Preparation and Evaluation of Hydroxypropyl Cellulose 5-ASA Conjugates for Colon Drug Delivery

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I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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ABSTRACT

Hydroxypropyl cellulose (HPC) with molecular weight 80,000 & 100,000 was selected as a macromolecular carrier for the designing of colon specific delivery of 5-aminosalicylic acid (5-ASA) due to its properties like safety, pH insensitivity and solubility both in water and polar organic solvents. The polymeric conjugates were synthesized with the participation of two-ester and one azo bond to accomplish the local delivery of the active compound, 5-ASA, to the terminal ileum and/or the colon for the treatment of Crohn's disease (CD) and ulcerative colitis (UC). *P*ara-aminobenzoic acid (PABA) and 5-ASA were used as spacers between the polymer, HPC and salicylic acid (SA). For this purpose, HPC was partially modified by varying the molar ratios of chloroacetyl chloride, a small azo compound PABA-SA was synthesized and its sodium salt was coupled to HPC functionalized with the chloroacetate groups. HPC functionalized with chloroacetate groups was also coupled with the sodium salt of 5-ASA and then SA was coupled to the diazonium salt of HPC-5-ASA ester conjugate to develop HPC-5-ASA-5-ASA conjugates. All the HPC-PABA-N=N-SA (**5-8**) conjugates and HPC-5-ASA-5-ASA conjugates (**17-19**) were synthesized in high % yield (79.8 – 83.7% for **5-8** and 71.6 – 76.7% for **17-19**) and well characterized by FT-IR, ^{1}H-NMR, XRD, SEM, DSC and TGA techniques. All the HPC-PABA-N=N-SA (**5-8**) conjugates were hydrolyzed to detach the PABA-SA azo moiety to calculate the percentage drug loading and the quantity of 5-ASA attached to

the polymer was determined by HPLC. The attachment of drug was increased as the molar ratio of chloroacetyl chloride was increased to HPC and 5-ASA was calculated i.e. 7.7 (**5**), 9.8 (**6**), 11.14 (**7**) and 13.69 % (**8**), respectively. HPC-5-ASA-5-ASA conjugates (**17**-**19**) were also hydrolyzed to detach the 5-ASA-N=N-ASA azo moiety for determining the percentage drug loading by HPLC and the quantity of 5-ASA was calculated such as 10.4 (**17**), 14.7(**18**) and 18.6 % (**19**), respectively. The ester and azo bonds of four HPC-PABA-N=N-SA and three HPC-5-ASA-5-ASA conjugates were found chemically stable at pH 1.2, 6.8 and 7.4 for 24 hr at 37 ^{o}C. The drug release profiles of the conjugates were compared to the drug release profiles of sulfasalazine, which was used as positive compound. In intestinal content, 5-ASA was found slowly released after 4 hr from sulfasalazine and finally reached 56.2 % in 24 hr incubation period. All the HPC-PABA-N=N-SA conjugates(**5**-**8**) started to give release after 6 hr and the % release of 5-ASA was calculated highest i.e. 36.0 % with the lowest drug attachment (**5**) and **6**, **7** and **8** showed release 30.74, 25.8 and 19.9 %, respectively after 24 hr incubation period with the intestinal content. The release in the colonic content demonstrated that 5-ASA released from sulfasalazine was up to 75.82 % after incubation within the first thirty minutes and the total amount of 5-ASA released within 24 hr was up to 86.09 %. The highest quantity of 5-ASA released from the conjugate **5** was found 65.68 % and **6**, **7**and **8** showed release 52.42, 44.38 and 37.05 %, respectively after 24 hr incubation period with the colonic content. In intestinal content, the quantity of 5-ASA released from sulfasalazine was found 49.47 % and the % release of 5-ASA was calculated highest i.e. 31.02 % with the lowest drug attachment (**17**) and the conjugates **18** and **19** showed release 23.98 and 16.50 %, respectively after 24 hr incubation period. In colonic content, 5-ASA released from sulfasalazine was in higher amount and faster than in the intestinal contents. The release in the colonic content demonstrated that 5-ASA released from sulfasalazine was up to 77.42 % and the highest quantity of 5-ASA released in case of HPC-5-ASA-5-ASA conjugate **17**

was found 45.03 %. HPC-5-ASA-5-ASA conjugates **18** and **19** showed 5-ASA release 34.35 and 25.53 %, respectively after 24 hr incubation period with the colonic content. The release kinetics of sulfasalazine, all HPC-PABA-N=N-SA and all HPC-5-ASA-5-ASA in intestinal were found to follow the first order model, whereas, the release kinetics following Korsemeyer-peppas model were observed for all samples in colonic content both of sulfasalazine and the HPC conjugates. The results from *in vitro* study suggested that the developed conjugates have high potential for using as colonic drug delivery systems, since the systems gave very little amount or no release of the active drug within the gastric emptying time of the intestine, whereas, rapidly release of the active drug was observed once the systems have reached the colon.

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The author alone assumes responsibility for discussion and conclusions of this thesis and any errors of it may contain.

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LIST OF ABBREVIATIONS AND SYMBOLS

PABA-SA	5-((4-carboxyphenyl)diazenyl)-2-hydroxybenzoic acid azo conjugate
5-ASA	5-Aminosalicylic acid
pH	A measure of the acidity or alkalinity
NH_2	Amino
-N=N-	Azo bond
C=O	Carbonyl
-COOH	Carboxylic
CFU	Colony Forming Unit
CD	Crohn's disease
Da	dalton
°C	Degree Celsius
DMSO-d_6	Deuterated dimethyl sulfoxide
D_2O	Deuterated water
CH_2Cl_2	Dichloromethane
DSC	Differential Scanning Calorimetry
DTG	Differential thermogravimetric
DMA	Dimethylacetamide
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
H_2O	Distilled water
FT-IR	Fourier Transform-Infrared Spectroscopy
GIT	Gastrointestinal tract
g	Gram
HPLC	High performance liquid chromatography
hr	Hour
HCl	Hydrochloric acid
H_2	Hydrogen gas
HPC	Hydroxypropyl cellulose

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

IBD	Inflammatory bowel disease
K	Kilo
L	Liter
LD_{50}	Median lethal dose
MeOH	Methanol
µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
mg	Milligram
mL	Milliliter
mmole	Millimole
MIC	Minimum inhibitory concentrations
M	Molar
MW	Molecular weight
HPMA	*N*-(2-hydroxypropyl) methacrylamide
DMAP	*N,N*-Dimethylaminopyridine
$-NO_2$	Nitro
N	normality
NMR	Nuclear magnetic resonance
Pd/C	Palladium on activated charcoal
PABA	*p*-Aminobenzoic Acid
PAH	*p*-Aminohipuric acid
ppm	Part per million
%	Percentage
PBS	Phosphate buffer solution
PDA	Photodiode-array detector
KI	Potassium iodide
lb	pound

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

RT	Room temperature
rpm	Rounds per minute
SEM	Scanning Electron Microscope
$NaNO_2$	Sodium nitrite
Na_2SO_4	Sodium Sulfate
SD	Standard deviation
THF	Tetrahydrofuran
TG	Thermogravimetric
TGA	Thermogravimetric Analysis
θ	Theta
TCA	Trichloroacetic acid
UC	Ulcerative colitis
cm^{-1}	wavenumber

GENERAL INTRODUCTION

The oral route is supported by a number of advantages such as high patient compliance, flexibility in designing of dosage form and adjusting in dose in comparison with other routes. However, oral administration of conventional dosage forms normally start to dissolve from the upper gastrointestinal tract (GIT) depending upon the physicochemical properties of the administered drug (Mrsny, 2012). It is a major problem in conditions where a drug is needed in a specific target of the GIT such as colon.

The gastrointestinal 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 (Fig.1). In anatomy of digestive system, the colon is the part of the intestine from the cecum to the rectum (Fig.2). The primary objective of the colon is to extract water from feces. 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 digestive tract (Friend, 2005).

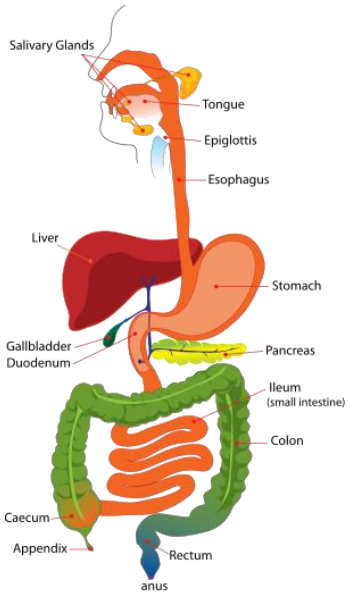


Figure 1 Diagram displayed human digestive system (From: (HelpingYouCare, 2012))

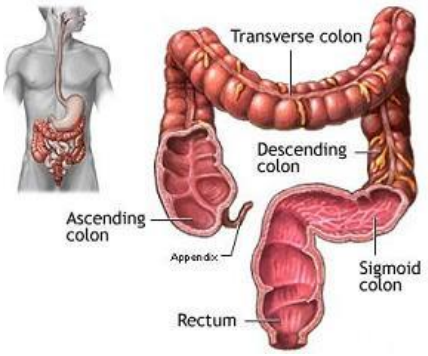


Figure 2 Picture of colon anatomy (From (Moshkovska, 2010))

The normal transit time of stomach is 1-2 hr and small intestine is 3-4 hr but in case of colon is 10 hr to several days (Fig.3). The transit time in the stomach is highly variable depending upon the condition of the subject such as fed or fasted but the transit time of small intestine is normally consistent (Table 1). Additionally, pathophysiological conditions can also accelerate the transit time of the gastrointestinal tract that can cause unpredictable drug release in case of time-dependent formulations and similarly pH variation between different segments of gastrointestinal tract can affect colon specific drug delivery system. There are a number of factors that can influence the slow and variable passage of drug through the colon such as stress, diet, mobility, dietary fiber content and disease. The transit time of dosage forms such as tablets and capsules through colon in adult males is approximately 20-30 hr but it can vary from male to female. Diseases can affect colonic transit time e.g. constipation can prolong and diarrhea can reduce colonic

transit time. However, in most disease states, transit time of colon remain practically stable (Friend, 2005).

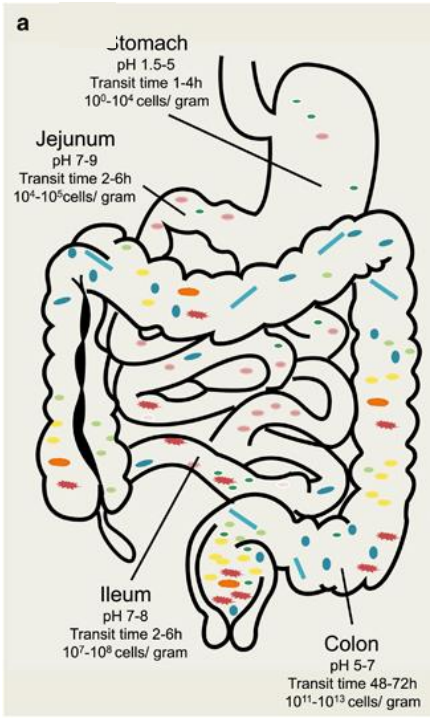


Figure 3 Schematic outline of the pH and transit time of the human digestive tract. (From:(Belzer and de Vos, 2012))

Table 1 The variation of pH in the GI tract which depending upon the fed and fasted conditions.

LOCATION	pH
1.STOMACH:	1.5-2.0
Fasted	3.0-5.0
Fed	5.0-6.5
2.SMALL INTESTINE:	6.0-7.5
Jejunum	6.4
Ileum	6.7-7.3
3.LARGE INTESTINE:	6.5-7.0
Right colon	6.6-7.0
Mid colon	6.6
Left colon	7.0

Colon specific drug delivery systems proffer a number of advantages such as availability of higher drug concentration at the target site, to extend the therapeutic effect and to reduce the toxic and unwanted side effects. The systems that can protect the drug from the hostile environment of the upper GIT and can deliver the drug directly to the colon are valuable in the treatment of diseases of colon such as ulcerative colitis, Crohn's disease, carcinomas and infections (Chourasia and Jain, 2003).

A number of approaches for colon specific drug delivery system have been established to deliver the drug locally to colon. They include: prodrugs, pH and time

dependent systems and enzyme dependent approaches. Prodrug (Fig.4) is an approach in which a drug is covalently linked to a low molecular weight or macromolecular carrier and it releases the active drug *in vivo* by enzymatic or chemical transformation (Mrsny, 2012). This technique has promising results over the parent drug molecule. The activation of site specific prodrug may be completed by the exploitation of some particular property at the target site, for example pH changes or higher no of microflora that release certain enzymes (Sinha and Kumria, 2001).

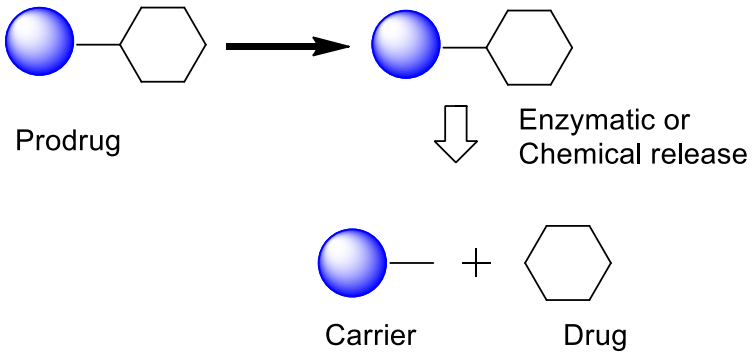


Figure 4 The concept of carrier linked prodrug consists of the attachment of a carrier group to the active drug to alter its physicochemical properties.

The presence of bacterial microflora in the stomach and small intestine is in order of 10^3–10^4 CFU/ml respectively that is mainly gram-positive facultative bacteria. The number of bacterial microflora in colon is many times higher i.e. 10^{11}–10^{12} CFU/ml consisting of mainly anaerobic bacteria (Fig.5). The main energy sources of these bacteria are di- and polysaccharides that were not digested in the small intestine (Belzer and de Vos, 2012).

During the fermentation process of undigested polysaccharides, the microflora of large bowel produces a wide number of enzymes such as azoreductase, β-galactosidase, β-xylosidase, nitroreductase, glycosidase deaminase, etc (Table 2).

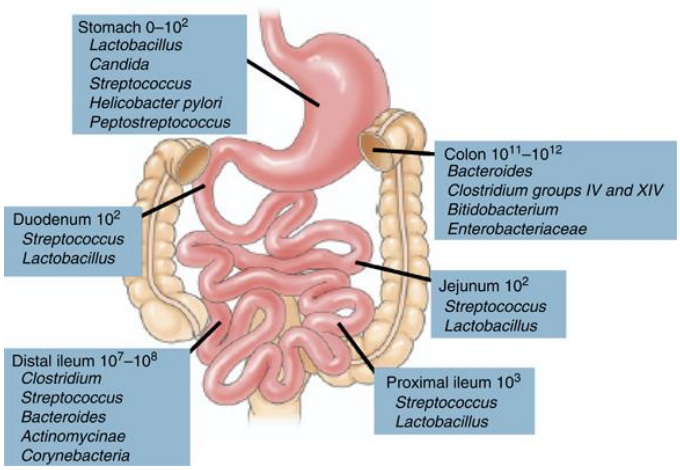


Figure 5 The presence of bacterial microflora in GI tract (From:(Sartor and Mazmanian, 2012))

Colonic microflora due to these enzymes is now preferably and largely being exploited for the designing of colon specific drug delivery systems. The conversion of prodrug to active drug also utilizes these enzymes and long transit time in the colon is helpful for the action of these enzymes on the prodrug substrates (Sinha and Kumria, 2001). The azo-conjugation approach was of interest in utilization of the colonic environment to cleave these conjugates and protects the drug from absorption or degradation in the upper gastrointestinal tract. It is believed that azo-reductase enzymes released from colonic bacteria are playing important role for the degradation of azo-aromatic compounds for site-specific delivery of the drug to the colon (Carrette *et al.*, 1995, Shantha *et al.*, 1995, Lu *et al.*, 2002).

Table 2 Enzymes in colon which play important role in major metabolic processes occurring in the colon are *hydrolysis* and *reduction*

Reducing enzymes	**Hydrolytic enzymes**
Nitroreductase	Amidases
N-oxide reductase	Glycosidases
Azoreductase	Glucuronidase

Sulphoxide reductase	Esterases
Hydrogenase	Sulfatase

Site specific drug delivery to the colon is extremely valuable for systemic and topical treatment of inflammatory bowel disease (IBD). The main two types of IBD are Crohn's disease (CD) and ulcerative colitis (UC) (Kopecek *et al.*, 1992, Colitis-Pathophysiology, 2003, Omar *et al.*, 2007). From 1955 corticosteroids were in use for patients with active CD and UC such as prednisone, prednisolone, methylprednisolone and more recently budesonide (Truelove and Witts, 1955). The maintenance therapy with long-term usage of theses corticosteroids is not suitable due to the unwanted side effects (e.g. adeno-suppression, immune-suppression, Cushinoid symptoms and bone resorption) lead to patient compliances (Klotz and Schwab, 2005). So, there is a need of some other choices for the treatment of IBD.

5-Aminosalicylic acid (5-ASA, Fig.6) is an anti-inflammatory agent (Martin and Greer, 1987). It has been exploited to relive pain and inflammation in patients having CD or UC. This drug is well known as a first choice agent for the treatment of colonic disease. However, it can be absorbed quickly and exclusively from the small intestine after oral administration (French and Mauger, 1993) . Moreover, 5-ASA irritates stomach (Wolfe *et al.*, 1999), therefore, using in conventional formulations is limited. For the treatment of IBD, an approach that specifically deliver 5-ASA to the lower gastrointestinal tract could be helpful to minimize side-effects of the drug and improve the quality of life for patients who suffering from colon-specific diseases (Omar *et al.*, 2007).

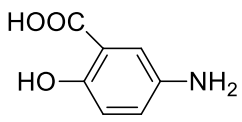


Figure 6 Chemical structure of 5-Aminosalicylic acid (5-ASA)

A well-known commercially available prodrug, sulfasalazine (Fig.7) was used for the management of rheumatoid arthritis and anti-inflammatory disease. Sulfasalazine is a 5-aminosalicylic acid prodrug which is reduced by enzyme azoreductase produced by the microflora of colon into 5-ASA and sulfapyridine (Klotz *et al.*, 1980). 5-ASA therapeutic action is mostly topical at the colonic site because its absorption rate from the colon is less than 20 %. In addition, sulfapyridine carrier absorption is almost 80 % from the colonic site and many serious systemic side effects were monitored due to the extensive absorption of sulfapyridine. That's why many patients are not capable to bear treatment with sulfasalazine (Qureshi and Cohen, 2005)and due to this reason many new techniques for the therapy of IBD have emerged.

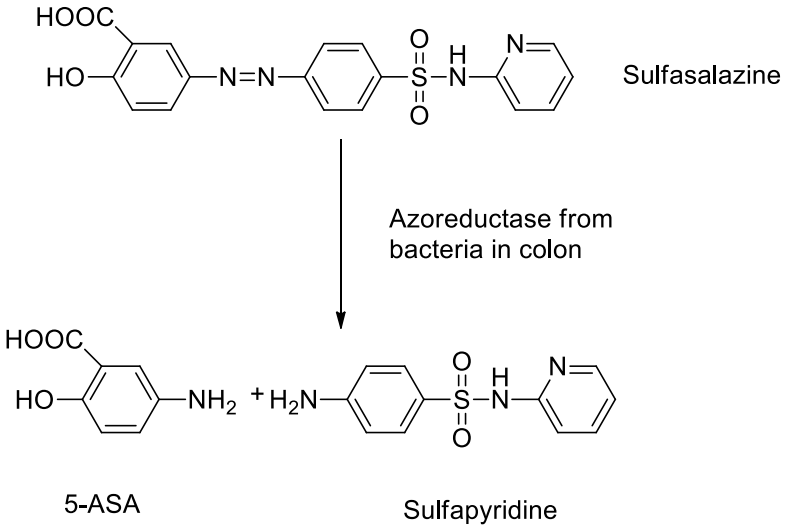


Figure 7 Reduction of sulfasalazine into 5-ASA and sulfapyridine (Chourasia and Jain, 2003)

The necessity for comparatively less toxic or non-toxic carrier than sulfapyridine opened a new door for researchers to synthesize and trial a number of

low molecular weight azo-bond prodrugs (Fig.8). There are some examples of 5-ASA azo linked prodrugs such as in case of ipsalazine, *p*-aminohippurate (4-amino benzoyl glycine) was employed as a carrier and for balsalazine, 4-amino benzoyl-β-alanine was used as a carrier of 5-ASA (Järnerot, 1994). The oral bioavailability of balsalazine is low and variable. The most attractive example is olsalazine in which one molecule of 5-ASA played a role of carrier for the other molecule of 5-ASA (Selby *et al.*, 1985). Olsalazine is an azo linked 5-ASA prodrug that is reduced in the colon by azoreductases and releasing two molecules of 5-ASA (Fig.8). Clinical trials of olsalazine showed better results as compared to sulfasalazine but almost 15% of IBD patients cannot tolerate treatment with olsalazine due to watery diarrhea (Ferry *et al.*, 1993). The oral bioavailability of olsalazine is only 2.4 % (Ryde *et al.*, 1991).

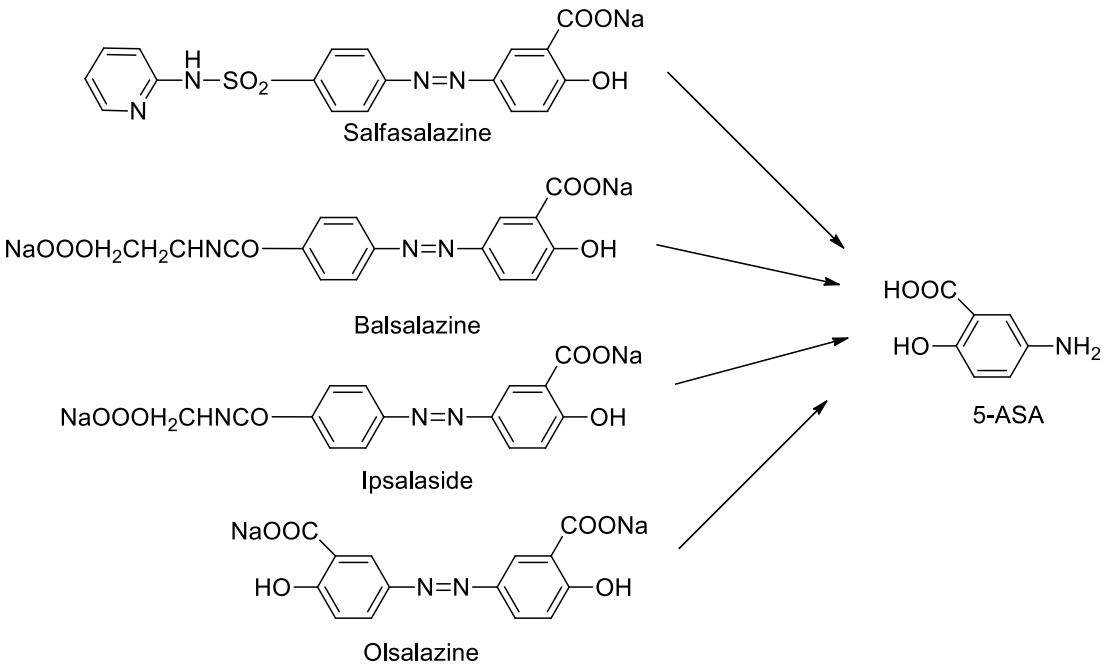


Figure 8 The chemical structures of sulfasalazine, balsalazide, ipsalazide and olsalazine presenting the cleavage of azo bond by azoreductases leading to the development of the active agent 5-ASA (Chourasia and Jain, 2003)

Polymeric prodrug approach in which drug is attached to the backbone of a high molecular weight polymer was also explored for colon targeted drug delivery (Clerici *et al.*, 1994, Davaran *et al.*, 1999, Jung *et al.*, 2001). In this case drug is released in the large intestine by breaking the linkage between the drug and polymer by an enzymatic attack in the large intestine. As compared to low molecular weight prodrugs, macromolecular prodrugs have the capability to not absorb from the upper GIT due to larger in size. Dynapol corporation synthesized a macromolecular compound that was developed on the salicylazosulphapyridine carrier concept i.e. sulfapyridine unit is coupled to an inert macromolecular backbone (Sinha and Kumria, 2001). In this research 5-ASA was linked with a carrier i.e. polysulfonamidoethylene by the development of an azo bond (Fig.9). The method of release of 5-ASA to the colon is effectively by reduction mechanism of azo bond by azoreductase enzymes produced by microflora reside in colon (Sinha and Kumria, 2001). In this case, the by-product i.e. sulfapyridine connected to the polymer backbone was excreted unabsorbed in the feces, resulting in no side effects of sulfapyridine.

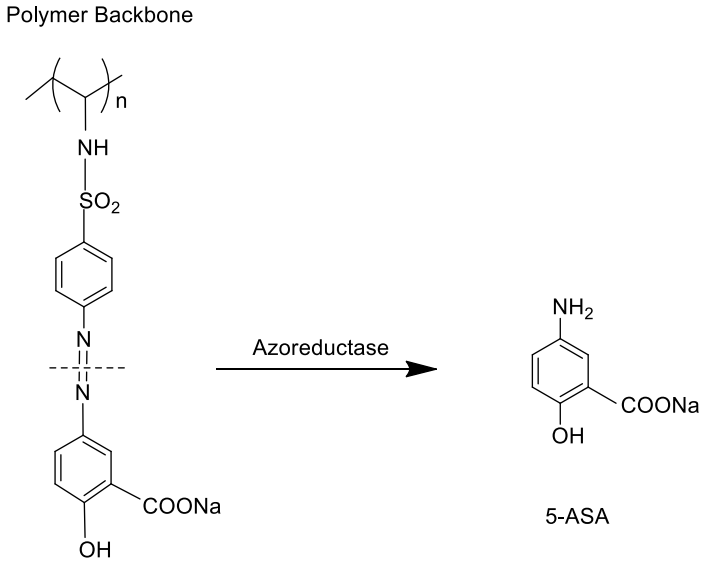


Figure 9 The chemical structure of polysulfonamidoethylene-ASA showing that azo bond is breaking down by azoreductases to release the active agent 5-ASA in the colon (Sinha and Kumria, 2001).

The importance of spacer in the polymer-drug conjugates was previously demonstrated that biodegradable spacer (Fig. 10) between the polymer carrier and the drug can be used to control the site of release of the therapeutic agent (Friend, 2005). Wiwattanaptapee and co-workers (2003) utilized water-soluble polyamidoamine (PAMAM) dendrimer (Fig. 11) macromolecule as a carrier because its structure is rich with a large number of functional groups. Salicylic acid (SA) was attached to the dendrimer by using two different spacers, *p*-aminobenzoic acid (PABA) and *p*-aminohippuric acid (PAH), containing azo bond. Incubation of 5-ASA-PAMAM dendrimer conjugates containing PABA and PAH spacers with rat gastrointestinal contents at 37 °C demonstrated that 5-ASA was specifically released in rat cecal contents. The active drug was slowly released and prolonged for at least 24 hr. 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. The amount of 5-ASA released from the commercial prodrug, suflasalazine was much faster than both the conjugates (80.2% in 6 hr). It was observed that the cleavage of azo bond in case of suflasalazine was much easier as compared to dendrimer conjugates. From these results, it was concluded that structure of carrier (low or high molecular weight) can affect the activity of azoreductase enzymes. Dendrimer conjugates were remained stable during chemical stability study with phosphate buffer pH 1.2 and 6.8 and no 5-ASA was found from the incubation of the dendrimer conjugates with the homogenate of the stomach content of the rat. 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 (Wiwattanapatapee *et al.*, 2003). However, the length of different spacers gave different pattern of drug release.

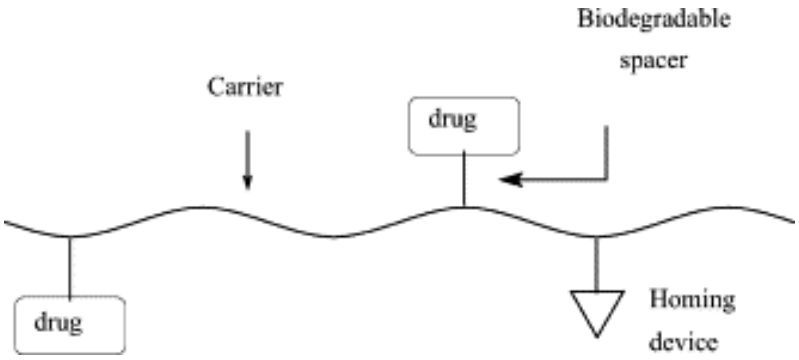


Figure 10 The concept of using spacer in CDDS (Friend, 2005).

Canevari and co-workers (2009) utilized poly(ethylene glycol) (PEG) as a macromolecular carrier and PABA as a spacer for the designing of colon specific delivery of 5-ASA. They exploited different molecular weights and structures of PEG as a carrier for the synthesis of three different 5-ASA-N=N-PEG conjugates (Fig. 12). All the conjugates were well characterized and found stable after incubation with the gastric and pancreatic artificial juices. Maximum 5-ASA was released in case of (biazo, PEG-(PABA-NN-SA)$_2$) conjugate and it may be due to availability of double azo bonds in this conjugate. It was also examined *in vitro* on mouse colonic epithelial cells (CMT-9) and *in vivo* on mice model with induced colitis. The release profiles of 5-ASA from these polymer-conjugates demonstrated that drug was released only in colon tract after the reduction of azo bond and the lowest molecular weight with double azo bonds conjugate was the most promising in case of release profile (Canevari *et al.*, 2009).

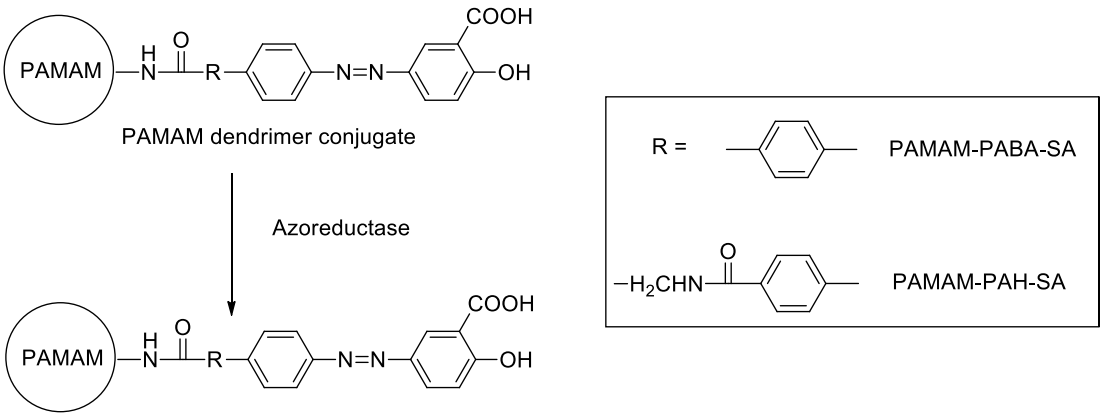


Figure 11 The chemical structure of PAMAM dendrimer-5-ASA conjugates in which R is representing PABA and PAH (Wiwattanapatapee *et al.*, 2003).

Cellulose derivatives like methyl-, ethyl-, hydroxypropyl-, hydroxypropylmethyl-, methylhydroxyethyl- cellulose solve the insolubility issue of cellulose (Reese *et al.*, 1950, Miyamoto *et al.*, 1989). These cellulose ether derivatives are still highly valuable even after many decades of their innovation. These derivatives have remarkable solubility property in aqueous and organic solvents that was really needed by researchers in the fields of pharmaceutical and biomedical sciences. Cellulose derivatives have been employed in drug delivery systems (Jenkins *et al.*, 1990, Chourasia and Jain, 2003, Edgar, 2007) as polymer-drug conjugates, vaccine bullets, delayed release of drug, as a disintegrating agent in the formulation of matrix tablets, as crystal clear media for liquid crystalline display technology and stabilization of dispersion polymerization (Hussain, 2008).

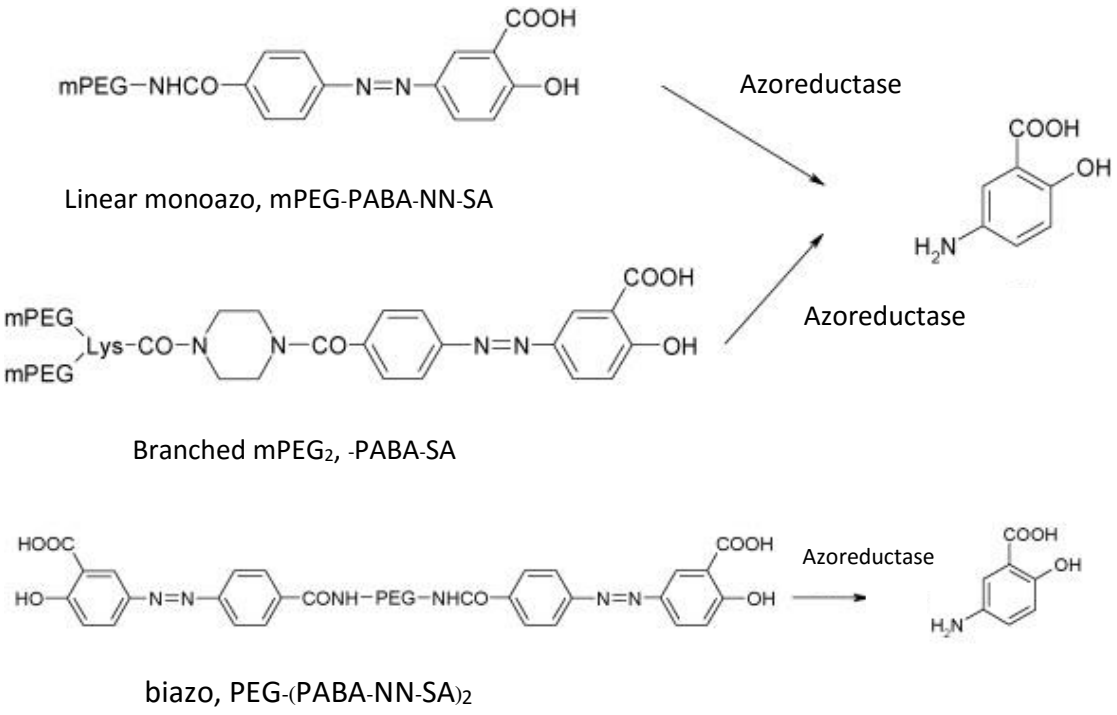


Figure 12 Structure of the three PEG-5-ASA conjugate and release of 5-ASA following azoreductase incubation: linear monoazo, mPEG-PABA-NN-SA biazo, PEG-(PABA-NN-SA)2 and branched mPEG2 -PABA-NN-SA (Canevari *et al.*, 2009).

Hydroxypropyl cellulose (HPC) is one of the pharmaceutically valuable cellulose ether due to its appropriate properties like cheap, easily available, non-toxic, pH insensitivity, odorless, thermoplastic and can be soluble both in water and polar organic solvents (Macleod *et al.*, 1999, Nunthanid *et al.*, 2008). HPC has also remarkable applications in textile, ceramics, paper and paint industries. HPC has also been widely used as a tablet binder, a thickening agent and as a film coating material in different pharmaceutical dosage forms. It has low oral toxicity (LD_{50} in rats of 10,200 mg/kg) (Khan *et al.*, 2008). In the chemical structure of HPC there are three hydroxyl groups per anhydroglucose unit (Fig. 13), which can be employed in the synthesis process of polymer-drug conjugates or these hydroxyl groups can play an important role in the fabrication of macromolecular reactive carrier for different applications in biological and medical sectors (Khan *et al.*, 2008).

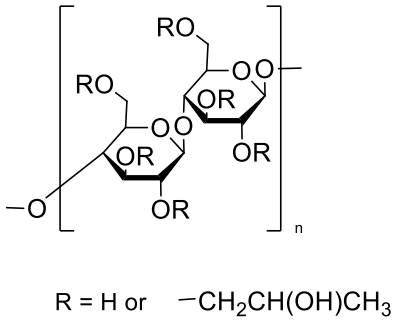


Figure 13 The chemical structure of hydroxypropyl cellulose (Anonymous, 2012).

From previously reported literatures, there were a few examples found for the chemical attachment of drugs on to the cellulose hydroxyl ethers as macromolecular prodrug. The following three ester products were reported, however, no azo bond conjugate products were found with HPC.

In 2005, Zou and co-workers reported ester prodrugs of hydroxypropyl cellulose-5-ASA and amide prodrug of chitosan-5-ASA (Fig. 14). The result from this study showed that these two prodrugs did not release active drug, 5-ASA both in the cecal or colonic contents of rats, therefore, respective ester and amide bonds connected to hydroxypropyl cellulose and chitosan were stable in gastrointestinal tract.

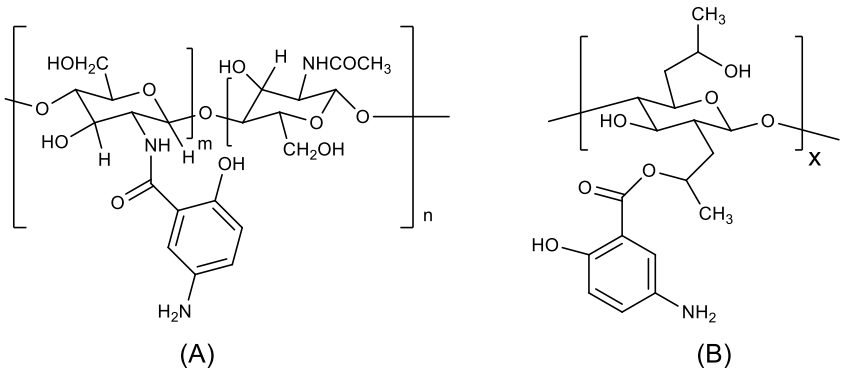


Figure 14 Structures of amide prodrugs of chitosan-5-ASA (A) and HPC-5-ASA ester prodrug (B).

In 2008, Hussain, M. A. synthesized highly pure HPC abietates conjugates by varying the molar ratios of abietic acid to HPC. All these conjugates were soluble in organic solvents but on the other hand in water these conjugates showed a swelling behavior. In the synthesis scheme (Fig. 9) abietic acid was activated by *p*-toluenesulfonyl chloride (Tos-Cl). HPC was dissolved in *N*,*N*-dimethylacetamide (DMAc) and it was reacted with abietic acid and Tos-Cl for 24 hr at 70 ºC. Tos-Cl reacted with abietic acid to make it as reactive intermediates, which can be then reacted with hydroxyl groups of HPC backbone, to synthesize polymer conjugate with ester linkage (Hussain, 2008).

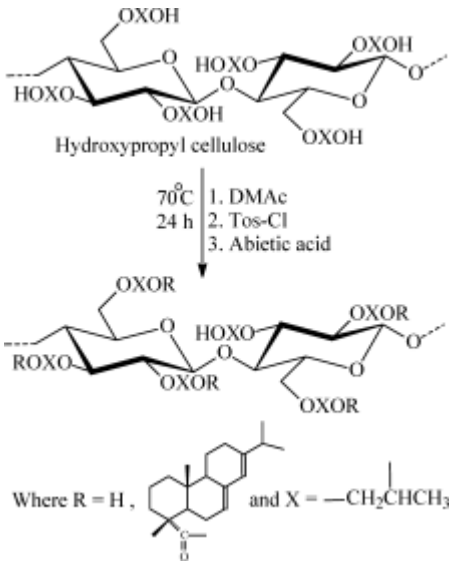


Figure 15 Synthesis scheme of HPC abietates conjugates (Hussain, 2008).

Khan and co-workers (2008) synthesized and characterized amino acid-functionalized HPC derivatives. The esterification reaction between HPC and different amino acids was accomplished by the participation of hydroxyl group of HPC and the carboxyl terminal of amino acids. A new family of biocompatible materials was synthesized in high % yield and all these conjugates have good solubility in organic

solvents such as dimethylsulfoxide (DMSO), dimethylformamide (DMF), dimetylacetamide (DMA) and tetrahydrofuran (THF) but these conjugates are not soluble in water. This esterification reaction (Fig. 16) was completed by using the *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC·HCl) as a coupling reagent and 4-(dimethylamino)-pyridine (DMAP) as a base at 30 °C for 48 hr.

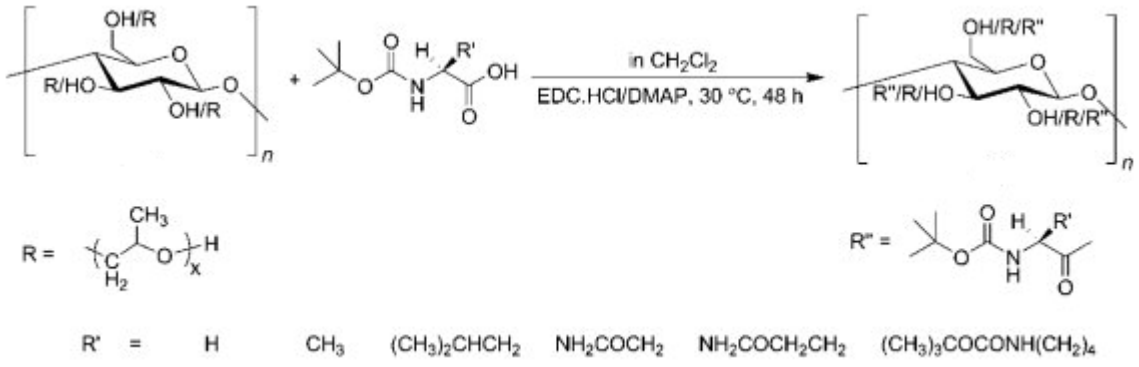


Figure 16 Synthesis of amino acid esters of hydroxypropyl cellulose (Khan *et al.*, 2008).

Since, there was no publication on utilizing HPC as a drug carrier to develop azo conjugate, we therefore, would like to develop colon-specific drug delivery system by using HPC as drug carrier by conjugating with 5-ASA using azo linkage via two types of spacers, PABA and 5-ASA (Fig.17). The resulting products will be characterized and determined for their stability in various buffer solutions (buffer pH 1.2, 6.8 and 7.4).

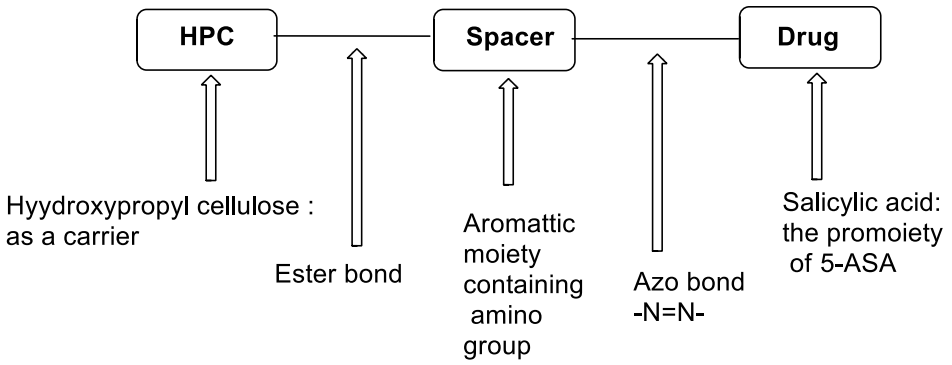


Figure 17 Rationale design for the HPC-azo-5-ASA conjugates of this study.

Furthermore, *in vitro* release study of 5-ASA from these conjugates will be performed with the gastrointestinal contents of the rat. *p*-Aminobenzoic acid (PABA) was selected in this study as a spacer due to its low toxicity i.e. LD_{50} in rat (oral administration) 6 g/kg (Scott and Robbins, 1942).

OBJECTIVES

1. To synthesize and characterization colon specific delivery of 5-aminosalicylic acid using hydroxypropyl cellulose as a drug carrier.
2. To study the stability of the conjugates in buffer solutions.
3. To study the release of 5-ASA from the conjugates in vitro.

RESULTS AND DISCUSSIONS

HPC with molecular weight 80,000 & 100,000 was selected as a macromolecular carrier for the designing of colon specific delivery of 5-aminosalicylic acid (5-ASA) due to its properties like safety, pH insensitivity and solubility both in water and polar organic solvents. HPC is cheap and biocompatible pharmaceutical excipient with three hydroxyl groups per anhydroglucose unit (Hussain, 2008). Zou and co-workers (2005) formulated HPC-5-ASA ester prodrugs by varying the molar ratios of HPC and 5-ASA (1:1, 1:2, 1:6, 1:10) and they reported that none of the prodrug released 5-ASA in any of the contents of the rat within 48 h due to insolubility and steric hindrance factors (Zou *et al.,* 2005). Keeping in mind these issues, we decided to synthesize HPC-5-ASA conjugates by bringing the three major changes i.e. decreasing the molar ratios between HPC and 5-ASA, developing a spacer arm between HPC and 5-ASA and an azo bond was developed between spacer and drug instead of ester. PABA is an aromatic naturally occurring non-protein amino acid and it was selected as a spacer in our synthesis scheme. It is safe and its absorption rate from the gastrointestinal tract is very fast and completely metabolized by the liver (Wan *et al.*, 1972). Structurally, PABA consists of a benzene ring substituted with an amino and a carboxyl group. PABA was also used as a spacer in the synthesis scheme of dendrimer-5-ASA azo conjugates (Wiwattanapatapee *et al.*, 2003) and polyethylene glycol-5-ASA azo conjugates and it could facilitate 5-ASA release from the polymeric conjugates (Wiwattanapatapee *et al.*, 2003, Canevari *et al.*, 2009).

1. Synthesis process of *p*-nitrobenzoylhydroxypropyl cellulose

In this study hydroxyl groups of HPC were esterified with *p*-nitrobenzoyl chloride under homogeneous reaction conditions in dried dichloromethane as a solvent similar to the previous reported for the synthesis of nitrobenzoyl cellulose derivatives (Talaba

et al., 1996). Pyridine was utilized as bases to attempt to capture the released hydrogen chloride according to the synthesis scheme displays in Figure 18. HPC (10.0 g, 29.8 mmol) was dissolved in a 160 ml of dried dichloromethane in a two-necked round bottom flask with constant mechanical stirring at room temperature. Pyridine 12 ml (149 mmol) was added to the flask as an acid acceptor and *p*-nitrobenzoyl chloride 27.6 g (149 mmol) was added lastly by parts. The reaction mixture was heated at 25 °C overnight under nitrogen atmosphere. The solution that was obtained after the required time was clear and viscous with light yellow color. The solution was evaporated for removing dichloromethane and dissolved in DMSO for transferring into the dialysis bag. The polymer product was purified by dialysis against distilled water (MWCO = 12,000-14,000 Da) for at least 3 days to remove the impurity, *pyridinium chloride* which was soluble in water. The purified *p*-nitrobenzoylhydroxypropyl cellulose ester conjugate was obtained by lyophilization[7]. Yield: 85 % based on DS 2.52.

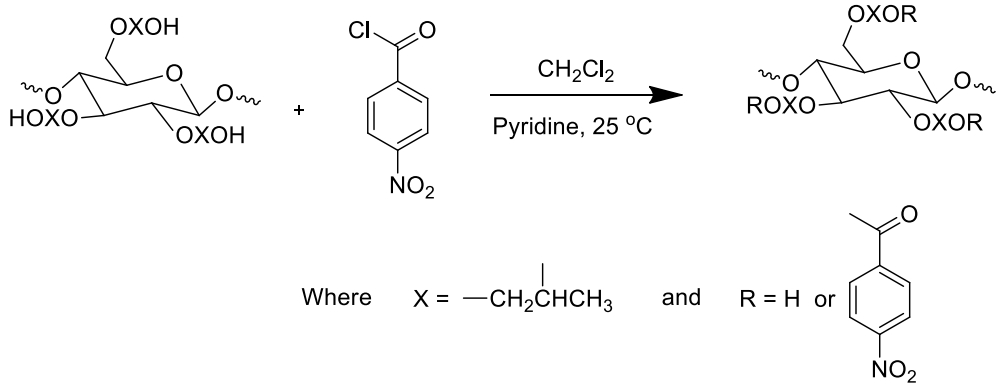


Figure 18 Reaction between HPC and *p*-nitrobenzoyl chloride to prepare and *p*-nitrobenzoyl hydroxypropyl cellulose

HPC was reacted with different molar ratios of *p*-nitrobenzoyl chloride but the maximum nitration was achieved with five folds excess of *p*-nitrobenzoyl chloride. The native HPC was soluble in water but after the incorporation of aromatic nitro groups, it could not preserve its water solubility property. HPC-NO_2 has solubility in

organic solvents like dimethylsulfoxide (DMSO), dimethylformamide (DMF), dimetylacetamide (DMA) and tetrahydrofuran (THF).

2. Reduction process of *p*-nitrobenzoylhydroxypropyl cellulose

The reduction of the aromatic nitro group to its respective amine can be completed by different ways. The reduction of HPC-NO_2 was carried out by using three different reducing agents i.e. catalytic hydrogenation using Pd/C (method 1) sodium dithionite (method 2) and tin(II)chloride dehydrate (method 3) (Fig 19).

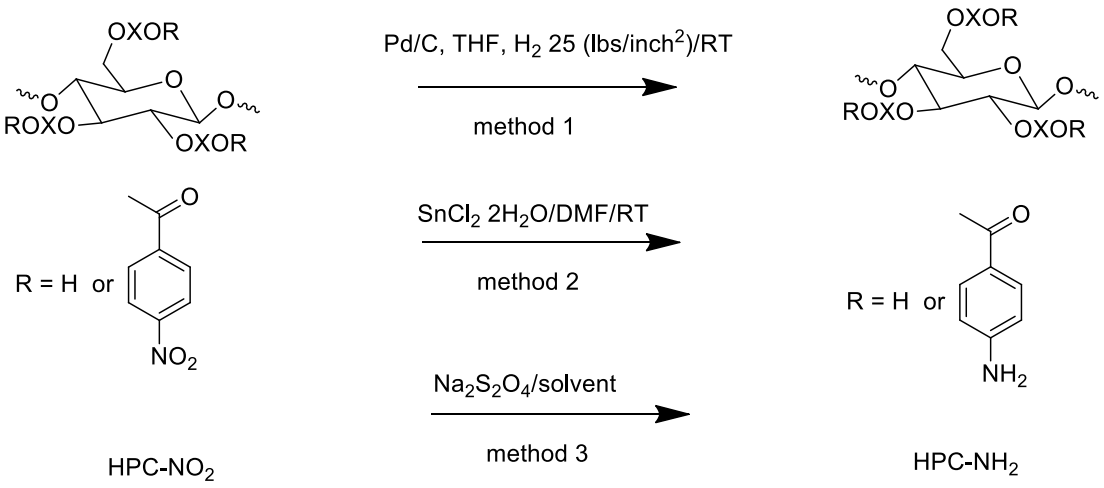


Figure 19 Reduction of HPC-NO_2 by Pd/C, $SnCl_2.2H_2O$ and $Na_2S_2O_4$.

The catalytic hydrogenation method (method 1) was used in this study because it is the cleanest and simplest. THF was selected as a solvent of choice because HPC-NO_2 product has solubility in THF and it can evaporate easily after completion the reaction. The reaction was carried out in heterogeneous phase because HPC-NO_2 was in solution phase but on the other hand palladium on activated charcoal was in solid phase. The reaction was run for different time intervals and at different pressures and finally H_2 gas pressure at 25 (lbs/inch^2) and time 12 hr were selected. Maximum HPC-

NO_2 was reduced to HPC-NH_2 but could not completely reduce (observed by FT-IR) because the reaction environment was heterogeneous. About 60% of the product yield (calculated by weight) was obtained. Moreover, some of the product was lost during filtration process through celite to remove palladium catalyst.

In case of tin (II) chloride dihydrate the reduction of HPC-NO_2 was carried out homogeneously in DMF (method 2) according to the process reported in (Scheuerman R.A. and Tumelty.D. 2000). HPC-NO_2 has good solubility in DMF along with tin (II) chloride dihydrate and that is why all the aromatic nitro groups were completely and cleanly converted to amino groups (detected by FT-IR). The HPC-NH_2 was obtained from the dialysis residue by lyophilization and the percentage yield of HPC-NH_2 (calculated by weight) was up to 140%. Despite the advantages of tin reduction, we observed that substantial quantities of tin by-products remain inside the dialysis bag due to its insolubility in distilled water. The product from this experiment was obtained in high yield since the product entrapped tin by-product which cannot remove from HPC-NH_2 by dialysis method.

Sodium dithionite was investigated as an alternative to tin (method 3) due to inexpensive, non-toxic, neutral and specific to the reduction of nitro compounds in the presence of other functionalities (Scheuerman R.A. and Tumelty.D. 2000). We carried out a detailed examination of HPC-NO_2 reduction by using sodium dithionite as a reducing agent. Optimum conditions for the reduction of HPC-NO_2 involving, type of solvent (water, organic solvents e.g. DMA, DMF, DMSO, THF, co-solvents etc), quantity of reducing agent (nitro product : reducing agent, 1:4, 1:6, 1:8, 1:10 and 1:12 by weight), reaction time (8, 12, 16, 20 and 24 hr) and reaction temperature (room temperature, 45, 60 and 85 $^{\circ}$C) were monitored. The optimum conditions for the reduction of HPC-NO_2 were determined as following: In water, sodium dithionite was

readily soluble but HPC-NO_2 was not soluble. To solve this issue we first prepared the homogeneous solution HPC-NO_2 in DMF and then added aqueous solution of sodium dithionite slowly. The pH was maintained at 8 to 9 because at this pH was reported to be the best condition for reduction by sodium dithionite and its consumption was determined such as 1 g of the HPC-NO_2 require almost 12 g of sodium dithionite and with 24 hr reaction time at 45 °C. Sodium dithionite did not give any reduction (observed by FT-IR) in organic solvents alone like DMA, DMF, DMSO and THF due to its insolubility in these solvents. Further studies are required with sodium dithionite by finding a suitable phase-transfer catalyst and solvent system to minimize its consumption during reduction process. The FT-IR results after HPC-NO_2 reduction using the above mentioned conditions are shown in Figure 20.

Figure 20 shows the FT-IR spectra of the native HPC (A) which its OH group stretching appeared in the range of 3600 to 3200 cm^{-1}, and OH bending appeared near ~ 1020 cm^{-1}. In case of the HPC-NO_2 (B) the strong absorption bands appeared at 1527 cm^{-1} and 1348 cm^{-1} correspond to NO_2 asymmetric and symmetric stretching, respectively. The absorption band at 1276 cm^{-1}correspond to C-N stretching and an absorption band appeared at 1722 cm^{-1} due to carbonyl stretching. These FT-IR results confirmed the esterification reaction between HPC and *p*-nitrobenzoyl chloride. Catalytic hydrogenation using 10 % Pd/C as a catalyst (C) reduced maximum NO_2 to NH_2 as in FT-IR results N-H stretching absorption bands appeared at 3460 cm^{-1} and 3364 cm^{-1} but on the other hand small peaks of NO_2 are still present at 1527 cm^{-1} and 1348 cm^{-1} indicated incomplete reduction. In case of tin (II) chloride dihydrate (D) and sodium dithionite (E) the NO_2 peaks were completely disappeared but no bands assigned to N-H bending in the range of 3500-3000 cm^{-1} and 1640-1560 cm^{-1}, respectively were observed. This may be due to the overlapping of -OH stretching band of unreacted hydroxyl groups, in the range of 3000-3600 cm^{-1}, and the -OH

bending of the adsorbed water molecules because HPC-NH_2 was purified by dialysis process in water. The aromatic sharp peak was observed at 1603 - 1607 cm^{-1} in all the cases except native HPC.

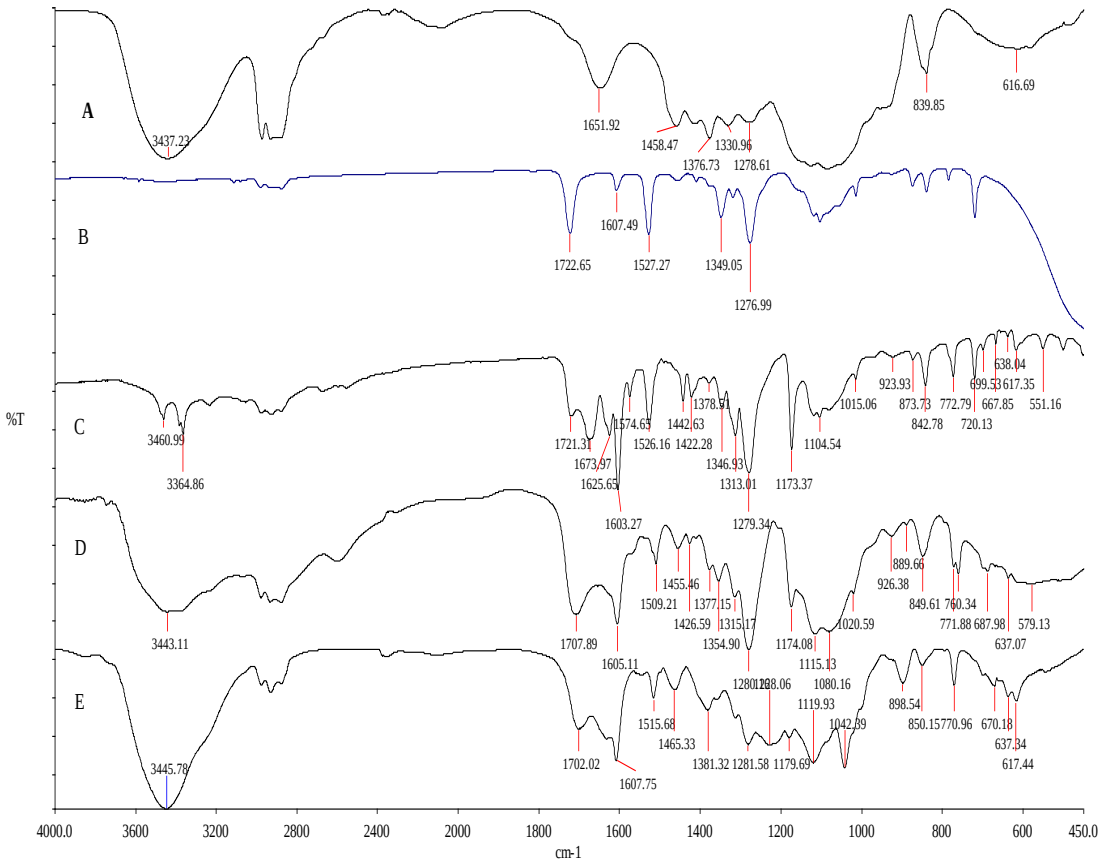


Figure 20 FTIR spectra of: (A) HPC, (B) HPC-NO_2 , (C) HPC-NH_2 by Pd/C, (D) HPC-NH_2 by tin (II) chloride dihydrate, (E) HPC-NH_2 by sodium dithionite.

The ^{1}H NMR spectrum further confirmed the synthesis of HPC-NO_2 (Fig. 20), the peak appeared at 2.49 ppm is due to DMSO-d_6. The spectrum of HPC- NO_2 ester product shows peaks at 1.0 – 1.25 ppm from the methyl groups in the hydroxypropyl moieties. Peaks in a range of 3.0 – 4.0 ppm belong to protons of cyclic glucose units. The characteristics resonance peaks at 7.5 to 8.5 ppm are ascribed to the protons of aromatic moiety of the HPC-NO_2 product. A tiny peak detected at 5.2 ppm belongs to the resonance of the methine protons of HPC. In case of HPC-NH_2 obtained from the

reduction reaction by using sodium dithionite (Fig. 21), the peaks of aromatic moiety appeared at 7.0 to 8.0 ppm. In case of HPC-NH_2 the aromatic amines was simply tested and confirmed by diazotization reaction gave deep red product which was not able to dissolve well in water. The product was not further utilized in the next synthesis process since there was some of the reducing agent, sodium dithionite also remained in the final product.

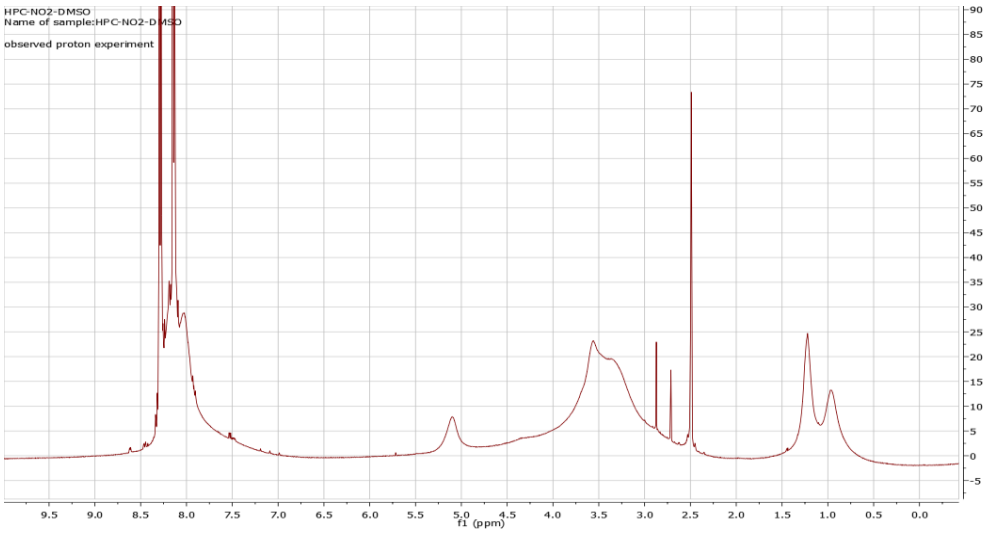


Figure 21 ^{1}H NMR spectra of HPC-NO_2 (500 MHz, in DMSO-d_6).

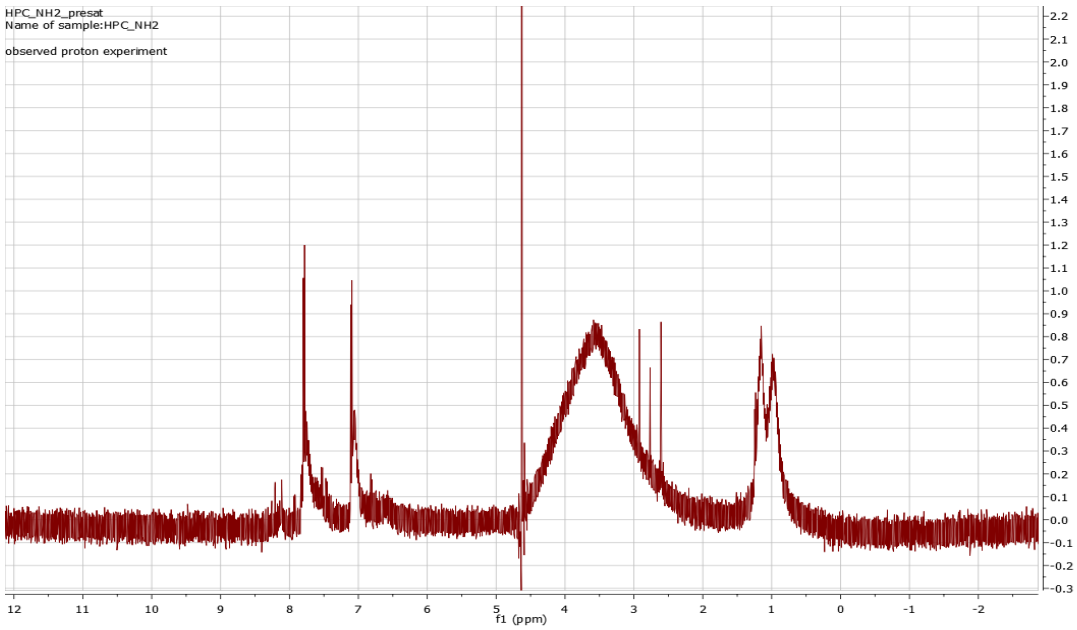


Figure 22 ^{1}H NMR spectra of HPC-NH_2 (500 MHz, in D_2O).

3. **Synthesis route of HPC-PABA ester conjugate**

For acquiring the primary amino functionality on HPC, we approached in another way that was esterification reaction between PABA and HPC. In this method, the primary amino group of PABA was first protected by 98% formic acid according to the method in the literature (Zou *et al.*, 2005). The protection of primary amino group was helpful to avoid from self-coupling of PABA during the reaction due to its bi-functional groups nature. 4-*N*-Formylaminobenzoic acid (4-*f*-PABA) was successfully esterified with HPC using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC.HCl) as a condensating agent and 4-(dimethylamino)pyridine (DMAP) as a base to provide the desired product in 63 % yield (Fig.23). Finally, the protecting group was tried to remove by stirring the product in 0.5 mol/l HCl according to the prescribed method (Zou *et al.*, 2005). However, HPC-PABA with free amino group was not successfully achieved by hydrolysis of HPC-4-*f*-PABA proved by FT-IR result demonstrated in Fig.24 which shows no ester bond character. The result demonstrated that the ester bond between HPC and protected PABA was broken, that may be due to the strong acid strength and high temperature assisted the hydrolysis of ester bond.

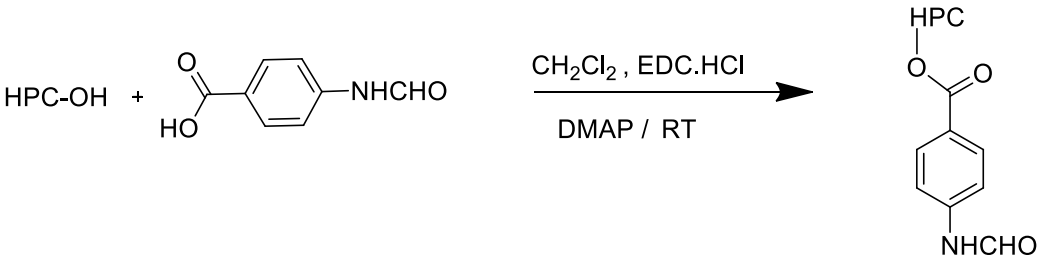


Figure 23 Coupling reaction between HPC & protected PABA (4-*f*-PABA).

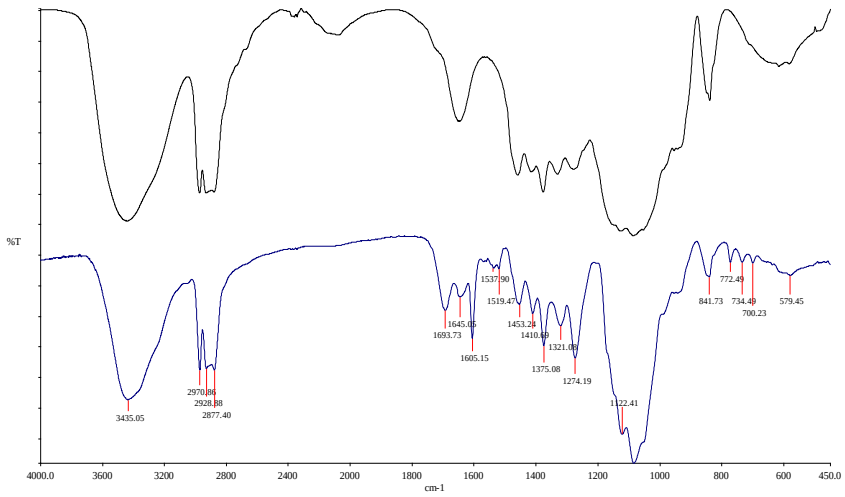


Figure 24 FT-IR spectra of HPC (above) and product form the reaction between HPC and protected PABA (4-*f*-PABA) (below).

Another attempt to use protected PABA was carried out using FMOC as a protecting agent. PABA was first protected with fluorenylmethyloxycarbonyl chloride (FMOC-Cl) and then utilized in the coupling reaction using similar condition as the previous reaction.

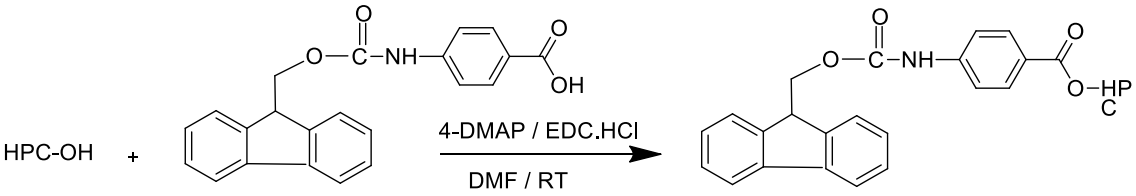


Figure 25 Coupling reaction between HPC & FMOC-protected PABA.

The result was once again proved that no desired product (no ester bond characteristic) was achieved as seen in the FT-IR result as displayed in Fig.26.

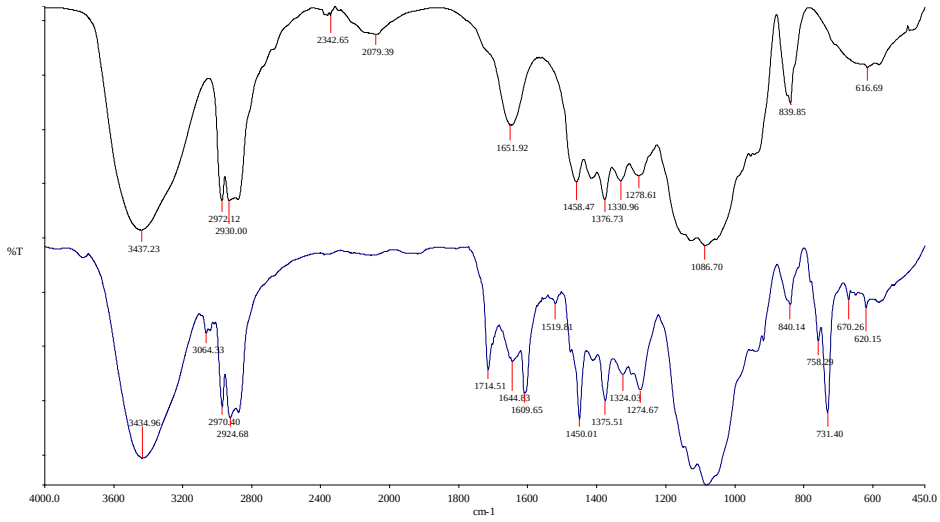


Figure 26 FT-IR spectra of HPC (above) and product form the reaction between HPC and FMOC-protected PABA (below).

Finally, a novel and the simplest synthesis route of HPC-PABA ester conjugate was adopted for achieving the macromolecular carrier with primary aromatic amino functionality. HPC was first modified with chloroacetyl chloride to provide chloroacetate groups (Fig.27) using similar process to the previously report with some modification (Jantas et al. 2007).

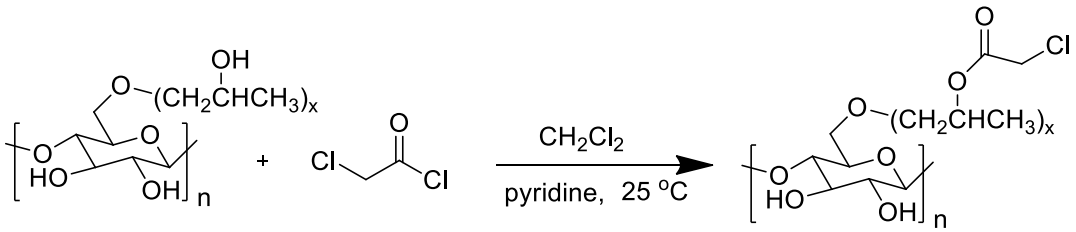


Figure 27 Reaction scheme for the synthesis of chloroacetylated HPC.

The coupling of bioactive carboxylic acid of PABA to HPC functionalized with chloroacetate groups was carried out by using the sodium-*p*-amino benzoate in DMSO (Fig.28). It was a novel methodology for the synthesis of a macromolecular carrier with primary amino functionality and interestingly there was no need of any

coupling agent and base for completing this type of esterification reaction. Highly pure HPC-PABA ester conjugate in 88.0 %yield was fabricated by this elegant method because the impurity (NaCl) was soluble in distilled water, hence removed simply by dialysis. In this strategy there were no hard challenges like complete reduction of nitro group or complete deprotection of protected amino group that were faced in the beginning of this research work. Regarding solubility, HPC-PABA ester conjugate was soluble in many organic solvents like DMSO, DMA, DMF, THF, MeOH and EtOH, but it was not soluble in water.

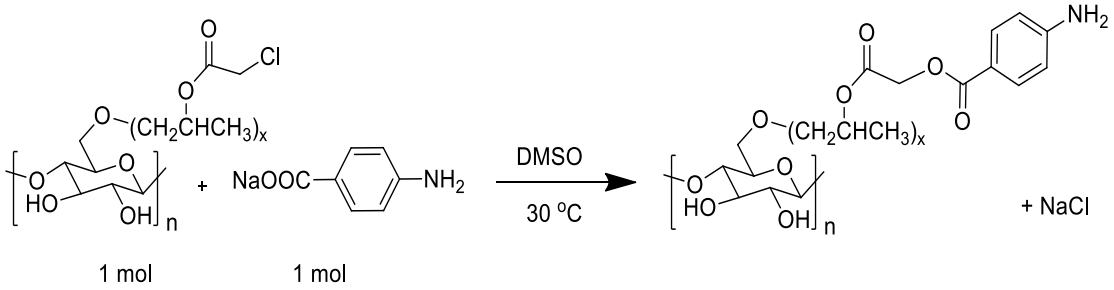


Figure 28 Synthesis scheme for the preparation of HPC-PABA ester conjugate.

Figures 29a-c show the FT-IR spectra of unmodified HPC (Fig. 29a), partially modified HPC with chloroacetate groups (Fig. 29b), and HPC-PABA ester conjugate (Fig. 29c). Unmodified HPC (Fig. 29a) shows broad transmission bands at 3600-3100 $cm^{-1}$of hydroxyl stretching vibration. This band is obviously reduced after modification in the spectrum of the obtained chloroacetylated HPC (Fig. 29b) . Moreover, FT-IR of chloroacetylated HPC displays new characteristic bands of the attached ester groups appeared at 1754 cm^{-1} belongs to the stretching vibration of carbonyl of the ester, at 1754 cm^{-1} belongs to –C-O stretching vibration, and at 781 $cm^{-1}$of -CH_2Cl. In addition, the FT-IR spectrum of HPC-PABA ester conjugate (Fig. 29c) demonstrated the absorption bands of –C=O stretching vibration at 1757 cm^{-1} (HPC-O-**C=O**) and a new band at 1711 cm^{-1} of -CH_2-O-**C=O**. Moreover, since the final product

of HPC-PABA ester conjugate contained 4-aminobenzoate moiety, therefore the characteristic peaks of primary amine which shows two N–H stretching vibration at 3357 and 3244 cm^{-1}, C–N stretching at 1281cm^{-1}, N–H bend at 1619 cm^{-1} and -C=C, and C-H in the benzene ring at 1605 and 771 cm^{-1} were observed. The FT-IR results indicated that the desired product was successfully obtained by this procedure.

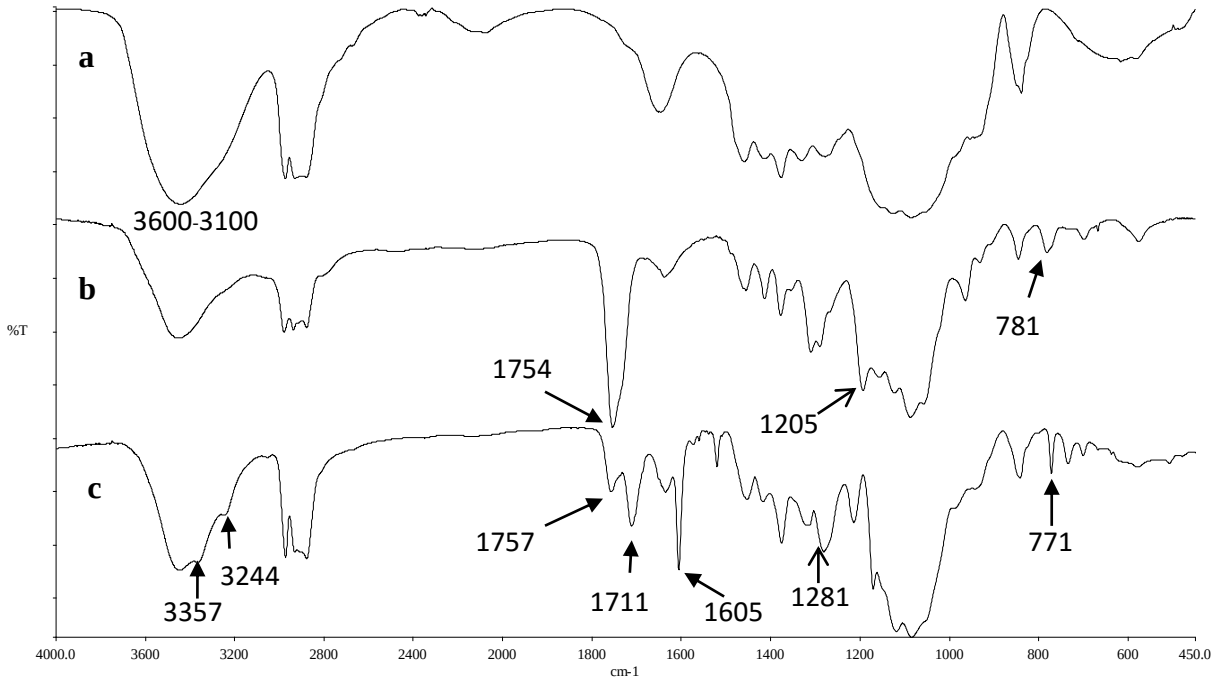


Figure 29 FTIR spectra of: HPC (a), Chloroacetylated HPC (b), HPC-PABA ester conjugate (c).

The structure of modified HPC was confirmed by ^{1}H NMR spectra also in DMSO-d_6 and displayed in Fig.30. The peak at 2.49 ppm is due to DMSO-d_6. The spectrum of HPC-PABA ester conjugate shows broad peaks at 1.0 – 1.25 ppm from the methyl groups in the hydroxypropyl moieties. Peaks in a range of 3.19 – 4.2 ppm belong to protons of cyclic glucose units. The characteristics resonance peaks at 7.67 and 6.59 ppm are ascribed to the protons of aromatic moiety of PABA. The peak at 5.86 ppm belongs to protons of the primary amino group of PABA and a characteristic

peak of protons of –CO-**CH**$_2$-O- appeared at 4.72 ppm. A tiny peak detected at 4.95 ppm belongs to the resonance of the methine protons of modified HPC. Both FT-IR and ^{1}H-NMR characterization results confirmed that HPC-PABA ester conjugate was synthesized successfully and could be obtained in purified form.

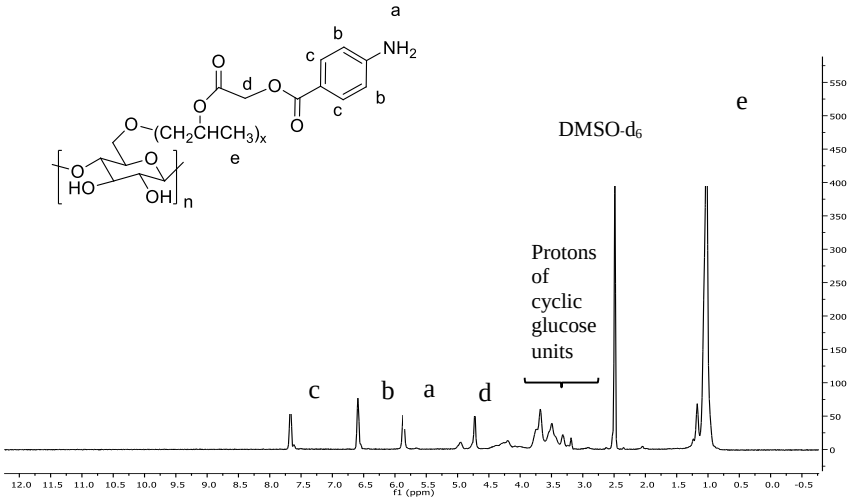


Figure 30 ^{1}H NMR spectra of HPC-PABA ester conjugate (500 MHz, in DMSO-d$_6$)

HPC-PABA ester conjugate contains primary amine functionality for the synthesis of final product i.e. HPC-PABA-NN-SA azo conjugate. For this purpose diazonium salt of HPC-PABA ester conjugates was prepared in 3 N HCl and SA was attempted to couple under basic conditions. During diazotization process HPC-PABA ester conjugates was in solution form in 3 N HCl and ester bond between HPC and PABA was broken due to strong acid strength and we could not successful in achieving the final product.

The resulting product was further utilized for the synthesis of HPC-PABA-NN-SA conjugates. The reaction was performed by using similar process as described by Wiwattanapattpee and co-workers (2003). The diazotization reaction was carried out by first dissolving HPC-PABA ester conjugate in DMSO and then used in the reaction with sodium nitrite in the presence of HCl at 3-5 °C followed by additional of salicylic acid. After the reaction, the reaction was neutralized by adding NaOH solution. The product obtained was finally purified by dialysis against distilled water

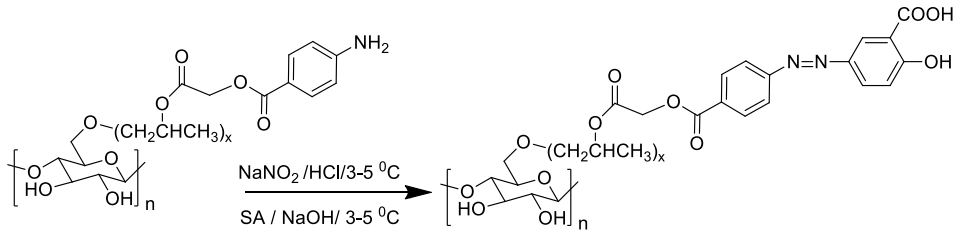


Figure 31 The synthesis scheme of HPC-PABA-NN-SA conjugates by using diazotization reaction between HPC-PABA ester conjugate and salicylic acid.

It was unfortunately after the reaction, the purified product was subjected to FT-IR analysis and found that no azo bond characteristic was observed. Moreover, the ester bond which was belonging to the HPC-PABA ester conjugate at 1757 cm^{-1} was also disappeared. The result indicated that under the strong acid condition, the ester bond of HPC-PABA ester conjugate was cleaved out. Therefore, by this method HPC-PABA-NN-SA conjugates were not successfully obtained.

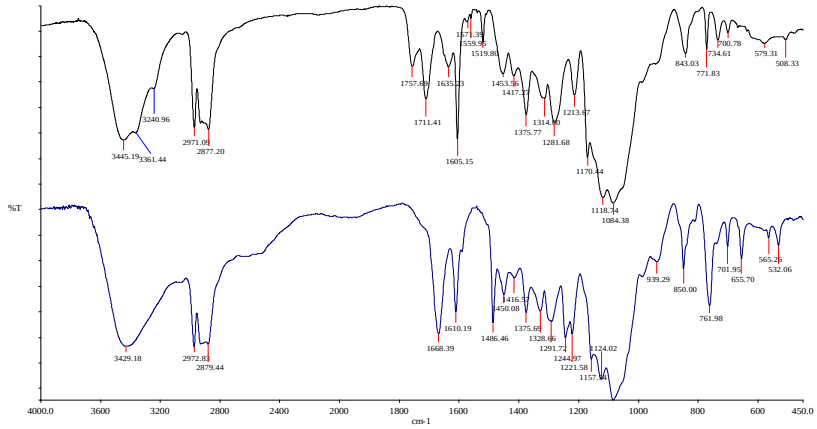


Figure 32 FTIR spectra of: HPC-PABA ester conjugate (above) and the product from the diazotization reaction with salicylic acid (below).

4. **Final synthesis route of HPC-PABA-NN-SA conjugates**

Finally, the synthesis route of HPC-PABA-NN-SA conjugates was adopted and are summarized in Figure 33.

Four HPC-PABA-NN-SA conjugates were successfully synthesized by modifying HPC (Mw ~80,000) by varying the molar ratios of chloroacetate groups. The hydroxyl groups of HPC were kept constant but on the other hand the feeding ratio of chloroacetate groups were varied such as 1:0.25 (**1**), 1:0.5 (**2**), 1:0.75 (**3**) and 1:1 (**4**) and light yellow color products (**1**), (**2**), (**3**), and (**4**) were obtained with good percentage yield (Table 3) i.e. 87.8, 82.09, 78.67 and 75.2 % respectively. The products were characterized by FT-IR. In the final process, the coupling of bioactive

carboxylic acid from para position of PABA-SA to HPC functionalized with chloroacetate groups was carried out by using the sodium salt of PABA-SA in DMSO. It was a novel methodology for the synthesis of a macromolecular azo conjugate and interestingly there was no need of any coupling agent and base for completing this type of coupling reaction.

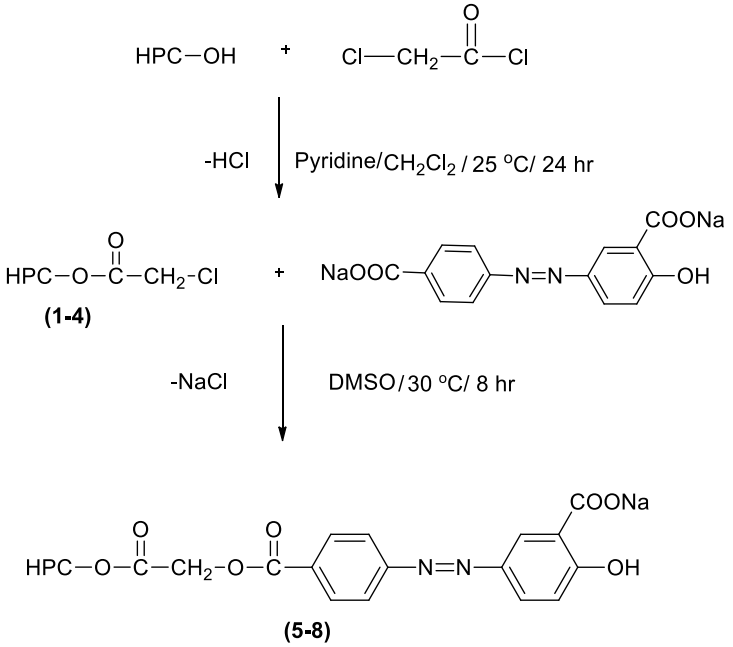
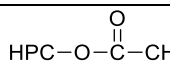
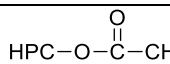
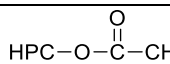
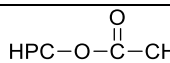
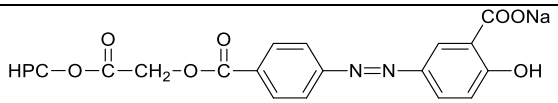
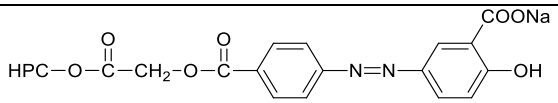


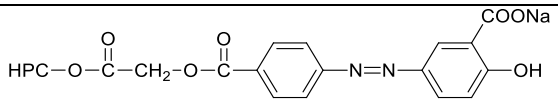
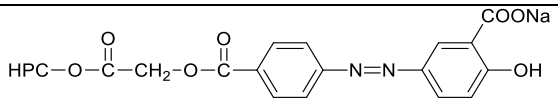
Figure 33 Synthesis scheme of HPC-PABA-NN-SA conjugates (**5-8**).

Preliminary experiments of coupling reaction suggested that sodium salt of PABA-SA is a better choice for the grafting of small azo compound onto the polymers as compared to carboxylic acid form. In case of salts of carboxylic acid, there is no complex reactive intermediate and after completing the reaction, the excessive PABA-SA can easily remove by dialysis due to good solubility in water. Highly purity of HPC-PABA-NN-SA azo conjugates was fabricated by this elegant method because the impurity (NaCl) was soluble in distilled water, hence removed simply by dialysis. Orange to deep reddish brown color HPC-PABA-NN-SA [**5** (from **1**), **6** (from **2**), **7** (from

3) and **8** (from **4**)] macromolecular azo conjugates (Fig. 34) were successfully achieved with good percentage yield i.e. 83.7, 81.89, 82.04 and 79.77 % respectively (Table 3). The SEM images of HPC-PABA-NN-SA (**5-8**) were observed and their images are shown in Fig. 35. The compact structure of HPC was changed to loose fiber like structure containing varied size of pores. All the HPC-PABA-NN-SA (**5-8**) conjugates were hydrolyzed to detach the PABA-SA azo moiety to calculate the percentage drug loading and the quantity of 5-ASA attached to the polymer was determined by HPLC. The attachment of drug was increased as the molar ratio of chloroacetyl chloride was increased to HPC and 5-ASA was calculated i.e. 7.7 (**5**), 9.8 (**6**), 11.14 (**7**) and 13.69 % (**8**) (Table 4).

Table 3 The %yields of the obtained products from the synthesis route of HPC-PABA-NN-SA conjugates

Entry	Product	% yield
1	 (**1**) From HPC: chloroacetate, 1:0.25 by mole)	87.8
2	 (**2**) From HPC: chloroacetate, 1:0.5 by mole)	82.1,
3	 (**3**) From HPC: chloroacetate, 1:0.75 by mole)	78.7
4	 (**4**) From HPC: chloroacetate, 1:1 by mole)	75.2
5	 (**5**) From **1**	83.7
6	 (**6**) From **2**	81.9

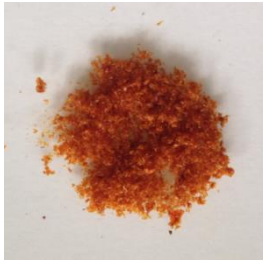
7		(**7**) From **3**	82.0
8		(**8**) From **4**	79.8

(a) HPC MW.80,000

(b) HPC-PABA-N=N-SA [**5**]

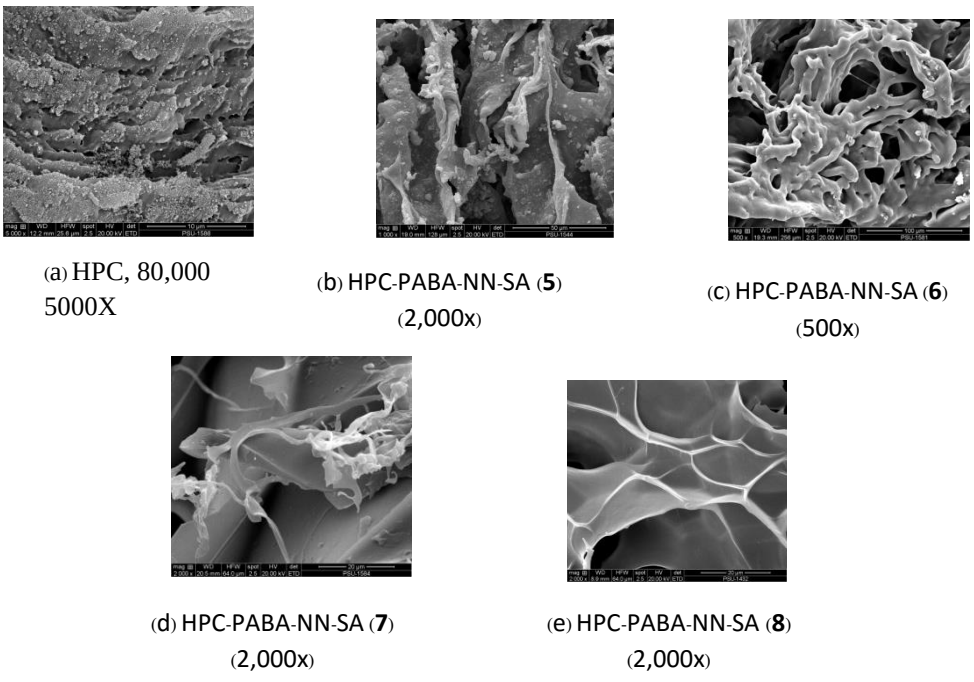
(c) HPC-PABA-N=N-SA [**6**]

(d) HPC-PABA-N=N-SA [**7**]



(e) HPC-PABA-N=N-SA [**8**]

Figure 34 Pictures of HPC (a) and HPC-PABA-N=N-SA conjugates (**5**-**8**) which were obtained from chloroacetylated-HPC (**1**, b), (**2**, c), (**3**, d) and (**4**, e), respectively.



(a) HPC, 80,000 5000X

(b) HPC-PABA-NN-SA (**5**) (2,000x)

(c) HPC-PABA-NN-SA (**6**) (500x)

(d) HPC-PABA-NN-SA (**7**) (2,000x)

(e) HPC-PABA-NN-SA (**8**) (2,000x)

Figure 35 SEM images of HPC (a) and HPC-PABA-N=N-SA conjugates (**5**-**8**) which were obtained from chloroacetylated-HPC (**1**, b), (**2**, c), (**3**, d) and (**4**, e), respectively.

Table 4 Drug loading capacity of 5-ASA (% w/w) which attached to the polymer determined by HPLC.

Entry	Product	5-ASA loading capacity (%W/W)
1	**5**	7.7
2	**6**	9.8
3	**7**	11.14
4	**8**	13.69

Figure 36 (a-c) shows the FT-IR spectra of unmodified HPC, partially modified HPC with chloroacetate groups and HPC-PABA-NN-SA azo conjugate. As can be seen, in the spectrum of chloroacetylated HPC (Fig. 34b), new, characteristic bands of the attached ester groups appeared at 1754 cm^{-1} (C=O) and at 781 cm^{-1} (-CH_2Cl). On the other hand, the band of hydroxyl groups in the range 3600 -3200 cm^{-1} partly disappeared. Moreover, in the spectrum of HPC-PABA-NN-SA azo conjugate (Fig. 34c) the absorption bands appeared at 1772 cm^{-1} (C=O), 1720 cm^{-1} (C=O), 1680 and 775 cm^{-1} (C=C, C-H in the benzene ring respectively), and the weak peak of azo (N=N) group appeared at 1483 cm^{-1} further confirmed the successful synthesis.

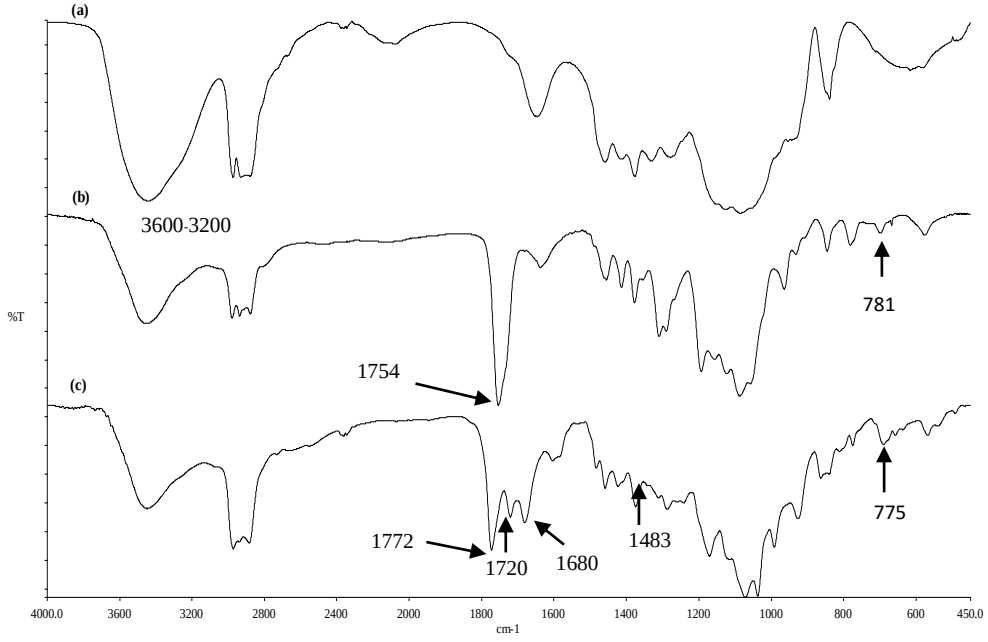


Figure 36 FTIR spectra of HPC (a), Chloroacetylated HPC (**4**) (b) and HPC-PABA-NN-SA conjugate (**8**) (c).

In the ^{1}H NMR spectrum of HPC-PABA-NN-SA azo conjugates (**5**) (Fig. 37), the characteristics resonance peaks from 7.11 ppm to 8.39 ppm are ascribed to the protons of azo moiety of PABA-SA. The characteristic peak of protons of -$COCH_2$-O- group appeared at 4.3 ppm. A tiny peak detected at 4.95 ppm belongs to the resonance of the methine protons of modified HPC. The peaks at 1.17 and 1.0 ppm are ascribed to methyl groups of the hydroxypropyl group after and before esterification and all other protons of HPC were merged in the broad multiplets from 3.19 to 4.0 ppm. Both FT-IR and ^{1}H NMR results confirmed that HPC-PABA-NN-SA azo conjugates were synthesized and characterized successfully.

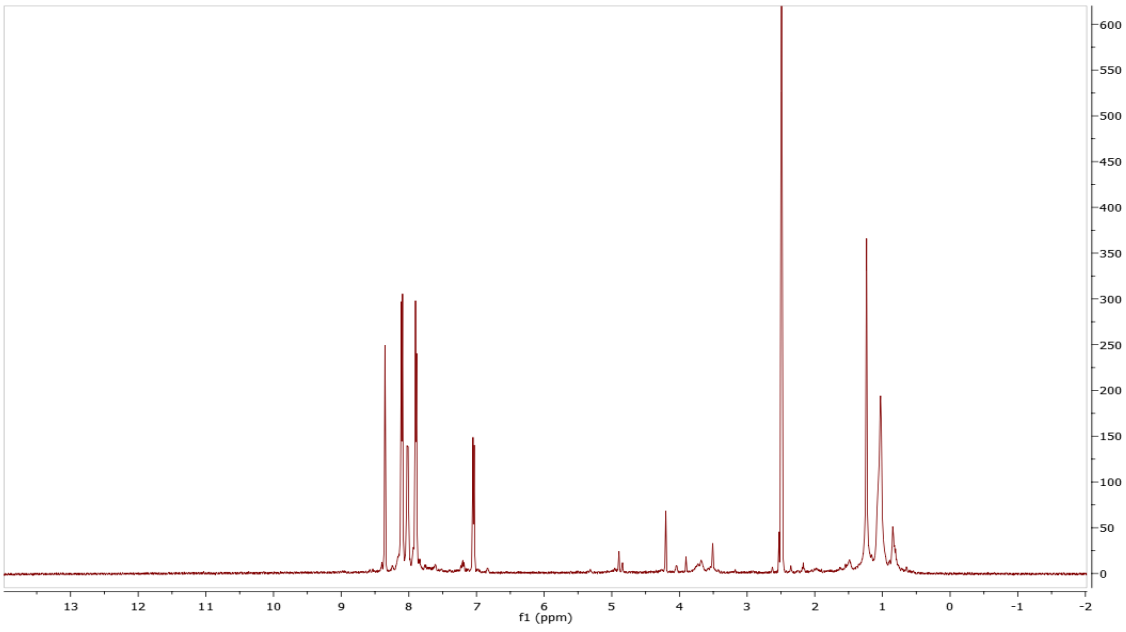


Figure 37 ^{1}H NMR spectra of HPC-PABA-NN-SA conjugate (**5**) (500 MHz, in DMSO-d_6).

5. Physicochemical properties of HPC-PABA-NN-SA conjugates

X-ray powder diffraction was used as a rapid analytical technique for phase identification and classification of materials in this study. Samples which have crystallinity property show a crystallinity pattern consisting of a series of sharp peaks, amorphous materials, however, produce broad background signals. The physical state of PABA-SA, HPC and HPC-PABA-NN-SA (**8**) was assessed by XRD technique. X-ray powder diffraction spectra of the samples were obtained at room temperature using an X-ray diffractometer (Philips X' Pert MPD, Netherlands) with Cu as an anode material and graphite monochromator operated at a voltage of 40 kV. The samples were analyzed in a 2θ angle range from 20-80° and the process parameters were set as a step size of 0.05° (θ) and scan step time of 1.0 second. The powder X-ray diffractometry pattern of pure PABA-SA shows a number of distinctive sharp peaks at 8.27, 9.90 13.22, 14.27, 15.56, 16.76, 18.71, 24.76, 26.70, 27.43, 35.30, 37.98, 41.98 and 50.25 (2θ) indicates crystalline structure (Fig 38a). In contrast to the results of X-ray powder diffraction patterns of pure HPC (Fig 38b) and PABA-NN-SA azo conjugate (Fig 38c) which amorphous state characteristic was observed. The result indicated that PABA-SA after conjugation to the chloroacetylated HPC, changed its crystallinity to amorphous state. Moreover, this demonstrated that no small molecules either PABA-SA, PABA or SA were remained in the conjugated product.

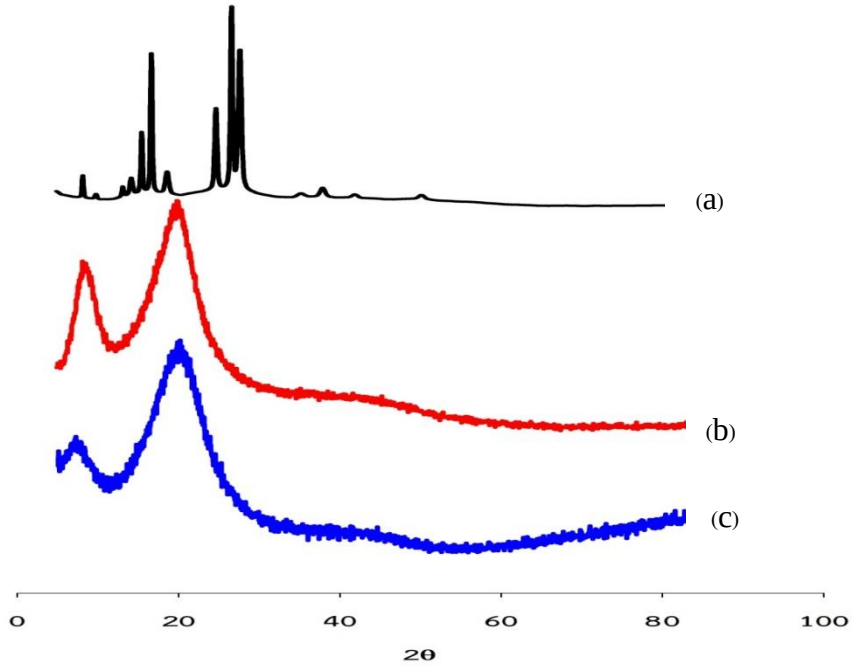


Figure 38 X-ray diffraction patterns of PABA-SA (a), HPC (b), HPC-PABA-NN-SA (**8**) (c)

Differential scanning calorimetry (DSC) is a thermal analysis technique used to measure changes in heat flows associated with material transition. DSC measurements can provide both qualitative and quantitative information on endothermic (heat absorption) and exothermic (heat evolving) process. DSC is commonly utilized to determine the glass transition temperature and crystalline melting point of polymeric material. In this study thermograms of pure 5-ASA, HPC, and HPC-PABA-NN-SA (**8**) were obtained using DSC7, Perkin Elmer, USA. The sample of 2-4 mg was accurately weighed into an aluminum pan with a cover sealed. The measurements were performed under nitrogen purge over 50-500 °C at a heating rate of 10 °C/min. The DSC thermogram of pure 5-ASA shows a melting endotherm at about 283 °C (Fig 39). It was however, no sharp peak in the DSC thermograms of pure HPC and HPC-PABA-NN-SA (**8**) were observed. It is therefore, both DSC and XRD results are confirming that after coupling PABA-NN-SA azo conjugate with HPC, it changed

from crystalline to amorphous state and no free PABA-NN-SA azo conjugate was detected in HPC-PABA-NN-SA (**8**) conjugate.

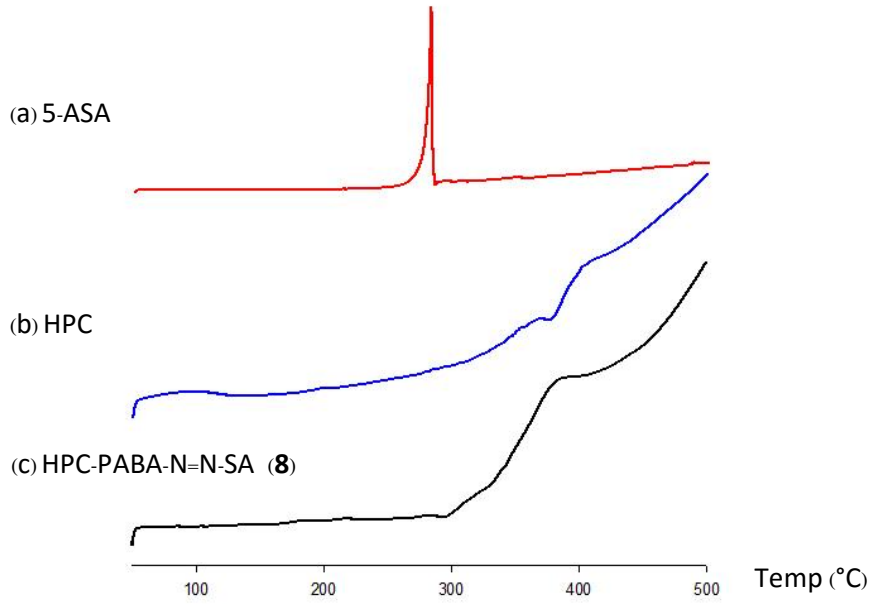


Figure 39 DSC thermograms of (a) HPC-PABA-NN-SA (**8**), (b) 5-ASA and (c) HPC.

For the designing and synthesis of newly developed polymer-drug conjugates, the thermal stability is an important consideration for attaining the longer shelf life of the therapeutic agent. While synthesis the polysaccharide-5-ASA conjugates, usually increased stability of the targeted molecule is required. Therefore, HPC-PABA-NN-SA conjugate was investigated by thermal analysis to find the difference in stability of pure 5-ASA and in conjugated form. Thermogravimetric analyzer, TGA7, Perkin, Elmer, USA was used for the measurement of thermogravimetric (TG) and differential thermogravimetric (DTG) curves. The measurements were performed under nitrogen purge over 50-500 °C at a heating rate of 10 °C/min. The overlay of TG and DTG curves of 5-ASA, HPC and HPC-PABA-NN-SA (**8**) conjugate are shown in Fig. 40 and Fig. 41, respectively.

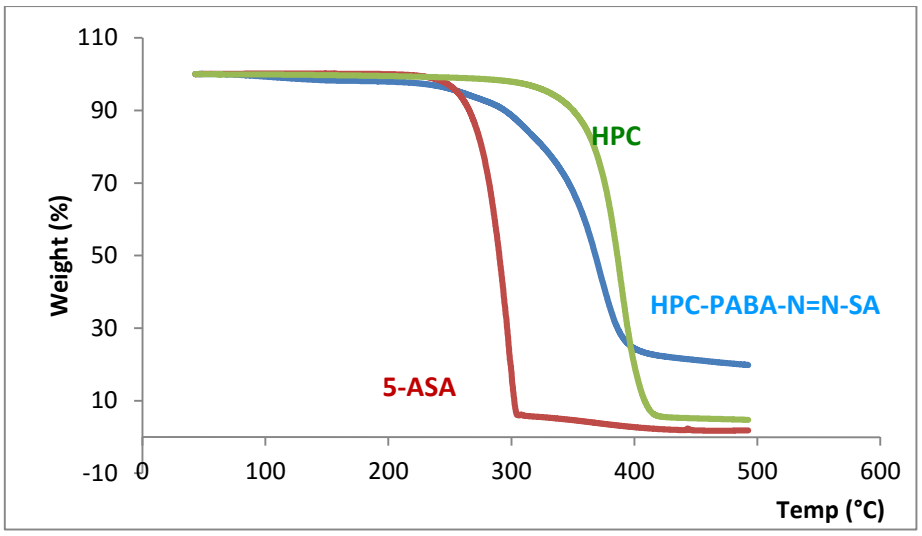


Figure 40 Overlay TG curves of 5-ASA, HPC and HPC-PABA-NN-SA conjugate (**8**).

The thermogravimetric analysis of 5-ASA, HPC and HPC-PABA-NN-SA (**8**) conjugate has shown that thermal stability of 5-ASA is increased significantly in conjugated form as compared to pure drug (Fig. 40). Thermal decomposition of HPC-PABA-NN-SA (**8**) starts at 293 °C (T_{di}) and ends at 438 °C with thermal decomposition maxima (T_{dm}) at 352 °C which is considerably higher than the thermal degradation of pure drug. In case of 5-ASA thermal decomposition ends at 337 °C with thermal decomposition maxima (T_{dm}) at 276 °C which is significantly lesser than the thermal degradation of conjugate and pure HPC thermal decomposition ends at 365 °C.

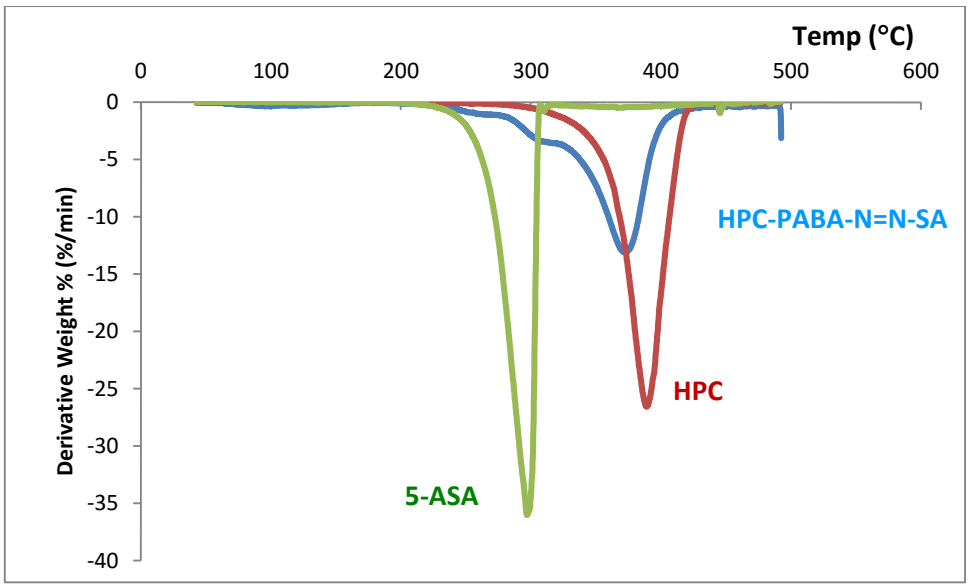


Figure 41 Overlay DTG curves of 5-ASA, HPC and HPC-PABA-NN-SA conjugate (**8**).

6. Chemical stability studies of HPC-PABA-NN-SA conjugates (5-8) in physiological conditions.

Four HPC-PABA-NN-SA conjugates (**5-8**) were designed with the participation of two ester and one azo bonds for colon specific delivery and for this purpose these three chemical bonds should be stable while passing through the stomach and small intestine. The in vitro stability study was investigated by incubating all the conjugates in phosphate buffer solutions of pH 1.2, pH 6.8 and pH 7.4. In order to evaluate the stability of the conjugates, samples were quantified during 24 hr experiments by HPLC analysis. It was observed that no free 5-ASA and PABA-SA were detectable, which confirmed that all the conjugates were chemically stable at pHs 1.2, 6.8 and 7.4.

7. 5-ASA Release Studies

In order to make sure the actual potential of the conjugates to release the 5-ASA into the colonic area, an in vitro release study was conducted by using the stomach, intestinal and colonic contents of male Wistar rats. The release profiles of the HPC-PABA-NN-SA (**5-8**) conjugates were determined up till to 24 hr after incubation at 37 °C with the gastrointestinal contents of the rat.

It was a remarkable comparison release study between low molecular weight molecule of commercially available sulfasalazine and macromolecular prodrug HPC-PABA-NN-SA synthesized in this work. Interestingly both sulfasalazine and HPC-PABA-NN-SA conjugates (**5-8**) were not soluble in water, but before mixing to the rat contents under nitrogen atmosphere, the homogenous solution of sulfasalazine was prepared by using few drops of 0.1 N NaOH and HPC-PABA-NN-SA conjugates were used heterogeneously. On incubation of sulfasalazine or conjugates (equivalent to 0.58 mg of 5-ASA) with the homogenate of the stomach content, no 5-ASA or PABA-SA was detected during a 24 hr period, which indicated that these HPC-PABA-NN-SA conjugates were chemically stable with the stomach contents of the rat. It was also clarified that the availability of azoreductases were not enough in the stomach to trigger the drug release from sulfasalazine or macromolecular conjugates.

When both sulfasalazine and macromolecular conjugates were incubated with the homogenate of the small intestine for 24 hr, it was clearly observed that 5-ASA could not release in the first 4 hr from sulfasalazine and in case of macromolecular conjugates, no 5-ASA was found during the first 6 hr of incubation period (Fig. 42). In intestinal content, 5-ASA was found slowly released after 4 hr from sulfasalazine and finally reached 48.74 % at about 24 hr after incubation. All the HPC-PABA-NN-SA conjugates (**5-8**) started to give release after 6 hr and the % release of 5-ASA was

calculated highest i.e. 31.22 % with the lowest drug attachment (**5**) and similarly **6**, **7** and **8** showed release 26.65, 22.38 and 17.23 % respectively after 24 hr incubation period.

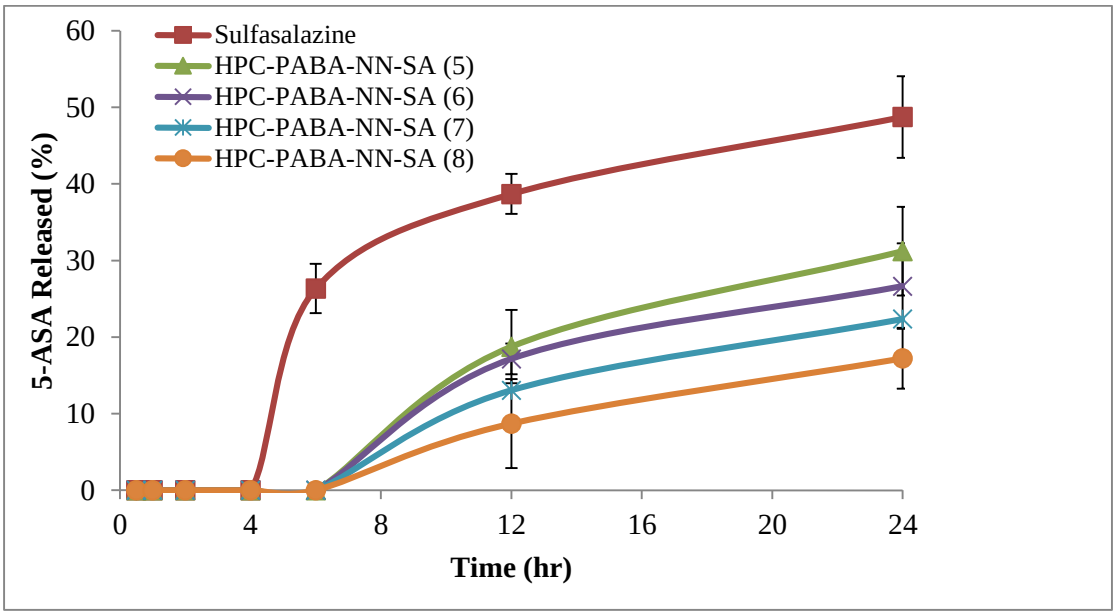


Figure 42 Release profiles of 5-ASA from the conjugates (**5**-**8**) and sulfasalazine during incubation with rat intestinal content at 37 °C. Error bars represent standard deviation, n = 3.

☐ The release profiles of all samples in intestinal and colonic contents during 24 hr were subjected to the kinetics analysis using Sigma Plot program version 12. The models used in this study were first order, Hixon and Crowell, Higuchi, Baker and Lonsdale and Korsemeyer-peppas models. The analysis results are summarized in Tables 5 and 6. The results revealed that in intestinal content sulfasalazine and all HPC-PABA-N=N-SA conjugates (**5**-**8**) fit well with the first order kinetics in which the release rate of the active drug is depend on its concentration. However, in colonic content sulfasalazine and all HPC-PABA-N= N-SA conjugates (**5**-**8**) fit well with Korsemeyer-peppas model. Sulfasalzine and HPC-PABA-N= N-SA conjugates (**5**-**7**)

showed Fickian diffusion release pattern whereas HPC-PABA-N=N-SA conjugates (**8**) was in non- Fickian diffusion release pattern. This indicated that the release of 5-ASA from sulfasalazine would be a result from azo-bond reduction only, while, the release of 5-ASA from HPC-PABA-N= N-SA conjugates (**5**-**8**) more than one type of release phenomena could be involved (Peppas *et al.*, 1980, Costa and Lobo, 2001, Dash *et al.*, 2010, Siepmann and Peppas, 2012).

Table 5 The kinetic releasing analysis results of sulfasalazine and HPC-PABA-N=N-SA conjugates (**5**-**8**) in intestinal content.

Sample	First order		Hixon and Crowell		Higuchi model		Baker and Lonsdale		Korsemeyer-peppas model		
	r^2	k	r^2	k	r^2	k	r^2	k	r^2	K	n
Sulfasalazine	0.8765	0.0384	0.8712	0.0115	0.7414	10.1869	0.7131	0.0020	0.8543	4.2053	0.8429
(**5**)	0.8699	0.0166	0.8780	0.0053	0.6214	5.0773	0.6039	0.0005	0.0000	8.70×10^{-8}	4.0710
(**6**)	0.8683	0.0142	0.8741	0.0046	0.6230	4.4285	0.6082	0.0003	0.0000	1.03×10^{-7}	3.6226
(**7**)	0.8793	0.0114	0.8847	0.0037	0.6203	3.6090	0.6081	0.0002	0.0000	5.35×10^{-8}	4.3901
(**8**)	0.8874	0.0083	0.8921	0.0027	0.6114	2.6702	0.6022	0.0001	0.0000	2.48×10^{-9}	2.6000

Table 6 The kinetic releasing analysis results of sulfasalazine and HPC-PABA-N=N-SA conjugates (**5**-**8**) in colonic content.

Samples	First order		Hixon and Crowell		Higuchi model		Baker and Lonsdale		Korsemeyer-peppas model		
	r^2	k	r^2	k	r^2	k	r^2	k	r^2	K	n
Sulfasalazine	0.0000	2.0418	0.0000	0.0636	0.0000	26.1241	0.0000	0.0275	0.7796	75.8076	0.0326
(**5**)	0.7936	0.0669	0.6923	0.0183	0.9715	14.5508	0.9884	0.0048	0.9830	16.7670	0.4427
(**6**)	0.5237	0.0456	0.4055	0.0129	0.9412	11.9042	0.9774	0.0031	0.9887	15.3662	0.3960
(**7**)	0.3749	0.0349	0.2707	0.0102	0.9106	10.1300	0.9457	0.0021	0.9727	13.4712	0.3836

(**8**)	0.8204	0.0255	0.7861	0.0077	0.9174	7.8920	0.9119	0.0012	0.9272	6.6474	0.5683

In colonic content, 5-ASA released from sulfasalazine was in higher amount and faster than in the intestinal contents. The release in the colonic content demonstrated that 5-ASA released from sulfasalazine was up to 65.32 % after incubation within the first thirty minutes and the total amount of 5-ASA released within 24 hr was up to 74.17 %. In case of PAMAM conjugates the release of 5-ASA from sulfasalazine was up to 80.2 % after incubation within the first 6 hr and the % release was higher as compared to our results and it may be due to variation in quantity of colonic microflora and higher water solubility property of their products (Wiwattanapatapee *et al.*, 2003). The release of 5-ASA from the synthesized HPC-PABA-NN-SA conjugates (**5**-**8**) showed a better release as compared to intestinal content could be due to higher number of azoreductase enzymes in the colonic region. However, the released amount of the drug from each conjugate was different. All the HPC-PABA-NN-SA conjugates (**5**-**8**) started to give release after thirty minutes of incubation period and it remained continuous release up to 24 hr. All the conjugates presented almost similar and sustained release profiles and the highest quantity of 5-ASA released from the conjugate **5** was found 56.58 % after 24 hr incubation period. HPC-PABA-NN-SA conjugates **6**, **7** and **8** showed release 45.16, 38.23 and 32.0 % respectively after 24 hr incubation period (Fig. 43).

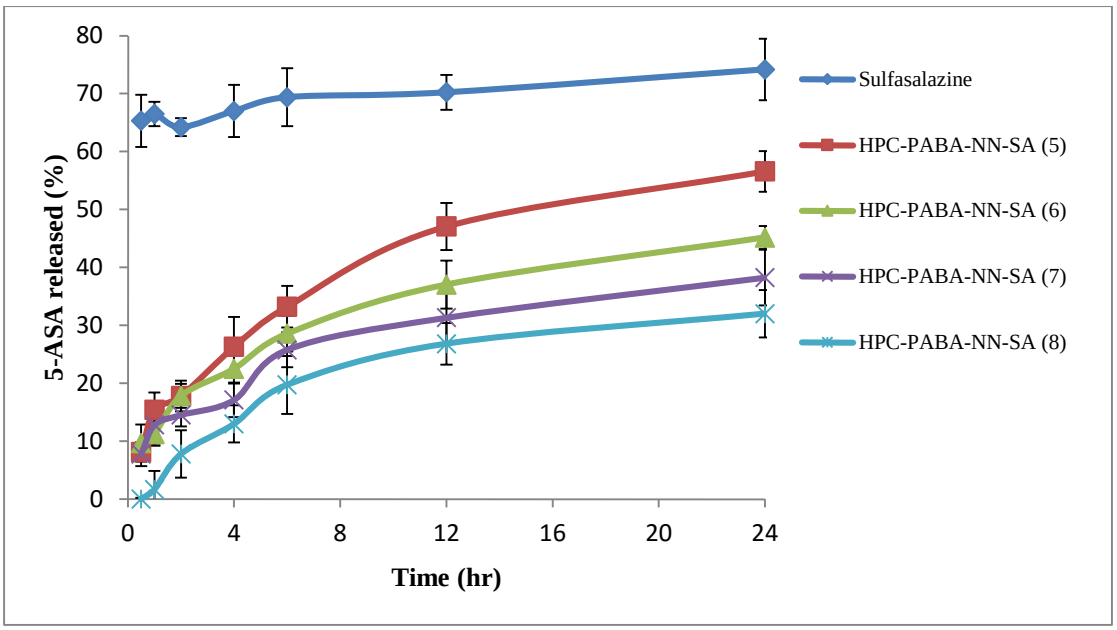


Figure 43 Release profiles of 5-ASA from the conjugates (**5-8**) and sulfasalazine during incubation with rat colonic content at 37 ^{o}C. Error bars represent standard deviation, n = 3.

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According to release study results demonstrated that all the HPC-PABA-NN-SA conjugates (**5-8**) could release 5-ASA in both intestinal and colonic contents but not in the gastric content of the rat. Moreover, the ester bonds of HPC-PABA-NN-SA conjugates (**5-8**) remained stable during stability and release studies since no PABA spacer was detected in all samples during incubation period. That could be due to insolubility of these conjugates or a steric effect of the bulky HPC moiety that esterase enzymes could not breakdown the ester bonds and 5-ASA was only released after the reduction of azo bond by the azoreductase enzyme. The mechanism of the release of 5-ASA from all HPC-PABA-NN-SA samples was proposed in Fig. 44. After the azo bond cleavage by azoreductase, active drug (5-ASA) was then released while PABA was still attached with the acetylated moiety on HPC.

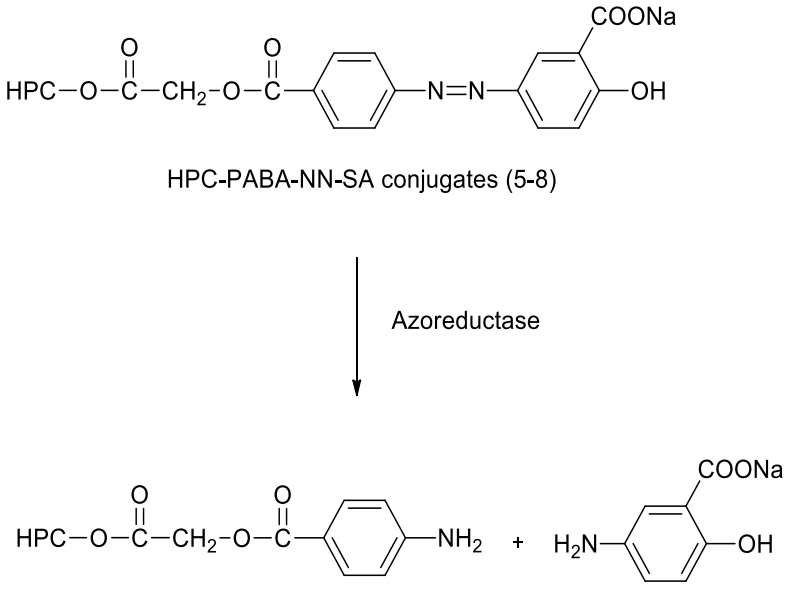


Figure 44 Proposed release mechamism of 5-ASA from HPC-PABA-NN-SA conjugates (**5-8**) in colonic content by azoreductase cleavage.

8. Synthesis of HPC-5-ASA-5-ASA conjugates (17-19)

Another group of HPC-5-ASA conjugates were aimed to synthesis, however, 5-ASA was used as a spacer instead of PABA.

First HPC-5-ASA ester conjugated was synthesized (Fig. 45) using similar process as described by Zou and co-worker (2005) by using 4-*N*-formyl-5aminosalicylic acid (4-*f*-5-ASA) to couple with HPC using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC.HCl) as a condensating agent and 4-(dimethylamino)pyridine (DMAP) as a base to provide the desired product. However, after deprotection process, no desired product was achieved. That may be due to a strong acid condition may cleave the obtained ester bond. It is therefore another approach was chosen to get the desired product. The similar procedure as the previously been used for the preparation of HPC-PABA-NN-SA was adopted to

conjugate 5-ASA to HPC. This part of the work was performed by using HPC with MW of 100,000.

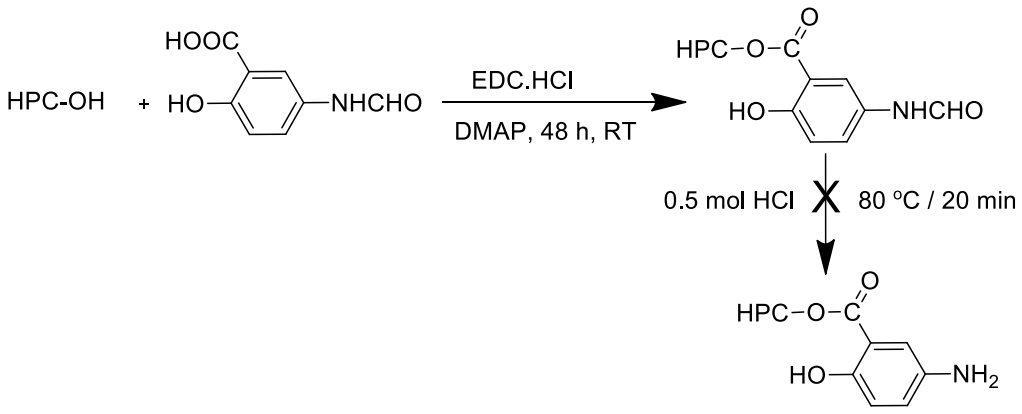


Figure 45 Synthesis scheme for the preparation of HPC-5-ASA ester conjugated by coupling reaction and de-protection processes.

The final synthesis route of HPC-5-ASA-5-ASA conjugates is shown in Figure 46.

Four HPC-5-ASA ester conjugates were synthesized by modifying HPC (Mw ~100,000) using varying the molar ratios of chloroacetate groups. The hydroxyl groups of HPC were kept constant but on the other hand the molar ratio of chloroacetate groups were varied such as 1:0.25 (**9**), 1:0.5 (**10**), 1:0.75 (**11**) and 1:1 (**12**).

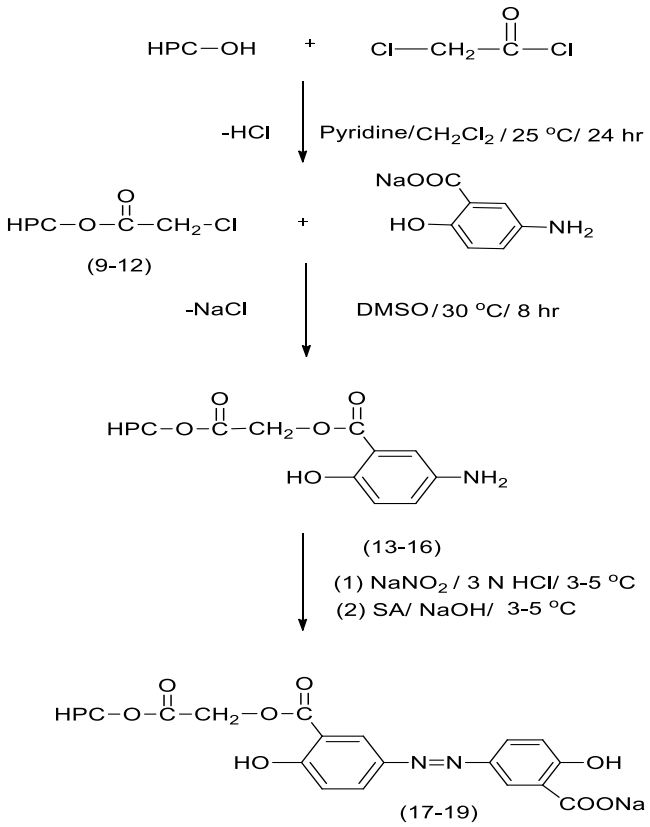


Figure 46 Synthesis scheme of HPC-5-ASA-5-ASA conjugates (**17**-**19**).

After the purification process, light yellow color products (**9**-**12**) were obtained with good percentage yield i.e. 89.6, 85.2, 87.9 and 82.4 %, respectively (Table 7).

Table 7 The %yields of the obtained chloroacetylated-HPC (MW.100,000) with different ratios of HPC and chloroacethyl chloride.

Entry	Product	% yield
1	HPC—O—C(=O)—CH2-Cl (**9**) From HPC: chloroacetate, 1:0.25 by mole)	89.6
2	HPC—O—C(=O)—CH2-Cl (**10**) From HPC: chloroacetate, 1:0.5 by mole)	85.2,
3	HPC—O—C(=O)—CH2-Cl (**11**) From HPC: chloroacetate, 1:0.75 by mole)	87.9

4	HPC−O−C(=O)−CH_2-Cl (**12**) From HPC: chloroacetate, 1:1 by mole)	82.4

5-ASA was selected as the spacer in this study due to its safety, suitability of functional groups and as the compound of choice for the treatment of IBD. It holds carboxylic group for coupling with polymer by esterification and amino group can be employed for the diazotization process with salicylic acid (SA) to give rise another molecule of 5-ASA. The coupling of bioactive carboxylic acid of 5-ASA to HPC functionlized with chloroacetate groups was carried out by using the sodium salt of 5-ASA in DMSO. This was a novel coupling technique of 5-ASA with HPC which could be performed just only in two steps without help of any coupling agent and base. Preliminary experiments of coupling reaction of 5-ASA with HPC were attempted by Zou and co-workers (2005). In their synthesis scheme consists of four steps, we followed the same scheme but in the final step i.e. deprotection of HPC-5-f-ASA, we could not find the optimum conditions for removing the protecting group (formic acid) completely. Many attempts have been made to find the optimum conditions such as time, strength of acid and temperature but characterizations by FT-IR and ^{1}H NMR did not support us. Sodium salt of 5-ASA was a better option for grafting onto polymer as compared to its carboxylic acid form. In case of salts of carboxylic acid, there is no complex reactive intermediate and after completing the reaction, the excessive 5-ASA can easily remove by dialysis due to good solubility in water. Highly purity of HPC-5-ASA ester conjugates (**13**-**16**) were fabricated by this efficient method because the impurity (NaCl) was soluble in distilled water, hence removed easily by dialysis. Dark brown color HPC-5-ASA ester conjugates [**13** (from **9**), **14** (from **10**), **15** (from **11**) and **16** (from **12**)] were successfully obtained with good percentage yield i.e. 84.1, 88.9, 83.4 and 81.6 %, respectively (Table 8).

Table 8 The %yields of the obtained HPC-acetylated-5-ASA (MW.100,000) obtained from **9**-**12**.

Entry	Product	% yield
1	**13** (from **9**)	84.1
2	**14** (from **10**),	88.9
3	**15** (from **11**),	83.4
4	**16** (from **12**),	81.6

Olsalazine comprises two molecules of 5-ASA that are linked covalently through an azo bond similar to that in sulfasalazine. After reduction of azo bond by azoreductases in colon, each mole of olsalazine provides two mole of 5-ASA. Olsalazine is usually used for the management of patients with ulcerative colitis but 5-10 % patients cannot tolerate it due to severe diarrhea (Wadworth and Fitton, 1991). In our research, we bring a new idea to develop an olsalazine like product based on polymer i.e. one molecule of 5-ASA previously linked with HPC can be further employed for coupling another molecule of 5-ASA by an azo bond. For this purpose diazonium salt of HPC-5-ASA ester conjugates (**13**-**16**) were prepared in 3 N HCl and SA was coupled under basic conditions to develop a dimer of 5-ASA. During diazotization process only HPC-5-ASA conjugate **13** was in solution form in 3 N HCl but the other three conjugates i.e. no **14**-**16** behaved like a suspension. HPC-5-ASA conjugate no **13** was completely soluble in 3 N HCl due to the lowest drug attachment i.e. 6.4 % only and that is why ester bond between HPC and 5-ASA was broken during diazotization reaction. In case of HPC-5-ASA conjugates **14**-**16** were successfully coupled during diazotization process and the purified reddish brown HPC-5-ASA-5-ASA conjugates [**17** (from **14**), **18** (from **15**), **19** (from **16**)] were achieved by lyophilization with good percentage yield i.e. 76.7, 73.9 and 71.6 %, respectively (Fig. 47 and Table 9).

Table 9 The %yields of the obtained HPC-5-ASA-5-ASA conjugates (**17**-**19**) (MW.100,000) obtained from **14**-**16**.

Entry	Product	% yield
1	**17** (from **14**)	76.7
2	**18** (from **15**),	73.9
3	**19** (from **16**),	71.6

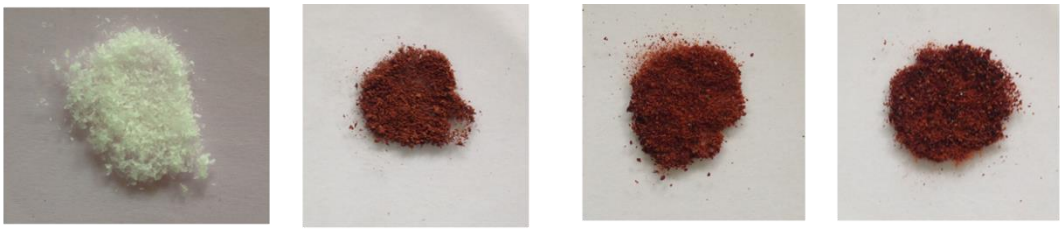


Figure 47 Pictures of HPC-5-ASA-5-ASA conjugates (17-19) obtained from the synthesis process.

The micro structural morphology of the HPC-5-ASA-5-ASA conjugates (**17-19**) was optimized and the SEM images are displayed in Fig.48. The obtained products showed different morphology when compared with the image of HPC (Fig.35). The HPC-5-ASA-5-ASA conjugates displayed loose network morphology containing varying sizes of pores. The most dense network was observed in the SEM image of 19 that could be due to the highest drug loading capacity (result in the below section) as well as the highest content of 5-ASA spacer.

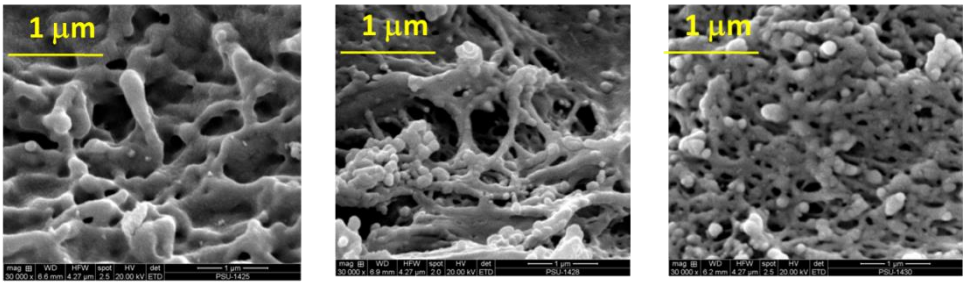


Figure 48 SEM images of HPC-5-ASA-5-ASA conjugates (**17**-**19**)

Figure 49 (a-d) shows the comparative FT-IR spectra of unmodified HPC (a), partially modified HPC with chloroacetate groups of **12** (b), HPC-5-ASA ester conjugate of **16** (c) and HPC-5-ASA-5-ASA azo conjugate of **19** (d). It can be observed that in the spectrum of chloroacetylated HPC (Fig. 27b), new characteristic band of carbonyl group (C=O) appeared at 1754 cm^{-1} and -CH_2Cl group at 781 cm^{-1}. On the other hand, the band of hydroxyl groups of HPC in the range 3600 - 3200 cm^{-1} partly disappeared. Moreover, in the spectrum of HPC-5-ASA ester conjugate (Fig. 27c) the absorption bands appeared at 1683 and 786 cm^{-1}, which resulted from scissoring vibrations bands of -C=C- and C-H in the benzene ring. The peak of -CH_2Cl group at 781 cm^{-1} completely disappeared, it means that all the chloroacetate groups were consumed during esterification reaction. In case of HPC-5-ASA-5-ASA azo conjugate (Fig. 27d), the azo (N=N) group weak peaks were appeared at 1593 & 1457 cm^{-1}.

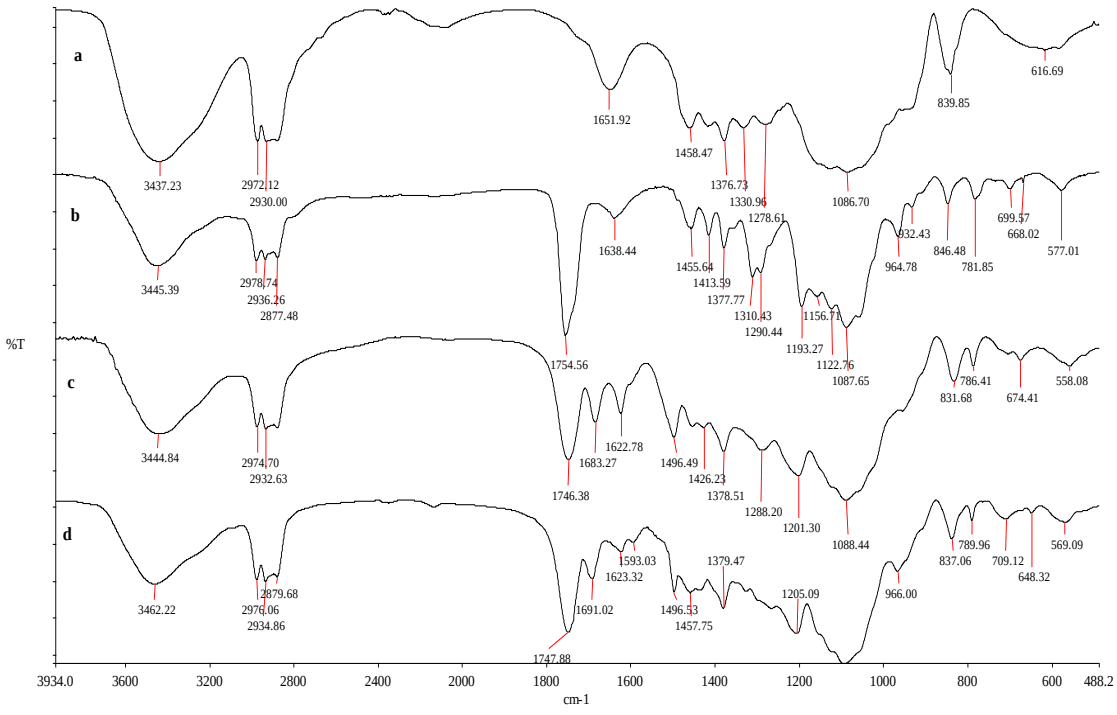


Figure 49 The comparative FT-IR spectra of HPC (a), Chloroacetylated HPC (b) HPC-5-ASA ester conjugate (c) and HPC-5-ASA-5-ASA azo conjugate (d).

In the ^{1}H NMR spectrum of HPC-5-ASA-5-ASA azo conjugate (**17**), the multiplet characteristics resonance peaks from 6.8 ppm to 8.4 ppm are ascribed to the protons of azo moiety of 5-ASA-5-ASA (Fig. 50). The characteristic peak of protons of –COO-CH_2-O- group appeared at 4.3 ppm. A tiny peak detected at 5.0 ppm belongs to the resonance of the methine protons of modified HPC. The peaks at 1.2 and 1.0 ppm are ascribed to methyl groups of the hydroxypropyl group after and before attachment of 5-ASA and all other protons of HPC were merged in the broad multiplets from 3.2 to 4.0 ppm. FT-IR and ^{1}H NMR results supported that HPC-5-ASA-5-ASA azo conjugates were synthesized and characterized successfully.

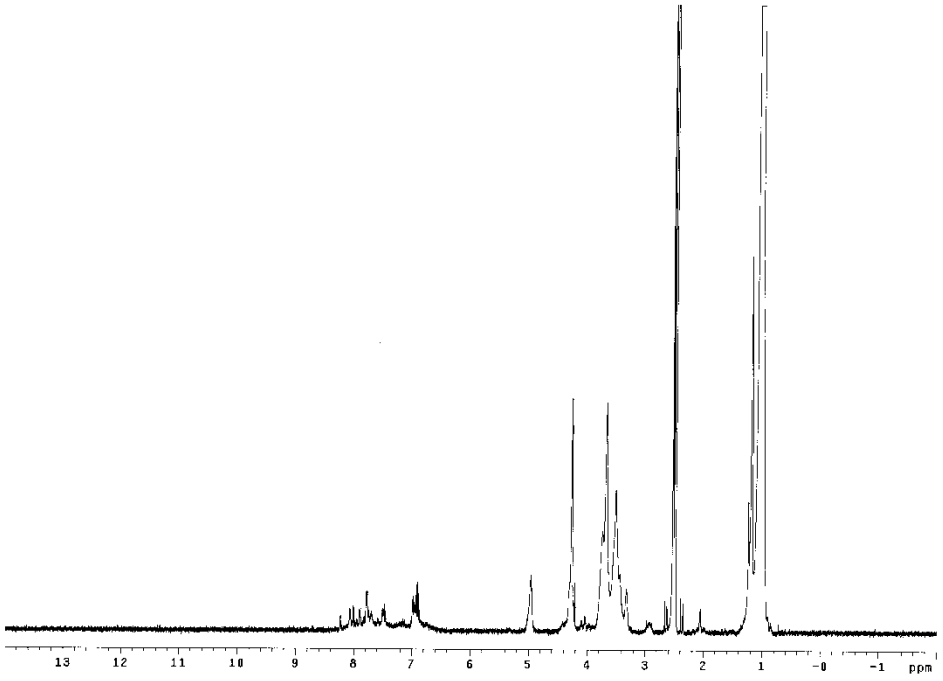


Figure 50 ^{1}H NMR spectra of HPC-5-ASA-5-ASA conjugate (**17**) (500 MHz, in DMSO-d_6).

In this study differential scanning calorimetry (DSC) thermograms of pure 5-ASA, HPC, HPC-5-ASA-5-ASA (**18**) and (**19**) were obtained using DSC7, Perkin Elmer, USA. The sample of 2-4 mg was accurately weighed into an aluminum pan with a cover sealed. The measurements were performed under nitrogen purge over 50-500 °C at a heating rate of 10 °C/min. The DSC thermograms of all samples are displayed in (Fig 51). Pure 5-ASA shows a melting endotherm at about 283 °C, it was however, no sharp peak in the DSC thermograms of pure HPC and HPC-5-ASA-5-ASA (**18**) and (**19**) were observed. This result is therefore indicated that after coupling 5-ASA with HPC to give HPC-5-ASA-5-ASA azo conjugate, it changed from crystalline to amorphous state and no free 5-ASA was remain in the conjugates.

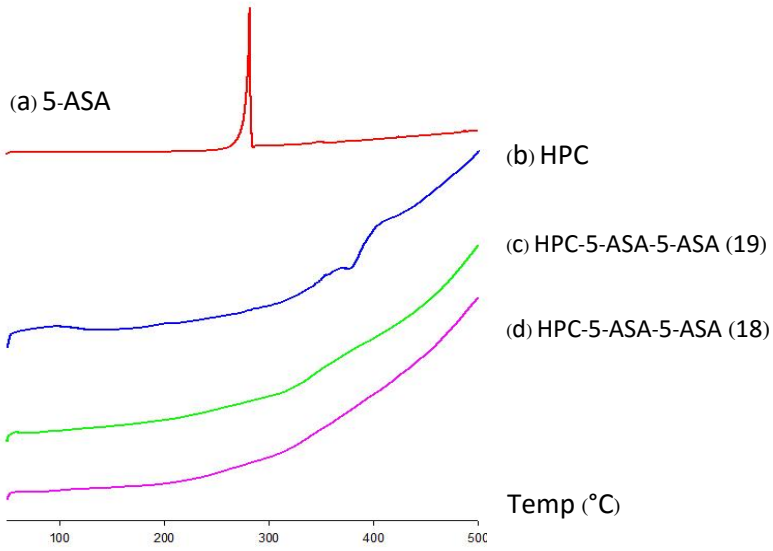


Figure 51 DSC thermograms of pure 5-ASA, HPC, HPC-5-ASA-5-ASA (**18**) and (**19**)

Thermal properties of HPC-5-ASA-5-ASA conjugates were evaluated by thermal analysis using thermogravimetric analyzer, TGA7, Perkin, Elmer, USA. The measurements were performed under nitrogen purge over 50-500 °C at a heating rate

of 10 °C/min. The overlay of TG and DTG curves of pure 5-ASA, HPC, HPC-5-ASA-5-ASA (**18**) and (**19**) conjugates are shown in Fig. 52 and 53, respectively.

The thermogravimetric analysis is normally used for determination degradation properties of polymer (Reich and Levi, 1967). In this study the thermogravimetric analysis of HPC-5-ASA-5-ASA (**18**) and (**19**) conjugates demonstrated that thermal stability of 5-ASA increased significantly in conjugated form as compared to pure drug (Fig. 52). However, thermal decomposition of HPC-5-ASA-5-ASA (**18**) and (**19**) started earlier than 5-ASA and HPC but ending later than 5-ASA at almost 400 °C which is considerably higher than the thermal degradation of pure drug. In case of 5-ASA thermal decomposition ends at 337 °C with thermal decomposition maxima (T_{dm}) at 276 °C which is significantly lesser than the thermal degradation of conjugate and pure HPC thermal decomposition ends at 365 °C.

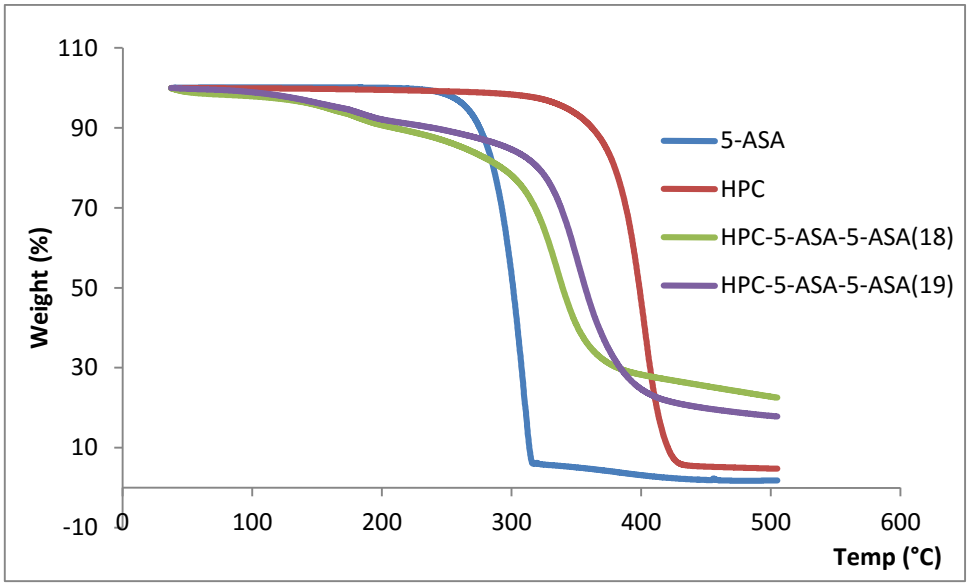


Figure 52 Overlay TG curves of pure 5-ASA, HPC, HPC-5-ASA-5-ASA (**18**) and (**19**)

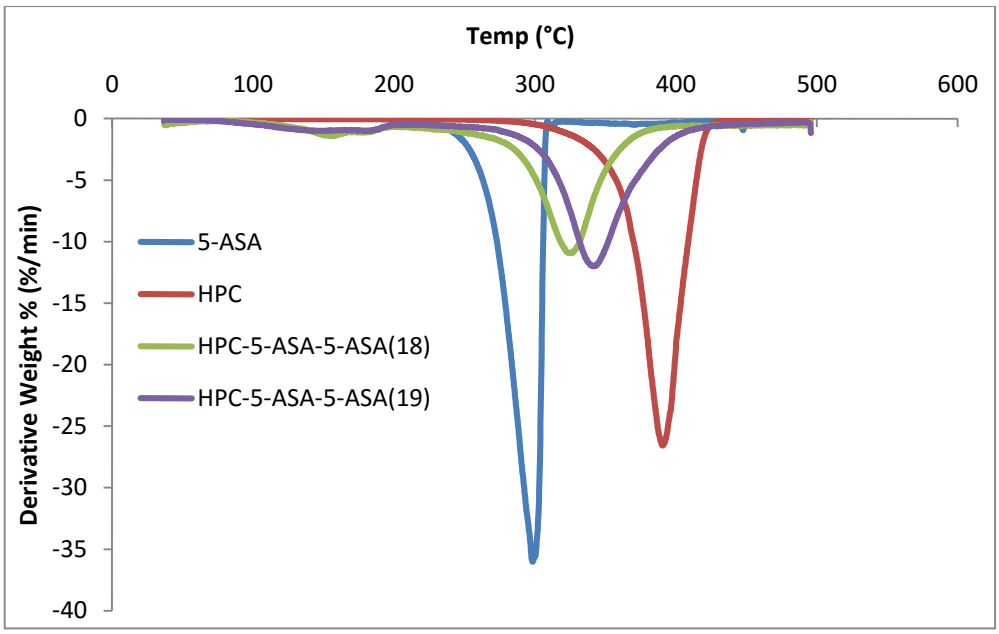


Figure 53 Overlay DTG curves of pure 5-ASA, HPC, HPC-5-ASA-5-ASA (**18**) and (**19**)

The quantity of 5-ASA content in the polymer conjugates was estimated by detaching the olsalazine moiety from the polymer conjugates after alkaline hydrolysis. Each conjugate (100 mg) was placed in 1 N NaOH solution (100 ml) and stirred at 50 °C for 2 hr. An aliquot of the clear solution (0.1 ml) was further diluted with mobile phase and 5-ASA was calculated by measuring the amount of olsalazine by HPLC at 348 nm (Jung *et al.*, 1998). The synthesized olsalazine was used as the standard comparison. The standard solutions of olsalazine were prepared in a concentration ranging from 2-10 μg/ml and analyzed by HPLC. The attachment of drug was found increasing as the molar ratio of chloroacetyl chloride was increased to HPC and the quantity of 5-ASA was calculated to give 10.4 (**17**), 14.7(**18**) and 18.6 % (**19**), respectively (Table 10).

Table 10 Drug loading capacity of 5-ASA (% w/w) which attached to the HPC-5-ASA-5-ASA conjugates (**17**-**19**) determined by HPLC.

Entry	Product	5-ASA loading capacity (%W/W)
1	**17**	10.4
2	**18**	14.7
3	**19**	18.6

9. HPC-5-ASA-5-ASA Conjugates Chemical Stability Studies

Three HPC-5-ASA-5-ASA conjugates (**17**-**19**) were designed with the participation of two-ester and one azo bond for colon specific delivery and for this purpose these three chemical bonds should be stable while passing through the stomach and small intestine. The in vitro stability study was investigated by incubating all the conjugates in phosphate buffer solutions of pH 1.2, pH 6.8 and pH 7.4. In order to evaluate the stability of the conjugates, samples were quantified during 24 h experiments by HPLC analysis. It was observed that no free 5-ASA and 5-ASA-NN-5-ASA small molecule of azo conjugate were detectable, which confirmed that all the conjugates were chemically stable at pHs 1.2, 6.8 and 7.4.

10. 5-ASA Release Studies of HPC-5-ASA-5-ASA conjugates (17-19)

HPC-5-ASA-5-ASA conjugates (**17**-**19**) were hydrolyzed to detach the 5-ASA-NN-ASA azo moiety for determining the percentage drug loading by HPLC. The attachment of drug was increased as the molar ratio of chloroacetyl chloride was increased to HPC and the quantity of 5-ASA was calculated such as 10.4 (**17**), 14.7 (**18**) and 18.6 % (**19**), respectively. In order to ensure the potential of these three macromolecular azo conjugates for colon specific release of 5-ASA, an ex vivo release study was conducted by using the gastrointestinal contents of male Wistar rat. The percentage release of 5-ASA from HPC-5-ASA-5-ASA conjugates (**17**-**19**) were determined within 24 hr period after incubation at 37 ^{o}C with the gastrointestinal contents of the rat.

It was an interesting comparison release study between the low molecular weight commercially available sulfasalazine and macromolecular HPC-5-ASA-5-ASA conjugates that were synthesized in this work. Both sulfasalazine and HPC-5-ASA-5-ASA conjugates (**17**-**19**) were not soluble in water that is why before mixing the rat contents under nitrogen atmosphere, the homogenous solution of sulfasalazine was prepared by using few drops of 0.1 N NaOH and HPC-5-ASA-5-ASA conjugates (**17**-**19**) were used heterogeneously. On incubation of sulfasalazine or conjugates (equivalent to 0.58 mg of 5-ASA) with the homogenate of the stomach, no 5-ASA or small molecule of 5-ASA-NN-5-ASA were detected during a 24 hr period, which proved that these HPC-5-ASA-5-ASA conjugates were chemically stable with the stomach contents of the rat. It was also confirmed that the number of azoreductase enzymes were not enough in the stomach to trigger the drug release from sulfasalazine or macromolecular conjugates.

In case of incubation with the homogenate of the small intestine for 24 hr, it was found that the release of 5-ASA started after 4 hr from sulfasalazine and macromolecular conjugates did not release 5-ASA during the first 6 hr (Fig. 54). The quantity of 5-ASA released from sulfasalazine was found 42.9 % after the incubation period of 24 hr. HPC-5-ASA-5-ASA conjugates (**17**-**19**) started to give release after 6 hr and the % release of 5-ASA was calculated highest i.e. 26.9 % with the lowest drug attachment (**17**) and the conjugates **18** and **19** showed release 20.8 and 14.3 % respectively after 24 hr incubation period.

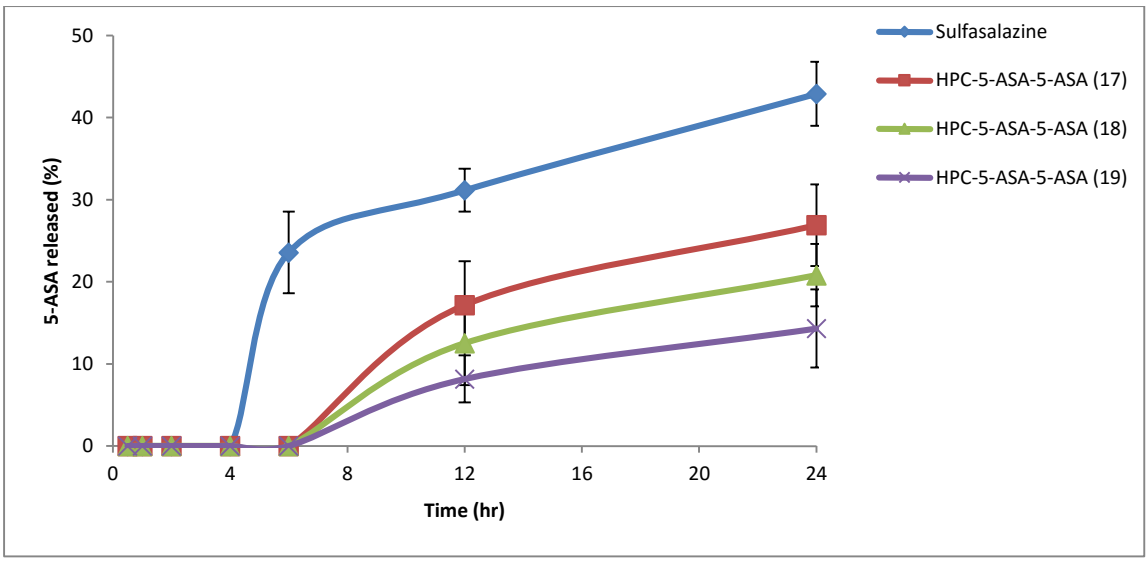


Figure 54 Release profile of 5-ASA from the conjugates (17-19) and sulfasalazine during incubation with rat intestinal content at 37 ºC. Error bars represent standard deviation, n = 3.

It can be monitored that 5-ASA was released from both sulfasalazine and macromolecular conjugates in the intestinal segment of the rat and it can be due to availability of microflora which produce azoreductase enzyme such as *Bacteroides* and *Clostridium* (Rafii *et al.*, 1990). Therefore, the reduction of azo bond can be possible in small intestine. It was authenticated by Wiwattanapatapee and co-workers in 2003 that the azoreductases cleaved the azo bond of the PAMAM conjugates in

small intestine and 5-ASA was released up till to 7.2 % after 12 h incubation at 37 ^{o}C (Wiwattanapatapee *et al.*, 2003).

In colonic content, 5-ASA released from sulfasalazine was in higher amount and faster than in the intestinal contents. The release in the colonic content demonstrated that 5-ASA released from sulfasalazine was up to 59.97 % after incubation within the first thirty minutes and the total amount of 5-ASA released within 24 hr was up to 66.7 % (Fig. 30). The release of 5-ASA from the synthesized HPC-5-ASA-5-ASA conjugates (**17**-**19**) showed a better release as compared to intestinal content due to higher number of azoreductase enzymes in the colonic region. However, the released amount of the drug from each conjugate was different. All the HPC-5-ASA-5-ASA conjugates (**17**-**19**) started to give release after thirty minutes of incubation period and it remained continuous up to 24 hr. All the conjugates presented almost similar and sustained release profiles and the highest quantity of 5-ASA released from the conjugate **17** was found 38.8 % after 24 hr incubation period. HPC-5-ASA-5-ASA conjugates **18** and **19** showed 29.6 and 22.0 % release after 24 hr incubation period (Fig. 55), respectively.

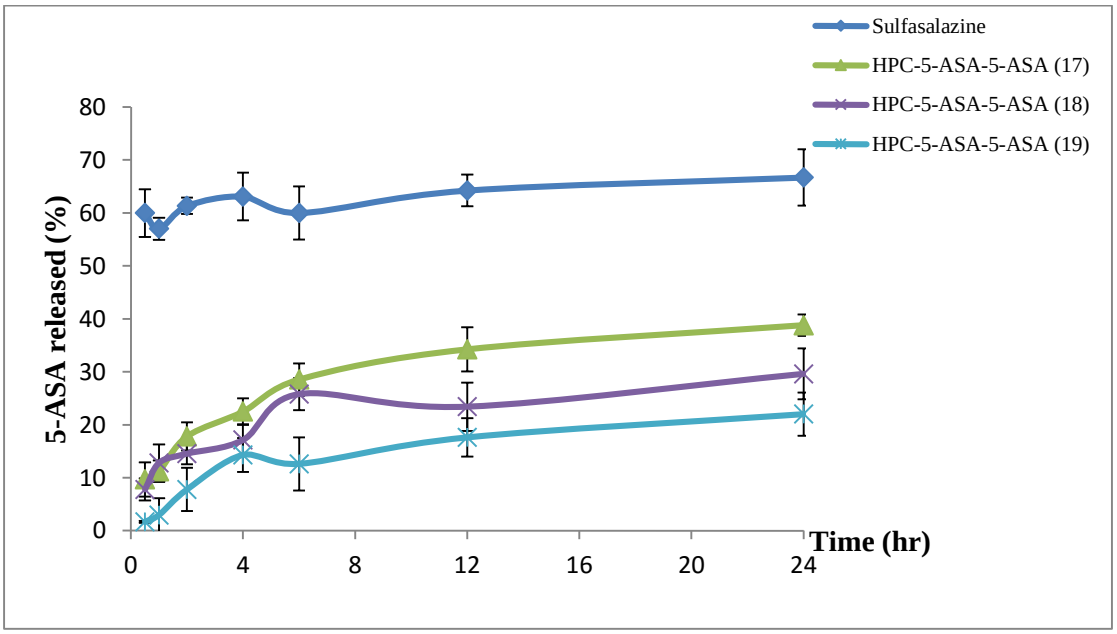


Figure 55 Release profiles of 5-ASA from the conjugates (**17**-**19**) and sulfasalazine during incubation with rat colonic content at 37 ^{o}C. Error bars represent standard deviation, n = 3.

☐ The release profiles of all samples in intestinal and colonic contents during 24 hr were subjected to the kinetics analysis using Sigma Plot program version 12. The models used in this study were first order, Hixon and Crowell, Higuchi, Baker and Lonsdale and Korsemeyer-peppas models. The analysis results are summarized in Tables 11 and 12. The results revealed that in intestinal content sulfasalazine and all HPC-5-ASA-N=N-5-ASA conjugates (**17**-**19**) fit well with the first order kinetics in which the release rate of the active drug is depend on its concentration (Siepmann and Peppas, 2012). However, in colonic content sulfasalazine and all HPC-5-ASA-N=N-5-ASA conjugates (**17**-**19**) fit well with Korsemeyer-peppas model. Sulfasalzine and all HPC-5-ASA-N=N-5-ASA conjugates (**17**-**19**) showed Fickian diffusion release pattern. This indicated that the release of 5-ASA from sulfasalazine would be a result from azo-bond reduction only, while, the release of 5-ASA from, while for HPC-5-ASA-N=N-5-ASA conjugates (**17**-**19**), more than one type of release phenomena could be involved (Peppas *et al.*, 1980, Costa and Lobo, 2001, Dash *et al.*, 2010, Siepmann and Peppas, 2012).

☐

Table 11 The kinetic releasing analysis results of sulfasalazine and HPC-5-ASA-N=N-5-ASA conjugates (**17**-**19**) in intestinal content.

Sample	**First order**		**Hixon and Crowell**		**Higuchi model**		**Baker and Lonsdale**		**Korsemeyer-peppas model**		
	r^2	k	r^2	k	r^2	k	r^2	k	r^2	K	n
Sulfasalazine	0.8797	0.0310	0.8749	0.0094	0.7441	8.7557	0.7199	0.0014	0.8634	3.4611	0.8588

(17)	0.8690	0.0143	0.8750	0.0046	0.6229	4.4561	0.6080	0.0003	0.0000	9.96×10^{-8}	3.6596
(18)	0.8787	0.0106	0.8833	0.0034	0.6214	3.3837	0.6102	0.0002	0.0000	5.84×10^{-8}	4.0570
(19)	0.8868	0.0070	0.8900	0.0023	0.6192	2.2919	0.6116	8.96×10^{-5}	0.0000	3.10×10^{-9}	4.7675

Table 12 The kinetic releasing analysis results of sulfasalazine and HPC-5-ASA-N=N-5-ASA conjugates (**17**-**19**) in colonic content.

Samples	First order		Hixon and Crowell		Higuchi model		Baker and Lonsdale		Korsemeyer-peppas model		
	r^2	k	r^2	k	r^2	k	r^2	k	r^2	K	n
Sulfasalazine	0.0000	1.2958	0.0000	0.0598	0.0000	23.6522	0.0000	0.0467	0.6777	68.8522	0.0314
(17)	0.1240	0.0393	0.0000	0.0112	0.8234	10.9474	0.8932	0.0026	0.9654	16.0647	0.3426
(18)	0.0000	0.0261	0.0000	0.0078	0.5489	8.4951	0.6360	0.0015	0.8955	14.2544	0.2855
(19)	0.5061	0.0162	0.4687	0.0051	0.9036	5.7073	0.9120	0.0006	0.9119	6.4744	0.4491

HPC-5-ASA-5-ASA conjugates (**17**-**19**) were developed by decreasing the molar ratios between HPC and 5-ASA, developing a spacer arm between HPC and 5-ASA and an azo bond was developed between spacer and drug. In our results, all the HPC-5-ASA-5-ASA conjugates (**17**-**19**) released 5-ASA in both intestinal and colonic contents of the rat. The maximum release of 5-ASA from HPC-5-ASA-5-ASA conjugates was supported by a higher number of azoreductases in the colonic region of the rat. Length of the spacer arm between polymer and drug was also helpful in the cleavage of azo bond by enzyme. The cleavage of azo bond in case of HPC-5-ASA-5-ASA conjugates was much slower as compared to sulfasalazine. Sulfasalazine azo bond was easily attacked and promptly reduced by azoreductases, which may be due to two factors that firstly carrier of 5-ASA was a low molecular weight instead of polymer and secondly sulfasalazine was in solution form before mixing the rat contents. Both the ester bonds of HPC-5-ASA-5-ASA conjugates (**17**-**19**) remained stable during stability and release studies and it may be due to insolubility of these conjugates or a steric effect of the bulky HPC moiety that esterase enzymes could not breakdown the ester bonds and 5-ASA was only released after the reduction of azo bon. The release

mechanism of 5-ASA from HPC-5-ASA-5-ASA conjugates (**17**-**19**) is proposed in Fig. 56 in which the release was only from azo-bond cleavage by azoreductase enzyme available in colonic content.

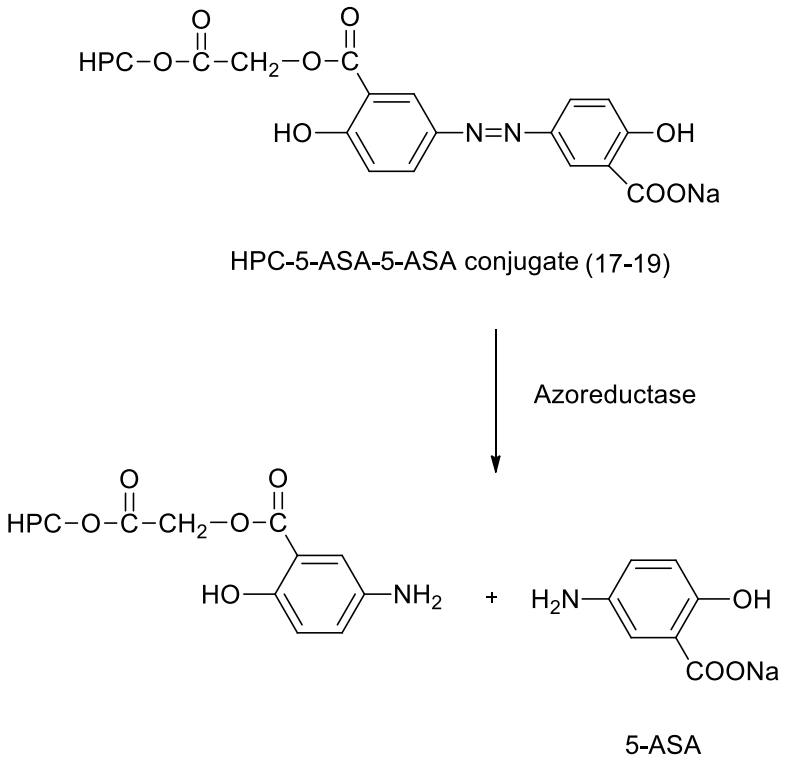
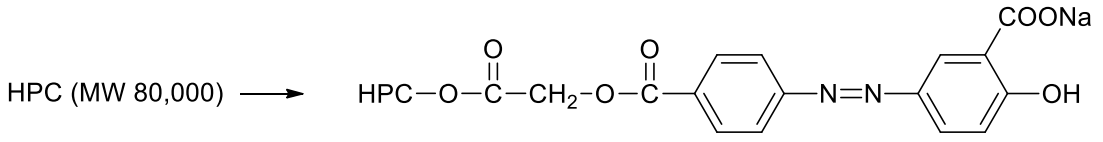


Figure 56 The proposed mechanism of 5-ASA release from HPC-5-ASA-5-ASA conjugates (**17**-**19**).

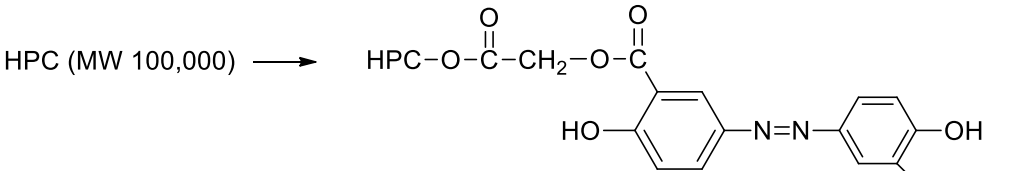
CONCLUSIONS

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. Hydroxypropyl cellulose, HPC, 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. HPC with molecular weight 80,000 & 100,000 was selected as a macromolecular carrier and PABA & 5-ASA was chosen as spacers in this study.

Two types of polymeric conjugates were synthesized with high percentage yields as summarized in the diagram below.



(5-8)



(17-19)

All conjugated products were well characterized by FT-IR and ^{1}H-NMR. For the synthesis of first type of polymeric conjugate i.e. HPC-PABA-NN-SA conjugate (**5-8**), HPC-PABA ester conjugate holding primary amine functionality was synthesized and its diazonium salt was prepared in 3 N HCl and SA was attempted to couple under basic conditions. During diazotization process HPC-PABA ester conjugates was

in solution form in 3 N HCl and ester bond between HPC and PABA was broken due to strong acid strength and we could not successful in achieving the final product. Finally, four HPC-PABA-NN-SA conjugates (**5**-**8**) were synthesized with the participation of two ester and one azo bond and these bonds were found chemically stable at pH 1.2, 6.8 and 7.4 for 24 hours at 37 ^{o}C. For this purpose, HPC was partially modified by varying the molar ratios of chloroacetyl chloride in dried dichloromethane by using pyridine as a catalyst. The hydroxyl groups of HPC were kept constant but on the other hand the feeding ratio of chloroacetate groups were varied such as 1:0.25 (**1**), 1:0.5 (**2**), 1:0.75 (**3**) and 1:1 (**4**). A small azo compound PABA-SA was synthesized and its sodium salt was coupled to HPC functionalized with chloroacetate groups in a homogenous reaction in dimethylsulfoxide (DMSO).

For the synthesis of second type of polymeric conjugates i.e. HPC-5-ASA-5ASA conjugates (**17**-**19**), HPC functionalized with chloroacetate groups was coupled with the sodium salt of 5-ASA and then SA was coupled to the diazonium salt of HPC-5-ASA ester conjugate to develop HPC-5-ASA-5-ASA conjugates. The ester and azo bonds of three HPC-5-ASA-5-ASA conjugates were found chemically stable at pH 1.2, 6.8 and 7.4 for 24 hr at 37 $^{\circ}$C.

The drug release profiles of all 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 of rat (within 1 hr), whereas the HPC conjugates presented the slow release profiles in the first 6 hr and continued to release up to maximum within 24 hr. Moreover, the HPC conjugates and sulfasalazine could also release 5-ASA after 24-hr incubation in intestinal content. The release study results suggested that the less drug attachment with the polymer will provide fast and maximum release as compared to more drug loading onto the polymer. The results from *in vitro* study suggested that all the macromolecular conjugates have a potential

to release 5-ASA in the lower part of the GIT tract in a sustained release profile for the treatment of IBD and HPC has potential for use as colon specific drug carrier.

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APPENDICES

Appendix 1 Reprints of papers and manuscripts

Appendix 2 Analytical HPLC method

Appendix 3 Acceptance letters for papers 1 and 2

Appendix 4 Animal Ethic Approval Document

Appendix 1 Reprints of papers and manuscripts

PAPER 1

Synthesis, Characterization and Reduction of

p-NitrobenzoylHydroxypropyl Cellulose

(Published in *Asian Journal of Chemistry, Vol.27, No. 5 (2015), 1875-1878.*)

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ASIAN JOURNAL OF CHEMISTRY

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Synthesis, Characterization and Reduction of *p*-Nitrobenzoyl Hydroxypropyl Cellulose

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Synthesis and structural characterizations of *p*-nitrobenzoyl and *p*-aminobenzoyl hydroxypropyl cellulose were reported in this work. Nitrobenzoyl-hydroxypropyl cellulose was synthesized in good yield *via* esterification reaction between *p*-nitrobenzoyl chloride and hydroxyl groups of hydroxypropyl cellulose (Mw 80,000 and 100,000) in dried dichloromethane using pyridine as a catalyst. *p*-Nitrobenzoylhydroxypropyl cellulose was then attempted many times for its reduction to *p*-aminobenzoylhydroxypropyl cellulose by using three different reducing agents like sodium dithionite, tin(II) chloride dihydrate and palladium on activated charcoal. The resulting *p*-nitrobenzoyl and *p*-aminobenzoyl hydroxypropyl cellulose were characterized by FT-IR and ^{1}H NMR.

Keywords: Hydroxypropyl cellulose, *p*-Nitrobenzoylhydroxypropylcellulose, *p*-Aminobenzoylhydroxypropyl cellulose.

INTRODUCTION

The purpose of the work described in this paper was the synthesis of hydroxypropyl cellulose (HPC) containing primary aromatic amino groups. In the synthesis of heterocyclic compounds the aromatic amines play a major role. Usually such type of aromatic amines are achieved by the reduction of polymer-supported nitro derivatives[1]. Cellulose derivatives with aromatic NH_2 groups can be used to immobilize enzyme or immunoprotiens *via* diazo coupling in bio-chemical sensor technology[2]. On diazotization NH_2-functionalized cellulose derivatives can couple to many aromatic hydroxyl and aromatic amines that can play a vital role in pharmaceutical and textile sectors[3]. However, the synthesis pathways are frequently very complex for achieving this type of a target[4].

In solution phase chemistry, the reduction of aromatic nitro compounds is not a difficult task as compared to heterogeneous phase[5]. Recently, many novel reduction methods have been reported in the literature for small nitro compounds and polymer-supported aromatic nitro groups. The reducing agents that are routinely used are zinc, iron, or tin, in the presence of an acid, catalytic hydrogenation using Ni or Pd/C and other reagents like sodium dithionite *etc*[6]. However, the reduction of aromatic nitro groups on solid supports is still challengeable. The reduction of polymers having aromatic nitro groups through esterification or etherification reactions is unpredictable. The major challenges in this area are solubility issue, cleanly and completely conversion of all aromatic nitro groups, sensitivity of other functional groups, purification and to find the optimum conditions for large scale.

The goal of the present study was to develop *p*-aminobenzoylhydroxypropyl cellulose (HPC-NH_2) in a two-step process. During the first step, hydroxypropyl cellulose was esterified with *p*-nitro-benzoyl chloride and in the second step the aromatic nitro group was subjected to the reduction process to give primary amino group. The comparative reducing effects of sodium dithionite, tin(II) chloride dihydrate and catalytic hydrogenation using Pd/C were observed in this study. This finding will contribute to the development of a novel polyaromatic polyamine macromolecular carrier.

EXPERIMENTAL

Hydroxypropyl cellulose (Mw 100,000 and 80,000) was purchased from Sigma Aldrich (USA) and dried at 50 °C under vacuum for 3 days before use. *p*-Nitrobenzoyl chloride and palladium on activated charcoal (10 %) was purchased from Fluka (Switzerland). Rest of the chemicals and solvents were of analytical grade. IR spectra were recorded on a Perkin-Elmer FT-IR model spectrum one spectrophotometer. Catalytic hydrogenation was completed by hydrogenation apparatus (Parr® Low Pressure, USA). ^{1}H NMR spectra were recorded by the Varian Nuclear Magnetic Resonance Spectrometer (500 MHz). ^{1}H NMR spectra of all the products (10 mg sample/mL) were measured in dimethyl sulfoxide (DMSO-d_6) at 60 °C.

Esterification of hydroxypropyl cellulose with *p*-nitrobenzoyl chloride: In the first step of the synthesis, 10 g (29.8 mmol) hydroxypropyl cellulose was dissolved in a 160 mL of dried dichloromethane in a two-necked round bottom flask with constant mechanical stirring at room temperature. Pyridine 12 mL (149 mmol) was added to the flask as an acid acceptor and *p*-nitrobenzoyl chloride 27.6 g (149 mmol) was added lastly by parts. The reaction mixture was heated at 25 °C overnight under nitrogen atmosphere. The solution that was obtained after the required time was clear and viscous with light yellow colour. The solution was evaporated for removing dichloromethane and re-dissolved in DMSO for transferring into the dialysis bag. The polymer product was purified by dialysis against distilled water (MWCO = 12,000-14,000 Da) for at least 3 days. The purified *p*-nitrobenzoylhydroxypropyl cellulose ester conjugate was obtained by lyophilization[7]. Yield: 85 % based on DS 2.52.

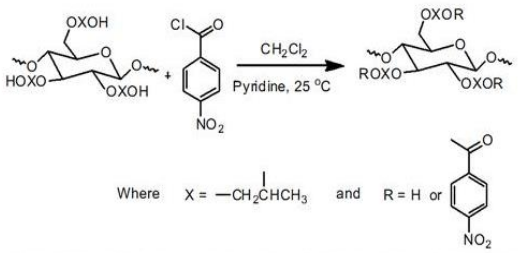
Reduction by hydrogenation: *p*-Nitrobenzoylhydroxypropyl cellulose ester conjugate was tried to reduce by catalytic hydrogenation using 10 % Pd/C as a catalyst. First of all, 40 mL dried THF was bubbled by nitrogen gas for few minutes and 10 % Pd/C (0.60 g) was added and activated by hydrogen gas for 10 min in the reaction flask. HPC-nitro (-NO_2) conjugate (2 g, 5.2 mmol) was then added and hydrogenated in a Parr Hydrogenator Apparatus by maintaining H_2 gas pressure at 25 (lbs/inch^2) for 12 h and the reaction mixture was then immediately filtered on a bed of celite to remove the catalyst. The dark yellow clear solution was then evaporated and dried in vacuum overnight[8].

Reduction by tin(II) chloride dihydrate: In this method, *p*-nitrobenzoylhydroxypropyl cellulose (2 g, 5.2 mmol) was dissolved in 30 mL DMF at 50 °C and 1 M tin(II) chloride dihydrate solution 30 mL prepared in DMF was added to the HPC-NO_2 solution slowly. The reaction mixture was homogeneous and stirred for 16 h at 50 °C. The resulting mixture was tried to purify by dialysis against distilled water (MWCO = 12,000-14,000 Da) for at least 3 days to remove excess tin and byproducts[5].

Reduction by sodium dithionite: *p*-Nitrobenzoylhydroxypropyl cellulose (2 g, 5.2 mmol) was dissolved in 30 mL DMF at 45 °C and sodium dithionite (24 g, 138 mmol) was dissolved in water and added dropwise to *p*-nitroben-zoylhydroxypropyl cellulose (HPC-NO_2) solution. The reaction medium was kept basic (pH 8-9) by $NaHCO_3$ and the reaction mixture was stirred at 45 °C for 24 h. The resulting product was purified by dialysis against distilled water (MWCO = 12,000-14,000 Da) for at least 24 h to remove excess sodium dithionite and byproducts. The product was obtained from the dialysis residue by lyophilization with 76 % yield[9].

RESULTS AND DISCUSSION

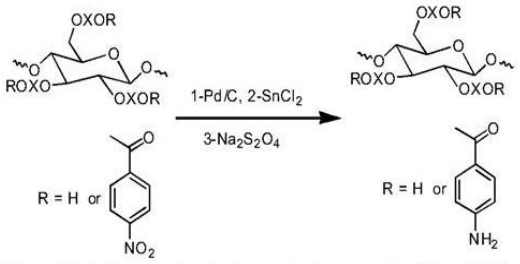
Hydroxypropyl cellulose with molecular weight 80,000 and 100,000 was selected as a backbone for the attachment of *p*-nitrobenzoyl chloride due to its properties like safety, pH insensitivity and solubility both in water and polar organic solvents. Hydroxypropyl cellulose is low-cost and biocompatible pharmaceutical excipient with three hydroxyl groups per anhydroglucose unit. In this study, these hydroxyl groups were esterified with *p*-nitrobenzoyl chloride under homogeneous reaction conditions in dry dichloromethane as a solvent and pyridine as bases to capture the released hydrogen chloride according to the synthesis given in **Scheme-I**. The product hydroxypropyl cellulose-NO_2 was easily purified by dialysis against distilled water because the impurity pyridinium chloride was soluble in water.



Scheme-I: Reaction between hydroxypropyl cellulose and *p*-nitrobenzoyl chloride

Hydroxypropyl cellulose was reacted with different molar ratios of *p*-nitrobenzoyl chloride but the maximum nitration was achieved with five fold excess of *p*-nitrobenzoyl chloride. The native hydroxypropyl cellulose was soluble in water but after the incorporation of aromatic nitro groups it could not preserve its water solubility property. Hydroxypropyl cellulose-NO_2 has solubility in organic solvents like dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), dimethyl acetamide (DMA), tetrahydrofuran (THF), *etc*,

The reduction of the aromatic nitro group to its respective amine can be completed by different ways. The reduction of HPC-NO_2 was carried out by using three different reducing agents *i.e.* sodium dithionite, tin(II) chloride dihydrate and catalytic hydrogenation using Pd/C according to the synthesis given in **Scheme-II**.



Scheme-II: Reduction of *p*-nitrobenzoylhydroxypropyl cellulose (HPC-NO_2) by Pd/C, $SnCl_2$ and $Na_2S_2O_4$

The complete and clean conversion of polymer supported aromatic nitro groups to primary amino groups is a high value task in organic synthesis. The reduction of aromatic nitro groups is readily accomplished in solution phase chemistry as compared to the solid phase. The major challenge is insolubility issue and in this situation the reducing agent has not complete access to -NO_2 groups. The reducing agent can reduce only outer surface of the polymer supported aromatic nitro groups but it cannot approach to the aromatic nitro groups that are present on the inner surface. *p*-Nitrobenzoylhydroxypropyl

cellulose (HPC-NO_2) product has not capability to swell in an aqueous environment but it has good solubility in organic solvents. We conducted a comparison study of three different reducing agents for the reduction of HPC-NO_2.

The catalytic hydrogenation method was used in this study because it is the cleanest and simplest. In the first attempt we used dried methanol as a solvent but HPC-NO_2 could not soluble in this solvent. THF was selected as a solvent of choice after examination of many solvents for this reaction. *p*-Nitrobenzoylhydroxypropyl cellulose (HPC-NO_2) has solubility in THF and it can evaporate easily after completion of the reaction. The reaction was carried out in heterogeneous phase because HPC-NO_2 was in solution phase but on the other hand palladium on activated charcoal was in solid phase. The reaction was run for different time intervals and at different pressures and finally H_2 gas pressure at 25 (lbs/inch^2) and reaction time of 12 h were selected. The result demonstrated that most of the nitro groups on the polymers have been reduced to give HPC-NH_2. Some of the nitro groups remained may be due to the heterogeneous reduction conditions. About 60 % of the product yield (calculated by weight) was obtained. Some of the product was lost during the filtration process through celite.

In case of tin(II) chloride dihydrate the reduction of HPC-NO_2 was carried out homogeneously in DMF. HPC-NO_2 has good solubility in DMF along with tin(II) chloride dihydrate and that is why all the aromatic nitro groups were completely and cleanly converted to amino groups. The HPC-NH_2 was obtained from the dialysis residue by lyophilization and the percentage yield of HPC-NH_2 (calculated by weight) was up to 140 %. Despite the advantages of tin reduction, we observed that substantial quantities of tin by-products remain inside the dialysis bag due to insolubility in distilled water.

Sodium dithionite was investigated as an alternative to tin due to inexpensive, non-toxic, neutral and specific to the reduction of nitro compounds in the presence of other functionalities. We carried out an examination of HPC-NO_2 reduction by sodium dithionite. Optimum conditions for the reduction of HPC-NO_2 involving, type of solvent (water, organic solvents *e.g.* DMA, DMF, DMSO, THF, cosolvents, *etc.*), quantity of reducing agent (nitro product : reducing agent, 1:4, 1:6, 1:8, 1:10 and 1:12 by weight), reaction time (8, 12, 16, 20 and 24 h), reaction temperature (room temperature, 45, 60 and 85 °C) and dialysis time (12, 18 and 24 h) were monitored. The optimum conditions for the reduction of HPC-NO_2 were determined as following : In water, sodium dithionite was readily soluble but HPC-NO_2 was not soluble. To solve this issue we first prepared the homogeneous solution HPC-NO_2 in DMF and then added aqueous solution of sodium dithionite slowly. The pH was maintained at 8 to 9 since it is known that the best reduction condition using sodium dithioniteas a reducing agent should be performed in basic condition[10] and its consumption was determined such as 1 g of the HPC-NO_2 require almost 12 g of sodium dithionite, with 24 h reaction time at 45 °C and dialysis time was 24 h. Sodium dithionite did not give any reduction in organic solvents alone like DMA, DMF, DMSO, THF, *etc.* due to the insolubility in these solvents.

In FT-IR spectra (Fig. 1) and data of the native hydroxypropyl cellulose (A), the OH group stretching of native hydroxypropyl cellulose appeared in the range of 3600-3200 cm^{-1} and OH bending appeared near ~1020 cm^{-1}. In case of HPC-NO_2 (B) the strong absorption bands appeared at 1527 and 1348 cm^{-1} correspond to NO_2 asymmetric and symmetric stretch, respectively. The absorption band at 1276 cm^{-1} correspond to C-N stretching and an absorption band appeared at 1722 cm^{-1} due to carbonyl stretching. These FT-IR results confirmed the esterification reaction between hydroxypropyl cellulose and *p*-nitrobenzoyl chloride. Catalytic hydrogenation using 10 % Pd/C as a catalyst (C) reduced maximum NO_2 to NH_2 as in FT-IR results N-H stretching absorption bands appeared at 3460 and 3364 cm^{-1} but on the other hand small peaks of NO_2 are still present at 1527 and 1348 cm^{-1}. In case of using tin(II) chloride dihydrate (D) and sodium dithionite (E) as the reducing agents,the NO_2 peaks were completely disappeared but no bands assigned to N-H stretching and bending in the range of 3500-3000 and 1640-1560 cm^{-1}, respectively. This may be due to the overlapping of OH stretching band of unreacted hydroxyl groups, in the range of 3600-3000 cm^{-1} and the bending of adsorbed water molecules at approximately 1650 cm^{-1} because HPC-NH_2 was purified by dialysis process against distilled water. The aromatic sharp peak was observed at 1607 cm^{-1} in all the cases except native hydroxypropyl cellulose.

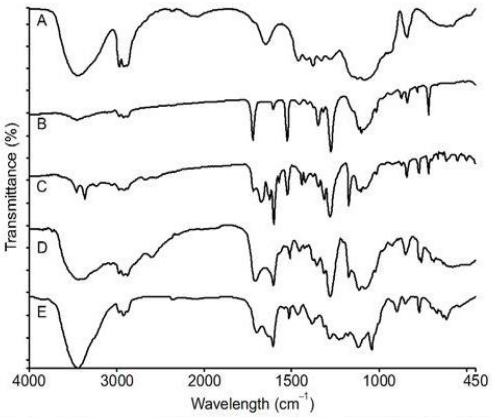


Fig. 1. FTIR spectra of: (A) HPC, (B) H.PC-NO_2, (C) HPC-NH_2 by Pd/C, (D) HPC-NH_2 by tin(II) chloride dihydrate, (E) HPC-NH_2 by sodium dithionite

^{1}H NMR spectrum (not shown) further confirmed the synthesis of HPC-NO_2 and HPC-NH_2 as the multiplet signals of protons of aromatic moiety appeared from 6.7 to 8.2 ppm. In case of HPC-NH_2 the aromatic amines was simply tested and confirmed by diazotization reaction.

Conclusion

p-Nitrobenzoylhydroxypropyl cellulose was synthesized by homogeneous reaction in dried dichloromethane in combination with *p*-nitrobenzoyl chloride and characterized successfully. Light yellow HPC-NO_2 was organo soluble and a comparison study of its reduction was conducted by using three different reducing agents. According to the reduction results sodium dithionite was found to be the better choice for nitro-reduction because it is safer and low cost as compared to tin(II)

chloride dihydrate and palladium on activated charcoal. Both the molecular weights of hydroxypropyl cellulose (Mw 80,000 and 100,000) behave similarly in case of esterification and reduction. Further studies are planned with the help of sodium dithionite by finding a suitable phase-transfer catalyst and solvent system to minimize its consumption during reduction process.

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PAPER 2

Synthesis and Characterization of Hydroxypropyl Cellulose-*p*-Aminobenzoic acid Ester Conjugate

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Synthesis and characterization of hydroxypropyl cellulose-*p*-aminobenzoic acid ester conjugate

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ABSTRACT

Hydroxypropyl cellulose (HPC) was modified to contain chloroacetate groups by the reaction of HPC with chloroacetyl chloride in dried dichloromethane by using pyridine as a catalyst. The coupling of para-aminobenzoic acid (PABA) to HPC functionalized with chloroacetate groups was carried out in a homogenous reaction between chloroacetylated HPC and sodium salt of PABA in dimethylsulfoxide (DMSO). The structures of chloroacetylated HPC and HPC-PABA conjugates were well characterized by FT-IR and ^{1}H-NMR techniques. The degree of substitution (DS) per anhydroglucose unit (AUG) of chloroacetylated HPC and HPC-PABA ester conjugate was calculated by using integrated area from ^{1}H-NMR spectra. Synthesis and structural characterizations of HPC-PABA ester conjugate was successfully carried out and this strategy will contribute to the development of a potential macromolecular carrier for biomedical and pharmaceutical applications.

Keywords: *p*-Aminobenzoic acid, Esterification, Hydroxypropyl cellulose, ^{1}H-NMR , FT-IR

INTRODUCTION

For many years pharmacists have been employing cellulose as the most abundant natural material in many aspects for drug development and research, but there is the disadvantage of its insolubility to limit its applications[1, 2]. Presently a number of water soluble cellulose derivatives are available in the market and that can be used in different pharmaceutical applications[3, 4]. HPC is one of the pharmaceutically valuable cellulose ether due to its suitable properties like safety, pH insensitivity and can be soluble both in water and polar organic solvents[5-8].HPC is routinely used as an excipient in a variety of pharmaceutical dosage forms as a binder, film for coating and thickening agent. Its applications in mucoadhesive delivery systems have also been reported for several different drugs[9-12].

HPC is cheap and biocompatible pharmaceutical excipient with three hydroxyl groups per anhydroglucose unit that can be used in organic synthesis reactions and which makes it a valuable tool for the fabrication of macromolecular prodrugs[13, 14].The bioactive compounds can be coupled to the polymeric backbone by the participation of hydroxyl and carboxyl groups of the polymers and compounds, respectively [15]. Carbohydrate polymers such as HPC can be modified into a useful reactive carrier for the attachment of the therapeutic agents and to introduce a spacer arm between the polymer and the therapeutic agents[16]. It was reported that therapeutic agents can be attached directly to the polymer chain are stable and could not split off easily due to the steric hindrance effect since enzyme could not reach and break down the covalent bond between drug and polymer[17]. PABA is an aromatic naturally occurring non-protein amino acid. It is safe and its absorption rate from the gastrointestinal tract is very fast and completely metabolized by the liver[18]. Structurally, PABA consists of a benzene ring substituted with an amino and a carboxyl group. Through substitution of its amino and carboxyl group, PABA has also been functioned as a structural moiety for many drugs with a wide range of therapeutic applications. PABA was used as a spacer in

the synthesis scheme of dendrimer-5-aminosalicylic acid (5-ASA)azo conjugates and polyethylene glycol-5-ASA prodrugs and it could facilitate 5-ASA release from the polymeric conjugates[19, 20].

The goal of the present study is to synthesize and characterize HPC-PABA ester conjugate in a two simple and homogeneous steps. During the first step, HPC was chloroacetylated with chloroacetyl chloride, while in the second step the chloroacetate groups were reacted with sodium-*p*-aminobenzoate to give the desired HPC-PABA ester conjugate. This strategy will contribute to the development of a novel polyaromatic polyamine macromolecular carrier containing a pendant primary amine functionality which can be used for different biomedical and pharmaceutical applications.

EXPERIMENTAL SECTION

Materials and instruments

HPC powder (Mw~100,000) was purchased from Sigma Aldrich (USA) and dried at 50°C under vacuum for 3 days before use. PABA and chloroacetyl chloride were analytical grade and obtained from Merck (Darmstadt, Germany). The rest of the chemicals and solvents were of analytical grade. FT-IR spectra were recorded on a Perkin-Elmer FT-IR model spectrum one spectrophotometer. ^{1}H-NMR spectra were recorded by the Varian Nuclear Magnetic Resonance Spectrometer (500 MHz). ^{1}H NMR spectra of the esters (10 mg sample/ml) were measured in deuterated dimethylsulfoxide (DMSO-d_6) at 60 °C.

Esterification of hydroxypropyl cellulose with chloroacetyl chloride

In the first step of the synthesis, 10.5 g of HPC(93.8 mmol eq. to-OH groups) was dissolved in 120 ml of dried dichloromethane in a two-necked round bottom flask with constant mechanical stirring at room temperature. Pyridine (7.5 ml, 93.8 mmol) was added to the flask as an acid acceptor. Dried dichloromethane solution (10 ml) containing 7.5 ml of chloroacetyl chloride (93.8 mmol) was then added drop wise at 0-5 °C with stirring. The reaction mixture was kept stirring at 25 °C for overnight under nitrogen atmosphere. The solution that was obtained after the required time gave clear and viscous with light yellow color. The solution was evaporated for removing dichloromethane and re-dissolved in DMSO in order to transfer into a dialysis bag(MWCO = 12,000-14,000 Da). The polymer product was purified by dialysis against distilled water for at least 3 days. The purified chloroacetylated HPC ester conjugate was finally obtained by lyophilization. The product was obtained in 78 % yield with 0.52 degree of substitution (DS).

Reaction of chloroacetylated HPC with sodium-*p*-aminobenzoate

In the second step of synthesis, the resulting chloroacetylated HPC (12.0 g, 31.58 mmoleq. to $ClCH_2CO$- groups) was dissolved in 120 ml DMSO at room temperature and then 6.0 g sodium salt of PABA (37.9 mmol) was added slowly while stirring. The reaction was performed at 30 °C and under intense stirring for about 8 h. The final product was purified by dialysis against distilled water for at least 2 days. The purified HPC-PABA adduct was obtained by lyophilization in 88 %yield, based on with 2.53 degree of substitution (DS).

Calculation process for the degree of substitution

The degree of substitution (DS) of chloroacetylated-HPC was calculated form the spectrum of ^{1}H-NMRby using the integration ratio of the peaks arising due to the methyl protons of hydroxypropyl pendants and protons of $ClCH_2CO$-group that incorporated into HPC through esterification [3]. On the other hand, the DS of HPC-PABA adduct was also calculated form the spectrum of ^{1}H-NMR by using the integration ratio of the peaks appearing due to protons of anhydroglucose unit of HPC and peaks appearing due to protons of aromatic moiety of PABA[21].

RESULTS AND DISCUSSION

Cellulose derivatives containing NH_2-functionalized aromatic groups has been of interest to great expectations concerning their suitability as support matrices for biocompounds. However, their synthesis pathways are frequently very complex and usually gave low product yield. In this study, we have tried to synthesis *p*-aminobenzoylhydroxypropyl cellulose by the reaction of HPC with *p*-nitrobenzoyl chloride using pyridine as a catalyst, the nitro group was then subjected to reduce to an amino group by using sodium dithionite as a non-toxic reducing agent[22].The original HPC was soluble in water but after the incorporation of aromatic nitro groups to the polymer chain, it could not preserve its water solubility property. *p*-Nitrobenzoylhydroxypropyl cellulose has solubility only in organic solvents like dimethylsulfoxide (DMSO), dimethylformamide (DMF), dimetylacetamide (DMA), tetrahydrofuran (THF) and etc., but on the other hand, sodium dithionite has not solubility in these organic solvents. Many efforts were performed to achieve complete conversion but could not find the satisfactory results, which might be caused by reduction in heterogeneous phase the reducing agent may not be in an active form.

For acquiring the primary amino functionality on HPC, we approached in another way that was esterification reaction between PABA and HPC. In this method, the primary amino group of PABA was first protected by 98% formic acid according to the method in the literature[23]. The protection of primary amino group was helpful to avoid from self-coupling of PABA during the reaction due to its bi-functional groups nature. 4-*N*-Formylaminobenzoic acid (4-f-PABA) was successfully esterified with HPC using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC.HCl) as a condensating agent and 4-(dimethylamino)pyridine (DMAP) as a base[2]. Finally, the protecting group was tried to remove by stirring the product in 0.5 mol/lHCl according to the prescribed method[23]. However, HPC-PABA with free amino group was not successfully achieved by hydrolysis of HPC-4-f-PABA. The result demonstrated that the ester bond between HPC and protected PABA was broken, that may be due to the strong acid strength and high temperature assisted the hydrolysis of ester bond.

Finally, a novel and the simplest synthesis route of HPC-PABA ester conjugate was adopted for achieving the macromolecular carrier with primary aromatic amino functionality. HPC was first modified with chloroacetyl chloride to provide chloroacetate groups (Fig.1) using similar process to the previously report with some modification [24].

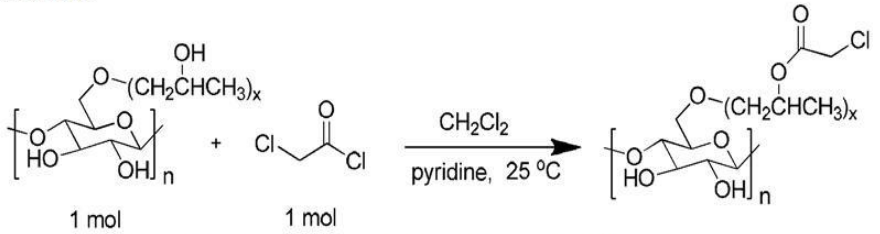

Figure 1 Reaction scheme for the synthesis of chloroacetylated HPC ester conjugate

For achieving the proper distribution of chloroacetate functionality in HPC chain,the esterification was performed in dried dichloromethane. The product, chloroacetylated HPC was easily obtained in high purity by dialysis against distilled water, since the by-product of the reaction, pyridinium chloride was soluble in water.

The coupling of bioactive carboxylic acid of PABA to HPC functionalized with chloroacetate groups was carried out by using the sodium-*p*-amino benzoate in DMSO (Fig.2). The sodium salt of PABA was easily prepared by reacting equivalent amounts of the acid with sodium hydroxide. The solution was left standing overnight to evaporate slowly. It was a novel methodology for the synthesis of macromolecular carrier and interestingly there was no need of any coupling agent and base for completing this type of esterification reaction. Highly pure HPC-PABA ester conjugate was fabricated by this elegant method because the impurity (NaCl) was soluble in distilled water, hence removed simply by dialysis. In this strategy there were no hard challenges like complete reduction of nitro group or complete deprotection of protected amino group that were faced in the beginning of this research work. Regarding solubility, HPC-PABA ester conjugate was soluble in many organic solvents like DMSO, DMA, DMF, THF, MeOH and EtOH, but it was not soluble in water.

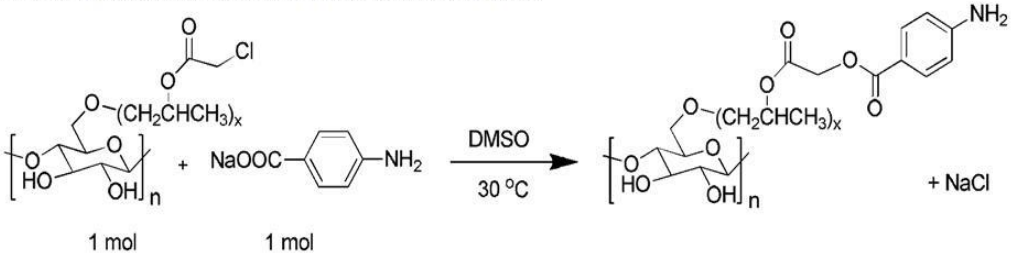

Figure 2 Synthesis scheme for the preparation of HPC-PABA ester conjugate

Figures 3a-c show the FT-IR spectra of unmodified HPC (Fig. 3a), partially modified HPC with chloroacetate groups (Fig. 3b), and HPC-PABA ester conjugate (Fig. 3c). Unmodified HPC (Fig. 3a) shows broad transmission bands at 3600-3100cm^{-1}of hydroxyl stretching vibration. This band is obviously reduced after modification in the spectrum of the obtained chloroacetylated HPC (Fig. 3b). Moreover, FT-IR of chloroacetylated HPC displays new characteristic bands of the attached ester groups appeared at 1754 cm^{-1}belongs to the stretching vibration of carbonyl of the ester, at 1754 cm^{-1}belongs to –C-O stretching vibration, and at 781 cm^{-1}of -CH_2Cl. In addition, the FT-IR spectrum of HPC-PABA ester conjugate (Fig. 3c) demonstrated the absorption bands of –C=O stretching vibration at 1757 cm^{-1} (HPC-O-**C=O**) and a new band at 1711cm^{-1} of -CH_2-O-**C=O**. Moreover, since the final product of HPC-PABA ester conjugate contained 4-aminobenzoate moiety, therefore the characteristic peaks of primary amine

which shows two N–H stretching vibration at 3357 and 3244cm^{-1}, C–N stretching at 1281cm^{-1}, N–H bend at 1619.cm^{-1} and -C=C, and C-H in the benzene ring at1605 and 771 cm^{-1} were observed. The FT-IR results indicated that the desired product was successfully obtained by this procedure.

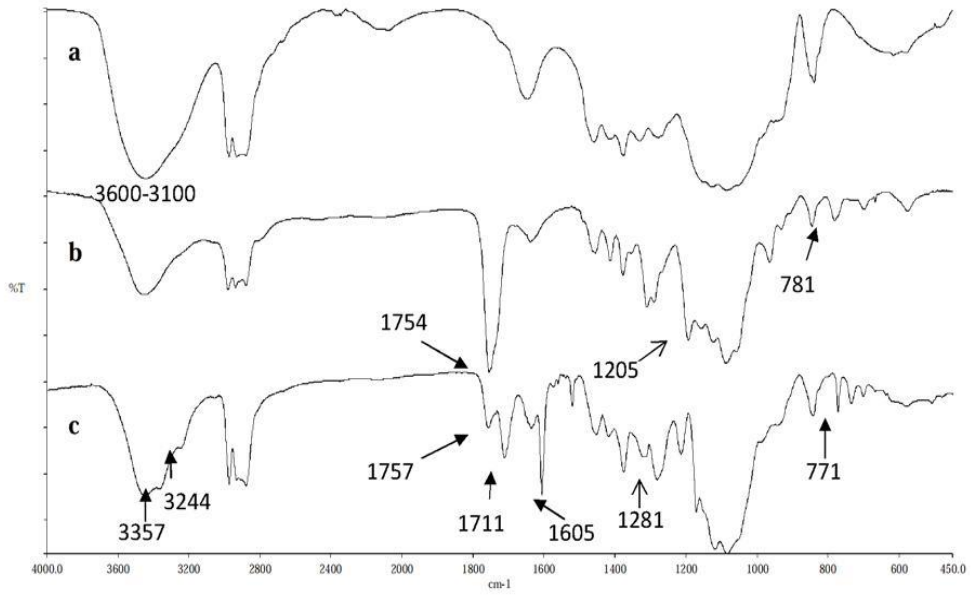

Figure 3FTIR spectra of: a) HPC, b) chloroacetylated HPC (DS 0.52), c) HPC-PABA ester conjugate (DS = 2.53)

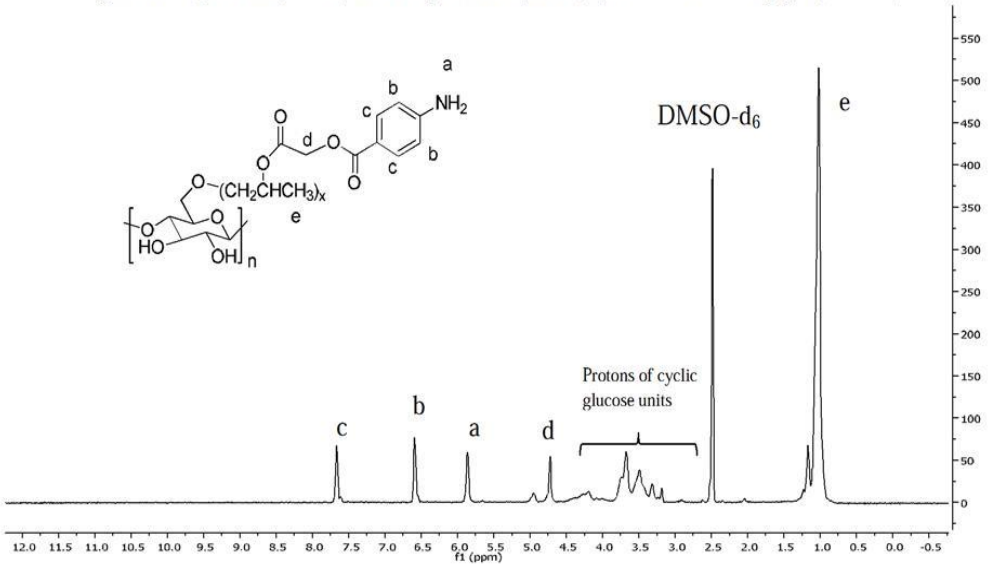

Figure 4[1]H NMR spectra of HPC-PABA ester conjugate (500 MHz, in DMSO-d_6)

The structure of modified HPC was confirmed by[1]H NMR spectra with a 500 MHz Unity Inova, Varian Nuclear Magnetic Resonance Spectrometer in DMSO-$d_6$and displayed in Fig.4. The peak at 2.49 ppm is due to DMSO-d_6. The spectrum of HPC-PABA ester conjugate shows broad peaks at 1.0 -1.25 ppm from the methyl groups in the hydroxypropyl moieties. Peaks in a range of 3.19 – 4.2 ppm belong to protons of cyclic glucose units. The characteristics resonance peaks at 7.67 and 6.59 ppm are ascribed to the protons of aromatic moiety of PABA. The peak at 5.86 ppm belongs to protons of the primary amino group of PABA and a characteristic peak of protons of –CO-**CH_2**-O- appeared at 4.72 ppm. A tiny peak detected at 4.95 ppm belongs to the resonance of the methine protons of modified HPC. The DS of HPC-PABA ester conjugate can be easily calculated form the spectrum of[1]H-NMR by using the integration ratio of the peaks appearing due to protons of the cyclic glucose units of HPC and

peaks appearing due to protons of aromatic moiety of PABA and the result demonstrated that the product was obtained with 2.53 degree of substitution (DS).

Both FT-IR and ^{1}H-NMR characterization results confirmed that HPC-PABA ester conjugate was synthesized successfully and could be obtained in purified form.

CONCLUSION

Fabrication of a novel macromolecular carrier that is HPC-PABA ester conjugate was completed in a two easy steps that were performed homogeneously. Organosoluble macromolecular conjugate with primary aromatic amine functionality were synthesized and characterized effectively with excellent percentage yield and purity. HPC-PABA ester conjugate can be a useful carrier for different biomedical and pharmaceutical applications.

Acknowledgments
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PAPER 3

Hydroxypropyl Cellulose Conjugates for Colonic Delivery of 5-Aminosalicylic acid

(Manuscript submitted to *Carbohydrate Polymers*)

Hydroxypropyl Cellulose Conjugates for Colonic Delivery of 5-Aminosalicylic acid

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Abstract

Hydroxypropyl cellulose (HPC) was used as a macromolecular carrier for the designing of colon specific delivery of 5-aminosalicylic acid (5-ASA) for the treatment of inflammatory bowel diseases. HPC was partially modified with chloroacetate groups by the reaction of HPC with chloroacetyl chloride in dried dichloromethane by using pyridine as a catalyst. A small azo compound PABA-SA was synthesized by coupling of salicylic acid (SA) to the diazonium salt of *p*-aminobenzoic acid (PABA). The coupling of PABA-SA to HPC functionalized with chloroacetate groups was carried out in a homogenous reaction between chloroacetylated HPC and sodium salt of PABA-SA in dimethylsulfoxide. HPC-PABA-NN-SA conjugates were chemically stable at pH 1.2, 6.8 and 7.4 and no 5-ASA was detected after incubation with the homogenate of the stomach of rat. All the conjugates released 5-ASA gradually after 24 hr incubation period at 37 ^{o}C with the rat intestinal and colonic contents and release of 5-ASA from sulfasalazine (commercial prodrug) was comparatively higher and faster. The results suggested that HPC-PABA-NN-SA macromolecular conjugates have potential for use as colon specific delivery of 5-ASA.

Keywords: Hydroxypropyl cellulose, 5-aminosalicylic acid, Macromolecular prodrug, Incubation, Colon-specific drug delivery

1. Introduction

Polymer-drug conjugates or polymeric prodrugs are novel and particular approach towards the improved colon specific drug delivery system (Pasut & Veronese, 2007; Plyduang, Lomlim, Yuenyongsawad & Wiwattanapatapee, 2014). During last decades, the site-specific drug delivery to the colon has received a great deal of attention for the treatment of bowel diseases such as inflammatory bowel disease, irritable bowel syndrome and colonic cancer (Van den Mooter & Kinget, 1995; Watts & Lllum, 1997; Yang, Chu & Fix, 2002). The major approaches that are available for colon-specific drug delivery are pharmaceutical formulation like coating with pH-sensitive or bacterially degradable polymers, time dependent and prodrugs (Chourasia & Jain, 2003; Friend, 2005). The polymeric prodrug approach is achieved by the chemical attachment of low molecular weight drugs to synthetic or natural polymers with the participation of functional groups such as hydroxyl or carboxyl which are present in the polymer chain (Jantas, Draczyński, Herczyńska & Stawski, 2012). In most cases, therapeutic agents bound directly to the polymer backbone exhibit either a reduced or zero therapeutic activity (Zou et al., 2005). For this reason, the polymer should be transformed into a suitable reactive derivative, in order to achieve the attachment of therapeutic agents and to introduce a spacer arm between the carrier and drug molecules (Jantas, Draczyński & Stawski, 2007).

Chronic inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are still an open challenge for better treatment. 5-ASA is the compound of choice for the long-term maintenance therapy to prevent relapses of Crohn's disease and ulcerative colitis (Azad Khan, Piris & Truelove, 1977; Qureshi & Cohen, 2005). However, 5-ASA is absorbed rapidly and extensively through the upper gastrointestinal tract, and hardly reaches to the colonic site, and causes unwanted systemic side effects (French & Mauger, 1993). Several low molecular weight azo

prodrugs of 5-ASA have been developed for the therapeutic treatment of inflammatory bowel diseases (IBDs) such as sulfasalazine, ipsalazide and balsalazide etc, but almost 30 % of patients are unable to tolerate the multiple adverse effects of these small azo conjugates (Jarner, 1989; Peppercorn, 1984; Wadworth & Fitton, 1991). Many macromolecular produgs of 5-ASA were also developed and studied for their colon specific release profile in the past (Brown, McGarraugh, Parkinson, Wingard Jr & Onderdonk, 1983). Dextran and cyclodextrin were investigated by linking the drug by means of an ester linkage (Jung, Lee, Kim, Kim & Kim, 1998; Zou et al., 2005) and polyethylene glycol and dendrimer via an azo bond, but polymeric prodrugs of 5-ASA are still not available for clinical use (Canevari et al., 2009; Wiwattanapatapee, Lomlim & Saramunee, 2003).

Carbohydrate polymers possess multiple primary and secondary hydroxyl groups can be widely employed for developing drug delivery technology to the colon (Kopecek et al., 1992). These polysaccharides are not absorbed in the physiological environment of the upper part of the gastrointestinal tract (GIT), but are selectively degraded by polysaccharidases of the colonic microflora (Mura et al., 2011; Sinha & Kumria, 2001). The advantages of HPC as a polymer-drug conjugates are well accepted, as is apparent from the literature data (Hussain et al., 2013; Zou et al., 2005). HPC has also been extensively employed in pharmaceutical formulations as a tablet binder, a thickening agent, a film coating material, an emulsifier and as a colloidal stabilizer (Khan, Shiotsuki, Sanda, Nishio & Masuda, 2008). HPC is a unique biopolymer with special characteristics such as cheap, biocompatible, odorless, thermoplastic and non-toxic in nature (Hussain, 2008). Additionally, HPC has excellent solubility in water and as well as in polar organic solvents and it possesses three hydroxyl groups per anhydroglucose unit, which makes it a valuable tool for fabrication of macromolecular prodrugs (Hussain, 2008).

The aim of this paper was to develop and characterize HPC-5-ASA azo conjugates as a potential colon-specific polymeric prodrug in a two-step procedure. During the first step, HPC was chloroacetylated with chloroacetyl chloride, while in the second step, chloroacetate groups were coupled with *p*-aminobenzoic acid-salicylic acid (PABA-SA) small azo conjugate. The final product consists of two ester and one azo bond and its chemical stability study was carried out in vitro in acid and basic buffers. The release of 5-ASA from HPC-PABA-NN-SA conjugates after incubation with gastrointestinal contents of rats was also compared to a well-known commercial prodrug of 5-ASA, sulfasalazine.

2. Materials and Methods

2.1. Materials and Instruments

HPC powder (average Mw ~80,000, 20 mesh particle size) was purchased from Sigma Aldrich (USA) and dried at 110 °C for 5 h before use. *P*-aminobenzoic acid (PABA) and chloroacetyl chloride were purchased from Merck (Germany). Sulfasalazine was purchased from Fluka (Switzerland). Methanol (HPLC grade) was from RCI Labscan (Bangkok, Thailand). Rest of the chemicals and solvents were of analytical reagent grade. Wistar rats were from Prince of Songkla University animal house. IR spectra were recorded on a Perkin-Elmer FT-IR model spectrum one spectrophotometer. ^{1}H NMR spectra were recorded by the Varian Nuclear Magnetic Resonance Spectrometer (500 MHz). ^{1}H NMR spectra of the samples (~10 mg /ml) were measured in dimethylsulfoxide (DMSO-d_6) at 60 °C. High-performance liquid chromatography (HPLC) was performed by using an Agilent HPLC system (HP 1100 series, Agilent, USA).

2.2. Synthesis of HPC-PABA-NN-SA conjugates

2.2.1. Esterification of HPC with Chloroacetyl Chloride

In the first step of the synthesis 5.25 g (47 mmol, OH groups) of HPC was dissolved in a 120 ml of dried dichloromethane in a two-necked round bottom flask with constant mechanical stirring at room temperature. Pyridine (0.95 ml, 11.75 mmol) was added to the flask as an acid acceptor. Dried dichloromethane solution (5 ml) containing 0.93 ml (11.75 mmol) chloroacetyl chloride was then added dropwise at 0-5 °C with stirring (Jantas, Draczyński, Herczyńska & Stawski, 2012). The homogeneous reaction mixture was heated at 25 °C overnight under nitrogen atmosphere. The solution that was obtained after the required time was clear and viscous with light yellow colour. The solution was evaporated for removing dichloromethane and dissolved in DMSO for transferring into the dialysis bag (MWCO = 12,000-14,000 Da). The polymer product was purified by dialysis against distilled water for at least 3 days. The purified chloroacetylated HPC ester conjugate was obtained by lyophilization and characterized by FT-IR and ^{1}H NMR. The yield was 87.8 %.

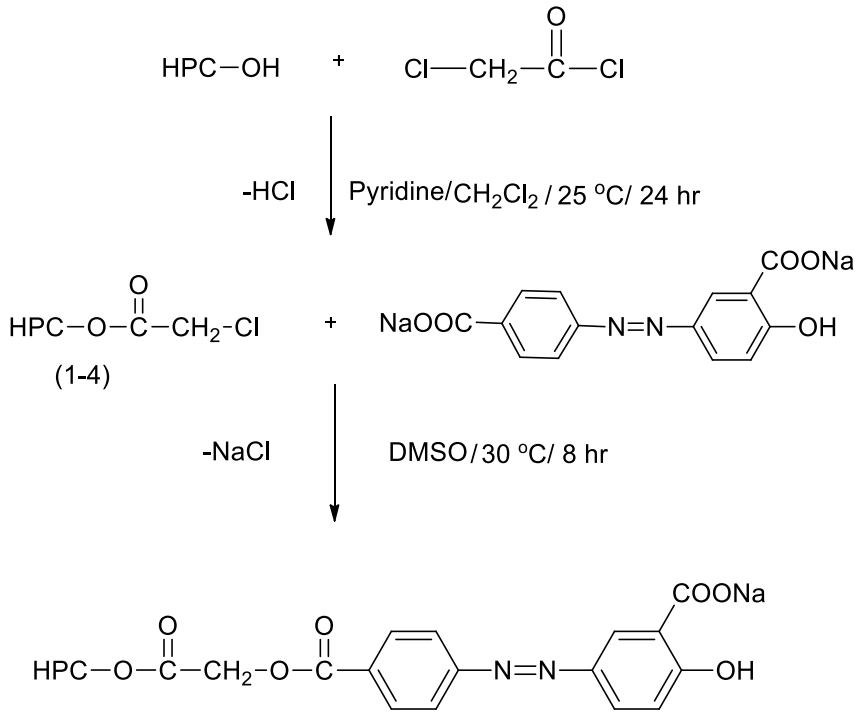
2.2.2. Synthesis of PABA-NN-SA Conjugate

PABA (5 g, 36.5 mmol) was suspended in 60 ml of 3 N HCl and stirred in ice bath (3-5 °C) for 5 minutes. A solution of $NaNO_2$ (3 g, 43.8 mmol) in 20 ml distilled water was added dropwise into the solution of PABA. The mixture was stirred in an 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 potassium iodide / starch indicator paper. Salicylic acid (5.5

g, 40 mmol) was dissolved in 40 ml of distilled methanol and added dropwise into the mixture while stirring at 3-5 °C for 30 minutes. Then 3 M NaOH solution was added to adjust pH 9-10, the reddish brown solution appeared and the solution was stirred for a further 30 minutes. The mixture was then acidified with 3 N HCl and precipitates were filtered, washed with distilled water and 50 % methanol in water (Wiwattanapatapee, Lomlim & Saramunee, 2003). Product was dried at 70 °C for 4 hours and characterized by FT-IR and ^{1}H NMR spectroscopy. The yield was 78 %.

2.2.3. Reaction of Chloroacetylated HPC with PABA-NN-SA Conjugate

The typical procedure of the coupling reaction was as follow: The chloroacetylated HPC (4 g, 11.7 mmol) was dissolved in 80 ml DMSO at room temperature and then 4.6 g (14 mmol) sodium salt of PABA-SA azo conjugate was added while stirring. The reaction was performed at 30 °C under intense stirring for about 8 h. The obtained product was purified by dialysis against distilled water (MWCO = 12,000-14,000 Da) and distilled water was changed every 6 hr for 3 days to remove un-conjugated PABA-NN-SA moiety (Khan and Ovatlarnporn, 2015). The purified HPC-PABA-NN-SA conjugate was obtained by lyophilization and characterized by FT-IR and ^{1}H NMR. The yield was 83.7 %.



Scheme 1. Synthesis of HPC-PABA-NN-SA conjugates (**5-8**).

2.3. Determination of drug content in conjugates

The quantity of 5-ASA was estimated by detaching the PABA-NN-SA moiety from the polymer conjugates after alkaline hydrolysis. Each conjugate (100 mg) was placed in 1 N NaOH solution (100 ml) and stirred at 50 °C for 2 h. An aliquot of the clear solution (0.1 ml) was further diluted with mobile phase and 5-ASA was calculated by measuring the amount of PABA-NN-SA by HPLC at 348 nm (Jung, Lee, Kim, Kim & Kim, 1998; Zou et al., 2005).

2.4. Chemical stability studies

The conjugates will be used in the release study of 5-ASA in rats gastrointestinal contents. The rat gastrointestinal content will be prepared in different pHs, such as 1.2 for stomach content, 6.8 for intestinal content and 7.4 for colon content. Each conjugate contains two ester and one azo bond and the chemical stability of these bonds were determined in these buffer solutions before release study. For this purpose each conjugate 100 mg was placed in a membrane dialysis bag with MWCO 12,000–14,000 and transferred into a beaker. Each beaker covered with a cover slip containing 70 ml of the buffer solution and was stirred in a thermostatic bath at 37 ºC for 24 h. The solution outside dialysis bag (1 ml) was taken at selected time intervals and replaced with the same buffer. The amount of 5-ASA which may release from these conjugates was analyzed by HPLC (Mura et al., 2011).

2.5. 5-ASA release studies

2.5.1. Incubation of HPC-PABA-NN-SA conjugates with the homogenate of the stomach or small intestine of rats

Male Wistar rats (250–300 g) were sacrificed with a midline incision. Sections of the stomach and small intestine were collected separately, weighed and homogenized. Homogenate was diluted with 1% peptone in hydrochloric acid buffer solution (pH 1.2) for stomach and with phosphate buffer solution (pH 6.8) for small intestine to yield a 10% w/v dilution under nitrogen atmosphere. To 900 µl of each homogenate, 100 µl of conjugates or sulfasalazine solution (equivalent to 0.58 mg of 5-ASA) was added under anaerobic system and the mixture was incubated for 24 hours at 37 ˚C. Aliquot amount of samples (500 µl) were taken at selected time intervals: 0.5, 1, 2, 4, 6, 12 and 24 hr after incubation. Fifty microliters of 30% (w/v) trichloroacetic acid (TCA) was added to

stop the enzymatic reaction and frozen at −20 ˚C until analyzed by HPLC (Wiwattanapatapee, Lomlim & Saramunee, 2003).

2.5.2. Incubation of HPC-PABA-NN-SA conjugates with the colonic content of the rats

Male Wistar rats (250–300 g) were sacrificed with a midline incision, the abdomen was opened, the cecum and colon isolated and surgically removed. The cecal segment of the intestine contents was collected, weighed and homogenized. Homogenate was diluted with 1% peptone in phosphate buffer solution (pH 7.4) for colon to yield a 10% w/v dilution under nitrogen atmosphere. To 900 µl of each homogenate, 100 µl of conjugates or sulfasalazine solution (equivalent to 0.58 mg of 5-ASA) was added under anaerobic system and the mixture was incubated for 24 hours at 37 ˚C. Aliquot amount of samples (500 µl) were taken at selected time intervals: 0.5, 1, 2, 4, 6, 12 and 24 hr after incubation. Fifty microliters of 30% (w/v) trichloroacetic acid (TCA) was added to stop the enzymatic reaction and frozen at −20 ˚C until analyzed by HPLC (Wiwattanapatapee, Lomlim & Saramunee, 2003).

2.5.3. Sample preparation for HPLC analysis

The frozen samples were taken and stored at room temperature for 1 hr before analysis and then centrifuged at 10,000 rpm (5 ˚C) for 25 minutes. Fifty microliters of methanol was added to the supernatant (200 µl), to precipitate the protein and sample solution was diluted with 750 µl of mobile phase and filtered through the 0.45 µm-membrane filter. The quantity of drug release (5-ASA) was then analyzed by HPLC.

2.5.4. Quantification of 5-ASA by HPLC analysis

Concentration of 5-ASA released from the conjugates was determined by HPLC using an Agilent HPLC system (HP 1100 series, Agilent, USA). Data analysis was performed by using Agilent software. A reverse phase C18 (ACE® HPLC Columns) (250 mm x 4.6 mm, 5 µm) column was eluted with the mobile phase A consisted of 5 % methanol in phosphate buffer pH 6.0 and methanol was used as mobile phase B. The flow rate of the mobile phase was 1.0 ml/min with a gradient program of 0/0, 7/0, 12/40, 22/40, 27/0, 30/0 (min/% B). The detection wavelength was set at 240 nm for the quantitation of released 5-ASA and 348 nm for detection of any released of PABA-NN-SA conjugate. The injection volume was 20 µl.

3. Results and discussion

3.1. Synthesis of HPC-PABA-NN-SA conjugates

The synthesis route of HPC-PABA-NN-SA conjugates is shown in scheme 1. For achieving the uniform distribution of chloroacetate groups along the polymer chain, the esterification was accomplished homogeneously in dried dichloromethane. Four HPC-PABA-NN-SA conjugates were synthesized by modifying HPC (Mw ~80,000) by varying the molar ratios of chloroacetate groups. The hydroxyl groups of HPC were kept constant but on the other hand the feeding ratio of chloroacetate groups were varied such as 1:0.25 (**1**), 1:0.5 (**2**), 1:0.75 (**3**) and 1:1 (**4**). The chloroacetylated HPC product was easily purified by dialysis against distilled water because the impurity *pyridinium chloride* was soluble in water. Light yellow color products **1**-**4** were synthesized with good percentage yield i.e. 87.8, 82.09, 78.67 and 75.2 %, respectively.

PABA was selected as the spacer because of its safety and suitability of functional groups (Canevari et al., 2009; Wiwattanapatapee, Lomlim & Saramunee, 2003). It

contains carboxylic group for ester bond formation and amino group can be utilized for the diazotization process with salicylic acid (SA). The coupling of bioactive carboxylic acid from para position of PABA-SA to HPC functionlized with chloroacetate groups was carried out by using the sodium salt of PABA-SA in DMSO. The sodium salt of PABA-SA was easily prepared by reacting equivalent amounts of the small azo compound and sodium hydroxide. The solution was left to stand overnight to evaporate slowly. It was a novel methodology for the synthesis of a macromolecular azo conjugate and interestingly there was no need of any coupling agent and base for completing this type of coupling reaction. Preliminary experiments of coupling reaction suggested that sodium salt of PABA-SA is a better choice for the grafting of small azo compound onto polymer as compared to carboxylic acid form. In case of salts of carboxylic acid, there is no complex reactive intermediate and after completing the reaction, the excessive PABA-SA can be easily removed by dialysis due to good solubility in water. Highly purity of HPC-PABA-NN-SA azo conjugates were fabricated by this elegant method because the impurity (NaCl) was soluble in distilled water, hence removed simply by dialysis. Reddish brown color HPC-PABA-NN-SA **5-8** macromolecular azo conjugates were synthesized with good percentage yield i.e. 83.7, 81.89, 82.04 and 79.77 %, respectively.

Figure 1 (a-c) shows the FT-IR spectra of unmodified HPC, partially modified HPC with chloroacetate groups and HPC-PABA-NN-SA azo conjugate. As can be seen, in the spectrum of chloroacetylated HPC (Fig. 1b), new characteristic bands of the attached ester groups appeared at 1754 cm^{-1} (C=O) and at 781 cm^{-1} (-CH_2Cl). On the other hand, the band of hydroxyl groups in the range 3600 - 3200 cm^{-1} partly disappeared. Moreover, in the spectrum of HPC-PABA-NN-SA azo conjugate (Fig. 1c), the absorption bands appeared at 1772 cm^{-1} (C=O), 1720 cm^{-1} (C=O), 1680 and 775 cm^{-1} (C=C, C-H in the benzene ring respectively), and the azo (N=N) group weak peak appeared at 1483 cm^{-1} further confirmed the successful synthesis. In the ^1H NMR

spectrum of HPC-PABA-NN-SA azo conjugates, the characteristics resonance peaks from 7.11 ppm to 8.39 ppm are ascribed to the protons of azo moiety of PABA-SA. The characteristic peak of protons of –CO-CH_2-O- group appeared at 4.3 ppm. A tiny peak detected at 4.95 ppm belongs to the resonance of the methine protons of modified HPC. The peaks at 1.17 and 1.0 ppm are ascribed to methyl groups of the hydroxypropyl group after and before esterification and all other protons of HPC were merged in the broad multiplets from 3.19 to 4.0 ppm (Fig. 2).Both FTIR and ^{1}H NMR results confirmed that HPC-PABA-NN-SA azo conjugates were synthesized and characterized successfully.

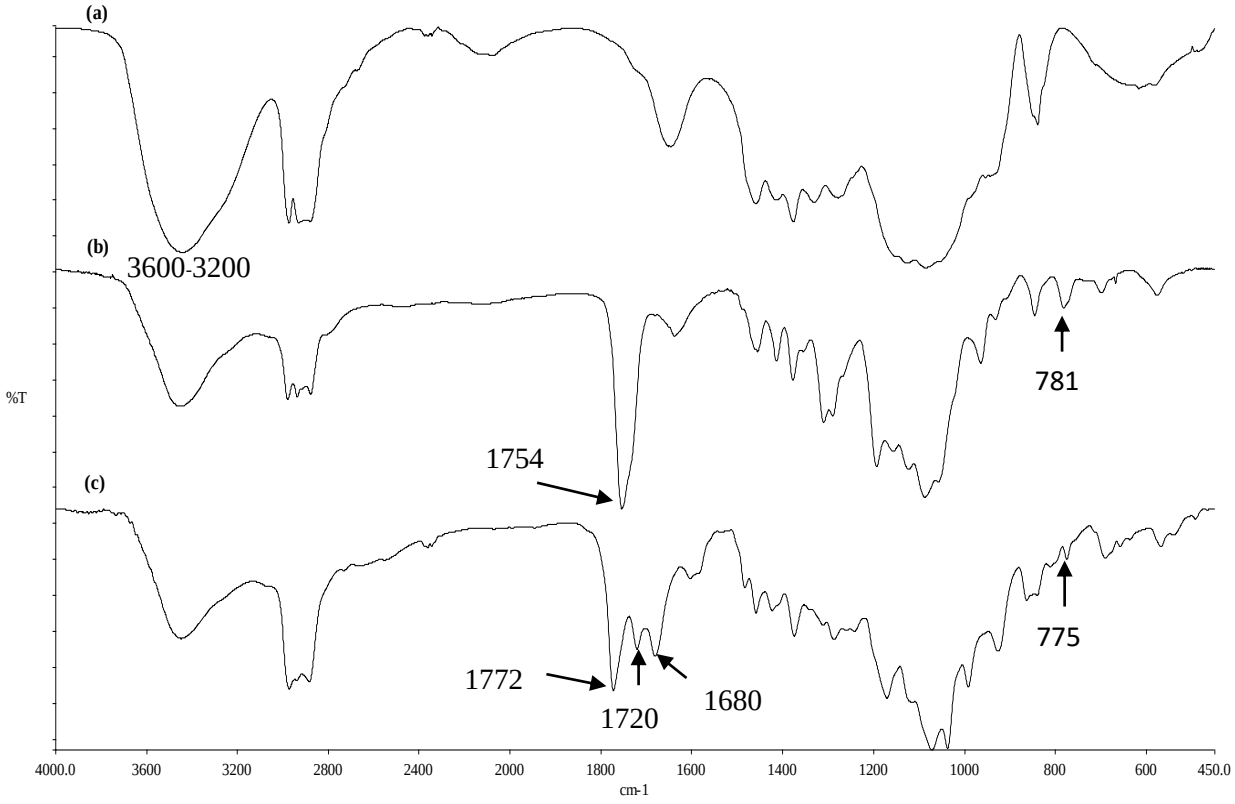


Figure. 1. FT-IR spectra of HPC (a), Chloroacetylated HPC (b) and HPC-PABA-NN-SA conjugate (c).

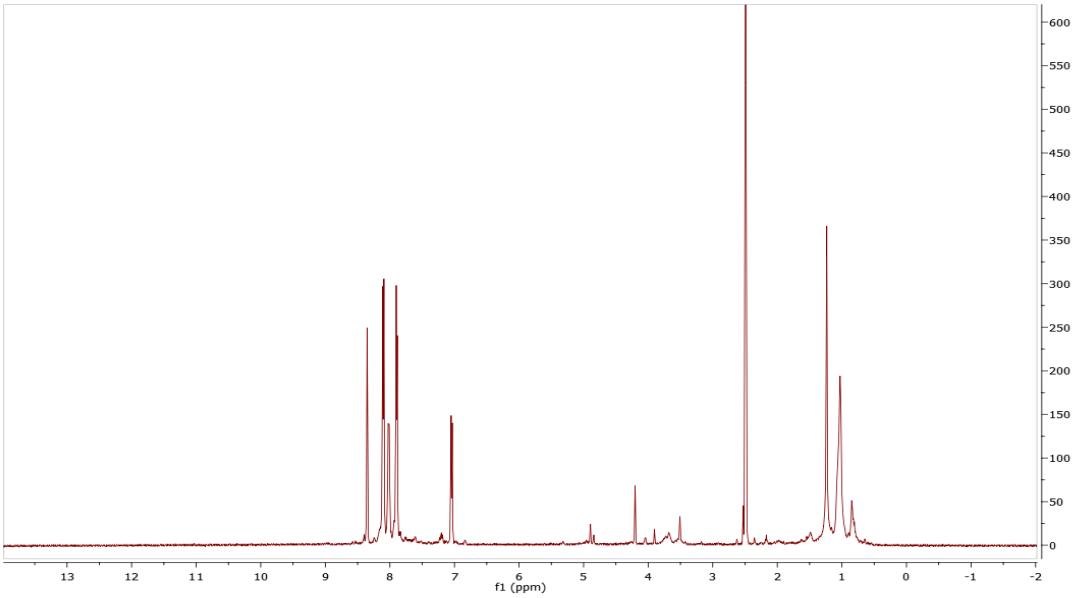


Figure. 2. ^{1}H NMR spectra of HPC-PABA-NN-SA conjugate (500 MHz, in DMSO-d_6)

3.2. Conjugates chemical stability studies

Four HPC-PABA-NN-SA conjugates (**5-8**) were designed with the participation of two ester and one azo bond for colon specific delivery and for this purpose these three chemical bonds should be stable while passing through the stomach and small intestine. Polymer-drug azo conjugates that were studied previously were found stable in gastrointestinal environment such as dendrimer-5-ASA azo conjugates (Wiwattanapatapee, Lomlim & Saramunee, 2003) and poly(ethylene glycol)-5ASA azo conjugates (Canevari et al., 2009). The in vitro stability study was investigated by incubating all the conjugates in buffer solution of pH 1.2, pH 6.8 and pH 7.4. In order to evaluate the stability of the conjugates, samples were quantified during 24 hr experiments by HPLC analysis. It was observed that no free 5-ASA and PABA-SA were detectable, which confirmed that all the conjugates were chemically stable at pHs 1.2, 6.8 and 7.4.

3.3. 5-ASA release studies

All the HPC-PABA-NN-SA conjugates (5-8) were hydrolyzed to detach the PABA-SA azo moiety to calculate the percentage drug loading and the quantity of 5-ASA attached to the polymer was determined by HPLC. The attachment of drug was increased as the molar ratio of chloroacetyl chloride was increased to HPC and 5-ASA was calculated i.e. 7.7 (**5**), 9.8 (**6**), 11.14 (**7**) and 13.69 % (**8**). In order to make sure the actual potential of the conjugates to release the 5-ASA into the colonic area, an in vitro release study was conducted by using the stomach, intestinal and colonic contents of male Wistar rat. It was previously investigated that 5-ASA linked with polyethylene glycol and dendrimer via an azo bond was selectively triggered into the colonic area by the azoreductases (Canevari et al., 2009; Wiwattanapatapee, Lomlim & Saramunee, 2003). In case of dendrimer-5-ASA azo conjugates the release study was carried out about 12 hr for stomach and intestinal contents and polyethylene glycol-5-ASA prodrug was studied only for colonic contents of the rat. HPC-PABA-NN-SA conjugates contain two ester bonds that are susceptible to enzymatic hydrolysis before reaching the colon target. The release profiles of the HPC-PABA-NN-SA conjugates (**5-8**) were determined up till to 24 hr after incubation at 37 $^{\circ}$C with the gastrointestinal contents of the rat.

It was a remarkable comparison release study between low molecular weight commercially available sulfasalazine and macromolecular prodrug HPC-PABA-NN-SA synthesized in this work. Interestingly both sulfasalazine and HPC-PABA-NN-SA conjugates (**5-8**) were not soluble in water, but before mixing the rat contents under nitrogen atmosphere, the homogenous solution of sulfasalazine was prepared by using few drops of 0.1 N NaOH and HPC-PABA-NN-SA conjugates were used heterogeneously. On incubation of sulfasalazine or conjugates (equivalent to 0.58 mg of 5-ASA) with the homogenate of the stomach, no 5-ASA or spacer PABA-SA was

detected during a 24 hr period, which indicated that these HPC-PABA-NN-SA conjugates were chemically stable with the stomach contents of the rat. It was also clarified that the availability of azoreductases were not enough in the stomach to trigger the drug release from sulfasalazine or macromolecular conjugates.

When both sulfasalazine and macromolecular conjugates were incubated with the homogenate of the small intestine for 24 hr, it was clearly observed that 5-ASA could not release in the first 4 hr from sulfasalazine and in case of macromolecular conjugates, no 5-ASA was found during the first 6 hr. In intestinal content, 5-ASA was found slowly released after 4 hr from sulfasalazine and finally reached 48.74 % at about 24 hr after incubation. All the HPC-PABA-NN-SA conjugates (**5**-**8**) started to give release after 6 hr and the % release of 5-ASA was calculated highest i.e. 31.22 % with the lowest drug attachment (**5**) and similarly (**6**-**8**) showed release 26.65, 22.38 and 17.23 % respectively after 24 hr incubation period (Fig. 3). It can be observed that 5-ASA was released from both sulfasalazine and macromolecular conjugates in the intestinal segment of the rat and it may be due to availability of some of bacteria which produce azoreductase enzyme such as *Bacteroides* and *Clostridium* in the distal part of small intestine i.e. ileum (Rafii, Franklin & Cerniglia, 1990). Therefore, the azo bond can also be reduced in this part of the GIT tract. It was also confirmed by Wiwattanapatapee and co-workers (2003) that the azoreductase enzymes could cleave the azo bond of the PAMAM conjugates and release 5-ASA in the simulated small intestine content up to 7.2 % after 12 h incubation at 37 ^{o}C. In addition, it was also reported in 2001 that the azo bond between 9-aminocamptothecin and HPMA copolymer was cleaved after 24 h incubation at 37 ^{o}C in the content of small intestine and the drug could be released up to 25 % (Sakuma, Lu, Kopeckova & Kopec • ek, 2001). The information provided above indicated that a number of macromolecular azo conjugates may release the attached drug in the small intestine before reaching the colon.

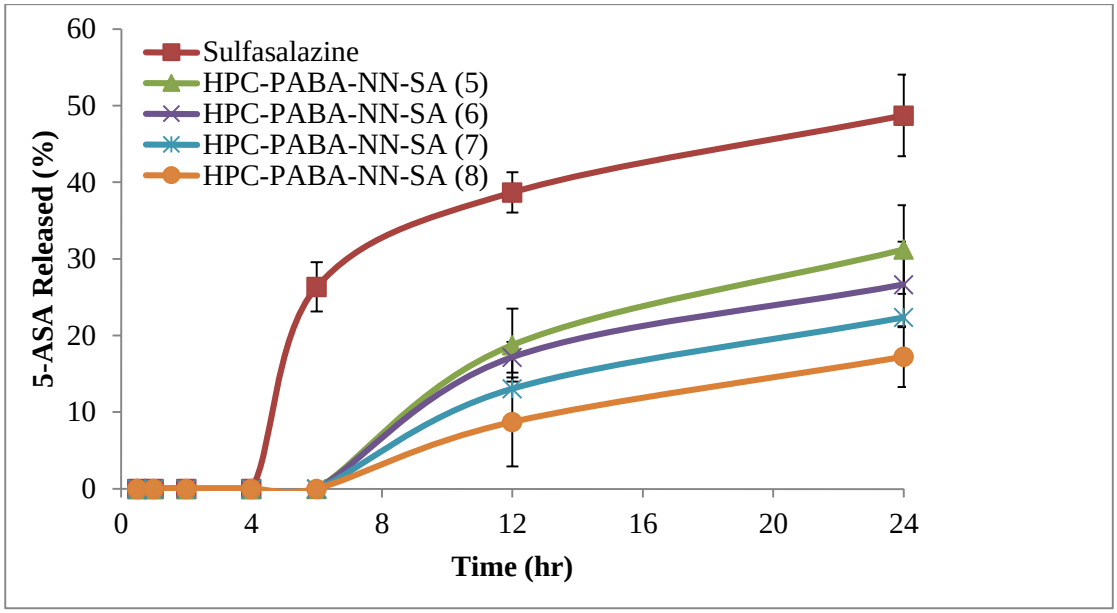


Figure 3. Release profiles of 5-ASA from the conjugates (5-8) and sulfasalazine during incubation in rat intestinal content at 37 ˚C. Error bars represent standard deviation, n = 3.

In colonic content, 5-ASA released from sulfasalazine was observed in higher amount and faster than in the intestinal contents. The release of 5-ASA from sulfasalazine in the colonic content was observed up to 65.32 % after incubation within the first thirty minutes and the total amount of 5-ASA released within 24 hr was up to 74.17 %. In case of PAMAM conjugates the release of 5-ASA from sulfasalazine was up to 80.2 % after incubation within the first 6 hr (Wiwattanapatapee, Lomlim & Saramunee, 2003) and the % release was higher as compared to our results and it may be due to variation in quantity of colonic microflora. The release of 5-ASA from the synthesized HPC-PABA-NN-SA conjugates (5-8) showed a better release in colonic content as compared to the intestinal content due to higher number of azoreductase enzymes available in the colonic region. However, the released pattern of the drug from each conjugate was different. All the HPC-PABA-NN-SA conjugates (**5-8**) started to give releasing after

thirty minutes of incubation period and it remained continuous up till to 24 hr. All the conjugates presented almost similar sustained release profile and the highest quantity of 5-ASA released 56.58 % was found from the conjugate **5** after 24 hr incubation period. HPC-PABA-NN-SA conjugates **6-8** showed release up to 45.16, 38.23 and 32.0 %, respectively after 24 hr incubation period (Fig. 4). According to the release results, an inversely proportional relationship was observed between the attachment of the drug to polymer and % release. The release of 5-ASA increased by decreasing the attachment of the drug onto the polymer.

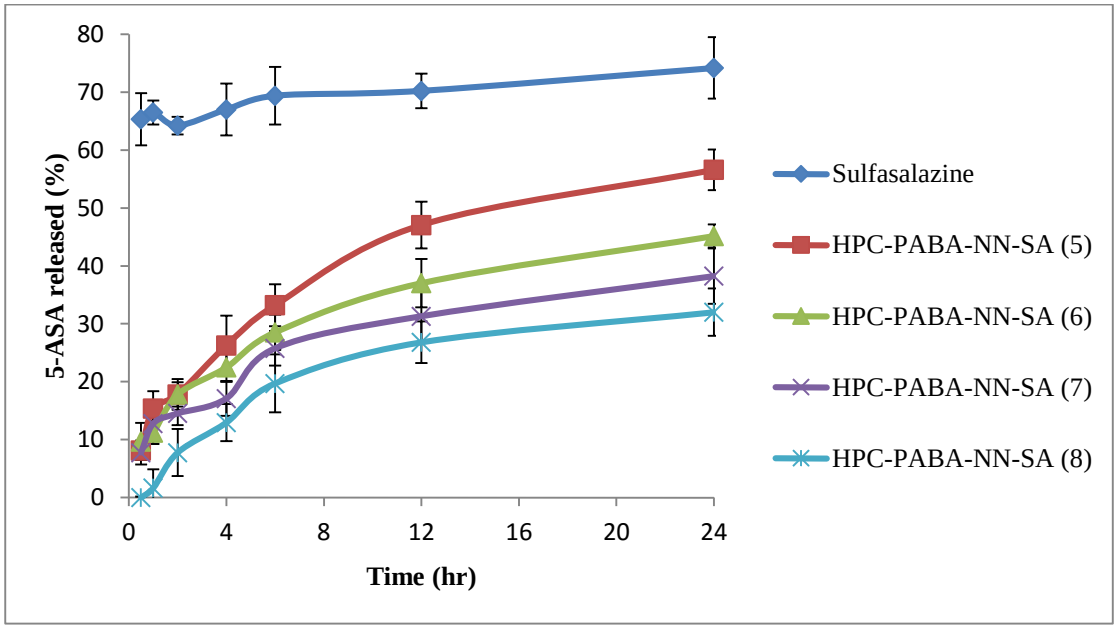


Figure. 4. Release profiles of 5-ASA from the conjugates (5-8) and sulfasalazine during incubation in rat colonic content at 37 ^{o}C. Error bars represent standard deviation, n = 3.

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Zou and co-workers (2005) formulated HPC-5-ASA ester prodrugs by varying the molar ratios of HPC and 5-ASA (1:1, 1:2, 1:6, 1:10) and they reported that none of the prodrug released 5-ASA in any of the contents of the rat within 48 hr due to insolubility issue of these prodrugs. Since solubility and steric hindrance factors were found to be important, we decided to synthesize HPC-PABA-NN-SA conjugates by

bringing the three major changes i.e. decreasing the molar ratios between HPC and 5-ASA, developing a spacer arm between HPC and 5-ASA and an azo bond was developed between polymer and drug. In our studies, all the HPC-PABA-NN-SA conjugates (**5-8**) released 5-ASA in both intestinal and colonic contents but no release in the gastric content of the rat. The release of 5-ASA from HPC-PABA-NN-SA conjugates was supported by a higher number of azoreductases as compared to estrases in the intestinal and colonic region of the rat. Length of spacer arm between polymer and drug was also helpful in the cleavage of azo bond by enzyme. The cleavage of azo bond in case of HPC-PABA-NN-SA conjugates was much slower as compared to sulfasalazine. Sulfasalazine azo bond was easily attacked and promptly reduced by azoreductases, which may be explained due to two reasons that firstly carrier of 5-ASA was a low molecular weight instead of polymer and secondly sulfasalazine was prepared in solution form before mixing with the rat contents. Both the ester bonds of HPC-PABA-NN-SA conjugates (5-8) remained stable during stability and release studies and it may be due to insolubility of these conjugates or a steric effect of the bulky HPC moiety that esterase enzymes could not breakdown the ester bonds and 5-ASA was only released after the reduction of azo bond (Fig. 5).

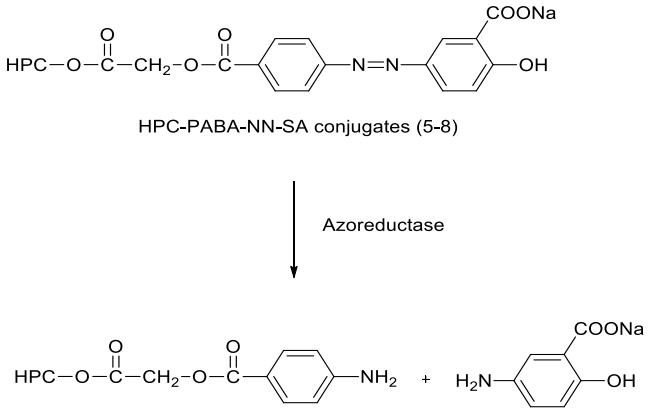

Figure 5. Release of 5-ASA from the conjugates (**5-8**).

4. Conclusions

HPC-PABA-NN-SA conjugates (**5-8**) were fabricated as a novel macromolecular prodrug of 5-ASA in a two easy and homogenous steps with high percentage yield and purity. Each conjugate was developed by the participation of two ester and one azo bond and both the ester bonds remained stable during stability and release studies. 5-ASA was liberated from each conjugate after incubation with the intestinal and colonic contents of the rat by the reduction of azo bond. In comparison study, the release of 5-ASA from sulfasalazine was significantly faster as compared to HPC-PABA-NN-SA conjugates. All the macromolecular conjugates have a potential to release 5-ASA in the lower part of the GIT tract in a sustained release profile for the treatment of IBD. The results suggested that HPC has potential for use as colon specific drug carrier.

Acknowledgments

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PAPER 4

Synthesis and Evaluation of Hydroxypropyl Cellulose-Olsalazine Conjugates for Colonic Delivery

(Manuscript submitted to *International Journal of Biological Macromolecules*)

Synthesis and Evaluation of Hydroxypropyl Cellulose-Olsalazine Conjugates for Colonic Delivery

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Abstract

Chronic inflammatory bowel (IBDs) diseases are mainly affect the lower part of the gastrointestinal tract and drug delivery approaches that can provide a local treatment which will give rise to minimize unwanted side effects of the systemic therapies. Macromolecular prodrugs are particularly promising for exploiting the colon specific enzyme azoreductase to trigger selective drug release at this site. The aim of the present study was to develop a colon targeted delivery system for 5-aminosalicylic acid (5-ASA) using hydroxypropyl cellulose (HPC) as a drug carrier. Three HPC-olsalazine conjugates (**6b-d**) were synthesized with good % yield and well characterized by FT-IR and ^{1}HNMR. The HPC-olsalazine conjugates were chemically stable at pH 1.2, 6.8 and 7.4 and no 5-ASA was detected after incubation with the homogenate of the stomach of rat. All the conjugates released 5-ASA gradually during 24 h incubation period at 37 °C with the rat intestinal and colonic contents. The maximum release of 5-ASA in the colonic content was found (38.8%) from HPC-5-ASA-5-ASA (**6b**) azo conjugate and sulfasalazine (commercial prodrug) released 5-ASA significantly faster (66.7%) as compared to the macromolecular conjugates at the same

incubation time. These results suggested that HPC-olsalazine conjugates have potential for use as colon specific delivery of 5-ASA.

Keywords: Hydroxypropyl cellulose, 5-aminosalicylic acid, Colon-specific drug delivery, Olsalazine

2. Introduction

Macromolecular prodrugs or polymer-drug conjugates offer the potential strategies for drug delivery towards the colon in a controlled manner for the treatment of bowel diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome and colonic cancer [1,2]. In this way, therapeutically effective concentration can be achieved and systemic side effects and toxicity can be minimized [3]. Many colon-specific drug delivery approaches are available nowadays such as pharmaceutical formulation like coating with pH-sensitive or bacterially degradable polymers, time dependent and prodrugs [4,5]. In polymeric prodrug approach, the drugs can be linked to synthetic or natural polymers with the participation of functional groups such as hydroxyl or carboxyl which are present in the polymer chain [6]. In most cases, therapeutic agents bound directly to the polymer backbone could not exhibit therapeutic activity [7]. For this reason, a spacer arm can be helpful in separating and splitting off drugs more readily from the polymeric backbone by means of the cleavage of a bond between a polymer-spacer and a drug by specific enzymes as prodrug reaches the target site [8].

Colon specific enzymes particularly azoreductases catalyze a reductive cleavage of azo bond and release 5-ASA in the colon from its azo-prodrugs. That could be due to the colonic mictoflora presence of much higher number (10^{11}–10^{12} CFU/ml) as compared to the small intestine (10^{4} CFU/ml) [9,10]. Some examples of low molecular weight azo prodrugs of 5-ASA available in the market are sulfasalazine, balsalazide and olsalazine (Fig. 1) [11]. Unfortunately, even these commercially available 5-ASA conjugates allow locally treatment of IBD but a lot of serious systemic side effects

have been reported from these conjugates [12]. In case of olsalazine, one molecule of 5-ASA was used as a carrier for the other and after reduction of azo bond two molecules of 5-ASA were locally available for the treatment of IBD, but due to severe diarrhea, many patients cannot tolerate it [13]. A number of macromolecular produgs of 5-ASA were also developed and studied for their colon specific release profiles in the past [14]. Dextran and cyclodextrin were investigated by linking the drug by means of an ester linkage [15,7] and polyethylene glycol and dendrimer via an azo bond, but polymeric prodrugs of 5-ASA are still not available for clinical use [2,1].

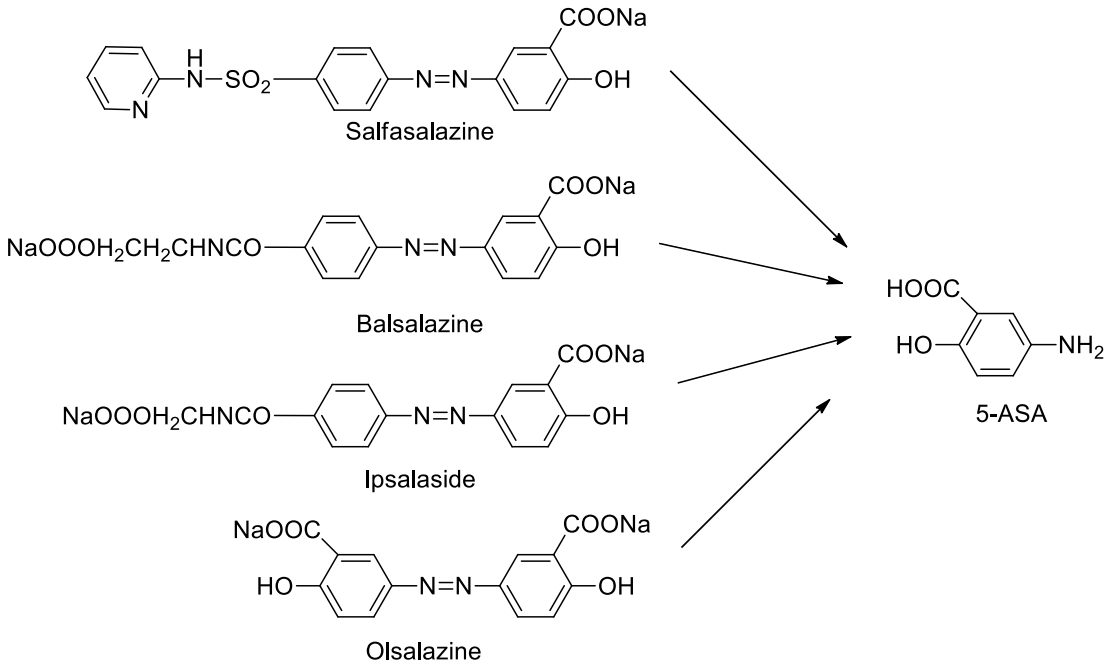


Figure 1: The chemical structures of sulfasalazine (SASP), balsalazide, ipsalazide and olsalazine (OSZ) presenting the cleavage of azo bond by azoreductases leading to the development of the active agent 5-ASA (Chourasia and Jain 2003).

Polysaccharides having multiple primary and secondary hydroxyl groups can be widely employed for developing drug delivery technology to the colon such as chitosan, cyclodextrin and cellulose derivatives [16]. These polysaccharides remain

unabsorbed in the upper environment of the gastrointestinal tract (GIT), but are selectively degraded by polysaccharidases of the colonic microflora [17]. The advantages of hydroxypropyl cellulose (HPC) as a polymer-drug conjugates are well known and it was previously used as a carrier of 5-ASA by Zou and co-workers in their study [7]. HPC has also been extensively utilized in pharmaceutical formulations as a tablet binder, a thickening agent, a film coating material, an emulsifier and as a colloidal stabilizer [18]. HPC is a unique biopolymer with special characteristics such as cheap, biocompatible, odorless, thermoplastic and non-toxic in nature [19]. Additionally, HPC has excellent solubility in water and as well as in polar organic solvents and it possesses three hydroxyl groups per anhydroglucose unit, which makes it a valuable tool for the fabrication of macromolecular prodrugs [20].

The aim of this report was to develop and characterize HPC-olsalazine conjugates as a potential colon-specific polymeric prodrug in a three-step procedure. During the first step, HPC was chloroacetylated with chloroacetyl chloride, while in the second step, chloroacetate groups were coupled with sodium salt of 5-ASA and in the final step salicylic acid (SA) was coupled to the diazonium salt of HPC-5-ASA product. The HPC-olsalazine conjugates final product consist of two ester and one azo bonds and their chemical stability study was carried out in vitro in acid and basic buffers. The release of 5-ASA from HPC-olsalazine conjugates after incubation with gastrointestinal contents of rats was also compared to a well-known commercial prodrug of 5-ASA, sulfasalazine.

2. Materials and Methods

2.1. Materials and Instruments

HPC powder (average Mw ~100,000, 20 mesh particle size) was purchased from Sigma Aldrich (USA) and dried at 110 °C for 5 h before use. 5-Aminosalicylic acid (5-ASA) and chloroacetyl chloride were purchased from Merck (Germany). Sulfasalazine was purchased from Fluka (Switzerland). Methanol (HPLC grade) was from RCI Labscan (Bangkok, Thailand). Rest of the chemicals and solvents were of analytical reagent grade. Wistar rats were from Prince of Songkla University animal house. IR spectra were recorded on a Perkin-Elmer FT-IR model spectrum one spectrophotometer. ^{1}H NMR spectra were recorded by the Varian Nuclear Magnetic Resonance Spectrometer (500 MHz). ^{1}H NMR spectra of the samples (~10 mg /ml) were measured in dimethylsulfoxide (DMSO-d_6) at 60 °C. High-performance liquid chromatography (HPLC) was performed by using an Agilent HPLC system (HP 1100 series, Agilent, USA).

2.2. Synthesis of HPC-olsalazine conjugates

HPC-olsalazine conjugates were synthesized in three steps procedure according to the Scheme 1.

2.2.1. Esterification of HPC with Chloroacetyl Chloride

In the first step of the synthesis 47 mmol of (**1**) HPC was dissolved in a 120 ml of dried dichloromethane in a two-necked round bottom flask with constant mechanical stirring at room temperature. Pyridine (11.75, 23.5, 35.25 and 47 mmol) was added to the flask as an acid acceptor. Dried dichloromethane solution containing (11.75, 23.5, 35.25 and 47 mmol) chloroacetyl chloride (**2**) was then added dropwise at 0-5 °C with stirring [21, 24]. The homogeneous reaction mixture was heated at 25 °C overnight under nitrogen atmosphere. The solution that was obtained after the required time was clear and viscous with light yellow colour. The solution was evaporated for removing dichloromethane and dissolved in DMSO for transferring into the dialysis bag (MWCO = 12,000-14,000 Da). The polymer product was purified by dialysis against

distilled water for at least 3 days. The purified chloroacetylated HPC ester conjugates (3a-d) were obtained by lyophilization and characterized by FT-IR and ^{1}H NMR.

2.2.2. Reaction of chloroacetylated HPC with sodium salt of 5-ASA

The typical procedure of the reaction was as follow. The chloroacetylated HPC (3a-d) (15.8 mmol) was dissolved in 120 ml DMSO at room temperature and then 3.3 g of sodium salt of 5-ASA (**4**) (18.8 mmol) was added while stirring. The reaction was performed at 30 °C and under intense stirring for about 8 hr. The obtained product was isolated by precipitation using distilled water as precipitant and then washed many times with distilled water to remove unreacted sodium5-aminosalicylate.The product was re-dissolved into DMSO for further purification by dialysis method to remove unwanted products that soluble in water and the purified product was obtained from the dialysis residue by lyophilization [24]. The products HPC-5-ASA (**5a-d**) were then characterized by FT-IR and ^{1}H NMR.

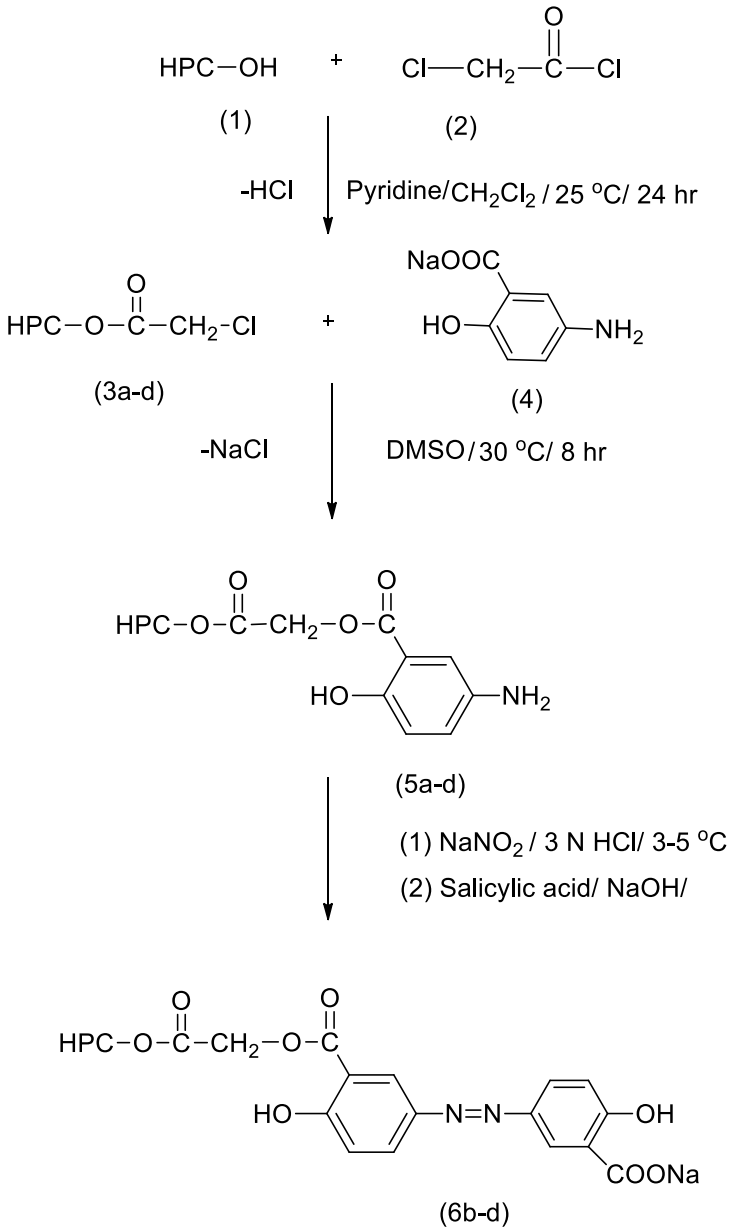
2.2.3. Synthesis of HPC-olsalazine conjugates

HPC-5-ASA conjugates (**5a-d**) (11.3 mmol) were suspended in 3 N HCl solution (60 ml) in an ice bath (3-5 °C). A solution of $NaNO_2$ (22.7 mmol) in distilled water (10 ml) was prepared and added dropwise into the solution of HPC–5-ASA conjugate. The mixture was continued stirring in the ice bath (3-5 °C) for another 5 minutes and then the diazonium salt was tested for the presence of excess nitrous acid using potassium iodide/starch indicator paper. Salicylic acid (22.7 mmol) was dissolved in methanol (20 ml) and added into the diazonium salt solution and stirred at 3-5 °C for thirty minutes. After that 3 M NaOH solution was added to adjust pH to 9-10. The reddish brown solution was obtained and the solution was continued stirring for another 30 minutes [1]. The obtained product was purified by dialysis against distilled water using dialysis

bag (MWCO = 12,000-14,000 Da). The distilled water outside dialysis bag was changed every 6 hr for 2 days. The purified HPC-olsalazine conjugates (**6b-d**) were obtained by lyophilization and characterized by FT-IR and ^{1}H NMR.

2.3 Synthesis of olsalazine

5-Aminosalicylate (0.5 g, 2.8 mmol) was suspended in 3 N HCl (20 ml). A solution of $NaNO_2$ (0.29 g, 4.2 mmol) in distilled water (10 ml) was prepared and added dropwise into the solution of 5-aminosalicylate in an ice bath (3-5 °C). The mixture was stirred in the ice bath (3-5 °C) for 5 another minutes and then the resulting diazonium salt was tested for the presence of excess nitrous acid using potassium iodide/starch indicator paper. Sodium salicylate (0.67 g, 4.2 mmol) was dissolved in distilled water (10 ml) and added dropwise into the diazonium salt solution of 5-aminosalicylate and continued stirring at 3-5 °C for another 30 minutes. After that 3 M NaOH solution was added to adjust pH to 9-10. The resulting reddish brown solution was kept stirring for a further 30 minutes. The resulting mixture was then acidified with 3 N HCl and precipitates were filtered, washed several times with water and 50 % methanol in water. The reddish brown solid product was dried at 70 °C for four hrs to give 79.2 %yield. FTIR spectra of olsalazine sodium (KBr) showed principal peaks at 1723 and 1772 cm^{-1} resulted from C=O stretching and the peak at 2900 cm^{-1} resulted from carboxylic O-H stretching and peaks at 3445, 3099 and 1592 cm^{-1} resulted from aromatic O-H stretching, aromatic C-H stretching and aromatic C=C stretching respectively. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.02 and 7.12 (-Ar-OH), 7.77- 8.29 (m, -Ar) and 11.23 (s, -OH of carboxylic group).



Scheme 1. Synthesis of HPC-olsalazine conjugates (**6b-d**).

2.4. Determination of drug content in conjugates

The quantity of 5-ASA was estimated by detaching the olsalazine moiety from the polymer conjugates after alkaline hydrolysis. Each conjugate (100 mg) was placed in 1 N NaOH solution (100 ml) and stirred at 50 °C for 2 hr. An aliquot of the clear solution (0.1 ml) was further diluted with mobile phase and 5-ASA was calculated by

measuring the amount of olsalazine by HPLC at 348 nm [15]. The synthesized olsalazine was used as the standard comparison. The standard solutions of olsalazine were prepared in a concentration ranging from 2-10 μg/ml and analyzed by HPLC.

2.5. Chemical stability studies

The releasing of 5-ASA from the obtained conjugates was performed in rats gastrointestinal contents. The rat gastrointestinal content was prepared in different pHs, such as 1.2 for stomach content, 6.8 for intestinal content and 7.4 for colon content. Since, each conjugate contains two ester and one azo bond and the chemical stability of these bonds need to be considered in these buffer solutions before release study. For this purpose each conjugate 100 mg was placed in a membrane dialysis bag with MWCO 12,000–14,000 and transferred into a beaker. Each beaker covered with cover slip containing 70 ml of each buffer solution and was stirred in a thermostatic bath at 37 ^{o}C for 24 hr. The solution outside dialysis bag (1 ml) was taken at selected time intervals and replaced with the same buffer. The amount of 5-ASA which may release from these conjugates was analyzed by HPLC [7,17].

2.6. The releasing study of 5-ASA from the HPC-olsalazine conjugates (6b-d).

2.6.1. Incubation of HPC-olsalazine conjugates with the homogenate of the stomach or small intestine of rats

Male Wistar rats (250–300 g) were sacrificed with a midline incision. Sections of the stomach and small intestine were collected separately, weighed and homogenized. The homogenate was diluted with 1% peptone in hydrochloric acid buffer solution (pH 1.2) for stomach and with phosphate buffer solution (pH 6.8) for small intestine to yield a 10% w/v dilution under nitrogen atmosphere. To 900 μl of each homogenate, 100 μl of

conjugates (**6b-d**) or sulfasalazine solution (equivalent to 0.58 mg of 5-ASA) was added under anaerobic system and the mixture was incubated for 24 hr at 37 °C. Aliquot amount of samples (500 µl) were taken at selected time intervals: 0.5, 1, 2, 4, 6, 12 and 24 hr after incubation. Fifty microliters of 30% (w/v) trichloroacetic acid (TCA) was then added to stop the enzymatic reaction and stored at –20 ºC until analyzed by HPLC [22].

2.6.2. Incubation of HPC-olsalazine conjugates with the colonic content of the rats

Male Wistar rats (250–300 g) were sacrificed with a midline incision, the abdomen was opened and the cecum and colon were isolated and surgically removed. The cecal segment of the intestine contents was collected, weighed and homogenized. Homogenate was diluted with 1% peptone in phosphate buffer solution (pH 7.4) for colon to yield a 10% w/v dilution under nitrogen atmosphere. To 900 µl of each homogenate, 100 µl of conjugates or sulfasalazine solution (equivalent to 0.58 mg of 5-ASA) was added under anaerobic system and the mixture was incubated for 24 hr at 37 °C. Aliquot amount of samples (500 µl) were taken at selected time intervals: 0.5, 1, 2, 4, 6, 12 and 24 hr after incubation. Fifty microliters of 30% (w/v) trichloroacetic acid (TCA) was then added to stop the enzymatic reaction and stored at –20 °C until analyzed by HPLC [22].

2.6.3. Sample preparation for HPLC analysis

The frozen samples were taken and keep at room temperature for 1 hr before use and then centrifuged at 10,000 rpm (5 °C) for 25 minutes. Fifty microliters of methanol was added to the supernatant (200 µl) to precipitate the protein and sample solution was diluted with 750 µl of mobile phase and filtered through the 0.45 µm-membrane filter. The quantity of drug release (5-ASA) was then analyzed by HPLC [22].

2.6.4. Quantification of 5-ASA by HPLC analysis

Concentration of 5-ASA released from the conjugates was determined by HPLC using an Agilent HPLC system (HP 1100 series, Agilent, USA). Data analysis was performed by using Agilent software. A reverse phase C18 (ACE®, HPLC Columns) (250 mm x 4.6 mm, 5 µm) column was eluted with the mobile phase A consisted of 5 % methanol in phosphate buffer pH 6.0 and methanol was used as mobile phase B. The flow rate of the mobile phase was 1.0 ml/min with a gradient program of 0/0, 7/0, 12/40, 22/40, 27/0, 30/0 (min/% B). The detection wavelength was set at 240 nm for the quantitation of released 5-ASA and 348 nm for detection of any released of olsalazine. The injection volume was 20 µl.

3. Results and discussion

3.1. Synthesis of HPC-olsalazine conjugates

The synthesis route of HPC-olsalazine conjugates was shown in scheme 1 according to our previous reported procedure [24]. For accomplishing the uniform distribution of chloroacetate groups along the polymer chain, the esterification was completed homogeneously in dried dichloromethane. Four chloroacetylated HPC conjugates (**3a-d**) were synthesized by modifying HPC (Mw ~100,000) by varying the molar ratios of chloroacetate groups to the hydroxyl groups of HPC. The hydroxyl groups of HPC were kept constant but on the other hand the molar ratio of chloroacetate groups were varied such as 1:0.25 (**a**), 1:0.5 (**b**), 1:0.75 (**c**) and 1:1 (**d**). The resulting chloroacetylated HPC products (**3a-d**) were easily purified by dialysis method against distilled water due to good solubility of *pyridinium chloride* by-product in water. Light yellow color

products of (**3a-d**) were synthesized with good percentage yield i.e. 89.6, 85.2, 87.9 and 82.4 %, respectively.

5-ASA was selected as the spacer due to its safety, suitability of functional groups and as the compound of choice for the treatment of IBD. It holds carboxylic group for coupling with polymer by esterification and amino group can be employed for the diazotization process with salicylic acid (SA) to give rise to another molecule of 5-ASA. The coupling of bioactive carboxylic acid of 5-ASA to HPC functionalized with chloroacetate groups was carried out by using the sodium salt of 5-ASA (**4**) in DMSO. The sodium salt of 5-ASA was simply prepared by reacting equivalent amounts of 5-ASA and sodium hydroxide and this solution was left to stand overnight to evaporate slowly. It worth to note that this reported procedure is a novel coupling technique of 5-ASA onto HPC by performing reaction just only in two steps without any help of coupling agent and base. Preliminary experiments of coupling reaction of 5-ASA onto HPC were attempted by Zou and co-workers method [7], which consisted of four steps. We have tried to follow the same scheme but in the final step i.e. deprotection of HPC-5-f-ASA, however, we could not find the optimum conditions for completely removing the protecting group (formic acid). We were then tried to find another optimum conditions in deprotection process such as time, strength of acid and temperature but characterizations by FT-IR and ^{1}H NMR did not support us. Sodium salt of 5-ASA (**4**) was another option for grafting onto polymer the as compared to its carboxylic acid form. In case of salts of carboxylic acid, there is no complex reactive intermediate and after completing the reaction, the excessive 5-ASA can be easily removed by dialysis due to its good solubility in water. Highly purity HPC-5-ASA ester conjugates (5a-d) were fabricated by this efficient method because the impurity (NaCl) from the reaction was soluble in distilled water, hence can be removed easily by dialysis. Dark brown color HPC-5-ASA ester conjugates (**5a-d**) were synthesized with good percentage yield i.e. 84.1, 88.9, 83.4 and 81.6 %, respectively.

Olsalazine comprises two molecules of 5-ASA that are linked covalently through an azo bond similar to that in sulfasalazine (Fig. 1). After reduction of azo bond by azoreductases in colon, each mole of olsalazine provides two moles of 5-ASA. Olsalazine is usually used for the management of patients with ulcerative colitis but about 5-10 % patients cannot tolerate it due to severe diarrhea [13]. In our research, we bring a new idea to develop an olsalazine like product based on polymer i.e. one molecule of 5-ASA was previously linked with HPC and can be further employed for coupling with another molecule of 5-ASA by an azo bond. For this purpose diazonium salt of HPC-5-ASA ester conjugates were prepared in 3 N HCl and SA was coupled under basic conditions to develop a dimer of 5-ASA. During diazotization process HPC-5-ASA conjugate (**5a**) was in solution form in 3 N HCl but the other three conjugates (5b-d) behaved like a suspension. 5a was completely soluble in 3 N HCl due to the lowest drug attachment (6.4 %) and that may cause ester bond between HPC and 5-ASA broken during diazotization reaction. In case of HPC-5-ASA conjugates (**5b-d**), SA was successfully coupled during diazotization process and the purified reddish brown HPC-olsalazine conjugates (**6b-d**) were achieved by lyophilization as characteristic with good percentage yield i.e. 76.7, 73.9 and 71.6 %, respectively.

Figure 2 (a-d) shows the comparative FT-IR spectra of unmodified HPC (a), partially modified HPC with chloroacetate groups (**3d**) (b), HPC-5-ASA ester conjugate (**5d**) (c) and HPC-olsalazine conjugate (6d) (d). It can be observed that in the spectrum of chloroacetylated HPC (Fig. 3b), new characteristic band of carbonyl group appeared at 1754 cm^{-1} (C=O) and -CH_2Cl group at 781 cm^{-1}. On the other hand the band of hydroxyl groups of HPC in the range 3600 - 3200 cm^{-1} partly disappeared. Moreover, in the spectrum of HPC-5-ASA ester conjugate (Fig. 3c) the absorption bands appeared at 1683 and 786 cm^{-1}, which results from scissoring vibrations bands of C=C and C-H bonds in the benzene ring. The peak of -CH_2Cl group at 781 cm^{-1} completely disappeared, it means that all the chloroacetate groups were consumed during

esterification reaction. In case of HPC-olsalazine conjugate (Fig. 3d), the azo (N=N) group weak peaks were appeared at 1593 & 1457 cm^{-1}.

In the ^{1}HNMR spectrum (data not shown) of HPC-olsalazine conjugates, the multiplets characteristics resonance peaks from 6.8 ppm to 8.4 ppm are ascribed to the protons of azo moiety of olsalazine. The characteristic peak of protons of –COO-CH_2-O- group appeared at 4.3 ppm. A tiny peak detected at 4.95 ppm belongs to the resonance of the methine protons of modified HPC. The peaks at 1.2 and 1.0 ppm are ascribed to methyl groups of the hydroxypropyl group after and before attachment of 5-ASA and all other protons of HPC were merged in the broad multiplets from 3.2 to 4.0 ppm. FT-IR and ^{1}H NMR results supported that HPC-olsalazine conjugates were synthesized and characterized successfully. Olsalazine was synthesized in good yield and was used as a standard comparison in the preparation of calibration curve for calculating the percentage drug loading. In case of olsalazine, azo bond (N=N) weak peaks were appeared at 1588 & 1486 cm^{-1} and it was further confirmed by ^{1}H NMR (data not shown) by finding the multiplet signals in aromatic groups region between 6.5-8.5 ppm.

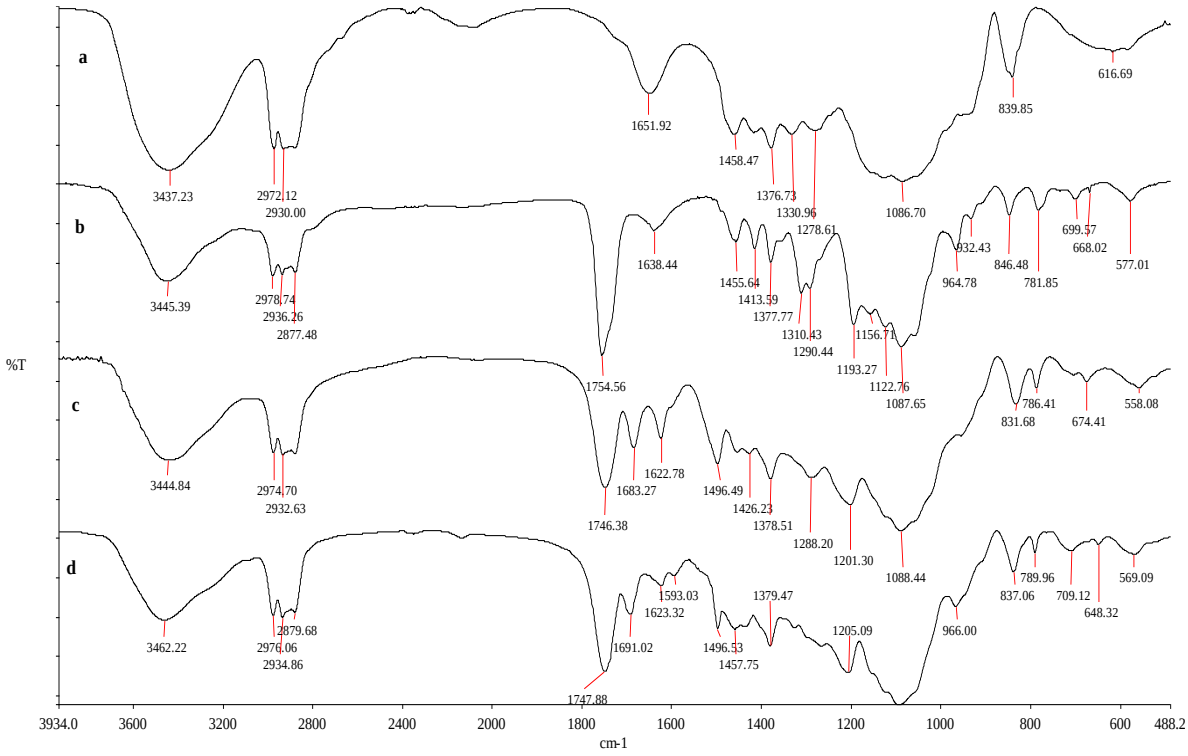


Figure. 2. The comparative FT-IR spectra of (a) HPC, (b) Chloroacetylated HPC (**3d**), (c) HPC-5-ASA ester conjugate (**5d**) and (d) HPC-olsalazine conjugate (**6d**).

3.2. Conjugates chemical stability studies

Three HPC-olsalazine conjugates (**6b-d**) were designed with the participation of two ester and one azo bond for colon specific delivery and for this purpose these three chemical bonds should be stable while passing through the stomach and small intestine. Polymer-drug azo conjugates that were studied previously were found stable in gastrointestinal environment such as dendrimer-5-ASA azo conjugates [1] and poly(ethylene glycol)-5ASA azo conjugates [2]. The in vitro stability study was investigated by incubating all of the conjugates in buffer solution of pH 1.2, pH 6.8 and pH 7.4. In order to evaluate the stability of the conjugates (**6b-d**), samples were quantified during 24 h experiments by HPLC analysis. It was observed that no free 5-ASA and olsalazine were detectable in all buffer solutions, which confirmed that all the conjugates were chemically stable at pHs 1.2, 6.8 and 7.4.

3.3. 5-ASA release studies

HPC-olsalazine conjugates (**6b-d**) were hydrolyzed to detach the olsalazine moiety for determining the percentage drug loading by HPLC. The attachment of drug was increased as the molar ratio of chloroacetyl chloride was increased to HPC and the quantity of 5-ASA was calculated to give 10.4 (**6b**), 14.7 (**6c**) and 18.6 % (**6d**). In order to ensure the potential of these three macromolecular azo conjugates for colon specific release of 5-ASA, an ex vivo release study was conducted by using the gastrointestinal contents of male Wistar rat. HPC-olsalazine conjugates contain two ester bonds that are susceptible to enzymatic hydrolysis before reaching the colon target. The percentage release of 5-ASA from 6b-d were determined up till to 24 hr after incubation at 37 ^{o}C with the gastrointestinal contents of the rat.

It was an interesting comparison release study between the low molecular weight commercially available sulfasalazine and macromolecular HPC-olsalazine conjugates that were synthesized in this work. Both sulfasalazine and HPC-olsalazine conjugates (**6b**-**d**) were not soluble in water that is why before mixing with the rat contents under nitrogen atmosphere, the homogenous solution of sulfasalazine was prepared by using few drops of 0.1 N NaOH and HPC-olsalazine conjugates were used heterogeneously. On incubation of sulfasalazine or conjugates (equivalent to 0.58 mg of 5-ASA) with the homogenate of the stomach, no 5-ASA or small molecule olsalazine was detected during a 24 hr period, which proved that 6b-d were chemically stable with the stomach contents of the rat. It was also confirmed that the number of azoreductases may not presence in the stomach to trigger the drug release from sulfasalazine or macromolecular conjugates.

In case of incubation with the homogenate of the small intestine for 24 hr (Fig. 3), it was found that sulfasalazine started to release 5-ASA after 4 hr incubation period. However macromolecular conjugates (**6b**-**d**) did not show any release of 5-ASA during the first 6 hr. The quantity of 5-ASA released from sulfasalazine was found (42.9 %) after the incubation period of 24 hr. HPC-olsalazine conjugates (**6b**-**d**) started to release 5-ASA after 6 hr and the % release of 5-ASA was found to be the highest (26.9 %) with the lowest drug attachment (**6b**) and the conjugates (**6c**-**d**) showed release up to 20.8 and 14.3 %, respectively after 24 hr incubation period. It can be observed that 5-ASA was released from both sulfasalazine and the macromolecular conjugates in the intestinal segment of the rat which could be due to availability of microflora in this GI section which produce azoreductase enzyme such as *Bacteroides* and *Clostridium* [9]. Therefore, the reduction of azo bond can be possible in small intestine. It was authenticated by Wiwattanapatapee and co-workers (2003) that the azoreductases could also cleave the azo bond of the PAMAM conjugates in small intestine and 5-ASA was found releasing up to 7.2 % after 12 hr incubation at 37 °C [1]. It was also

reported in 2001 that the azo bond between 9-aminocamptothecin and HPMA copolymer was cleaved after 24 h incubation at 37 ˚C in the content of small intestine and released the drug was detected in 25 % [23]. According to the above literatures data, it was supported that a number of macromolecular azo conjugates could release the drug in the small intestine before reaching the colon.

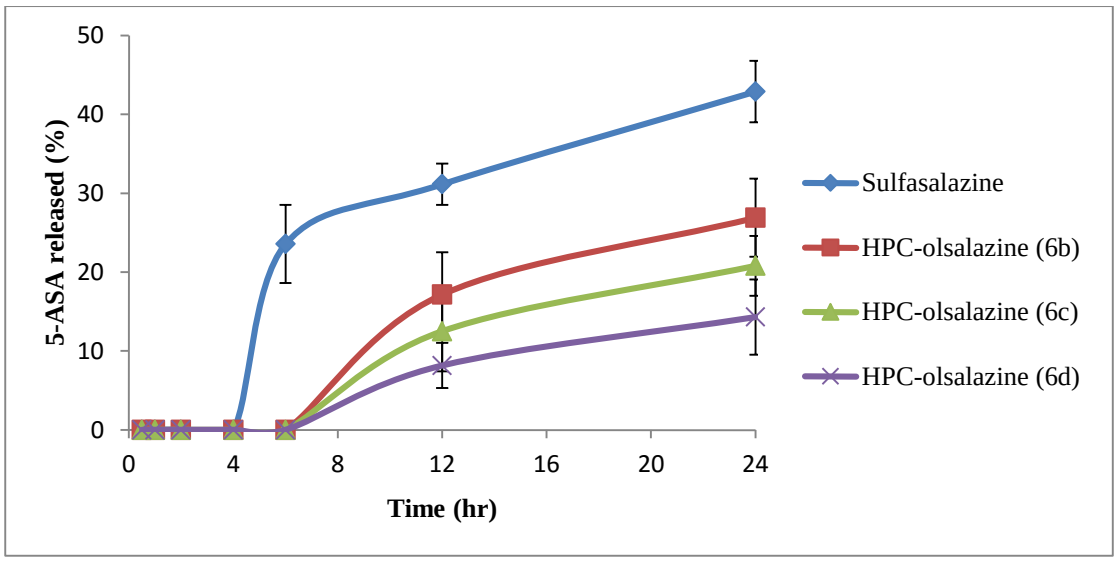


Figure. 3. Release profiles of 5-ASA from the conjugates and sulfasalazine during incubation with rat intestinal content at 37 ˚C. Error bars represent standard deviation, n = 3.

In colonic content, 5-ASA released from sulfasalazine was in a higher amount and faster than in the intestinal content. The release in the colonic content demonstrated that 5-ASA could release from sulfasalazine upto 59.97 % after incubation within the first thirty minutes and the total amount of 5-ASA released within 24 hr was upto 66.7 % (Fig. 4). In case of PAMAM conjugates the release of 5-ASA from sulfasalazine was up to 80.2 % after incubation within the first 6 hr and the % release was higher as compared to our results and it may be due to variation in quantity of colonic microflora and availability of an azoreductase. The release of 5-ASA from the

synthesized HPC-olsalazine conjugates (**6b-d**) showed a better release as compared to intestinal content due to higher number of azoreductase enzymes in the colonic region. However, the released amount of the drug from each conjugate was in different patterns. All the HPC-olsalazine conjugates (**6b-d**) started to give releasing of 5-ASA after thirty minutes of incubation period and showed continuous releasing until 24 hr of incubation period. All the conjugates presented almost similar and sustained release profiles and the highest quantity of 5-ASA released from the conjugate **6b** was found to be 38.8 % after 24 hr incubation period. HPC-olsalazine conjugates (**6c-d**) found to release 5-ASA upto 29.6 and 22.0 %, respectively after 24 hr incubation period (Fig. 4). HPC polymer has good solubility in water but on the other hand 5-ASA is slightly soluble in water and as the attachment of drug was increased to the polymer, HPC could not preserve its water soluble property. These results suggested that the lowest drug attachment on macromolecule can provide the highest release due to less steric hindrance effect.

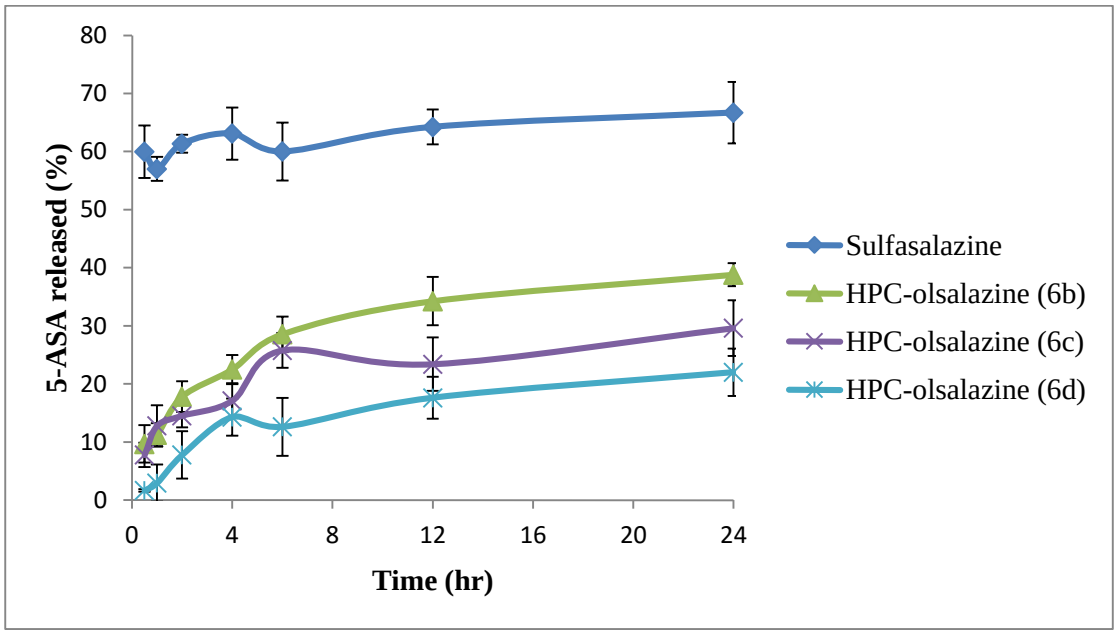


Figure 4. Release profiles of 5-ASA from the conjugates and sulfasalazine during incubation with rat colonic content at 37 ^{o}C. Error bars represent standard deviation, n = 3.

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Zou and co-workers (2005) synthesized HPC-5-ASA ester prodrugs by varying the molar ratios of HPC and 5-ASA (1:1, 1:2, 1:6, 1:10) and they reported that the ester bond between HPC and 5-ASA could not be broken by esterase enzymes in any part of the GI tract of the rat due to insolubility issue [7]. PAMAM-PABA-SA & PAMAM-PAH-SA were two water soluble macromolecular conjugates of 5-ASA which were synthesized by Wiwattanapatapee and co-workers and they reported that during the release study the amide bond between PAMAM and spacer were degraded [1]. Zou and co-workers also synthesized water soluble γ-cyclodextrins-5-ASA ester prodrugs and found 5-ASA releasing property dependent on the solubility of the prodrugs [7]. From the above results, it was concluded that degradation of amide or ester bond between polymer and drug can be dependent on the water solubility property of the prodrugs. Drug release from macromolecular conjugates may depend upon a number of factors such as type of enzyme, solubility, steric hindrance and carrier etc. HPC-olsalazine conjugates (**6b-d**) were developed by decreasing the molar ratios between HPC and 5-ASA, insert a spacer arm between the HPC and 5-ASA and an azo bond was developed between spacer and drug. In our results, all the conjugates (**6b-d**) could release 5-ASA in both intestinal and colonic contents of the rat. The maximum release of 5-ASA from the conjugates was supported by a higher number of azoreductases in the colonic region of the rat. Length of the spacer arm between polymer and drug was also helpful in the cleavage of azo bond by the enzyme. The cleavage of azo bond in case of HPC-olsalazine conjugates was much slower as compared to sulfasalazine. Sulfasalazine azo bond was easily attacked and promptly reduced by azoreductases, which may be due to two factors that firstly carrier of 5-ASA was a low molecular weight instead of polymer and secondly sulfasalazine was in solution form before

mixing the rat contents. Both the ester bonds of HPC-olsalazine conjugates (**6b-d**) was found stable during stability and release studies and it may be due to insolubility of these conjugates or a steric effect of the bulky HPC moiety that esterase enzymes could not breakdown the ester bonds and 5-ASA was only released after the reduction of azo bond (Fig. 5).

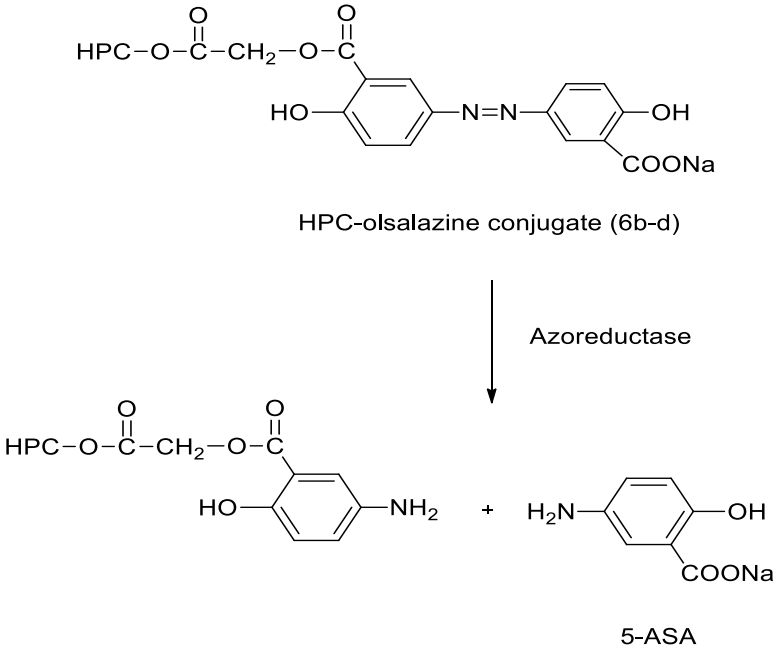

Figure 5. Release of 5-ASA from the conjugates (**6b-d**)

4. Conclusions

From our investigation, HPC-olsalazine conjugates (**6b-d**) were fabricated as a novel macromolecular prodrugs of 5-ASA with high percentage yield and purity. Each conjugate was developed by the participation of two ester and one azo bond and both the ester bonds remained stable during stability and release studies. The release of 5-ASA from each conjugate was higher in colonic contents as compared to the intestinal contents of the rat. It was observed that the cleavage of azo bond is more easy and faster in the colonic region due to higher number of azoreductases. In comparison study, the release of 5-ASA from sulfasalazine was significantly faster as compared to

HPC-olsalazine conjugates. All the macromolecular conjugates have a potential to release 5-ASA in the lower part of the GI tract in a sustained release profile during 24 hr incubation period and that could be useful for the prolong treatment of IBD. The results suggested that HPC has potential for use as colon specific drug carrier.

Acknowledgments

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Appendix 2 Analytical HPLC method

HPLC method use for quantification of 5-ASA in the release study

1. **Chromatographic conditions**

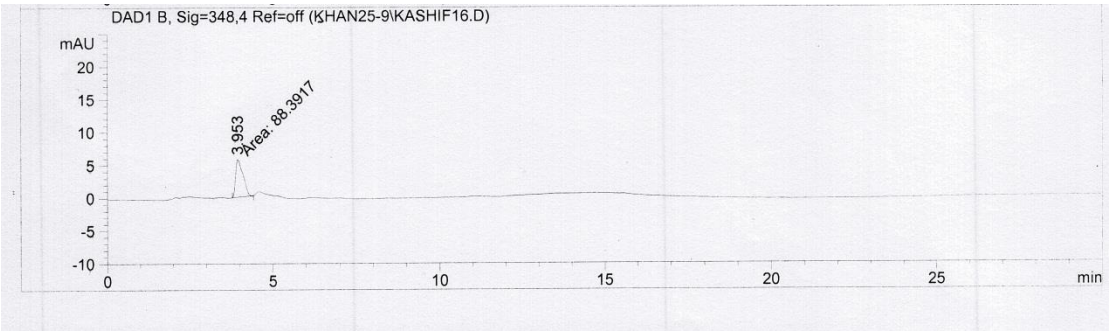
Concentration of 5-ASA released from the conjugates was determined by HPLC using an Agilent HPLC system (HP 1100 series, Agilent, USA). Data analysis was performed by using Agilent software. A reverse phase C18 (ACE®, HPLC Columns) (250 mm x 4.6 mm, 5 µm) column was eluted with the mobile phase A consisted of 5 % methanol in phosphate buffer pH 6.0 and methanol was used as mobile phase B. The flow rate of the mobile phase was 1.0 ml/min with a gradient program of 0/0, 7/0, 12/40, 22/40, 27/0, 30/0 (min/% B). The detection wavelength was set at 240 nm for the quantitation of released 5-ASA and 348 nm for detection of any released of olsalazine. The injection volume was 20 µl.

2. **Calibration and solutions**

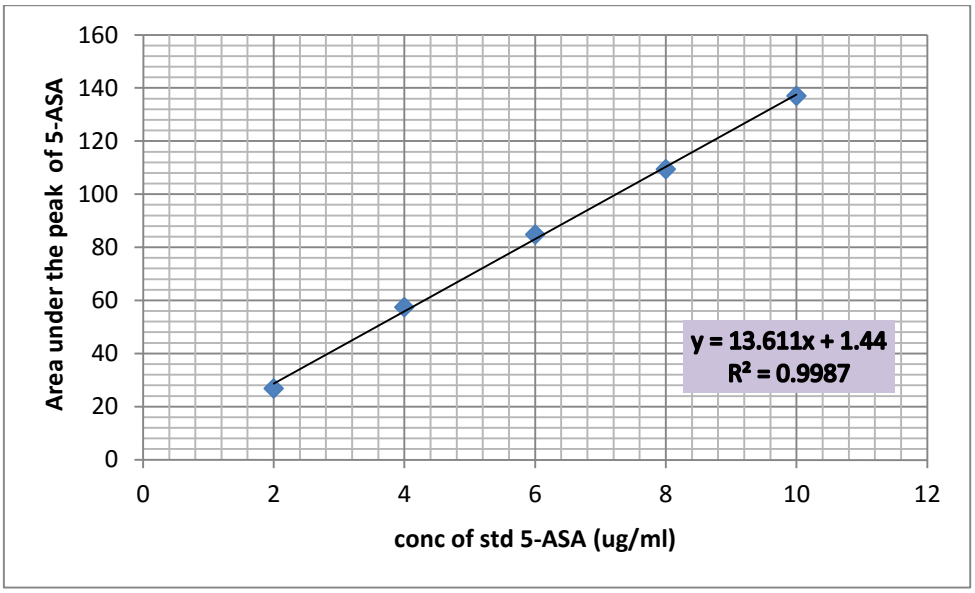
A stock solution of 5-aminosalicylic acid (5-ASA) and olsalazine was prepared in a mixture of 0.1 N NaOH and methanol which gave clear solutions having concentration of about 100 µg/ml. The stock solutions were serially diluted in methanol to give a series of standard solutions having concentration in a range of 1-10 µg/ml. The resulting solutions were subjected to analyze by HPLC.

The Chromatogram of standard solution of 2 µg/ml of 5-ASA is shown below.

The retention time of 5-ASA was 3.9 – 4.1 .min.

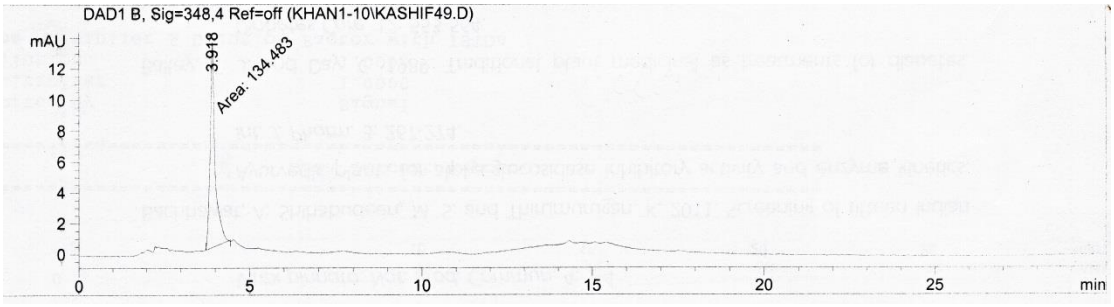


The selected standard calibration curve of 5-ASA was linear between 2 and 10 µg/mL as shown below. Slope and intercept value for calibration curve was $Y = 13.61x + 1.44$ ($R^2 = 0.9987$).

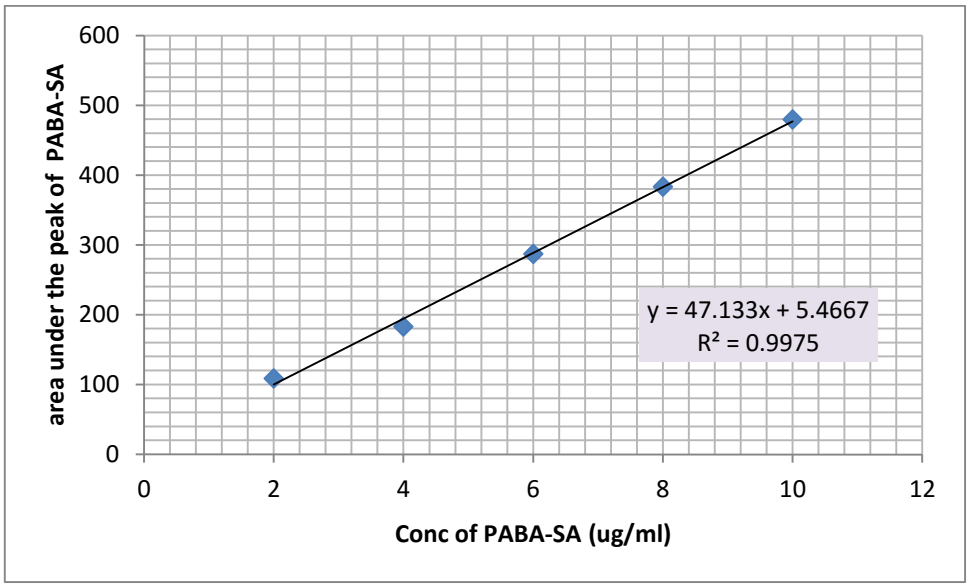


Standard curve of 5-ASA at different concentrations

The Chromatogram of sample solution of 5-ASA that was released from HPC-PABA-N=N-SA conjugate after incubated with colonic content for 6 hr is shown below.

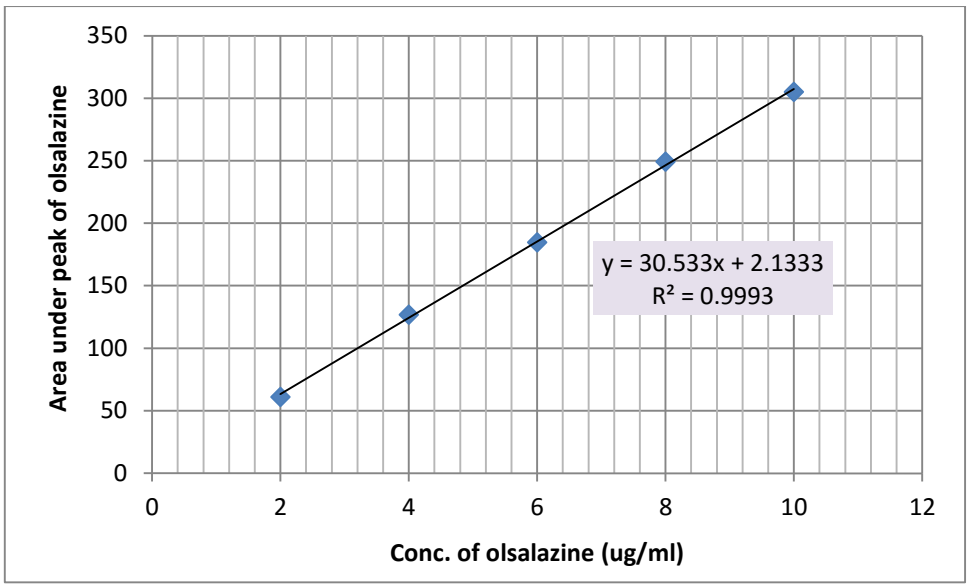


The selected standard calibration curve of PABA-SA was linear between 2 and 10 µg/mL as shown below. Slope and intercept value for calibration curve was $Y = 47.13x + 5.467$ ($R^2 = 0.9975$).



Standard curve of PABA-SA **at different concentrations**

The selected standard calibration curve of olsalazine was linear between 2 and 10 µg/mL as shown below. Slope and intercept value for calibration curve was $Y = 30.53x + 2.13$ ($R^2 = 0.9993$).



Standard curve of olsalazine at different concentrations

Appendix 3 Acceptance letters for paper 1 and 2

Acceptance letters for paper 1

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Prof. R. K. AGARWAL
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Circulation/Database Manager
Mrs. Anjul Agarwal

Dated: 6th September 2014

Dr. Chitchamai Ovatlarnpon
Department of Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
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Hat Yai, Songkhla, 90112
Thailand

Dear Dr. Chitchamai Ovatlarnpon,

We are pleased to inform you that your research article No. 18353/2014 entitled, *"Synthesis, Characterization and Reduction of p-Nitrobenzoyl Hydroxypropyl Cellulose"* has been accepted for publication in Asian Journal of Chemistry. The manuscript will appear in Volume 26 (2014) of Asian Journal of Chemistry. Here, we are enclosing an invoice bill No. 6983 dated 6th September 2014 of US $ 300=00 (US Dollars Three Hundred Only) towards the Printing/Publication charges.

You are requested to remit the payment at the earliest in favour of Asian Journal of Chemistry. Your early response will be highly appreciated.

Thanks

Sincerely Yours

Dr. Himanshu Agarwal
Executive Editor
Asian Journal of Chemistry

Acceptance letters for paper 2

JCPR-5556
Dear Author,

You will glad to know that your research work has been recommended for publication by the concerning reviewer but at the same time, there are some corrections like:

Organize the manuscript as abstract, key words, introduction, experimental section, results and discussion, conclusion, acknowledgements and finally references.

There are some typological/spelling mistakes.

Manuscript should be in **Times new roman and font size should be 12** throughout the paper

Arrange the reference section at the end as per the **instructions to authors** e.g.

For a Journal:

[1] CS Sharma; RK Nema; SN Meyyanathan, *Academic J. Cancer Res.*, **2009**, 2(1), 19-24.

For a Book:

[2] RK Nema, SN Meyyanathan, CS Sharma. A Practical Approach to Pharmaceutical Analysis, 1st Edition, CBS Publishers & Distributors, New Delhi, **2008**, 89-90.

You are requested to revise the manuscript as per the above mentioned points and send us within 24 hrs so that we can further proceed in the matter.

Thanks & Regards

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Appendix 4 Animal Ethic Approval Document

PRINCE OF SONGKLA UNIVERSITY
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Website : www.psu.ac.th

MOE 0521.11/ 734 Ref.13/2013

July 11 , 2013

This is to certify that the research project entitled " Preparation of chitosan (or hydroxypropyl cellulose) anti-inflammatory agent conjugates for colonic drug delivery" which was under conducted by Asst. Prof. Dr. Chitchamai Ovatlarnporn, Faculty of Pharmaceutical Sciences , Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

Kitja Sawangjaroen

Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University

VITAE

Name: Mr. Kashif-ur-Rehman Khan Student ID: 5410730019

Educational Attainment

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M. Phil	Bahauddin Zakariya University, Multan, Pakistan	2008
B. Pharm	Bahauddin Zakariya University, Multan, Pakistan	2003

Scholarship Awards during Enrolment

1. Thesis Grant, Education fee and Salary
   The Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (Grant no. PHA540545g).
2. Thesis Grant
   The Graduate School, Prince of Songkla University.
3. Thesis Grant
   The Nanotec-PSU Excellence Center on Drug Delivery System, Faculty of Pharmaceutical Sciences, Prince of Songkla University.
4. Conference Scholarship
   Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Work-Position and Address

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List of Publication and Proceeding

Publications

1. **Kashif-ur-Rehman Khan**; Sirinporn Nalinbenjapun; Natee Sakorn and Chitchamai Ovatlarnporn, 2015. Synthesis, Characterization and Reduction of *p*-Nitrobenzoyl Hydroxypropyl Cellulose. Asian Journal of Chemistry 27 (5): 1875-1878.
2. **Kashif-ur-Rehman Khan** and Chitchamai Ovatlarnporn, 2015. Synthesis and Characterization of Hydroxypropyl Cellulose-*p*-Aminobenzoic acid Ester Conjugate. Journal of Chemical and Pharmaceutical Research 7 (3): 727-731.
3. **Kashif-ur-Rehman Khan** and Chitchamai Ovatlarnporn, 2015. Hydroxypropyl Cellulose Conjugates for Colonic Delivery of 5-Aminosalicylic acid. Submitted to Carbohydrate Polymers.
4. **Kashif-ur-Rehman Khan** and Chitchamai Ovatlarnporn, 2015. Synthesis and Evaluation of Hydroxypropyl Cellulose Conjugates for Colonic Delivery of 5-Aminosalicylic acid. Submitted to International Journal of Biological Macromolecules.

Proceedings

1. Presented oral presentation on the topic "Synthesis and Characterization of Hydroxypropyl Cellulose-5-Aminosalicylic Acid Conjugates," CDD 2014, Pavillion Queen's Bay, Ao Nang, Krabi, Thailand, May 1-3, 2014.
2. Presented oral presentation on the topic "Esterification of Hydroxypropyl Cellulose and Determination the Optimum Conditions for Its Reduction," PharmaTech 2012, Kuala Lumpur Convention Centre, Malaysia, November y20-21, 2012.