiles of sulfasalazine, which was used as positive compound. Sulfasalazine released 5-ASA rapidly in colonic content (within 1 hour), whereas the PEG conjugates presented the slow release profiles in the first 6 hours and continued to release up to maximum within 24 hours. Moreover, the PEG conjugates and sulfasalazine could also release 5-ASA after 12-hour incubation in intestinal content. The results from *in vitro* study demonstrated

that the developed polymer conjugates have potential for use as intestinal and colonic drug delivery systems.

Keywords: Poly(ethylene) glycol (PEG); 5-Aminosalicylic acid (5-ASA); *p*-Aminobenzoic acid (PABA); Colonic drug delivery

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Pathamaporn Sakaopraiphu

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
2^{o} amide	Secondary amide
4f-Spacer	4-Formylated spacer
4-NO_2-PABA-Cl	*p*-Nitro benzoylchloride
5-ASA	5-Aminosalicylic acid
5-HETE	5-Hydroxyeicosatetraenoic acid
ABBC	4-[4-(chlorocarbonyl) phenylazo] benzoylchloride
BA	Benzoylated
BTC	bis(trichloromethyl) carbonate
C. perfringens	*Clostridium perfringens*
C=O	Carbonyl
$CaCl_2.H_2O$	Calcium chloride dehydrate
C_{actual}	Actual concentration of standard solution of each standard
CD	Crohn's disease
CDI	*N*, *N*-Carbonyldiimidazole
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
C_{obs}	Determined concentration of each standard
-COOH	Carboxyl
^{o}C	Degree celcius
d	Doublet
Da	Dalton
dd	Doublet of doublet
ddd	Doublet of doublet of doublet
DMAP	*N*, *N*-Dimethylaminopyridine
DMCC	Direct microscopic clump counts
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
E. coli	*Escherichia coli*

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

ERPF	Effective renal plasma flow
Ether	Diethyl ether
EtOH	Ethanol
g	Gram
g/l	Gram per liter
G3	Third generation
GI tract	Gastrointestinal tract
H_2	Hydrogen (gas)
H_2O	Distilled water
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPMA	*N*-(2-hydroxypropyl) methacrylamide
hr	Hour
IBD	Inflammatory bowel disease
$inch^2$	Square inch
J	Coupling value
kDa	Kilo Dalton
kg	Kilogram
KI	Potassium iodide
lb	Pound
LD_{50}	Median lethal dose
LOD	Limit of detection
LOQ	Limit of quantitation
LTB4	Leukotriene B4
M	Molar
MeOH	Methanol
mg	Milligram
min	Minute
ml	Milliliter

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

mmol	Millimole
mPEG	Monomethoxy poly(ethylene) glycol
MRP2	Multi-drug resistance protein 2
Mw/Mn	Polydispersity
N	Normal
-N=N-	Azo bond
N_2	Nitrogen (gas)
Na_2SO_4	Sodium sulfate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NAT1	*N*-acetyltransferase 1
$-NH_2$	Amino
NH_4OH	Ammonium hydroxide
nm	Nanometer
$-NO_2$	Nitro
PABA	*p*-Aminobenzoic acid
PABA-SA	5-((4-carboxyphenyl)diazenyl)-2-hydroxybenzoic acid azo conjugate
PAH	*p*-Aminohipuric acid
PAH-SA	5-((4-(carboxymethylcarbamoyl)phenyl)diazenyl)-2-hydroxybenzoic acid azo conjugate
Pd/C	Palladium on activated charcoal
PDA	Photodiode-array detector
PEG	Poly(ethylene) glycol
P-gp	P-glycoprotein
pH	A measure of the acidity or alkalinity of a solution, numerically equal to 7 for neutral solutions, increasing with increasing alkalinity
PHEA	Poly [*N*-(2-hydroxyethyl)-*DL*-aspartamide]

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

pKa	The value that is defined from Ka and can be calculated from the Ka value from the equation $pKa = -Log_{10}(Ka)$
ppm	Part per million
PRAS	Prereduced anaerobically sterilized
PTMO	Bis-aminopropyl poly (tetraethyleneoxide)
QC	Quantity controlled
rpm	Rounds per minute
RSD	Relative standard deviation
RT	Room temperature
R^2	Determination coefficient
s	Singlet
S/N	Signal to noise
SA	Salicylic acid
SD	Standard deviation
SHIME	Simulated human intestinal microbial ecosystem reactor
SiO_2	Silica or Silicon dioxide
$SOCl_2$	Thionyl chloride
t	Triplet
TCA	Trichloroacetic acid
TD	Toxic dose
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Tm	Transport maximum
UC	Ulcerative colitis
v/v	Volume by volume
Zn	Zinc
δ	Chemical shift
λ_{max}	Maximum wavelength

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

μg	Microgram
μl	Microliter
μm	Micrometer
ν	Wavenumbers

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

The gastrointestinal (GI) tract is a continuous mucous membrane lined tube extending from the oral cavity to the anus including four main parts: esophagus, stomach, small intestine, and large intestine (Figure 1-1).

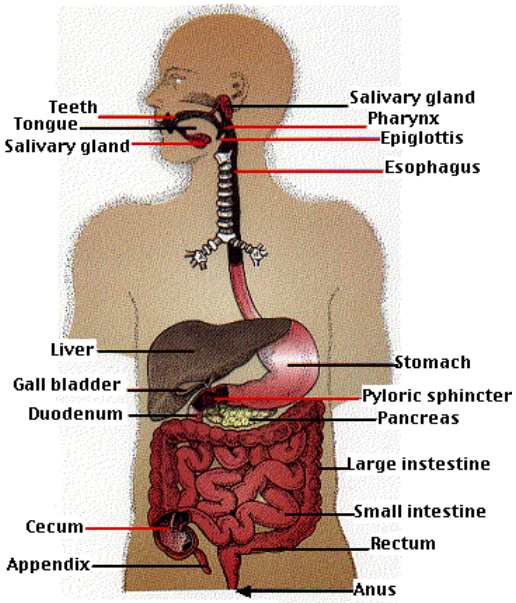


Figure 1-1 Simple anatomy of gastrointestinal tract (from Kaufman and McKee, 1996)

The stomach is the beginning digestive organ, whereas, the small intestine is the site where most of digestion is completed. The latter is the longest part of the digestive tract and is divided into three parts: duodenum, jejunum and ileum. In anatomy of digestive system, the colon is the part of the intestine from the cecum (‘caecum’ in British English) to the rectum. Its’ primary propose is to extract water from feces. In mammals, it consists of the ascending colon, transverse colon, the descending colon, the sigmoid colon and the rectum (Kaufman and McKee, 1996).

Many conditions, such as infections, inflammatory diseases and tumor, affect both the small and large intestines. These two organs are therefore considered together. Collectively, disorders of the intestines account for a large portion of human disease. For example, two inflammatory disorders of unknown etiology affecting the intestinal tract are Crohn's disease (CD) and ulcerative colitis (UC). These diseases share many common features and are collectively known as inflammatory bowel disease (IBD). Both CD and UC are chronic, relapsing inflammatory disorders of obscure origin. CD is a granulomatous disease that may affect any portion of the gastrointestinal tract from mouth to anus but most often involves the small intestine and colon. UC is a non-granulomatous disease limited to colonic involvement (Figure 1-2) (Cotran *et al*., 1994).

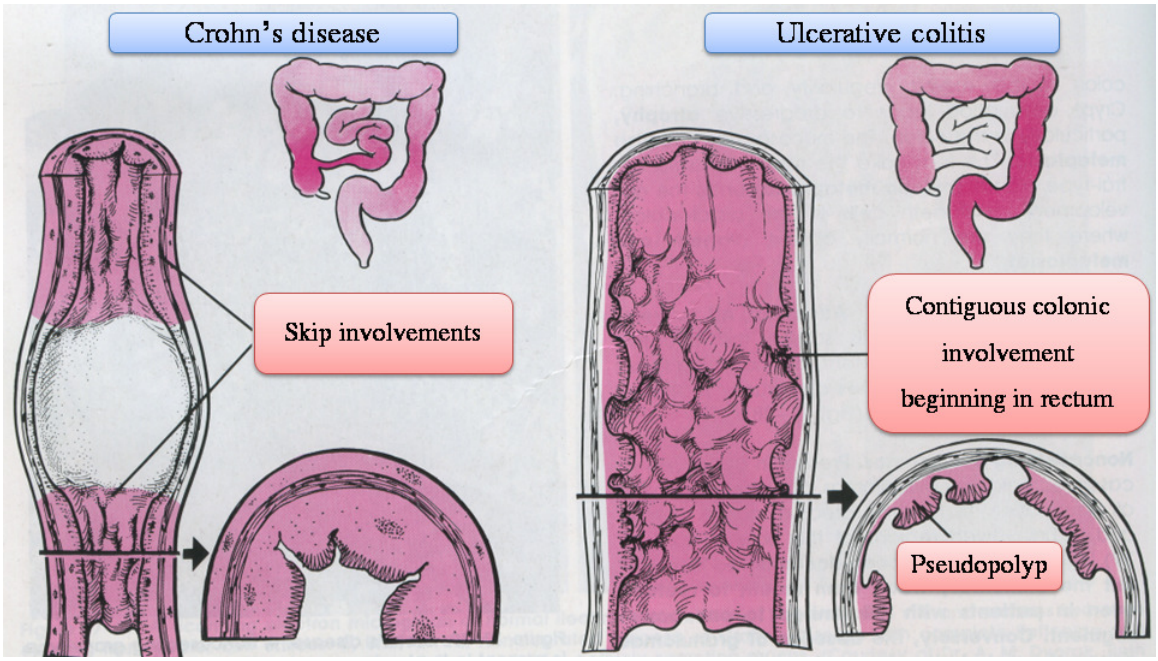


Figure 1-2 The distribution patterns of Crohn's disease and ulcerative colitis are compared as well as the different conformations of the ulcers and wall thickenings (from Cotran *et al*., 1994)

Corticosteroids have been used in treatment of IBD since 1955. The various compounds (e.g. prednisone, prednisolone, methylprednisolone and more recently budesonide)

are applied for systemic and local short-term treatment (induction of remission) in patients with active CD and UC. However, due to the serious side effects during long-term usage, they are not suitable for maintenance therapy (Klotz and Schwab, 2005).

5-Aminosalicylic acid (5-ASA) or mesalamine is the active drug released from sulfasalazine, first-line therapy for patients with IBD for decades (Selby, 2000). The identification of mesalamine as the active moiety responsible for the luminal anti-inflammatory effects of the prodrug, as well as patient allergy, intolerance, or unresponsiveness to this agent (Peppercorn, 1984) has led to the development of multiple mesalamine conjugates (Qureshi and Cohen, 2005), each with its own specific delivery system. Due to small molecular and simple chemical structure of this effective drug, mesalamine or 5-ASA was used as a model drug in many researches, such as TIME CLOCK® system (Pozzi *et* al., 1994; Steed *et al*., 1997), pH- and time-dependent system (Gupta *et al*., 2001), CODES™ (Katsuma *et al*., 2002) and etc.

Targeting pharmaceutical drugs to the colon makes it possible to guarantee local or systemic drug delivery to this site. To deliver the compounds in a non-degraded form to the last part of the gastrointestinal tract, they must first of all pass through the stomach, the upper part of the intestine and must use the characteristics of the colon to specifically release the drugs in this part of the digestive tract. Usual methods for the specific delivery of the drugs to the colon are based on the chemical or technological modification of excipients, as mentioned above. Due to the effectiveness of sulfasalazine releasing property in colon (Peppercorn, 1984), the azo bond (-N=N-) which could be cleaved by azoreductase enzyme, produced by intestinal microflora, and release the drug, 5-ASA, as a result, is another interesting system for improvement of colonic delivery. Thus, the polymeric conjugate system consists of carrier-spacer-active drug was subjected to be synthesized (Figure 1-3). Poly(ethylene) glycols, such as $mPEG_{5000}$, PEG_{4000}, PEG_{5106} diamino and PEG_{3677} diamino would be used as the carrier. We expected that these polymers could protect the conjugates from degradation in stomach and release the active drug, 5-ASA, in the colon specifically after enzymatic cleavage. *p*-Aminobenzoic acid (PABA) and *p*-aminohippuric acid (PAH) would be used as the spacer since they contained amino functional group that could be able to form azo bond with salicylic acid (SA), the promoeity drug. *In vitro*

drug release study of the active drug (5-ASA) from the products would be examined in the rat gastrointestinal contents.

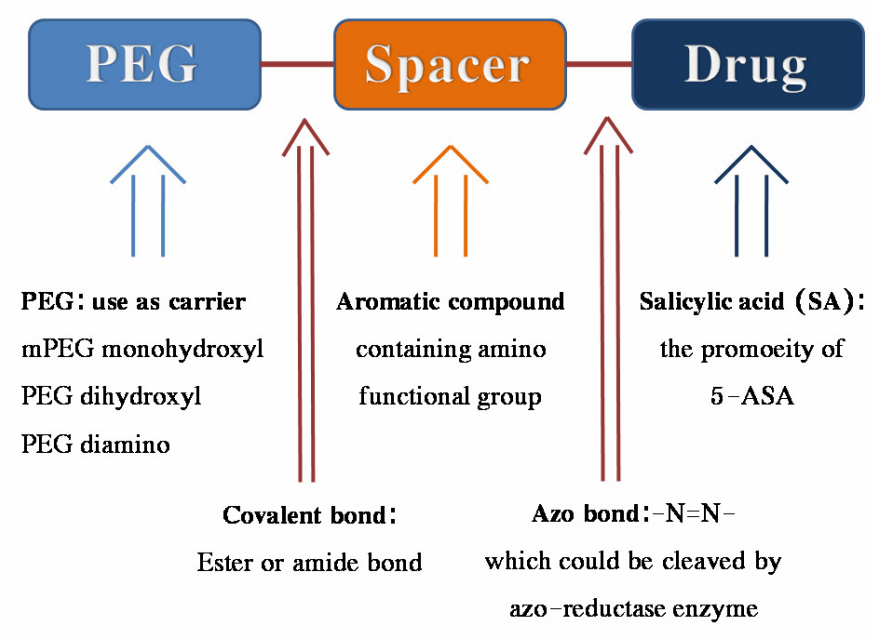


Figure 1-3 The simple structure of the tripartate products

1.2 Objectives

The objectives of this study were as follows:

1.2.1 To synthesize colonic specific delivery of 5-aminosalicylic acid (5-ASA) by using poly(ethylene) glycols (PEGs) as drug carriers.

1.2.2 To study the stability of the conjugates in buffer solutions.

1.2.3 To study the release of 5-ASA from the conjugates *in vitro*.

CHAPTER 2

LITERATURES REVIEW

Currently, the interest of the development of prodrugs and conjugates is shown increasingly in the attachment of drugs to polymers. The objectives of these prodrugs and conjugates are to increase the duration of activity through slow release, or by means of target-directing drugs in the body. The conjugates such as polymeric conjugates of penicillin, aspirin, amphetamine, quinidine and atropine have been developed for improvement of their efficacy (Zalipsky *et al*., 1983). Among those included the development of polymeric conjugates of 5-aminosalicylic acid (5-ASA) which is susceptible to azoreductase enzyme.

2.1 5-Aminosalicylic acid (5-ASA)

5-Aminosalicylic acid (5-ASA), or mesalamine (Figure 2-1), is an anti-inflammatory agent with molecular weight of 153.14 g/mol (http://www.fda.gov/). It has been used to relieve pain and inflammation in patients. This drug is well known as one of agents for the treatment of colonic diseases, such as ulcerative colitis (UC) and Crohn's disease (CD). Mesalamine is a potent inhibitor of the cyclooxygenase pathway, inhibiting the production of the prostaglandin E2 in inflamed intestinal specimens (Collier *et al*., 1976). Blockage of the lipooxygenase pathway also has been shown inhibiting both 5-lipooxygenase and 5-lipooxygenase activation protein, which in-turn blocks the production and chemotactic activity of leukotrienes such as leukotriene B4 (LTB4) and 5-hydroxyeicosatetraenoic acid (5-HETE). Its efficacy as an anti-inflammatory agent is also thought to be due to effects on leukotrienes metabolism. 5-ASA is rapidly and completely absorbed from the upper intestine when administered orally, but poorly absorbed from the colon. This is due to its solubility property in water is slightly soluble, 0.84 g/l at 25 $^{\circ}$C (French and Mauger, 1993). After oral or rectal administration of the various 5-ASA formulations (or cleavage of azo-prodrugs in the colon), the released 5-ASA is taken up by the epithelial cells in the small and large bowel. During absorption, capacity-limited intestinal acetylation occurs. Both 5-ASA and the metabolite are partly secreted back into

the intestinal lumen (Klotz and Schwab, 2005). Active transport of the two compounds from the basolateral to the epical site is probably accomplished by membrane-bound drug transporter such as multi-drug resistance protein 2 (MRP2) or P-glycoprotein (P-gp) (Zhou *et al.*, 1999). Thus, the intra-individual and inter-individual variability in the expression and activity of both N-acetyltransferase 1 (NAT1) and drug efflux pumps will affect mucosal concentrations of 5-ASA and consequently drug response. The impact of genetic polymorphisms of NAT1 and/or MRP2 on local/systemic availability of 5-ASA is presently unknown (Klotz and Schwab, 2005).

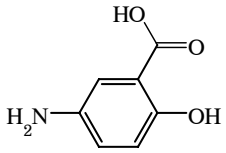


Figure 2-1 Chemical structure of 5-aminosalicylic acid (5-ASA) or mesalamine

However, because 5-ASA irritates the stomach (Wolfe *et al.*, 1999), its use in conventional formulations is limited. To overcome these problems and allow this drug to reach the inflamed colon, a variety of 5-ASA delivery systems has been developed. These include administrating 5-ASA as an enema (Rowasa®) or suppository (Canasa™). Pentasa® is produced in moisture-sensitive coated tablet. Azacol® tablet coated with a pH-sensitive resin which degraded in the basic environment of the distal ileum and colon. Other approaches are creating a larger unabsorbed molecule (prodrug or conjugate) by binding 5-ASA to carriers via an azo-bond, which subsequently undergoes cleavage in the colon releasing the active 5-ASA moiety. The examples of the latter system are sulfasalazine (Azulfidine®, Azulfidine EN-tabs®), olsalazine sodium (Dipentum®) and balsalazide disodium (Colazal®) (Peppercorn, 1984; Qureshi and Cohen, 2005).

Moreover, 5-ASA could be combustible at high temperature to give carbon oxides and nitrogen oxides. Therefore, 5-ASA products should be stored in cool places, dried and tight containers. This agent should be kept away from strong oxidizing agents, acids, acid anhydrides, acid chlorides and chloroformates due to incompatibility property (http://www. sciencelab.com/).

2.2 Sulfasalazine

Sulfasalazine was originally developed for the treatment of rheumatoid arthritis and it was fortuitously discovered in 1942 to be effective against ulcerative colitis. It was the first widely accepted effective therapeutic agent for active inflammatory colitis over a decade later, and subsequently for maintenance of remission (Qureshi, and Cohen, 2005). Sulfasalazine itself is poorly absorbed (3-12%) and its elimination half-life of about 5-10 hours is probably affected by the absorption process. The major part of sulfasalazine is split by bacterial azo-reduction process in the colon into 5-ASA and sulfapyridine (Figure 2-2). The latter is accounting for the most of the agent's adverse effects. The effective cleavage of sulfasalazine depends on an intact colon and transit time. It is markedly reduced in patients taking antibiotics and after removal of the large bowel. Sulfapyridine is almost completely absorbed from the colon and it is metabolized by hydroxylation and excreted in the urine. The acetylation rate of sulfapyridine is genetically controlled, and depending on the genetic phenotype. The elimination half-life and apparent oral clearance of sulfapyridine ranges from 14 hours (40 ml/min, for slow acetylators) to 6 hours (150 ml/min, for fast acetylators). The 5-ASA released from sulfasalazine in the colon, at least 25% is absorbed and after acetylation is subsequently excreted in the urine, while at least 50% is eliminated in the feces (Klotz, 1985). Therefore, increased intestinal transit results in a decrease in the proportion of acetylated-5-ASA to 5-ASA and increased excretion of un-split parent drug. Mean urinary and fecal excretion of total 5-ASA are 11-33% and 23-75%, respectively (Qureshi and Cohen, 2005).

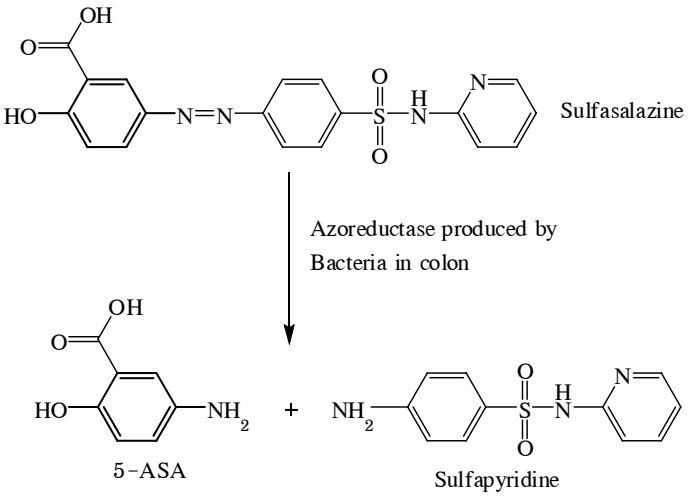


Figure 2-2 The reduction pattern of sulfasalazine in colon

Although sulfasalazine is useful in treatment of the diseases, especially in the colon, the side effects of this prodrug are usually found. Mosts are due to the sulfapyridine moiety. Many are idiosyncratic but others are dose related, which limited the use of sulfasalazine in 20-45% of the patients. Common dose related side effects, which include headache, nausea and fatigue, often respond to reduction in the dose. Hypersensitivity reactions to the sulfa-moiety commonly found are rash, fever, Steven-Johnson syndrome, hepatitis, pneumonitis, hemolytic anemia and bone marrow suppression. The incidence of adverse events increases with daily doses of 4 g or more, or total serum sulfapyridine levels above 50 μg/ml. Although sulfasalazine has been shown to be safe in pregnancy and nursing mothers, the adverse effects of the sulfa-drug is an important factor limiting its frequency of use (Qureshi and Cohen, 2005).

2.3 Polymer-5-aminosalicylic acid conjugates

Polymers containing 5-ASA conjugates have previously been reported. Most of those conjugates, 5-ASA was linked to non-absorbable polymers and used for specific delivery of drug to colon. Schacht and co-workers (1996) reported the rational design of systems for the delivery of 5-aminosalicylic acid (5-ASA) to colon by using polymeric conjugates of 5-ASA containing azo groups in their backbone. The polymers utilized in this study were vinyl pyrrolidone-maleic anhydride copolymer (Figure 2-3A, Pato *et al*., 1982), poly[*N*-(2-hydroxyethyl)-*DL*-aspartamide] (PHEA, Figure 2-3B, Antoni *et al*., 1974) and bis-aminopropyl poly(tetraethyleneoxide), PTMO (Figure 2-3C, Schacht *et al*., 1996). The release of 5-ASA from the conjugates was tested *in vitro* in the reductive buffer or in the bioreactor medium (Figure 2-5A). The result demonstrated that for the hydrophobic polymer (4-[4-(chlorocarbonyl) phenylazo] benzoylchloride/α, ω-bis-aminopropyl poly(tetra-methylene oxide), ABBC/PTMO, Figure 2-4), the apparent reduction was about 50% and stopped at the hydrazine stage (Figure 2-5C) whereas for a more hydrophilic analogue (ABBC/Jeffamine ED-600, Figure 2-4), reduction with formation of amines occurred (Figure 2-5B, Schacht *et al*., 1996). Moreover, other polymers have been utilized as carriers for colonic specific delivery such as *N*-(2-hydroxypropyl) methacrylamide (HPMA, Figure 2-6) for 9-aminocamptothecin (Sakuma *et al*., 2001), polysulfoamidoethylene (Figure 2-7) for sodium 5-aminosalicylate (Brown *et al*., 1983) and dextran (Figure 2-8) for methylprednisolone and dexamethasone

(McLeod *et al*., 1993). The result from those studies showed that these conjugates are good potential carriers system for colon-specific drug delivery and demonstrated that the active drugs were released slowly. Therefore, this could reduce the frequency of drug administration orally.

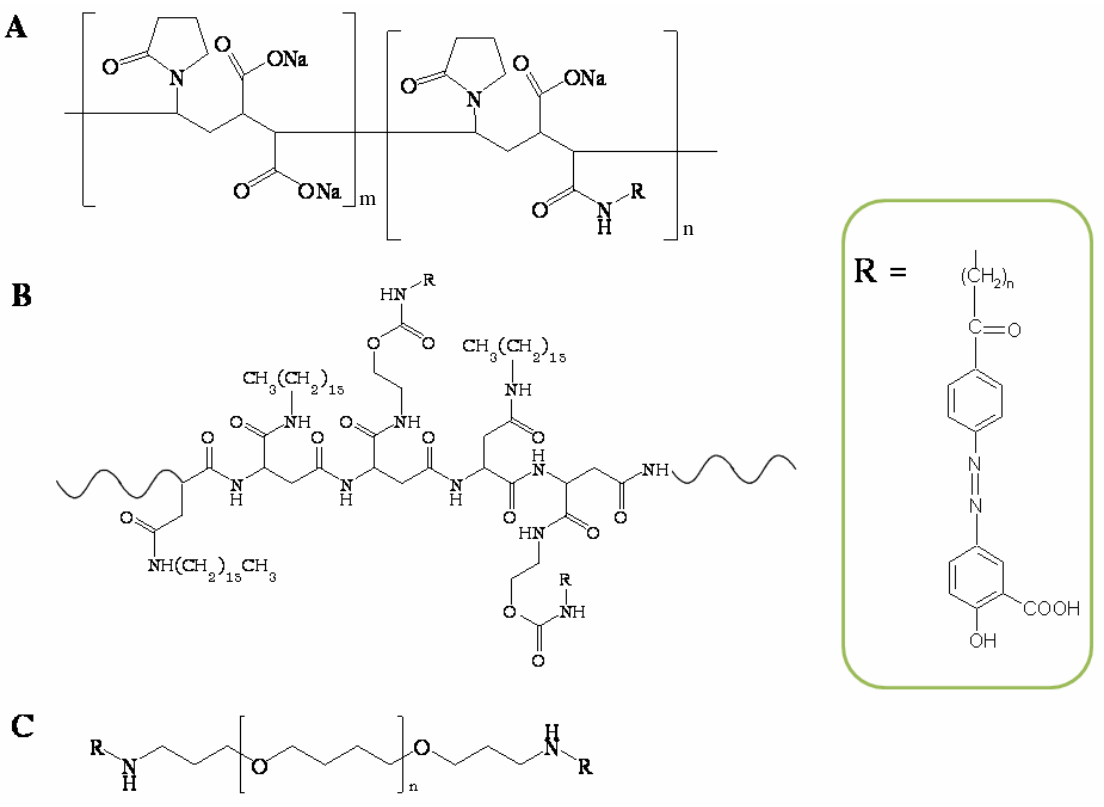


Figure 2-3 Polymeric conjugates containing 5-ASA conjugate covalently linked to poly (1-vinyl-2-pyrrolidone co-maleic anhydride), **A** (Pato *et al*., 1982), poly[*N*-(2-hydroxyethyl)-aspartamide, B (Antoni *et al*., 1974) and α, ω-bis-aminopropyl poly(tetramethylene oxide), PTMO, **C** (Schacht *et al*., 1996)

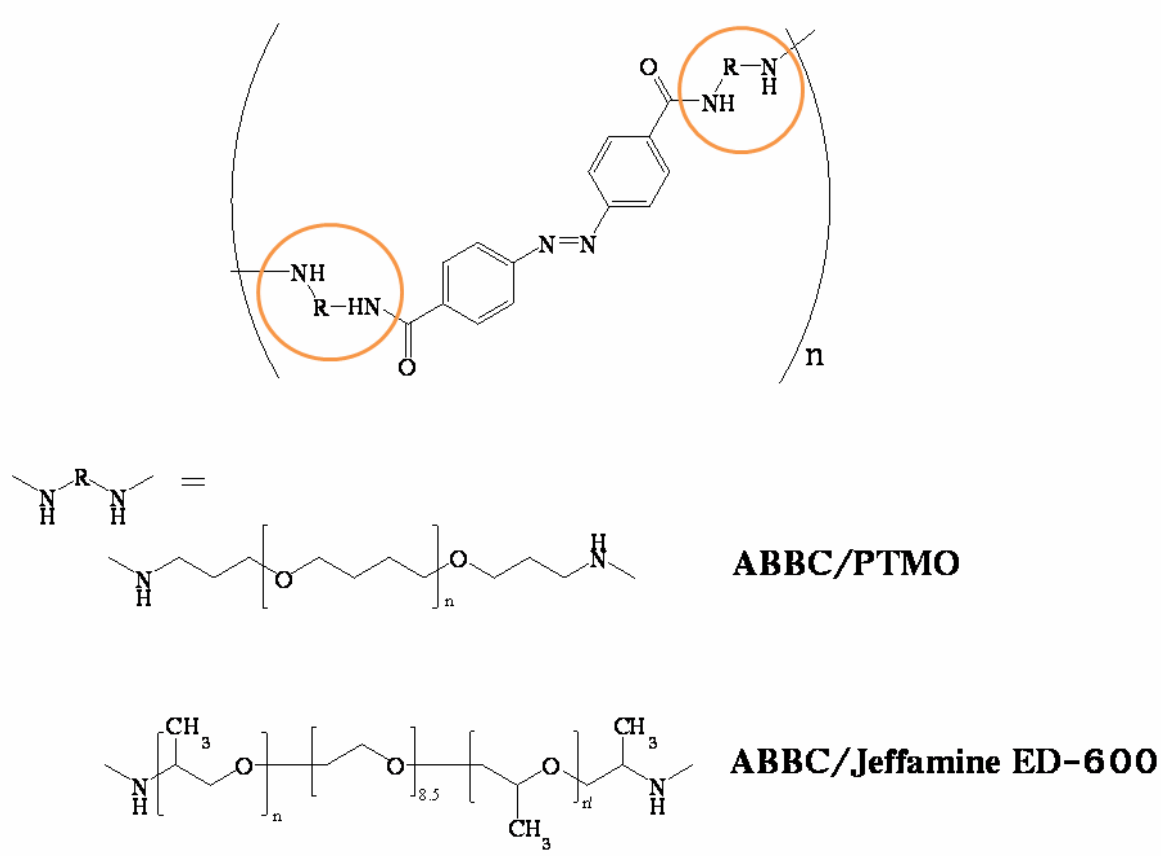


Figure 2-4 Chemical structure of (4-[4-(chlorocarbonyl phenylazo] benzoylchloride/α, ω-bis-aminopropyl poly(tetramethylene oxide), ABBC/PTMO, and ABBC/ Jeffamine ED-600 (Schacht *et al.*, 1996)

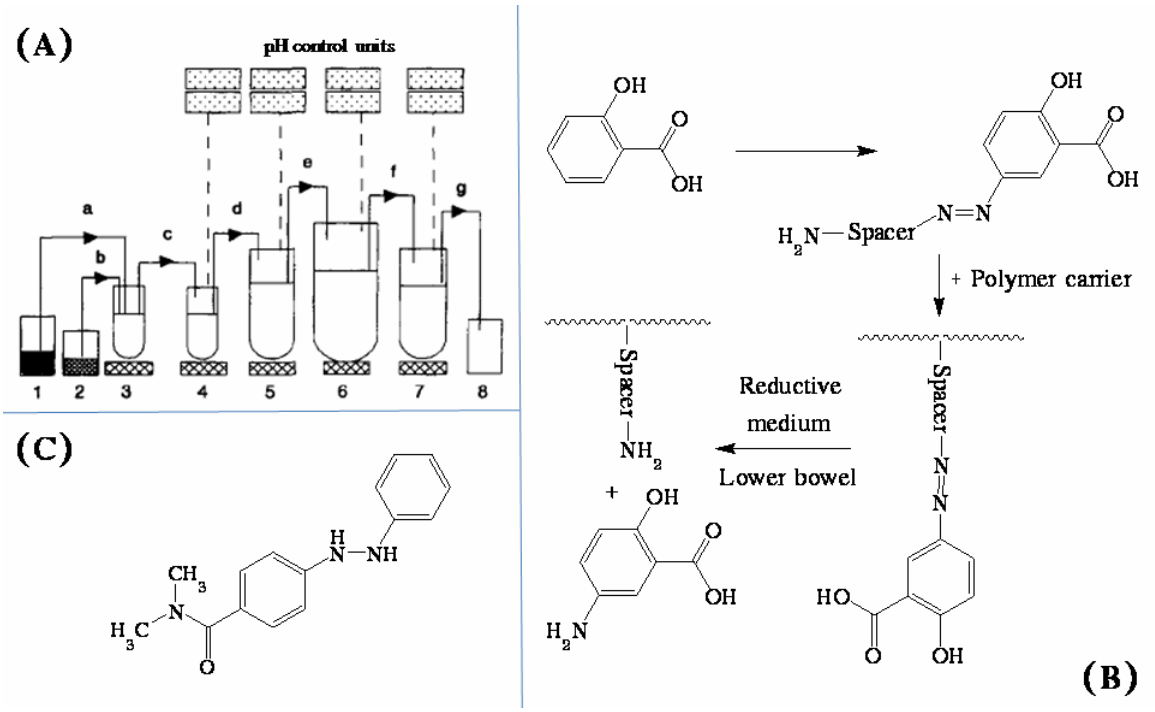


Figure 2-5 (**A**) Set-up of the simulated human intestinal microbial ecosystem reactor (SHIME): 1, feed; 2, pancreas acetone powder; 3, reactor 1 (duodenum and jejunum); 4, reactor 2 (ileum); 5, reactor 3 (caecum and ascending colon); 6, reactor 4 (transverse colon); 7, reactor 5 (descending colon); 8, effluent. Pumps a-d were operated semi-continuously; pumps e-g were operated continuously, (**B**) The general scheme for the preparation and biodegradation of the macromolecular azo-conjugates, and (**C**) Chemical structure of hydrazine containing by product after reduction of azo bond in the hydrophobic polymer (4-[4-(chlorocarbonyl) phenylazo]benzoylchloride/α, ω-bis-aminopropyl poly (tetramethylene oxide), ABBC/PTMO (Schacht *et al.*, 1996)

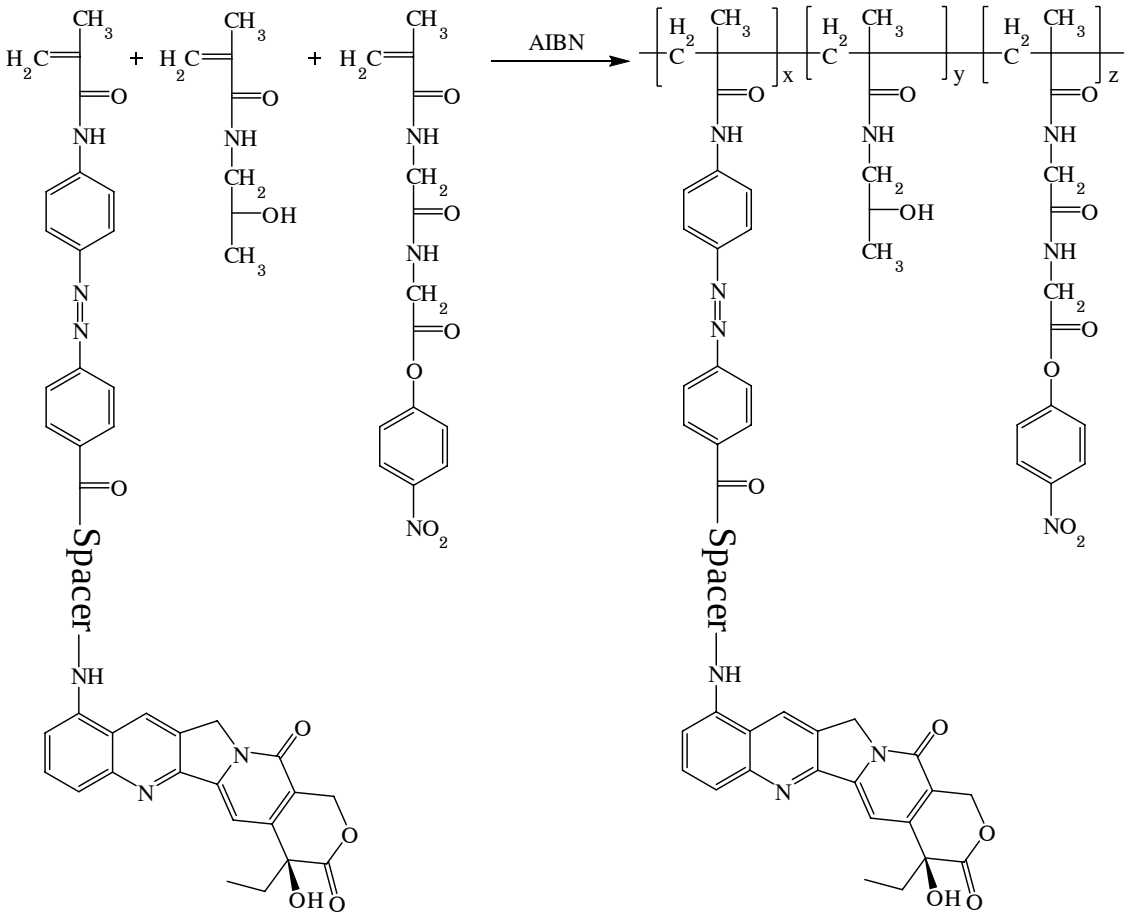


Figure 2-6 Synthesis of HPMA copolymer-9-aminocamptothecin conjugates (**IX** and **X**) (Sakuma *et al.*, 2001)

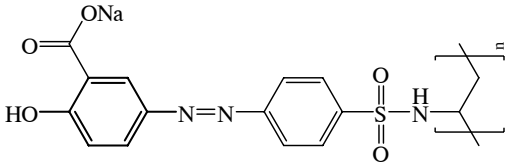


Figure 2-7 Chemical structure of polysulfoamidoethylene-5-ASA sodium conjugate (Brown *et al.*, 1983)

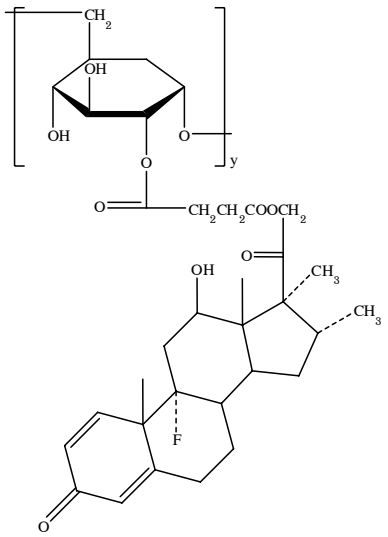


Figure 2-8 Chemical structure of dextran-dexamethasone conjugate (McLeod *et al.*, 1993)

2.4 PAMAM dendrimer-5-aminosalicylic acid conjugates

Since increasing numbers of interest are focused on polymeric drug conjugation, PAMAM dendrimers which are macromolecular compounds, have a defined structure with a large number of functional groups at their multiple chain ends (Figure 2-9) were utilized. Wiwattanapatapee and co-workers (2003) utilized salicylic acid (SA) bound to the dendrimer using two different spacers, *p*-aminobenzoic acid (PABA) and *p*-aminohippuric acid (PAH), containing azo bond (Figure 2-10). Incubation of 5-ASA-PAMAM dendrimer conjugates containing PABA or PAH spacers with rat gastrointestinal contents at 37 °C, 5-ASA was specifically released in rat cecal contents. The active drug was slowly released and prolonged for at least 24 hours. The amount of drug released from PAMAM-PABA-SA conjugates and PAMAM-PAH-SA conjugates were 45.6% and 57.0% of the loaded contents, respectively. However, only a small number of 5-ASA linked was found after characterization of both PAMAM dendrimer conjugates. One molecule of this dendrimer could carry only 3-10 molecules of the drug, whereas there were 32 amino functional side chains are available for conjugation per 1 molecule of this polymer. The spacer-SA which was linked to this polymer could also be cleaved in small amount after incubation in the contents of small intestine (3.8-12.5% released) and cecum

contents (0.2-0.4% released) at 37 °C. So the large amount of polymer would be inconvenient taken orally for each therapeutic dose.

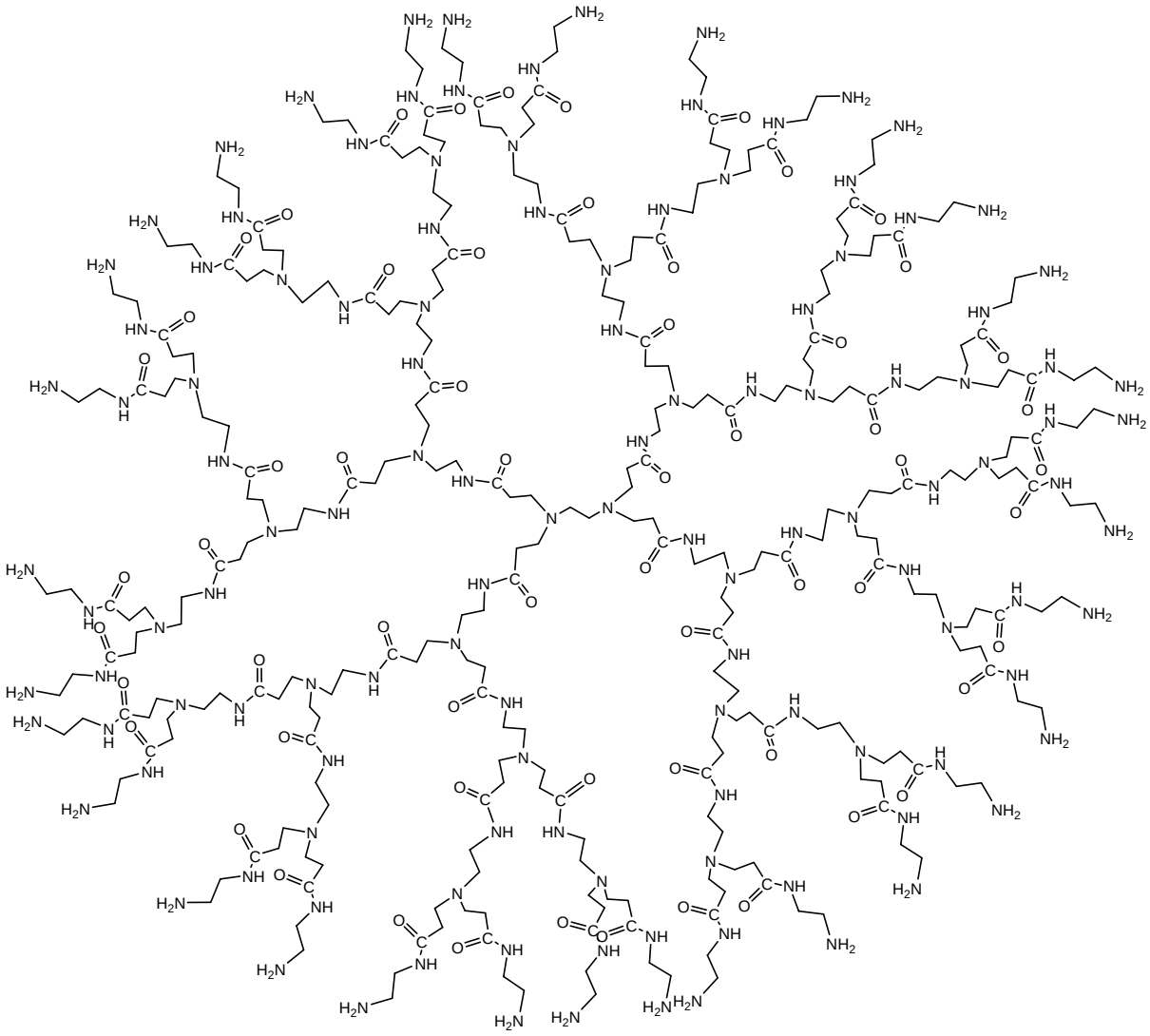


Figure 2-9 Structure of the third generation (G3) PAMAM dendrimers (Wiwattanapatapee, *et al*., 2003)

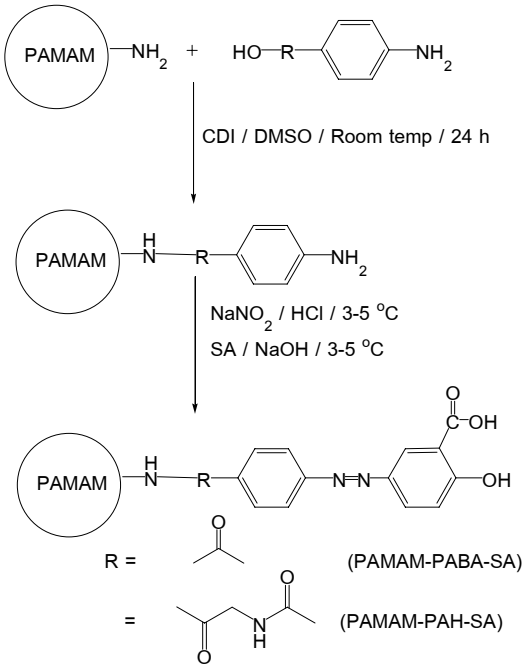


Figure 2-10 Synthetic Scheme for PAMAM-Spacers-SA conjugates (Wiwattanapatapee, *et al.*, 2003)

2.5 Poly(ethylene) glycol

Poly(ethylene) glycol (PEG) is the one among other polymers which has suitable properties as carrier for improvement of drug delivery as conjugates. PEG has been used as the carrier polymer in many studies, for example theophylline (Zacchigna *et al.*, 2003). Two theophylline esters with two different PEGs, PEG_{1500} and methoxy PEG_{1900} ($mPEG_{1900}$), were prepared (Figure 2-11). Quantitative yields of pure products were obtained. Unlike the free drug, the polymer-drug conjugates were freely water-soluble at room temperature. *In vitro* and *in vivo* release experiments demonstrated that both conjugates were stable in buffer pH 7.4 and 1.2, and theophylline conjugates displayed a sustained release of the parent drug. Besides, various drugs had been attached to PEG such as procaine, atropine, and various salicylates (Zalipsky *et al.*, 1983). Furthermore, the polymer-drug conjugates showed longer duration of activity, due to their slow release properties. Insulin and small molecule protein had also been attached to PEG intended to improve the physiological properties, enhance water solubility, or prolong half-life for short

half-life drug which could be inactivated before reaching to the active site (Zalipsky *et al*., 1983).

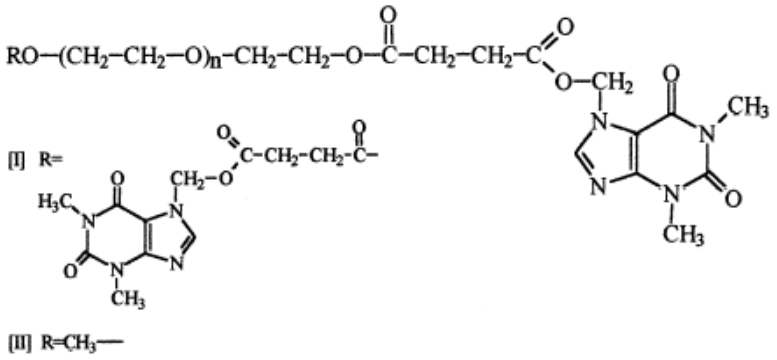


Figure 2-11 PEG_{1500}-[7-(hydroxymethyl)-theophylline]$_2$ conjugate (I) and $mPEG_{1900}$-[7-(hydroxymethyl)-theophylline] conjugate (II) (from Zacchigna *et al*., 2003)

Polyethyleneglycol, PEG, was chosen as the carrier in this research due to its non-toxic, non-antigenic, non-teratogenic, non-immunogenic and biocompatible properties (Zalipsky *et al*., 1983). They are available in a variety of molecular weights from 200 to over 10,000 Da (Fruijtier-Pölloth, 2005). PEG is a linear, uncharged, amphiphilic polymer that is soluble in water and in most organic solvents. It also has solubilizing properties. Due to the non-biodegradable property of PEG, it is used widely in pharmaceutical products. Moreover, PEG is rapidly eliminated from the body, and has been approved for a wide range of biomedical applications (Zalipsky *et al*., 1983; Zacchigna *et al*., 2002) such as emulsifying agent or surfactant in perfume, mouthwash, toothpaste, lipsticks, etc. It is also used as vehicle for drugs as an ointment bases, capsules, tablets and pill binders, suppositories, liquid prescriptions, and in veterinary drugs, including parenteral, topical, ophthalmical, oral, and rectal preparations (Fruijtier-Pölloth, 2005).

PEG is a unique polyether diol, usually manufactured by the aqueous anionic polymerization of ethylene oxide, although other polymerization initiators can be employed. Initiation of ethylene oxide polymerization using anhydrous alkanols such as methanol or derivatives including methoxy ethoxy ethanol results in a mono-alkyl capped polyethylene glycol, such as methoxy PEG (mPEG). Hence, the polymerization reactions

can be modulated and a variety of molecular weights (1,000-50,000) can be obtained with low polydispersities (Mw/Mn), less than 1.05 (Greenwald *et al*., 2003). These properties make it a convenient polymeric base for immobilizing various biologically active compounds. Different ways of introducing biologically active compounds into PEG are considered. The traditional way is to modify PEG by the end of hydroxyl groups leading to synthesis of telechelics. There are many methods for modification of different functional groups for PEG available (Topchiyeva, 1990). PEG is possibly excreted from the body after intake. The elimination of PEG is combined from renal to eliminate PEG with molecular weight less than 30 kDa, and by hepatic pathways to eliminate less than 20 kDa of PEG. The toxicological information of PEG demonstrated LD_{50} of PEG_{4000} in rats and rabbits after oral administration is 50 g/kg and of PEG_{20000} is 31.6 g/kg (http://www.atcc.org/). Moreover, the study of PEG_{4000} showed no concentration of this polymer has been absorbed in rat intestine after 5 hours and it was eliminated within 24 hours. However, there are some reports mentioned that the absorption of PEG_{3350} is less than 2%. There was no evidence that PEG_{8000} was absorbed at all (Fruijtier-Pölloth, 2005). Because of these properties, it is widely used in biochemistry and molecular biology. It have also been commercially used in pharmaceutical products, cosmetics, and food, or used as drug carrier into body, inclusively. PEG itself also induces apoptosis in HT-29 colorectal cancer cells (Zalipsky *et al*., 1983). Thus, PEG is chosen in present study for using as a drug carrier to improve colonic specific drug delivery.

2.6 *p*-Aminobenzoic acid (PABA) and *p*-aminohippuric acid (PAH)

p-Aminobenzoic acid (PABA: toxic dose (TD) 8 g/day) (Figure 2-12) (http://www.thirdage.com/) was chosen as a spacer linked between PEG and the drug, SA. PABA is an organic compound. It is a white crystalline substance that is only slightly soluble in water. It consists of a benzene ring substituted with an amino group and a carboxylic acid.

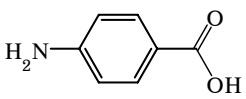


Figure 2-12 Chemical structure of *p*-aminobenzoic acid (PABA)

PABA is an essential nutrient for some bacteria and is sometimes called Vitamin B_x. However, PABA is not essential for humans and it varies in its activity from other B vitamins. Although humans lack the ability to synthesize folate from PABA, it is sometimes marketed as an essential nutrient under the premise that it can stimulate intestinal bacteria (http://www.thirdage.com/).

Sulfonamides (sulfa drugs) are chemically similar to PABA, and their antibacterial activity is due to their ability to interfere with PABA utilization by bacteria (http://www.thirdage.com/).

PABA also finds use in the manufacture of esters, folic acid, and azo dyes (http://www.thirdage.com/).

There are, however, some proposed medicinal uses of oral PABA supplements. PABA is sometimes suggested as a treatment for various diseases of the skin and connective tissue, as well as for male infertility. However, most of the clinical data on PABA comes from very old studies, some from the early 1940s (http://www.thirdage.com/).

PABA has been suggested as a treatment for scleroderma, a disease that creates fibrous tissue in the skin and internal organs. A 4-month double-blind, placebo-controlled study of 146 people with long-standing, stable scleroderma did not support this, failing to find any evidence of benefit. However, half the participants in this trial dropped out before the end, making the results unreliable. It has also been suggested for other diseases in which abnormal fibrous tissue is involved, such as Peyronie′s disease, a condition in which the penis becomes bent owing to the accumulation of such tissue. However, no double-blind studies have yet been performed that could show PABA effective for Peyronie's disease (http://www. thirdage.com/).

Based on one small World War II-era study, PABA has been suggested for treating male infertility as well as vitiligo, a condition in which patches of skin lose their pigment, resulting in pale blotches (http://www.thirdage.com/). A recent study suggests that high dosages of PABA can cause vitiligo (http://www.thirdage.com/).

PABA is probably safe when taken at a dosage up to 400 mg daily. Possible side effects at this dosage are minor, including skin rash and loss of appetite (http://www.thirdage.com/).

PABA is nontoxic, readily absorbed from the gastrointestinal tract and is extensively metabolized in the liver by phase II conjugation pathways, independent of the cytochromes P450. PABA is safe, convenient, and affordable for use in children. Side effects such as nausea, vomiting, rash, pruritis, fever, hepatitis, acidosis have been reported only when 8-48 times the recommended dose (5 mg/kg, maximum 170 mg) was ingested (Furuya *et al.*, 1995). There has been one reported case of severe liver toxicity in a woman taking 12 g daily of PABA. Fortunately, her liver recovered completely after she discontinued her use of this supplement. Also, a recent study suggests that 8 g daily of PABA can cause vitiligo, the patchy skin disease described previously. Moreover, safety in young children, pregnant or nursing women, or those with serious liver or kidney disease has not been determined (http://www.thirdage.com/).

p-Aminohippuric acid (PAH, Figure 2-13) is the glycine conjugated derivative and metabolite of PABA which has longer carbon chain (Furuya *et al.*, 1995). It could be synthesized in human body as a metabolite of PABA in liver function test (Deiss and Cohen, 1950). *p*-Aminohippuric acid (PAH) is a commonly used marker of renal plasma flow. A compound of high extraction across the kidney, PAH, has also been utilized extensively in the study of organic anion renal secretion (Savant *et al.*, 2001).

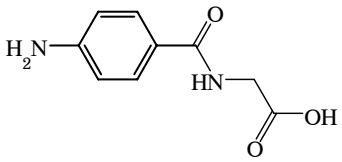


Figure 2-13 Chemical structure of *p*-aminohippuric acid (PAH)

PAH is commercially available as Aminohippurate Sodium "PAH" Injection (Merck®). Aminohippurate sodium is another form of this agent used to measure effective renal plasma flow (ERPF). It is the sodium salt of *p*-aminohippuric acid. It is water soluble, lipid-insoluble, and has a pKa of 3.83. The empirical formula of the anhydrous salt is $C_9H_9N_2NaO_3$. PAH is filtered by the glomeruli and is actively secreted by the

proximal tubules. At low plasma concentrations (1.0 to 2.0 mg/100 ml), an average of 90 percent of PAH is cleared by the kidneys from the renal blood stream in a single circulation. It is ideally suited for measurement of ERPF since it has a high clearance, is essentially nontoxic at the plasma concentrations reached with recommended doses, and its analytical determination is relatively simple and accurate (http://www.drugs.com/).

PAH is also used to measure the functional capacity of the renal tubular secretory mechanism or transport maximum (Tm_{PAH}). This is accomplished by elevating the plasma concentration to levels (40-60 mg/100 ml) sufficient to saturate the maximal capacity of the tubular cells to secrete PAH (http://www.drugs.com/).

Long-term studies in animals have not been done to evaluate any effects upon fertility or carcinogenic potential of PAH. It is categorized in pregnancy category C. Animal reproduction studies have not been done with PAH. It is also not known whether PAH can cause fetal harm when given to a pregnant woman or can affect reproduction capacity. PAH should be given to a pregnant woman only if clearly needed. It is neither known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when PAH is administered to a nursing woman. Safety and effectiveness in pediatric patients have not yet been established. Moreover, clinical studies of PAH did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects. Other reported clinical experience has not identified differences in responses between the elderly and younger patients (http://www.drugs.com/).

The adverse reactions which could be found in using this agent are hypersensitivity reactions including anaphylaxis, angioedema and urticaria, vasomotor disturbances, flushing, tingling, nausea, vomiting, and cramps may occur. Patients may have a sensation of warmth or the desire to defecate or urinate during or shortly following initiation of infusion. The intravenous LD_{50} in female mice is 7.22 g/kg (http://www.drugs.com/).

2.7 Intestinal normalflora and enzymes

Generally, the bacteria could be classified into 2 groups, aerobic and anaerobic bacteria. Anaerobic bacteria, normally, cannot grow in the presence of oxygen (strict or obligate anaerobes). However, some of anaerobic bacteria can grow either in the conditions which containing oxygen rich and without oxygen. They are well known as facultative anaerobic bacteria (e.g. Streptococci, Enterobacteriaceae-*Escherichia coli* (*E. coli*)).

Anaerobic bacteria are present in soil and are a part of the normal flora of humans and all other animals, as well as the insects examined so far. In humans, anaerobes are found on all mucous membranes particularly in the oral cavity and the lower intestinal tract. In the mouth, their numbers are approximately equal to the numbers of aerobic bacteria present. The numerical proportion of anaerobic bacteria to facultative bacteria increases, proceeding distally in the intestinal tract to such an extent that, in the colon, anaerobic bacteria outnumber facultative bacteria by a thousand to one. Anaerobes occur at a concentration of two hundred billion to four hundred billion organisms per gram dry weight of fecal material. Anaerobic bacteria can also be isolated as predominant organisms from the human skin where they occur primarily in the sebaceous glands. Anaerobic bacteria are those bacteria to which oxygen is, in some way, toxic. These bacteria range considerably in their ability to tolerate oxygen; many survive quite well in the presence of oxygen but are unable to initiate growth in its presence. Most bacteriologists define ″anaerobic bacteria″ as bacteria that grow better in the absence of oxygen than in its presence (Silver, 1980).

2.7.1 Anaerobic Methodology

Normally, the lumen of GI tract is anaerobic and maintains a highly reduced atmosphere: any oxygen entering with food or in swallowed air is quickly utilized. The gas mixture in the lumen headspace is approximately 63% CO_2, 27% CH_4, 7% N_2 with traces of H_2 and H_2S and small transient amounts of O_2 (Pridmore and Silley, 1998). To generate the anaerobic system in laboratory, three systems are currently in use for anaerobic culture: the anaerobic jar (Figure 2-14) (GasPak® or evacuation replacement),

prereduced anaerobically sterilized (Figure 2-15, PRAS) roll tubes and the anaerobic glove box (Figure 2-16). Several studies on the yield of anaerobes from clinical specimens indicate that all three give comparable results. Each has its advantages and disadvantages, depending on the personnel, volume, and resources of the laboratory (Table 2-1).

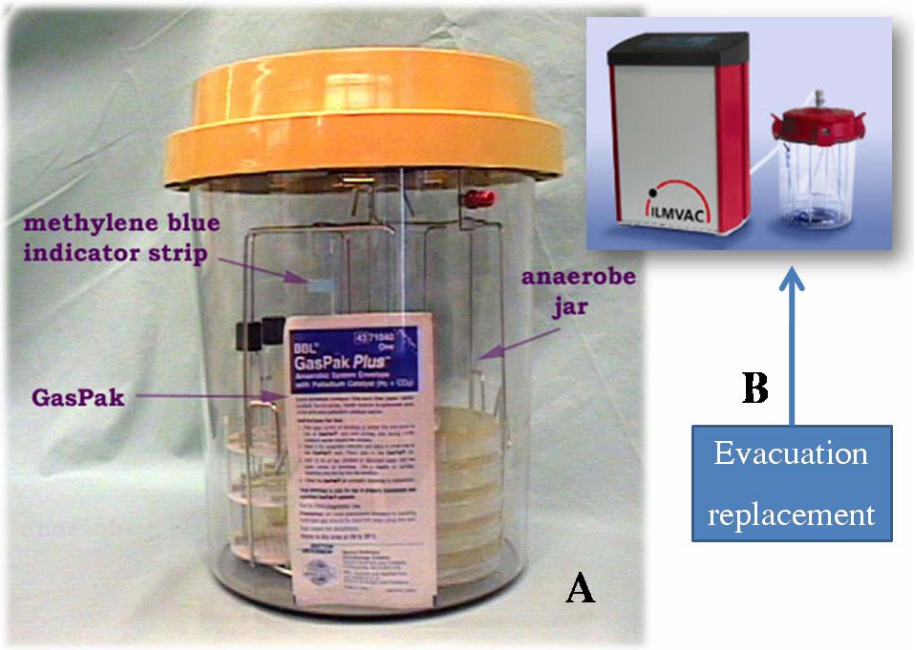


Figure 2-14 (**A**) Anaerobic system generated in anaerobic jar using Gaspak® to generate oxygen-free gas and detected oxygen with methylene blue indicator strip (from http://www.mc.maricopa.edu/.../Dbiochem/oxy.html), (**B**) evacuation-replacement technique (from http://www.industrialvacuum.net/p/p0054.htm/)

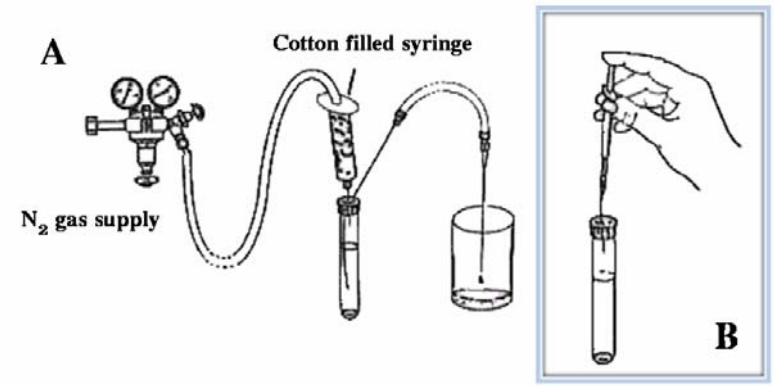


Figure 2-15 (A) Producing of a prereduced anaerobically sterilized (PRAS) roll tube, (**B**) Inoculation of fresh culture medium (from Malik, 1989)

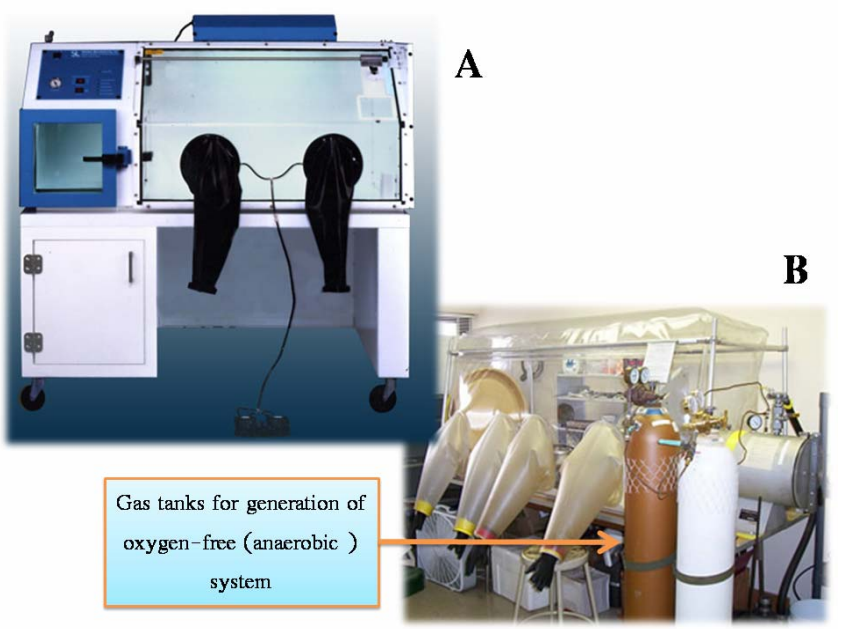


Figure 2-16 (A) Anaerobic glove box (from http://www.epsovens.com/Anaerobic-Chamber/), (**B**) Anaerobic glove box (bag) including gas tank for oxygen-free gas generation (from http://www.cme.msu.edu/tiedjelab/labresources.shtml/)

Table 2-1 Comparison of anaerobic systems

	GasPak®	Roll Tubes	Anaerobic glove box
Initial cost	Moderate	Moderate	High
Maintenance cost	Variable	Moderate-due to cost of PRAS tubes	Moderate-mainly gases
Space requirements	Variable	Small	High-depends on size of chamber
Training	Minimal	VPT course or equivalent	Moderate
Technique	Conventional bench	Specialized equipments	Conventional bench
Daily inspection of growth	Yes	Yes	Yes
Colonial morphology	Detectable	Difficult	Detectable
Use of rapid testing method	Possible	Not possible	Possible

2.7.1.1 Anaerobic Jar Techniques

When performing an anaerobic incubation with anaerobic jar techniques, an anaerobic gas generating kit and anaerobic indicator are needed. Gas generating kit (GasPak®) will provide the correct atmosphere in which to affect culture of anaerobes to ensure that the proportion of oxygen to carbondioxide is suitable for the growth of microaerophillic organisms.

The components using in this technique are: a polycarbonate jar with lid; a hydrogen-carbon dioxide generator; a catalyst; and an indicator (Figure 2-14A). A GasPak *Plus*® sachet was used to generate oxygen-free gas in the jar by decomposition of pellets of bicarbonate and sodium borohydride in the presence of citric acid and cobalt chloride forming carbon dioxide and hydrogen (Silver, 1980; Pridmore and Silley, 1998). The oxygen in the jar was detected by methylene blue indicator strip, which would turn from blue to be white color when the system inside the jar was anaerobe (http://www.mc.maricopa.edu/.../Dbiochem/oxy.html).

Another technique to introduce anaerobic atmosphere in an aerobic jar is evacuation-replacement technique. This technique involves the replacement of air in the jar with a gas that contains no oxygen instead of using anaerobic sachet to generate the anaerobic system (http://www.industrial-vacuum.net/p/p0054.htm/). The gas usually employed is 10% hydrogen, 5% carbon dioxide and 85% nitrogen (Figure 2-14B).

2.7.1.2 Roll Tube Technique

In this technique, prereduced anaerobically sterilized (PRAS) roll tubes are made, sterilized, stored and sealed tightly (Figure 2-15), and inoculated or used in an oxygen-free environment (anaerobic glove box). Tube culture of anaerobes is based on procedures developed by Hungate (1950) for culture of sewage and intestinal bacteria. In concept and in practice, this method is one of the easiest and fastest available for culture of even the most sensitive anaerobes (Silver, 1980).

Medium and the condition are designed for anaerobes which could be culture under low-oxygen atmosphere. For fecal bacteria, the media most commonly used are based on media developed by Bryant and Robinson (1961). These media contain a small amount of peptone, yeast extract, sugars (glucose, starch and cellobiose) in low concentration, and a mixture of volatile fatty acids, added either in rumen fluid (Bryant and Robinson, 1961) or as a defined supplement (Caldwell and Bryant, 1966). These media permit culture of 30 to more than 90% of the fecal bacteria seen in direct microscopic clump counts (DMCC).

For culture of clinical specimens, media are prepared in the absence of air and placed in stopped tubes. Air is prevented from entering the tubes when they are opened by placing a gentle stream of oxygen-free gas into each tube when it is opened. When the tube is restoppered, it is ready for incubation. Agar in tubes is used either to streak or to inoculate when molten (Holdeman and Moore, 1972).

For intestinal studies, it is necessary to mix the intestinal contents with media under CO_2 in a plastic bag and place in the tubes. Tubes are restoppered, and filled with CO_2 (Holdeman and Moore, 1972).

2.7.1.3 Anaerobic glove box

With this system, a complete anaerobic environment is supplied and maintained. Specimens are introduced, media inoculated and incubated, biochemical testing of isolates and antibiotic sensitivity tests are all performed in the glove box (Figure 2-16) (http://www.epsovens.com/Anaerobic-Chamber/). Generally, the anaerobic chamber would be contained with 85% N_2, 10% CO_2 and 5% H_2, generated from the gas tanks. Traces of oxygen inside the chamber must be continuously removed and replaced with inert gas (Figure 2-16B) (http://www.cme.msu.edu/tiedjelab/labresources.shtml/; Silver, 1980).

2.7.2 Intestinal microflora

As mentioned earlier that anaerobic bacteria are presented also in lower part of gastrointestinal tract, the intestine. The duodenum and jejunum of normal people contain few bacteria ($<10^3$ viable cells/ml of intestinal contents). Streptcocci and lactobacilli are the bacteria most usually isolated. In the ileum, a richer and more permanent flora is found (Skinner and Carr, 1976).

Gastric acid controls the entry of bacteria into the small intestine; the distribution of bacteria within the small intestine is determined by the movement of intestinal contents. The type of diet and frequency of feeding affects the secretion of gastric acid and the rate of gastric emptying thus indirectly influences the distribution of intestinal bacteria (Skinner and Carr, 1976).

The fecal flora is assumed to refer to that of the large intestine. A small number of parallel studies suggested that the flora of the large intestine is similar to that of the feces though the intestinal contents are more dilute than those of the rectum. The colon can be thought of as a fermenter but the composition of the medium depends upon the activity of the small intestine and is thus unknown (Skinner and Carr, 1976).

2.7.3 Azoreductase enzyme produced by intestinal microflora

As mentioned above, one of the first prodrugs on the market that used colonic enzymatic biodegradation as its active principle was sulfasalazine (salazosulfapyridine, Figure 2-17). More modern successor products are olsalazine®, balsalazine® and ipsalazine® (Figure 2-18), all of them are used for the treatment of inflammatory bowel diseases and containing two molecules linked by azo bond (Figure 2-18). The prodrugs pass the stomach and the small intestine unchanged and unabsorbed. Reaching the cecum, they are reduced and cleaved by specific azoreductases of the microflora. During this process of enzymatic decomposition, the drug (5-ASA sodium) is released in a micro-fine physical state that promotes rapid and extensive dispersion, and thus guarantees maximal topical and systematic activity (Schreier, 2001).

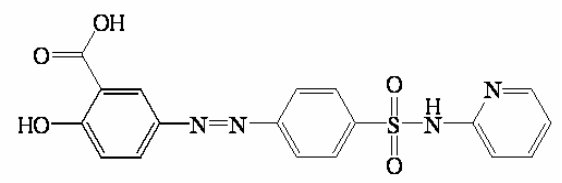


Figure 2-17 Chemical structure of sulfasalazine

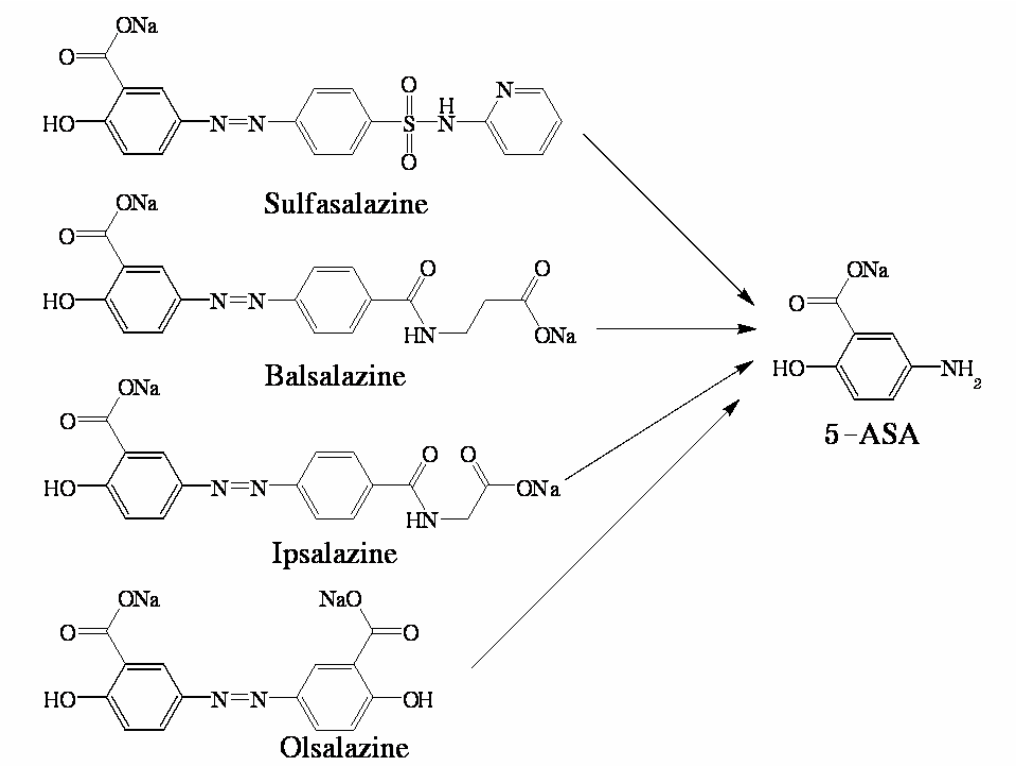


Figure 2-18 Chemical structure of sulfasalazine, olsalazine®, balsalazine® and ipsalazine®, and 5-ASA, the drug released from the compounds (Chourasia and Jain, 2003)

Several anaerobic bacteria from human intestinal tract are capable of reducing azo dyes and nitropolycyclic aromatic hydrocarbons to the corresponding aromatic amines with enzymes that have azoreductase and nitroreductase activities. The majority of bacteria with these activities belong to the genera *Clostridium* (Figure 2-19) and *Eubacterium* (e.g. *Enterococcus faecalis* which was formerly classified as *Streptococcus faecalis*, Figure 2-21). The enzymes from various bacteria had different electrophoretic mobilities. The azoreductases from all of the bacteria had immunological homology, as was evident from the cross-reactivity of an antibody raised against the azoreductase of

Clostridium perfringens (Rafii and Cerniglia, 1995; Semdé *et al*., 1998; Figure 2-20) with azoreductases from other bacteria. Furthermore, the nitroaromatic compounds competitively inhibited the azoreductase activity. The data indicated that the reduction of both nitroaromatic compounds and azo dyes may be carried out by the same enzyme, which is possibly a flavin adenine dinucleotide dehydrogenase that is synthesized throughout the cell and not associated with any organized subcellular structure (Rafii and Cerniglia, 1995).

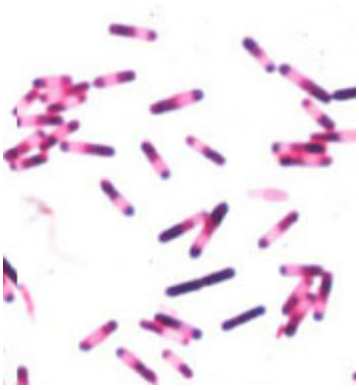


Figure 2-19 *Clostridium difficile*, Gram stain (from http://textbook ofbacteriology.net/normalflora.html.htm/)

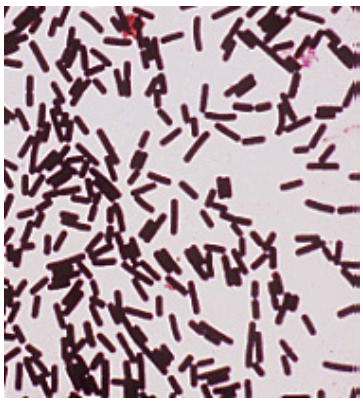


Figure 2-20 *Clostridium perfringens*, Gram stain (from http://textbook ofbacteriology.net/normalflora.html.htm/)

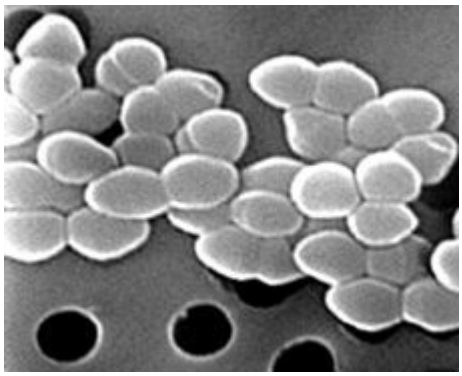


Figure 2-21 Vancomycin Resistant *Enterococcus faecalis*, Scanning E.M. CDC (from http://textbook ofbacteriology.net/normalflora.html.htm/)

Moreover, the exact number of bacteria which could generate azoreductase enzyme is still unknown. The bacterial flora of the GI tract of animals has been studied more extensively than that of any other site. The proximal small intestine has a relatively sparse Gram-negative flora, consisting mainly of lactobacilli and *Enterococcus faecalis*. This region has about 10^5-10^7 bacteria per ml of fluid. The distal part of small intestine contains greater numbers of bacteria (10^8/ml) and additional species including coliforms and *Bacteroides*, in addition to lactobacilli and enterococci (http://textbookofbacteriology.net/normalflora.html. htm/). Moreover, Skinner and Carr (1976) reported that Clostridia which could produce azoreductase enzyme (Bragger *et al*., 1997) were isolated from the contents of the terminal ileum of 2 of 12 control subjects at counts in the range of 10^4-10^5/ml. There are many other studies that support these observations in fasting subjects (Skinner and Carr, 1976). The flora of the large intestine (colon) is qualitatively similar to that found in faeces. Populations of bacteria in the colon reach levels of 10^{11}/ml faeces. Coliforms become more prominent, and enterococci, clostridia and lactobacilli can be regularly found, but the predominant species are anaerobic *Bacteroides* and anaerobic lactic acid bacteria in the genus *Bifidobacterium* (*Bifidobacterium bifidum*). These organisms may outnumber *E. coli* by 1,000:1 to 10,000:1. It is known that significant numbers of anaerobic methanogenic bacteria (up to 10^{10}/gm) also reside in the colon of humans (http://textbookofbacteriology.net/normalflora.html.htm/; Silver, 1980). The composition differs between various animal species, and within an animal species. In humans, there are differences in the composition of the flora which are influenced by age, diet, cultural conditions, and the use of antibiotics. The latter greatly perturbs the composition of the intestinal flora. The following table shows the distribution of some common intestinal bacteria in various animal species including humans. (Table 2-2)

The data from Table 2-2 demonstrated that bacteria produced azo-reductase enzyme found in human faeces were similar to the bacteria found in feces of other animals including mice. Therefore, to study the releasing property of the synthesized polymeric conjugates (Figure 1-3) which containing azo bond, animal models can be used.

Table 2-2 Numbers of viable bacteria found in one gram faeces of adult animals (medial value from 10 animals, modified from Rosebury, T. 1962)

Animal	*E. coli*	*C. perfringens*	Enterococci	*Bacteroides*	Lactobacilli
Cattle	$1.99x10^4$	$1.99x10^2$	$1.99x10^5$	1	$2.51x10^2$
Sheep	$3.16x10^6$	$1.99x10^4$	$1.25x10^6$	1	$7.94x10^3$
Horses	$1.25x10^4$	1	$6.30x10^4$	1	$1.00x10^7$
Pigs	$3.16x10^6$	$3.98x10^3$	$2.51x10^6$	$5.01x10^5$	$2.51x10^8$
Chickens	$3.98x10^6$	$2.51x10^2$	$3.16x10^7$	1	$3.16x10^8$
Rabbits	$5.01x10^2$	1	$1.99x10^4$	$3.98x10^8$	1
Dogs	$3.16x10^7$	$2.51x10^8$	$3.98x10^7$	$5.01x10^8$	$3.98x10^4$
Cats	$3.98x10^7$	$2.51x10^7$	$1.99x10^8$	$7.94x10^8$	$6.30x10^8$
Mice	$6.30x10^6$	1	$7.94x10^7$	$7.94x10^8$	$1.25x10^9$
Humans	$5.01x10^6$	$1.58x10^3$	$1.58x10^5$	$5.01x10^9$	$6.30x10^8$

The polymeric conjugates were designed to contain ester or amide bond between the hydroxyl group of PEG and carboxyl group of spacer (Figure 2-22). Though the ester bond is well-known that it is weaker than the amide bond and ester hydrolysis could occur easier in GI tract, still there were some researches chose this covalent bond linked between the polymer and the drug or spacer. Moreover, those ester polymeric conjugates could release the drug in colon without ester bond breaking before reaching the lower part of GI tract (Kamada *et al.*, 2002; Zou *et al.*, 2005). Thus, the ester bond was also chosen as mentioned above. In addition, the amino functional group of spacer would be conjugated with salicylic acid (SA) by the azo bond which expected to be cleaved by azoreductase enzyme in the colon.

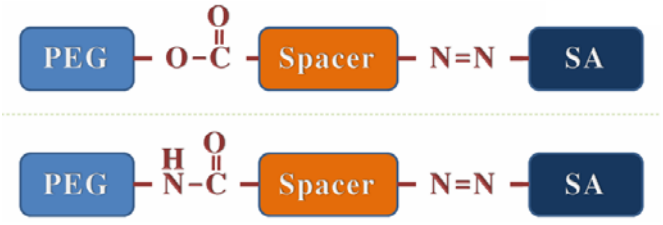


Figure 2-22 The design of ester and amide polymeric conjugates

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials: Chemicals, reagents and specimens

Commercial grade of dichloromethane, diethyl ether, tetrahydrofuran, *n*-hexane, analytical grade of 37% hydrochloric acid, sodium hydroxide, glacial acetic acid, methanol, formic acid, dimethylsulfoxide and dimethylformamide were purchased from Labscan Asia (Thailand). Analytical grade ammonia solution was obtained from Guangdong Chemical (China). Analytical grade of triethylamine and absolute ethanol, were received from Prolabo (Paris, France). Water was purified by passing through a Milli-Q purification system. Analytical grade trichloroacetic acid and KI/Stach indicator paper were purchased from Riedel-de Haën (Germany). Monomethoxy poly(ethylene) glycol (Mw 5000), poly(ethylene) glycol (Mw 4000), diamino poly(ethylene) glycol (Mw 3000), *p*-aminobenzoic acid, *p*-aminohippuric acid, 5-aminosalicylic acid, pyridine, palladium on activated charcoal, zinc powder and sulfasalazine were obtained from Fluka (Switzerland). Triphosgene or bis(trichloromethyl) carbonate, *N*, *N*-Carbonyldiimidazole and *N*, *N*-dimethyl aminopyridine were purchased from Fluka (China). *p*-Nitro benzoylchloride was purchased from Fluka (Germany). Celite® 500 fine was purchased from Fluka (United State). Diamino poly(ethylene) glycol (Mw 5106) and Diamino poly(ethylene) glycol (Mw 3677) were obtained from NOF (Singapore). Salicylic acid was purchased from M&B Laboratory chemicals (England). Sodium nitrite, Karl Fisher grade dried methanol, toluene, Potassium dihydrogen phosphate, anaerobic system indicator, microbiology Anaerotest® and TLC aluminium sheets, Silica gel 60 F_{254} were purchased from Merck (Germany). Anhydrous sodium sulfate was obtained from Fisher Scientific (United Kingdom). Citric acid, calcium chloride dihydrate and disodium hydrogen orthophosphate anhydrous were received from Ajax Finechem (Australia). Dialysis bag, regenerated cellulose tubular membrane MW cut off 3500 was purchased from Celu Sep (USA). Bacteriological peptone and Anaerogen® were received from Oxoid (England). Silica gel 60 was obtained from Scharlau (Spain). pH indicator paper was purchased from Macherey-Nagel (Germany). 0.2 μm-Membrane syringe filter nylon was obtained from

Vertical Chromatography (Thailand). Thionyl chloride was available in laboratory of the department of pharmaceutical chemistry, Prince of Songkla University. Wistar rats were purchased from Prince of Songkla University animal house.

3.2 Instruments and equipments

Mettler Toledo 320 pH meter was used for pH measurement. In the synthesis experiments, rotary evaporator (BÜCHI, Switzerland), centrifuge, Hettich zentrifugen universal 16/16 R, vortex-2 GINIE Scientific Industries (USA Model G-560E), hot plate and magnetic stirrer (Cemarec® 2 Thermolyne SP46920-33, USA), vacuum oven, Precision®, hot air oven (Memmert UNV-500, S.V. Medico), hydrogenation apparatus (Parr® Low Pressure, USA), micropipettes (Biorad, USA) and homogenizer (Moulinex, Romania) were used. Anaerobic system was generated in an anaerobic bag (Fluka, Switzerland). UV spectra were obtained from UV-visible spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrophotometer). IR spectra were recorded by use of neat and KBr pellets on a FT-IR Spectrometer (Perkin Elmer FT-IR Spectrometer 73561). UVGL-58 handheld UV lamp (UVP, United Kingdom) was use as UV detector. High performance liquid chromatography (HPLC) was carried out using HPLC Agilent 1100 series and HPLC analytical column (Restek, pinnacle II C18 5 μm, 250 x 4.6 mm).

3.3 Methods

3.3.1 Solubility test of starting materials

The reactions were performed using several types of reagents. Therefore, in order to choose the proper solvent for performing the reaction, the solubility of each compound in selected solvent should be known. The solubility was performed by accurately weighing about 25 mg of each compound and transferred to screw-capped tube (10 ml), solvent was then added into each of the test tubes using micropipette (200 μl each time) and swirled until completely dissolved. The solubility values were calculated in the unit of g/l.

3.3.2 Procedure for synthesis of polymer conjugates

Poly(ethylene) glycol-spacer-salicylic acid (PEG-spacer-SA) conjugates were prepared according to the scheme displayed in Figure 3-1. Eight methods were attempted and used in the preparation of PEG-spacer-SA conjugates. Two types of products are expected from the study, first PEG-spacer-SA conjugate having ester bond between PEG polymer and spacer and second, PEG-spacer-SA conjugate having amide bond between PEG polymer and spacer.

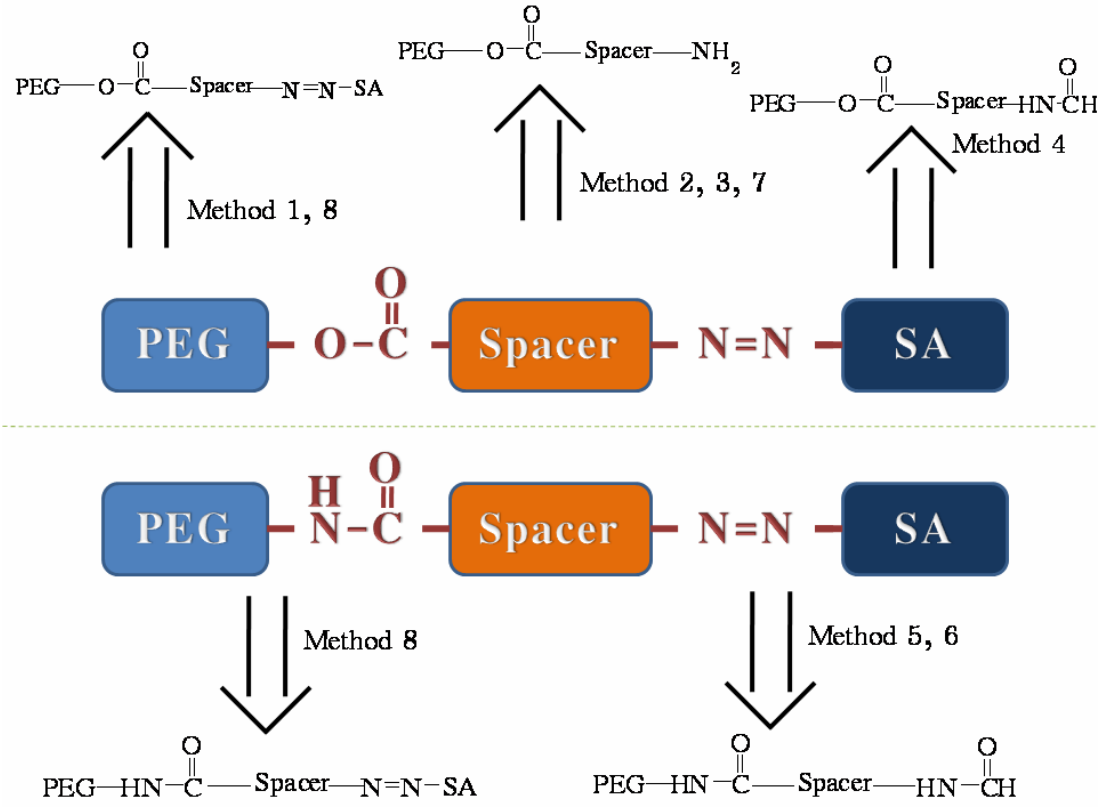
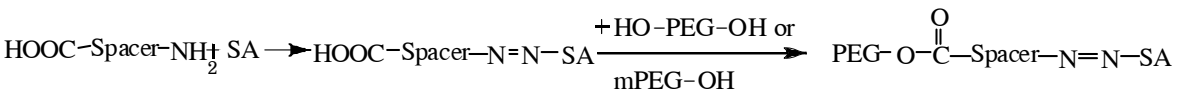


Figure 3-1 Synthesis protocols for PEG-spacer-SA conjugates

3.3.2.1 Synthesis of poly(ethylene) glycol-spacer-SA ester conjugates (Method 1)



Since azo compounds could be synthesized easily by diazotization reaction, the synthesis of the expected ester products was started from synthesis of the small

molecule, azo products. This method included synthesis of spacer–SA conjugates which will be used to conjugate with PEG in the later step.

3.3.2.1.1 Synthesis of PABA–SA and PAH–SA azo conjugates

PABA (**1**, 0.66 g, 4.8 mmol) was dissolved in 10 ml of 3 *N* HCl and stirred in ice bath (3–5 °C) for 5 minutes. A solution of $NaNO_2$ (0.36 g, 5 mmol) in 5 ml water was added dropwise into the solution of PABA. The mixture was stirred in ice bath (3–5 °C) for a further 5 minutes and then the diazonium salt was tested for the presence of excess nitrous acid using KI/starch indicator paper). Salicylic acid (**3**, 0.69 g, 5 mmol) was dissolved in 10 ml of MeOH and added dropwise into the mixture while stirring at 3–5 °C for 30 minutes (Wiwattanapatapee *et al.*, 2003). Then 3 M NaOH solution was added to adjust pH to 10, the reddish brown solution appeared and the solution was stirred for a further 20 minutes. The mixture was then acidified with 3 *N* HCl and reddish–brown solid was separated. The solid was filtered and washed with water. Product (**4**) was dried at 70 °C for 4 hours and identified by UV–visible, FT–IR, ^{1}H–NMR spectroscopy and melting points. Synthesis of PAH–SA azo conjugate was also performed in the same method but PAH was added instead of PABA.

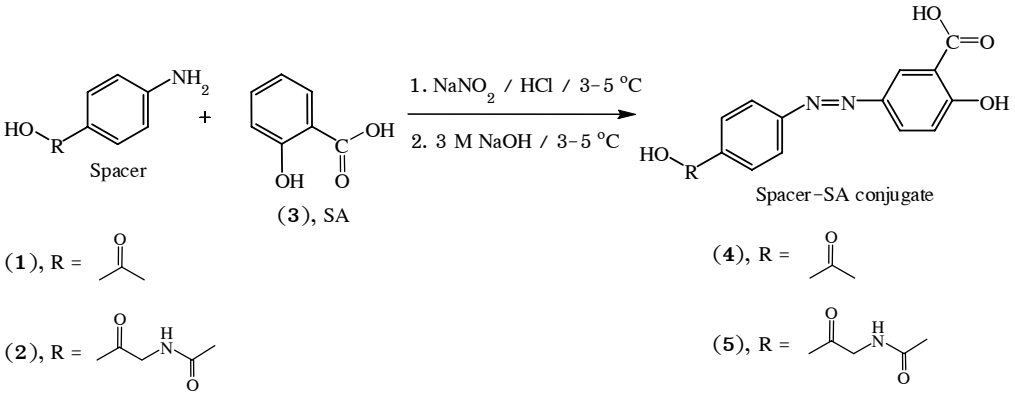


Figure 3–2 Synthetic scheme for spacer–SA azo conjugates

3.3.2.1.1.1 Determination of % free salicylic acid and spacers

Since excess amount of salicylic acid was used in the synthesis procedure, there may be some free salicylic acid remained in the final azo products which may have an

effect on the next step of the synthesis procedure. Therefore, the free salicylic acid and other by products in each of the conjugates were determined by the following procedure.

Each of the spacer-azo conjugates (**4** or **5**, 10 mg) was weighed in accurate amount and dissolved in 5 *N* NH_4OH (5 ml) in 10-ml volumetric flask. The solution was then adjusted to volume by mobile phase A (mixture of 85% of acetate buffer pH 3.3 and 15% methanol, v/v) as the stock solution. The stock solution was subsequently diluted to 10 μg/ml with mobile phase A and analyzed using validated HPLC system described later.

3.3.2.1.2 Synthesis of poly(ethylene) glycol-spacer ester conjugates

Poly(ethylene) glycol-spacer ester conjugates (**8-11**) were synthesized employing the method described by Topchiyeva (Topchiyeva, 1990), by using *N*, *N*-carbonyldiimidazole (CDI) as the coupling reagent (Wiwattanapatapee, *et al*., 2003).

Purified and dried $mPEG_{5000}$ (**6**, 1 g, 0.2 mmol) or PEG_{4000} (**7**, 1 g, 0.25 mmol) was dissolved in 20 ml CH_2Cl_2 and stirred at room temperature. Solutions of PABA-SA (**4**, 0.46 **g**, 1.6 mmol for **6**; or 0.72 g, 2.5 mmol for **7**) or PAH-SA (**5**, 0.55 g, 1.6 mmol for **6**; or 0.86 g, 2.5 mmol for **7**) in DMSO (5 ml) and CDI (0.3 g, 2 mmol for **6**; or 0.5 g, 3 mmol for **7**) in DMSO (5 ml) were added while stirring. The mixture was continued stirred at room temperature for 24 hours (Bayer and Mutter, 1972; Rajasekharan Pillai *et al*., 1979; Wiwattanapatapee *et al*., 2003). The resulting products were extracted with distilled water twice in order to remove DMSO. The organic solvent was then removed by rotary evaporator. The residue was re-dissolved in CH_2Cl_2 and precipitated by ether. FT-IR and ^{1}H-NMR spectral data of the resulting compound were determined.

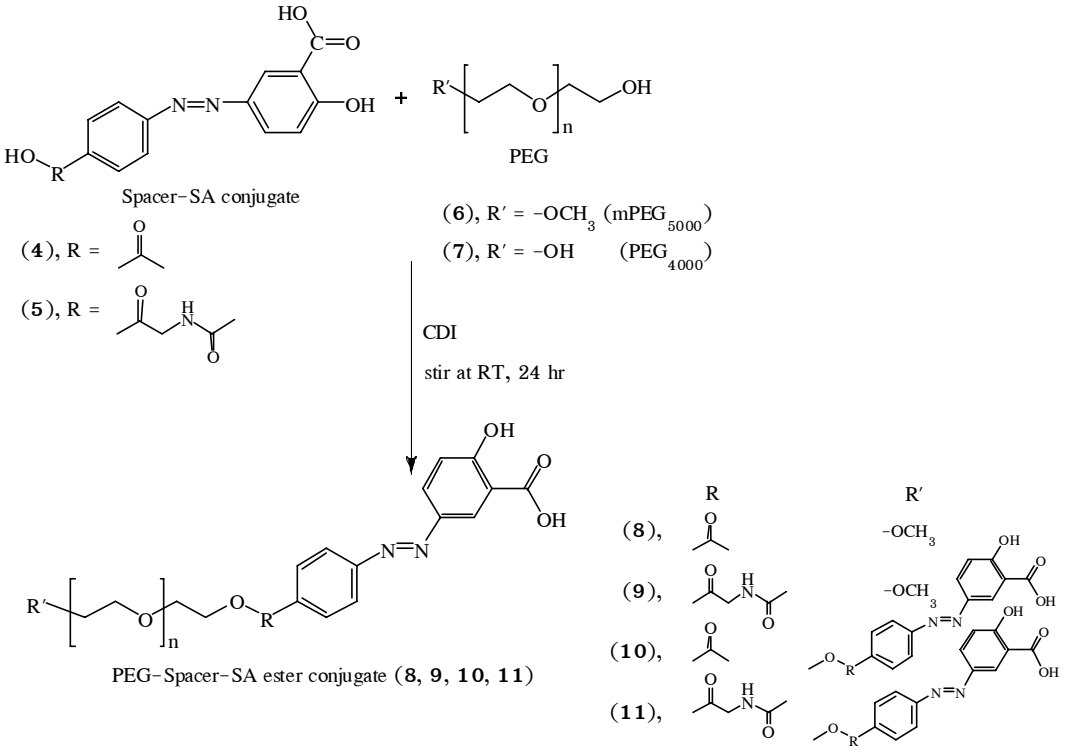


Figure 3-3 Synthetic scheme of $mPEG_{5000}$-O-C(=O)-PABA-SA conjugate (**8**), $mPEG_{5000}$-O-C(=O)-PAH-SA conjugate (**9**), PEG_{4000}-O-C(=O)-$(PABA-SA)_2$ conjugate (**10**) and PEG_{4000}-O-C(=O)-$(PAH-SA)_2$ conjugate (**11**)

3.3.2.2 Synthesis of PEG-O-C(=O)-spacer conjugates (Method 2, Figure 3-4)

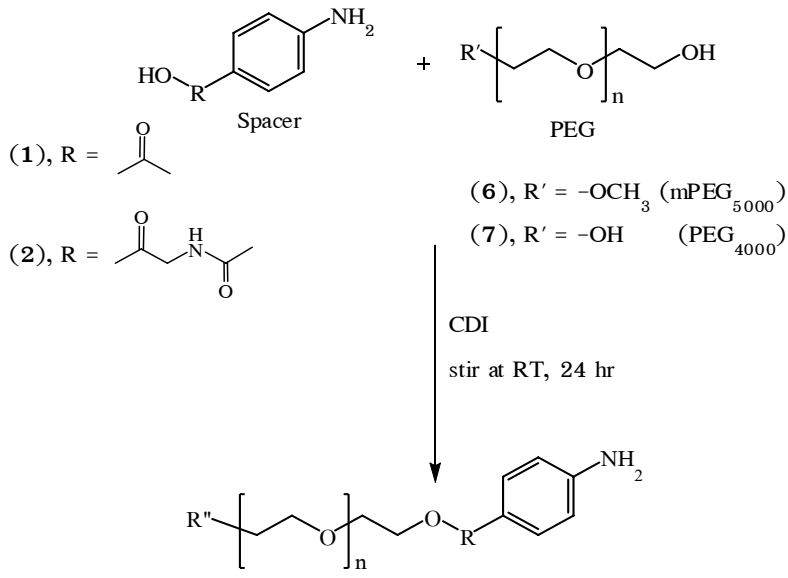
The ester conjugates of PEGs (**12**-**15**) were synthesized by the following procedure by using *N*, *N*-carbonyldiimidazole (CDI) as the coupling reagent (Peng *et al*., 2006).

One gram of purified and dried $mPEG_{5000}$ (**6**, 0.2 mmol) or PEG_{4000} (**7**, 0.25 mmol) was dissolved in 20 ml CH_2Cl_2. Solutions of PABA (**1**, 0.22 **g**, 1.6 mmol for **6**; or 0.35 g, 2.5 mmol for **7**) or PAH (**2**, 0.32 **g**, 1.6 mmol for **6**; or 0.49 g, 2.5 mmol for **7**) in DMSO (5 ml) and CDI (0.48 **g**, 3 mmol for **6**; or 0.82 g, 5 mmol for **7**) in DMSO (5 ml) were added while stirring at room temperature. The mixture was

continuously stirred at room temperature for 24 hours (Bayer and Mutter, 1972; Rajasekharan Pillai *et al*., 1979; Wiwattanapatapee *et al*., 2003). The resulting products were precipitated by ether. FT-IR and ^{1}H-NMR spectral data of the resulting compound were determined.

3.3.2.3 Synthesis of PEG-O-C(=O)-spacer conjugates (Method 3, Figure 3-5)

Since there was no product obtained from synthesis procedure in section 3.3.2.2 (method 2), dimethylaminopyridine (DMAP) was added in the reaction mixture as a catalyst as previously described (Xi *et al*., 2005) intended to obtain the PEG-spacer ester conjugates. The synthetic route is shown in Figure 3-5. The spacer (**1**, 0.22 **g**, 1.6 mmol for **6** or 0.36 g, 2.5 mmol for **7**; or **2**, 0.31 **g**, 1.6 mmol for **6** or 0.48 g, 2.5 mmol for **7**) was dissolved in 25 ml DMF and stirred in ice-bath for 5 minutes. A solution of CDI coupling reagent (0.48 **g**, 3 mmol for **6**; or 0.83 g, 5 mmol for **7**) in 10 ml of pre-dried and distilled CH_2Cl_2 was then added while stirring and continue stirring in ice-bath for 4 hours. Pre-dried $mPEG_{5000}$ (**6**, 1 g, 0.2 mmol) or PEG_{4000} (**7**, 1 g, 0.25 mmol) was dissolved in 20 ml dried CH_2Cl_2 and added to the mixture. The solution of dimethylaminopyridine (DMAP: 0.03 **g**, 0.2 mmol for **6**; or 0.04 g, 0.3 mmol for **7**) in 10 ml dried CH_2Cl_2 was added as the catalyst of the reaction (Zou *et al*., 2005). The final mixture was stirred at room temperature for 24 hours. Then, the solution mixture was extracted twice by equal volume of distilled water to remove the residual DMF. After that the residual of free spacer was removed by extraction twice with 0.1 *N* HCl, and once with 0.1 *N* NaOH. Then the organic portion was dried by anhydrous Na_2SO_4, filtered and evaporated, and the resulting product was characterized preliminarily by FT-IR spectroscopy.



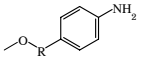
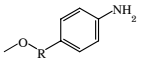
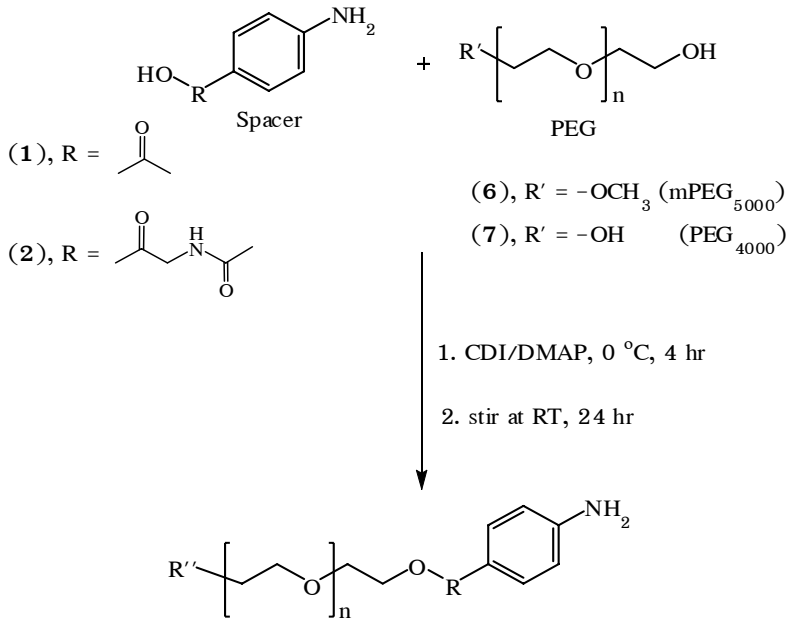
		R	R″
12	$mPEG_{5000}$–PABA ester conjugate		$-OCH_3$
13	$mPEG_{5000}$–PAH ester conjugate		$-OCH_3$
14	PEG_{4000}–$(PABA)_2$ ester conjugate		
15	PEG_{4000}–$(PAH)_2$ ester conjugate		

Figure 3-4 Synthetic scheme of $mPEG_{5000}$–O–C(=O)–PABA conjugate (**12**), $mPEG_{5000}$–O–C(=O)–PAH conjugate (**13**), PEG_{4000}–O–C(=O)–$(PABA)_2$ conjugate (**14**) and PEG_{4000}–O–C(=O)–$(PAH)_2$ conjugate (**15**)



PEG-spacer ester conjugate (**12, 13, 14, 15**)

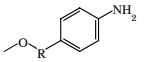
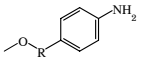
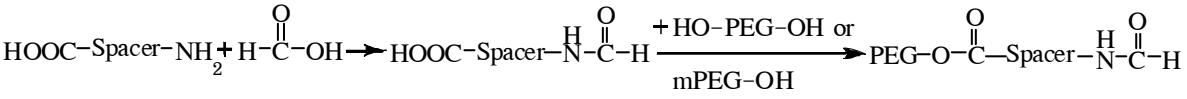
		R	R″
12	$mPEG_{5000}$-PABA ester conjugate		$-OCH_3$
13	$mPEG_{5000}$-PAH ester conjugate		$-OCH_3$
14	PEG_{4000}-$(PABA)_2$ ester conjugate		
15	PEG_{4000}-$(PAH)_2$ ester conjugate		

Figure 3-5 Synthetic scheme of PEG-O-$\overset{O}{\overset{\|}{C}}$-spacer conjugates (**12, 13, 14, 15**)

3.3.2.4 Synthesis of PEG-O-$\overset{O}{\overset{\|}{C}}$-4f-spacer conjugates (Method 4)



Since the desired products, PEG-O-$\overset{O}{\overset{\|}{C}}$-spacer prepared by method described in section 3.3.2.3 could not be obtained. It may be due to self-conjugation of the spacer during conjugation process. Therefore, in order to prevent that, protection of amino group on PABA and PAH was performed using formic acid (Zou *et al.*, 2005).

3.3.2.4.1 Synthesis of protected PABA and protected PAH (Figure 3-6)

Each of spacers, PABA (**1**, 1.37 g, 10 mmol) or PAH (**2**, 1.95 g, 10 mmol), was dissolved in 50 ml formic acid (excess) and refluxed for 30 minutes (Zou *et al.*, 2005). Then the reaction mixture was left to room temperature and cold water was added, resulting in precipitation. The precipitate (4f-PABA, **16**; or 4f-PAH, **17**) was filtered and washed with cold water. Melting point, FT-IR and ^{1}H-NMR spectral data of the resulting compounds were determined.

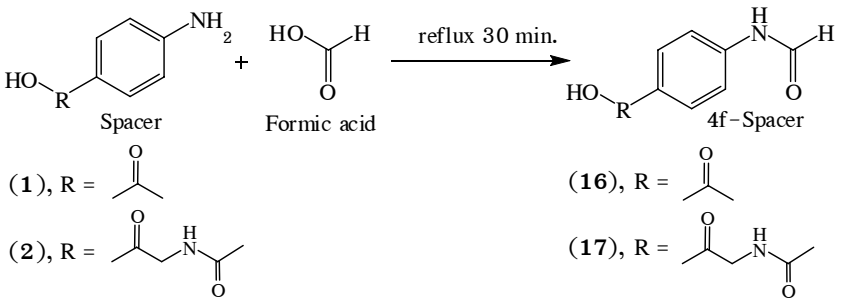


Figure 3-6 Synthetic scheme of 4f-PABA (**16**) and 4f-PAH (**17**)

3.3.2.4.2 Synthesis of PEG-O-C(=O)-4-formylated spacer conjugates (Figure 3-7)

mPEG$_{5000}$ (**6**, 1 g, 0.2 mmol) or PEG$_{4000}$ (**7**, 1 g, 0.25 mmol) was washed by hexane and dried by azeotropic distillation before used. 4f-Spacer (**16**, 0.27 g, 1.6 mmol for **6** or 0.53 g, 3.2 mmol for **7**; or **17**, 0.35 g, 1.6 mmol for **6** or 0.71 g, 3.2 mmol for **7**) was dissolved in 20 ml dried pyridine (or TEA). CDI (0.49 g, 3 mmol for **6**; or 0.97 g, 6 mmol for **7**) in dried CH_2Cl_2 (20 ml) were then added. Dried PEG (**5**, 1 g, 0.2 mmol or **6**, 1 g, 0.25 mmol) in 20 ml CH_2Cl_2 was added dropwise while stirring at room temperature. The solution mixture was stirred at room temperature overnight until the white precipitant appears. Then precipitate was filtered, the resulting filtrate was extracted 3 times by distilled water to remove excess pyridine. Then, the organic portion was extracted twice with 1 M HCl to remove excess 4f-spacer. The organic portion was quenched by extraction with 0.5 M NaOH and washed with distilled water,

respectively (Zou *et al.*, 2005). The organic fraction was dried over anhydrous Na_2SO_4. The solvent was evaporated and dried overnight under vacuum. FT-IR and ^{1}H-NMR spectral data of the resulting product were determined.

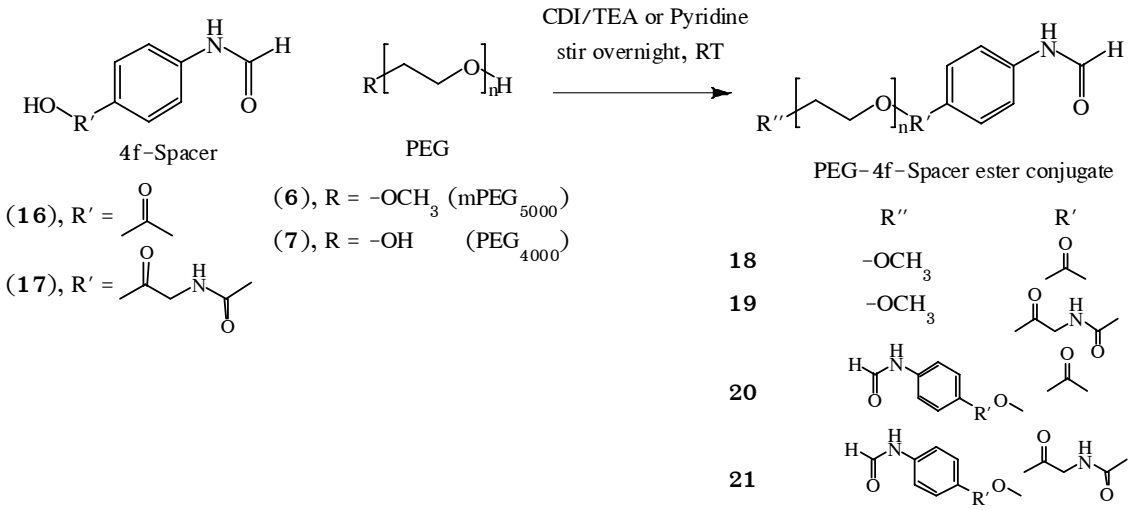


Figure 3-7 Synthetic scheme of $mPEG_{5000}$-O-C(=O)-4f-PABA (**18**), $mPEG_{5000}$-O-C(=O)-4f-PAH (**19**), PEG_{4000}-O-C(=O)-(4f-PABA)$_2$ (**20**) and PEG_{4000}-O-C(=O)-(4f-PAH)$_2$ (**21**) conjugates

3.3.2.5 Synthesis of PEG_{3000}-N(H)-C(=O)-4-formylated PABA conjugate (Method 5, Figure 3-8)

Purified and dried PEG_{3000} diamino (**22**, 0.15 g, 0.05 mmol) was dissolved in 10 ml CH_2Cl_2. Solutions of 4f-PABA (**16**, 0.33 g, 0.2 mmol) in DMSO (5 ml) and 0.2 mmol CDI (0.33 g) in DMSO (5 ml) were added while stirring at room temperature. The mixture was stirred at room temperature for 24 hours (Bayer and Mutter, 1972; Rajasekharan Pillai *et al.*, 1979; Wiwattanapatapee *et al.*, 2003). The resulting product was precipitated by ether. FT-IR and ^{1}H-NMR spectral data of the resulting compound were determined.

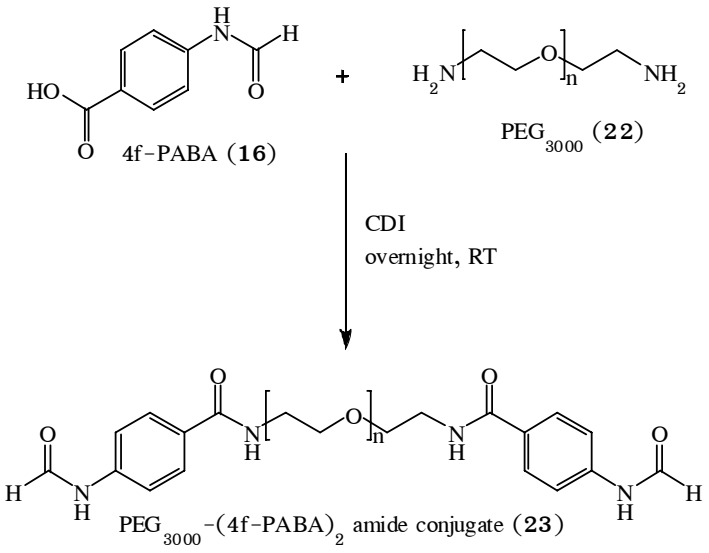


Figure 3-8 Synthetic scheme of PEG_{3000}-$\overset{H}{N}$-$\overset{O}{\overset{\|}{C}}$-(4f-PABA)$_2$ conjugate (**23**)

3.3.2.6 Synthesis of PEG_{3000}-$\overset{H}{N}$-$\overset{O}{\overset{\|}{C}}$-4-formylated PABA conjugate (Method 6, Figure 3-9)

In this method, acid chloride of 4f-PABA was generated in situ using triphosgene prior to react with PEG_{3000} diamino. Triphosgene or bis(trichloromethyl) carbonate (BTC: 0.18 g, 0.6 mmol) in THF (5 ml), and dried pyridine (1.5 ml, 1.8 mmol) was mixed. Solution of 4f-PABA (**16**, 0.31 g, 1.8 mmol) in 20 ml THF was then added to the mixture at 0 ^{o}C while stirring under N_2 for 1 hour. The reaction was elevated to room temperature, followed by the slowly addition of PEG_{3000} diamino (**22**, 0.18 g, 0.06 mmol) solution in 10 ml THF. The reaction mixture was then stirred at room temperature for another 2 hours (Hardie *et al*. 2001). The resulting precipitate was filtered and the solvent was removed by rotary evaporator under vacuum. The product was precipitated by CH_2Cl_2/ether. FT-IR and ^{1}H-NMR spectroscopy of the resulting compound were determined.

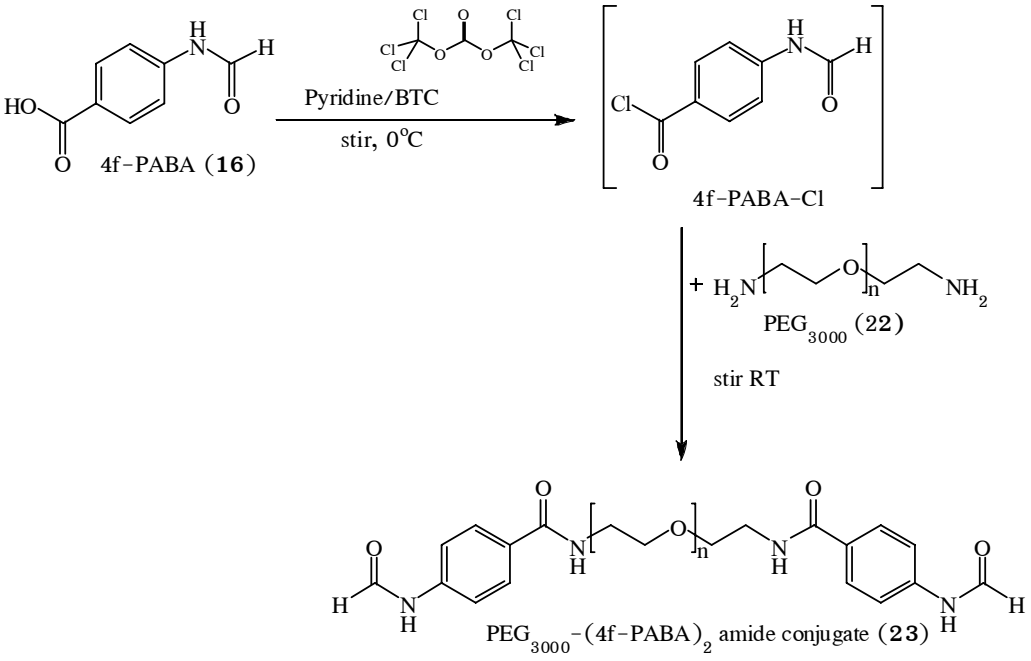


Figure 3-9 Synthetic scheme of PEG_{3000}-$\overset{H}{N}$-$\overset{O}{\overset{\|}{C}}$-$(4f\text{-}PABA)_2$ conjugate (**23**)

3.3.2.7 Synthesis of PEG_{4000}-O-$\overset{O}{\overset{\|}{C}}$-PABA conjugate (Method 7)

To generate ester bond between the hydroxyl group of PEG and carboxyl group of a spacer, acid chloride intermediate was synthesized by using thionyl chloride according to the reported procedures (Zalipsky *et al.*, 1983; Topchiyeva, 1990).

3.3.2.7.1 Synthesis of PEG_{4000}-O-$\overset{O}{\overset{\|}{C}}$-4-formylated PABA conjugate (Figure 3-10)

4f-PABA (**16**, 1.65 g, 10 mmol) was dissolved in dried pyridine (30 ml). Thionyl chloride ($SOCl_2$: 1.1 ml, 15 mmol) was added dropwise while the reaction mixture was being refluxed. The reaction was further refluxed for 30 minutes. The excess $SOCl_2$ was then removed by evaporation before adding the reaction mixture into dried PEG_{4000} (**7**, 4 g, 1 mmol) solution in dried pyridine (10 ml). The mixture was refluxed for 4 hours followed by stirring at room temperature for 24 hours (Rawat *et al.*, 2007).

The solvent was removed and re-dissolve the residue in CH_2Cl_2. The product was then precipitated using diethyl ether.

Then the resulting product was re-dissolved again in CH_2Cl_2 and extracted 3 times each by 1 M HCl, 0.5 M NaOH and distilled water, respectively. The organic fraction was dried over anhydrous Na_2SO_4, evaporated and dried overnight under vacuum. FT-IR and ^{1}H-NMR spectral data of the synthesized compound were determined.

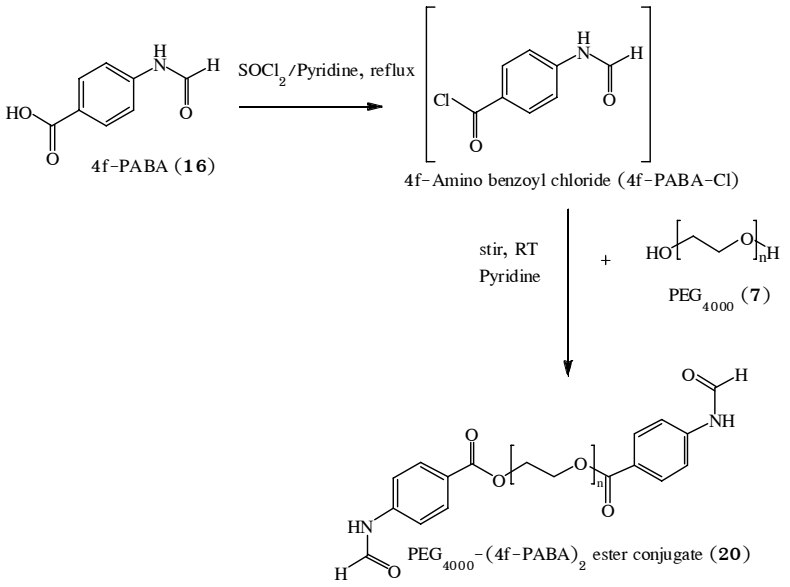


Figure 3-10 Synthetic scheme of PEG_{4000}-O-$\overset{O}{\overset{\|}{C}}$-$(4f\text{-}PABA)_2$ conjugate (**20**)

3.3.2.7.2 Hydrolysis of formaldehyde in PEG_{4000}-O-$\overset{O}{\overset{\|}{C}}$- 4-formylated PABA conjugate (Figure 3-11)

The resulting PEG_{4000}-$(4f\text{-}PABA)_2$ ester conjugate (**20**, 1 g, 0.23 mmol), was hydrolyzed by stirring vigorously while refluxing in 15% ethanolic HCl solution (30 ml, excess) for 15 minutes (Zou *et al.*, 2005) to obtain amino functional group of **14** (Figure 3-11). Then ethanol was removed and extracted 3 times with CH_2Cl_2. The organic fraction was dried over anhydrous Na_2SO_4, filtered, removed the solvent and kept in vacuum overnight. The product was purified by SiO_2 gel column

chromatography, using CH_2Cl_2: MeOH (9:1) as mobile phase. FT-IR and ^{1}H-NMR spectroscopy of the resulting compound were determined.

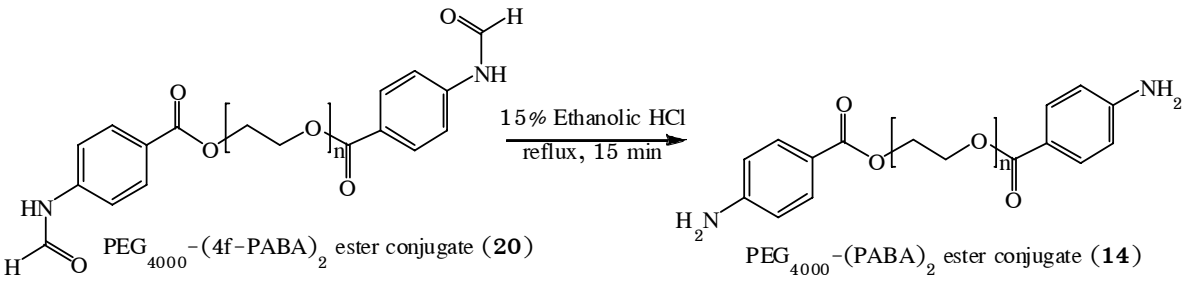
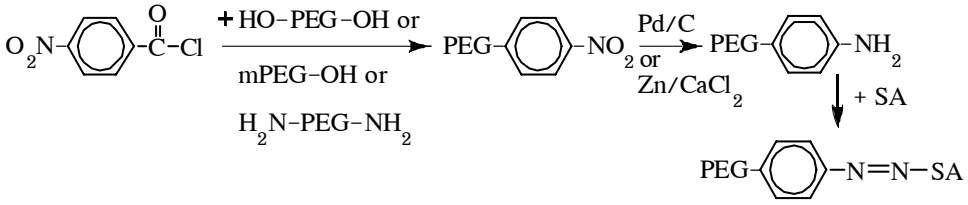


Figure 3-11 Hydrolysis of formaldehyde in PEG_{4000}-O-$\overset{O}{\overset{\|}{C}}$-(4f-PABA)$_2$ conjugate (**14**)

3.3.2.8 Synthesis of poly(ethylene) glycol-PABA-SA ester and amide conjugates (Method 8)



Due to the effectiveness of using acid chloride derivative of 4f-PABA conjugated to PEG_{3000} diamino (method 6) and PEG_{4000} dihydroxyl (method 7), more stable acid chloride derivative of *p*-aminobenzoic acid (PABA, **1**) was used in this procedure.

3.3.2.8.1 Synthesis of poly(ethylene) glycol-4-nitro benzoylated ester and amide conjugates (Figure 3-12)

Each PEG (**6**, **7**, **22**, **24**, **25**) was dissolved in dried tetrahydrofuran (THF). Solution of *p*-nitro benzoylchloride (**26**) in dried THF was added to the PEG solution while stirring in ice bath. Then, pyridine was added dropwise. Mole ratios of the compounds used in this synthesis procedure were shown in Table 3-1. The reaction mixture was stirred at room temperature overnight (Callant and Schacht, 1990; Schiavon *et al.*, 2004). The precipitate was filtered off. The solution was evaporated to dryness and re-

dissolved in CH_2Cl_2. The resulting product was obtained by precipitation in diethyl ether. The amide products were purified again by column chromatography using CH_2Cl_2/MeOH (9:1) as mobile phase, in case of free *p*-nitro benzoic acid was detected. The organic fraction was dried over anhydrous Na_2SO_4 and removed the solvent. The residue was dried overnight in vacuum. FT-IR and ^{1}H-NMR spectroscopy of the synthesized compounds (**27, 28, 29, 30, 31**) were determined.

Table 3-1 Mole ratios of the compounds and amount of solvent used in the synthesis procedure of poly(ethylene) glycol-4-nitro benzoylated ester and amide conjugates (3.3.2.8.1)

PEGs (g, mmol)	26 (g, mmol)	Pyridine (ml, mmol)	THF (ml)	Product No.
6, $mPEG_{5000}$ (10, 2)	2.1, 12	1.0, 12	40	**27**
7, PEG_{4000} (8 , 2)	3.3, 20	1.7, 20	40	**28**
22, PEG_{3000} diamino (0.8 , 0.25)	0.42, 2.5	0.2, 2.5	20	**29**
24, PEG_{5106} diamino (0.9 , 0.2)	3.4, 2	0.2, 2.5	20	**30**
25, PEG_{3677} diamino (0.8 , 0.2)	3.4, 2	0.2, 2.5	20	**31**

3.3.2.8.2 Reduction of nitro functional group in poly(ethylene) glycol-4-nitro benzoylated ester and amide conjugates

There were two procedure used to reduce the nitro functional group in the PEG conjugates (**27, 28, 29, 30, 31**).

3.3.2.8.2.1 Reduction by $Zn/CaCl_2.2H_2O$ (Figure 3-13)

Aromatic nitro ($-NO_2$) functional site chain was subjected to reduction to amino ($-NH_2$) group prior to the following diazotization reaction with salicylic acid by $Zn/CaCl_2.2H_2O$ (10:1 mole ratio) in $EtOH/H_2O$ (Callant and Schacht, 1990). The amount in gram and mmol of substrates were shown in Table 3-2.

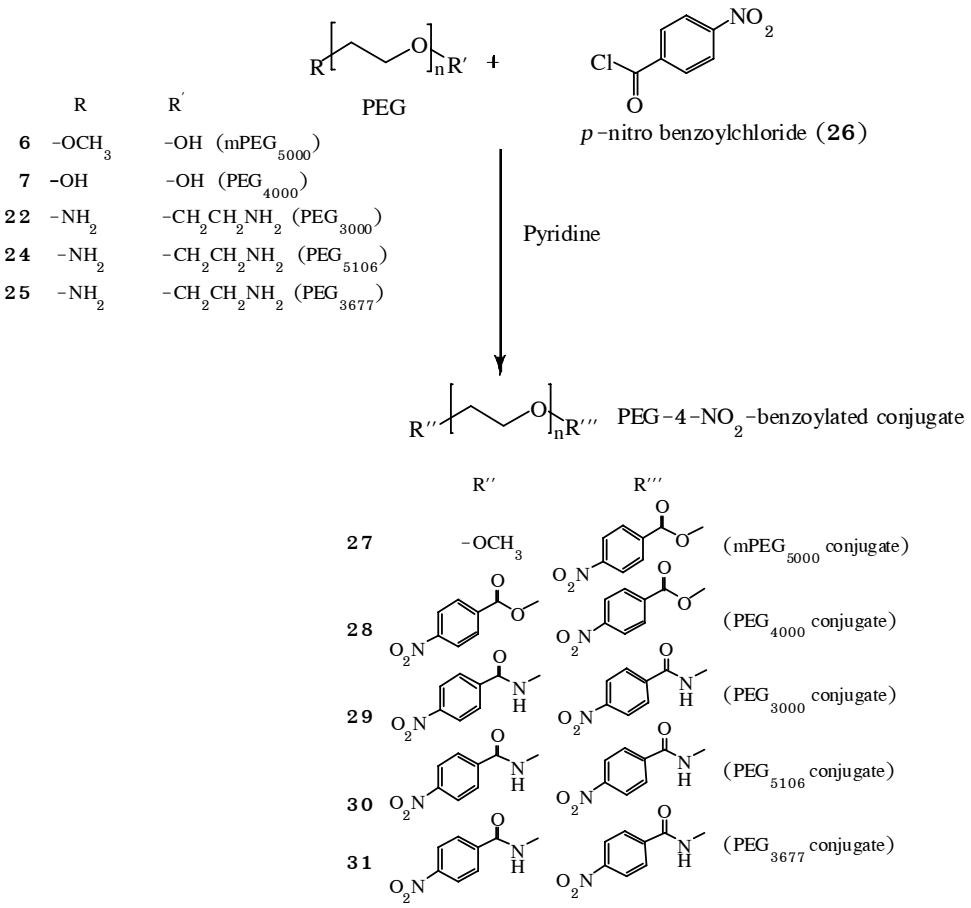


Figure 3-12 Synthetic scheme of PEG-4-NO_2-benzoylated conjugates (**27, 28, 29, 30, 31**)

PEG-4-NO_2-benzoylated or PEG-4-NO_2-BA conjugate (**27, 28, 29**) was weighed in exact amount, then, dissolved in 4:1 EtOH/H_2O solution and reflux for 5 minutes. Zinc powder (Zn) and calcium chloride dihydrate ($CaCl_2.2H_2O$) were mixed before adding to the reaction flask. The amount of substrate and reducing agents used in this process were listed in Table 3-2. The reaction mixture was refluxed for 3 hours (Callant and Schacht, 1990). After cooled down to room temperature, the clear solution was filtered and evaporated, then re-dissolved in CH_2Cl_2 and washed with distilled water. Dried and evaporated the organic fraction to obtain the expected products. FT-IR and ^{1}H-NMR spectroscopy of the resulting compounds were determined.

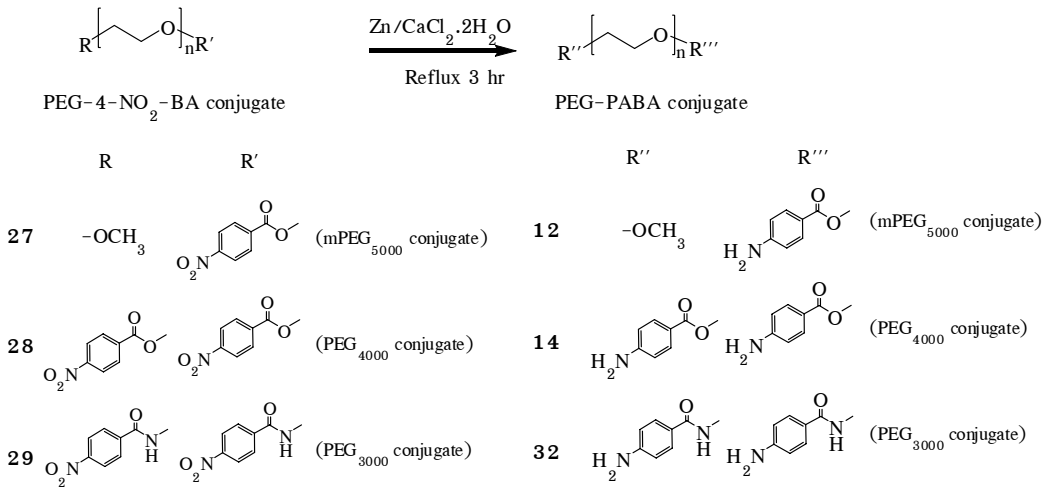


Figure 3-13 Reduction of nitro functional group in PEG-4NO_2-BA conjugates

Table 3-2 The amount of substrates used in reduction procedure of nitro group

PEG conjugates	**PEG conjugate (g, mmol)**	**Zn (g/mmol)**	**$CaCl_2.2H_2O$ (g/mmol)**	$EtOH/H_2O$ 4:1 (ml)
27, $mPEG_{5000}$-4-NO_2-BA ester	2.60, 0.5	0.32, 5	0.08, 0.5	30
28, PEG_{4000}-(4-NO_2-BA)$_2$ ester	2.18, 0.5	0.65, 10	0.15, 1.0	30
29, PEG_{3000}-(4-NO_2-BA)$_2$ amide	0.60, 0.2	0.27, 4	0.06, 0.4	20

3.3.2.8.2.2 Reduction by hydrogenation (Figure 3-14)

After reduction by $Zn/CaCl_2.2H_2O$, the ester and amide bond between PEG and spacer was degraded. It showed that this reduction condition was too strong. Therefore, other reduction procedure was used to reduce the nitro functional group, so that the amino functional group could be obtained and ready for the final process, diazotization with SA.

Aromatic nitro ($-NO_2$) functional site chain was reduced to amino ($-NH_2$) functional group before the following diazotization reaction with SA by hydrogenation reaction in dried methanol. The amount in gram and mmol of substrates, and reaction conditions used in this procedure were shown in Table 3-3.

PEG-4-NO_2-benzoylated conjugate (**27**, **28**, **30**, or **31**) was weighed in exact amount and dissolve in dried Methanol. Palladium on activated charcoal (Pd/C) was added. The reduction of nitro functional group was run under $H_{2\ (g)}$ using Parr® hydrogenation apparatus for 10 minutes (Tjoeng and Hodges, 1979; Zalipsky *et al.*, 1983; Carceller *et al.*, 2001). After the reaction, the suspension mixture was immediately filtered through Celite®. The clear solution was then evaporated and dried in vacuum overnight. FT-IR and ^{1}H-NMR spectroscopy of the synthesized compounds (**12**, **14**, **33**, **34**) were determined.

Table 3-3 The amount of substrates used in reduction procedure of nitro group

PEG conjugates	**PEG-4-NO_2-benzoylated conjugate (g, mmol)**	**Pd/C (g), H_2 pressure ($lbs/inch^2$)**
27, $mPEG_{5000}$-4-NO_2-benzoylated ester	1.20, 0.23	0.25, 5
28, PEG_{4000}-(4-NO_2-benzoylated)$_2$ ester	1.05, 0.24	0.22, 10
30, PEG_{5106}-(4-NO_2- benzoylated)$_2$ amide	0.52, 0.10	0.15, 8
31, PEG_{3677}-(4-NO_2- benzoylated)$_2$ amide	0.50, 0.13	0.15, 8

3.3.2.8.3 Synthesis of poly(ethylene) glycol-PABA-SA ester and amide conjugates (Figure 3-15)

The final products of this method were achieved by diazotization reaction of the products obtained from previously described in section 3.3.2.8.3. Mole ratios of the compounds used in this synthesis procedure were shown in Table 3-4.

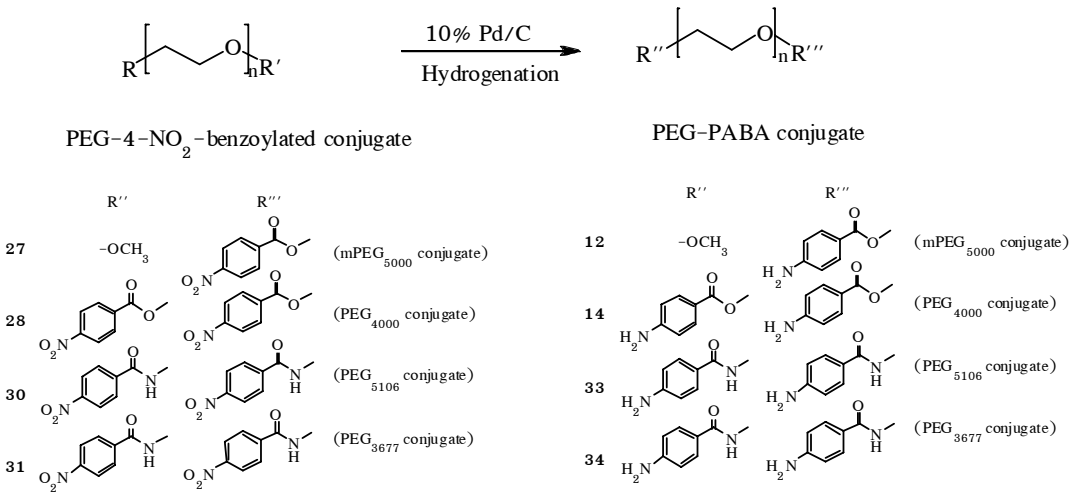


Figure 3-14 Reduction procedure of PEG-4-NO_2-benzoylated conjugates (**27, 28, 30, 31**)

A solution of each PEG-spacer conjugates (**12, 14, 33** or **34**) was dissolved in distilled water and stirred in ice bath (3-5 °C) for 5 minutes. The pH was adjusted to 1-2 with 3 M HCl. A solution of $NaNO_2$ in water was added dropwise into the solution of each PEG-spacer conjugate. The mixture was stirred in ice bath (3-5 °C) for 5 minutes further and then the diazonium salt was tested for the presence of excess nitrous acid using KI/starch indicator paper. Salicylic acid was dissolved in MeOH and stirred in ice bath. Then the diazonium salt solution was added into the SA solution while stirring at 3-5 °C (Wiwattanapatapee *et al.*, 2003). One molar NaOH solution was added to adjust pH to 9-10. The reddish brown solution appeared and the solution was stirred for a further 10 minutes. The mixture was then purified by dialysis in distilled water for 15-30 minutes. The product in water solution was extracted 3 times with CH_2Cl_2. The organic portion was dried over anhydrous Na_2SO_4, removed solvent by vacuum evaporation. The products were dried in vacuum overnight to obtain the reddish orange amorphous solid. UV-visible spectrophotometry, FT-IR and ^{1}H-NMR spectroscopy of the synthesized compound (**8, 10, 39, 40**) were determined.

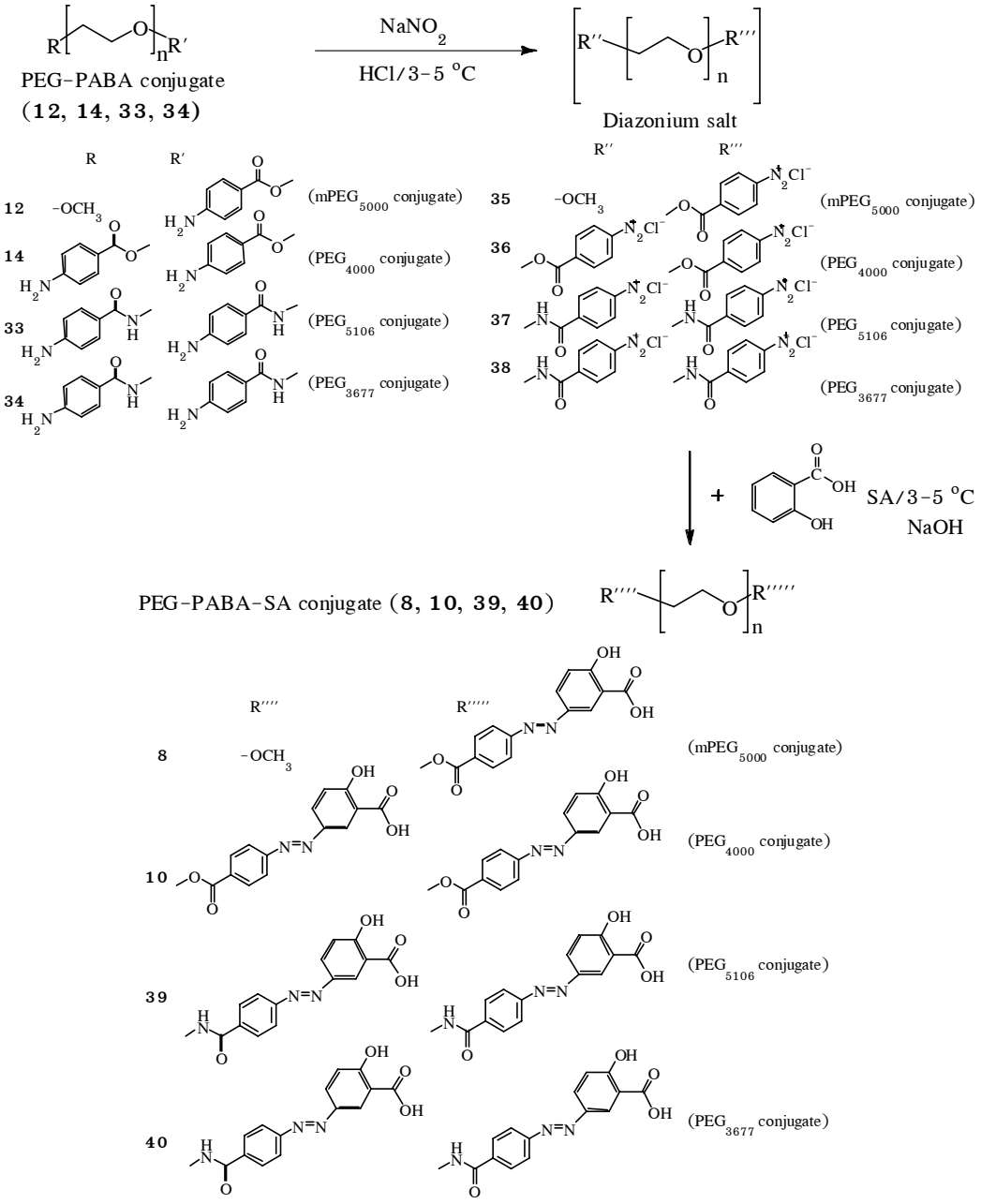


Figure 3-15 Synthetic scheme of $mPEG_{5000}$-O-C(=O)-PABA-SA conjugate (**8**), PEG_{4000}-O-C(=O)-$(PABA-SA)_2$ conjugate (**10**), PEG_{5106}-N(H)-C(=O)-$(PABA-SA)_2$ conjugate (**39**) and PEG_{3677}-N(H)-C(=O)-$(PABA-SA)_2$ conjugate (**40**)

Table 3-4 Mole ratios of the substrates used in method 8 (3.3.2.8.3)

PEG conjugates (g, mmol)	$NaNO_2$ (g, mmol)	SA (g, mmol)
12, $mPEG_{5000}$-PABA ester (1.02, 0.21)	0.04, 0.6	0.05, 0.4
14, PEG_{4000}-$PABA_2$ ester (0.85, 0.20)	0.13, 1.5	0.08, 0.6
33, PEG_{5106}-$PABA_2$ amide (0.48 , 0.09)	0.07, 1.0	0.06, 0.4
34, PEG_{3677}-$PABA_2$ amide (0.41, 0.10)	0.07, 1.0	0.06, 0.4

3.3.3 Quantitative analysis

According to the aims of our study, the released of the active drug (5-ASA) out from the polymeric conjugates need to be determined after incubation in different conditions. However, not only the active drug is released from the conjugates, other compounds such as spacers (PABA, **1** and PAH, **2**) as well as small molecule of azo conjugates (PABA-SA, **4** and PAH-SA, **5**) may be released in the test conditions. It is therefore, the suitable method that could be used to determine the exact amount of these compounds must be investigated and validated. High performance liquid chromatography (HPLC) has been utilized in our study, since it could provide good separation between compounds with different polarity. The modified procedure from previously reported methods (She *et al.*, 1997; Bareggi and Benedittis, 1998; Saito *et al.*, 2006) was used in the study and the method is validated based on ICH guideline (1996) as followed:

3.3.3.1 HPLC conditions

HPLC analysis was carried out using Agilent 4100 series equipped with an Agilent 1100 series Photodiode-array detector (PDA) and autosampler. Data analysis was performed using Agilent software (Agilent, USA). The chromatographic system was reverse phase HPLC (250 mm x 4.6 mm i.d. C-18 column) with gradient system of mobile phase A, pH 3.3 acetate buffer/MeOH (85:15 v/v) and mobile phase B, MeOH. The % ratio of mobile phase A/mobile phase B were 100/0 (0-5 minutes), 60/40 (5.01-9 minutes), 100/0 (9.01-11 minutes), 33/67 (11-18 minutes) and 100/0 (18.01-25 minutes). Flow rate was maintained at 0.8 ml/min and at 25 $^{\circ}C$. The

injection volume was 20 µl. The quantitation wavelength was set at 295 nm, for detection of 5-ASA, PAH (**2**), PABA (**1**) and SA, and 365 nm, for detection of PAH-SA (**5**) and PABA-SA conjugates (**4**).

3.3.3.2 Preparation of standard solution

Standard stock solution of 5-ASA, PAH (**2**), PABA (**1**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**) were prepared by accurately weighting each standard (10 mg) and dissolved in methanol to yield a final volume of 10 ml (final concentration 1 mg/ml). The conjugate (**4** or **5**) was dissolved in 5 *N* NH_4OH (0.5 ml) before continually added methanol, resulting in the clear solutions having concentration of 1 mg/ml as the stock solution. The stock solutions were used in preparation of working standard solutions for analyses. Working solution of the standards were subsequently prepared in methanol and further diluted in mobile phase A which would be described later, to provide serial solutions for using in constructing calibration curves for each of analysis.

3.3.3.3 Preparation of mobile phase

In HPLC gradient system, mobile phase A and B were prepared as described below. Mobile phases were filtered and sonicated before use.

Mobile phase A was the mixture of 85% acetate buffer pH 3.3 and 15% methanol (v/v). One liter of acetate buffer pH 3.3 was prepared by pipette 1.14 ml of analytical grade of glacial acetic acid and mix with 500 ml of distilled water and subsequently adjusted to 1000 ml by distilled water. The pH was adjusted to 3.3 by 5 *N* NH_4OH. Mobile phase B was 95% methanol. Each mobile phase, A and B, was kept in separated bottles and freshly prepared before use.

3.3.3.4 Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Identification of retention time of the peaks of 5-ASA, PAH (**2**), PABA (**1**), SA (**3**), PAH-SA conjugate (**5**) and

PABA-SA conjugate (**4**) was carried out using each standard solution. By products from synthesized azo conjugates procedure and the released compounds in gastrointestinal contents in 1% peptone buffer solutions after drug release study were investigated using the same conditions. Ten milligrams of each standards; 5-ASA, PAH (**2**), PABA (**1**) and SA (**3**); were weighed and dissolved in methanol, then adjusted to 10 ml. PAH-SA (**5**, 10 mg) or PABA-SA (**4**, 10 mg) conjugates in 0.5 ml 5 *N* NH_4OH was further diluted to 10 ml. The final concentration of each standard; 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**); in the mixture were 6 μg/ml, whereas the concentration of PABA (**1**) in the mixture was 0.6 μg/ml. The experiments were performed in triplicate. The analyte should not be interfered from other constituents and well resolved from those constituents (http://www.ich.org/cache/compo/276-254-1.html).

3.3.3.5 Limit of detection (LOD) and limit of quantitation (LOQ)

The detection limit of an analytical method is the lowest amount of the analyte that can be detected. LOD value indicates sensitivity of an analytical method. The quantitation limit of an analytical method is the lowest amount of the analyte that can be quantitatively determined with suitable precision and accuracy. In this study, LOD and LOQ were determined using standard solution of 5-ASA, PAH (**2**), PABA (**1**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**). Each compound was accurately weighed and diluted to result the solutions having concentration in range of 0.2-10 μg/ml. The signal to noise (S/N) ratio of each standard signal was calculated. LOD and LOQ were determined by analysis serial dilution of the mixture of standards solution and obtained S/N ratio of 3:1 and 10:1, respectively (http://www.ich.org/cache/compo/276-254-1.html).

3.3.3.6 Linearity and range

Calibration curves were constructed by analysis working standard at different concentrations covering the range of 2-10 μg/ml for determination of 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**), and of 0.2-1.0 μg/ml for determination of PABA (**1**). The concentration of each standards; 5-ASA,

PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**); were 2, 4, 6, 8 and 10 μg/ml, whereas PABA (**1**) standard solutions were 0.2, 0.4, 0.6, 0.8 and 1.0 μg/ml. The analysis was performed repeatedly for at least 5 determinations per concentration. Peak area was plotted subsequently against the concentration of each reference standards solution. The linearity of the peak area of the standard was assessed using linear regression. The curves were considered linear if coefficient of correlation (R^2) was equal to, or more than 0.99 (http://www.ich.org/cache/compo/276-254-1.html).

3.3.3.7 Precision

The precision of an analytical method is the similarity of the test results to the actual value such as the concentration of the analyte. The accuracy of the current analytical method was demonstrated by recovery studies. Three concentrations (low, medium and high) of working standard solution were prepared as quantity controlled (QC) samples in concentration of 2, 6 and 10 μg/ml, for determination of 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**), and 0.2, 0.6 and 1.0 μg/ml, for determination of PABA (**1**). These QC samples were analyzed according to conditions described in section 3.3.3.1, for calibration curve analysis. QC samples were determined 5 times per concentration and compared to calibration curve (http://www.ich.org/cache/compo/276-254-1.html). Recovery percentage in mobile phase was determined using the equation.

$$\text{Recovery (\%)} = \frac{C_{obs}}{C_{actual}} \times 100$$

which C_{obs} refers to determined concentration of each standard

C_{actual} refers to actual concentration of standard solution of each standard

3.3.3.8 Repeatability

Repeatability of analytical method is the similarity of individual measures of an analyte when the procedure is performed repeatedly (http://www.ich.org/cache/

compo/276-254-1.html). Accuracy was evaluated based on relative standard deviation (% RSD) determined using the equation.

$$\% \text{ RSD} = \frac{\text{SD}}{\text{mean}} \times 100$$

3.3.3.8.1 Within-run analysis

Within-run analysis study was determined by analysis of QC samples having concentrations of 2, 4, 6, 8 and 10 μg/ml, for determination of 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**); and 0.2, 0.4, 0.6, 0.8 and 1.0 μg/ml, for determination of PABA (**1**). Each QC sample was determined five times repeatedly. Repeatability of the analytical method was evaluated using RSD (%), which should be lower than 15% (http://www.ich.org/cache/compo/276-254-1.html).

3.3.3.8.2 Between-run analysis

Between-run analysis studies were determined by analysis of QC samples as same as within-run analysis. QC samples were 2, 4, 6, 8 and 10 μg/ml of 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**); and 0.2, 0.4, 0.6, 0.8 and 1.0 μg/ml of PABA (**1**). Each QC sample was determined five times repeatedly for three times of analysis. Reproducability of analytical method was evaluated using RSD (%), which was considered if % RSD was lower than 15% (http://www.ich.org/cache/compo/276-254-1.html).

3.3.4 % Free SA and other related compounds study

As mentioned earlier that SA could irritate stomach after oral administration, then the amount of SA and other related compounds in synthesized products were determined by validated HPLC system.

The PEG-PABA-SA conjugates (**8**, **10**, **39**, **40**) were weighed in exact amount equivalent to 5 mg of 5-ASA and dissolved by distilled water. The volume of clear

orange solution was adjusted to 5 ml (1 mg/ml). Three diluted samples of each product (100 μg/ml) were prepared and determined by validated HPLC. % Free of SA and other related compounds were calculated. SA, PABA and PABA-SA were used as standards.

3.3.5 Water solubility test

Since 5-ASA is a very poor water soluble compound, therefore, when conjugate 5-ASA to PEG, water soluble polymer, it may improve water solubility property of this drug. The solubility test is described below.

Each of the PEG-PABA-SA conjugates (**8**, **10**, **39**, **40**) was accurately weighed and transferred to each test tube. Then 200 μl of distillated water was added into each test tubes using micropipette at room temperature. The mixture was shaken and checked for dissolution. If the compound was not dissolved, another 200 μl of distilled water was added and performed in the same manner. The distilled water was repeatedly added until clear solution was obtained. According to the solubility property of 5-ASA (French and Mauger, 1993), if the total volume of added distilled water was over than 120 ml, insoluble property would be recorded. The accurate solubility value was calculated.

3.3.6 Stability test

Since there are two chemical bonds in the final products of these tripartate compounds, ester or amide bond between PEG and PABA, and azo bond between PABA and SA, chemical stability of each product was studied.

The PEG-PABA-SA conjugates (**8**, **10**, **39**, **40**) were weighed in exact amount equivalent to 14 mg of 5-ASA and transferred to 10-ml volumetric flask. The volume was then adjusted to 10 ml with distilled water resulting in clear orange solution (1.4 mg/ml). The solution (100 μl) was taken and transferred to eppendorf tube and adjusted to 1 ml by adding 900 μl of solvent (H_2O or citrate buffer pH 1.2, or phosphate buffer pH 6.8 or 7.5). The products were incubated at 37 ^{o}C for 6 hours in citrate buffer (pH 1.2), 12 hours in phosphate buffer (pH 6.8), and 24 hours in phosphate buffer pH

7.5 and distilled water. The samples (100 µl) were taken at selected time intervals; 0, 0.5, 1, 2 and 6 hours for incubation in citrate buffer pH 1.2; 0, 0.5, 1, 2, 6 and 12 hours for incubation in phosphate buffer pH 6.8; and 0, 0.5, 1, 2, 6, 12 and 24 hours for incubation in phosphate buffer pH 7.5 and distilled water (Wiwattanapatapee *et al*., 2003; Zou *et al*., 2005). The diluted samples of each product (14 µg/ml) were prepared and determined by validated HPLC system. 5-ASA, SA (**3**), PABA (**1**) and PABA-SA (**4**) were used as standards.

3.3.7 Study of drug release in gastrointestinal contents

3.3.7.1 Samples preparation

The PEG-PABA-SA conjugates (**8**, **10**, **39**, **40**) were weighed in exact amount equivalent to 14 mg of 5-ASA and transferred to 10-ml volumetric flask. The volume was adjusted to 10 ml with distilled water, resulting in clear orange solution (1.4 mg of 5-ASA/ml).

Sulfasalazine solution was prepared in similar manner but 0.5 ml of 0.1 *N* NaOH was used as the solvent and used as the positive reference. Distilled water was then added to adjust the volume to 10 ml, resulting in clear red solution (1.4 mg of 5-ASA/ml). All sample solutions were blown with $N_{2\,(g)}$ before used in the release study.

3.3.7.2 Gastrointestinal contents preparation

The experimental protocols described in the present study were approved and guided by the Animal Ethical Committee of the Prince of Songkla University (Ref.05/49). Two male Wistar rats (about 200 g) were sacrificed and a midline incision was made in anaerobic system using anaerobic bag (Figure 3-16) in preparation of the gastrointestinal contents and incubation process. Sections of stomach, small intestine and colon were collected separately and homogenized (Wiwattanapatapee *et al*., 2003; Zou *et al*., 2005). Each homogenate was diluted with 1% peptone in citrate buffer solution pH 1.2, and phosphate buffer solution pH 6.8 and pH 7.5, respectively, to yield 1:2 ratio

suspension. The suspensions were centrifuged at 5000 rpm (5 °C) for 5 minutes and kept in ice bath before added to the sample solutions of **8**, **10**, **39**, **40** and sulfasalazine.

Figure 3-16 Two-hand anaerobic glove bag (Aldrich® AtmosBag, from www.sigma-aldrich.com)

3.3.7.3 Drug release study

To 900 µl of each homogenate, 100 µl of the PEG-PABA-SA conjugate (**8**, **10**, **39**, **40**) or sulfasalazine solution was added under anaerobic system. The mixture was incubated for 24 hours at 37 °C. Aliquot amount of samples (140 µg/ml) were taken at selected time intervals: 0.5, 1, 2, 4, and 6 hours after incubation in 1% peptone in citrate buffer solution pH 1.2; 0.5, 1, 2, 4, 6 and 12 hours after incubation in 1% peptone in phosphate buffer solution 6.8; and 0.5, 1, 2, 4, 6, 12, 16 and 24 hours after incubation in 1% peptone in phosphate buffer solution 7.5 (Wiwattanapatapee *et al.*, 2003; Zou *et al.*, 2005). Fifty microliters of 30% (w/v) trichloroacetic acid (TCA) was added to stop the enzymatic reaction. The sample was centrifuged at 10000 rpm (5 °C) for 25 minutes. Fifty microliters of methanol was added to the supernatant (300 µl), to precipitate the protein, and filtered through the 0.2 µm-membrane filter (Zou *et al.*, 2005). A hundred microliters of the clear sample solution was diluted with 900 µl of mobile phase. 5-ASA and other related compounds were determined by validated HPLC system. The % release of each compounds were calculated. 5-ASA, SA, PABA and PABA-SA were used as standards.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Solubility test of starting materials

The solubility values of each compound used in selected solvents were calculated. The results are shown in Table 4-1.

Table 4-1 Solubility values of the chemicals used in synthesis process

Chemicals	Solubility (g/l)					
	THF	**Acetone**	**DMF**	**DMSO**	**$CHCl_3$**	**Ether**
$mPEG_{5000}$	86	101	< 5	< 9	272	< 5
PEG_{4000}	157	223	< 4	< 3	38	< 6
$PEG_{3000}NH_2$	108	145	5	2	10	< 1
PABA (**1**)	193	109	77	156	< 6	9
PAH (**2**)	10	< 10	34	69	< 8	< 1
SA	585	261	580	4100	209	103
PABA-SA (**4**)	23	< 2	16	9	< 1	< 1
PAH-SA (**5**)	< 1	< 3	27	7	< 1	< 3
CDI	137	235	79	20	116	8
DMAP	111	188	91	21	228	21

The polymers were very well soluble in $CHCl_3$, acetone and THF. PABA could be dissolved very well in DMSO, THF, acetone and DMF, whereas PAH was dissolved in DMSO and DMF. Therefore, the best solvent for PABA and PAH was DMSO. However, DMF could also be used to dissolve these two compounds. CDI coupling reagent was dissolved in acetone, THF, $CHCl_3$ and DMF. DMAP was dissolved in $CHCl_3$, acetone, THF and DMF. The azo conjugates, PABA-SA (**4**) and PAH-SA (**5**) could be dissolved well THF and DMF, respectively. Since, the homogeneous reaction system is required for performing the conjugate reaction, therefore, DMSO or DMF or THF, and $CHCl_3$ were

chosen in the process of ester or amide bond synthesis. Dichloromethane (CH_2Cl_2) was preferred to be used in place of $CHCl_3$ due to lower toxicity (Brown-Woodman *et al.*, 1998).

4.2 Synthesis of poly(ethylene) glycol-spacer-SA conjugates (8-11)

4.2.1 Synthesis of poly(ethylene) glycol-spacer-SA ester conjugates (8-11, Method 1)

In order to avoid ester or amide bond-breaking between PEG and spacer during the diazotization reaction, each spacer, PABA and PAH, was prior linked to SA by azo bond. Two azo products were synthesized successfully resulting in reddish brown amorphous solid of PABA-SA (**4**) and PAH-SA (**5**) conjugates (Figure 4-1). The % yields of both products were 74.86 and 77.70%, respectively. The melting point of **4** and **5** were more than 286 °C (melting point of PABA, **1**, PAH, **2**, and SA, **3**, are 187, 198-199 and 159 °C, respectively; http://www.mpbio.com).

[4] **[5]**

Figure 4-1 PABA-SA **[4]** and PAH-SA **[5]** conjugates

Since the remaining of SA (**3**), PABA (**1**) or PAH (**2**) in the obtained azo products may interfere in the conjugation process, the determination for free substrate was performed by HPLC method. SA (**3**), PABA (**1**) and PAH (**2**) solutions were prepared and subjected to inject to HPLC system as described in section 3.3.3.1 separately. The chromatogram demonstrated that PABA (**1**, Figure 4-3), PAH (**2**, Figure 4-4) and SA (**3**, Figure 4-2) had the retention time at 10.56, 7.02 and 13.60 min., respectively. PABA-SA (**4**) and PAH-SA (**5**) conjugates solutions were also prepared and subjected to

inject into the same HPLC conditions. The standard curves of *p*-aminohippuric acid (PAH, **2**), *p*-aminobenzoic acid (PABA, **1**) and salicylic acid (SA, **3**) were constructed in ranges of 2-10 μg/ml with R^2 = 0.9995, 0.9997 and 0.9994, respectively. The results demonstrated that chromatogram of PABA-SA (**4**) and PAH-SA (**5**) conjugates showed their peaks at 20.74 and 14.01 min., respectively (Figure 4-3 and 4-4). SA (**3**) was not detected in the chromatogram of both azo products. However, small amount of PAH (**2**) and PABA (**1**) were found remaining in the azo products in 0.45 and 0.96%, respectively. This indicated that the azo conjugates were purely isolated from the reaction. % Purity of PABA-SA (**4**) and PAH-SA (**5**) were 99.55 and 99.04%, respectively (Table 4-2).

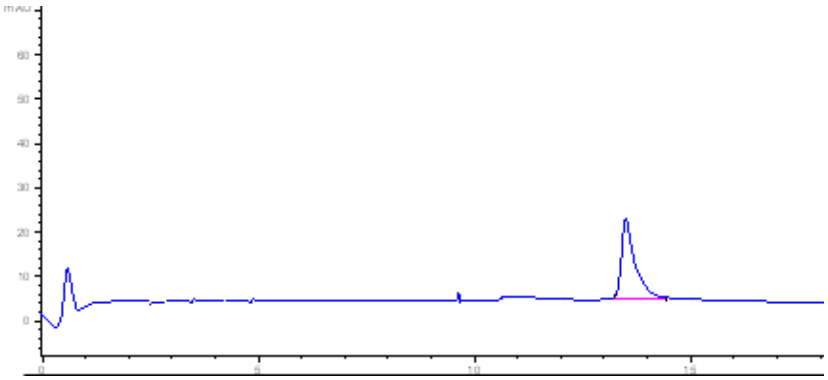


Figure 4-2 HPLC chromatogram of SA (**3**) detected at 295 nm

Table 4-2 % Content of PABA (**1**), PAH (**2**), SA (**3**), PABA-SA conjugate (**4**) and PAH-SA conjugate (**5**) in azo products (**4**, **5**)

Azo compound	% PAH	% PABA	% SA	% Content of Azo compound
PABA-SA (**4**)	-	0.45	0	99.55
PAH-SA (**5**)	0.96	-	0	99.04

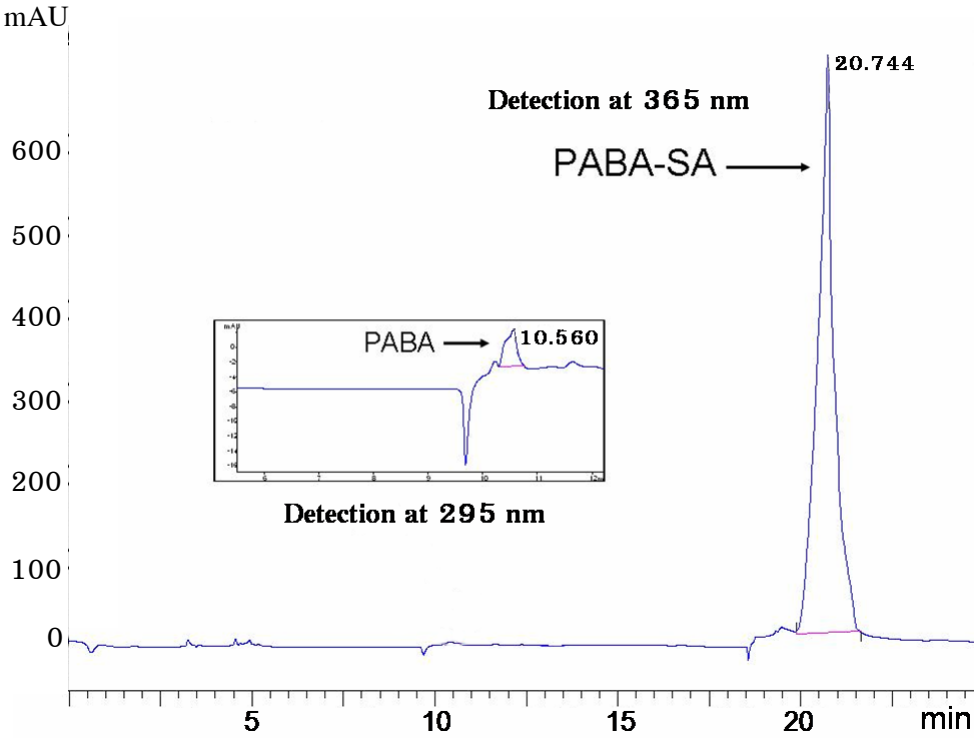


Figure 4-3 HPLC chromatogram of PABA-SA conjugate (**4**)

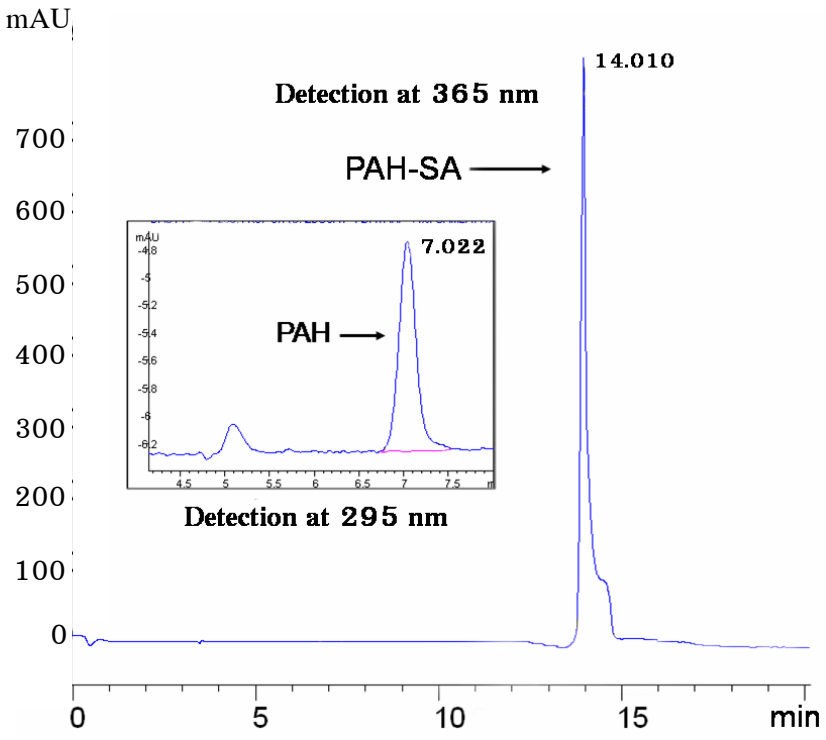


Figure 4-4 HPLC chromatogram of PAH-SA conjugate (**5**)

The UV absorption spectrum of the azo products, PABA-SA (**4**) and PAH-SA (**5**) conjugates were recorded by dissolving in 5 *N* ammonium hydroxide (NH_4OH) and further diluted with distilled water. Distilled water was used as a blank solution. The maximum wavelengths (λmax) of **4** and **5** were at 356 and 358 nm, respectively (Figure 4-5).

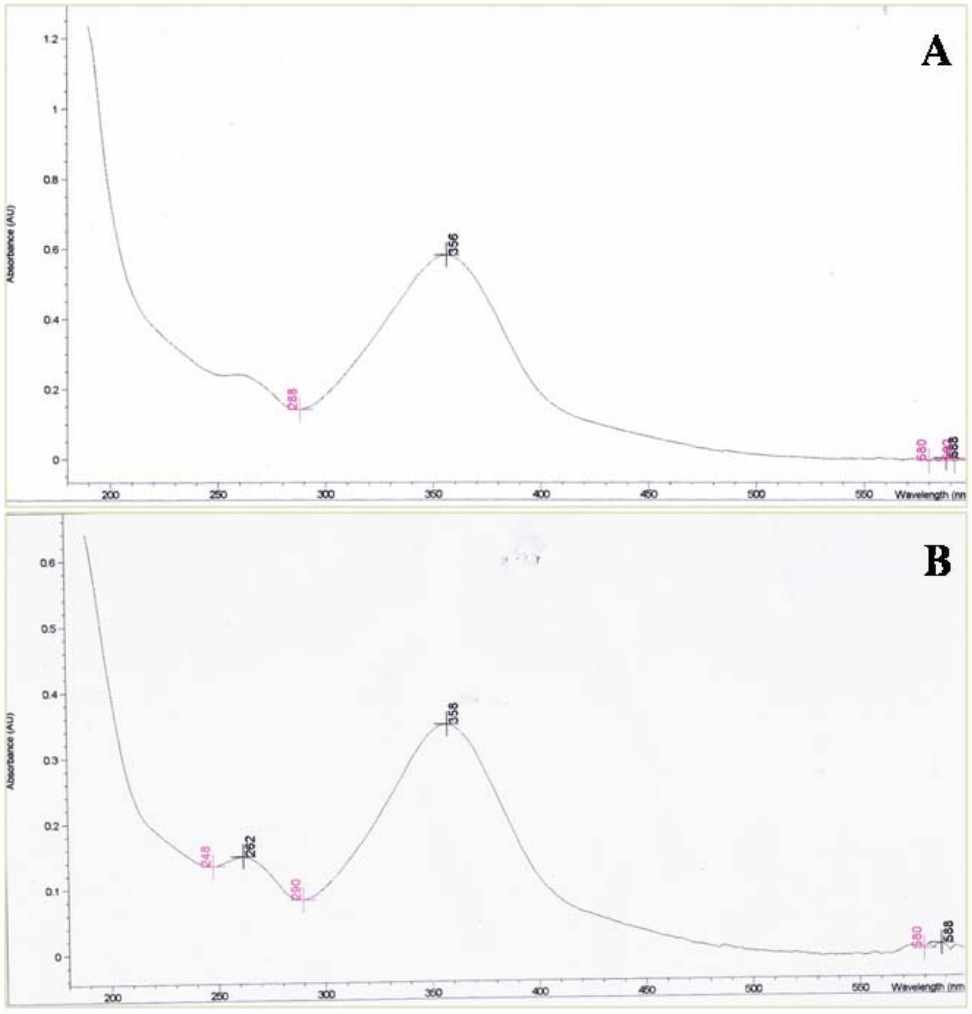


Figure 4-5 UV spectra of PABA-SA (**4, A**) and PAH-SA (**5, B**) conjugates

Table 4-3 FT-IR spectral data of products (**4**, **5**) and substrates (**1**, **2**)

Sample	FT-IR spectral data (cm^{-1})
PABA (**1**)	3364, 3461 (N-H stretching of NH_2); ~3175-2505 (O-H stretching); 1667 (C=O stretching)
PAH (**2**)	3384, 3473 (N-H stretching of NH_2); ~3175-2423 (O-H stretching); 1744, 1650 (C=O stretching)
PABA-SA (**4**)	1578, 1427 (-N=N-); ~3450-2300 (O-H stretching); 1667 (C=O stretching)
PAH-SA (**5**)	1575, 1429 (-N=N-); ~3478-2390 (O-H stretching); 1707, 1657, 1638 (C=O stretching)

FT-IR spectrum of the azo products, (**4**, Figure 4-6) and (**5**, Figure 4-7) showed the N=N weak peaks at 1578, 1427 and 1575, 1429 cm^{-1}, respectively. N-H stretching of amino group of **1** at 3364, 3461 cm^{-1} and **2** at 3384, 3473 cm^{-1} were disappeared, indicated the azo products were obtained. Both azo products (**4** and **5**) presented O-H stretching broadening band ~3450-2300 cm^{-1}. The C=O stretching of **4** and **5** were found at 1667 and 1707, 1657 and 1638 cm^{-1}, respectively. FT-IR spectral data were shown in Table 4-3.

^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **4** (Figure 4-8), δ (ppm) 7.151-7.169 (d, J = 9.0 Hz, 1 H, C_1), 7.914-7.931 (dd, J = 8.5, 2.0 Hz, 1 H, C_2), 8.100 (s, 1 H, C_3), 13.186 (s, 1 H, -COOH), 8.085-8.090 (d, J = 2.5 Hz, 1 H), 8.102-8.113 (d, J = 5.5 Hz, 1 H), 8.107-8.117 (d, J = 5.0 Hz, 1 H) and 8.350-8.355 (d, 1 H, J = 2.5 Hz) are CH-aromatic of PABA (C_4-C_7). Broad band of -COOH of PABA appeared at 13.186 ppm (s, 1 H), whereas broad band of -COOH and -OH of SA appeared at 3.639 (s, 2 H). The ^{1}H-NMR spectral data (**1**, **4**) were listed in Table 4-4.

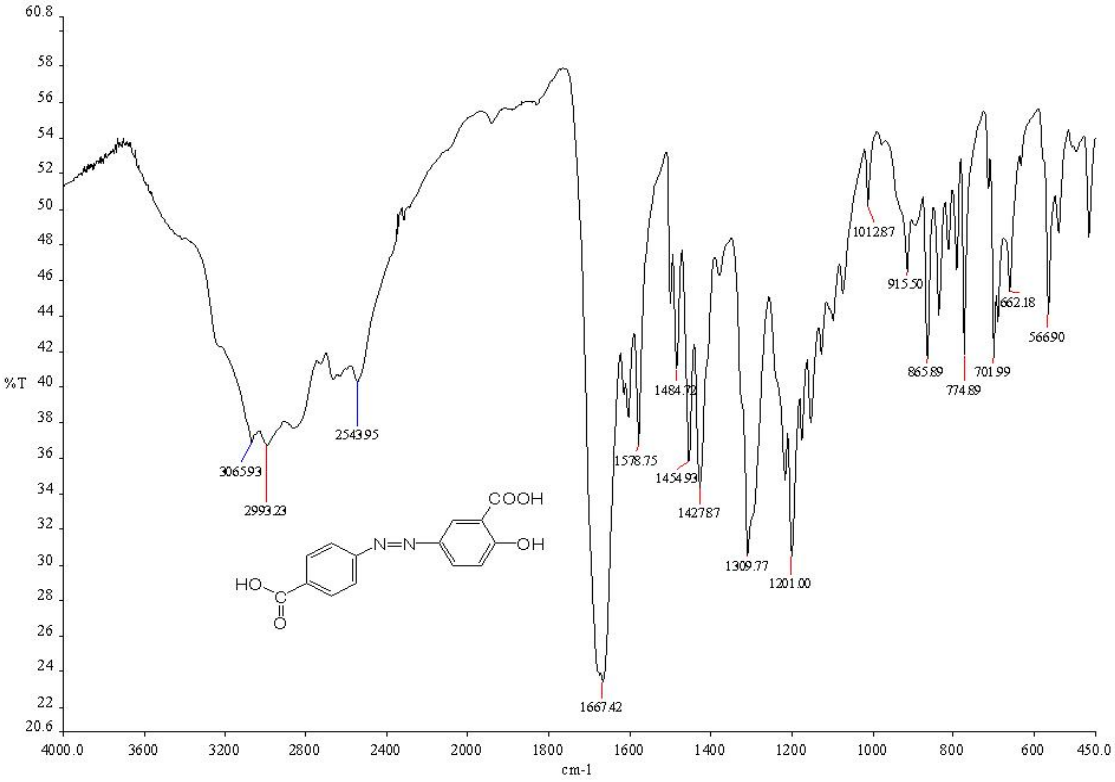


Figure 4-6 FT-IR spectrum of PABA-SA conjugate (**4**)

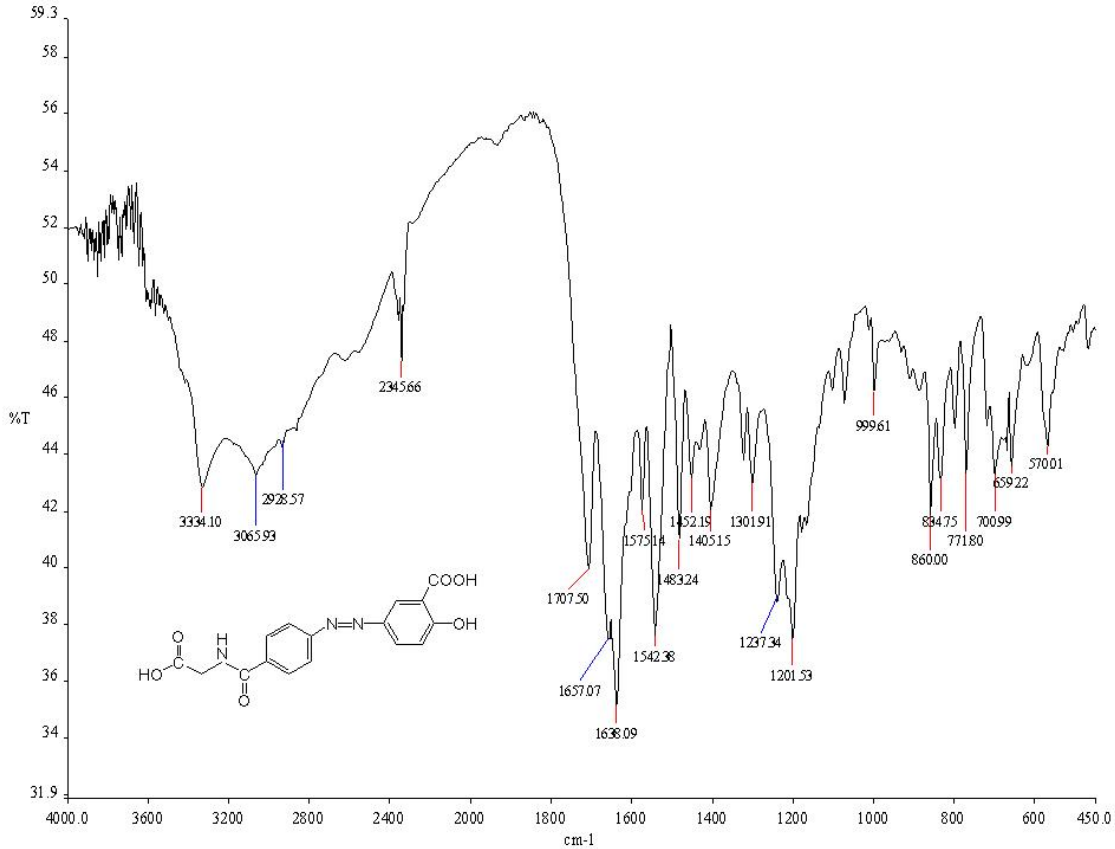


Figure 4-7 FT-IR spectrum of PAH-SA conjugate (**5**)

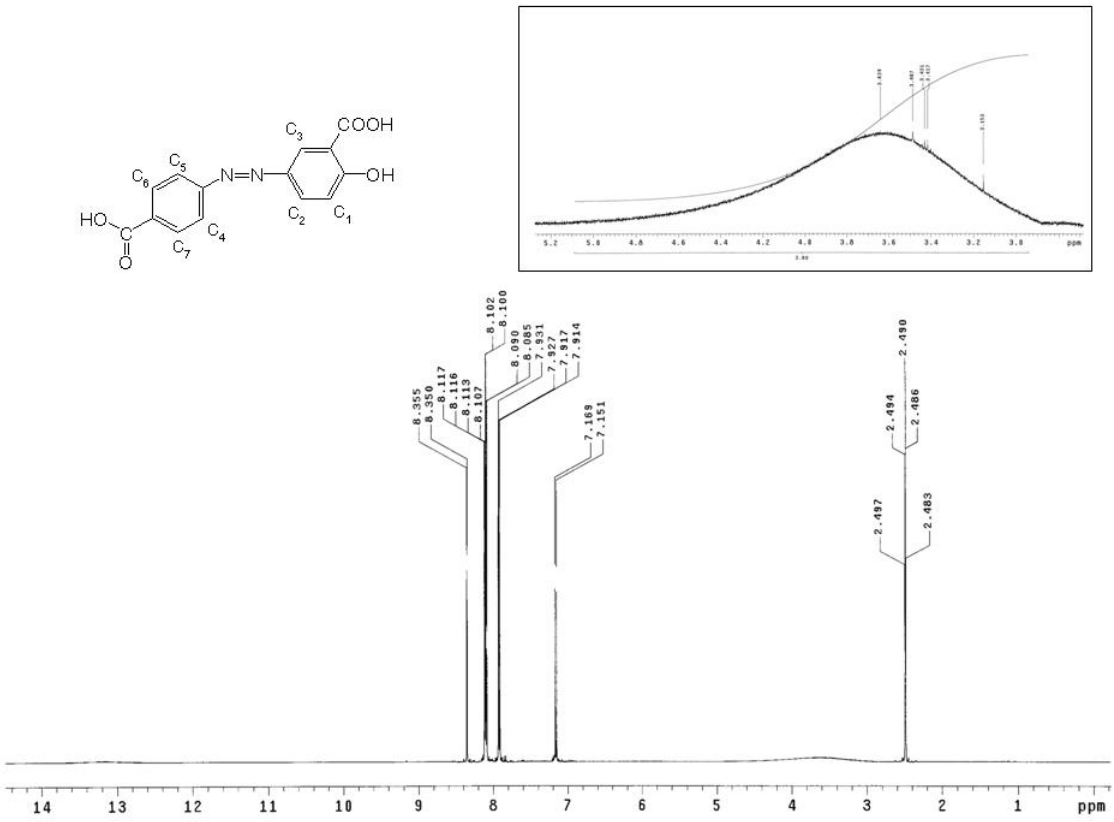


Figure 4-8 NMR signal of PABA-SA conjugate (**4**)

^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **5** (Figure 4-9), δ (ppm) 7.071-7.088 (d, *J* = 9.0 Hz, 1 H), 8.038-8.056 (d, *J* = 9.0 Hz, 1 H) and 8.347-8.352 (d, *J* = 2.5 Hz, 1 H) are the CH-aromatic of SA, 7.915-7.932 (dd, *J* = 8.5, 2.0 Hz, 2 H) and 8.034-8.051 (dd, *J* = 8.5, 2.0, 2 H) are CH-aromatic of PAH, 9.006-9.030 ppm (t, *J* = 6.0, 1 H, -CH_2-NH-CO-), 3.941-3.953 ppm (d, *J* = 6.0 Hz, 2 H, -CO-CH_2-), 12.672 (br, s, 1 H, -COOH of PABA) and 3.486 (br, s, 2 H, -COOH and -OH of SA). The ^{1}H-NMR spectral data (**2**, **5**) were demonstrated in Table 4-4.

^{1}H-NMR spectral data of both **4** and **5** confirmed that PABA-SA (**4**) and PAH-SA (**5**) conjugates were synthesized successfully.

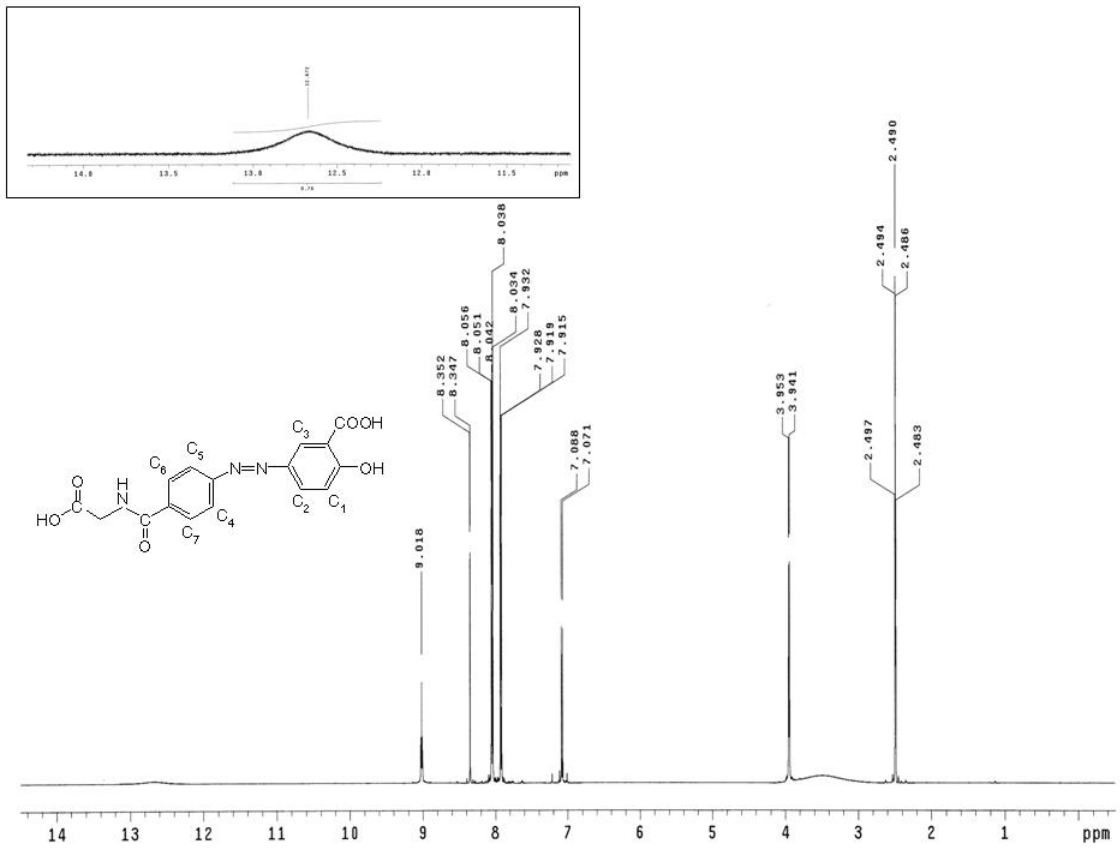


Figure 4-9 NMR signal of PAH-SA conjugate (**5**)

Ester conjugates of PEGs were further synthesized from hydroxyl PEGs (**6**, $mPEG_{5000}$ or **7**, PEG_{4000}) and spacer-SA conjugates (**4**, **5**) by using CDI as coupling reagent (Figure 3-3). However, after the reaction, the asymmetric stretching of the activated acid imidazolide carbonyl (Lee *et al.*, 2006) appeared in the precipitated polymers (Figure 4-10) at wavenumber 1744 cm^{-1}. The starting PEGs were recovered, confirmed by ^{1}H-NMR spectral data. Therefore, the expected conjugates could not be obtained via this procedure.

FT-IR spectrum of the precipitated product (Figure 4-10) indicated that the imidazolide intermediate of the carboxyl group was formed. However, the expected conjugates could not be synthesized as mentioned above. It may be due to the effect of the excess CDI which was still presented in the reaction flask. CDI could continue react with the target groups and thereby prevent their acylation. The excess CDI could irreversibly

Table 4-4 ^{1}H-NMR spectral data of azo conjugates (**4**, **5**) and substrates (**1**, **2**)

Sample	Chemical shift (δ, ppm)	*J* value (Hz)
PABA (1)**	11.940 (s, 1 H, -COOH)	-
	7.630-7.648 (ddd, 2 H, CH-aromatic)	9.0, 2.5, 2.0
	6.580-6.598 (ddd, 2 H, CH-aromatic)	9.0, 2.5, 2.0
	5.5.848-5.852 (d, 2 H, $-NH_2$)	2.0
PABA-SA (4)**	13.186 (s, 1 H, -COOH of PABA)	-
	8.350-8.355 (d, 1 H, CH-aromatic of PABA)	2.5
	8.107-8.117 (d, 1 H, CH-aromatic of PABA)	5.0
	8.102-8.113 (d, 1 H, CH-aromatic of PABA)	5.5
	8.085-8.090 (d, 1 H, CH-aromatic of PABA)	2.5
	8.100 (s, 1 H, CH-aromatic of SA)	-
	7.914-7.931 (dd, 1 H, CH-aromatic of SA)	8.5, 2.0
	7.151-7.169 (d, 1 H, CH-aromatic of SA)	9.0
	3.639 (s, 2 H, -COOH and -OH of SA)	-
PAH (2)**	12.420 (br, s, 1 H, -COOH)	-
	8.308-8.358 (t, 1 H, $-CH_2-NH-CO-$)	2.5
	7.585-7.635 (ddd, 2 H, CH-aromatic)	2.5, 2.0, 1.5
	6.546-6.586 (ddd, 2 H, CH-aromatic)	2.5, 2.0, 1.5
	2.628 (br, s, 1 H, $-NH_2$)	-
	3.863-3.923 (d, 2 H, $-CO-CH_2-$)	2.5
PAH-SA (5)**	12.672 (s, 1 H, -COOH of PAH)	-
	9.006-9.030 (t, 1 H, $-CH_2-NH-CO-$)	6.0
	8.347-8.352 (d, 1 H, CH-aromatic of SA)	2.5
	8.038-8.056 (d, 1 H, CH-aromatic of SA)	9.0
	7.071-7.088 (d, 1 H, CH-aromatic of SA)	9.0
	8.034-8.051 (dd, 2 H, CH-aromatic of PAH)	8.5, 2.0
	7.915-7.932 (dd, 2 H, CH-aromatic of PAH)	8.5, 2.0
	3.941-3.953 (d, 2 H, $-CO-CH_2-$)	6.0
	3.486 (s, 2 H, -COOH and -OH of SA)	-

** Solvent: DMSO-d_6

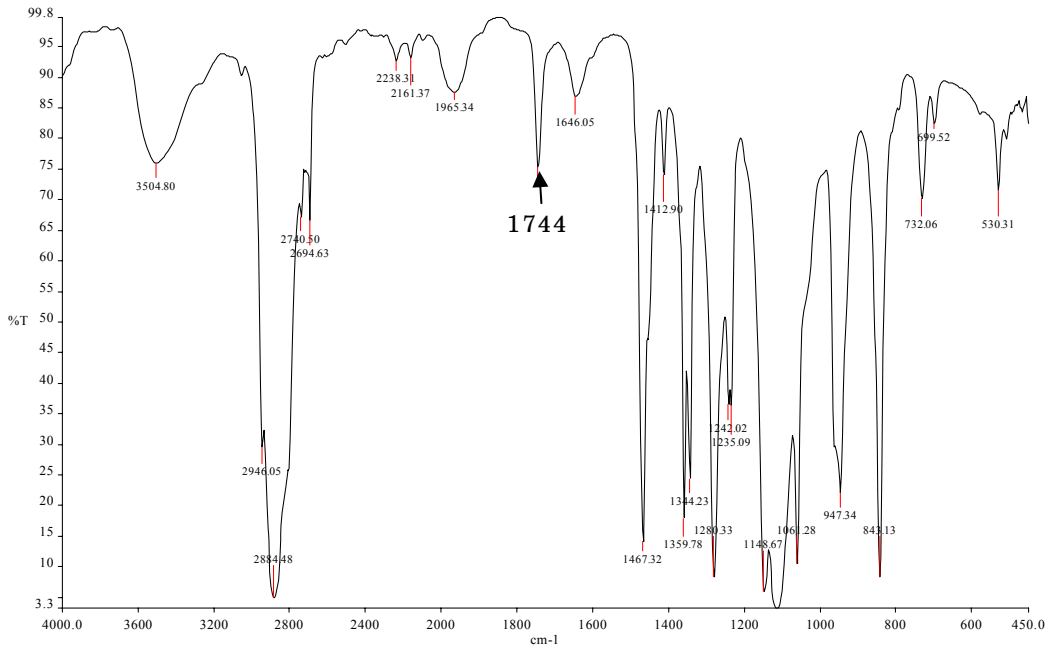


Figure 4-10 FT-IR spectrum of precipitated product of $mPEG_{5000}$ (**6**) demonstrated an imidazolide carbonyl stretching at wave number 1744 cm^{-1}

block the hydroxyl group of PEG (Morton *et al.*, 1988). In addition, Morton and co-workers (1988) reported that the rate of reaction of CDI with hydroxyl group was approximately 175 times faster than that of the imidazolide (Figure 4-19). So the acylation reaction must therefore be performed in the absence of CDI after the acid imidazolide derivative will be obtained (Morton *et al.*, 1988). However, each azo conjugate contains two aromatic carboxyl groups which can react with the coupling reagent and form mixture of two kinds of imidazolide derivatives after *N*-acylation. Moreover, the carboxyl group of the smaller chemical structure of each spacer (**1**, **2**) might be more reactive than those in the azo conjugates (**4**, **5**) due to electron delocalization in the conjugated structure of both azo conjugates (**4**, **5**) made it more stable than spacers (**1**, **2**). Therefore the conjugation of PEG with carboxylic group of each spacer by esterification was performed.

4.2.2 Synthesis of poly(ethylene) glycol-spacer ester conjugates (Method 2)

CDI, coupling reagent, was used in this method. Generally, this reagent is used in peptide bond synthesis. However, there were a number of reports used this reagent to generate ester bond (Cecchi *et al.*, 1981; Caprariis *et al.*, 1994; Peng *et al.*, 2006).

The reaction was performed by conjugation of PEG (**6**, **7**) with PABA (**1**) or PAH (**2**) using CDI as the coupling reagent. However, after the reaction, the starting PEGs (**6**, **7**) were recovered, confirmed by FT-IR and ^{1}H-NMR spectral data. Therefore, the expected conjugates could not be obtained via this procedure. The FT-IR spectra of the precipitated products were similar to that presented in method 1 (Figure 4-10). There was only the imidazolide carbonyl stretching peak appeared at wavenumber 1748 cm^{-1}. Moreover, Vaidyanathan and co-workers (2004) reported the possible mechanism which the acid imidazolide (Figure 4-19) could form anhydride intermediate of the expected product after *O*-acylation and obtain the expected conjugate after rearrangement. As many molecules of the anhydride intermediate are presented, more percentage of the ester conjugate will be obtained (Morton *et al*., 1988). Morton and co-workers (1988) indicated that addition of a base as catalyst in the presence of CDI could enhance formation of the anhydride intermediate. Therefore the base would be added in the following method.

4.2.3 Synthesis of poly(ethylene) glycol-spacer ester conjugates (Method 3)

PEG-ester conjugate (**12-15**) were prepared from PEG (**6**, **7**) with PABA (**1**) or PAH (**2**) using CDI as coupling reagent. DMAP was selected as a catalyst in the reaction according to the previous report (Xi *et al*., 2005). FT-IR spectral data of the precipitated product represented carbonyl stretching peak of ester at wavenumber 1701 cm^{-1} and primary amino at 3360 and 3242 cm^{-1} (Figure 4-11). However, after confirm by ^{1}H-NMR spectral data, the starting PEGs (**6**, **7**) were recovered. Therefore, the expected conjugates could not be obtained via this procedure.

Due to the chemical structure of spacers (**1**, **2**), the intermolecular self-conjugation between carboxyl and amino functional groups in themselves may occur. The conjugation between carboxyl group of each spacer and hydroxyl group of PEG might be interfered by this self-conjugation. Therefore the protection of amino group of each spacer before esterification was performed and the protected spacers will be used in the conjugation step.

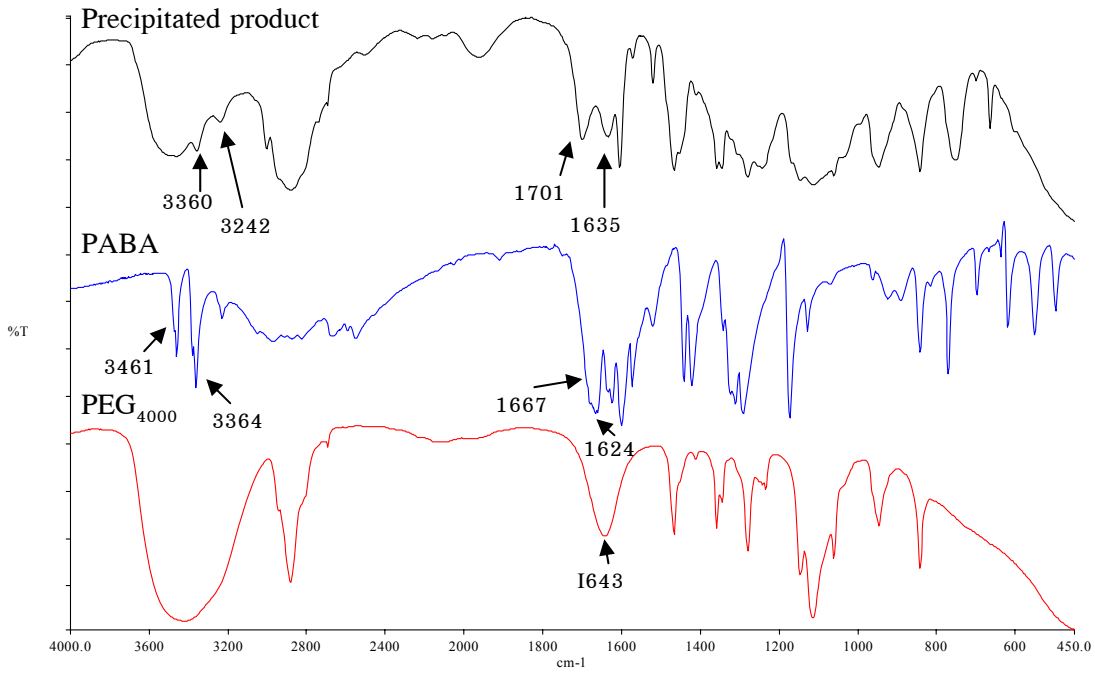
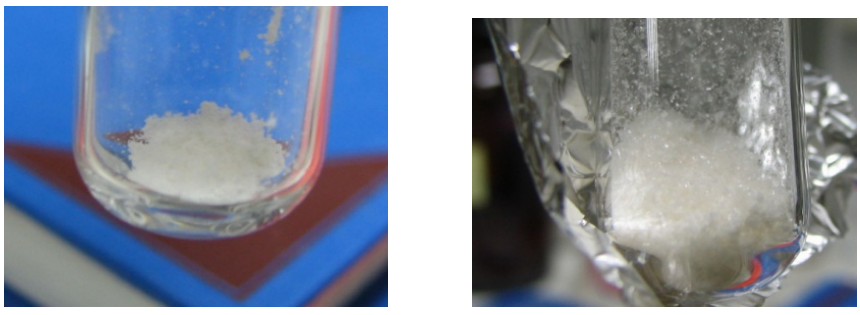


Figure 4-11 FT-IR spectra of precipitated product and the starting materials

4.2.4 Synthesis of poly(ethylene) glycol-4 formylated spacer ester conjugates (Method 4)

Formic acid was used for protection of amino group of PABA (**1**) and PAH (**2**), in order to prevent intermolecular self-conjugation of spacer (**1**, **2**) during conjugation process (Zou *et al*., 2005).



(a) (b)

Figure 4-12 The resulting products of (a) 4f-PABA (**16**) and (b) 4f-PAH (**17**)

White crystal of 4f-PABA (**16**, Figure 4-12a) was obtained in 95.11% yield. The melting point of the product was 257-258 °C.

FT-IR spectrum (KBr) showed disappearance of N-H stretching of amino group of PABA (**1**) at wavenumber (ν) = 3364 and 3461 cm^{-1}. Single weak absorption of N-H (2° amide, -Ar-NH-CO-) was presented at 3356 cm^{-1}. The strong carbonyl band of -NH-CO-H at ν= 1710-1680 cm^{-1}, which overlapped with C=O stretching of carboxyl group was observed. Weak "W" shape absorption bands of C-H stretching of -HC=O at 2925 and 2883 cm^{-1} was observed. The characteristic of aldehyde functional group -HC=O overlapped with broadening absorption of carboxyl O-H stretching at ν= 3267-2520 cm^{-1} were observed (Figure 4-13). FT-IR spectral data were demonstrated in Table 4-5.

^{1}H-NMR (300 MHz, DMSO-d_6) spectrum of **16** is displayed in Figure 4-14, δ (ppm): 12.78 (s, 1 H, -COOH); 10.62 (s, 1 H, -NH-CO-); 8.34 (s, 1 H, -CO-H); 7.88-7.90 (dd, *J* = 6.0, 4.0 Hz, 2 H, CH-aromatic); 7.66-7.70 (dd, *J* = 6.0, 4.0 Hz, 2 H, CH-aromatic).

^{1}H-NMR (Figure 4-14, Table 4-6) and IR spectral data demonstrated above confirmed that 4f-PABA (**16**) was synthesized successfully.

Table 4-5 FT-IR spectral data of products (**16**, **17**) and substrates (**1**, **2**)

Sample	FT-IR spectral data (cm^{-1})
PABA (**1**)	3364, 3461 (N-H stretching of NH_2); ~3175-2505 (O-H stretching); 1667 (C=O stretching)
PAH (**2**)	3384, 3473 (N-H stretching of NH_2); 3252 (2° amide, -NH-CO-Ar-); ~3175-2423 (O-H stretching); 1744, 1650 (C=O stretching)
4f-PABA (**16**)	3356 (2° amide, -Ar-NH-CO-); ~3267-2520 (O-H stretching); 1710-1680 (C=O stretching); 2925, 2883 (-HC=O)
4f-PAH (**17**)	3197 (2° amide, -NH-CO-Ar-); 3302 (2° amide, -Ar-NH-CO-); ~3300-2500 (O-H stretching); 1722-1714, 1638 (C=O stretching); 2769, 2736 (-HC=O)

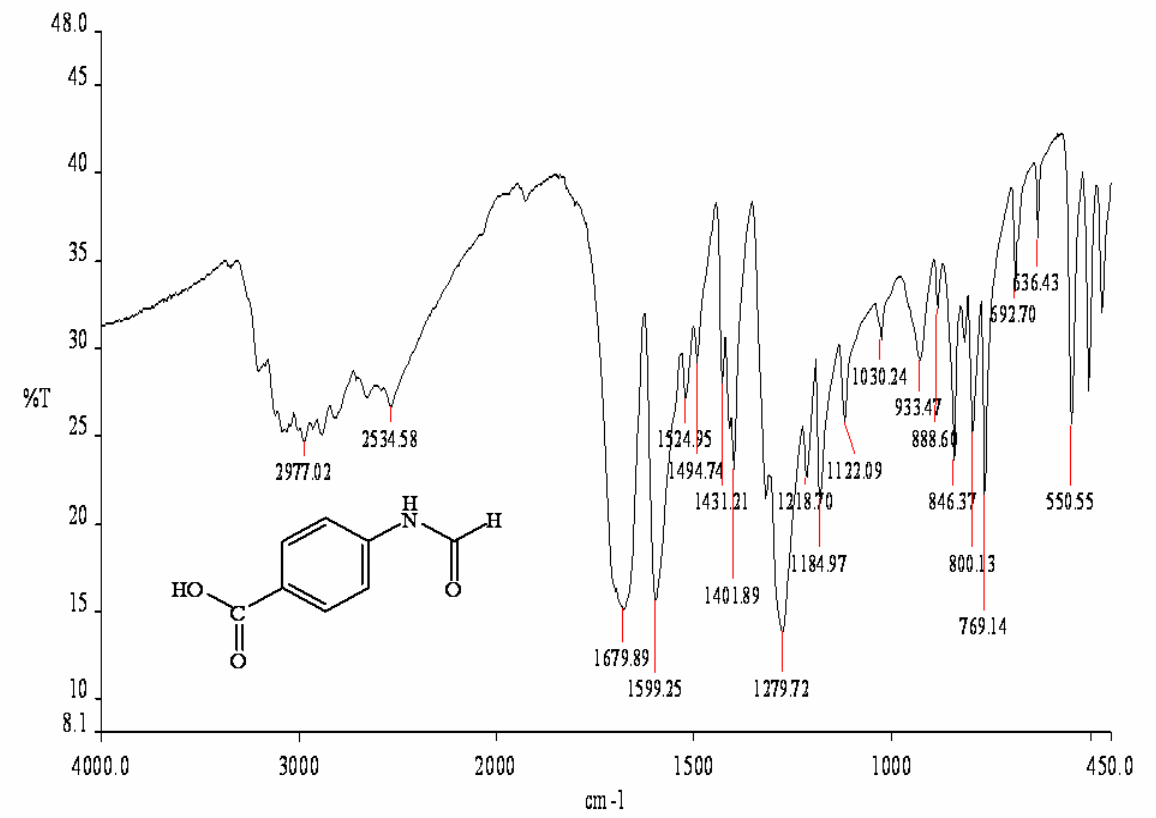


Figure 4-13 FT-IR spectrum of 4f-PABA (**16**)

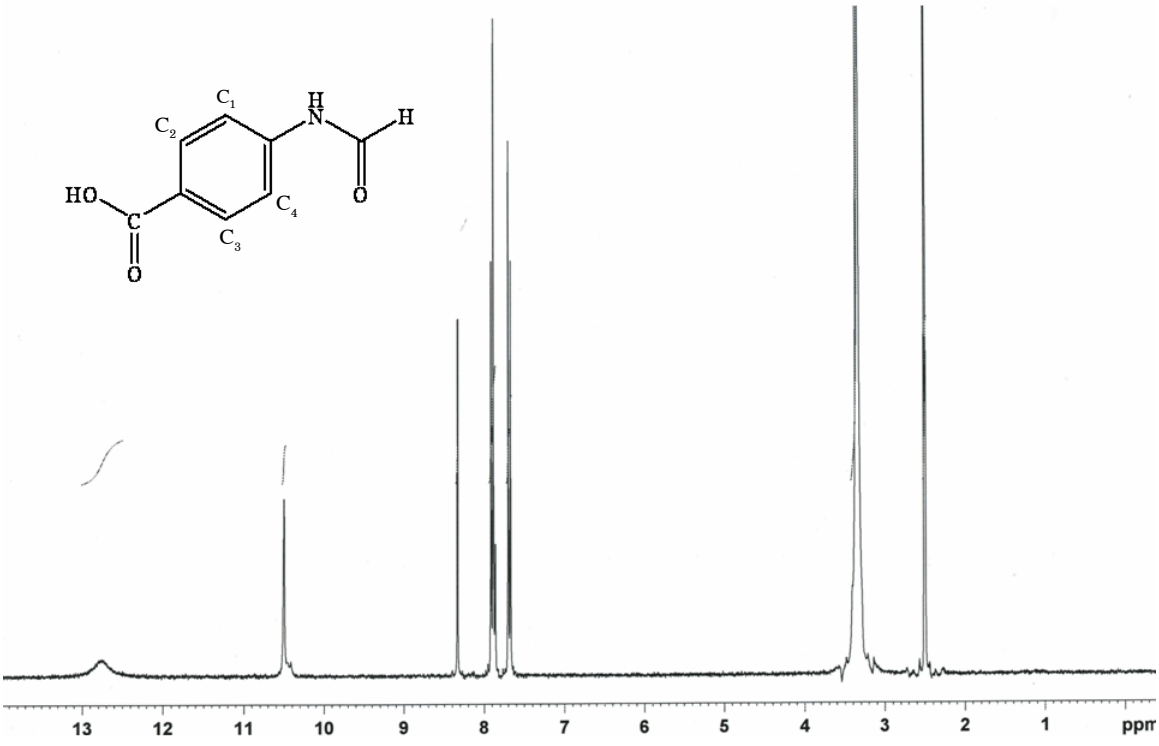


Figure 4-14 ^{1}H-NMR signal of 4f-PABA (**16**)

White crystal of 4f-PAH (**17**, Figure 4-12b) was obtained in 60.21% yield. The melting point of the product was 208.5-210 °C.

FT-IR spectrum (KBr) of **17** showed disappearance of N-H stretching of amino group in **2**, starting material, at wavenumber (ν)= 3384 and 3473 cm^{-1}. Single weak absorption of N-H (2° amide, -NH-CO-Ar-) shifted from ν = 3252 cm^{-1} of **2** to 3197 cm^{-1}. Single medium sharp absorption of N-H (2° amide, -Ar-NH-CO-) appeared at ν= 3302 cm^{-1}. The strong carbonyl splitted end band of -NH-CO-Ar- and -NH-CO-Ar- at ν= 1722-1714 cm^{-1}, which overlapped with C=O stretching of carboxyl group were observed. Weak C-C stretching absorption (-CO-CH_2-NH-) presented at ν= 2890 cm^{-1}. Weak "W" shape absorptions of C-H stretching of -HC=O were shown at 2769 and 2736 cm^{-1}. However, this character of aldehyde functional group was found overlapped with broadening absorption of carboxyl O-H stretching at ν= 3300-2500 cm^{-1} (Figure 4-15). FT-IR spectral data were demonstrated in Table 4-5.

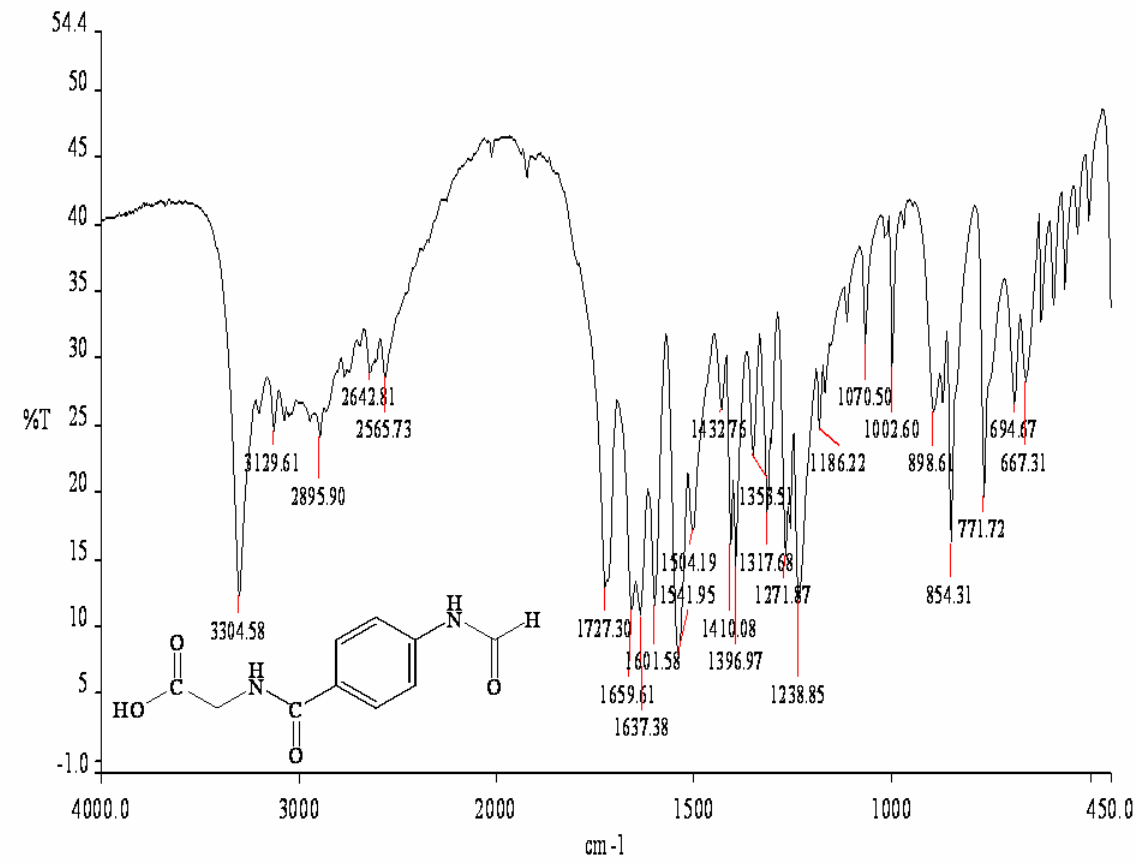


Figure 4-15 FT-IR spectrum of 4f-PAH (**17**)

^{1}H-NMR (300 MHz, DMSO-d_6) spectrum of **17** is demonstrated in Figure 4-16, δ (ppm): 12.56 (s, 1 H, -COOH); 10.43 (s, 1 H, -CO-H); 8.74 (t, *J* = 6.0 Hz, 1 H, -NH-CO-Ar); 8.32 (s, 1 H, -Ar-NH-CO-); 7.82-7.84 (dd, *J* = 6.0, 3.0 Hz, 2 H, CH-aromatic); 7.66-7.68 (dd, *J* = 6.0, 3.0 Hz, 2 H, CH-aromatic); 3.90-3.92 (d, *J* = 6.0 Hz, 2 H, -CO-CH_2-).

^{1}H-NMR (Figure 4-16, Table 4-6) and IR spectral data demonstrated above confirmed that 4f-PAH (**17**) was synthesized successfully.

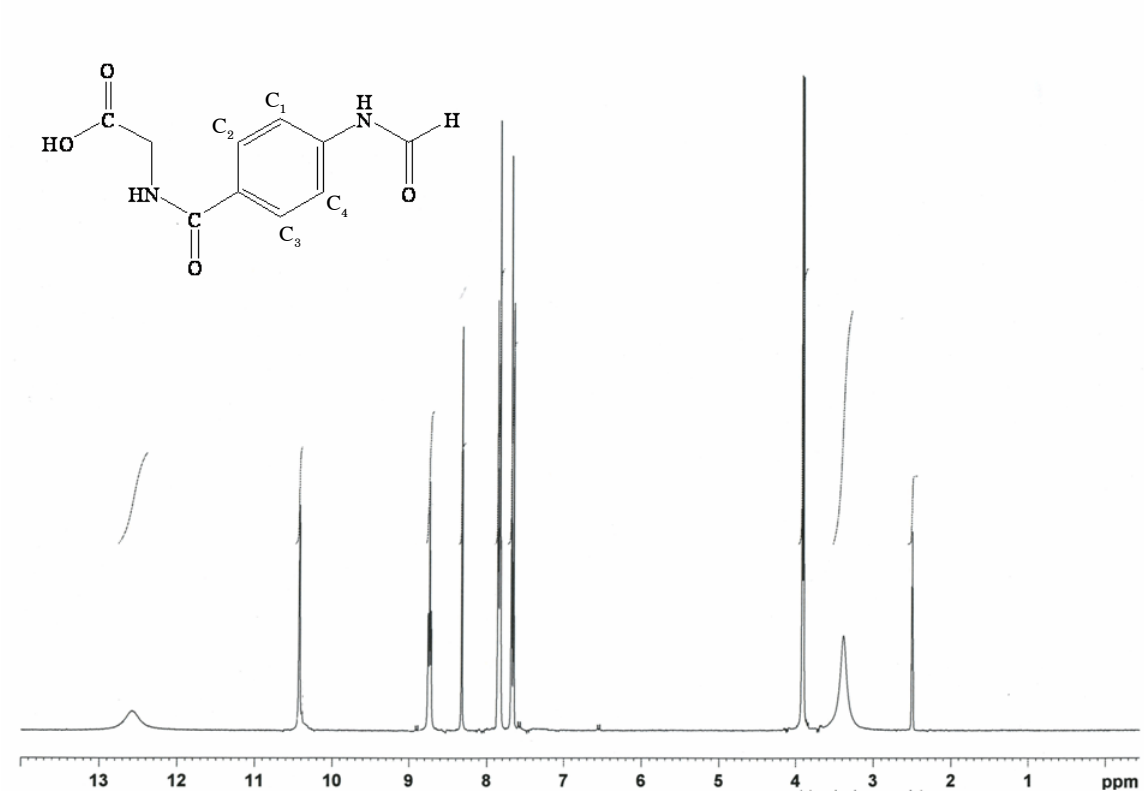


Figure 4-16 ^{1}H-NMR signal of 4f-PAH (**17**)

The resulting 4f-PABA (**16**) and 4f-PAH (**17**) were utilized in the coupling reaction with $mPEG_{5000}$ (**6**) and PEG_{4000} (**7**) using CDI as coupling reagent. Pyridine or triethylamine (TEA) was used as a catalyst in the reaction similar to that reported in 2005 (Zou *et al*., 2005).

Table 4-6 ^{1}H-NMR spectral data of 4f-spacers (**16, 17**) and substrates (**1, 2**)

Sample	Chemical shift (δ, ppm)	*J* value (Hz)
PABA (1)**	11.940 (s, 1 H, -COOH)	-
	7.630-7.648 (ddd, 2 H, CH-aromatic)	9.0, 2.5, 2.0
	6.780-6.798 (ddd, 2 H, CH-aromatic)	9.0, 2.5, 2.0
	5.5.848-5.852 (d, 2 H, $-NH_2$)	2.0
4f-PABA (16)**	12.78 (s, 1 H, -COOH)	-
	10.62 (s, 1 H, -NH-CO-)	-
	8.34 (s, 1 H, -CO-H)	-
	7.88-7.90 (dd, 2 H, CH-aromatic)	6.0, 4.0
	7.68-7.70 (dd, 2 H, CH-aromatic)	6.0, 4.0
PAH (2)**	12.420 (br, s, 1 H, -COOH)	-
	8.308-8.358 (t, 1 H, $-CH_2$-NH-CO-)	2.5
	7.585-7.635 (ddd, 2 H, CH-aromatic)	2.5, 2.0, 1.5
	6.546-6.586 (ddd, 2 H, CH-aromatic)	2.5, 2.0, 1.5
	2.628 (br, s, 1 H, $-NH_2$)	-
	3.863-3.923 (d, 2 H, -CO-CH_2-)	2.5
4f-PAH (17)**	12.56 (s, 1 H, -COOH)	-
	10.43 (s, 1 H, -CO-H)	-
	8.74 (t, 1 H, -NH-CO-Ar-)	6.0
	8.32 (s, 1 H, -Ar-NH-CO-)	-
	7.82-7.84 (dd, 2 H, CH-aromatic)	6.0, 3.0
	7.66-7.68 (dd, 2 H, CH-aromatic)	6.0, 3.0
	3.90-3.92 (d, 2 H, -CO-CH_2-)	6.0

** Solvent: DMSO-d_6

However, the FT-IR spectroscopic data indicated that ester bond between PEG and protected spacer could not be synthesized and $mPEG_{5000}$ (**6**) and PEG_{4000} (**7**) were recovered. FT-IR spectrum of the recovered product (Figure 4-17) after work up by precipitation demonstrated that no ester bond found. The C=O stretching which used for identification of imidazolide intermediate of 4f-PABA and CDI could not be detected

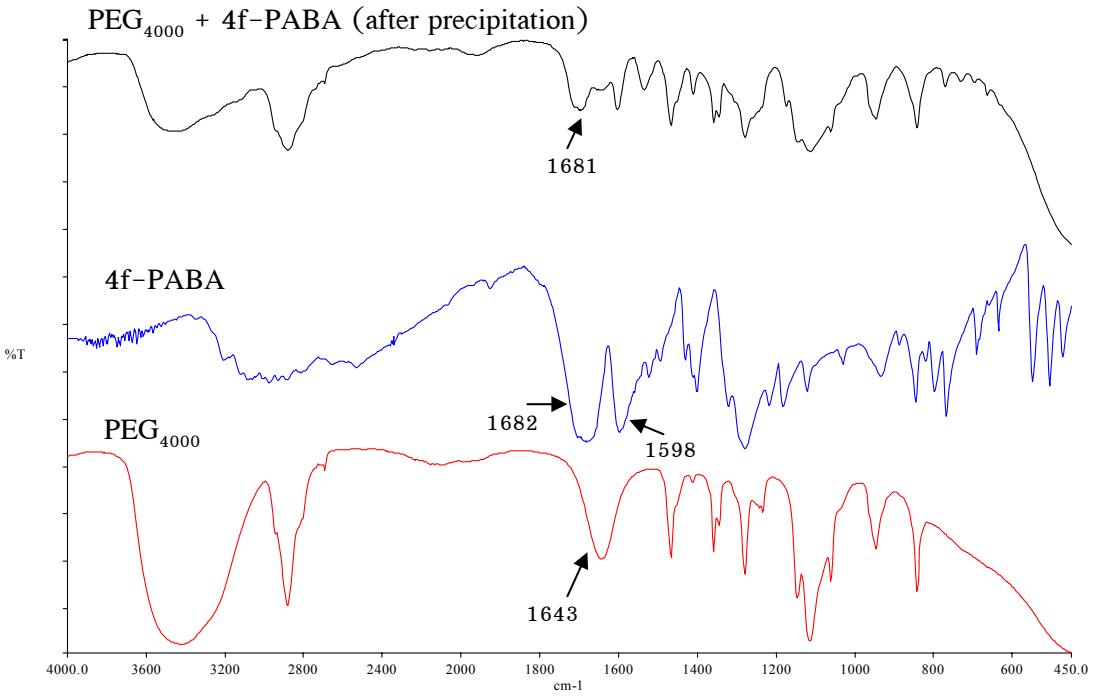


Figure 4-17 FT-IR spectra of physical mixture of PEG_{4000} (**7**) and 4f-PABA (**16**)

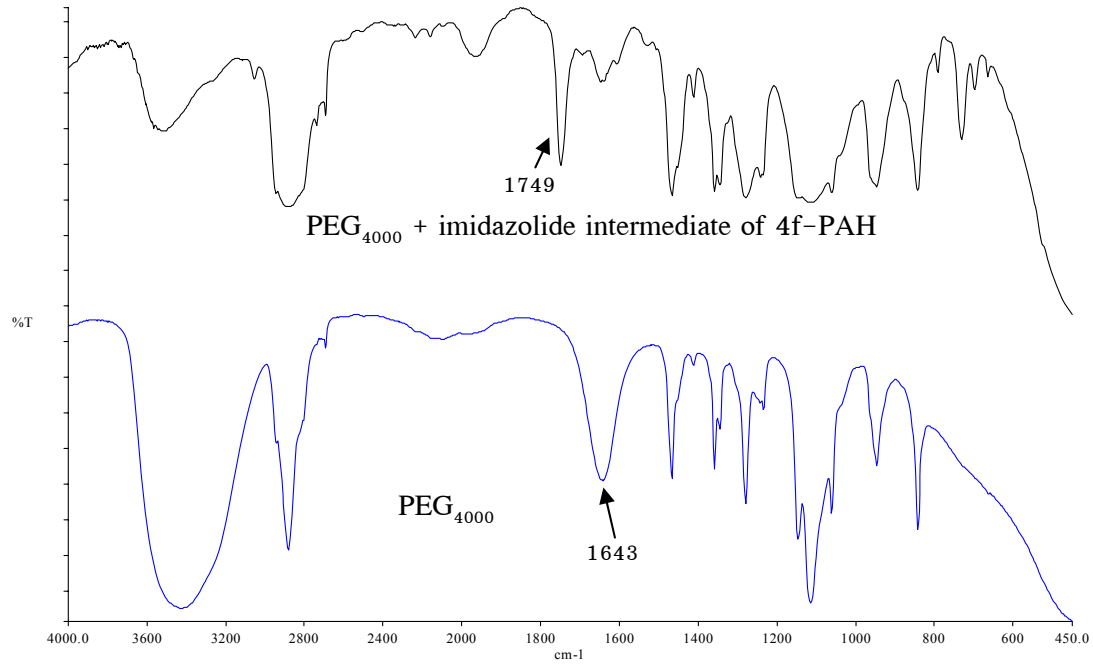


Figure 4-18 FT-IR spectra of imidazolide intermediate of 4f-PAH (**17**) and PEG_{4000} (**7**)

(Figure 4-17). However, the FT-IR spectral data of the precipitated product after the reaction of PEG_{4000} (**7**) and 4f-PAH (**17**) indicated that the imidazolide derivative of 4f-PAH could be generated (Figure 4-18). The C=O stretching of this derivative appeared at wavenumber 1749 cm^{-1}. There was no ester carbonyl peak detected still after more than 72-hour reaction time. As mentioned before that the excess CDI could block the hydroxyl group of PEG irreversibly (Morton *et al.*, 1988), the reported results from a research of Tang and co-workers (2004) supported the former one that aliphatic primary alcohol reacted with CDI afforded the corresponding carbamate which need very high temperature (170 ^{o}C) for breaking this bond. Thus, PEG-4f-spacer ester conjugates could not be obtained by this method.

4.2.5 Synthesis of poly(ethylene) glycol-4-formylated spacer amide conjugates (Method 5)

In this procedure, poly(ethylene) glycol polymer containing amino groups were used instead of PEG containing hydroxyl group in order to compare the suitability of the coupling conditions. CDI was used to generate imidazolide derivative of 4f-spacer which could react with amino functional side chain of PEG_{3000} diamino. This method, same reaction condition as previous reported (Bayer and Mutter, 1972; Rajasekharan Pillai *et al.*, 1979; Wiwattanapatapee *et al.*, 2003) were utilized.

The spectroscopic data showed that PEG_{3000} diamino (**22**) was recovered. Then pyridine was added as a catalyst. However, **22** was again recovered after worked up by precipitation. Therefore, PEG_{3000}-$(4f\text{-}PABA)_2$ amide conjugate (**23**) could not be synthesized by this method. Vaidyanathan and co-workers (2004) mentioned that CO_2 retained in the solution after imidazolide formation step could catalytic enhance the rate of amidation (Figure 4-19) by increasing formation of alkyl ammonium *N*-alkyl carbamate intermediate which would further react with the imidazolide intermediate to form anhydride derivative. However, in this reaction, CO_2 was removed from the reactor under N_2. Therefore, the rate of reaction would be slower as a result. Due to slow rate of amidation, another coupling reagent was considered.

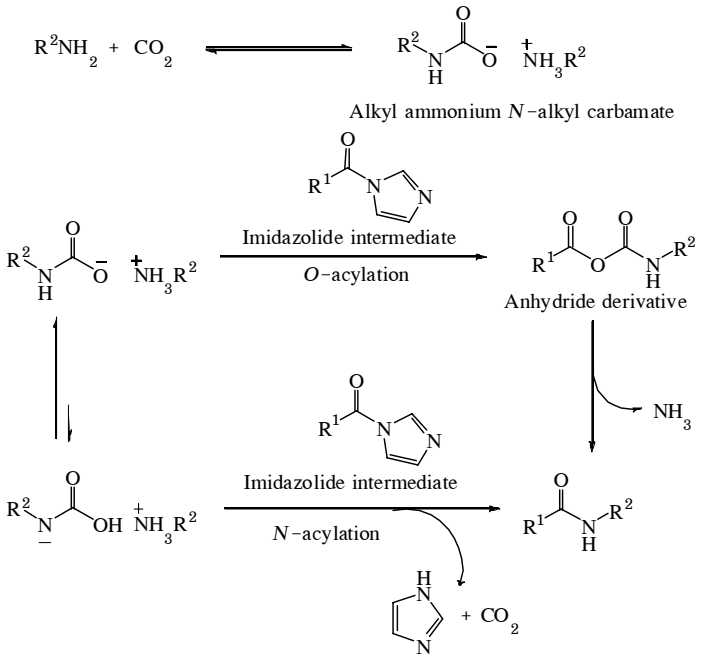


Figure 4-19 Mechanism in amide formation (Vaidyanathan *et al*., 2004)

4.2.6 Synthesis of poly(ethylene) glycol-4-formylated spacer amide conjugates (Method 6)

PEG_{3000}-(4f-PABA)$_2$ amide conjugate (**23**) was expected to be synthesized by this method. Bis(trichloromethyl) carbonate or triphosgene was used to generate 3 molecules of phosgene which could react with carboxylic group of 4f-PABA (**16**), resulting in the acid chloride intermediate which was immediately used in the next step. The resulting acid chloride was expected to further react with amino side chain of PEG_{3000} diamino (**22**) to yield amide conjugate of the polymer (**23**).

Yellowish white amorphous solid of **23** was obtained by precipitation with diethyl ether, resulting in 22.01% yield.

The FT-IR spectroscopic data in Figure 4-20 revealed that the resulting product was PEG_{3000}-(4f-PABA)$_2$ amide conjugate (**23**). The strong carbonyl band of -NH-CO-Ar- at ν= 1712 cm^{-1}, which overlapped with C=O stretching of carboxyl group and -NH-CO-H was observed. FT-IR spectral data were demonstrated in Table 4-7.

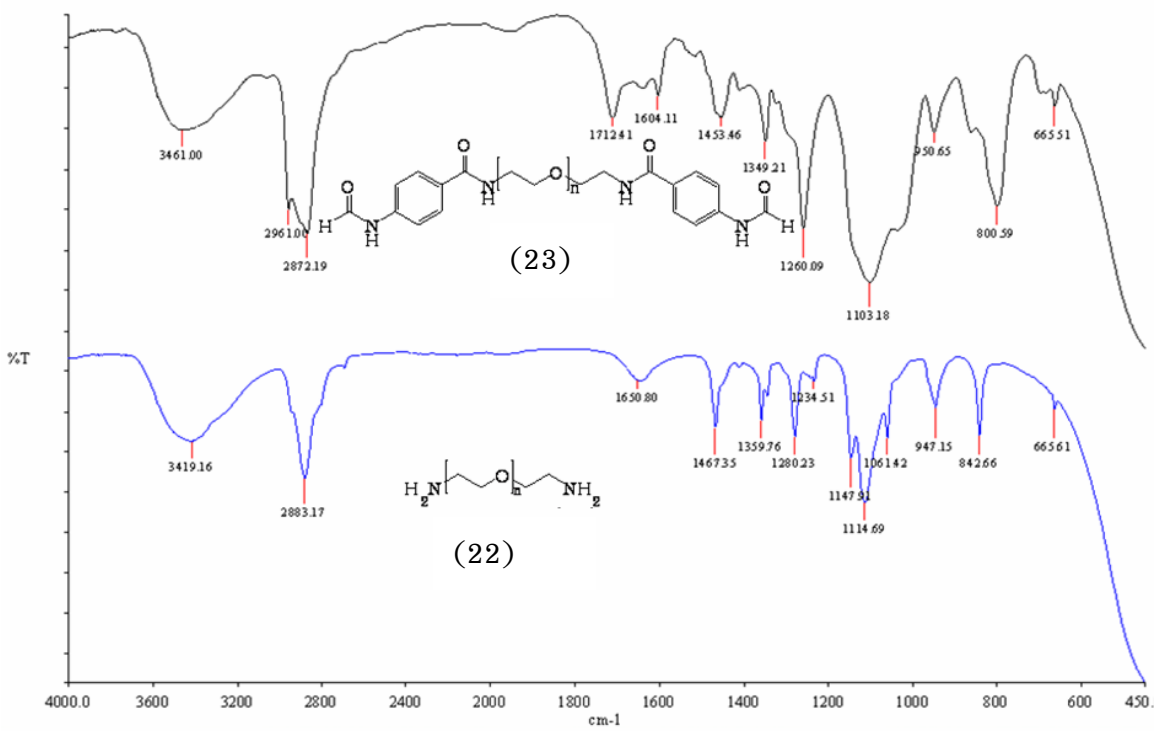


Figure 4-20 FT-IR spectrum of PEG_{3000} (**22**) and PEG_{3000}-(4f-PABA)$_2$ amide conjugate (**23**)

Table 4-7 FT-IR spectral data of product (**23**) and substrates (**16, 22**)

Sample	FT-IR spectral data (cm^{-1})
4f-PABA (**16**)	3356 (2^{o} amide, -Ar-NH-CO-); ~3267-2520 (O-H stretching); 1710-1680 (C=O stretching); 2925, 2883 (-HC=O)
Product (**23**)	1712, 1640 (C=O stretching)

^{1}H-NMR (300 MHz, DMSO-d_6) spectrum of **23**, δ (ppm): 10.52 (s, 4 H, -Ar-NH-CO-), 8.34 (s, 4 H, -CO-H), 7.88-7.92 (d, J = 12.0 Hz, 4 H, CH-aromatic); 7.68-7.72 (d, J = 12.0 Hz, 4 H, CH-aromatic); 4.26 (t, J = 6.0 Hz, 2 H, -NH-CO-Ar); 3.22-3.74 (m, 268 H, -CH- of $(CH_2CH_2-O)_n$).

Table 4-8 ^{1}H-NMR spectral data of product (**23**) and substrates (**16**, **22**)

Sample	Chemical shift (δ, ppm)	*J* value (Hz)
PEG_{3000} (22)*	3.46-3.95 (m, 272 H, -CH- of $\leftarrow CH_2CH_2-O\rightarrow_n$)	-
	1.74-2.05 (m, 4 H, $-NH_2$)	-
4f-PABA (16)**	12.78 (s, 1 H, -COOH)	-
	10.62 (s, 1 H, -NH-CO-)	-
	8.34 (s, 1 H, -CO-H)	-
	7.88-7.90 (dd, 2 H, CH-aromatic)	6.0, 4.0
	7.68-7.70 (dd, 2 H, CH-aromatic)	6.0, 4.0
Product (23)*	12.55 (s, 2 H, -COOH of **16**)	-
	10.52 (s, 4 H, -Ar-NH-CO-)	-
	8.34 (s, 4 H, -CO-H)	-
	7.88-7.92 (d, 4 H, CH-aromatic)	12.0
	7.68-7.72 (d, 4 H, CH-aromatic)	12.0
	7.86-7.88 (d, 4 H, CH-aromatic of **16**)	6.0
	7.68-7.70 (d, 4 H, CH-aromatic of **16**)	6.0
	4.26 (t, 2 H, -NH-CO-Ar-)	6.0
	3.22-3.74 (m, 268 H, -CH- of $\leftarrow CH_2CH_2-O\rightarrow_n$)	-

* Solvent: $CDCl_3$; ** Solvent: DMSO-d_6

^{1}H-NMR spectral data of **23** (Table 4-8) showed that the resulting product was not pure due to the remaining of **16** and pyridine in the resulting product, then liquid extraction purification by 1 M HCl to wash pyridine residue and 0.5 M NaOH was used to remove **16**, then washed with distilled water. After purification process, the conjugated product was found degraded resulting in recovery of PEG_{3000} diamino (**22**) by ^{1}H-NMR. This was due to the condition was too harsh to use in purification process. It is therefore lower concentration of HCl or other method of purification such as SiO_2 column chromatography, dialysis purification, etc, should be used instead.

4.2.7 Synthesis of poly(ethylene) glycol-4-formylated spacer ester conjugates (Method 7)

Another method to prepare PEG-4-formylated ester conjugates was performed by in situ generation of 4f-PABA acid chloride using thionyl chloride. The acid chloride generated in this reaction could further react with hydroxyl side chain of PEG_{4000} (**7**), resulting in ester conjugate of the polymer (**20**). Yellow amorphous solid of **20** was obtained by precipitation with diethyl ether, resulting in 48.47% yield (Figure 4-21).

Figure 4-21 PEG_{4000}-$(4f\text{-}PABA)_2$ ester conjugate (**20**)

Table 4-9 FT-IR spectral data of product (**20**) and substrates (**16, 7**)

Sample	FT-IR spectral data (cm^{-1})
PEG_{4000} (**7**)	~3434 (O-H stretching)
4f-PABA (**16**)	3356 (2^{o} amide, -Ar-NH-CO-); ~3267-2520 (O-H stretching); 1710-1680 (C=O stretching); 2925, 2883 (-HC=O)
Product (**20**)	~3524 (O-H stretching); 1725, 1635 (C=O stretching)

From FT-IR spectral data (Table 4-9) of PEG_{4000}-$(4f\text{-}PABA)_2$ ester conjugate (**20**), ester C=O stretching was detected at wavenumber 1725 cm^{-1} (Figure 4-22).

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of **20**, δ (ppm): 8.43-8.50 (s, 1 H, H-CO-); 7.62-7.66 (ddd, *J* = 9.0, 6.0, 3.0 Hz, 2 H, CH-aromatic); 7.44-7.48 (ddd, *J* = 9.0, 6.0, 3.0 Hz, 2 H, CH-aromatic); 5.10 (s, 1 H, Ar-NH-); 3.34-4.40 (m, 323 H, -CH- of $\leftarrow CH_2CH_2\text{-}O\rightarrow_n$).

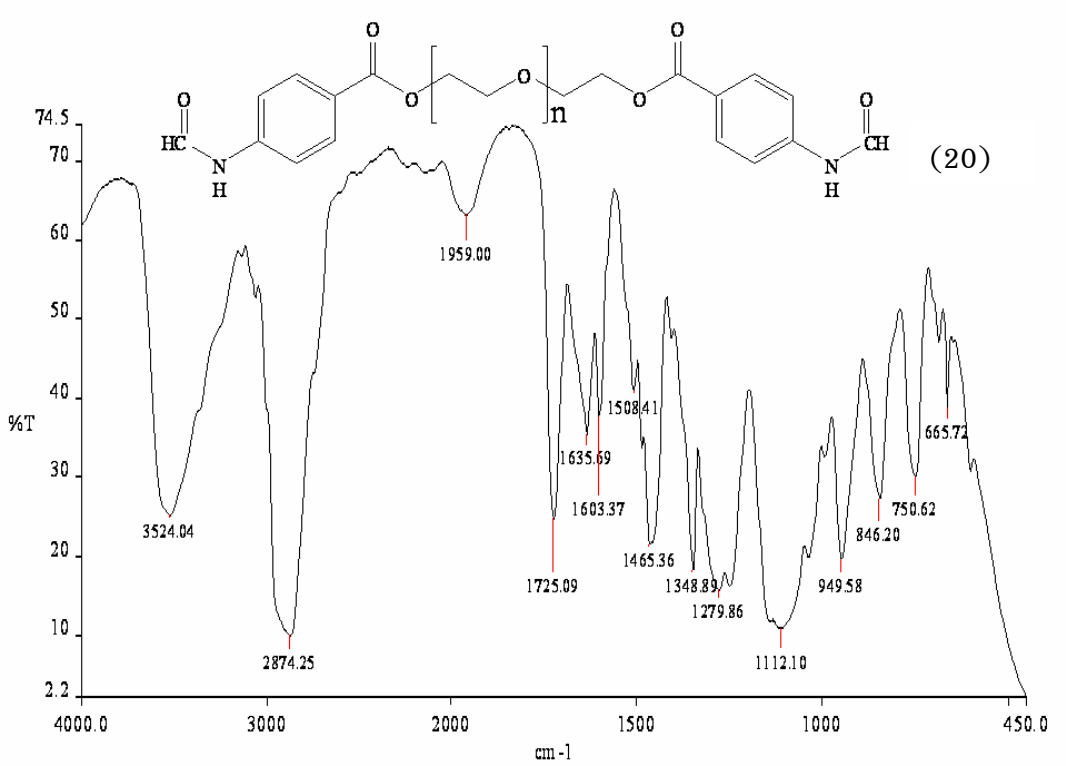


Figure 4-22 FT-IR spectrum of PEG_{4000}-(4f-PABA)$_2$ ester conjugate (**20**)

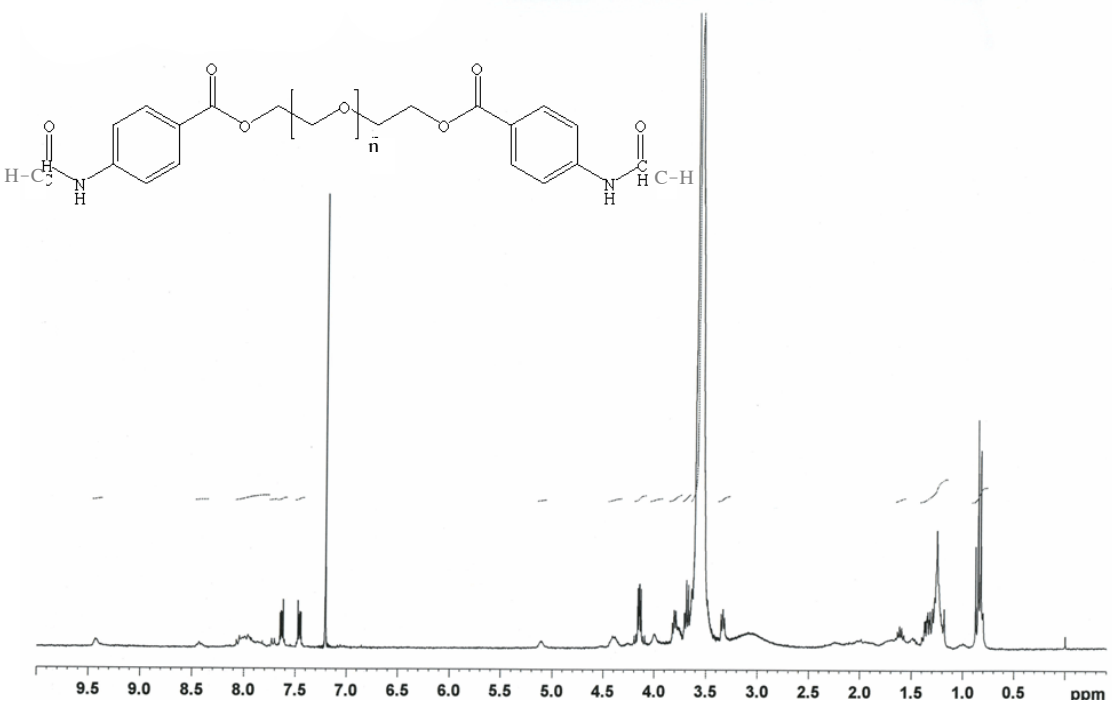


Figure 4-23 ^{1}H-NMR spectrum of PEG_{4000}-(4f-PABA)$_2$ ester conjugate (**20**)

Table 4-10 ^{1}H-NMR spectral data of product (**20**) and substrates (**16**, **7**)

Sample	Chemical shift (δ, ppm)	*J* value (Hz)
PEG_{4000} (7)[*a]	3.800-3.491 (m, 362 H, -CH- of $\leftarrow CH_2CH_2-O\rightarrow_n$)	-
	2.378 (s, 2 H, -O-H)	-
4f-PABA (16)**	12.78 (s, 1 H, -COOH)	-
	10.62 (s, 1 H, -NH-CO-)	-
	8.34 (s, 1 H, -CO-H)	-
	7.88-7.90 (dd, 2 H, CH-aromatic)	6.0, 4.0
	7.68-7.70 (dd, 2 H, CH-aromatic)	6.0, 4.0
Product (20)*	9.43 (s, 1 H, H-CO-)	-
	7.62-7.66 (ddd, 2 H, CH-aromatic)	9.0, 6.0, 3.0
	7.44-7.48 (ddd, 2 H, CH-aromatic)	9.0, 6.0, 3.0
	5.10 (s, 1 H, Ar-NH-)	-
	3.34-4.40 (m, 323 H, -CH- of $\leftarrow CH_2CH_2-O\rightarrow_n$)	-

* Solvent: $CDCl_3$; ** Solvent: DMSO-d_6; [a]: 500 MHz

The conjugate (**20**) was successfully synthesized optimized by FT-IR and ^{1}H-NMR spectral data. The % conjugation of 4f-PABA calculated from ^{1}H-NMR spectral data was 55.97%.

The resulting PEG_{4000}-$(4f\text{-}PABA)_2$ ester conjugate (**20**), was then hydrolyzed by stirring vigorously while refluxing in 15% ethanolic HCl solution for 15 minutes (Zou *et al*., 2005) to obtain amino functional group of **14** (Figure 3-11).

The FT-IR spectroscopic data revealed that PEG_{4000}-$(PABA)_2$ ester conjugate (**14**) was synthesized successfully. Carbonyl stretching of ester bond of **14** still remained at 1723 cm^{-1}. NH stretching of primary amino functional group of **14** was detected at wavenumber 3357 and 3252 cm^{-1} (Figure 4-25). The product was obtained in brownish white amorphous solid (Figure 4-24). The % crude yield of the product was 68.62%.

Figure 4-24 The synthesized products of PEG_{4000}-$(PABA)_2$ ester conjugate (**14**)

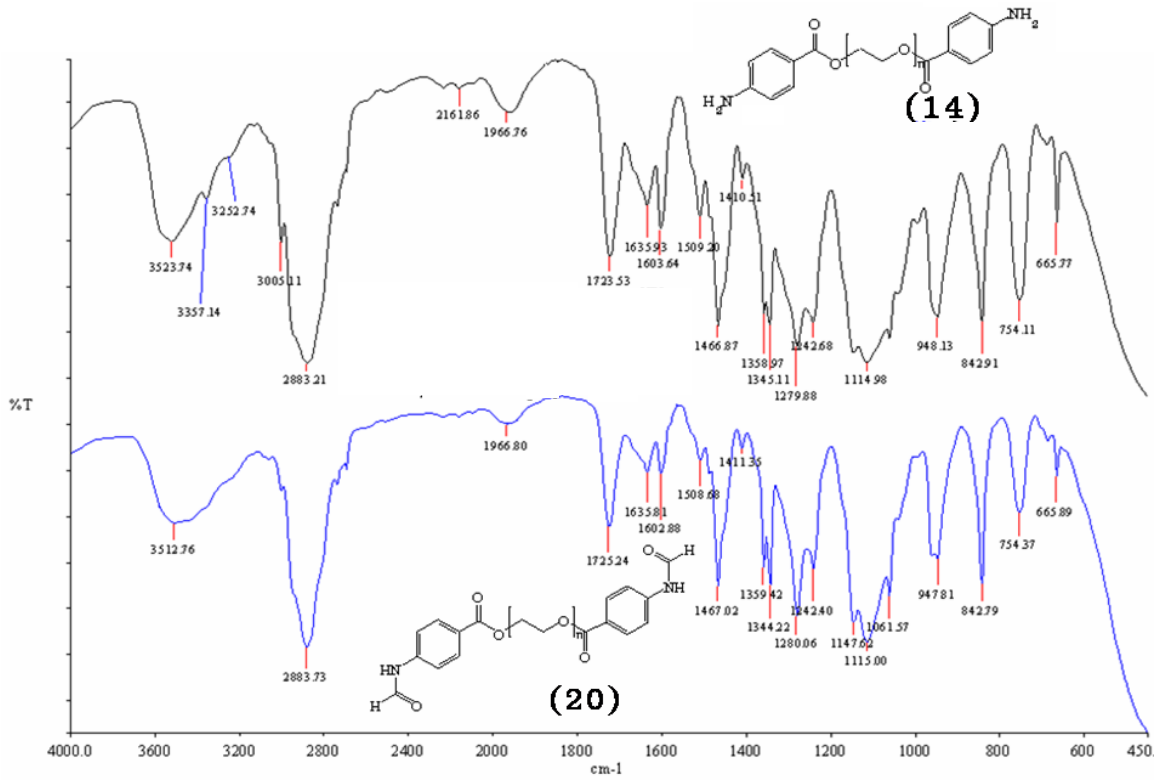


Figure 4-25 FT-IR spectra of **14** and **20**

However, ^{1}H-NMR spectral data of the synthesized product (**14**) demonstrated mixture of the starting material (**20**) and **14**. Therefore, SiO_2 gel column chromatography, using CH_2Cl_2: MeOH (9:1) as mobile phase was utilized in purification process. The ester conjugate of **14** was degraded to starting material (**7**), confirmed by ^{1}H-NMR spectral data. SiO_2 can act as a Lewis acid. The surface of silicon dioxide can function as a catalyst in many chemical reactions including ester hydrolysis. Therefore, purification with SiO_2 column chromatography is not suitable for the ester product.

4.2.8 Synthesis of poly(ethylene) glycol-PABA-SA ester and amide conjugates (Method 8)

p-Nitro benzoylchloride (**26**), a more stable commercial product of acid chloride derivative of PABA was conjugated to PEG (**6**, **7**, **22**, **24**, **25**) to obtain ester and amide products of PEG-(4-NO_2-BA) in the presence of triethylamine or pyridine.

After the reaction, the PEG-4NO_2-BA ester (**27**, **28**) and amide (**29**, **30**, **31**) conjugates were precipitated by ether and dried in vacuum. The yellowish white amorphous solid (Figure 4-26) were obtained. The products were stored at room temperature and protected from moisture. The % yield of PEG-(4NO_2-BA) conjugates (**27-31**) and % conjugation of 4NO_2-BA in PEG-(4NO_2-BA) conjugates (**27-31**) are displayed in Table 4-11.

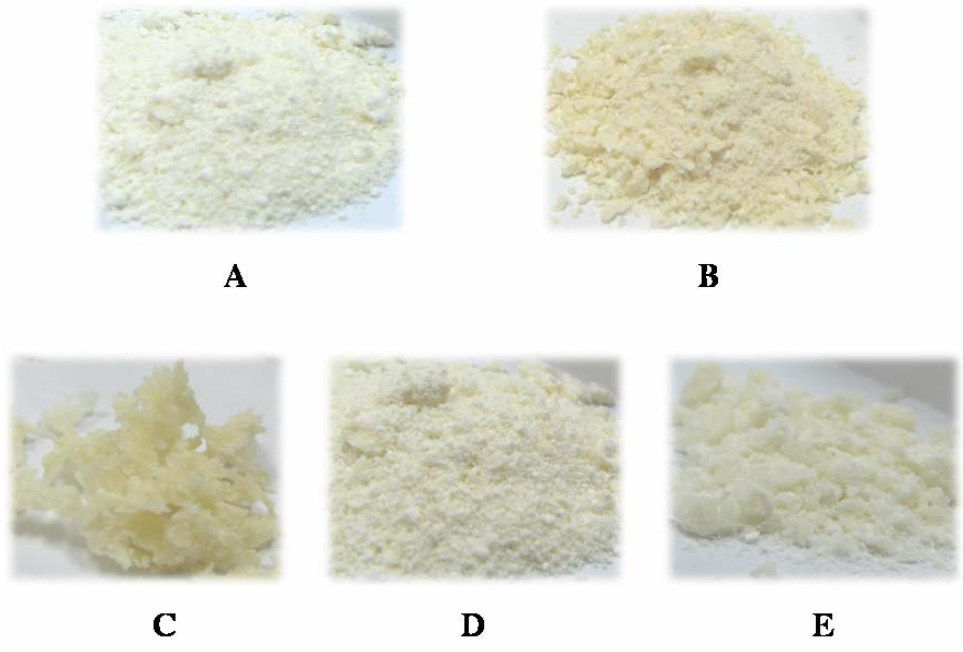


Figure 4-26 The synthesized products of mPEG_{5000}-4NO_2-BA ester (A, **27**), PEG_{4000}-(4NO_2-BA)$_2$ ester (B, **28**), PEG_{3000}-(4NO_2-BA)$_2$ amide (C, **29**), PEG_{5106}-(4NO_2-BA)$_2$ amide (D, **30**) and PEG_{3677}-(4NO_2-BA)$_2$ amide (E, **31**) Conjugates

Table 4-11 % Yield of PEG-($4NO_2$-BA) conjugates and % conjugation of $4NO_2$-BA in PEG-($4NO_2$-BA) conjugates (**27-31**)

PEG-($4NO_2$-BA) conjugates	**% yield**	**% conjugation of $4NO_2$-BA**
$mPEG_{5000}$-($4NO_2$- BA) ester (27)	79.73	86.07
PEG_{4000}-($4NO_2$- $BA)_2$ ester (28)	69.35	90.70
PEG_{3000}-($4NO_2$- $BA)_2$ amide (29)	86.04	59.36
PEG_{5106}-($4NO_2$- $BA)_2$ amide (30)	90.12	97.30
PEG_{3677}-($4NO_2$- $BA)_2$ amide (31)	93.27	95.56

FT-IR spectral data (Figure 4-27 and 4-28) of the synthesized compounds were shown in Table 4-12. However, the O-H stretching peak of hydroxyl functional group still remained. Therefore, the % conjugation was calculated (Table 4-11).

Table 4-12 FT-IR spectral data of PEG-($4NO_2$-BA) conjugates (**27-31**)

PEG-($4NO_2$-BA) conjugates	**Wavenumber (cm^{-1})**	
	C=O stretching	$-NO_2$
$mPEG_{5000}$-($4NO_2$- BA) ester (27)	1724	1529, 1467
PEG_{4000}-($4NO_2$-$BA)_2$ ester (28)	1726	1529, 1467
PEG_{3000}-($4NO_2$-$BA)_2$ amide (29)	1661	1525, 1467
PEG_{5106}-($4NO_2$-$BA)_2$ amide (30)	1655	1525, 1467
PEG_{3677}-($4NO_2$-$BA)_2$ amide (31)	1655	1525, 1467

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of $mPEG_{5000}$-($4NO_2$-BA) ester conjugate (**27**, Figure 4-29) δ (ppm): 8.21-8.26 (dd, J = 12.0, 3.0 Hz, 2 H, CH-aromatic); 8.15-8.19 (dd, J = 12.0, 3.0 Hz, 2 H, CH-aromatic); 3.35-4.49 (m, 451 H, -CH- of $(CH_2CH_2-O)_n$); 3.32 (s, 3 H, -O-CH_3). The % conjugation was 86.07%.

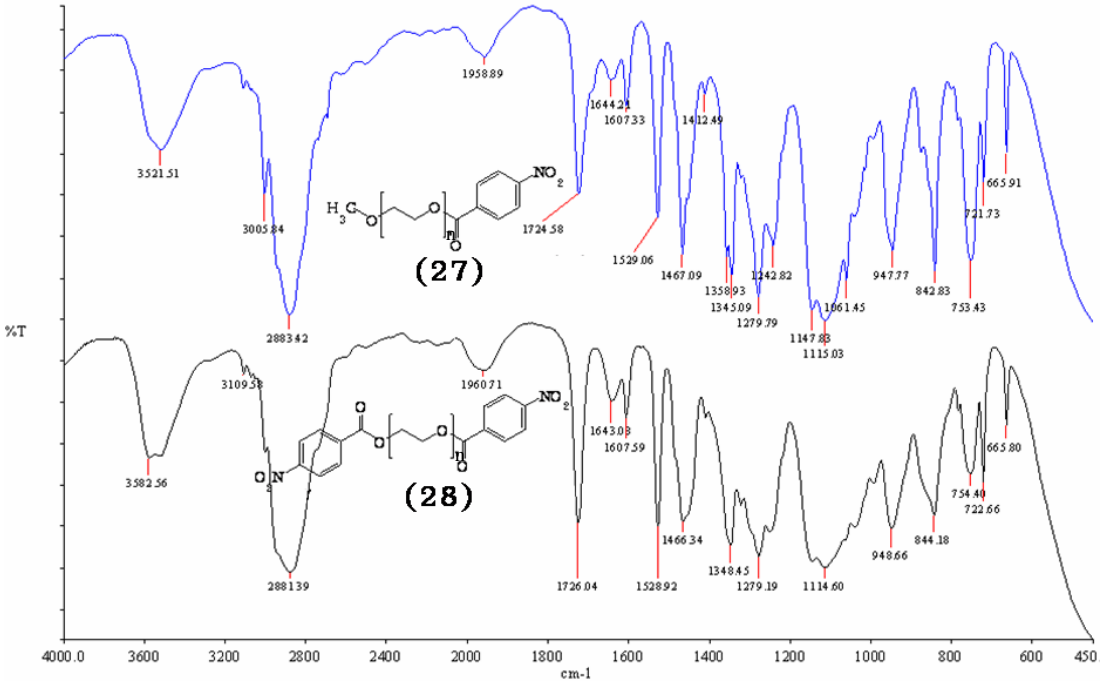


Figure 4-27 FT-IR spectra of **27** and **28**

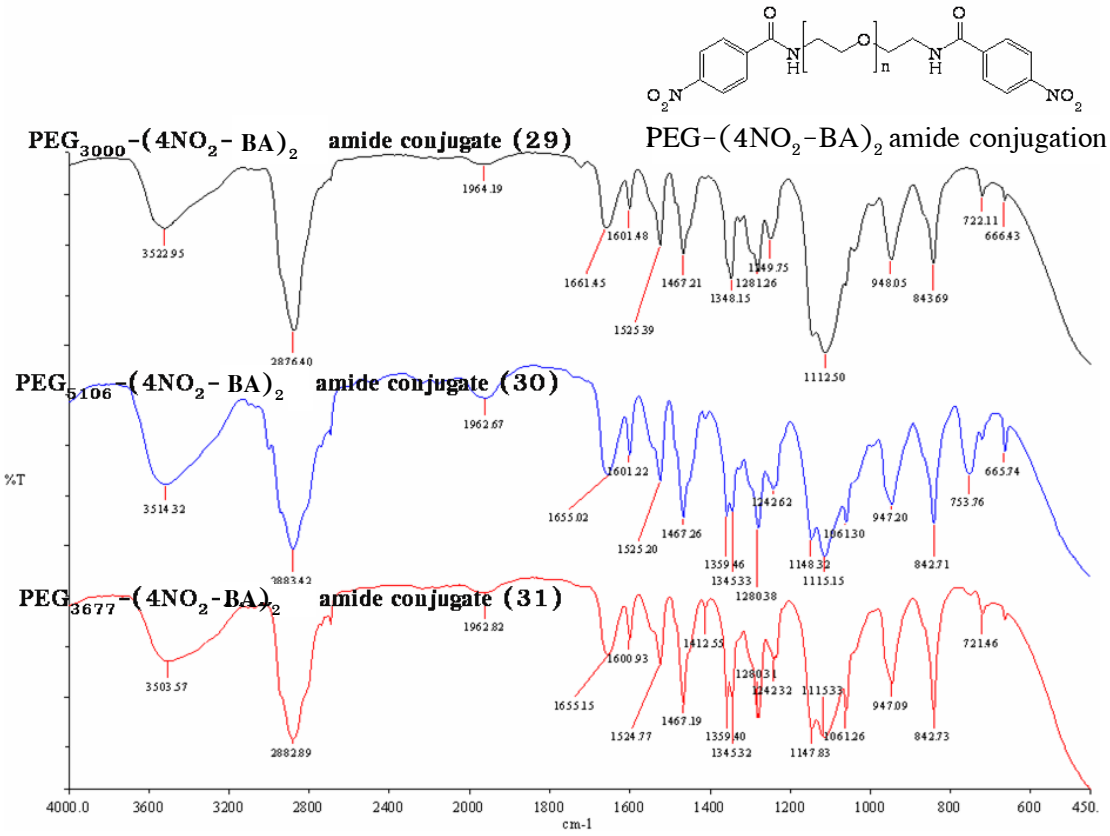


Figure 4-28 FT-IR spectra of **29**, **30** and **31**

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of PEG_{4000}-$(4NO_2$-BA$)_2$ ester conjugate (**28**, Figure 4-29) δ (ppm): 8.20-8.25 (dd, *J* = 12.0, 3.0 Hz, 4 H, CH-aromatic); 8.12-8.16 (dd, *J* = 12.0, 3.0 Hz, 4 H, CH-aromatic); 3.08-4.49 (m, 362 H, -CH- of $(CH_2CH_2-O)_n$). The % conjugation was 90.70%.

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of PEG_{3000}-$(4NO_2$-BA$)_2$ amide conjugate (**29**, Figure 4-29) δ (ppm): 8.18-8.22 (dd, *J* = 12.0, 3.0 Hz, 4 H, CH-aromatic); 8.00-8.03 (dd, *J* = 12.0, 3.0 Hz, 4 H, CH-aromatic); 3.30-3.86 (m, 268 H, -CH- of $(CH_2CH_2-O)_n$). The % conjugation was 59.36%.

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of PEG_{5106}-$(4NO_2$-BA$)_2$ amide conjugate (**30**, Figure 4-29) δ (ppm): 8.26-8.31 (d, *J* = 20.0 Hz, 4 H, CH-aromatic); 8.04-8.09 (d, *J* = 20.0 Hz, 4 H, CH-aromatic); 3.44-4.30 (m, 458 H, -CH- of $(CH_2CH_2-O)_n$). The % conjugation was 97.30%.

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of PEG_{3677}-$(4NO_2$-BA$)_2$ amide conjugate (**31**, Figure 4-29) δ (ppm): 8.28-8.32 (d, *J* = 12.0 Hz, 4 H, CH-aromatic); 8.02-8.08 (d, *J* = 12.0 Hz, 4 H, CH-aromatic); 3.30-4.40 (m, 328 H, -CH- of $(CH_2CH_2-O)_n$). The % conjugation was 95.56%.

^{1}H-NMR spectral data of the synthesized compounds, **27**, **28**, **29**, **30** and **31** (Figure 4-29), confirmed that the PEG-(4-NO_2-BA) conjugates were synthesized successfully.

After attempting to reduce the nitro functional group by refluxing with $Zn/CaCl_2.2H_2O$ in 4:1 EtOH/H_2O as previously reported by Callant and Schacht in 1990, the ester or amide bond between PEG and spacer was found degraded (confirmed by FT-IR and ^{1}H-NMR spectral data). It may be due to the reduction condition was too strong. PEG (**6**, **7**, **22**), the starting material were obtained after purification by liquid-liquid extraction.

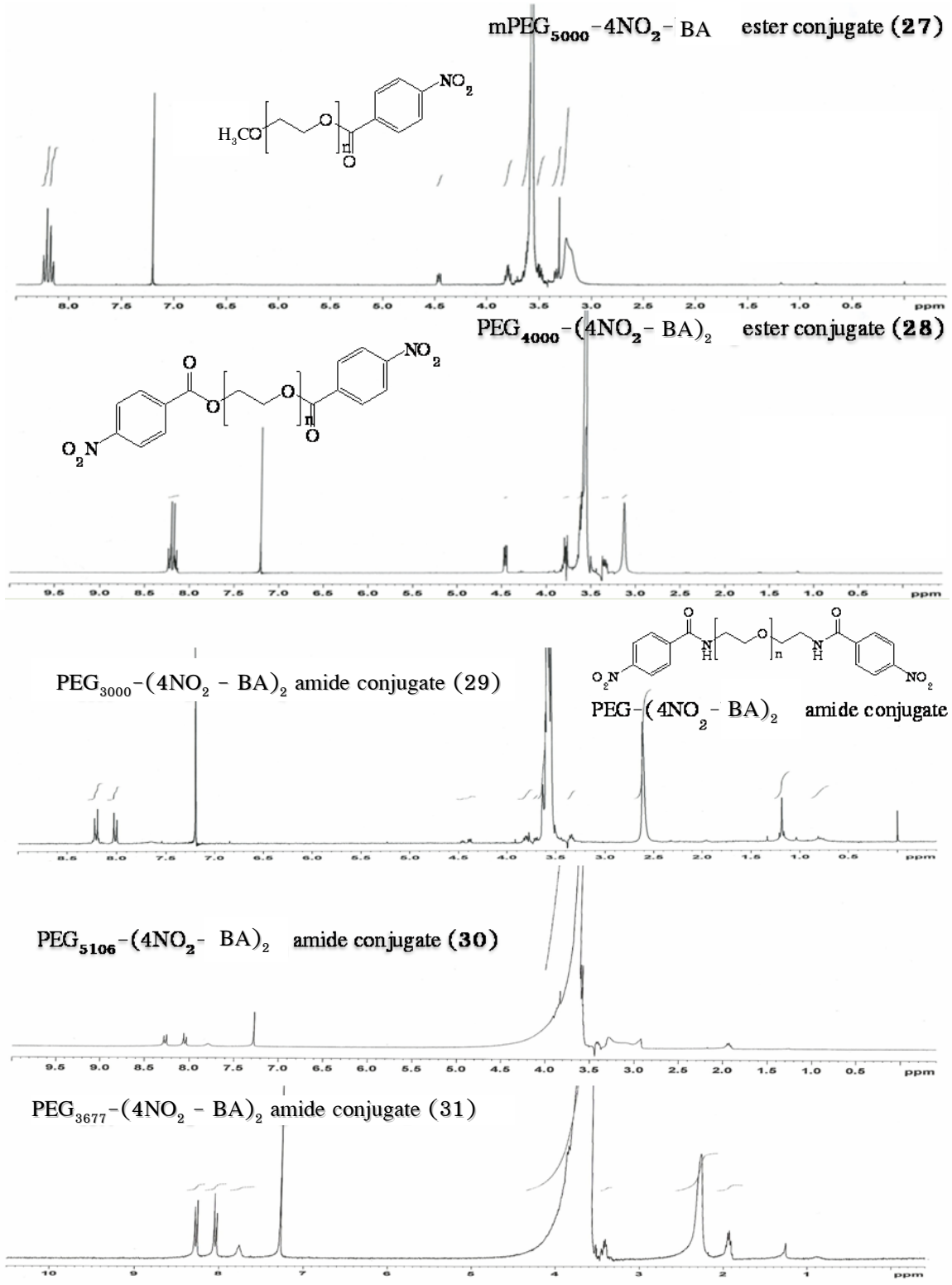


Figure 4-29 ^{1}H-NMR spectra of PEG-(4-NO_2-BA) conjugates (**27, 28, 29, 30, 31**)

Since the well known method for reduction of nitro group is catalytic hydrogenation by transition metal such as palladium or platinum (Carruthers and Coldham, 2004), therefore the reduction of the nitro group in ester and amide conjugates (**27**, **28**, **30** and **31**) using catalytic hydrogenation in the presence of palladium on activated charcoal was used instead of the previous method. Moreover, PEG with molecular weight less than 3350 Da could be absorbed in GI tract (Fruijtier-Pölloth, 2005). Hence, PEG_{3000} amide conjugate (**29**) was not utilized in the reduction process.

The aromatic nitro of **27**, **28**, **30** and **31** was then reduced to amino functional group by hydrogenation using Pd/C as catalyst, resulting in bright yellow amorphous solid after purification (Figure 4-30). The % yield of PEG-PABA conjugates (**12**, **14**, **33**, **34**) and % conjugation of PABA in PEG-PABA conjugates (**12**, **14**, **33**, **34**) are shown in Table 4-13.

FT-IR spectral data (Figure 4-31 and 4-32) of the synthesized compounds are shown in Table 4-14. The nitro functional peak of **27**, **28**, **30** and **31** at wavenumber ~1525 disappeared, means that nitro group of 4-NO_2-BA was reduced. However, the O-H stretching peak of hydroxyl functional group still remained. Therefore, the % conjugation was calculated (Table 4-13).

Table 4-13 % Yield of PEG-PABA conjugates and % conjugation of PABA in PEG-PABA conjugates

PEG-PABA conjugates	% yield	% conjugation of PABA
$mPEG_{5000}$-PABA ester (12)	59.30	78.44
PEG_{4000}-$(PABA)_2$ ester (14)	71.15	79.07
PEG_{5106}-$(PABA)_2$ amide (33)	82.98	91.35
PEG_{3677}-$(PABA)_2$ amide (34)	88.55	72.03

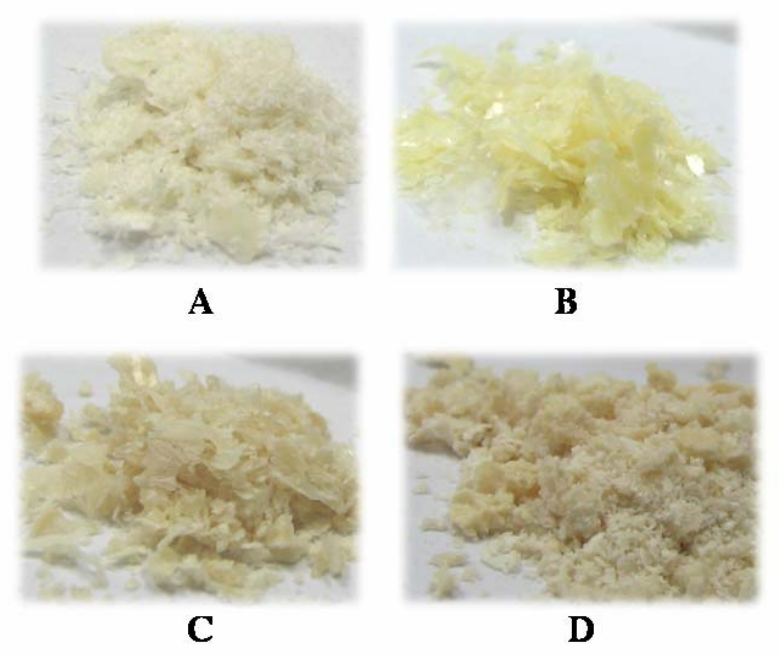


Figure 4-30 The synthesized products of $mPEG_{5000}$-PABA ester (A, **12**), PEG_{4000}-$(PABA)_2$ ester (B, **14**), PEG_{5106}-$(PABA)_2$ amide (C, **33**) and PEG_{3677}-$(PABA)_2$ amide (D, **34**) conjugates

Table 4-14 FT-IR spectral data of PEG-PABA conjugates

PEG-PABA conjugates	**Wavenumber (cm^{-1})**	
	C=O stretching	N-H stretching
$mPEG_{5000}$- PABA ester (12)	1701	3362, 3242
PEG_{4000}-$(PABA)_2$ ester (14)	1701	3360, 3236
PEG_{5106}-$(PABA)_2$ amide (33)	1634	3361, 3236
PEG_{3677}-$(PABA)_2$ amide (34)	1635	3361, 3241

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of $mPEG_{5000}$-PABA ester conjugate (**12**, Figure 4-33) δ (ppm): 8.12-8.15 (dd, J = 12.0, 3.0 Hz, 2 H, CH-aromatic); 7.78-7.81 (dd, J = 12.0, 3.0 Hz, 2 H, CH-aromatic); 3.42-4.54 (m, 451 H, -CH- of $(CH_2CH_2-O)_n$); 3.35 (s, 3 H, -O-CH_3). The % conjugation was 78.44%.

^{1}H-NMR (300 MHz, $CDCl_3$) spectrum of PEG_{4000}-$(PABA)_2$ ester conjugate (**14**, Figure 4-33) δ (ppm): 7.25-7.28 (dd, *J* = 9.0, 3.0 Hz, 4 H, CH-aromatic); 8.10-7.14 (dd, *J* = 9.0, 3.0 Hz, 4 H, CH-aromatic); 3.45-4.50 (m, 362 H, -CH- of $\leftarrow CH_2CH_2-O\rightarrow_n$). The % conjugation was 79.07%.

^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of PEG_{5106}-$(PABA)_2$ amide conjugate (**33**, Figure 4-33) δ (ppm): 9.369 (s, 2 H, -Ar-CO-NH-); 7.887-7.935 (dd, *J* = 8.5, 7.5 Hz, 4 H, CH-aromatic); 7.098-7.115 (dd, *J* = 8.5, 7.0 Hz, 4 H, CH-aromatic); 5.244 (s, 4 H, $-NH_2$); 3.457-4.767 (m, 458 H, -CH- of $\leftarrow CH_2CH_2-O\rightarrow_n$). The % conjugation was 91.35%.

^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of PEG_{3677}-$(PABA)_2$ amide conjugate (**34**, Figure 4-33) δ (ppm): 7.620-7.638 (dd, *J* = 9.0, 8.5 Hz, 4 H, CH-aromatic); 6.544-6.562 (dd, *J* = 9.0, 8.5 Hz, 4 H, CH-aromatic); 3.436-3.754 (m, 328 H, -CH- of $\leftarrow CH_2CH_2-O\rightarrow_n$). The % conjugation was 72.03%.

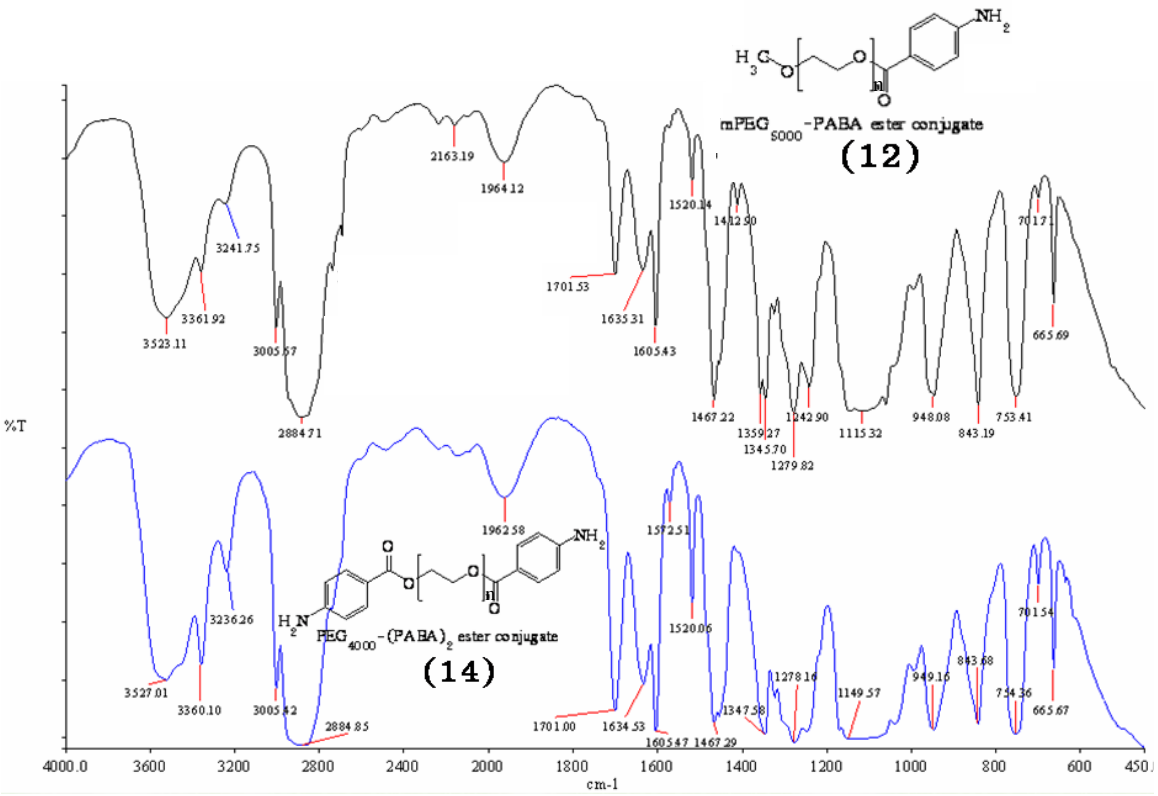


Figure 4-31 FT-IR spectra of **12** and **14**

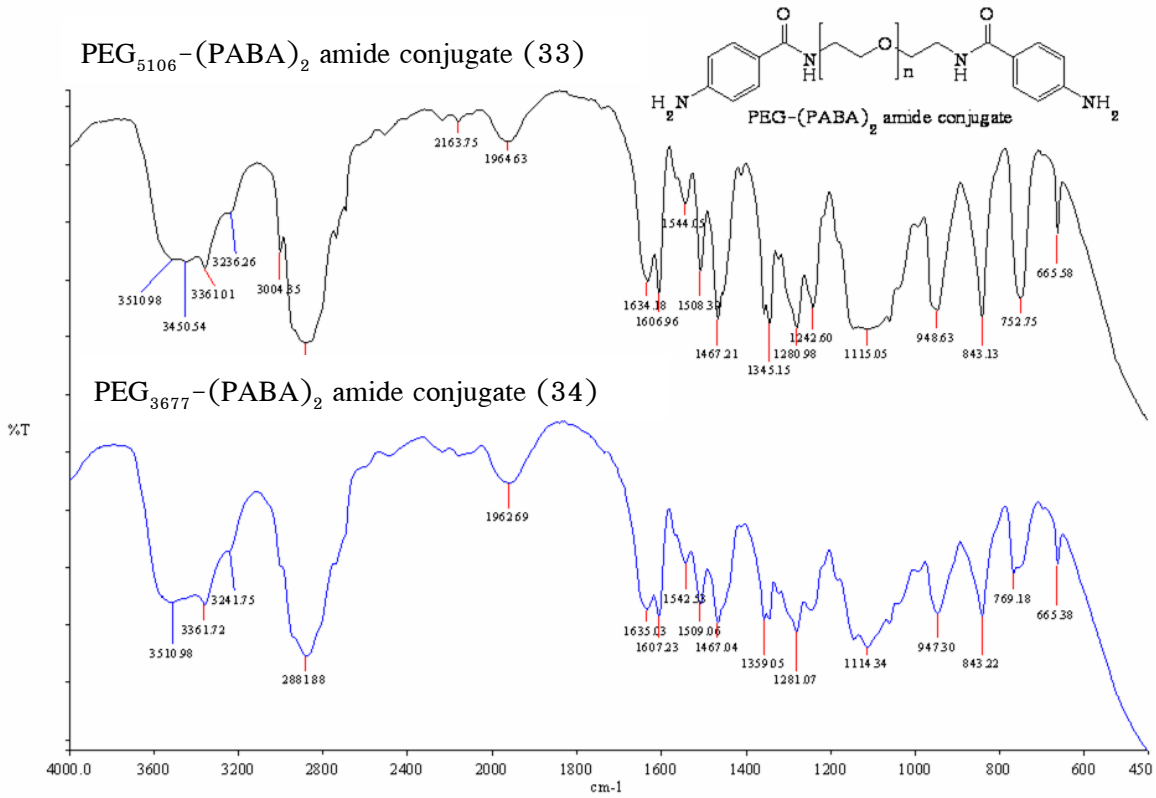


Figure 4-32 FT-IR spectra of **33** and **34**

^{1}H-NMR spectral data of the synthesized compounds, **12**, **14**, **33** and **34**, confirmed that the PEG-PABA conjugates were synthesized successfully.

After diazotization reaction with SA, compounds (**8**, **10**, **39**, **40**) were obtained, resulting in reddish orange amorphous solid after purification (Figure 4-34). The % yield of PEG-PABA-SA conjugates (**8**, **10**, **39**, **40**) and % conjugation of PABA-SA in PEG-PABA-SA conjugates (**8**, **10**, **39**, **40**) are shown in Table 4-15.

Table 4-15 % Yield of PEG-PABA-SA conjugates and % conjugation

PEG-PABA-SA conjugates	% yield	% conjugation
mPEG$_{5000}$-PABA-SA ester (8)	98.12	37.39
PEG$_{4000}$-(PABA-SA)$_2$ ester (10)	96.75	73.29
PEG$_{5106}$-(PABA-SA)$_2$ amide (39)	99.05	91.94
PEG$_{3677}$-(PABA-SA)$_2$ amide (40)	99.13	72.24

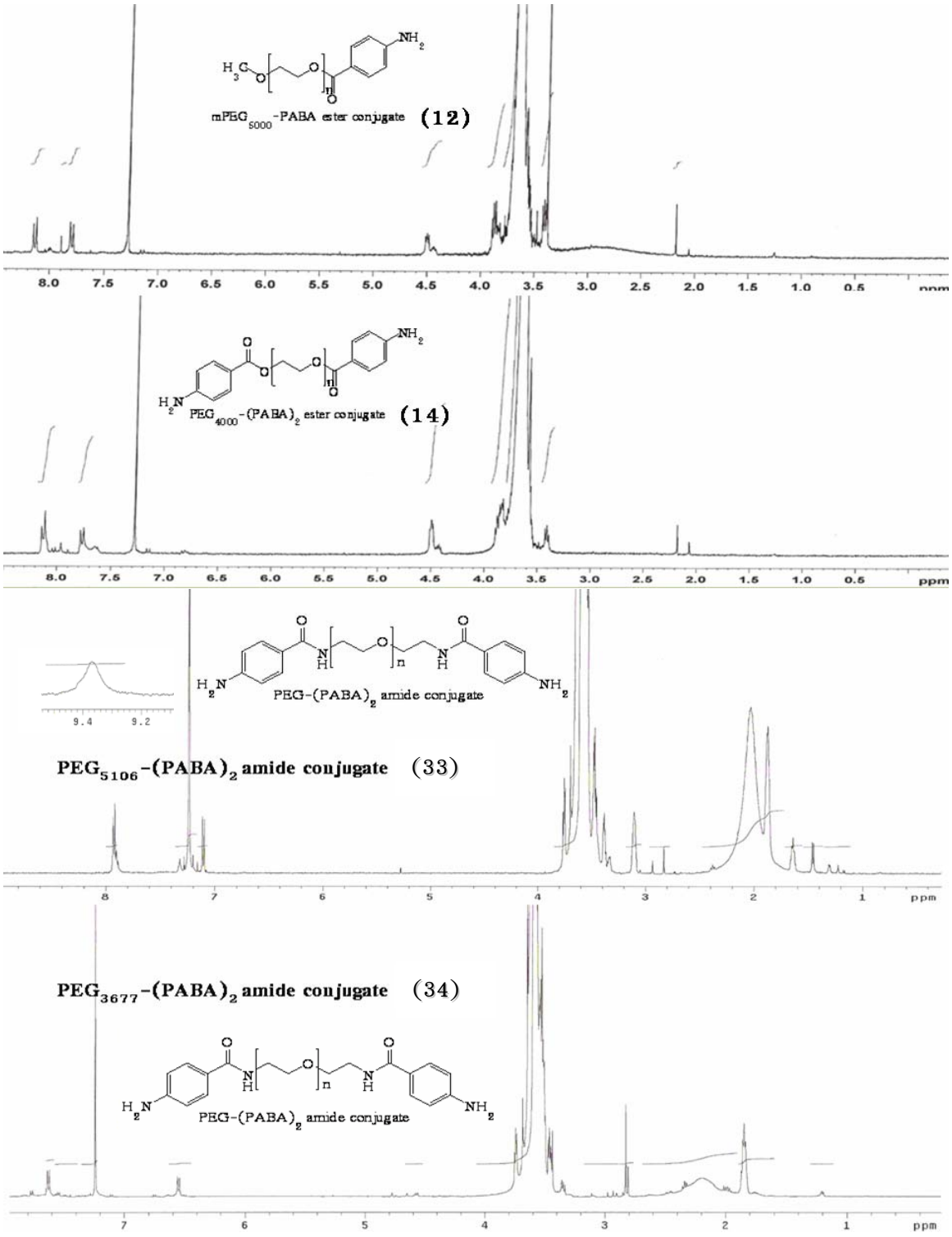


Figure 4-33 ^{1}H-NMR spectra of PEG-PABA conjugates (**12, 14, 33, 34**)

The UV-Visible absorption spectra of the PEG-PABA-SA conjugates (**8**, **10**, **39**, **40**) were determine by dissolving and diluting the products in distilled water. Distilled water was used as blank solution. The maximum wavelengths (λmax) of **8**, **10**, **39** and **40** were at 362, 362, 365 and 356 nm, respectively (Figure 4-35).

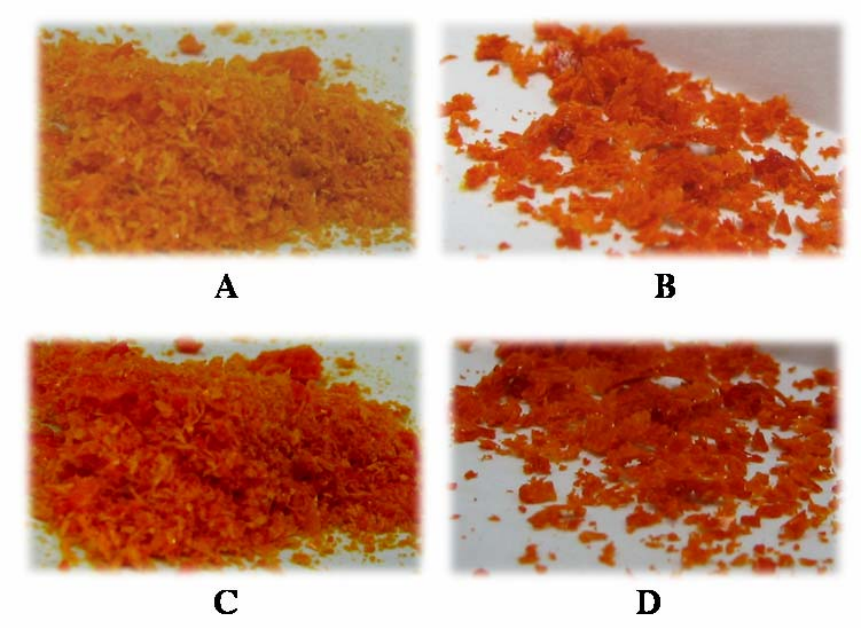


Figure 4-34 The synthesized products of $mPEG_{5000}$-PABA ester (A, **8**), PEG_{4000}-$(PABA)_2$ ester (B, **10**), PEG_{5106}-$(PABA)_2$ amide (C, **39**) and PEG_{3677}-$(PABA)_2$ amide (D, **40**) conjugates

FT-IR spectral data (Figure 4-36 and 4-37) of the synthesized compounds are shown in Table 4-16. The O-H stretching peak of hydroxyl and carboxyl functional groups was found at wavenumber ~3523 cm^{-1} and the N-H stretching of amino functional group of PEG-PABA disappeared. Therefore, the expected products were obtained.

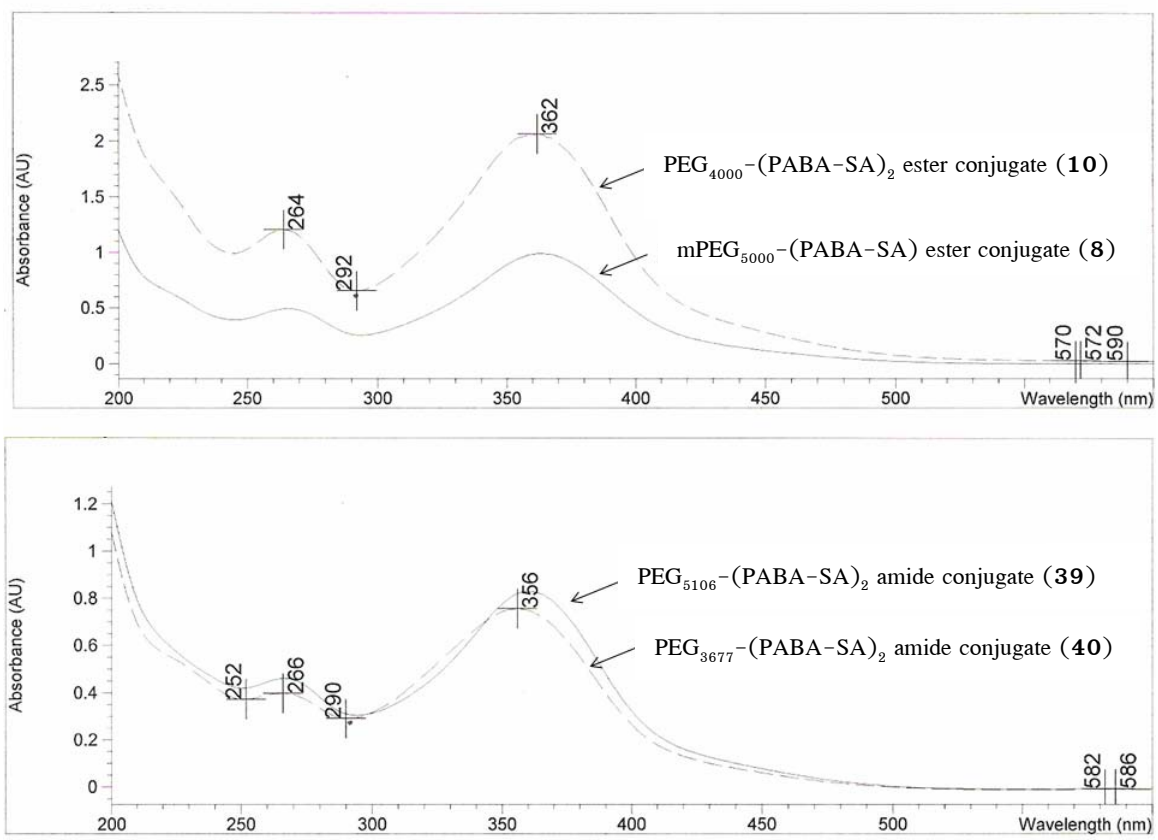


Figure 4-35 UV spectra of **8**, **10**, **39** and **40**

Table 4-16 FT-IR spectral data of PEG-PABA-SA conjugates

PEG-PABA conjugates	Wavenumber (cm^{-1})	
	C=O stretching	-N=N-
$mPEG_{5000}$- PABA-SA ester (8)	1718	1599
PEG_{4000}-$(PABA-SA)_2$ ester (10)	1717	1599
PEG_{5106}-$(PABA-SA)_2$ amide (39)	1653	1577 (weak)
PEG_{3677}-$(PABA-SA)_2$ amide (40)	1651	1577 (weak)

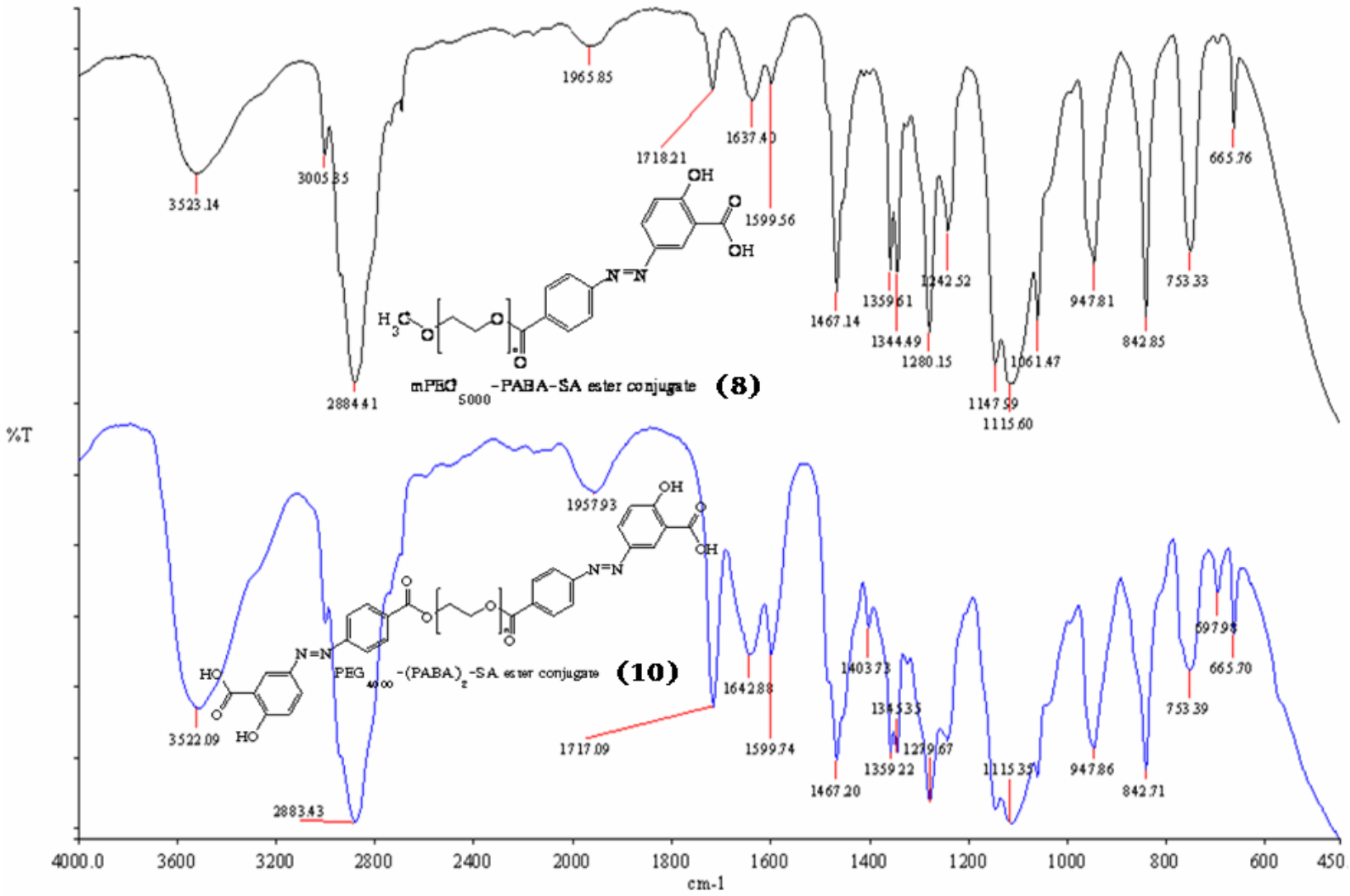


Figure 4-36 FT-IR spectra of **8** and **10**

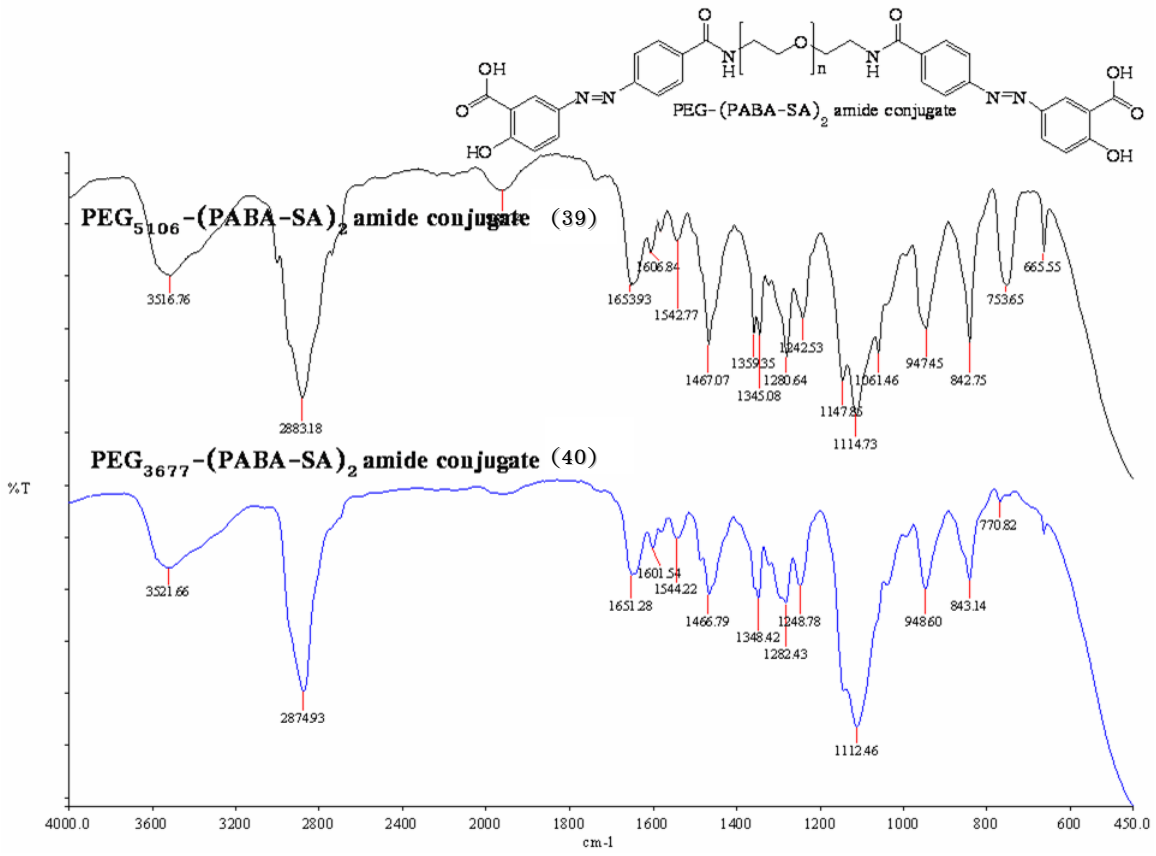


Figure 4-37 FT-IR spectra of **39** and **40**

^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of $mPEG_{5000}$-PABA-SA ester conjugate (**8**) δ (ppm): 8.654-8.659 (d, *J* = 2.5 Hz, 2 H, CH-aromatic of SA); 8.146-8.188 (ddd, J = 8.5, 3.5, 0.5 Hz, 4 H, CH-aromatic of PABA); 8.098-8.080 (d, *J* = 9.0 Hz, 2 H, CH-aromatic of SA); 7.885-7.990 (ddd, *J* = 8.5, 2.5, 0.5 Hz, 4 H, CH-aromatic of PABA); 7.065-7.083 (dd, *J* = 9.0, 0.5 Hz, 2 H, CH-aromatic of SA); 3.436-4.493 (m, 451 H, -CH- of $(CH_2CH_2-O)_n$); 3.348 (s, 3 H, $-O-CH_3$). The % conjugation was 37.39%.

^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of PEG_{4000}-$(PABA-SA)_2$ ester conjugate (**10**) δ (ppm): 11.670 (s, 2 H, Ar-OH); 8.499-8.503 (d, *J* = 2.0 Hz, 2 H, CH-aromatic of SA); 8.169-8.228 (dd, *J* = 8.5, 8.5 Hz, 4 H, CH-aromatic of PABA); 7.969-8.072 (d, *J* = 9.0 Hz, 2 H, CH-aromatic of SA); 7.883-7.986 (dd, *J* = 8.5, 8.0 Hz, 4 H, CH-aromatic of PABA); 7.063-7.081 (d, *J* = 9.0 Hz, 2 H, CH-aromatic

of SA); 3.444-4.511 (m, 362 H, -CH- of $(CH_2CH_2-O)_n$). The % conjugation was 73.29%.

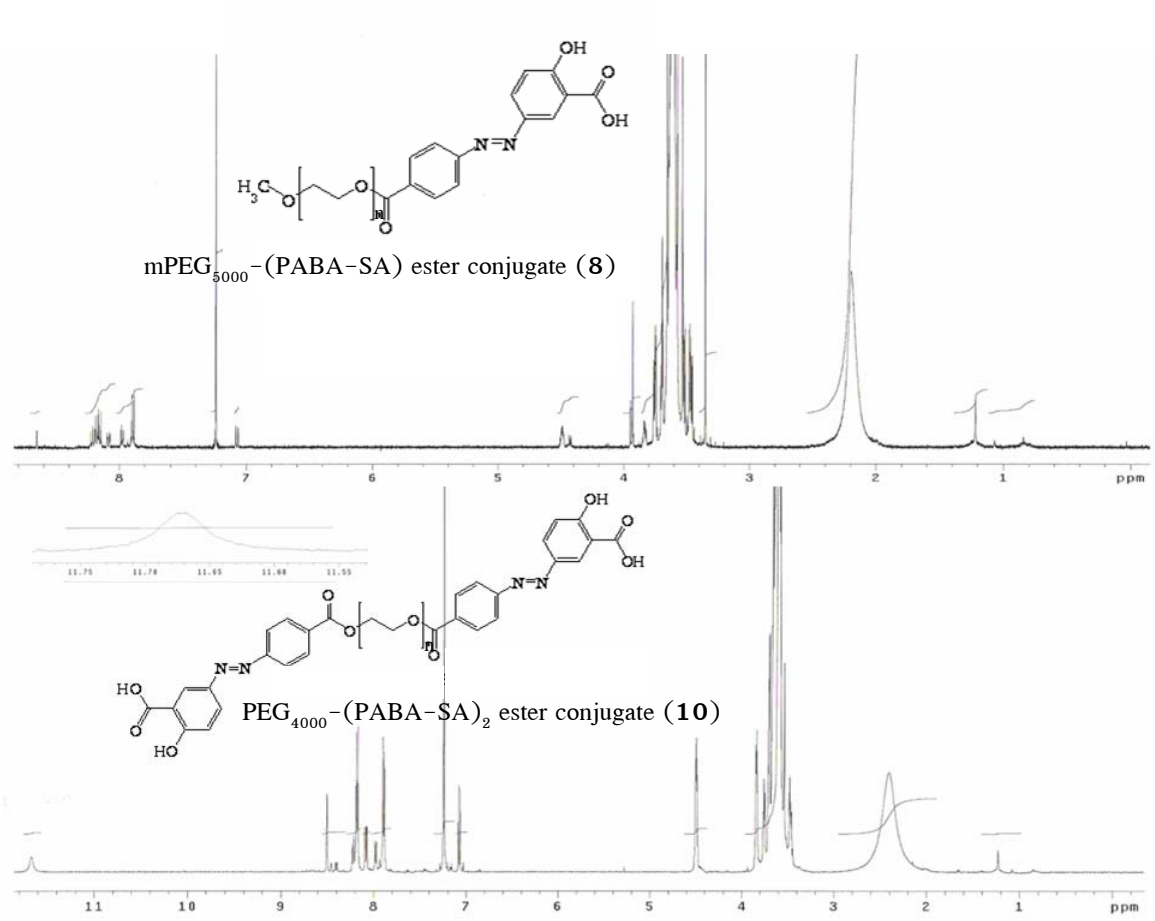


Figure 4-38 ^{1}H-NMR spectra of PEG-(PABA-SA) ester conjugates (**8, 10**)

^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of PEG_{5106}-$(PABA-SA)_2$ amide conjugate (**38**) δ (ppm): 8.530 (s, 2 H, CH-aromatic of SA); 7.934-7.951 (dd, *J* = 8.5, 7.5 Hz, 4 H, CH-aromatic of PABA); 7.858-7.875 (dd, *J* = 8.5, 8.5 Hz, 4 H, CH-aromatic of PABA); 7.746-7.763 (d, *J* = 8.5 Hz, 2 H, CH-aromatic of SA); 7.007-7.025 (d, *J* = 9.0 Hz, 2 H, CH-aromatic of SA); 3.342-3.798 (m, 458 H, -CH- of $(CH_2CH_2-O)_n$). The % conjugation was 91.94%.

^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of PEG_{3677}-$(PABA-SA)_2$ amide conjugate (**39**) δ (ppm): 8.554 (s, 2 H, CH-aromatic of SA); 7.900-7.936 (dd, *J* = 8.5, 9.5 Hz, 4 H, CH-aromatic of PABA); 7.812-7.829 (d, *J* = 8.5 Hz, 4 H, CH-

aromatic of PABA); 7.576-7.594 (d, J = 9.0 Hz, 2 H, CH-aromatic of SA); 6.926-6.944 (d, J = 8.5 Hz, 2 H, CH-aromatic of SA); 3.424-3.751 (m, 328 H, -CH- of $(CH_2CH_2-O)_n$). The % conjugation was 72.24%.

^{1}H-NMR spectral data of the synthesized compounds, **8**, **10**, **38** and **39**, confirmed that the PEG-PABA-SA conjugates were synthesized successfully.

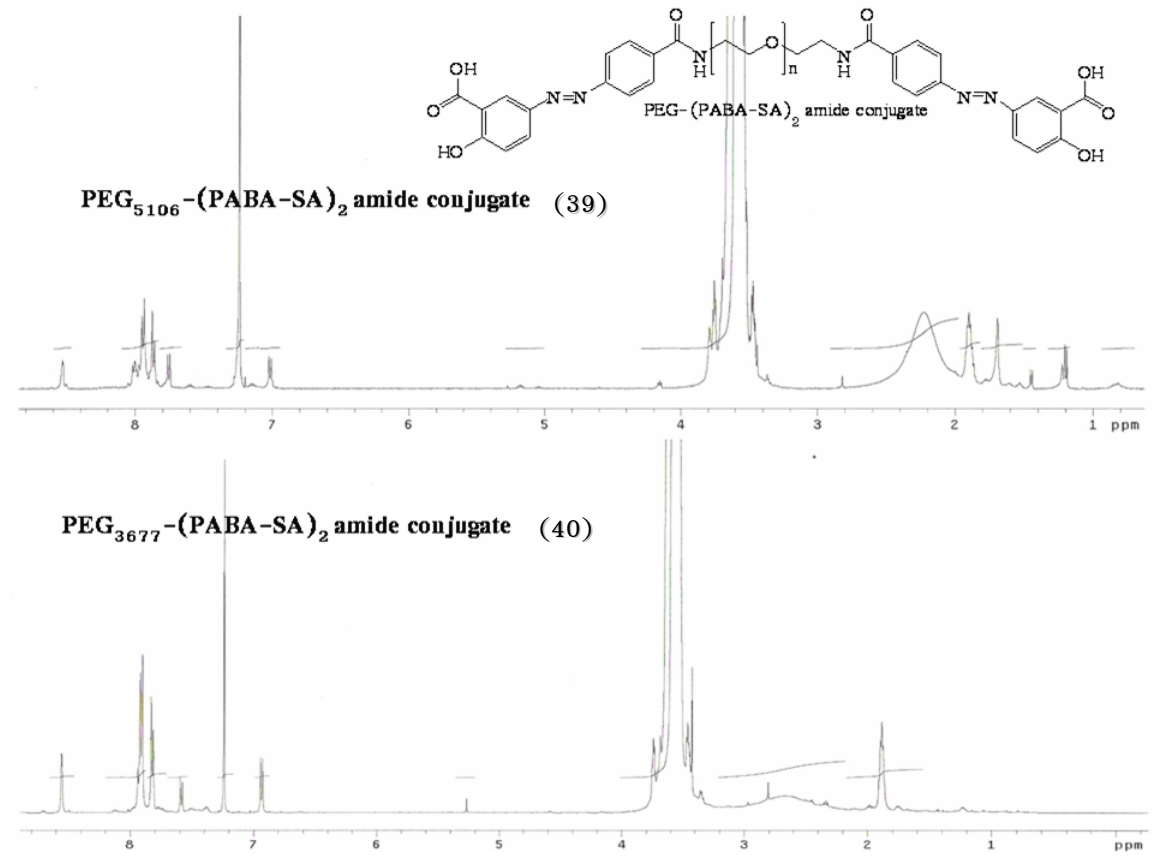


Figure 4-39 ^{1}H-NMR spectra of PEG-(PABA-SA) amide conjugates (**39**, **40**)

4.3 Quantitative analysis

4.3.1 Validation of HPLC method for quantitative analysis

The analytical method for quantification of the compounds released from the synthesized polymer conjugates was developed from previous reports (She *et al.*, 1997; Bareggi and Benedittis, 1998; Saito *et al.*, 2006).

The HPLC chromatographic condition was performed for quantitative analysis. The method validation was performed. HPLC analysis was carried out using Agilent 4100 series equipped with an Agilent 1100 series Photodiode-Array Detector (PDA) and autosampler. Data analysis was performed using Agilent software (Agilent, USA). The chromatographic system was reverse phase HPLC (250 mm x 4.6 mm i.d. C-18 column) with gradient system of mobile phase A, pH 3.3 acetate buffer/MeOH (85:15 v/v) and Mobile phase B, MeOH. The % ratio of mobile phase A/mobile phase B were 100/0 (0-5 minutes), 60/40 (5.01-9 minutes), 100/0 (9.01-11 minutes), 33/67 (11-18 minutes) and 100/0 (18.01-25 minutes). Flow rate was maintained at 0.8 ml/min and at 25 °C. Flow rate was 0.8 ml/min at 25 °C. The injection volume was 20 µl. The quantitation wavelength was set at 295 nm, for detection of 5-ASA, PAH (**2**), PABA (**1**) and SA, and 365 nm, for detection of PAH-SA (**5**) and PABA-SA conjugates (**4**).

In this study, HPLC method was developed to give a sensitive, reproducible and accurate analytical procedure for quantification of 6 compounds, 5-ASA, PAH (**2**), PABA (**1**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**). The developed method was then used to determine % free substrates of the products from synthesis reaction, the chemical stability of the conjugates and released free drug from release study. The quantitative wavelength was set at 295 nm, for detection of 5-ASA, PAH, PABA and SA, and 365 nm, for detection of PAH-SA and PABA-SA conjugates. The retention time of mobile phase, 5-ASA, PAH, PABA, SA, PAH-SA conjugate and PABA-SA conjugate were 1.18, 4.73, 6.19, 10.07, 13.30, 13.55 and 17.06 minutes, respectively. All peak were well separated (Figure 4-40). The standard curves of 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**) were in ranges

of 2-10 μg/ml and PABA (**1**) was in range of 0.2-1.0 μg/ml, R^2 = 0.9993, 0.9999, 0.9992, 0.9992, 0.9994 and 0.9994, respectively (Table 4-18).

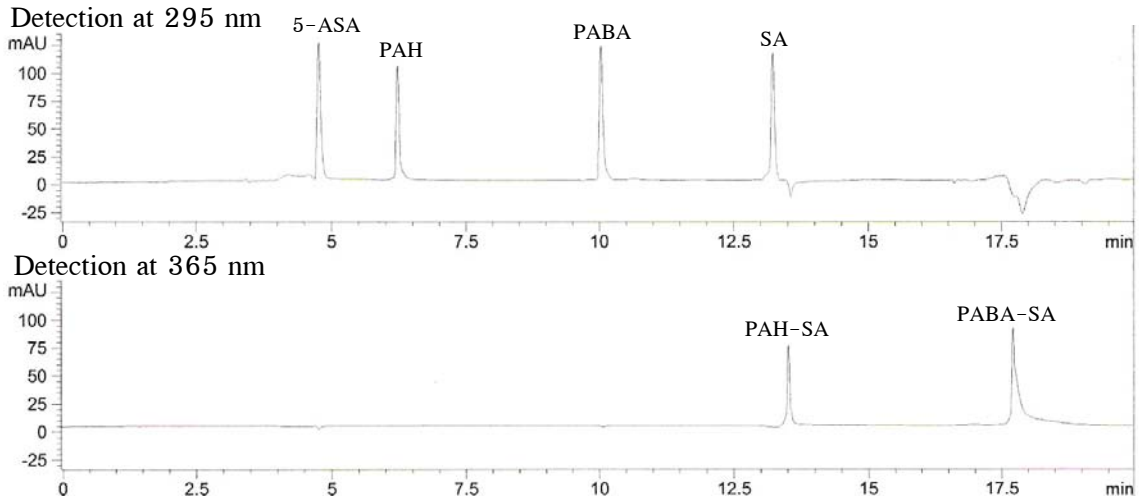


Figure 4-40 HPLC chromatograms of standard 5-ASA, PAH (**2**), PABA (**1**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**)

4.3.2 Selectivity

The developed HPLC method for PAH, PABA and SA was previously tested for its specificity by HPLC analysis of those 3 compounds in PAH-SA conjugate and PABA-SA conjugate prepared in mobile phase A (Figure 4-3 and 4-4). The same method was utilized in determination for its selectivity of 5-ASA, PABA, SA and PABA-SA conjugate in gastrointestinal contents treated with 1% peptone in buffer solutions (Figure 4-41). All chromatogram of the samples showed no peak as interferences. This study confirmed the stability indicating power of the method and showed that these chromatographic conditions can be used for investigation of the compounds both in buffer solutions and gastrointestinal contents.

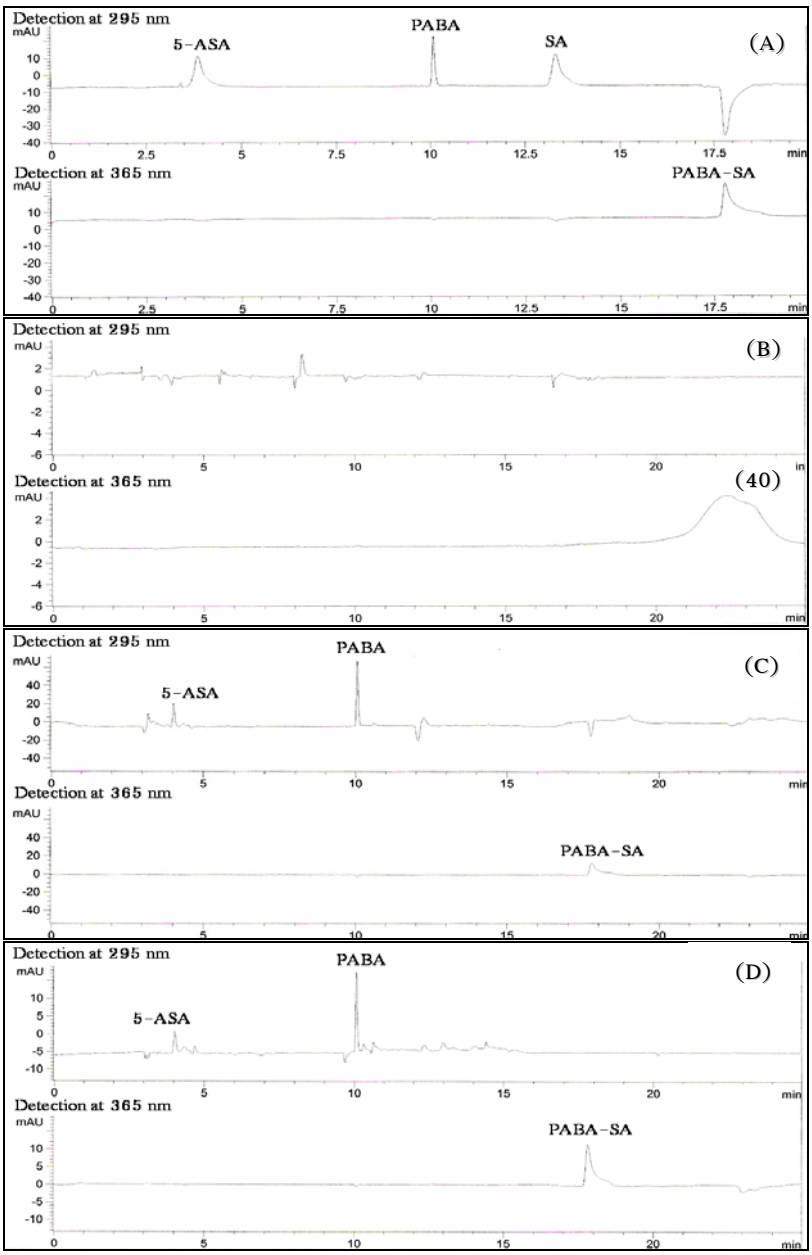


Figure 4-41 HPLC chromatograms of standard 5-ASA, PABA (**1**), SA (**3**) and PABA-SA conjugate (**4**) in mobile phase A (**A**), in gastric content treated with 1% peptone in citrate buffer pH 1.2 (**B**), in intestinal content treated with 1% peptone in phosphate buffer pH 6.8 (**C**) and in colonic content treated with 1% peptone in phosphate buffer 7.5 (**D**)

4.3.3 Limit of detection (LOD) and limit of quantitation (LOQ)

Since the developed method is used for determination of released compounds after incubation in different conditions, therefore, even the small amount of compounds, the method should be able to detect. The limit of detection (LOD) and LOQ of each standards, 5-ASA, PAH (**2**), PABA (**1**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**), are displayed in Table 4-17. The results demonstrated that LOD of 5-ASA and SA (**3**) were 1.8 μg/ml; PAH (2), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**) were 1.6 μg/ml; and PABA (**1**) was 0.2 μg/ml. LOQ of 5-ASA, PAH (**2**), SA (**3**) PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**) were 2.0 μg/ml, and PABA (**1**) was 0.2 μg/ml.

In the release study, 140 μg/ml of 5-ASA in tested conjugates were originally added into the releasing medium. And after diluted with mobile phase A, the final concentration of each sample was not more than 10 μg/ml of 5-ASA. Therefore, the actual amount of the released compounds was accurately calculated by our developed method due to the low LOD (about 18% of the final concentration) and LOQ (about 20% of the final concentration).

Table 4-17 LOD and LOQ (μg/ml) of the analytes

Standard	LOD (μg/ml)	LOQ (μg/ml)
5-ASA	1.8	2.0
PAH (**2**)	1.6	2.0
PABA (**1**)	0.2	0.2
SA (**3**)	1.8	2.0
PAH-SA (**5**)	1.6	2.0
PABA-SA (**4**)	1.6	2.0

4.3.4 Linearity and range

The solutions of 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**) were prepared in the range of 2-10 μg/ml, whereas, PABA (**1**) standard solution was prepared in the range of 0.2-1.0 μg/ml, and utilized in the linearity testing. The analytical method was performed as previously described. The peak areas obtained from each run were plotted against the concentration range of each standard. The linear fitted equation (Y = aX + b) and correlation coefficient (R^2) were summarized in Table 4-18. The results demonstrated that all compounds displayed linear response correlated to concentrative range (Figure 4-42).

Since the amount of the released compounds from the release study were fallen into the selected concentration range, hence, the range of standard solution was suitable for release study.

Table 4-18 Linear ranges and correlation coefficients of the calibration curves

Standards	**Y = aX + b linear model**[a]	**R^2**	**Concentration (μg/ml)**
5-ASA	Y = 26.42X - 23.10	0.9993	2-10 μg/ml
PAH (2)	Y = 8.481X + 21.91	0.9999	2-10 μg/ml
PABA (1)	Y = 15.24 X - 2.624	0.9994	0.2-1.0 μg/ml
SA	Y = 27.96 X - 0.046	0.9992	2-10 μg/ml
PAH-SA (4)	Y = 17.89X + 4.754	0.9994	2-10 μg/ml
PABA-SA (3)	Y = 25.27 X - 31.92	0.9992	2-10 μg/ml

[a]Y = peak area, X = concentration (μg/ml)

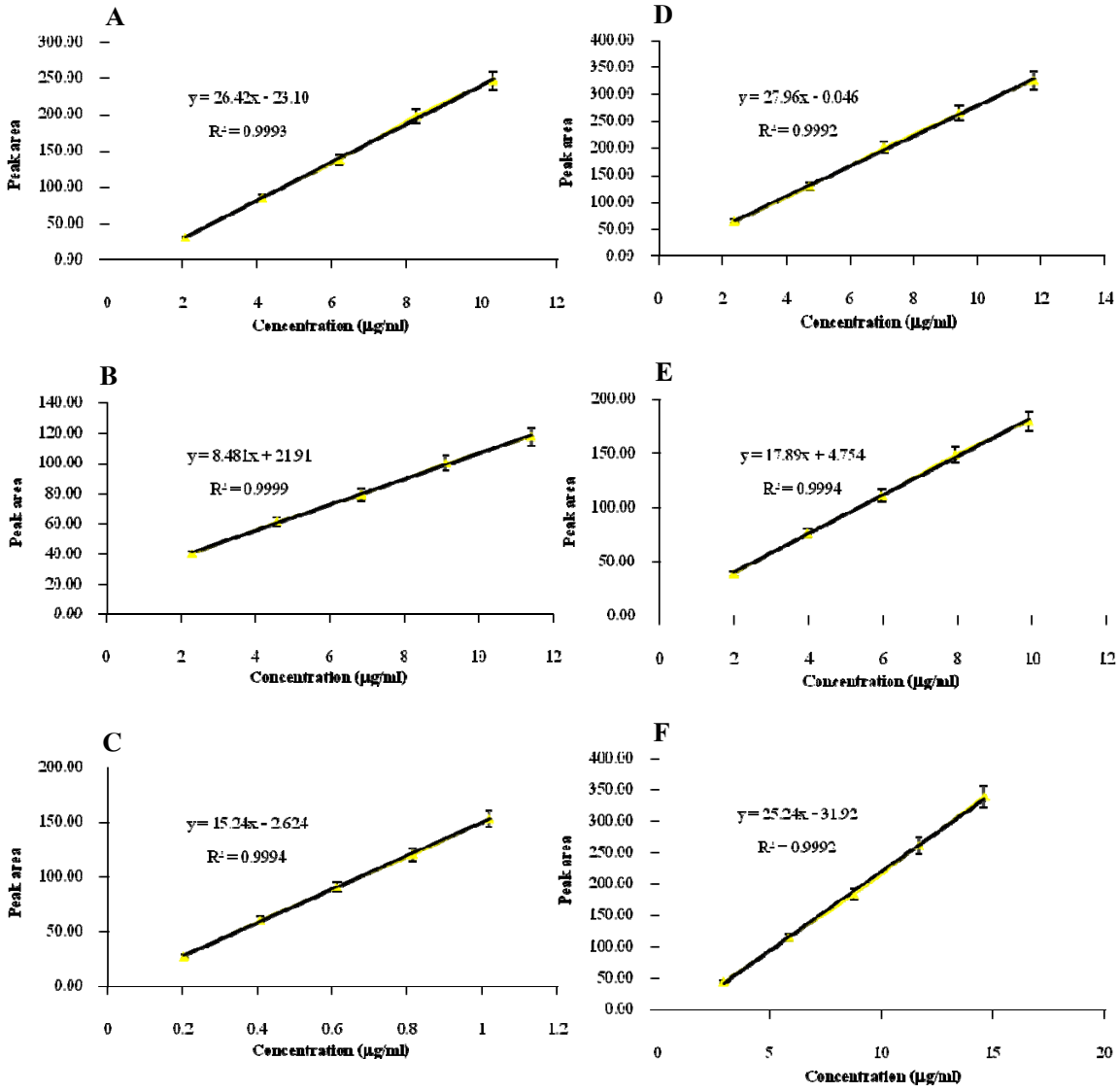


Figure 4-42 Calibration curves of standard solutions; (**A**) 5-ASA, (**B**) PAH, (**C**) PABA, (**D**) SA, (**E**) PAH-SA conjugate and (**F**) PABA-SA conjugate (mean ± SD; n=5)

4.3.5 Precision

The mean percentage recovery (mean ± SD) of the 6 standards in mobile phase were calculated and reported in Table 4-19 to 4-24.

Table 4-19 Recovery (%) of 5-ASA in working standard solutions (QC samples)

QC sample (μg/ml)	Recovery (%) on each QC sample					Recovery (%) mean ± SD
	1	2	3	4	5	
2	100.20	97.87	98.76	101.22	99.97	99.64 ± 1.31
6	100.35	99.95	98.94	100.56	101.03	100.17 ± 1.79
10	96.07	100.32	100.05	99.96	99.56	99.19 ± 1.76
Average	98.87	99.38	99.25	100.58	100.18	99.65 ± 0.70

Table 4-20 Recovery (%) of PAH (2) in working standard solutions (QC samples)

QC sample (μg/ml)	Recovery (%) on each QC sample					Recovery (%) mean ± SD
	1	2	3	4	5	
2	98.76	99.35	99.88	100.43	99.97	99.67 ± 0.64
6	100.02	99.74	101.05	98.77	98.25	99.56 ± 1.09
10	100.24	98.88	99.95	102.68	100.80	100.51 ± 1.40
Average	99.67	99.32	100.29	100.63	99.67	99.92 ± 0.53

Table 4-21 Recovery (%) of PABA (1) in working standard solutions (QC samples)

QC sample (μg/ml)	Recovery (%) on each QC sample					Recovery (%) mean ± SD
	1	2	3	4	5	
0.2	102.11	99.86	99.95	98.67	101.66	100.45 ± 1.41
0.6	100.02	103.55	102.77	99.46	101.13	101.38 ± 1.75
1.0	98.77	98.93	98.78	100.76	99.93	99.43 ± 0.88
Average	100.30	100.78	100.50	99.63	100.91	100.42 ± 0.50

Table 4-22 Recovery (%) of SA (**3**) in working standard solutions (QC samples)

QC sample (μg/ml)	Recovery (%) on each QC sample					Recovery (%) mean ± SD
	1	2	3	4	5	
2	102.55	100.07	100.17	102.87	100.36	101.20 ± 1.28
6	99.99	101.44	100.36	102.55	99.42	100.75 ± 1.24
10	98.82	99.41	99.65	101.34	99.76	99.79 ± 0.94
Average	100.45	100.30	100.06	102.25	99.85	100.58 ± 0.96

Table 4-23 Recovery (%) of PAH-SA conjugate (**5**) in working standard solutions (QC samples)

QC sample (μg/ml)	Recovery (%) on each QC sample					Recovery (%) mean ± SD
	1	2	3	4	5	
2	102.55	100.42	98.25	102.87	100.36	100.89 ± 1.88
6	99.78	101.26	100.36	102.55	99.42	100.67 ± 1.26
10	100.33	99.99	99.65	101.34	99.76	100.21 ± 0.68
Average	100.89	100.56	99.42	102.25	99.85	100.59 ± 1.09

Table 4-24 Recovery (%) of PABA-SA conjugate (**4**) in working standard solutions (QC samples)

QC sample (μg/ml)	Recovery (%) on each QC sample					Recovery (%) mean ± SD
	1	2	3	4	5	
2	98.88	98.46	99.42	98.73	101.24	99.45 ± 1.15
6	102.25	100.51	99.36	100.43	98.77	100.26 ± 1.33
10	98.57	98.74	101.45	102.33	99.56	100.13 ± 0.68
Average	99.90	99.24	100.24	100.50	99.86	99.95 ± 1.38

The results demonstrated that the developed method gave an excellent precision values. It is therefore, the method was suitable to determine the amount of each compound from the chemical stability and release studies.

4.3.6 Repeatability

The repeatability of the method was determined by between-run analysis and within-run analysis. The repeatability of the method was expressed as % relative standard deviation (% RSD) of a series of measurements. The experimental values obtained in the determination of the 6 compounds in standard solutions were presented in Table 4-25 and 4-26. The RSD values demonstrated in Table 4-25 and 4-26 were lower than 2% (http://www.ich.org/cache/compo/276-254-1.html), which showed high repeatability for the method.

Table 4-25 RSD (%) of between-run analysis and within-run analysis of 5-ASA, PAH (**2**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**)

Conc. (μg/ml)	RSD (%)									
	5-ASA		PAH		SA		PAH-SA		PABA-SA	
	Between-run	Within-run	Between-run	Within-run	Between-run	Within-run	Between-run	Within-run	Between-run	Within-run
2	0.41	0.43	0.35	0.18	0.51	0.92	1.66	1.32	1.77	1.13
4	0.87	0.26	0.44	0.92	1.07	1.34	1.87	1.95	1.77	1.99
6	0.40	0.19	0.21	0.16	0.43	0.27	1.06	1.45	1.04	1.62
8	1.35	0.98	1.16	0.99	0.56	0.88	1.45	1.28	1.86	1.59
10	0.29	0.30	0.19	0.85	0.30	0.46	1.96	1.64	1.09	1.81

The HPLC method validation results showed that determination of 5-ASA, PAH (**2**), PABA (**1**), SA (**3**), PAH-SA conjugate (**5**) and PABA-SA conjugate (**4**) in QC samples could be performed by validated HPLC method described above with accepted accuracy and precision.

Table 4-26 RSD (%) of between-run analysis and within-run analysis of PABA (**1**)

RSD (%)	Concentration (μg/ml)				
	0.2	**0.4**	**0.6**	**0.8**	**1.0**
Between-run	1.06	0.48	0.71	1.89	1.71
Within-run	0.62	1.85	0.85	0.78	1.99

After attempted to synthesize PEG-PABA-SA conjugates, it was shown two ester cnjugates consisted of $mPEG_{5000}$-(PABA-SA) ester conjugate (**8**) and PEG_{4000}-$(PABA-SA)_2$ ester conjugate (**10**), and two amide conjugates consisted of PEG_{5106}-$(PABA-SA)_2$ amide conjugate (**39**) and PEG_{3677}-$(PABA-SA)_2$ amide conjugate (**40**) were synthesized successfully by using synthetic method No. 8. The chemical reaction firstly started from ester or amide bond synthesis of *p*-nitro benzoylchloride (**26**) and each PEG.

The aromatic nitro functional group was then reduced to amino group by hydrogenation reaction. Finally, salicylic acid (SA) was added in diazotization reaction to obtain the reddish-orange amorphous solid.

All azo conjugates were obtained more than 96% yield. The % conjugation of PABA-SA in each conjugate calculated from ^{1}H-NMR spectral data were demonstrated in Figure 4-43. However, $mPEG_{5000}$ conjugate had % conjugation of PABA-SA only 37.39% due to degradation while purification by dialysis. The ester bond of **8** degraded due to hydrolysis.

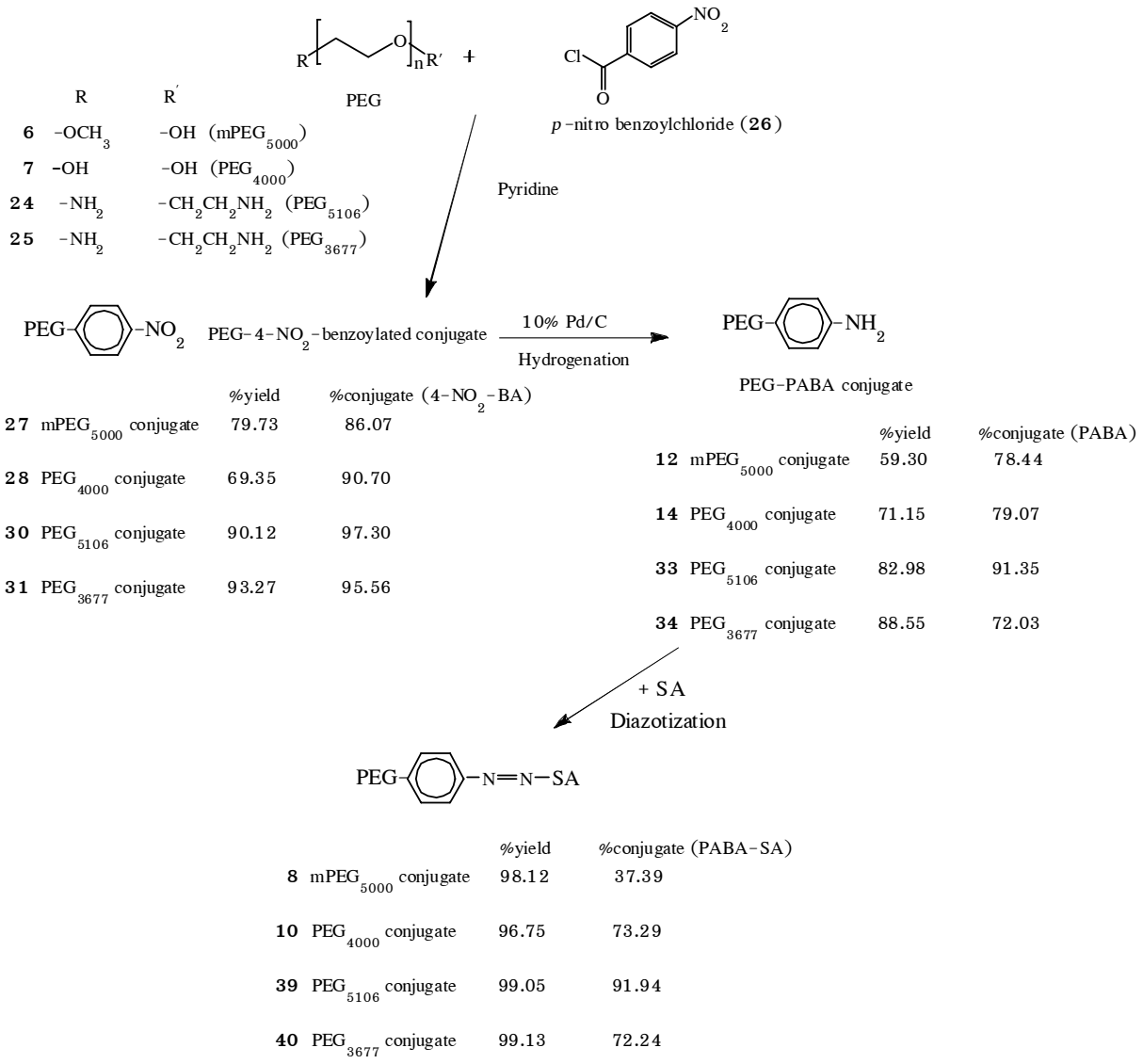


Figure 4-43 Synthetic scheme of PEG-PABA-SA conjugates (Method 8)

4.4 The determination of % free SA and other related compounds in the conjugate products

PEG-PABA-SA conjugates (**8, 10, 39, 40**) were tested for any free compounds which may remained from the synthesized procedures using the developed HPLC method. The results demonstrated that none of any free compound remained in the

conjugated products after purification by dialysis and precipitation (Table 4-27). The % purity of each conjugates was 100% for all tested compound.

Table 4-27 % Purity of PEG-(PABA-SA) conjugates

PEG-PABA-SA conjugate	%PABA	%SA	%PABA-SA	% Purity
$mPEG_{5000}$-PABA-SA ester (8)	0.00	0.00	0.00	**100.00**
PEG_{4000}-$(PABA-SA)_2$ ester (10)	0.00	0.00	0.00	**100.00**
PEG_{5106}-$(PABA-SA)_2$ amide (39)	0.00	0.00	0.00	**100.00**
PEG_{3677}-$(PABA-SA)_2$ amide (40)	0.00	0.00	0.00	**100.00**

4.5 Solubility test

The aqueous solubility property of products was studied. The quantitative results in unit of g/l at 25 °C were calculated. The data (Table 4-28) showed that PEG could enhance aqueous solubility of the active drug, 5-ASA, by conjugation system. The water solubility of 5-ASA was improved over 200 times by PEG conjugates. These results were similar to that previously reported by utilizing PEG conjugate to improve water solubility of poor water soluble compounds such as theophylline (Zacchigna *et al*., 2003), insulin and small molecule protein (Zalipsky *et al*., 1983), pyrene (Yang *et al*., 2004), paclitaxel (Greenwald *et al*., 1994), acyclovir (Zacchigna *et al*., 2002) etc.

Table 4-28 Aqueous solubility of PEG-PABA-SA conjugates

PEG-PABA-SA conjugate	Aqueous solubility (g/l), 25 °C
$mPEG_{5000}$-PABA-SA ester (8)	233.00
PEG_{4000}-$(PABA-SA)_2$ ester (10)	176.67
PEG_{5106}-$(PABA-SA)_2$ amide (39)	215.37
PEG_{3677}-$(PABA-SA)_2$ amide (40)	171.83
5-ASA	0.84

The aqueous solubility values from Table 4-28 demonstrated that the solubility value increased when 5-ASA conjugated with longer chain polymer. This is due to the water solubility property of PEG which containing a number of oxygen in its chemical structure. Each monomer (ethylene oxide unit) can bind three molecules of water resulting in segmental flexibility in aqueous solution (Hinds and Kim, 2002; Zalipsky, 1995). Therefore, higher molecular weight-PEG could increase the water solubility of the conjugate. However, the %conjugation of the drug also affected the solubility property of this kind of polymer similar to the information reported by Zou and co-worker (2005). The solubility of 5-ASA conjugated with $mPEG_{5000}$ was more than the drug which conjugated with PEG_{5106} diamino. As described previously that the % drug conjugation of $mPEG_{5000}$ was only 37%, whereas PEG_{5106} diamino containing 91% conjugate (Table 4-15). Therefore, the increasing of % drug conjugate decreased the aqueous solubility of the polymeric conjugate as a result.

In addition, Zou and co-worker (2005) reported that 5-ASA conjugated with the hydrophobic polymers such as polyamide polymer, did not release the drug in any part of the gastro-intestinal contents of rats after 48-hour incubation time. It was confirmed by the results reported by Schacht and co-workers (1996), and Chourasia and Jain (2003) that after tested the polyamides containing azo bond in the backbone in a reductive buffer or in the reactor medium, reduction stopped at the hydrazine stage (Figure 2-5C) whereas for the hydrophilic polymer, reduction with formation of amine occurred and released 5-ASA as a result. It was also demonstrated that 5-ASA conjugated with higher aqueous solubility polymer could release the drug in higher amount (Zou *et al.*, 2005). Therefore, the solubility property of the polymeric conjugate may be one of the factors affect the release of 5-ASA.

However, as described above that each ethylene oxide unit of PEG can bind 3 molecules of water resulting in segmental flexibility in aqueous solution (Hinds and Kim, 2002; Zalipsky, 1995). The higher number of the monomer of PEG, the smaller amount of 5-ASA released from the PEG conjugates. This may be due to the flexibility of chemical structure in aqueous solution which can prevent the enzyme to approach the azo bond (Zalipsky, 1995).

4.6 Stability test

Prior to test the release of the obtained conjugated in gastrointestinal content, the conjugates (**8**, **10**, **38**, **39**) (Figure 4-43) were subjected to test for their stability in gastrointestinal simulated conditions. The conditions used were buffer solution having pH 1.2 (gastric), 6.8 (intestinal) and 7.5 (colon). The incubation times were 6, 12 and 24 hours at 37 °C, respectively. The results (Table 4-29) illustrated that the ester and azo bonds of conjugates **8** and **10**, and amide and azo bonds of conjugate **39** and **40** were stable in all tested conditions.

The results revealed that PEG could prevent the degradation of the ester and amide bonds from hydrolysis in absence of biological enzymes (Zacchigna *et al*., 2002; Wiwattanapatapee *et al*., 2003; Zou *et al*., 2005) due to its property in solution. PEG is a highly hydrated polymer, where each monomer (ethylene oxide unit) can bind three molecules of water, as described before. The former reported results show the polymer exhibiting a large degree of segmental flexibility in aqueous solution (Hinds and Kim, 2002). In addition, PEG typically transfers many of the polymer's favorable characteristics to the resultant conjugates. The well known propensity of PEG is exclusion of particulates or molecules from its surrounding. This is one of the principal reasons for the use of this polymer for preparation of various conjugates. These properties of PEG have been explained by its chains' high mobility associated with conformational flexibility and water-binding ability as previously described (Zalipsky, 1995). Moreover, the azo bond is not reduced in the absence of gastrointestinal contents.

Table 4-29 % 5-ASA, PABA and PABA-SA conjugate released from PEG-(PABA-SA) conjugates after incubation in citrate buffer pH 1.2, phosphate buffer pH 6.8 and 7.5 for 6, 12 and 24 hours at 37 °C, respectively

Conjugate	Buffer pH 1.2*			Buffer pH 6.8**			Buffer pH 7.5***		
	% 5-ASA	%PABA	%PABA-SA	% 5-ASA	%PABA	%PABA-SA	% 5-ASA	%PABA	%PABA-SA
8	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0	0

* 6 hours, ** 12 hours, *** 24 hours

4.7 Drug release study in gastrointestinal contents

The obtained conjugates (**8**, **10**, **39**, **40**) (Figure 4-43) were then utilized for their release study in the presence of gastrointestinal contents of rats. Sulfasalazine was used as the positive reference under the same conditions. In the release study of sulfasalazine, the amount of 5-ASA in each condition at different incubation times was determined and calculated as % release by mole compare to the starting amount of sulfasalazine and the results are demonstrated in Figure 4-44.

After incubation of sulfasalazine in gastric content, the drug, 5-ASA, could not be detected in all samples during incubation period. In intestinal content, 5-ASA was found slowly released in the first 4 hours and finally reached 81.48 ± 6.35% at about 6 hours after incubation. However, the rate of drug release in colonic content was faster than in the intestinal content. 5-ASA could be released 94.17 ± 27.60% within 2 hours after incubation in colonic content.

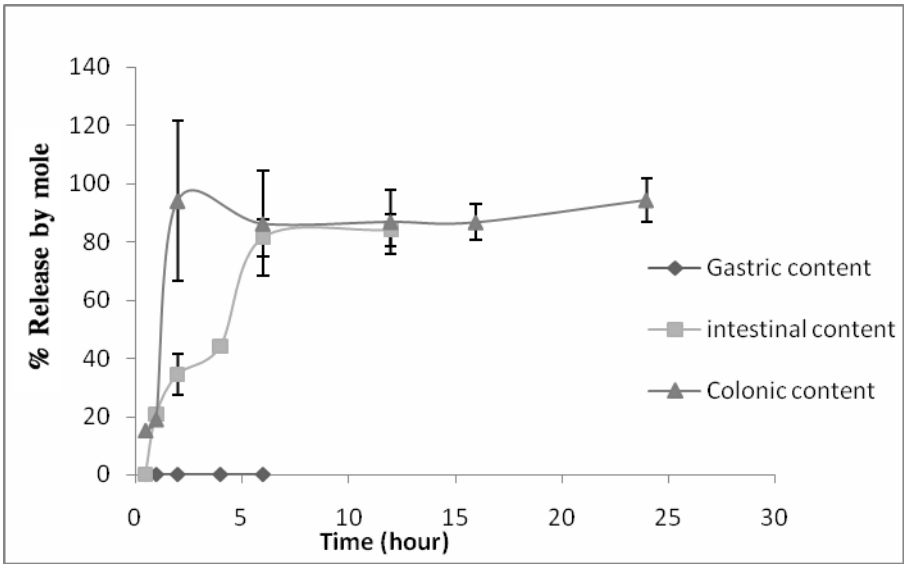


Figure 4-44 Release profiles of 5-ASA from sulfasalazine after incubation in gastrointestinal contents treated with 1% peptone in citrate buffer pH 1.2 (simulated gastric content), phosphate buffer pH 6.8 (simulated intestinal content) and 7.5 (simulated colonic content) for 6, 12 and 24 hours at 37 °C, respectively (mean ± SD; n=3)

The PEG_{5000}-PABA-SA conjugate (**8**) was performed for its release study by incubation in the same conditions as sulfasalazine, and its release profiles are displayed in Figure 4-45. 5-ASA was not detected during 6 hours of incubation period when incubate **8** in pH 1.2 simulated gastric content (Figure 4-45A). However, 5-ASA, PABA (**1**) and PABA-SA conjugate (**4**) were detected after incubation of **8** in both intestinal (Figure 4-45B) and colonic (Figure 4-45C) contents. PABA-SA conjugate was recovered up to 10.76 ± 0.43% in intestinal content after incubated for 2 hours and 9.96 ± 0.82% in colonic content after incubated for 4 hours. This indicated that the ester bond between PEG and PABA of **8** could be cleaved by the enzyme in small intestine almost as well as in colon. This compound was further disappeared after 10 hours of the incubation period, since its azo bond between PABA and SA might be further degraded similar to that found in sulfasalazine (Chungi *et al*., 1989) and previous reported (Wiwattanapatapee *et al*., 2003).

Moreover, the azo bond was slowly reduced to give 5-ASA up to 26.05 ± 0.67% after incubated in intestinal content for 12 hours and up to 30.38 ± 1.78% after incubated in colonic content for 24 hours. Free PABA was found slowly presence in intestinal content up to 26.57 ± 0.75% after 12-hour incubation, and up to 10.30 ± 0.78% after 24-hour incubation in colonic content. The free PABA (**1**) might be resulted from the degradation of azo bond both from released PABA-SA, and from degradation of the ester bond between PEG and PABA in PEG-PABA conjugate which remained after reduction of azo bond.

The PEG_{4000}-$(PABA-SA)_2$ ester conjugates (**10**) was also performed for its release profiles by incubation in the same conditions as PEG_{5000}-PABA-SA conjugate (**8**). Its release profiles are demonstrated in Figure 4-46. Similar to the release profiles of **8** (Figure 4-45A), none of expected molecules (5-ASA, **1** and **4**) that could be released from the polymer conjugates were detected in gastric content during first 6 hours of incubation period (Figure 4-46A). Moreover, there was no free PABA-SA released after 12-hour incubation in intestinal content (Figure 4-46B). However, the ester bond between PEG and PABA was reduced to give PABA-SA in very small amount, up to 6.17 ± 0.41% after the first hour incubation time in colonic content. Free PABA was then

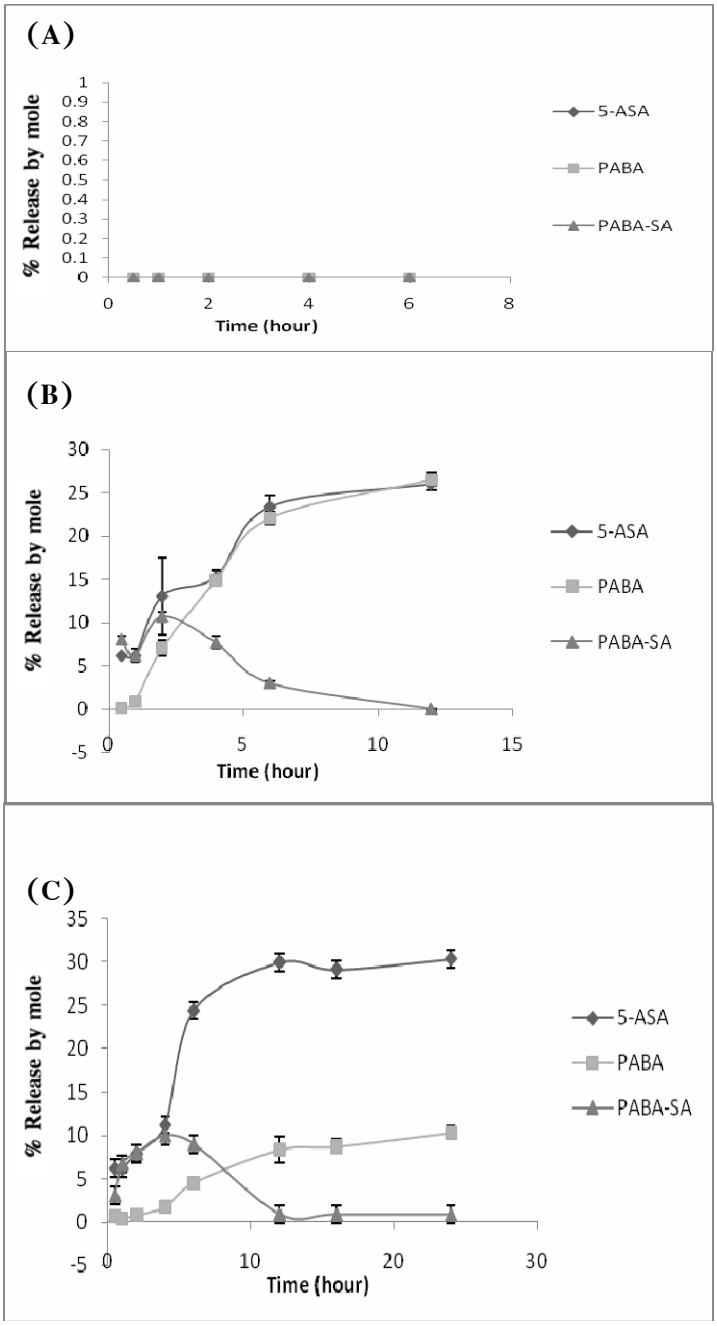


Figure 4-45 Release profiles of 5-ASA, PABA and PABA-SA conjugate from $mPEG_{5000}$-PABA-SA ester conjugate (**8**) after incubation in gastrointestinal contents treated with 1% peptone in **(A)** citrate buffer pH 1.2 (simulated gastric content), **(B)** phosphate buffer pH 6.8 (simulated intestinal content) and **(C)** phosphate buffer pH 7.5 (simulated colonic content) for 6, 12 and 24 hours at 37 °C, respectively (mean ± SD; n=3)

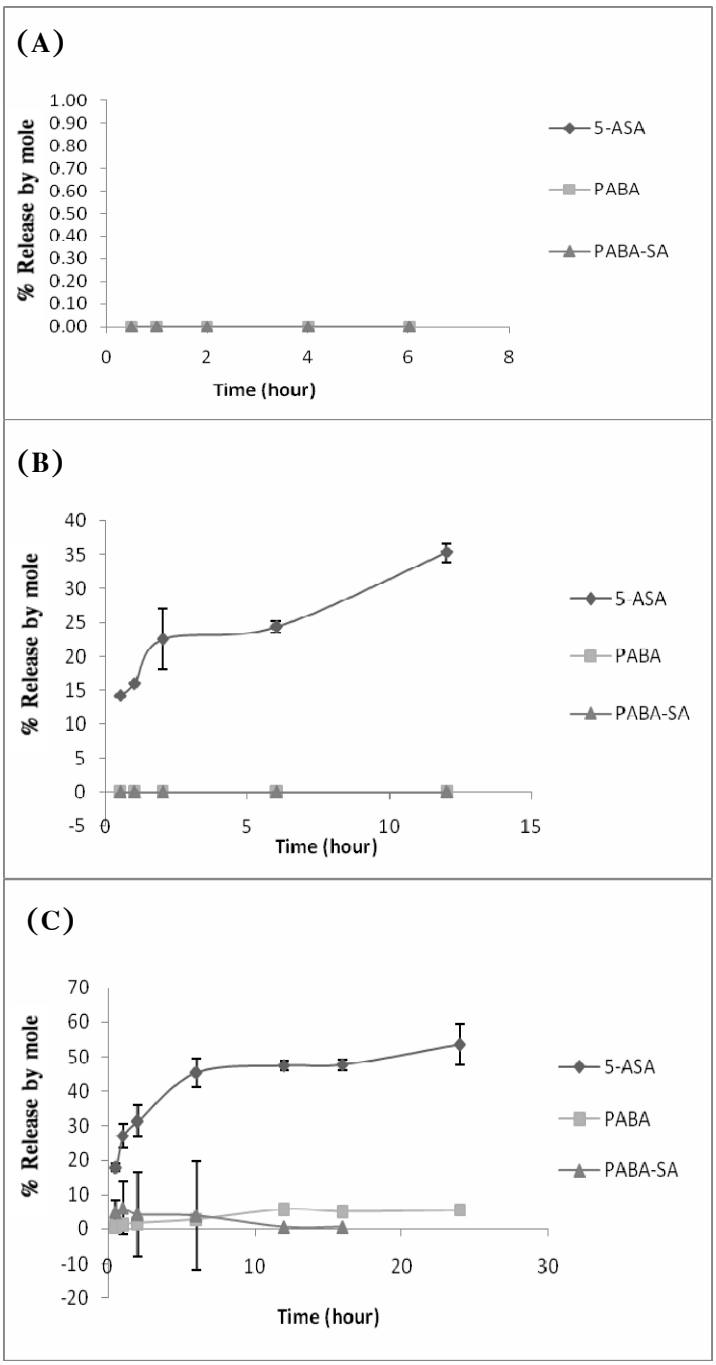


Figure 4-46 Release profiles of 5-ASA, PABA and PABA-SA conjugate from PEG_{4000}-$(PABA-SA)_2$ ester conjugate (**10**) after incubation in gastrointestinal contents treated with 1% peptone in **(A)** citrate buffer pH 1.2 (simulated gastric content), **(B)** phosphate buffer pH 6.8 (simulated intestinal content) and **(C)** phosphate buffer pH 7.5 (simulated colonic content) for 6, 12 and 24 hours at 37 ^{o}C, respectively (mean ± SD; n=3)

detected up to 5.78 ± 0.58% only in colonic content after first 12 hours incubation time (Figure 4-46C). This could be concluded that in colonic content, free PABA was released after the degradation of azo bond from released PABA-SA. Moreover, the reduction of azo bond between PEG-PABA and SA give 5-ASA up to 35.30 ± 1.56% after 12-hour incubation in intestinal contents, and up to 53.62 ± 5.92% after 24-hour incubation in colonic content. The results showed that the product, **10**, was more stable in esterase enzyme than **8**. However, it was then confirmed that ester bond was not stable in rat colonic content as previously reported by Kamada and co-workers (2002) due to ester hydrolysis.

PEG_{5106}-$(PABA-SA)_2$ conjugate (**39**) was then performed for its release profile by incubation in the same conditions used in release study of sufasalazine and both ester products (**8** and **10**). Its release profiles are demonstrated in Figure 4-47. The results demonstated that none of expected molecules (5-ASA, **1**, **4**) that could be released from the polymer conjugates (**39**) were detected in gastric content during first 6 hours of incubation in intestinal content (Figure 4-47B), and also after 24-hour incubation in colonic content (Figure 4-47C). Therefore, amide bond between PEG and PABA was stable in incubation period (Figure 4-47A). Since there was no free PABA-SA released after 12-hour quite stable to degradation process by amidase enzyme in gastrointestinal contents. Moreover, the reduction of azo bond between PEG-PABA and SA give 5-ASA up to 34.07 ± 0.42% after 12-hour incubation in intestinal contents, and up to 36.02 ± 12.37% after 24-hour incubation in colonic content.

Since it was demonstrated that the azo bond between PABA and SA of PEG_{5106}-$(PABA-SA)_2$ conjugate (**39**) could be cleaved and released 5-ASA both in intestinal and colonic contents, therefore, the rat intestinal content might have azoreductase enzyme. The results from this study were similar to previous report (Schacht *et al.*, 1996). Molly and co-workers developed the 5-step multi-chamber reactor (SHIME, Figure 2-5A) as a simulation of the human intestinal microbial system in 1993. This system was used in determination of 5-ASA released from the azo products by Schacht and co-workers in 1996. The results showed that 5-ASA could be released in the ileum, caecum and ascending colon parts whereas there was no free 5-ASA detected in duodenum and jejunum part, which meant that the azo bond could degrade in some part of the small intestine

(Schacht *et al*., 1996). However, the release profiles of 5-ASA from the product (**39**) in intestinal content was almost as same as the release profiles in colonic content.

The release profiles of PEG_{3677}-$(PABA\text{-}SA)_2$ amide conjugate (**40**) was demonstrated in Figure 4-48. It was incubated in the same conditions used in release study of sufasalazine and the PEG conjugates described above (**8**, **10** and **39**). The results were similar to the results of **39**. The result demonstrated that none of expected molecule (5-ASA) was detected in gastric content during 6 hours of incubation period (Figure 4-48A). PABA-SA conjugate was not released during 12-hour incubation in intestinal content (Figure 4-48B), and up to 24-hour incubation in colonic content (Figure 4-48C). This confirmed that amide bond between PEG and PABA was stable to the degradation process by enzyme in gastrointestinal contents. Moreover, the reduction of azo bond between PEG-PABA and SA give 5-ASA up to 79.69 ± 4.71% after 12-hour incubation in intestinal contents, and up to 96.17 ± 11.54% after 24-hour incubation in colonic content. The results showed that the azo bond between PABA and SA of product (**40**) could be cleaved both in intestinal and colonic contents. And the release profiles of 5-ASA from the product (**40**) in intestinal content was similar to the release profiles in colonic content. The release profiles of **40** was similar to the results from the release profiles of **39** (Figure 4-47). All percentage of 5-ASA and other related compounds released from the contents were demonstrated in Table 4-30.

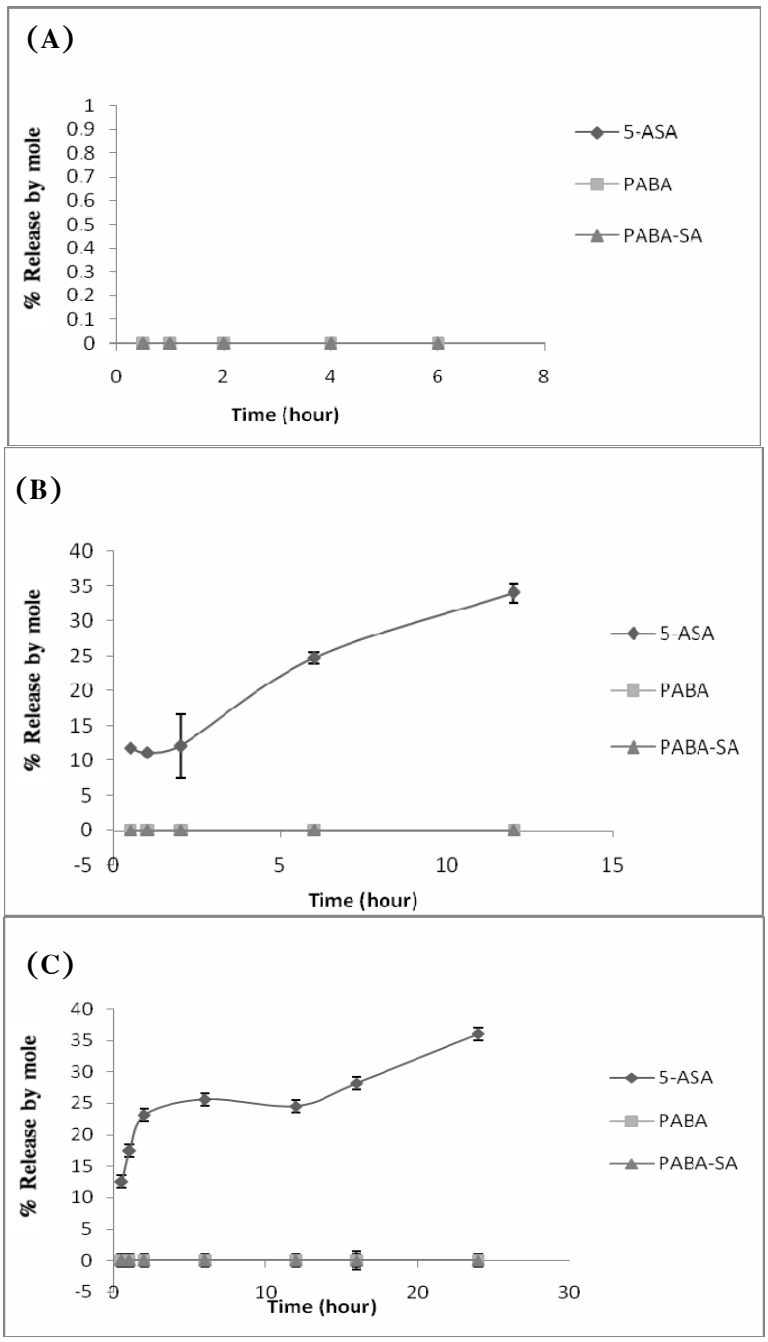


Figure 4-47 Release profiles of 5-ASA, PABA and PABA-SA conjugate from PEG_{5106}-$PABA\text{-}SA)_2$ amide conjugate (**39**) after incubation in gastrointestinal contents treated with 1% peptone in **(A)** citrate buffer pH 1.2 (simulated gastric content), **(B)** phosphate buffer pH 6.8 (simulated intestinal content) and **(C)** phosphate buffer pH 7.5 (simulated colonic content) for 6, 12 and 24 hours at 37 °C, respectively (mean ± SD; n=3)

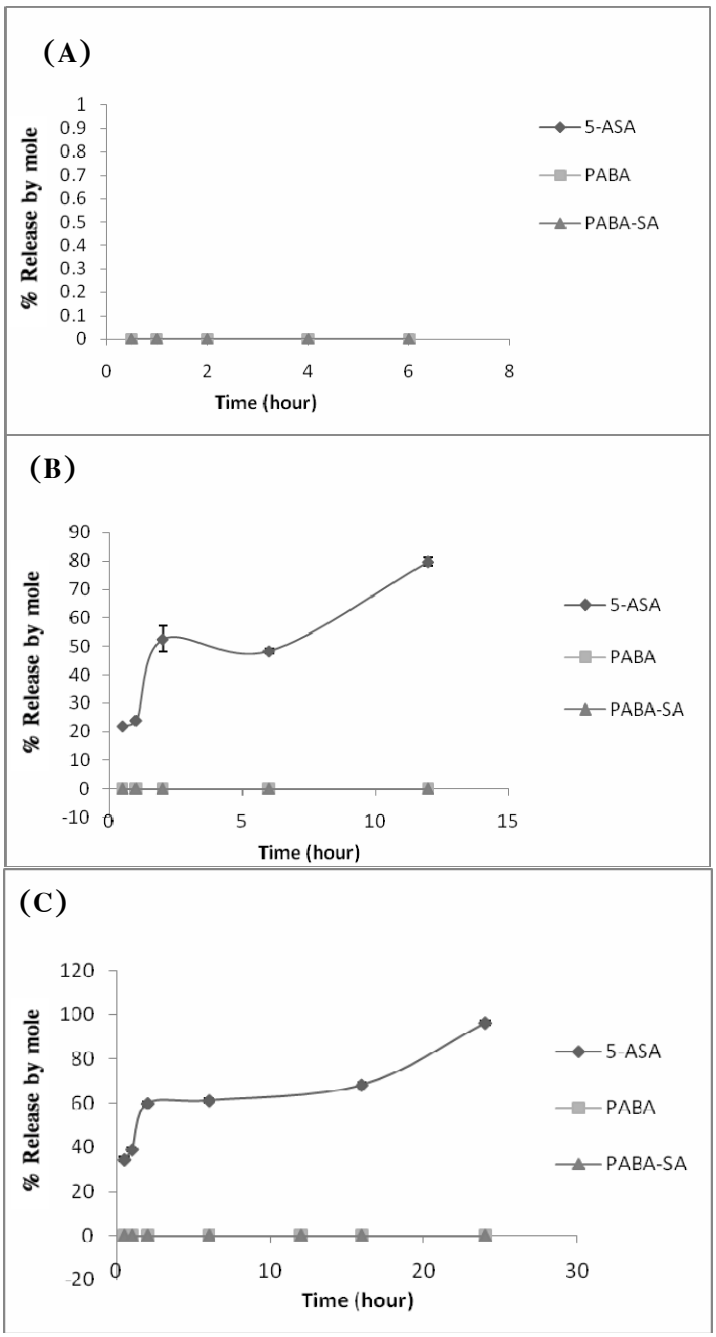


Figure 4-48 Release profiles of 5-ASA, PABA and PABA-SA conjugate from PEG_{3677}-$(PABA-SA)_2$ amide conjugates (**40**) after incubation in gastrointestinal contents treated with 1% peptone in **(A)** citrate buffer pH 1.2 (simulated gastric content), **(B)** phosphate buffer pH 6.8 (simulated intestinal content) and **(C)** phosphate buffer pH 7.5 (simulated colonic content) for 6, 12 and 24 hours at 37 °C, respectively (mean ± SD; n=3)

Table 4-30 % 5-ASA, PABA and PABA-SA conjugate released by mole from PEG-(PABA-SA) conjugates after incubation in gastrointestinal contents treated with 1% peptone in citrate buffer pH 1.2 (simulated gastric content), phosphate buffer pH 6.8 (simulated intestinal content) and phosphate buffer pH 7.5 (simulated colonic content) for 6, 12 and 24 hours, respectively, at 37 °C (mean ± SD; n=3)

Conjugate	Maximum mean of % release by mole ± SD (n=3)								
	Gastric content*			Intestinal content**			Colonic content***		
	%5-ASA	%PABA	%PABA-SA	%5-ASA	%PABA	%PABA-SA	%5-ASA	%PABA	%PABA-SA
8	0	0	0	26.05 ± 0.67	26.57 ± 0.75	10.76 ± 0.43	30.38 ± 1.78	10.30 ± 0.78	9.96 ± 0.82
10	0	0	0	35.30 ± 1.56	0	0	53.62 ± 5.92	5.24 ± 0.55	6.17 ± 0.41
39	0	0	0	34.07 ± 0.42	0	0	36.02 ±12.37	0	0
40	0	0	0	79.69 ± 4.71	0	0	96.17 ±11.54	0	0

* 6 hours, ** 12 hours, *** 24 hours

From the results reported in this study, 5-ASA could be released both in the intestinal and colonic contents (Figure 4-44 to 4-48). However, after incubation of sulfasalazine, **8**, **10**, **39** and **40** in 6-hour incubation in gastric content, no free 5-ASA was detected. This means that the ester and amide bond between PEG and PABA of all products (**8**, **10**, **39** and **40**) were stable in gastric content. Whereas the ester bond of product **8** and **10** degraded in the intestinal and colonic contents, in contrast, the amide bond of other two products (**39** and **40**) was more stable.

The release profiles of all samples in intestinal and colonic contents were gathered in Figure 4-49. It demonstrated that all samples gave 5-ASA released both in intestinal and colonic contents.

In intestinal content, the PEGylated drug with molecular weight of the polymer $\geq$ 4000 Da could release 5-ASA in smaller amount (not more than 36%) than the PEGylated drug with molecular weight of the polymer < 4000 Da (>79% of active drug

was released) after incubation for 12 hours. It demonstrated that higher molecular weight of PEG could also protect the drug 5-ASA from the reductase enzyme due to the longer chain of PEG. 5-ASA could be released rapidly from sulfasalazine after incubation in intestinal content within 1 hour. The drug recovered after releasing from sulfasalazine was more than the drug released from the PEGylated products.

The results from this experiment could be described by the information reported by Peppercorn (1984) and Klotz (1985). From clinical studies of sulfasalazine, it was found in patients with a permanent ileostomy that there was a less effective cleavage of sulfasalazine in such patients. Most of the unchanged sulfasalazine (70%) could be recovered in the ileostomy effluent (Klotz, 1985). Moreover, some of bacteria which produce azoreductase enzyme, such as *Bacteroides* and *Clostridium*, also locate in the distal part of small intestine, ileum (Vandamme *et al*., 2002; http://textbookofbacteriology.net/normalflora.html.htm/). Therefore, the azo bond could be reduced in this part. It was confirmed by Wiwattanapatapee and co-workers (2003) that the azoreductase enzyme could cleave the azo bond of the PAMAM conjugates and release 5-ASA in the 10% content of simulated small intestine up to 7.2 ± 1.8% after 12-hour incubation at 37 $^{\circ}$C (Wiwattanapatapee, *et al*., 2003). In addition, it was also reported in 2001 that azo bond between 9-aminocamptothecin and HPMA copolymer was cleaved after 24-hour incubation at 37 $^{\circ}$C in both simulated small intestine and large intestine up to 60% and 25%, respectively (Sakuma *et al*., 2001).

The information above indicates that a number of the azo conjugate may be degraded in the small intestine before it reaches the colon.

The release profiles of 5-ASA in intestinal content of sulfasalazine (Figure 4-49A) revealed that more than 85% of 5-ASA was released after 6-hour incubation period. Since it is well known that 5-ASA could be absorbed very well in small intestine (Klotz, 1985), then, at least 80% of 5-ASA released from sulfasalazine would be absorbed into blood circulation. Therefore, the active drug, 5-ASA, which should affect locally in treatment of inflammatory disorders would be less effective than the product that could deliver most of 5-ASA to colonic part. Moreover, 5-ASA released rapidly in small intestine might be a cause of irritation which is one of the side effects that the patients

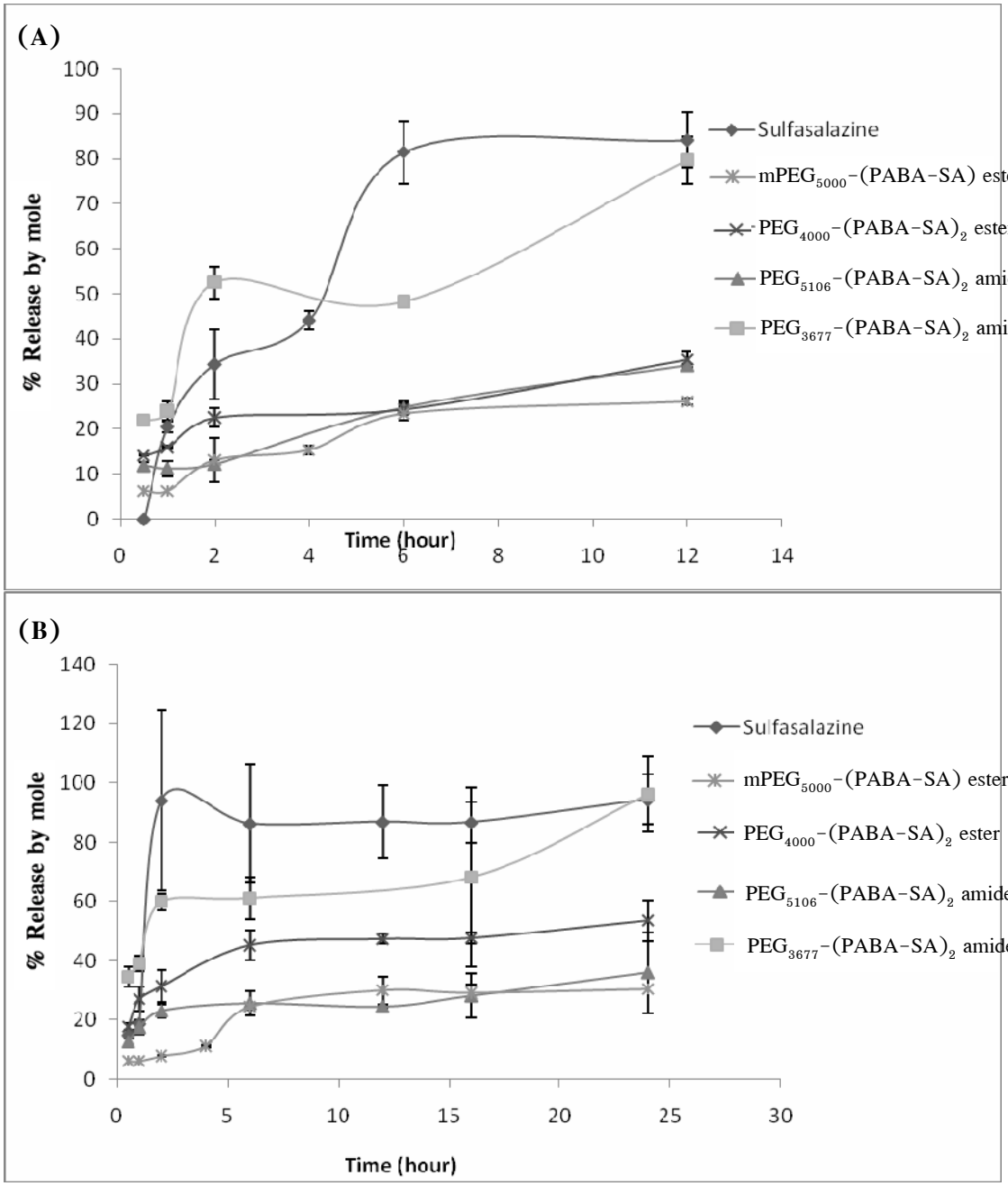


Figure 4-49 Release profiles of 5-ASA from PEG-PABA-SA conjugates (**8, 10, 39, 40**) and sulfasalazine after incubation in **(A)** intestinal and **(B)** colonic content treated with 1% peptone in phosphate buffer pH 6.8 (simulated intestinal content) and phosphate buffer pH 7.5 (simulated colonic content) for 12 and 24 hours at 37 °C, respectively (mean ± SD; n=3)

could not continue using the drug. From the experiment, it was shown that conjugation of the 5-ASA to the water soluble polymer, PEG, could prevent some of the product from degradation process by azoreductase enzyme in the small intestine. Hence, the drug could be delivered more specific to colon and reduce the irritation side effect.

In colonic content (Figure 4-49B), 5-ASA was released in higher amount and faster than in the intestinal contents. However, the PEGylated drug with molecular weight of the polymer $\geq$ 4000 Da could release 5-ASA in smaller amount (less than 50%) than the PEGylated drug with molecular weight of the polymer < 4000 Da (which could release the drug more than 60%) after incubation for 12 hours. PEG with molecular weight of 3677 Da could release 5-ASA about 60%, whereas sulfasalazine gave 5-ASA about 100% after incubation at the first two-hour.

Prolonged release of 5-ASA conjugated to the polymer (Figure 4-49) could reduce the frequency of drug administration and side effect of 5-ASA, mainly, irritation in the gastrointestinal tract. This behavior of PEG conjugates related to the aqueous solubility property and % drug conjugation. As mentioned before in the solubility test, 5-ASA conjugated with the higher molecular weight-PEG could increase water solubility of the conjugates (Zou *et al*., 2005) and the drug could be released after reduction by azo-reductase enzyme. Moreover, PEG with higher molecular weight could release 5-ASA in smaller amount due to protection of the flexible chemical structure of PEG from the enzyme (Zalipsky, 1995). However, PEG_{5106} diamino could release 5-ASA more than $mPEG_{5000}$ monohydroxyl despite the molecular size of the former one is longer. This could be described by the percentage of drug conjugation. $mPEG_{5000}$-(PABA-SA) ester conjugate (**8**) contained only 37% drug conjugate whereas 91% 0f 5-ASA conjugated with PEG_{5106} (Table 4-15). Thus, the free $mPEG_{5000}$ polymer which has flexible chemical structure could form hydrogen bonding with molecules of water and reduced the release rate in aqueous contents (Figure 4-49). As a result, PEG_{5106}-$(PABA-SA)_2$ amide conjugate could release the drug in higher percentage than the drug conjugated to $mPEG_{5000}$.

The release profiles of 5-ASA from the synthesized PEG conjugates indicate that the amide conjugates (**39**, **40**) are more suitable for intestinal and colonic

delivery than the ester conjugates (**8**, **10**) because there was no amide bond breaking in those contents whereas the ester bond was hydrolyzed immediately, in contrast. As it was demonstrated in Figure 4-49, both PEG_{5106}-$(PABA-SA)_2$ amide conjugate (**39**) and PEG_{3677}-$(PABA-SA)_2$ amide conjugate (**40**) could release 5-ASA in intestinal and colonic contents. However, the released amount of the drug from each conjugate was different. Conjugate No. **39** could release 5-ASA in intestinal content up to 20% within first 6-hour incubation period and released up to 30% after 24-hour incubation in colonic content. Due to the evidence of the inflammation area which is mainly in the distal part of colon, this conjugate may be suitable for treatment of ulcerative colitis because of its release profiles. Conjugate No. **40** could release 5-ASA more than 50% within the first 6-hour incubation in intestinal content. In addition, it could release up to 96% of 5-ASA after 24-hour incubation in colonic content. Therefore, conjugate of 5-ASA with poly(ethylene) glycol with molecular weight 3677 Da (**40**) may be suitable for treatment of Crohn's disease which usually shows the scattered evidence in both small and large intestine. Due to the release profile in colonic content, perhaps, conjugate No. **40** can also be used in treatment of ulcerative colitis similar to sulfasalazine (Klotz, 1985).

As mentioned before in section **2.3**, there were many reports used polymers as drug carriers. Brown and co-workers (1983) conjugated SA with polysulfanilamide resulting in salicylate salt of polymeric azo conjugate. They studied *in vitro* metabolism in cecal content of rats. The results indicated that the azo bond could be cleaved in the content. However, they did not study the release profile in gastric or intestinal contents. They also gave single oral dose to non-fasted rats and quantify for 5-ASA and its metabolites in biological samples (*in vivo*) compared to the drug release from sulfasalazine. The results demonstrated that the mean peak serum concentration of 5-ASA and metabolites (i.e. *N*-Acetyl-5-ASA) was significantly lower in the polymeric conjugate-treated group than in sulfasalazine-treated group, while the mean urinary excretion of 5-ASA and metabolites was significantly higher in the polymeric conjugate-treated rats ($8.0 \pm 0.81\%$, $p < 0.05$). Zhou and co-workers (1999) reported the intestinal metabolism and transport of 5-ASA by using Caco-2 cells. The results indicated that only 5-ASA could be absorbed completely in the duodenum and jejunum, but it was less permeable in ileum. Moreover, 5-ASA was poorly absorbed in colon. The drug could be metabolized mainly in the upper part of small intestine and in the intestinal cell cytosol.

Then 5-ASA and its metabolites would be eliminated completely in the urine. Therefore, this latter study confirms that polysulfanilamide-5-ASA azo conjugate may release the drug in small amount in the distal part of small intestine (8.0 ± 0.81%). However, most of 5-ASA could be delivered to colon. The polymeric conjugate had been shown to be more effective than sulfasalazine in reducing the inflammation of guinea pig ulcerative colitis model (Brown *et al*., 1983).

In 1990, Kopecek generated HPMA-5-ASA azo conjugate and studied the release of the drug from HPMA copolymers. The study was carried out in stomach and colonic contents of guinea pigs. The results revealed that HPMA copolymer contained both water solubility and bioadhesive properties which could be a potential polymeric carrier for colonic specific drug delivery. However, it was lag of release study in small intestinal content. So we still do not know the release profile of this polymeric conjugate in small intestine.

A number of macromolecular conjugates of 5-ASA was reviewed by Chourasia and Jain (2003). The polymers utilized in this review were vinyl pyrrolidone-maleic anhydride copolymer (Figure 2-3A, Pato *et al*., 1982), poly[*N*-(2-hydroxyethyl)-*DL*-aspartamide] (PHEA, Figure 2-3B, Antoni *et al*., 1974) and bis-aminopropyl poly(tetraethyleneoxide), PTMO (Figure 2-3C, Schacht *et al*., 1996). The release of 5-ASA from the conjugates was tested *in vitro* in the reductive buffer or in the bioreactor medium (Figure 2-5A). The result demonstrated that for the hydrophobic polymer (4-[4-(chlorocarbonyl) phenylazo] benzoylchloride/α, ω-bis-aminopropyl poly(tetra-methylene oxide), ABBC/PTMO, Figure 2-4), the apparent reduction was about 50% and stopped at the hydrazine stage (Figure 2-5C) whereas for a more hydrophilic analogue (ABBC/Jeffamine ED-600, Figure 2-4), reduction with formation of amines occurred (Figure 2-5B, Schacht *et al*., 1996). Despite of the fact that all these polymeric conjugates could deliver 5-ASA successfully to the distal part of the small intestine, ileum, and the large intestine, 5-ASA might not be the drug of choice for these systems. Moreover, the solubility property of each water-soluble polymeric conjugate was limited by the percentage of drug conjugation. Indeed, the required dose of 5-ASA ranges from 0.5 to 3 g daily. And since the drug makes less than 10% of the total weight of the conjugate, a very large amount of the polymer would need to be taken orally.

Wiwattanapatapee and co-workers (2003) reported PAMAM-PABA-SA and PAMAM-PAH-SA conjugates which could release 5-ASA in both homogenate of the small intestine and the cecal content. Twenty three percentage and thirty eight percentage of 5-ASA was detected after 12-hour incubation in cecal content of rats. 5-ASA could be also released up to 4.5 and 7.2 % after 12-hour incubation in the small intestine. Moreover, amide bond between amino group of PAMAM and carboxyl group of PABA-SA and PAH-SA degraded and released PABA-SA and PAH-SA up to 4.3 and 12.5 % after 12-hour incubation in the small intestine. The release of both spacer-SA in the homogenate of colon was less than 0.4% (Wiwattanapatapee *et al.*, 2003). However, in comparison to the original oral dose of 5-ASA (1500 mg/day), the patients need to take about 24 g of PAMAM-PABA-SA conjugate daily. In our case, PEG_{3677}-$(PABA-SA)_2$ (40) and PEG_{5106}-$(PABA-SA)_2$ (**39**) amide conjugates required to give 5-ASA 1500 mg/day are 26 and 28 g, respectively. The oral dose of the conjugates of PEG is a slightly higher that PAMAM-PABA-SA conjugate. However, there was no amide bond breaking found after incubation of both PEG amide conjugates in the small intestine and the colon contents. This indicated that PEG could prevent degradation of the amide bond better than PAMAM due to the flexibility of its chemical structure to surround and protect from amide degradation process. Furthermore, from the release study profiles of PEG conjugates (**39**, **40**), it could be observed that the active drug was slowly released from the developed conjugates and could prolong the release up to 24 hours. Therefore, less frequency will be required to take orally.

As mentioned above that conjugates No. **39** and **40** may have potential for using as intestinal and colonic drug delivery system in treatment of ulcerative colitis and Crohn's disease, respectively, the improvement of % drug conjugation should be investigated in order to increase the percentage of the drug content. This will lead to reduction of the amount of the polymer, PEG, taken orally. However, the aqueous solubility property and *in vitro* release profile of those designed conjugates should be further studied in order to evaluate the suitability for using as the drug carrier.

CHAPTER 5

CONCLUSIONS

Although sulfasalazine is useful in treatment of inflammatory bowel disease, especially in the colon, the side effects of this prodrug are usually found. Most are due to the sulfapyridine moiety (Qureshi and Cohen, 2005). Since the stomach irritation of 5-ASA is presented, therefore, its use in conventional formulations is limited (Klotz, 1985). To overcome these problems and allow this drug to reach the target site in colon, polymers conjugate with 5-ASA as the delivery systems has been developed.

The model of polymeric conjugate was designed to be similar to the chemical structure of sulfasalazine in order to reduce the side effect of 5-ASA and controlled the delivery system to deliver the drug, 5-ASA, at the active sites. Poly(ethylene) glycol, PEG, was chosen as the carrier in this research due to its non-toxic, non-antigenic, non-teratogenic, non-immunogenic and biocompatible properties. The synthesis of polymeric conjugate system consists of carrier-spacer-active drug was performed. Poly(ethylene) glycols, such as $mPEG_{5000}$, PEG_{4000}, PEG_{5106} diamino and PEG_{3677} diamino were used as the carrier.

In this study, eight methods were attempted to prepare the expecting polymer conjugates. However, only method No. 8 gave the desired products. In this method, *p*-nitro benzoylchloride was conjugated to each PEG polymer to obtain ester and amide products of PEG-(4-NO_2-BA) in the presence of triethylamine or pyridine, resulting in yellowish white amorphous solid. It was confirmed by ^{1}H-NMR and FT-IR spectral data that PEG-(4-NO_2-BA) conjugates were synthesized successfully. The conjugation percentages of 4-NO_2-BA in $mPEG_{5000}$, PEG_{4000}, PEG_{5106} and PEG_{3677} conjugates were 86.07, 90.70, 97.30 and 95.56%, respectively.

The aromatic nitro of PEG-(4-NO_2-BA) conjugates was then reduced to amino functional group by hydrogenation using Pd/C as catalyst, resulting in bright yellow amorphous solid of PEG-PABA conjugates. ^{1}H-NMR and FT-IR spectral data confirmed that PEG-PABA conjugates were synthesized successfully. The %conjugation of PABA in

$mPEG_{5000}$, PEG_{4000}, PEG_{5106} and PEG_{3677} conjugates were 78.44, 79.07, 91.35 and 72.03%, respectively.

After diazotization reaction of PEG-PABA conjugates with SA, PEG-(PABA-SA) conjugates of $mPEG_{5000}$, PEG_{4000}, PEG_{5106} and PEG_{3677} were obtained, resulting in reddish orange amorphous solid. The ester conjugates of $mPEG_{5000}$ and PEG_{4000}, and the amide conjugates of PEG_{5106} and PEG_{3677} were synthesized and obtained in 98.12, 96.75, 99.05 and 99.13 % yield, respectively. The conjugation of PABA-SA as di-ester (PEG_{4000}) or di-amide PEG products (PEG_{5106} and PEG_{3677}) were obtained in 73.29, 91.94 and 72.24%, respectively, whereas the mono-ester PEG product of $mPEG_{5000}$ resulting in PABA-SA conjugation in 37.39% conjugate. All products were resulted in reddish orange amorphous solid. The FT-IR and ^{1}H-NMR spectral data confirmed that the ester and amide tripartate products were synthesized successfully.

The % free SA and other related compounds in the resulting PEG-PABA-SA conjugates were determined using the validated HPLC conditions. The results demonstrated that none of any free or related compound remained in the conjugated products after purification. The % purity of each conjugates was 100%. Moreover, the water solubility of 5-ASA in this study was improved over 200 times by PEG conjugates.

The degradation of the ester and amide bonds from hydrolysis pathway after incubation in buffer solution having pH 1.2, 6.8 and 7.5 for 6, 12 and 24 hours at 37 ^{o}C, respectively, was protected by PEG conjugation. The results were similar to the previous reports (Zacchigna *et al*., 2002; Wiwattanapatapee *et al*., 2003; Zou *et al*., 2005). Moreover, the results demonstrated that the azo bond is not reduced in the absence of gastrointestinal contents.

From release study in rats gastrointestinal contents, sulfasalazine, which was used as positive reference, released 5-ASA slowly in first 4 hours in the intestinal content, and finally reached the maximum at about 80% after 6 hour-incubation period. However, the release profiles of the drug in colonic content was 3 times faster than in the intestinal content and almost all of 5-ASA could be released within 2 hours. After incubation of sulfasalazine or polymer-(PABA-SA) conjugates in gastric content for 6

hours, no free 5-ASA was detected by HPLC. Hence, the ester and amide bond between PEG and PABA of all products were stable in gastric content. Whereas the ester bond of $mPEG_{5000}$-(PABA-SA) and PEG_{4000}-$(PABA-SA)_2$ ester conjugates degraded in the intestinal and colonic content resulting in PABA-SA, the amide bond of other two products (PEG_{5106}-$(PABA-SA)_2$ and PEG_{3677}-$(PABA-SA)_2$ amide conjugates) was more stable. It is worth to note that 5-ASA could be released from the PEG ester and amide conjugates both in the intestinal and colonic contents similar to previous report (Schacht *et al.*, 1996).

The release profiles of all PEG conjugates demonstrated that all samples could release 5-ASA both in intestinal and colonic contents. In intestinal content, the PEGylated drug with molecular weight of the polymer $\geq$ 4000 Da could release 5-ASA in less than 36% which was lower than the PEGylated drug with molecular weight of the polymer < 4000 Da, which could release the drug more than 79% after incubation for 12 hours. It could be noted that the higher molecular weight of PEG, the better protection the drug 5-ASA from the reductase enzyme. In contrast to the release of 5-ASA from sulfasalazine, almost 100% could be released after incubation in intestinal content for 1 hour.

The release profiles in colonic content demonstrated that 5-ASA was released from sulfasalazine in higher amount and faster than in the intestinal contents. However, the PEGylated drug with molecular weight of the polymer $\geq$ 4000 Da could slowly release 5-ASA in smaller amount up to less than 50%, than the PEGylated drug with molecular weight of the polymer < 4000 Da, which could release the drug more than 96% after incubation for 12 hours. Compared to sulfasalzine, PEG with molecular weight of 3677 Da could release 5-ASA up to 60%, whereas sulfasalazine gave 5-ASA up to 100% after incubation within the first two-hour. PEG_{3677}-$(PABA-SA)_2$ amide conjugate could release 5-ASA up to 96% within 24 hours. The release profiles of 5-ASA from the synthesized PEG conjugates indicate that the amide conjugates are more suitable for intestinal and colonic delivery than the ester conjugates because there was no amide bond breaking in those contents.

Due to the evidence of the inflammation area which is mainly in the distal part of colon, PEG_{5106}-(PABA-SA) amide conjugate may be suitable for treatment of

ulcerative colitis because of its release profiles. Moreover, the results revealed that poly(ethylene) glycol with molecular weight 3677 Da is a polymer that may be suitable for treatment of Crohn's disease which usually shows the scattered evidence in both small and large intestine. Due to the release profile in colonic content, perhaps, this conjugate can also be used in treatment of ulcerative colitis.

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